

# Lovesick: immunological costs of mating to male sagebrush crickets

J. C. LEMAN\*, C. B. WEDDLE, S. N. GERSHMAN, A. M. KERR, G. D. OWER, J. M. ST JOHN, L. A. VOGEL & S. K. SAKALUK

Department of Biological Sciences, Illinois State University, Normal, IL, USA

## Keywords:

crickets;  
*Cyphoderris strepitans*;  
insect immunity;  
life history trade-offs;  
lipopolysaccharides;  
mating success;  
nuptial food gifts;  
phenoloxidase.

## Abstract

A growing body of evidence suggests that resources invested in reproduction often come at the expense of the ability to mount an immune response. During mating, female sagebrush crickets, *Cyphoderris strepitans*, consume the ends of the male's hind wings and ingest his haemolymph. Previous research has shown that this behaviour impairs the ability of males to secure additional matings. One hypothesis to account for this effect is that wing wounding triggers an energetically costly immune response, such that nonvirgin males are unable to sustain the costly acoustical signalling needed to attract additional females. To test this hypothesis, we injected virgin males with lipopolysaccharides (LPS) to provoke an immune response, and monitored their mating success in the field. LPS-injected virgin males took significantly longer to mate than sham-injected virgin males, and spent significantly less time calling. We also compared virgin, nonvirgin and experimentally wing-wounded virgin males with respect to: (1) their ability to encapsulate a foreign invader via the accumulation of haemocytes and deposition of melanin and (2) baseline levels of phenoloxidase (PO), a key enzyme in the biochemical cascade leading to the production of melanin. Although encapsulation ability did not differ with reproductive experience, virgin males had significantly higher levels of PO than either nonvirgin or experimentally wing-wounded virgin males. These results suggest that wing-wounding alone is sufficient to impair male immunity, and that males trade-off investment in reproduction and immunity.

## Introduction

Individual organisms maximize their lifetime fitness by balancing the demands of growth, maintenance and reproduction. Each of these components of life history competes with the others for a limited amount of energy (Stearns, 1989; Roff, 1992). Trade-offs in investment between reproductive and immunological aspects of life history have garnered increasing attention from evolutionary biologists (Sheldon & Verhulst, 1996; Zuk &

Stoehr, 2002; Viney *et al.*, 2005). Disease resistance is an important aspect of life history, and even though the costs of maintaining immune physiology can be relatively low, activation of immune responses can be very costly because of energy loss and damage to self tissue (Lochmiller & Deerenberg, 2000). Various sexual dimorphisms in immunological traits have been observed (Zuk & McKean, 1996; Siva-Jothy, 1999; Gershman, 2008) and underscore the functional links between immunity and mating (Zuk & Stoehr, 2002). Moreover, mating has a demonstrated negative effect on immunity in many insects (Adamo *et al.*, 2001; McKean & Nunney, 2001; Rolff & Siva-Jothy, 2003).

Insect immunity is a particularly burgeoning area of focus within the context of trade-offs between reproduction and maintenance (see Rolff & Siva-Jothy, 2003; Schmid-Hempel, 2005). Insects possess innate immunity,

*Correspondence:* Scott K. Sakaluk, Behavior, Ecology, Evolution and Systematics Section, Department of Biological Sciences, Illinois State University, Normal, IL 61790-4120, USA.

Tel.: +1 (309) 438 2161; fax: +1 (309) 438 3722;

e-mail: sksakal@ilstu.edu

\*Present address: J. C. Leman, Biology Department, Augustana College, Rock Island, IL 61201, USA.

comprised of both humoral and cellular components, but lack the antibodies that promote the acquired immunity of vertebrates described as adaptive immunity, presumably because the initial acute response is more important in short-lived organisms. Insects are well suited for evaluating the cost of reproduction and trade-offs with immunity, not only because they lack an antibody response, but also because they are short-lived and abundant, making it possible to secure reasonable estimates of lifetime fitness (Lawniczak *et al.*, 2006).

The sagebrush cricket, *Cyphoderris strepitans*, is one of only seven extant species of a relatively unknown orthopteran family, the hump-winged grigs (Haglidae) (Kumala *et al.*, 2005), and occurs in high-altitude sagebrush meadows in mountainous areas of Colorado and Wyoming (Morris & Gwynne, 1978). Males emit acoustical signals that function to attract females (Snedden & Irazuzta, 1994) and that appear to be the primary means of pair formation (Snedden & Sakaluk, 1992). Copulation is initiated when a female climbs onto the dorsum of the male, at which time he attempts to transfer a spermatophore, a small gelatinous packet containing sperm. During copulation, the female feeds on the male's fleshy hind wings and the haemolymph that oozes from the wounds she inflicts. After the spermatophore has been transferred, the male actively pulls away from the female, terminating wing feeding (Eggert & Sakaluk, 1994).

Virgin males secure more matings than their relative abundance in the population would predict, a population-wide pattern that has been described as the 'virgin-male mating advantage' (Morris *et al.*, 1989; Snedden, 1996). Mating appears to be costly to males: not only do they lose a significant portion of their hind wing tissue and haemolymph, they must also produce another spermatophore if they are to mate again. Previous work has shown that nonvirgin male calling time is reduced relative to virgin males (Sakaluk *et al.*, 1987; Sakaluk & Snedden, 1990). Females, however, do not appear to discriminate between virgin and nonvirgin males based on the structure of their calls (Sakaluk & Ivy, 1999). Given the importance of calling in pair formation and the marked decrease in calling time of nonvirgin males, it seems likely that it is the decrease in calling time that is responsible for the reduced mating success of nonvirgin males.

