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Give 'til it hurts: trade-offs between immunity and male reproductive effort in the decorated cricket, *Gryllodes sigillatus*

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trade-off;
reproductive effort;
spermatophylax.

Abstract

Trade-offs between life-history variables can be manifested at either the phenotypic or genetic level, with vastly different evolutionary consequences. Here, we examined whether male decorated crickets (*Gryllodes sigillatus*) from eight inbred lines and the outbred founder population from which they were derived, trade-off immune effort [lytic activity, phenoloxidase (PO) activity or encapsulation] to produce spermatophylaxes: costly nuptial food gifts essential for successful sperm transfer. Canonical correlation analysis of the outbred population revealed a trade-off between spermatophylax mass and lytic activity. Analysis of our inbred lines, however, revealed that although PO activity, encapsulation, body mass, spermatophylax mass and ampulla (sperm capsule) mass were all highly heritable, lytic activity was not, and there was, therefore, no negative genetic correlation between lytic activity and spermatophylax mass. Thus, males showed a phenotypic but not a genetic trade-off between spermatophylax mass and lytic activity, suggesting that this trade-off is mediated largely by environmental factors.

Introduction

Both mating effort and immunity represent substantial costs for most organisms in terms of energy investment. Given a limited pool of resources, individuals cannot simultaneously maximize effort devoted to reproduction and immunity, and thus trade-offs between these traits can occur (Adamo *et al.*, 2001; Rolff & Siva-Jothy, 2002; Baer *et al.*, 2006). For example, an immune challenge may cause a subsequent decrease in courtship quality or quantity (Jacot *et al.*, 2004; Fedorka & Mousseau, 2007; Leman *et al.*, 2009). Alternatively, increased reproductive activity has been found to impair immunity (Siva-Jothy *et al.*, 1998; McKean & Nunney, 2001; Ahtiainen *et al.*, 2004, 2005). Trade-offs between sexually selected and immune traits have also been demonstrated at the genetic level (Simmons & Roberts, 2005; McKean & Nunney, 2008), suggesting that antagonistic pleiotropy may play an important role in regulating this important life-history trade-off (Houle, 1991; Roff, 2002), as it does in trade-offs between immunity and other important life-history traits (Luong & Polak, 2007; Schwarzenbach & Ward, 2007; McKean *et al.*, 2008; Voordouw *et al.*, 2009; Ye *et al.*, 2009).

Mating can be especially expensive for males in taxa with costly nuptial food gifts (Vahed, 1998). In the ensiferan Orthoptera, the provision of such gifts often is required to secure matings or to ensure sperm transfer once mating has occurred (Vahed, 2007). Because these gifts constitute a considerable investment of male resources, males that produce gifts may do so at the expense of other functions, including somatic maintenance and immunity. Indeed, mating causes depressed immune function in male ground crickets, *Allonemobius socius* and sagebrush crickets, *Cyphoderris strepitans*, species in which females consume portions of the male's soma and ingest his haemolymph during mating (Fedorka *et al.*, 2004; Fedorka & Zuk, 2005; Leman *et al.*, 2009).

In male decorated crickets, *Gryllodes sigillatus*, the spermatophore transferred by a male at mating is comprised of a large gelatinous mass, the spermatophylax, surrounding a smaller sperm-containing ampulla. The spermatophore remains secured outside of the

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female's body at the base of her ovipositor by a narrow spermatophore tube threaded into her genital opening. Immediately after mating, the female removes the spermatophylax with her mandibles and consumes it. After consuming the spermatophylax, the female removes and eats the sperm-containing ampulla, thereby terminating sperm transfer. Smaller spermatophylaxes require less time to consume, and males providing such gifts experience premature ampulla removal and reduced sperm transfer (Sakaluk, 1984, 1985, 1987). The decrease in the number of sperm transferred often results in a decline in a male's fertilization success, particularly when females mate with multiple males (Sakaluk, 1986; Sakaluk & Eggert, 1996; Calos & Sakaluk, 1998; Eggert *et al.*, 2003). Thus, for successful mating, a male must produce a spermatophylax that is sufficiently large to permit transfer of the sperm from the ampulla. However, the spermatophylax is not a trivial investment, comprising approximately 2.2% of a male's body mass and leading to longer refractory periods relative to species without a spermatophylax (Sakaluk, 1985, 1997). Although the orthopteran spermatophylax is often considered a costly courtship gift (review in Vahed, 1998), no previous study has determined whether males experience a trade-off in effort devoted to immunity vs. spermatophylax production.

