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Experimentally induced spermatophore production and immune responses reveal a trade-off in crickets

Angela M. Kerr, Susan N. Gershman, and Scott K. Sakaluk
Behavior, Ecology, Evolution and Systematics Section, School of Biological Sciences, Illinois State University, 315 West College Avenue, Normal, IL 61790-4120, USA

The energetic demands of the immune system and reproduction are often high and can lead to trade-offs between these 2 life-history traits. In decorated crickets, *Gryllodes sigillatus*, much of a male's reproductive effort is devoted to calling, and to the synthesis of a spermatophylax, a large, gelatinous, non–sperm-containing mass forming part of the spermatophore and consumed by the female after mating. We employed a reciprocal design in which we experimentally induced an immune response in some males by injecting them with lipopolysaccharides and experimentally induced increased spermatophore production in others to determine if male *G. sigillatus* trade off immunity and reproduction. Immune-challenged males produced significantly smaller spermatophores, although they did not call less than controls. We also found that males that synthesized more spermatophores exhibited lower immunity. These results reveal a fundamental trade-off between immunity and reproductive effort in male *G. sigillatus*, specifically with respect to a male's ability to synthesize a costly nuptial food gift. However, we found no correlation between the mass of a male's gift and his ability to survive an acute bacterial infection; this result, coupled with the trade-off identified above, raises the possibility that the spermatophylax serves as a Zahavian handicap. *Key words:* crickets, *Gryllodes sigillatus,* immunity, life-history trade-offs, nuptial food gifts, spermatophore. *[Behav Ecol 21:647–654 (2010)]*

An individual's lifetime fitness is maximized when it optimally allocates resources among its various life-history traits. However, when resources are limited, an individual may need to trade off the costs of one trait with those of another (Roff 1992). Trade-offs occurring between immunity (a vital aspect of bodily maintenance) and other life-history traits, such as growth or reproduction, are of relatively recent interest to behavioral ecologists (Sheldon and Verhulst 1996; Lochmiller and Deerenberg 2000; Zuk and Stoehr 2002; Rantala and Roff 2005). An effective immune system is costly both in terms of its maintenance and in its activation against parasites and pathogens (Moret and Schmid-Hempel 2000; Rolff and Siva-Jothy 2003).

Evidence of a life-history trade-off between reproduction and immunity comes largely from studies seeking evidence of "good genes" sexual selection, particularly in birds (Sheldon and Verhulst 1996). The basic idea is that, assuming investment in immunity is sufficiently costly, only those males in especially good condition can invest in the elaborate ornamentation attractive to females, while maintaining a viable immune system. The coevolutionary arms race between parasites and hosts is believed to maintain the genetic variation in immunity needed to provide indirect genetic benefits to female mate choice (Hamilton and Zuk 1982).

In vertebrates, the immune system is composed of both innate and adaptive immunity. The innate immune system involves various cell types and substances that respond rapidly and nonspecifically against an array of parasites. The adaptive immune system involves the production of antibodies specific to components of invading cells and provides individuals with the capability of immunological memory (Martin et al. 2008). Invertebrates such as insects possess only innate immunity and lack the antibodies that confer the acquired immunity of vertebrates. Innate immunity in insects involves both a cellular response, which results in the encapsulation of macroparasites through the deposition of melanin and accumulation of hemocytes, and a humoral response, which involves the activation of antimicrobial peptides in the hemolymph (Gillespie et al. 1997; Söderhäll and Cerenius 1998; Rolff and Siva-Jothy 2003; Lawniczak et al. 2006).

The decorated cricket (*Gryllodes sigillatus*) is an ideal organism with which to study trade-offs between reproduction and immunity. Male *G. sigillatus* synthesize nuptial gifts and produce acoustic signals, both of which are crucial to their reproductive success and which entail significant costs. Nuptial food gifts are a common device by which males of various insect species seek to increase their fertilization success. These are gifts of food that males offer to females before or during mating and consist of prey items that males have captured, glandular secretions, or even parts of the male's soma (Vahed 1998, 2007). In *G. sigillatus*, as in many other species of ensiferan Orthoptera, such gifts come in the form of a spermatophylax, a large, gelatinous, non–sperm-containing component of the 2-part spermatophore transferred by the male at mating and consumed by the female after copulation (Vahed 1998). The entire spermatophore, composed of both the spermatophylax and a sperm-containing ampulla, remains secured to the female's genital opening outside her body after mating. Immediately after copulation, the female detaches the spermatophylax from the ampulla with her mouthparts and begins to consume it, while the ejaculate contained within the ampulla is evacuated into her reproductive tract (Sakaluk 1984). The spermatophylax functions to deter the female from removing the ampulla before sperm transfer is complete, which takes about 50 min (Sakaluk 1984). In addition, the spermatophylax provides the female with important

Address correspondence to Scott K. Sakaluk. E-mail: sksakal@ilstu.edu.