One possible proximate mechanism underlying the reduction in nonvirgin male calling time is the activation of the male's immune system that presumably results from the wing wounding inflicted by females at copulation, decreasing the amount of energy available for calling. Life history theory suggests that an optimal balance of immunological functions with other important life history traits is vital to an individual's long-term reproductive success, assuming there is a finite amount of resources to invest in physiological processes. Many studies have documented such costs as a suppression of secondary sexual characteristics or reduced sperm viability resulting from immune system activation (Faivre

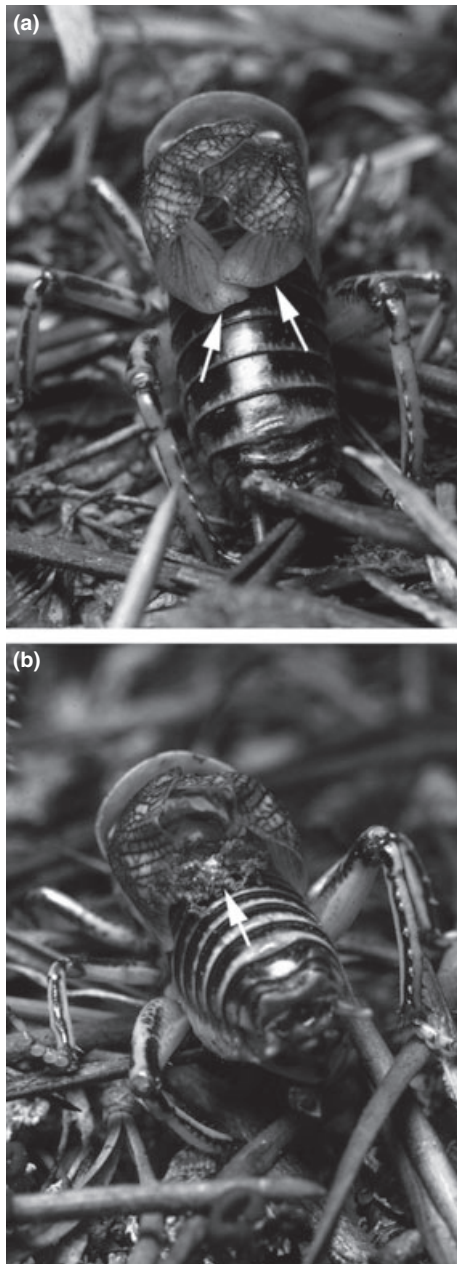
*et al.*, 2003; Simmons & Roberts, 2005). Of particular relevance to our study system, an induced immune response in field crickets has been shown to result in a reduction in calling time (Jacot *et al.*, 2005).

The objective of this study was to determine the effects of an induced immune response on the mating success of free-living male sagebrush crickets and to compare the immune responses of virgin and nonvirgin males. In the first set of experiments, the immunity of males was impaired to determine whether there was a concurrent effect on components of reproductive success. If males are forced to trade off reproduction with immunity, then experimentally immune-challenged males should call less and secure fewer matings than sham-treated males. In the second experiment, males of different mating experience were assayed to determine whether mating had a negative effect on measures of immunity. If wing wounding has a negative effect on male immunity, males that have mated or that have been experimentally wounded should show a reduced ability to combat future immune challenges compared to virgin males, as assayed by phenoloxidase (PO) and encapsulation assays.

## Methods

### Experiment 1: the effect of an experimental immune challenge on the mating success of free-living males

We conducted a mark-recapture study over 2 years at two different locations in Grand Teton National Park, Wyoming: (1) Deadman's Bar, a 3.2-ha rectangular study plot in sagebrush meadow habitat adjacent to the Snake River at Deadman's Bar (2006) and (2) Pacific Creek, a collection of several smaller contiguous study plots (total area = 9.15 ha) in sagebrush meadow habitat adjacent to the intersection of Pacific Creek road and John D. Rockefeller, Jr highway (2007). The larger study area in 2007 was necessitated by the lower density of crickets at this location. At the beginning of each breeding season, we attempted to capture all of the virgin males present in the study area. The initial collection period took place from 21 May to 24 May in 2006 and 20 May to 27 May in 2007. Males initiate pair formation by acoustically signalling to females from perches in sagebrush (Snedden & Sakaluk, 1992; Snedden & Irazuzta, 1994), so we located males by orienting to their calls. The mating status of males was determined by examining their hind wings for evidence of wing wounding by females. Only virgin males, as evidenced by their intact hind wings (Fig. 1a), were used in the experiment. A numbered flag marked the location of each virgin male collected, so that experimental males could be returned to their respective locations of capture after treatment. Males were held in collecting vials and transported to the University of Wyoming-National Park Service Research Station (UW-NPS), less than 30 km away, for processing.



**Fig. 1** (a) Intact hind wings of virgin male sagebrush cricket (denoted by white arrows). (b) Hind wings of nonvirgin male show extensive wounding and melanization. Photos by David Funk.

Males were weighed the morning after their capture to the nearest 0.1 mg and assigned to one of two treatments, one in which males were experimentally injected with lipopolysaccharides (LPS) and a sham-control treatment. LPS in this experiment were derived from the cell walls of *Serratia marcescens*, a Gram-negative bacterium that is a common insect pathogen (Bucher, 1959; Adamo *et al.*, 2001). Although LPS elicits an immune response in crickets and other insects (Jacot *et al.*, 2004, 2005), it is

itself nonpathogenic. Experimental males were injected with 50  $\mu\text{g}$  of bacterial LPS (L6136 Sigma-Aldrich Inc., St Louis, MO, USA) dissolved in 10  $\mu\text{L}$  of Grace's insect medium (Sigma, G8142), thus presenting males with an immunological challenge. Sham-control males were injected with 10  $\mu\text{L}$  of Grace's insect medium only. Injections were given approximately 12 h after males had been captured, and administered between the second and third abdominal sternites with a 10- $\mu\text{L}$  Hamilton syringe (#8003; Hamilton Co., Reno, NV, USA) after swabbing the abdomen with a cotton ball soaked in 70% ethanol. Each male was individually marked with a numbered tag secured to his pronotum with cyanoacrylic glue. Fluorescent paint (Testors Co., Rockford, IL, USA) was also applied around the pronotum and to the femora to facilitate the recapture of marked individuals with portable fluorescent lanterns. Marked males were returned to their respective sites of capture that evening at sunset, approximately 24 h after capture. We marked and released a total of 86 males in 2006 (43 LPS-injected and 43 sham-control) and 93 males in 2007 (47 LPS-injected and 46 sham-control).