One approach to examine trade-offs between reproductive and immune traits is to study variation within and between inbred lines. Inbred lines represent samples of the standing genetic variation in the source population and can be used to assess the heritability of reproductive and immunological traits, and thus, the potential for selection to act on these traits (David *et al.*, 2005). Further, inbred lines can be used to calculate genetic correlations among traits (David *et al.*, 2005), providing information about the degree to which the genes governing two traits are linked or are genetically independent and thus relatively free to evolve. This genetic covariance between traits is key to understanding whether any trade-offs between reproduction and immunity are under genetic control (Roff, 2002).

In addition to preserving samples of the standing genetic variation in an outbred population, inbred lines can also be used to evaluate the consequences of natural population bottlenecks. Inbreeding can have negative effects on overall health, as genetic drift allows disadvantageous traits to reach fixation (Keller & Waller, 2002). Inbred individuals may have disabled immune pathways or fewer resources available with which to respond to an immune challenge (Reid *et al.*, 2003; Calleri *et al.*, 2006; Ilmonen *et al.*, 2008). Inbreeding may also deprive a population of the genetic diversity necessary to counter rapidly coevolving pathogens and parasites. Further, inbred individuals may have reduced or impaired reproductive effort compared to outbred individuals (Keller & Waller, 2002).

The objective of our study was to identify possible phenotypic and genotypic trade-offs between spermatophore production and immunity using male *G. sigillatus* from eight highly inbred lines and the outbred founder population from which they were derived. In addition, we compared inbred and outbred males to determine the effects of inbreeding on male reproductive effort and immunity, and used the inbred lines to estimate the heritabilities of reproductive and immunological traits, as well as the genetic correlations between these traits.

Methods

Creation of inbred lines

Gryllodes sigillatus used in this study were the descendents of approximately 500 adult crickets collected in Las Cruces, New Mexico in 2001, and used to initiate a laboratory colony maintained at a population size of approximately 5000 and allowed to breed panmictically (hereafter, the outbred population). Nine inbred lines (designated A–I), eight of which (A, B, D–I) were used in this study, were created by subjecting crickets, randomly selected from the large, panmictic population described earlier, to 23 generations of full-sib mating followed by 3–4 generations of panmixis within each line (Ivy *et al.*, 2005). For both the colony and inbred lines, two boxes per line were maintained to mitigate box effect, and boxes were rotated in their positions within the incubator. However, because of logistical constraints, we were not able to track the boxes from which each animal was collected in this study, and thus we could not calculate variation caused by rearing in different boxes. However, if any box effects did exist, they would be unlikely to create a systematic bias in our experimental results because individuals were drawn from the two boxes at random.

Measurements made in 2007 revealed evidence of significant inbreeding depression in the inbred lines even after 17 generations of inbreeding: inbred lines all showed lower hatching success, decreased offspring production and longer developmental times compared to the outbred population (J.M. St. John & S.K. Sakaluk, unpublished). However, using methods similar to those described in this manuscript, male and female crickets from the eight inbred lines had higher phenoloxidase (PO) activity (ANOVA $F_{1,424} = 70.0$, $P < 0.0001$) and implant darkness (ANOVA $F_{1,424} = 57$, $P < 0.0001$), and higher lytic activity (ANOVA $F_{1,424} = 3.9$, $P = 0.05$) than outbred crickets (Gershman *et al.*, 2010). It is likely that our inbreeding design purged some of the least viable genotypes, raising average immunity when compared to outbred individuals.

Crickets were held in 55-L plastic storage bins in an environmental chamber maintained at 32 ± 1 °C on a 14 h : 10 h light/dark cycle. Crickets were provisioned with Flukers® cricket chow (Fluker Laboratories, Port Allen, LA, USA), water provided in 40-mL plastic tissue

culture flasks plugged with cotton dental rolls and egg cartons to provide shelter and to increase surface area. Moistened peat moss provided in small plastic containers was made available both as an oviposition substrate and a source of additional water.