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hydration benefits (Ivy et al. 1999), although it is generally lacking in nutritional value (Will and Sakaluk 1994; Ivy and Sakaluk 2005). Females remove and eat the ampulla soon after consuming the spermatophylax, and because larger spermatophylaxes take longer to consume, males that provide larger gifts are rewarded with increased sperm transfer (Sakaluk 1985). Thus, a male that synthesizes a smaller spermatophylax incurs a fitness cost because sperm transfer will be prematurely terminated by the female (Sakaluk 1985). The synthesis of the spermatophore imposes an additional reproductive cost on males: the spermatophore represents up to 6% of a male's total body mass, and males require a refractory period of more than 3 h to manufacture a new spermatophore after a previous mating. The cost incurred by males is the number of mating opportunities lost during this time (Sakaluk 1984, 1985; Vahed 1998).

Male *G. sigillatus* also produce a long-range acoustic signal to attract sexually receptive females. In the field, males begin calling shortly after sunset and continue throughout the night (Sakaluk 1987). Time spent calling is a determinant of the mating success of male crickets (Simmons 1986; Cade WH and Cade ES 1992) but represents a significant investment of energy (Prestwich and Walker 1981; Hoback and Wagner 1997; Hack 1998; Wagner and Hoback 1999). Previous studies have demonstrated that experimental immune challenges negatively affect male calling activity (Jacot et al. 2004; Leman et al. 2009) and song characteristics (Fedorka and Mousseau 2007).

In this study, we employed a reciprocal experimental approach to determine if male *G. sigillatus* trade off immunity and reproduction, using spermatophylax mass and time spent calling as proxies for male reproductive effort. In our first set of experiments, we administered an immune challenge to assess the effect of an invoked immune response on male reproductive effort. If some males are forced to invest more in immunity than other males, then immune-challenged males should produce smaller spermatophylaxes and call less frequently compared with sham-treated males. In our second experiment, we examined the relationship between reproductive effort and the ability to survive an acute bacterial infection. If a trade-off occurs between immunity and reproduction, then males that allocate more energy toward reproduction (i.e., make larger spermatophylaxes) than other males will be less likely to survive. In our third experiment, we forced males to synthesize additional spermatophores to assess the effect of increased reproductive effort on immunity. If some males synthesize more spermatophylaxes over a given time period compared with other males, then those that increase their reproductive investment should exhibit a reduced ability to combat immune challenges, as demonstrated by immune assays.

MATERIALS AND METHODS

Cricket rearing

Experimental males were randomly selected from a laboratory colony initiated with 500 individuals collected at Las Cruces, NM in 2001. Colony crickets were housed in plastic storage containers (59 × 43× 30.5 cm), maintained at 30 °C under a 14:10 h light:dark photoperiod, and provisioned with Fluker's cricket chow ad libitum, water supplied in 40-ml plastic tissue culture flasks plugged with cotton dental rolls, egg cartons for shelter and to increase rearing surface area, and moistened peat moss as an oviposition substrate and as an additional source of water. Experimental males were housed under the same environmental conditions as the stock colony and given food and water ad libitum.

Insect immunity

A number of orthopteran species are susceptible to a wide variety of pathogens and parasites (Walker and Masaki 1989). Their innate immunity involves both humoral and cellular responses to infection (Gillespie et al. 1997), and although they appear to lack the antimicrobial peptides found in other insect orders (Hoffmann et al. 1996), they are able to phagocytize or nodulate small microorganisms, encapsulate larger parasites or foreign objects, and invoke proteolytic cascades that lead to hemolymph clotting and melanization (Hoffmann et al. 1996). We examined both humoral and cellular responses in our study with methods commonly used to assess insect immunity. In our first experiment, we injected male *G. sigillatus* with lipopolysaccharides (LPS), a nonliving, nonpathogenic, molecular component of the outer membrane of the cell wall of gram-negative bacteria such as *Serratia marcescens*. When LPS is injected into the hemocoel of an insect, it stimulates several pathways of the humoral immune response without causing an infection, thereby enabling the assessment of immune system activation costs independent of infection costs (Imler et al. 2000; Ahmed et al. 2002). Although LPS is cleared from insect hemolymph within hours (Kato et al. 1994), it has been shown to cause long-term upregulation of immunity (Jacot et al. 2005; Fedorka and Mousseau 2007). In our second experiment, we infected male *G. sigillatus* with live *S. marcescens*, a gram-negative bacterium potentially lethal to, and commonly found in, wild cricket populations (Bucher 1959). This pathogen has been used to assess the immunocompetence of crickets and other insect species (Adamo et al. 2001; Inglis et al. 2003; Adamo 2004a; Lazzaro et al. 2006; Gershman 2008).