At intervals of two nights thereafter, weather permitting, we recaptured marked males to ascertain their mating status. Mating activity was inferred by loss of hind wing material in all treatments. Wing wounds were classified as 'fresh' (visibly wet wounds with no discoloration indicating that the male had mated on the night of recapture) or 'old' (dry, darkened wounds indicating that the male had mated at least one night previous to the night of recapture) (Fig. 1b).

### Experiment 2: the effect of an experimental immune challenge on male calling time

In a companion laboratory study conducted in 2006, we used time-lapse video recording to measure the effects of an induced immune response on male calling effort. Virgin males were collected from Pacific Creek and maintained at the UW-NPS Research Station in individual plastic containers (9 cm diameter  $\times$  8 cm height) misted with water daily and provisioned with a piece of apple and a sagebrush gall. Each male was assigned to one of the two experimental treatments: (1) LPS-injected males or (2) sham-control males. Males were treated the morning after capture and used in video trials that same evening.

Each night of the study beginning at 20:00 hours, one to four males from each of the two treatments were placed individually in Plexiglas viewing chambers (10.1  $\times$  7.1  $\times$  3.8 cm), each containing a short stick as a calling perch, and their calling activity monitored over the next 6 h using time-lapse video photography. Although the number of males recorded from night to night varied, we always monitored the same number of males in each treatment on any given night to control for any seasonal effects. Calling behaviour was recorded for each male over two consecutive nights. Night-time

recording was facilitated by illumination from a 25-W red light bulb. Upon review of video recordings, we determined the time spent calling by each male during the trial, measured as the number of 5-min intervals within which stridulation occurred (one-zero sampling; Altman, 1974).

### Experiment 3: the effect of wing wounding on encapsulation and PO activity

To determine the effect of wing wounding on the male's immune system, we compared the immune responses of three groups of males: virgin males ( $n = 50$ ), experimentally wounded virgin males ( $n = 49$ ) and nonvirgin males ( $n = 48$ ). Virgin and nonvirgin males (as evidenced by old wing wounds) were captured in 2007 at a third collecting site in Bridger Teton National Forest, adjacent to Grand Teton National Park, and transported to the UW-NPS Research Station. Half of the virgin males were experimentally wounded by removing a small portion (approximately 1/5) of the distal part of the fleshy hind wings with micro-scissors on the evening they were captured. This procedure created wounds resembling those of males that have recently mated.

For all males, we assayed two parameters of immune function widely used in other studies of insects: (1) encapsulation and (2) PO activity (Lawniczak *et al.*, 2006). The encapsulation response is the primary insect immune response against a foreign object present in the haemocoel (Gillespie *et al.*, 1997). Encapsulation occurs as a result of the aggregation of haemocytes that leads to the deposition of melanin and hardening of the resultant capsule. This capsule eventually kills the pathogen, either through asphyxiation or through the production of cytotoxic substances (Cerenius & Söderhäll, 2004). The magnitude of the encapsulation response can be measured by experimentally implanting an inert object into the haemocoel of the insect and quantifying the resulting accumulation of melanin and haemocytes (Ryder & Siva-Jothy, 2000). We examined the encapsulation response of male sagebrush crickets by implanting a 2-mm long nylon filament (0.2 mm diameter) that had been abraded using sandpaper. On the morning following their capture, the filament was inserted dorso-ventrally between the second and third abdominal sclerites in a small puncture made with a sterilized needle. A small knot was tied at the end of the filament to aid in its removal. This implant was allowed to melanize for 24 h, then was removed and photographed on a white background with a digital camera (Nikon, Melville, NY, USA) through the ocular of a stereomicroscope (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). To control for variation in lighting, the implant was photographed side-by-side with a control (i.e. nonimplanted) filament. The degree of melanization of the implant was measured using image-analysis software (IMAGE J) freely available from the NIH (<http://rsbweb.nih.gov>). This program compares the

number of black and white pixels in any section of the image to produce a greyscale value. The portion of the image containing the implant was evaluated by the program to obtain the greyscale value, with values of each pixel ranging from 0 (completely dark) to 256 (completely white). We determined the darkness of the implant as the difference in greyscale values between the implant and control filaments.

Phenoloxidase is a key enzyme in the biochemical cascade leading to the production of melanin, which is the key component in the encapsulation response (Söderhäll & Cerenius, 1998). We measured PO activity using methods adopted from Rantala & Kortet (2003), Fedorka & Zuk (2005) and Bailey & Zuk (2008). Immediately after removing the nylon filament, we drew 6  $\mu$ L of haemolymph from the site of filament removal, added this to 60  $\mu$ L of phosphate-buffered saline (PBS), and then froze the samples for several weeks at  $-25$  °C to disrupt haemocyte membranes. Five microlitres of the PBS/haemolymph solution were then added to 7  $\mu$ L of bovine pancreas  $\alpha$ -chymotrypsin ( $1.3$  mg mL $^{-1}$ ; Sigma C7762) and allowed to react at room temperature for 20 min. Alpha-chymotrypsin converts all of the pro-PO enzyme present in the haemolymph to PO (Cerenius & Söderhäll, 2004; Bailey & Zuk, 2008). We measured the resulting PO activity by adding 90  $\mu$ L of a 15-mM L-Dopa solution. Because the same amount of L-Dopa substrate was added to each sample, resulting differences in melanin production must be due to individual differences in PO enzyme activity. We used a spectrophotometer (Power Wave 340; BioTek, Winooski, VT, USA) to record the change in optical density (OD) at 490 nm for 130 min of the active phase of the reaction. Male PO activity was recorded as the change in OD over that span of time. The same calculation was performed on 10–12 control wells containing only PBS and L-Dopa. The average value of control samples was then subtracted from the PO value of an individual subject to obtain a final PO level.