Measurement of immune responses and reproductive effort

The insect immune system is comprised of both humoral and cell-mediated components. Humoral responses include the production of antimicrobial compounds that defend against bacterial, viral or fungal pathogens (Abbas & Lichtman, 2003). Cell-mediated responses include the encapsulation or melanization of foreign objects within the body cavity, which occurs as a result of the accumulation of melanin and hemocytes. In this study, we examined both cell-mediated and humoral responses. First, we estimated the amount of the inactive haemolymph-bound enzyme, pro-PO. PO catalyses the reaction of dopamine into melanin, a key step in the encapsulation response pathway (Söderhäll & Cerenius, 1998). Second, we estimated the amount of a haemolymph-bound enzyme lysozyme, which defends against bacteria. Third, we measured the degree to which a foreign body was engulfed by hemocytes and melanized, providing a measure of macroparasitic defence. Encapsulation ability was measured *in vivo* as the response of individuals to a novel immune challenge, and thus represents a 'realized' immune response. However, estimates of lysozyme and PO are 'potential' responses because they measure *in vitro* the amount of enzyme present, providing information about the potential of an individual to mount an immune response. PO activity (Adamo *et al.*, 2001; Siva-Jothy *et al.*, 2001; Fedorka & Zuk, 2005; Leman *et al.*, 2009), lytic activity (Rantala & Kortet, 2003) and implant encapsulation (Siva-Jothy *et al.*, 2001; Rantala & Kortet, 2003; Zuk *et al.*, 2004; Leman *et al.*, 2009) are commonly used to assess insect immunity.

Data for lytic activity, PO activity and implant darkness were obtained for 39–47 individuals from each of the inbred lines and for 33 individuals from the outbred population, representing a total of 249 males. Crickets were removed from the outbred population and inbred lines within 48 h of adult eclosion. To ensure that crickets were sexually experienced, males and females of the same age cohort were housed together for 6 days. To increase the probability that males had a spermatophore available for collection, males were then housed separately from females 24 h before spermatophore collection. Eight to nine days after adult eclosion, crickets were weighed and housed individually. Spermatophores were removed by gently squeezing the male's abdomen, causing the spermatophore to be extruded from the male's spermatophoric pouch. The spermatophore was removed with forceps, and its two constituent components, the spermatophylax and ampulla, separated and weighed to the nearest 0.001 mg using a Cahn® microbalance (Cahn Measurements Inc., Cerritos, CA, USA). Twenty-four hours later, crickets were implanted with a 3-mm long segment of 0.255-mm diameter nylon monofilament fishing line that had been abraded with sandpaper. A small hole was made ventrally between the fifth and sixth abdominal segments with a 27-gauge syringe needle, and the implant was inserted until it was completely contained within the cricket's abdominal cavity. Prior to implantation, crickets were cold-anesthetized for 10 min at 6 °C in a refrigerator, and implants and needles were sterilized in 70% ethanol. These procedures were similar to those used in other studies (Siva-Jothy *et al.*, 1998; Rantala *et al.*, 2000; Doums *et al.*, 2002; Fedorka *et al.*, 2004; Zuk *et al.*, 2004; Rantala & Roff, 2007). After implantation, crickets were returned to their individual containers in the environmental chamber and allowed to resume normal activity. A pilot study of 10 crickets demonstrated that 48 h was the most appropriate duration for the implants to remain in the crickets to provide a range of variation in melanization. Exactly 48 h after each cricket was implanted, haemolymph samples were drawn, after which crickets were frozen and stored in a −80 °C freezer. Three microlitres of haemolymph were mixed with 40 μL phosphate-buffered saline (PBS) solution and frozen in the −80 °C freezer to induce cell lysis and to prevent enzymatic reactions from proceeding. Haemolymph samples were stored at −80 °C for at least 1 week prior to analysis.

Implants were dissected out of the frozen crickets and any clumps of tissue removed. Each implant was photographed three times from three different sides next to a clean implant control using a Nikon Coolpix 4500 digital camera (Nikon, Inc., Melville, NY, USA) mounted on a Wild Heerbrugg dissecting microscope. Each implant and control was outlined using the ImageJ (http://rsbweb.nih.gov/ij/) polygon tool. The darkness of each implant and control was measured as the average gray-scale value of all of the pixels within each image using the same software. The darkness score for each individual was calculated as the average difference of the three implants' darkness scores subtracted from the controls. Thus, darker implants yielded higher darkness scores.

To measure PO activity of haemolymph samples drawn from experimental individuals, a known quantity of 3,4-Dihydroxy-L-phenylalanie (L-DOPA) was added to the haemolymph to replace the naturally occurring substrate. Because the amount of L-DOPA is not a limiting factor in the experimental reaction leading to melanization and was constant across samples, the subsequent melanization of the haemolymph must be because of variation in the amount of PO present in the haemolymph of individuals. In preliminary studies of *G. sigillatus*, males had undetectably low levels of standing PO. Therefore, we activated the pro PO of all males using the enzyme alpha-chymotrypsin to estimate the total PO activity for each cricket (Adamo, 2004a). We combined 5 μL of haemolymph solution with 7 μL of