In our third experiment, we measured the degree to which males are able to melanize a foreign body (encapsulation response) and estimated the amount of 2 enzymes, phenoloxidase (PO) and lysozyme, present within their hemolymph. Insects utilize the encapsulation response to combat parasites that enter the hemocoel. This response invokes both cellular and humoral defenses that lead to the formation of a capsule consisting of layers of dead melanized hemocytes that isolate, cover, and kill invaders (Gillespie et al. 1997; Ryder and Siva-Jothy 2000). The melanization reaction, also used in wound healing (Gillespie et al. 1997), is produced by the prophenoloxidase (PPO) cascade, a nonself recognition mechanism found in invertebrates (Hoffmann et al. 1996; Gillespie et al. 1997; Söderhäll and Cerenius 1998) that can be stimulated by LPS, bacteria, and fungi (Söderhäll and Cerenius 1998). PPO is a zymogen found in insect hemolymph and is the inactive form of PO, an important active enzyme that catalyzes the oxidation of phenols to quinones, leading to melanization (Söderhäll and Cerenius 1998). Lysozyme is another enzyme that recognizes and attacks cell walls of gram-positive bacteria (Adamo 2004a; Rantala and Roff 2005) and is important in Orthoptera because they seem to lack antimicrobial peptides (Adamo 2004a). The encapsulation response and challenges with LPS and *S. marcescens* occur in vivo and represent real immune responses. PO and lysozyme assays are performed in vitro, and because they measure the amount of enzymes present, they provide information about an individual's potential immune responses.

Effect of experimental immune challenge on male reproductive effort

Experiment 1A: effect of LPS on spermatophore size

To determine if an immune challenge alters a male's subsequent reproductive effort, we injected males with LPS and determined the effect of this treatment on the mass of spermatophores synthesized by males. We collected newly

eclosed (Day 1) adult virgin males every other day, creating cohorts of a 2-day age range, and housed them individually in 710-ml plastic containers. On Day 7–8, we introduced 2 females into each male's container for a 24-h period to afford males some sexual experience prior to treatment. On Day 9–10, we weighed males to the nearest 0.1 mg and examined them for the presence of a spermatophore; males normally produce their spermatophores prior to encountering females (Loher and Dambach 1989). We removed spermatophores by gently palpating the male's abdomen, causing the spermatophore to be extruded from the male's spermatophoric pouch. We removed the spermatophore with forceps and weighed its 2 constituent components, the spermatophylax and ampulla, each to the nearest 0.001 mg using a Cahn microbalance. We randomly assigned 72 males to 1 of 2 treatments: 1) LPS-injected ($n = 36$), in which we injected 25 μg of LPS (Sigma-Aldrich, St. Louis, MO) in 5 μl Grace's insect medium (Sigma-Aldrich) into the hemocoel between the sixth and seventh sternites, using a 10-μl Hamilton syringe (Hamilton Co., Reno, NV) equipped with a 31 gauge, 15-mm needle or 2) sham-control-injected ($n = 36$), in which we injected males with 5 μl Grace's insect medium only. On Day 10–11, we removed posttreatment spermatophores and separated and weighed the components as described above. All dependent variables met assumptions of normality and homoscedasticity.

Experiment 1B: effect of LPS on calling activity

In a companion study, we used time-lapse video recording to measure the effect of an induced immune response on male calling effort. We randomly assigned 60 crickets to 1 of 2 treatments, 1) LPS-injected ($n = 30$) or 2) sham-control-injected ($n = 30$) and recorded calling activity over 4 consecutive nights, 2 pretreatment and 2 posttreatment. We collected and housed cohorts of virgin males as described above. On Day 7–8, we recorded their calling activity over the 10-h dark portion of the photoperiod by housing them individually in Plexiglas viewing chambers (10.5 × 7.5 × 3.0 cm) and using time-lapse video photography. We repeated this procedure on Day 8–9. On Day 9–10, we injected them as described above and repeated the video recording procedure that evening as well as the next (Day 10–11). The illumination provided by a 25-W red light bulb facilitated nighttime video recording. On review of the video, we determined the time spent calling by each male, measured as the number of 5-min intervals in which stridulation occurred (i.e., one-zero sampling; Altmann 1974).