## Results

### Experiment 1: the effect of an experimental immune challenge on the mating success of free-living males

A two-way ANOVA showed no difference between treatments in the mean ( $\pm$  SE) initial mass of males in either year of the study (2006: sham-control =  $772 \pm 11.2$  mg, LPS =  $766 \pm 9.0$  mg; 2007: sham-control =  $778 \pm 9.5$  mg, LPS =  $772 \pm 7.8$  mg; ANOVA,  $F_{3,175} = 0.24$ ;  $P = 0.87$ ).

Seventy-one per cent of marked males were recaptured at least once in 2006 (61/86) and 68% in 2007 (63/93). There was no significant difference between treatments in the percentage of males recaptured in either year (2006: sham-control = 72% (31/43), LPS = 70% (30/43), likelihood ratio  $\chi^2 = 0.06$ ,  $P = 0.81$ ; 2007: sham-control = 67% (31/46), LPS = 68% (32/47), likelihood ratio  $\chi^2 = 0.01$ ,  $P = 0.94$ ). Likewise, the number of

times males were recaptured (excluding males that were never recovered) did not differ across treatments (median recapture frequency (range); 2006: sham-control = 3 (1–6), LPS = 2 (1–9), Kruskal–Wallis  $\chi^2_1 = 0.09$ ,  $P = 0.77$ ; 2007: sham-control = 2 (1–8), LPS = 2 (1–7), Kruskal–Wallis  $\chi^2_1 = 0.06$ ,  $P = 0.81$ ).

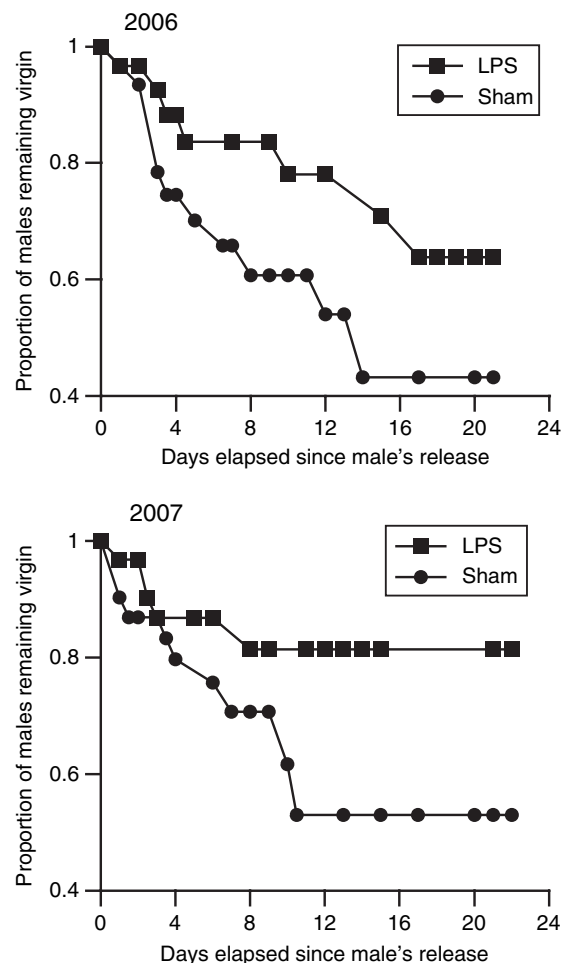
Time to mating was determined as the number of nights from the time a male was first released until he was recaptured as a nonvirgin. Nonvirgin males bearing fresh wing wounds were assumed to have mated on the night they were recaptured. Nonvirgin males bearing old wing wounds were assumed to have mated at least one night previous to their recapture or, if they had not been captured in the previous census, we recorded the night of mating as the mid-point between the earliest time they could have mated and the last time they could have mated. Males that had still not mated by the time of their last recapture were included as ‘censored’ observations.

We used failure-time analysis to compare time to mating across treatments (Allison, 1995), specifically, a Cox regression as implemented by PROC PHREG in SAS/STAT software (SAS Institute Inc., 2004). Treatment (LPS or sham) and year (2006 or 2007) were entered as covariates, and the EXACT option was specified in the model statement to handle ties, instances in which different males had the same time to mating. This option was employed because it assumes that mating times are, in reality, continuous and ordered, assumptions that are almost certainly met by our data. The analysis revealed a significant effect of treatment (Wald  $\chi^2 = 4.39$ ,  $P = 0.036$ ), but no effect of year (Wald  $\chi^2 = 0.44$ ,  $P = 0.51$ ) on time to mating (see Fig. 2). Sham-injected virgin males mated sooner than LPS-injected virgin males, and had an instantaneous probability of mating about twice that of LPS males (hazard ratio = 2.13).

We also used failure-time analysis as outlined above to compare survival across treatments. Survival was measured as the number of nights from the night on which a male was first captured to the night on which he was last recaptured. Males that were never recaptured after their initial release were excluded from the analysis because these males may have lost their tags or immediately left the study area upon their release. Males recaptured on the last night of the study were included as right-censored values. The proportion of males remaining alive declined linearly with time in both years (Fig. 3), but somewhat less rapidly for the population at Deadman’s Bar in 2006 (~3–4% mortality per night) than for the population at Pacific Creek in 2007 (~4.6% mortality per night). We acknowledge that we cannot determine whether this is a year effect or a site effect.