bovine pancreas alpha-chymotrypsin (1.3 mg mL^{-1}; Sigma-Aldrich, Inc., Steinheim, Germany, C7762) in each well of a spectrophotometer microplate and incubated the mixture for 20 min at room temperature (20°C). We then added 90 μL of 15 mM L-DOPA (Sigma-Aldrich, Inc., D9628-5G) and recorded optical density (OD) at 490 nm using a PowerWave 340 Microplate Spectrophotometer with Kc4 data analysis software (Bio-Tek Instruments Richmond, VA, USA). This method estimates the total change in OD over the course of the reaction, ranging from an OD of 0 (transparent) to 4 (opaque). OD readings were taken every 10 min for 210 min. Preliminary tests indicated that readings taken between 30 and 180 min best describe the fastest rate of change in OD over time. The PO activity rate was calculated as the change in OD over time (OD/time). These protocols were adapted from Adamo (2004a), Fedorka & Zuk (2005), Shoemaker *et al.* (2006) and Bailey & Zuk (2008).

To estimate lytic activity, 3 mg of *Micrococcus lysodeikticus* (Sigma-Aldrich, Inc., M3770-5G) per 10 mL of PBS buffer were added to the haemolymph to determine the ability of a given lysozyme to lyse the bacterial cells. Preliminary tests showed that this amount of *M. lysodeikticus* caused visible cell lysis and activity to level off after 3 h. To quantify lytic activity, 10 μL of the thawed frozen haemolymph sample, along with 90 μL of PBS buffer containing *M. lysodeikticus*, were added to each spectrophotometer microplate well and changes in OD recorded. This method estimates the total change in OD from opaque to clear as lysozymes or lysozyme-like enzymes lyse the bacterial cells. OD readings were recorded every 5 min for 180 min. Preliminary tests indicated that readings between 30 and 120 min produced a measurable and consistent change in OD/time. Although OD decreases as more bacterial cells are lysed, lytic activity is given as a positive number for clarity. This experimental design does not permit the characterization of the specific lysozyme responsible for cell lysis (Schneider, 1985); the observed lytic activity is thus attributed to a lysozyme-like enzyme.

Eight to twelve control samples lacking insect haemolymph were included in the spectrophotometric analysis of each 96-sample plate to detect any nonexperimental change in OD/time across plates. Control samples did not differ significantly among sample plates for lytic activity (ANOVA $F_{6,72} = 1.56$, $P = 0.18$). There was, however, an effect of plate on PO OD over time (ANOVA $F_{7,82} = 43.1$, $P < 0.0001$). To control for differences among plates, lytic activity and PO OD/time was subtracted from the average control OD/time of the plate.

Statistical analyses

Outbred population

To determine whether there were any phenotypic tradeoffs between spermatophore variables and immune variables in the outbred population, as well as within inbred lines, we employed canonical correlation analysis. Canonical correlation analysis determines whether, and how, two sets of variables (i.e. the reproductive variables, spermatophylax mass and ampulla mass and the immune variables, implant darkness, PO activity and lytic activity) are related and whether they are related in more than one way. This analysis creates pairs of canonical variates that explain the relationship between the two sets of variables. For each pair of canonical variates, we can then determine the contribution of each variable to the opposite canonical variate. Thus, we can determine the strength and direction of relationships between each variable and the opposite set of variables (Tabachnick & Fidell, 2001). With the exclusion of two data points as extreme outliers, all dependent variables were normally distributed and homoscedastic. For two individuals (one outbred and one line F inbred), PO activity scores were an order of magnitude larger than that of other individuals and caused a violation of the assumption of normality. For the multivariate analyses and PO activity analyses, these individuals were omitted entirely, but for the remaining univariate ANOVAS (everything else other than PO activity), these individuals were included in the analysis. To examine the relationships between different components of immunity and reproductive effort in the outbred population, we calculated pairwise Pearson correlations between the individual traits (i.e. lytic activity, PO activity, implant darkness, spermatophylax weight and ampulla weight). We used the sequential Bonferroni correction for multiple tests.

Inbred vs. outbred crickets

For comparisons between inbred and outbred crickets, it was necessary to correct for the possibility that results could have been confounded by the levels of genetic relatedness between individuals within a line. Individuals within inbred lines share a single genetic origin and so cannot be considered genetically independent. Individuals from outbred populations are likely to be more heterogeneous and so are less likely to share as high a number of genes compared to inbred individuals. Inbred and outbred crickets were compared by performing a multivariate contrast between multivariate average components of reproduction and immunity of each line vs. the components of reproduction and immunity for outbred individuals. This contrast allows us to compare inbred to outbred individuals without pseudoreplication caused by using each inbred individual within a given line as a separate sample and without the sampling error associated with using only one individual to represent the trait values of each line.