Relationship between male reproductive effort and ability to survive an immune challenge

Experiment 2: experimental infection with Serratia marcescens

To determine whether a male's reproductive effort is related to his ability to combat an actual pathogen, we examined the relationship between the mass of spermatophores synthesized by males and their subsequent survival when experimentally infected with a bacterial pathogen, *S. marcescens*. We collected newly eclosed (Day 1) adult virgin males over 4 consecutive days ($n = 49$), housing them individually until experimental manipulation. To provide males with some sexual experience prior to the experiment, we confined each male with 2 females for 4 days. Twenty-four hours after we removed the females, we weighed males (Days 7–10) and collected spermatophores as described previously. We continued to collect spermatophores each day for the following 3 days, drying all spermatophylaxes and ampullae to constant weight at 50 °C for 24 h. On the day after we collected the fourth spermatophore (Days 11–14), we injected males with 5 μl of 5×10^2 cells/5 μl *S. marcescens* into the hemocoel between the sixth and seventh sternites, using a 10-μl Hamilton syringe equipped with a 31 gauge, 15-mm needle. In earlier trials, we had determined this dosage to be the LD-50 that killed approximately half the number of injected males ($n = 40$). We prepared bacterial solutions from freeze-dried *S. marcescens* (Carolina Biological Supply, Burlington, NC) that contained approximately 1×10^5 bacterial cells per μl of broth. We performed counts with an Ultraplane Improved Neubauer hemocytometer (Hausser Scientific, Pittsburgh, PA) and produced the 5×10^2 cells/5 μl solution by serially diluting 2.7 μl of bacterial broth in 997.3 μl of insect saline (121 mM sodium chloride, 4.1 mM calcium chloride, 1.37 mM dibasic potassium phosphate, 198 μM monobasic potassium phosphate, and 23.6 mM Tris–hydrochloride, pH 7.4). We inspected crickets daily for 4 days and recorded date of death for each. Nearly all the crickets that die from experimental *S. marcescens* infection do so within 4 days of injection; in a previous study, none of the experimentally infected crickets that died after this period showed any of the obvious signs of *S. marcescens* infection and presumably died from other age-related sources of mortality (Adamo et al. 2001).

Effect of experimentally induced reproductive effort on immunity

Experiment 3: encapsulation ability, PO activity, and lytic activity

To assess the effect of increased reproductive effort on immunity, we randomly assigned 87 males to 1 of 3 treatments in which we forced them to produce varying numbers of spermatophores over 5 days: 1) "5-removed"—removal of a spermatophore on each of 5 consecutive days ($n = 26$), 2) "3-removed"—removal of a spermatophore every other day (abdomen palpated on the 2 "off" days) ($n = 32$), or 3) "0-removed"—no spermatophores removed, but abdomen palpated every day ($n = 29$). Based on mating frequencies recorded under natural conditions (Sakaluk et al. 2002), an average mating frequency of one per night is likely close to the maximum that males can achieve, although they can mate at more than twice this frequency when confined with females in a laboratory setting (Burpee and Sakaluk 1993). We collected newly eclosed (Day 1) adult virgin males and housed them collectively until Day 6, after which we housed them individually until the end of the experiment. We began experimental removal of spermatophores on Day 6 and continued through Day 10. We adapted the following protocols for implantation and enzyme assays from Adamo (2004a), Shoemaker et al. (2006), Bailey and Zuk (2008), and Gershman (2008). On Day 11, we implanted each cricket with a 2-mm long, sandpaper-roughened piece of 0.254-mm diameter nylon monofilament fishing line. Using a 27-gauge needle, we made a hole between the sixth and seventh sternites and inserted the implant completely into the hemocoel. Prior to use, the needle and implants were sterilized with 70% ethanol. Forty-eight hours later, we collected hemolymph samples from experimental subjects, after which we freeze-killed and stored them in a −80 °C freezer. We mixed 3 μl of hemolymph from each cricket with 40 μl phosphate-buffered saline (PBS) and stored the samples in a −80 °C freezer for several weeks to halt enzymatic reactions and to induce cell lysis.

We dissected the implants from the frozen crickets, removing any clumps of tissue, and photographed each one using a Nikon digital camera mounted on a stereomicroscope (Wild Heerbrugg Ltd, Heerbrugg, Switzerland). We photographed each implant next to a control (nonimplanted) filament to account for variation in lighting. We measured the degree of implant melanization using ImageJ image-analysis software freely available from the National Institutes of Health

(http://rsbvweb.nih.gov). We outlined the implant using the polygon tool, which produced an average grayscale value from the pixels within the implant image. Darkness scores range from 0 (completely white) to 256 (completely black). We determined the darkness score for each implanted filament as the difference between it and the control filament.

