## Lovesick: Immunological Costs of Mating of Male Sagebrush Crickets

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### ♦ ABSTRACT

A growing body of evidence suggests that resources invested in sexual signals and other reproductive traits often come at the expense of the ability to mount an immune response. Male sagebrush crickets, Cyphoderris strepitans, offer an unusual nuptial food gift to females during mating: females chew on the tips of males' fleshy hind wings and ingest hemolymph seeping from the wounds they inflict. Previous research has shown that once a male has mated, his probability of obtaining an additional copulation is reduced relative to that of a virgin male seeking his first mating. 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 signaling needed to attract additional females. To test this hypothesis, we injected virgin males with lipopolysaccharides (LPS), a non-living component of bacterial cell walls that leads to upregulation of the insect immune system. Males were released in the field and recaptured over the course of the breeding season to monitor their mating success. Over two breeding seasons, LPS-injected males took significantly longer to secure matings than sham-injected virgin males. An encapsulation rate assay showed no difference in the encapsulation response of males of different mating status, but virgin males had significantly higher levels of phenoloxidase than non-virgin males. These results suggest that males trade off investment in reproduction and investment in immunity.

### INTRODUCTION

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). C. strepitans occurs exclusively in high-altitude sagebrush meadows in mountainous areas of Colorado and Wyoming (Morris and Gwynne 1978). Mating occurs in the late spring after the snow melts. Males climb into sagebrush or lodgepole pine shortly after sunset to secure a perch, from where they emit acoustical signals that function to attract females (Snedden and Irazuzta 1994) and appear to be the primary means of pair formation (Snedden and 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 hemolymph 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 and 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 hemolymph, they must also produce another spermatophore if they are to mate again. Previous work has shown that non-virgin male calling time is reduced relative to virgin males (Sakaluk et al. 1987; Sakaluk and Snedden 1990). Given the importance of calling in pair formation and the marked decrease in calling time of non-virgin 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 non-virgin 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. 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 non-virgin males. If the immune response resulting from female wing feeding during copulation is responsible for the decline in non-virgin male mating success, then virgin males subjected to a similar immunological challenge should exhibit a similar decline in the incidence of matings.

### ✦ 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 two seasons at two different locations in Grand Teton National Park, Wyoming: 1) Deadman's Bar, a 4.1-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 hectares) 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 May 21 to May 24 in 2006, and May 20 to May 27 in 2007. Males initiate pair formation by acoustically signaling to females from perches in sagebrush (Snedden and Irazuzta 1994, Snedden and Sakaluk 1992), and we located males by orienting to their calls. The mating status of males was determined by examining the hind wings for evidence of wing wounding by females. Only virgin males, as evidenced by their intact hind wings, were used in the experiment. A numbered flag marked the location of each virgin male collected, so that experimental males could be returned back 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.

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 are derived from the cell wall of Serratia marcesens, a gram-negative bacterium that is a common insect pathogen (Adamo et al. 2001, Bucher 1959). 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 µg of bacterial lipopolysaccharides (LPS, Sigma, L6136 Sigma-Aldrich Inc., St. Louis, USA) dissolved in 10µl of Grace's insect medium (Sigma, G8142), thus presenting males with an immunological challenge. Sham control males were injected with 10 µl of Grace's insect medium only (N = 43 in 2006, N = 46in 2007). Injections were given approximately 12 hours after males were captured, administered between the 2nd and 3rd abdominal sternites with a 10-µl Hamilton syringe (#8003, Hamilton Co., Reno, Nevada, 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, Illinois, 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 hours 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 Shamcontrol).

At intervals of 2 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 capture) or "old" (dry, darkened wounds indicating that the male had mated at least one night previous to the night of capture).

## *Experiment 2: 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 old-wound non-virgin males (N = 48). Virgin and non-virgin 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 wing from virgin males with micro-scissors on the evening they were captured. This procedure created wounds resembling those of males that have recently mated with a female.

For all males, we assayed two parameters of immune function widely used in studies of other insects: 1) encapsulation and 2) phenoloxidase 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 leads to the asphyxiation or death of the pathogen through the production of cytotoxic substances (Cerenius and Söderhäll 2004). The magnitude of the encapsulation response can be measured by experimentally implanting an insect with an inert object (Ryder and Siva-Jothy 2000). We examined the encapsulation response of male sagebrush crickets by implanting them with 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 2<sup>nd</sup> and 3<sup>rd</sup> abdominal sclerites in a small puncture made with a sterilized sewing 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 hours before being removed and then photographed on white background with a digital camera (Nikon, Melville, New York, USA) through the ocular of a stereomicroscope (Wild, Heerbrugg Co, 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 freely available from (Image J) the NIH (http://rsb.info.nih.gov/ij/download.html). This program compares the number of black and white pixels in any section of the image to produce a grayscale value. The portion of the image containing the implant was evaluated by the program to obtain the grayscale 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 grayscale

values of the implant and control filament.