Genetic architecture of immunological and reproductive traits

We estimated the heritability of immune and reproductive traits from our inbred lines by calculating the

coefficient of intraclass correlation (t) (Hoffmann & Parsons 1988; David *et al.*, 2005) as:

$$t = \frac{nV_b - V_w}{nV_b + (n-1)V_w}$$

where n is the number of lines (in our case 8) and V_b and V_w are the between-line and within-line variance components, respectively, estimated directly from an ANOVA including line as the main effect. The standard error of the intraclass correlation [SE(t)] was calculated according to Becker (1984) as:

$$SE(t) = \sqrt{\frac{2(1-t)^2[1+(k-1)t]^2}{k(k-1)(n-1)}}$$

where k is the number of individuals sampled within each line (in our case 31). The heritability (h^2) of each phenotypic trait was then estimated according to Hoffmann & Parsons (1988) as:

$$h^2 = \frac{2}{\left(\frac{1}{t} - 0.5\right)}$$

The SE of this estimate, [SE(h^2)], was calculated according to Hoffmann & Parsons (1988) as:

$$SE(h^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2} SE(t)$$

Genetic correlations and their SEs were estimated using the jackknife method of Roff & Preziosi (1994). In short, this procedure first estimates the genetic correlation between two traits using mean estimates for each line. A sequence of N (in our case 8) pseudovalues is then computed by dropping, in turn, each of the lines, estimating the resulting correlations and using the formula:

$$S_{N,i} = Nr_N - (N-1)r_{N-1,i},$$

where $S_{N,i}$ is the ith pseudo-value, r_N is the genetic correlation estimated using the means of all N inbred lines and $r_{N-1,i}$ is the genetic correlation obtained by dropping the ith inbred line alone (Roff & Preziosi, 1994). The jackknife estimate of the genetic correlation (r_j) is then simply the mean of the pseudovalues, and an estimate of the SE is given by:

$$SE = \frac{\sum_{i=1}^{i=N} (S_{N,i} - r_j)^2}{N(N-1)}$$

Using simulation models, Roff & Preziosi (1994) showed that this jackknife approach provides better genetic estimates than those based on conventional inbred line means when the number of inbred lines contained in the analysis is small (< 20 lines). It is important to note that estimates of genetic (co)variance from inbred lines contain variance because of dominance and/or epistasis and therefore should be considered broad-sense estimates (Falconer & Mackay, 1996). Moreover, because crickets from each inbred line were reared in one or two containers, it is possible that some of the observed variance between inbred lines is because of common environmental effects. However, we believe that such common environmental effects were minimal in our experiment because each inbred line was maintained under identical conditions within the same growth chamber and rotated within the growth chamber. Heritability estimates and genetic correlations were considered statistically significant if values divided by SEs were > 1.96, rejecting the null hypothesis of no correlation with a two-tailed t-distribution and infinite degrees of freedom.

A mismatch between genetic and phenotypic trade-offs for a pair of traits may occur whenever a large environmental correlation exists between these traits (Roff, 2002). As the majority of variation within each of our inbred lines is expected to be environmental in origin (i.e. all individuals within a line are genetically homogeneous), examining the trade-off between traits within each of our lines provides an important test of whether these trade-offs are environmentally determined. Consequently, for each pair of traits showing a significant phenotypic trade-off (see Results), we determined whether there was an environmental trade-off within each of our inbred lines using both the canonical correlation analysis and pairwise Pearson correlations outlined earlier.

Inbred lines

To determine whether there were any phenotypic trade-offs between spermatophore variables and immune variables within each inbred line, we employed canonical correlation analysis as previously described for the outbred population.

Results

Outbred population

The first canonical correlation was 0.62 (39% overlapping variance) and was statistically significant (Wilks $\lambda = 0.50$, d.f. = 6, 44, $P = 0.015$). For the first canonical correlation, both spermatophylax mass and ampulla mass contributed to the reproductive canonical variate; and lytic activity, PO activity and implant darkness all contributed to the immunological variate (correlations > 0.3). Correlations between each variable and the opposite canonical variate showed that males with larger spermatophylaxes (0.60) and ampullae (0.48) had lower lytic activity (−0.53), but higher PO activity (0.37) and darker implants (0.23) than males producing smaller spermatophores. The second canonical correlation was not statistically significant (Wilks $\lambda = 0.82$, d.f. = 2, 23, $P = 0.11$). Univariate regression showed a negative correlation between spermatophylax mass and lytic activity (Fig. 1; $r^2 = 0.21$, $n = 30$, $P = 0.0106$). There were no