To estimate PO activity, we added a known quantity of L-3,4-Dihydroxyphenylalanine (L-DOPA) to replace the naturally occurring substrate. Because the amount of L-DOPA was constant across samples, any resulting differences in melanin production were due to individual differences in PO activity. We added 5 µl of thawed hemolymph sample and 7 µl of bovine pancreas α-chymotrypsin (Sigma-Aldrich) to each well of a microplate and allowed them to react at room temperature for 20 min. α-Chymotrypsin acts as a catalyst and converts all PPO present in the hemolymph into PO (Bailey and Zuk 2008). We added 90 µl of a 15 mM L-DOPA solution to each well and used a spectrophotometer (Power Wave 340; BioTek, Winooski, VT) to record change in optical density (OD) at 490 nm for 210 min. We recorded individual PO activity as change in OD over time. We performed the same calculation on 9 control wells containing only PBS and L-DOPA and then subtracted the average value of control samples from individual cricket values to obtain a final PO level.

We used 3 mg of *Micrococcus lysodeikticus* (Sigma-Aldrich), a gram-positive bacterium, per liter of PBS to determine the ability of a lysozyme-like enzyme to induce bacterial cell lysis. To estimate lytic activity, we added 5 µl of thawed hemolymph sample and 90 µl of PBS/*M. lysodeikticus* solution to each well of a spectrophotometer microplate and recorded change in OD at 490 nm for 165 min. The change in OD proceeds from opaque to clear as bacterial cells are lysed. We recorded individual lytic activity as change in OD over time. We performed the same calculation on 9 control wells containing only PBS/*M. lysodeikticus* solution and then subtracted the average value of control samples from individual cricket values to obtain a final level of lytic activity. This experimental design does not allow the characterization of the specific lysozyme responsible for cell lysis (Schneider 1985); the observed lytic activity is thus attributed to a lysozyme-like enzyme. All dependent variables met assumptions of normality and homoscedasticity.

RESULTS

Effect of experimental immune challenge on male reproductive effort

Experiment 1A: effect of LPS on spermatophore size

Of the 72 crickets recruited, 23 were eliminated from further consideration (13 LPS-injected and 10 sham-control-injected) because they did not produce a posttreatment spermatophore; there was no difference between treatments in the proportion of males that failed to produce a posttreatment spermatophore (likelihood ratio $\chi^2 = 0.58$, $P = 0.45$). Of the 49 remaining crickets, 23 were LPS-injected and 26 were sham-control-injected.

We used a multivariate analysis of covariance (MANCOVA) to assess the effects of treatment and male body mass (covariate) on the mass of pre- and posttreatment spermatophylaxes and ampullae, after testing for equal slopes. The interaction was not statistically significant, indicating slopes did not differ; hence, we omitted the interaction from the final model (MANCOVA, Pillai's trace: $F_{4,42} = 1.04$, $P = 0.3956$). We interpreted MANCOVA results using standardized canonical coefficients as described by Scheiner (1993). MANCOVA revealed significant treatment and body mass effects on spermatophores (Table 1). The magnitudes of standardized canonical coefficients showed that the effect of LPS on posttreatment spermatophore size contributed the most to the significant treatment effect. LPS-injected males produced smaller posttreatment spermatophores than sham-control-injected males, with spermatophylaxes being the primary contributor (Table 1 and Figure 1). Larger males also produced significantly larger spermatophylaxes and ampullae compared with smaller males (Table 1).

Table 1
MANCOVA of the effect of LPS and body mass on the mass of the spermatophylax and ampulla produced by male decorated crickets

	df	Pillai's Trace	*P*	Standardized canonical coefficients			
				Spx pre	Amp pre	Spx post	Amp post
Treatment	4, 43	3.20	0.0219	−0.0217	−0.3469	+1.0062	+0.6562
Body mass	4, 43	2.78	0.0385	+0.0215	+0.3267	+0.4769	+0.8436

The magnitudes of standardized canonical coefficients indicate the magnitude of the contribution of that variable to the significant MANCOVA effect (Scheiner 1993). Spx, spermatophylax; amp, ampulla; pre, pretreatment; post, posttreatment; df, degrees of freedom.

Experiment 1B: effect of LPS on calling activity

We analyzed calling activity using a repeated-measures MANOVA, with treatment as the between-subjects effect and time (night of recording) as the repeated factor. There was no significant time × treatment interaction ($F_{3,56} = 1.44$, $P = 0.2413$). However, there was an unexpected preexisting difference between groups, with LPS-injected males showing higher calling activity than sham-control-injected males (ANOVA, $F_{1,58} = 4.27$, $P = 0.0433$). In addition, there was a significant difference in calling activity across nights, with calling increasing over the course of the experiment for males in both treatments ($F_{3,56} = 21.62$, $P = < 0.0001$, Figure 2).