### Experiment 2: the effect of an experimental immune challenge on male calling time

Calling times were analysed using a repeated-measures ANOVA, with treatment as a main effect and night of

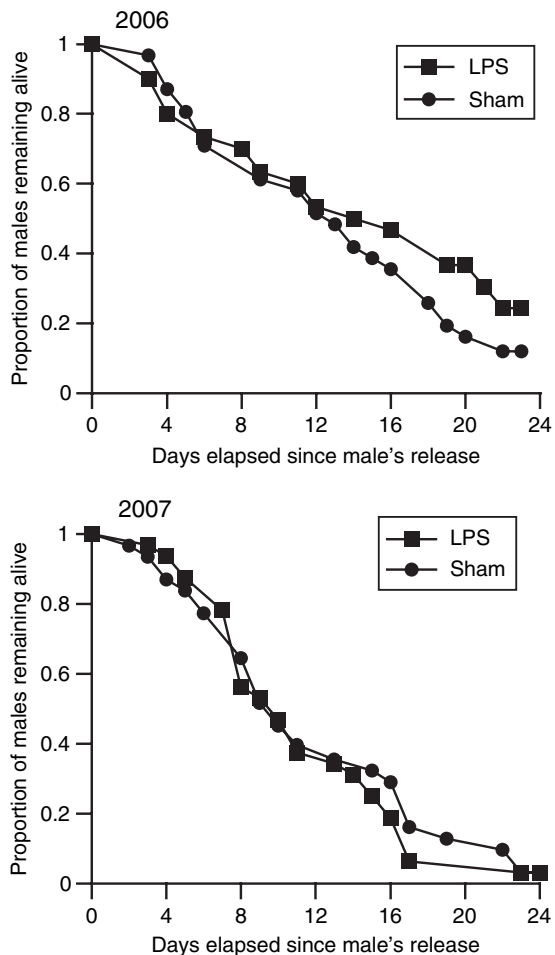


**Fig. 2** The proportion of male sagebrush crickets remaining unmated as a function of time elapsed since their initial release. Sham-injected males mated significantly sooner than lipopolysaccharides-injected males in both years (Wald  $\chi^2 = 4.39$ ,  $P = 0.036$ ).

recording (first or second) as the repeated factor. The data were arc-sin transformed to meet the assumption of normality. Control males spent significantly more time calling than LPS-injected males (ANOVA,  $F_{1,39} = 5.67$ ,  $P = 0.02$ ). There was no significant difference in calling time across nights ( $F_{1,39} = 1.99$ ,  $P = 0.17$ ), nor was there a significant time  $\times$  treatment interaction ( $F_{1,39} = 0.32$ ,  $P = 0.57$ , see Fig. 4).

### Experiment 3: the effect of wing wounding on encapsulation and PO activity

There was no significant difference in the mean encapsulation response of virgin, experimentally wounded virgin, and nonvirgin males (ANOVA:  $F_{2,140} = 0.01$ ,  $P = 0.99$ ). The mean encapsulation value was  $79.9 \pm 2.2$  ( $\pm$  SE) for virgin males (range = 29.2–121.0),  $79.4 \pm 2.9$  for experimentally wounded virgin males

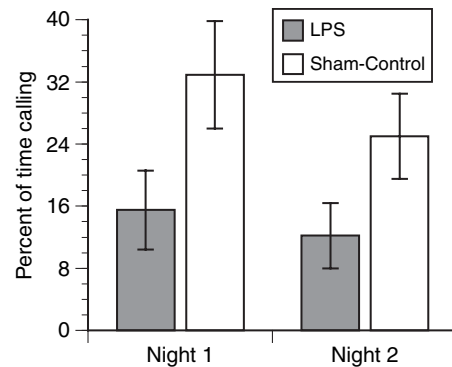


**Fig. 3** The proportion of male sagebrush crickets remaining alive as a function of time elapsed since their initial release. There was no effect of treatment on male survival (Wald  $\chi^2 = 0.15$ ,  $P = 0.70$ ), but there was an effect of year (Wald  $\chi^2 = 6.44$ ,  $P = 0.01$ ).

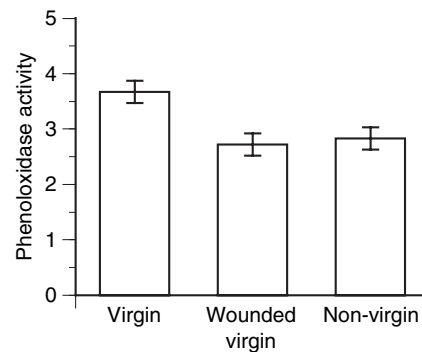
(range = 32.4–111.8) and  $79.5 \pm 2.9$  for nonvirgin males (range = 39.7–117.4). There was a significant difference in PO activity of virgin, experimentally wounded virgin and nonvirgin males (ANOVA:  $F_{2,146} = 8.37$ ,  $P < 0.001$ ). Virgin males had significantly higher PO activity than both experimentally wounded virgin males and nonvirgin males (Ryan–Einot–Gabriel–Welsch multiple range test,  $P < 0.05$ ), but there was no difference in the PO activity of experimentally wounded virgin males and nonvirgin males ( $P > 0.05$ , Fig. 5).

## Discussion

In this study, we investigated how the immune responses resulting from mating may constrain male mating success and thereby explain the well-documented virgin-male mating advantage in sagebrush crickets (Morris *et al.*, 1989; Snedden, 1996). In support of the hypothesis, we



**Fig. 4** The mean ( $\pm$  SE) proportion of time spent calling by male sagebrush crickets over two consecutive nights as measured by the number of 5-min intervals in which calling activity was recorded.



**Fig. 5** Mean ( $\pm$  SE) phenoloxidase activity (change in OD units  $\times 10^{-4}$ ) of male sagebrush crickets.

found that immunochallenged virgin males spent significantly less time engaged in calling behaviour compared to control males, and took significantly longer to secure a mating than control males. We also found that virgin male sagebrush crickets had significantly higher PO activity than either experimentally wounded virgin males or naturally wounded, nonvirgin males.

Calling appears to be essential to pair formation and hence mating in sagebrush crickets (Snedden & Sakaluk, 1992). Studies of other crickets have also shown that an immunochallenge with LPS results in reduced signalling behaviour of males (Rantala & Kortet, 2003; Jacot *et al.*, 2004; Fedorka & Mousseau, 2007). The production of cricket acoustical signals is energetically costly (Prestwich & Walker, 1981; Hoback & Wagner, 1997), such that any diversion of energy to mounting an immune response likely comes at the expense of energy devoted to calling (Jacot *et al.*, 2004).