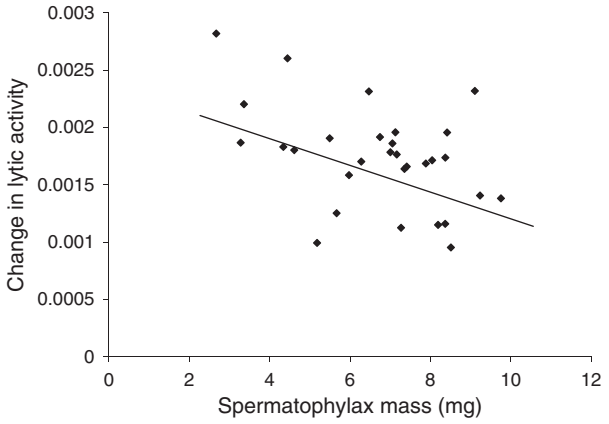

Fig. 1 Linear regression of change in lytic activity with spermatophylax mass in male *Gryllodes sigillatus* from an outbred population.

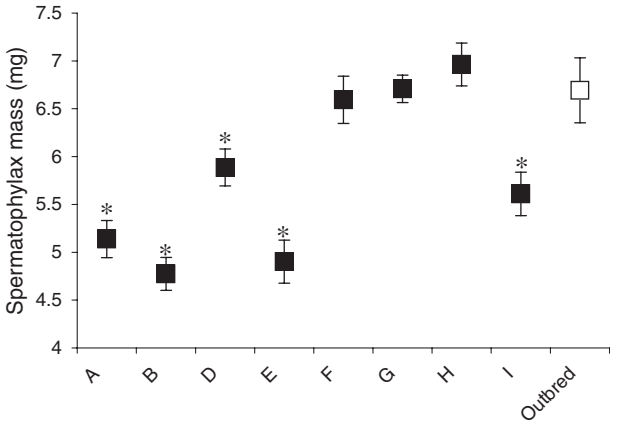

Fig. 2 The effect of genotype on spermatophlyax mass. Means and standard errors of spermatophylax mass from inbred lines A, B, D, E, F, G, H and I (dark squares) and the outbred colony (white square). Asterisks indicate lines that are significantly different from the outbred population at a level of $\alpha < 0.05$ with a sequential Bonferroni correction.

significant correlations between lytic activity, PO activity and implant darkness (all $P > 0.05$). Spermatophylax mass and ampulla mass were highly correlated ($r^2 = 0.36$, $n = 30$, $P = 0.0005$). The relationship between spermatophylax and ampulla mass was even stronger when the effect of body mass was removed by correlating the residuals of spermatophylax mass and ampulla mass on body mass ($r^2 = 0.99$, $n = 30$, $P = 0.0001$).

Inbred vs. outbred crickets

Inbred males had smaller spermatophylaxes than outbred males (Table 1). In univariate contrasts, males in the outbred colony had larger spermatophylaxes than males in seven of the eight inbred lines (Fig. 2). Inbreeding had no effect on ampulla mass (Fig. 3) or body mass (Fig. 4, univariate contrast between line mean and outbred: $F_{1,372} = 3.62$, $P = 0.058$).

Genetic architecture of immunological and reproductive traits

PO activity, implant darkness, body mass, spermatophylax mass and ampulla mass were all highly heritable

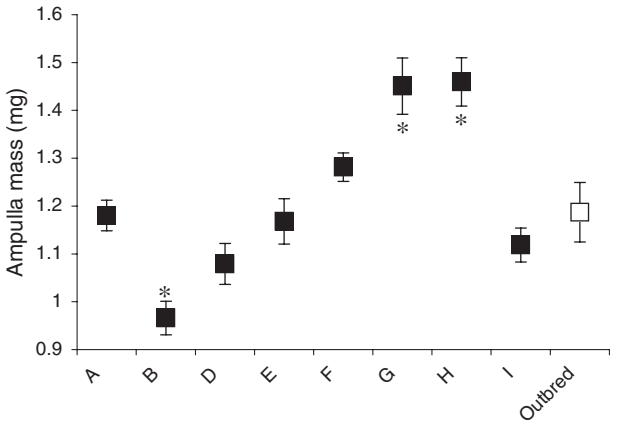

Fig. 3 The effect of genotype on ampulla mass. Means and standard errors of ampulla mass from inbred lines A, B, D, E, F, G, H and I (dark squares) and the outbred population (white square). Asterisks indicate lines that are significantly different from the outbred population at a level of $\alpha < 0.05$ with a sequential Bonferroni correction.