Relationship between male reproductive effort and ability to survive an immune challenge

Experiment 2: experimental infection with S. marcescens

Over the first 4 days postinfection, 22 crickets survived and 27 died. We used logistical regression to predict the probability that a male would survive more than 4 days after bacterial

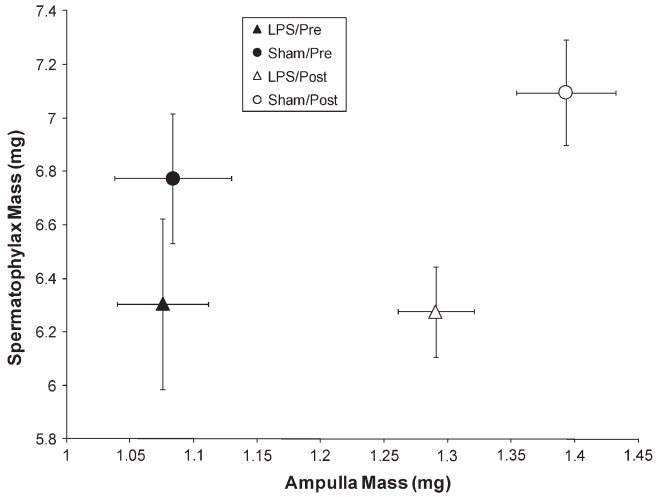

Figure 1
Bivariate least-square means (±standard error) of spermatophylax mass and ampulla mass of LPS-injected and sham-injected male decorated crickets. Pre, pretreatment and post, posttreatment. Significant MANCOVA effects are as described in Table 1.

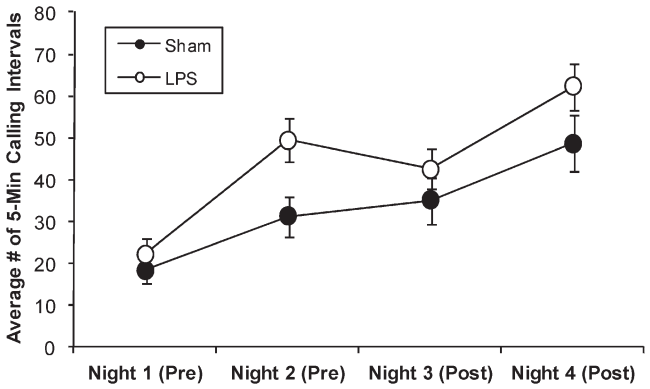

Figure 2
Average calling time (±standard error) of LPS-injected and sham-injected male decorated crickets. Pre, pretreatment and post, posttreatment.

infection, based on the total mass of the spermatophores he produced. We found that spermatophore investment was not a significant predictor of survival of an acute infection with *S. marcescens*, for either the spermatophylax (Wald $\chi^2 = 0.9314$, $P = 0.3345$) or the ampulla (Wald $\chi^2 = 0.0841$, $P = 0.7719$). Male mass was also not a significant predictor of survival (Wald $\chi^2 = 2.0817$, $P = 0.1491$).

Effect of experimentally induced reproductive effort on immunity

Experiment 3: encapsulation ability, PO activity, and lytic activity
We used MANOVA to assess the effects of increased spermatophore production on implant darkness, change in PO activity, and change in lytic activity. MANOVA indicated an overall significant effect of treatment (Table 2). We evaluated follow-up contrasts using the sequential Bonferroni method, with experiment-wise $\alpha = 0.05$ (Table 2). The magnitudes of standardized canonical coefficients revealed that the change in lytic activity was the primary contributor to the significant treatment effect (Table 2 and Figure 3).

DISCUSSION

The results of this study reveal a fundamental trade-off between immunity and reproductive effort in male *G. sigillatus*, specifically with respect to a male's ability to synthesize a costly nuptial food gift, the spermatophylax. When an immune response was experimentally elicited in males by injecting them with LPS, they produced significantly smaller spermatophores relative to control males. When the reciprocal experiment was conducted and males were manipulated into increasing their reproductive effort, males with increased spermatophore production exhibited lower immunity than males producing fewer spermatophores. Because the provision of a spermatophylax is a common form of nuptial feeding in the ensiferan Orthoptera (Vahed 1998, 2007), a trade-off between gift giving and immunity is likely to be pervasive in this group of insects. Although a previous study showing a negative correlation between lytic activity and spermatophylax mass in *G. sigillatus* is consistent with this hypothesis (Gershman et al. 2010a), our study is the first to provide evidence of such a trade-off using a manipulative experimental approach.