The cost of mounting an immune response has been shown in other cricket species to last for most of an individual's adult lifetime (Jacot *et al.*, 2005; Fedorka & Mousseau, 2007). Our study is among the first to show

not only a cost to sexual signalling of mounting an immune response, but also the corollary costs to reproduction of free-living males studied under natural conditions (see also Jacot *et al.*, 2004). Unlike some other studies (e.g. Moret & Schmid-Hempel, 2000; Armitage *et al.*, 2003; Jacot *et al.*, 2004), however, we found no evidence of reduced survival of immune-challenged males. It may be that male *C. strepitans* are able to secure sufficient resources after mating to negate any potential longevity cost or, alternatively, the short lifespan of this species may make it particularly difficult to detect any such costs.

The results of our PO assay demonstrate that an immune response in males occurs as a result of wing wounding by females. Our assay measured both pro-PO and active PO, and thus represents the total PO present (Adamo, 2004a; Bailey & Zuk, 2008). Prophenoloxidase gene expression is not up-regulated during an immune challenge (Ahmed *et al.*, 1999), so less PO activity should indicate that an immune response has occurred. Virgin males had higher PO activity than either experimentally wounded virgin males or naturally wounded, nonvirgin males, presumably because the latter two groups had experienced some depletion of their PO in responding to the immune challenge posed by wing wounding. In *Drosophila*, an increase in sexual activity has been linked to immunosuppression in males, as evidenced by their ability to clear a bacterial infection (McKean & Nunney, 2001). In our study, however, experimentally wounded virgin males and old-wound nonvirgin males exhibited roughly the same level of PO activity, suggesting that it is wing wounding *per se*, and not other aspects of mating (e.g. genitalic contact, spermatophore transfer), that leads to diminished PO activity.

The encapsulation response occurs because of melanin formation in the pro-PO to PO enzymatic cascade (Rantala & Kortet, 2003; Cerenius & Söderhäll, 2004) and is a reliable way to measure realized immunity (Gillepsie *et al.*, 1997; Fedorka & Zuk, 2005). In this study, the ability to encapsulate a foreign body was unaffected by male reproductive experience. Although PO activity can contribute to the encapsulation response, the two measures of immunity may (Rantala & Roff, 2007) or may not be correlated with one another because of interactions or intervening factors (Fedorka & Zuk, 2005; Leclerc *et al.*, 2006; Gershman, 2008). For example, Rantala *et al.* (2003) found that nutritional stress leads to a decrease in PO activity of grain beetles, *Tenebrio molitor*, but no corresponding effect on the encapsulation response. Although they suggested that the lack of an effect could be due to a time-lag in the effect of stress on encapsulation, this is unlikely to be the case for *C. strepitans* because many of our nonvirgin males would have mated long before the encapsulation assay was performed and presumably had sufficient time for any detrimental effects of wing wounding to accrue. It should also be acknowledged that encapsulation and other

measures of cricket immunity can only provide evidence of an immune response, not overall immunocompetence (Adamo, 2004b). Immunity is undoubtedly more complex than a single trait, and individual aspects of immunity may be traded off against each other as part of an optimal allocation strategy (Zuk & Stoehr, 2002; Rolff & Sivy-Jothy, 2003; Rantala & Roff, 2005; Lawniczak *et al.*, 2006).

The virgin-male mating advantage in sagebrush crickets appears to reduce the opportunity for sexual selection in males, compared to other species in which mating is not so costly (Snedden, 1996). If the immune responses elicited by wing wounding constrain a male's ability to acquire future mates, it seems possible that the trade-off between reproduction and immunity could increase the operational sex ratio and promote a sex-role reversal in which males become more selective of prospective mating partners (e.g. Gwynne, 1981; Gwynne & Simmons, 1990). However, male *C. strepitans* do not appear to be at all choosy, as males court virtually all the females they contact, at least in encounters staged in the laboratory, and we have never seen males pull away from a mounted female before the spermatophore has been transferred. In contrast, we have frequently witnessed females actively pulling away from males that they have mounted (Sakaluk *et al.*, 1995). Thus, whatever the extent to which immune responses constrain male mating success, a paucity of receptive females seems to mitigate against any overt choosiness on the part of males. Indeed, a significant proportion of males often remain unmated by the end of the mating season (Sakaluk & Snedden, 1990; Snedden, 1996), which might maintain the intensity of sexual selection despite the high costs of mating.

Understanding the trade-off between immunity and reproduction is essential to ascertaining how organisms optimally allocate resources to competing aspects of life history in a way that maximizes fitness (Lochmiller & Deerenberg, 2000; Viney *et al.*, 2005). Our data support the hypothesis that wing wounding during copulation leads to an energetically costly immune response, such that mated males are less able to sustain the acoustical signalling needed to attract additional mates. This suggests that the investment in an immune response is a cost of reproduction that directly constrains the future mating success of male sagebrush crickets. It must be acknowledged, however, that the cost of an immune response is not the only cost accruing to wing wounding: previous work suggests that the energetic cost of replenishing lost haemolymph may also impose a constraint on mating (Sakaluk *et al.*, 2004).

Although a number of studies have shown trade-offs between investment in immune responses and investment in secondary sexual characteristics (Sheldon & Verhulst, 1996; Faivre *et al.*, 2003; Jacot *et al.*, 2004), few have shown that mating itself can lead to immune responses that compromise future mating success.

Indeed, a recent study of parasite resistance in female field crickets revealed that mating actually enhances females' resistance to infection (Shoemaker et al., 2006). This study provides evidence that an immune response associated with wing wounding constitutes a cost of reproduction (Harshman & Zera, 2007) and reduces the competitiveness of previously mated males relative to virgin male sagebrush crickets.