Table 1 MANOVA with multivariate contrasts between mean of each line vs. outbred spermatophore component masses and ANOVAS of the effect of this contrast on spermatophylax mass, ampulla mass and body mass. *P*-values indicated by an asterisk are statistically significant.

	d.f.	*F*	*P*
MANOVA (Wilks' λ)	2, 258	8.31	< 0.0001*
ANOVAS			
Spermatophylax mass	1, 259	13.1	< 0.0001*
Ampulla mass	1, 259	0.28	0.60
Body mass	1, 259	2.54	0.11

(Table 2). Lytic activity was more variable within than between inbred lines and therefore yielded a negative heritability estimate (that was set to zero). Consequently, no genetic correlations were calculated for comparisons involving lytic activity. Spermatophylax and ampulla mass were highly correlated, and both were strongly genetically correlated with body mass. This relationship was not driven by body mass, as re-analysis using trait residuals to correct for their relationship with body mass revealed that these spermatophore components were still positively genetically correlated (0.66 ± 0.07). Both

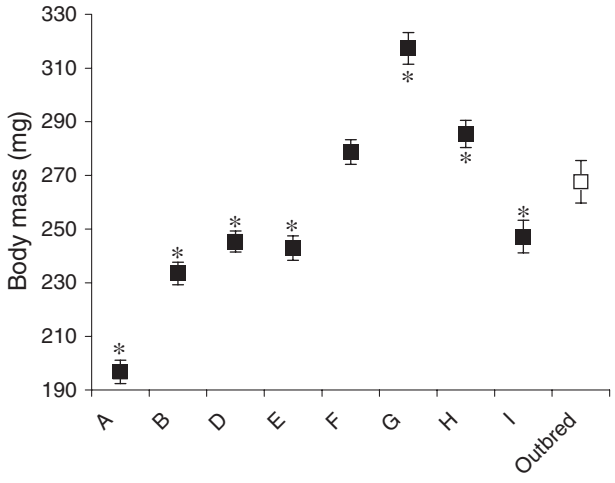

Fig. 4 The effect of genotype on body mass. Means and standard errors of body mass from inbred lines A, B, D, E, F, G, H and I (dark squares) and the outbred population (white square). Asterisks indicate lines that are significantly different from the outbred population at a level of $\alpha < 0.05$ with a sequential Bonferroni correction.

Table 2 Heritability (±SE) of body mass, immunological and reproductive variables and genetic correlations. Lytic activity had a negative heritability estimate and therefore was not used in estimates of genetic correlations. Significant values are indicated by asterisks.

Trait			
Body mass	0.98	±	0.01*
Heritability			
Immunological variables			
Lytic activity		–	
PO activity	0.76	±	0.10*
Implant darkness	0.78	±	0.09*
Reproductive variables			
Spermatophylax mass	0.94	±	0.03*
Ampulla mass	0.94	±	0.03*
Genetic correlations			
Spermatophylax × ampulla	0.86	±	0.01*
Spermatophylax × body mass	0.79	±	0.001*
Spermatophylax × implant	0.76	±	0.03*
Ampulla × body mass	0.73	±	0.03*
Ampulla × implant	0.72	±	0.04*
Implant × body mass	0.48	±	0.10*
PO × spermatophylax	0.09	±	0.18
PO × implant	–0.06	±	0.10
PO × ampulla	–0.05	±	0.25
PO × body mass	0.04	±	0.10

PO, phenoloxidase.

spermatophore components were positively genetically correlated with implant darkness. Likewise, re-analysis using residuals showed that both spermatophylax mass (0.65 ± 0.06) and ampulla mass (0.54 ± 0.08) were positively correlated with implant darkness, independent of body mass. PO was not significantly correlated with implant darkness, body mass or either of the spermatophore components (Table 2).

Inbred lines

Given that we found a significant trade-off between lytic activity and spermatophylax weight at the phenotypic level but not at the genetic level, we determined whether there was an environmental trade-off between these traits within each of our inbred lines. With the exception of inbred line B, none of the canonical correlations were statistically significant (all *P*-values exceeded 0.05). For line B, the first canonical correlation was 0.70 (with 49% overlapping variance) and was statistically significant (Wilks $\lambda = 0.40$, d.f. = 6, 54, $P < 0.0001$). For the first canonical correlation, spermatophylax mass and ampulla mass contributed to the reproductive canonical variate, and lytic activity contributed to the immunological variate (based on correlations > 0.3); PO activity and lytic activity did not contribute significantly to the immunological variate. Correlations between each variable and the opposite canonical variate, showed that males with larger ampullae (0.69) and spermatophylaxes (0.26) had lower lytic activity (−0.64). The second canonical correlation was 0.45 (with 23% overlapping variance) and was also statistically significant (Wilks $\lambda = 0.78$, d.f. = 2, 28, $P = 0.03$). For the second canonical correlation, spermatophylax mass contributed to the reproductive canonical variate; and lytic activity and implant darkness contributed to the immunological variate (based on correlations > 0.3); ampulla mass did not contribute significantly to the reproductive variate and PO activity did not contribute significantly to the immunological variate. Correlations between each variable and the opposite canonical variate showed that males with larger spermatophylaxes (0.43) had darker implants (0.45).