Analysis of the effect of LPS injection on spermatophore production showed that the treatment had a greater effect on mass of the spermatophylax than on the mass of the ampulla. This suggests that immune-challenged males may be unable to produce spermatophylaxes of sufficient mass to deter females from prematurely removing the sperm ampulla before complete sperm transfer has occurred (Sakaluk 1984, 1985). This in turn should lead to a decrease in male reproductive success because a male's fertilization success is influenced greatly by the number of sperm he transfers to the female (Sakaluk 1986; Sakaluk and Eggert 1996; Calos and Sakaluk 1998; Eggert et al. 2003). A similar result has been found in male striped ground crickets, *Allonemobius socius*, in which females

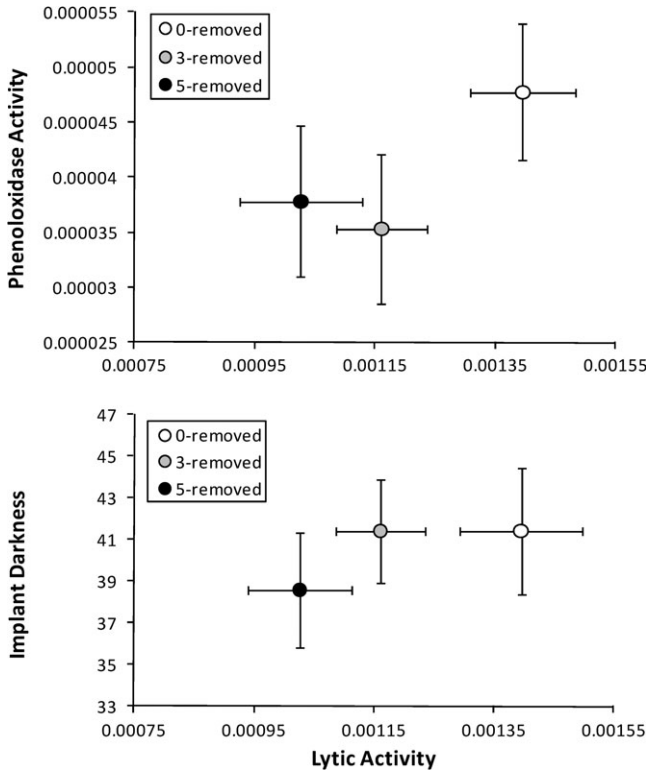

Figure 3
Multivariate means ± standard error for immune responses of male decorated crickets producing varying numbers of spermatophores (shown as number of spermatophores removed). Results of the MANOVA are reported in Table 2. Bivariate plot for PO activity and lytic activity (top graph). Bivariate plot for the encapsulation response and lytic activity (bottom graph).

Table 2
MANOVA of the effect of increased spermatophore production on 3 measures of immunity in male decorated crickets

	df	Pillai's Trace	*P*	Standardized canonical coefficients		
				ID	PO	LA
Treatment	**6, 166**	**2.17**	**0.0484**	**+0.4278**	**+0.4125**	**+1.0137**
0 versus 3	3, 82	2.09	0.1072	+0.2292	+0.6111	+0.9215
3 versus 5	3, 82	0.76	0.5192	+0.7323	−0.1168	+0.9374
0 versus 5	**3, 82**	**4.07**	**0.0095**	**+0.4654**	**+0.3667**	**+1.0231**

Follow-up contrasts assess the differences among the 3 treatment groups and were performed with a sequential Bonferroni correction. The numbers in the treatment groups represent the number of spermatophores removed over 5 days: 0 = none removed, 3 = three removed, and 5 = five removed. Bold values are statistically significant. The magnitudes of standardized canonical coefficients indicate the magnitude of the contribution of that variable to the significant MANOVA effect (Scheiner 1993). ID, implant darkness; LA, lytic activity.

chew on specialized spurs on males' hind tibiae during copulation and ingest hemolymph leaking from the spur (Fedorka and Mousseau 2002a). As is the case with the spermatophylax, the size of this hemolymph-based nuptial gift is positively correlated with the amount of sperm transferred by the male at mating (Fedorka and Mousseau 2002b). When juvenile male *A. socius* were injected with LPS, they too produced smaller nuptial gifts (as adults) compared with control males (Fedorka and Mousseau 2007).