## Acknowledgments

This research was conducted in Grand Teton National Park under the auspices of Scientific Research and Collecting Permits (GRTE-2006-SCI-0030 and GRTE-2007-SCI-0033) issued by the National Park Service. We thank Shelly Adamo for advice on immune measurements, Steve Juliano for statistical advice, and the members of JCL's M.S. thesis advisory committee, Rachel Bowden and Chris Horvath, for helpful suggestions. We thank the University of Wyoming and the National Park Service field station for providing bench space and other assistance. This research was funded by grants from the National Science Foundation to SKS and C. G. Hamaker (IOS-0543254 and IOS-0718140), and grants from the Beta Lambda chapter of the Phi Sigma Biological Sciences Honor Society to JCL. Additional support to JCL was provided by an NSF GK-12 award to C. J. Moore. JMS was supported by an NSF Research Experiences for Undergraduates supplemental award to SKS. SNG was partially supported by a postdoctoral fellowship from the Program of Excellence in Neuroscience and Behavior at Illinois State University.

## References

- Adamo, S.A. 2004a. Estimating disease resistance in insects: phenoloxidase and lysozyme-like activity and disease resistance in the cricket *Gryllus texensis*. *J. Insect Physiol.* **50**: 209–216.
- Adamo, S.A. 2004b. How should behavioral ecologists interpret measures of immunity? *Anim. Behav.* **68**: 1443–1449.
- Adamo, S.A., Jensen, M. & Younger, M. 2001. Changes in lifetime immunocompetence in male and female *Gryllus texensis* (formerly *G. integer*): trade-offs between immunity and reproduction. *Anim. Behav.* **62**: 417–425.
- Ahmed, A., Martin, D., Mannetti, A.G.O., Han, S.-J., Lee, W.-J., Mathiopoulou, K.D., Müller, H.-M., Kafatos, F.C., Raikhel, A. & Brey, P.T. 1999. Genomic structure and ecdysone regulation of the prophenoloxidase 1 gene in the malaria vector *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA.* **96**: 14795–14800.
- Allison, D. 1995. *Survival Analysis Using the SAS System: A Practical Guide*. SAS Institute, Cary, NC.
- Altmann, J. 1974. Observational study of behavior: sampling methods. *Behaviour* **49**: 227–267.
- Armitage, S.A.O., Thompson, J.J.W., Rolf, J. & Siva-Jothy, M.T. 2003. Examining costs of induced and constitutive immune investment in *Tenebrio molitor*. *J. Evol. Biol.* **16**: 1038–1044.
- Bailey, N.W. & Zuk, M. 2008. Changes in immune effort of male field crickets infested with mobile parasitoid larvae. *J. Insect Physiol.* **54**: 96–104.
- Bucher, G.E. 1959. Bacteria of grasshoppers of western Canada: III. Frequency of occurrence, pathogenicity. *J. Insect Pathol.* **1**: 391–405.
- Cerenius, L. & Söderhäll, K. 2004. The prophenoloxidase-activating system in invertebrates. *Immunol. Rev.* **198**: 116–126.
- Eggert, A.-K. & Sakaluk, S.K. 1994. Sexual cannibalism and its relation to male mating success in sagebrush crickets, *Cyphoderris strepitans* (Orthoptera: Haglidae). *Anim. Behav.* **47**: 1171–1177.
- Faivre, B., Grégoire, A., Prévault, M., Cézilly, F. & Sorci, G. 2003. Immune activation rapidly mirrored in a secondary sexual trait. *Science* **300**: 103.
- Fedorka, K.M. & Mousseau, T.A. 2007. Immune system activation affects male sexual signal and reproductive potential in crickets. *Behav. Ecol.* **18**: 231–235.
- Fedorka, K.M. & Zuk, M. 2005. Sexual conflict and female immune suppression in the cricket, *Allonemobious socius*. *J. Evol. Biol.* **18**: 1515–1522.
- Gershman, S.N. 2008. Sex-specific differences in immunological costs of multiple mating in *Gryllus vocalis* field crickets. *Behav. Ecol.* **19**: 810–815.
- Gillespie, J.P., Kanost, M.R. & Tresczek, T. 1997. Biological mediators of insect immunity. *Ann. Rev. Entomol.* **42**: 611–643.
- Gwynne, D.T. 1981. Sexual difference theory: Mormon crickets show role reversal in mate choice. *Science* **213**: 779–780.
- Gwynne, D.T. & Simmons, L.W. 1990. Experimental reversal of courtship roles in an insect. *Nature* **346**: 172–174.
- Harshman, L.G. & Zera, A.G. 2007. The cost of reproduction: the devil in the details. *Trends Ecol. Evol.* **22**: 80–86.
- Hoback, W.W. & Wagner, W.E. 1997. The energetic cost of calling in the variable field cricket, *Gryllus lineaticeps*. *Physiol. Entomol.* **22**: 286–290.
- Jacot, A., Scheuber, H. & Brinkhof, M.W.G. 2004. Costs of an induced immune response on sexual display and longevity in field crickets. *Evolution* **58**: 2280–2286.
- Jacot, A., Scheuber, H., Kurtz, J. & Brinkhof, M.W.G. 2005. Juvenile immune system activation induces a costly up-regulation of immunity in adult field crickets *Gryllus campestris*. *Proc. R. Soc. Lond. B* **272**: 63–69.
- Kumala, M., McLennan, D.A., Brooks, D.R. & Mason, A.C. 2005. Phylogenetic relationships within hump-winged grigs, *Cyphoderris* (Insecta, Orthoptera, Tettigoniodea, Haglidae). *Can. J. Zool.* **83**: 1003–1011.
- Lawniczak, M.K.N., Barnes, A.I., Linklater, J.R., Boone, J.M., Wigby, S. & Chapman, T. 2006. Mating and immunity in invertebrates. *Trends Ecol. Evol.* **22**: 48–55.
- Leclerc, V., Pelte, N., El Chamy, L., Martinelli, C., Ligoxygakis, P., Hoffmann, J.A. & Reichhart, J.-M. 2006. Prophenoloxidase activation is not required for survival to microbial infections in *Drosophila*. *EMBO Reports* **7**: 231–235.
- Lochmiller, R.L. & Deerenberg, C. 2000. Trade-offs in evolutionary immunology: just what is the cost of immunity? *Oikos* **88**: 87–98.
- McKean, K.A. & Nunney, L. 2001. Increased sexual activity reduces male immune function in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA.* **98**: 7904–7909.
- Moret, Y. & Schmid-Hempel, P. 2000. Survival for immunity: the price of immune system activation for bumblebee workers. *Science* **290**: 1166–1168.
- Morris, G.K. & Gwynne, D.T. 1978. Geographical distribution and biological observations of *Cyphoderris* (Orthoptera: Haglidae) with a description of a new species. *Psyche* **85**: 147–167.