Phenoloxidase (PO) is a key enzyme in the biochemical cascade leading to the production of melanin, which is the key component in the encapsulation response (Söderhäll and Cerenius 1998). We measured phenoloxidase activity using methods adopted from Rantala and Kortet (2003), Fedorka and Zuk (2005) and Bailey and Zuk (2008). Immediately after removing the nylon filament, we drew 6 µl of hemolymph from the site of filament removal, added it to 60 µl of phosphate buffered saline, and then froze the samples for several weeks at -25°C to disrupt hemocyte membranes. Five ul of the PBS/hemolymph solution were then added to 7  $\mu$ l of bovine pancreas  $\alpha$ chymotrypsin (1.3 mg/mL, Sigma C7762) and allowed to react at room temperature for 20 minutes. Alphachymotrypsin converts all of the pro-PO enzyme present in the hemolymph to PO (Bailey and Zuk 2008, Cerenius and Söderhäll 2004). We measured the resulting PO activity by adding 90 ul of a 15 millimolar 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, BIO-Tek) to record the change in optical density (OD) at 490 nm for 130 minutes 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 for each individual to obtain a final PO level.

#### ✦ RESULTS

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

Time to mating was determined as the number of nights from the time a male was first released until he was captured as a non-virgin. Non-virgin males bearing fresh wing wounds were assumed to have mated on the night they were captured. Non-virgin males bearing old wing wounds were assumed to have mated at least one night previous to their capture or, if they had not been captured in the previous census, we recorded the night of mating as the mid-point of 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 capture 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 = 0.0362), but no effect of year (Wald  $\chi^2 = 0.44$ , p=0.505) on time to mating (see Figure 1). Sham-injected males mated sooner than LPS injected virgin males, and were more than twice as likely to secure a mating as were LPS males (Hazard Ratio = 2.129).



**Days Elapsed Since Male's Release** 

Figure 1. Leman et al. The proportion of male sagebrush crickets remaining unmated as a function of time elapsed since their initial release. LPS-males took significantly longer to secure a mating than sham-control males over both years of the study (Wald  $\chi^2 = 4.39$ , P = 0.0362).

*Experiment 2: 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 old-wound males (ANOVA:  $F_{2,140} = 0.0104$ , p = 0.99). The mean encapsulation value was  $79.9 \pm 2.2$  ( $\pm$  SE) for virgin males,  $79.4 \pm 2.9$  for experimentally wounded virgin males, and  $79.5 \pm 2.9$  for old-wound males.

There was a significant difference in PO activity of virgin, experimentally wounded virgin, and old-wound non-virgin males (ANOVA:  $F_{2, 146}$  = 8.37, p = 0.0004). Virgin males had significantly higher PO

activity than both experimentally wounded virgin males and old-wound 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 old-wound non-virgin males (p > 0.05, Figure 2).



Figure 2. Mean (+SE) phenoloxidase activity (change in OD units x 10-4) of male sagebrush crickets. Figure 2. Leman et al.

### DISCUSSION

Immunochallenged virgin male sagebrush crickets took significantly longer to secure a mating than control males. Virgin males had significantly higher PO activity than either experimentally wounded virgin males or naturally wounded, non-virgin males. 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 signaling needed to attract additional mates. This suggests that the investment in an immune response imposes a cost to reproduction that directly constrains the future mating success of male sagebrush crickets.

The results of our PO assay demonstrate that an immune response in males occurs as a result of wingwounding by females. Our assay measured both pro-PO and active PO, and thus represents the total PO present Zuk (Adamo 2004, Bailey and 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, non-virgin males,

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presumably because the latter two groups had experienced some depletion of their PO in responding to the immune challenge posed by wing wounding.

The encapsulation response occurs because of melanin formation in the pro-PO to PO enzymatic cascade (Cerenius and Söderhäll 2004, Rantala and Kortet 2003) and is a reliable way to measure realized immunity (Gillepsie et al. 1997, Fedorka and Zuk 2005). However, PO activity is not correlated with encapsulation ability (Fedorka and Zuk 2005, Gershman 2008). Contrary to our expectation, there was no difference between virgin males, experimentally wounded virgin males, and naturally wounded, nonvirgin males in their ability to encapsulate a filament.

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 tradeoff 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 and 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 (pers. obs.). 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.

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