In contrast to the effect of LPS injection on spermatophore mass, we found no effect of LPS injection on calling behavior in *G. sigillatus*, a result inconsistent with other cricket studies that have examined the effect of LPS on acoustic signaling behavior (Jacot et al. 2004; Fedorka and Mousseau 2007; Leman et al. 2009). In fact, crickets assigned to the LPS-injected group called more than those assigned to the control group, both before and after the injection (Figure 2). We have no obvious explanation as to why males assigned to the LPS-injected group would have called more than control males prior to injections because males were randomly assigned to the treatments. We found a significant general trend of increased calling activity for both control and LPS-injected males over the course of the experiment (Figure 2), which may be due to the crickets becoming more acclimated to the viewing chambers in which they were housed during that time period.

The MANOVA of the effects of increased spermatophore production on male immunity revealed that lytic activity was the primary contributor to the significant treatment effect. Similarly, Gershman (2008) demonstrated that when female vocal field crickets, *Gryllus vocalis*, mated more frequently, they experienced reduced lytic activity, but mating frequency did not affect PO activity or implant darkness. Simmons and Roberts (2005) found that sperm viability in male field crickets, *Teleogryllus oceanicus*, was positively correlated with encapsulation ability, but negatively correlated with lytic activity, and concluded that males would experience decreased paternity if they needed to mobilize their immune system against bacterial infection. Thus, it appears that different components of immunity do not necessarily vary in concert with increased reproductive effort, perhaps because different components are involved in the defense against different types of pathogens and parasites and also because the cost of activating one component of immunity may be traded off with one or more of the others as part of an optimal allocation strategy (Adamo 2004b; Rantala and Roff 2005; Lawniczak et al. 2006). For example, other studies have demonstrated a negative correlation between encapsulation rate and lytic activity (Rantala and Kortet 2003; Rantala and Roff 2005; Simmons and Roberts 2005; Väänänen et al. 2006) and between PO activity and lytic activity (Bailey and Zuk 2008).

We predicted that if males trade off energy invested in reproduction versus that invested in immunity, then those that produced larger spermatophores would be less likely to survive an acute bacterial infection. Instead, we found no correlation between the mass of a male's spermatophore and his likelihood of survival after injection with *S. marcescens*. We also found that a male's mass was not correlated with his survival ability, a result consistent with Adamo et al. (2001). It may be that males that produce larger food gifts, typically the larger males (Sakaluk 1985, 1997), are of intrinsically higher vigor than males that produce smaller gifts and thus are equally capable of combating a bacterial infection despite the higher reproductive effort. Indeed, Fedorka et al. (2005) found that male *A. socius* that invested more in immunity (i.e., exhibited higher lytic activity) actually transferred larger hemolymph-based nuptial gifts to females, suggesting that this association was due to natural selection for overall male vigor. Additionally, crickets in our experiment were provided with food ad libitum, and it is possible that they were able to balance the energetic demands of immune system activation with those devoted to reproduction because of a surplus of food resources. Any detectable trade-off between gift size and immunity may actually be condition dependent and, as suggested by Armitage et al. (2003), might be more pronounced in individuals that are food limited and thus nutritionally stressed.

The lack of an association between the mass of the spermatophylax and a male's immunocompetence, coupled with the finding that increased spermatophore production results in decreased immunity, raises the possibility that the spermatophylax serves as a Zahavian handicap (Zahavi 1975, 1977). Males providing larger food gifts to females appear to be able to maintain the same level of immunity as males providing smaller food gifts, and thus, the cost of the gift would appear to fall more heavily on the latter, an essential element of the model (Nur and Hasson 1984). Although the spermatophylax seems designed specifically to invoke a gustatory response in females (Sakaluk 2000; Warwick et al. 2009), thereby deterring them from prematurely removing the sperm ampulla, there is no obvious reason why a female should automatically remove the sperm ampulla as soon as she has consumed the spermatophylax. The handicap hypothesis offers a possible explanation because females that terminate sperm transfer after consuming the spermatophylax may increase the insemination success of those males of the highest vigor and overall genetic quality. In support of this possibility, a recent study has shown that both PO activity and the encapsulation response are significantly heritable in *G. sigillatus* (Gershman et al. 2010b). Thus, females accepting a greater number of sperm from males providing larger food gifts may enhance the immunocompetence of their offspring. Alternatively, there could be direct selection on female mating preferences because parasitic nematodes are sexually transmitted in *G. sigillatus* (Luong et al. 2000), and infected males produce significantly smaller spermatophylaxes (Luong and Kaya 2005). Thus, females that prematurely remove the sperm ampullae of males providing smaller food gifts may reduce their own chances of acquiring a sexually transmitted disease.

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