- Morris, G.K., Gwynne, D.T., Klimas, D.E. & Sakaluk, S.K. 1989. Virgin male mating advantage in a primitive acoustic insect (Orthoptera: Haglidae). *J. Insect Behav.* **2**: 173–185.
- Prestwich, K.N. & Walker, T.J. 1981. Energetics in singing crickets: effect of temperature in three trilling species (Orthoptera: Gryllidae). *J. Comp. Physiol. B.* **143**: 199–212.
- Rantala, M.J. & Kortet, R. 2003. Courtship song and immune function in the field cricket *Gryllus bimaculatus*. *Biol. J. Linn. Soc.* **79**: 503–510.
- Rantala, M.J. & Roff, D.A. 2005. An analysis of trade-offs in immune function, body size and development time in the Mediterranean field cricket, *Gryllus bimaculatus*. *Funct. Ecol.* **19**: 323–330.
- Rantala, M.J. & Roff, D.A. 2007. Inbreeding and extreme outbreeding cause sex differences in immune defence and life history traits in *Epirrita autumnata*. *Heredity* **98**: 329–336.
- Rantala, M.J., Kortet, R., Kotiaho, J.S., Vainikka, A. & Suhonen, J. 2003. Condition dependence of pheromones and immune function in the grain beetle *Tenebrio molitor*. *Funct. Ecol.* **17**: 534–540.
- Roff, D.A. 1992. *The Evolution of Life Histories: Theory and Analysis*. Chapman & Hall, New York, NY.
- Rolff, J. & Siva-Jothy, M.T. 2003. Invertebrate ecological immunology. *Science* **301**: 472–475.
- Ryder, J. & Siva-Jothy, M.T. 2000. Male calling song provides reliable information about immune function in a cricket. *Proc. R. Soc. Lond. B* **267**: 1171–1175.
- Sakaluk, S.K. & Ivy, T.M. 1999. Virgin-male mating advantage in sagebrush crickets: differential male competitiveness or non-independent female mate choice? *Behaviour* **136**: 1335–1346.
- Sakaluk, S.K. & Snedden, W.A. 1990. Nightly calling durations of male sagebrush crickets, *Cyphoderris strepitans*: size, mating and seasonal effects. *Oikos* **57**: 153–160.
- Sakaluk, S.K., Morris, G.K. & Snedden, W.A. 1987. Mating and its effect on acoustic signalling behavior in a primitive orthopteran, *Cyphoderris strepitans* (Haglidae): the cost of feeding females. *Behav. Ecol. Sociobiol.* **21**: 173–178.
- Sakaluk, S.K., Bangert, P.J., Eggert, A.-K., Gack, C. & Swanson, L.V. 1995. The gin trap as a device facilitating coercive mating in sagebrush crickets. *Proc. R. Soc. Lond. B* **261**: 65–71.
- Sakaluk, S.K., Campbell, M.T.H., Claek, A.P., Johnson, J.C. & Keorpes, P.A. 2004. Hemolymph loss during nuptial feeding constrains male mating success in sagebrush crickets. *Behav. Ecol.* **15**: 845–849.
- SAS Institute Inc. 2004. *SAS OnlineDoc® 9.1.3*. SAS Institute Inc., Cary, NC.
- Schmid-Hempel, P. 2005. Evolutionary ecology of insect immune defenses. *Ann. Rev. Entomol.* **50**: 529–551.
- Sheldon, B.C. & Verhulst, S. 1996. Ecological immunology: costly parasite defences and trade-offs in evolutionary ecology. *Trends Ecol. Evol.* **11**: 317–321.
- Shoemaker, K.L., Parsons, N.M. & Adamo, S.A. 2006. Mating enhances parasite resistance in the cricket *Gryllus texensis*. *Anim. Behav.* **71**: 371–380.
- Simmons, L.W. & Roberts, B. 2005. Bacterial immunity traded for sperm viability in male crickets. *Science* **309**: 2031.
- Siva-Jothy, M.T. 1999. Male pigmentation may affect reproductive success via female choice in a calopterygid damselfly (Zygoptera). *Behaviour* **136**: 1365–1377.
- Snedden, W.A. 1996. Lifetime mating success in male sagebrush crickets: sexual selection constrained by a virgin male mating advantage. *Anim. Behav.* **51**: 1119–1125.
- Snedden, W.A. & Irazuzta, S. 1994. Attraction of female sagebrush crickets to male song: the importance of field bioassays. *J. Insect Behav.* **7**: 233–236.
- Snedden, W.A. & Sakaluk, S.K. 1992. Acoustical signalling and its relation to male mating success in sagebrush crickets. *Anim. Behav.* **44**: 633–639.
- Söderhäll, K. & Cerenius, L. 1998. Role of the prophenoloxidase-activating system in invertebrate immunity. *Curr. Opin. Immunol.* **10**: 23–28.
- Stearns, S.C. 1989. Tradeoffs in life history evolution. *Func. Ecol.* **3**: 259–268.
- Viney, M.E., Riley, E.M. & Buchanan, K.L. 2005. Optimal immune responses: immunocompetence revisited. *Trends Ecol. Evol.* **20**: 665–669.
- Zuk, M. & McKean, K.A. 1996. Sex differences in parasite infections: patterns and processes. *Int. J. Parasitol.* **26**: 1009–1024.
- Zuk, M. & Stoehr, A.M. 2002. Immune defense and host life history. *Am. Nat.* **160**: S9–S22.

Received 21 August 2008; revised 27 September 2008; accepted 29 September 2008