There was a significant trade-off between lytic activity and spermatophylax mass in inbred lines B ($r^2 = 0.15$, $n = 33$, $P = 0.027$) and I ($r^2 = 0.14$, $n = 29$, $P = 0.046$), but neither would be significant if we adjusted for multiple comparisons (adjusted critical *P*-value = 0.006). However, no significant trade-off between these variables was detected in the remaining six inbred lines (A, D, E, F, G and H). Collectively, these results provide limited evidence for an environmental trade-off between lytic activity and spermatophylax mass.

Discussion

The spermatophylax produced by male *G. sigillatus* constitutes a large investment in reproduction: each spermatophylax weighs approximately 2.2% of a male's body mass (Sakaluk, 1997) and males produce at least one spermatophylax per night over the course of their adult

lifetime (Sakaluk *et al.*, 2002). Furthermore, it is essential for males to produce spermatophylaxes that are sufficiently large to prevent females from interrupting the transfer of sperm and negating the male's mating effort (Sakaluk, 1984). The results of this study suggest that male *G. sigillatus* experience a phenotypic trade-off between reproductive effort, as manifest in the size of their nuptial gift, and immunological effort, as males that produced larger spermatophylaxes subsequently exhibited lower lytic activity. However, using eight highly inbred lines, we showed that this phenotypic trade-off does not occur at the genetic level due, in part, to the low genetic variance for lytic activity in this species. Our results highlight, therefore, the importance of examining trade-offs at both the phenotypic and genetic levels when understanding how they evolve (Roff, 2002).

Similar phenotypic trade-offs between reproductive effort and lytic activity have been documented in other invertebrates. Gershman (2008) showed that female crickets, *Gryllus vocalis*, that mated more frequently had lower lytic activity, and Simmons & Roberts (2005) documented a negative genetic correlation between sperm viability and lytic activity in another cricket species, *Teleogryllus oceanicus*. Ahtiainen *et al.* (2004, 2005) showed that male wolf spiders of greater mobility and a higher rate of sexual signalling (i.e. drumming behaviour) had lower lytic activity, reduced PO activity and a lower encapsulation response. However, our study is the first to address whether costly male spermatophylaxes, pervasive across the ensiferan Orthoptera, are subject to a trade-off with immune function.

We found that the mass of the spermatophylax was highly heritable, but lytic activity was not, so that there was little genetic covariance between these two traits. Similarly, Rantala & Roff (2006) found lytic activity in male *Gryllus firmus* not to be heritable (broad-sense heritability $H^2 = 0.03$, SE not given), although Simmons & Roberts (2005) found lytic activity to be heritable in *T. oceanicus* ($h^2 = 0.31 \pm 0.05$). Thus, our study shows that although there is a phenotypic trade-off between investment in spermatophylax mass and immunity, this trade-off is not under genetic control. Consequently, there is no single optimal solution governing how much effort a male should devote to spermatophylax mass vs. effort allocated to lytic activity that can evolve over time. Instead, our results suggest that the optimal amount of effort that males should spend on each trait will depend on environmental factors. This is, perhaps, not totally unexpected because harmful bacterial outbreaks may occur irregularly, and it may benefit males to be able to allocate more or less effort to lytic activity as necessary. Alternatively, rather than being a trade-off for common resources (e.g. Fedorka *et al.*, 2004), spermatophylax production may directly impair male lytic activity. For example, Rolff & Siva-Jothy (2002) have shown that mating reduces PO activity in the mealworm beetle (*Tenebrio molitor*) and that this down-regulation is mediated by juvenile hormone that has direct and antagonistic physiological effects on immune function in this species. Clearly, more work is needed before we can distinguish between these alternatives in *G. sigillatus*.

The finding that the phenotypic trade-off between lytic activity and spermatophylax mass does not have a genetic basis may indicate that environmental factors or variation because of other unmeasured male traits may mediate this relationship. Indeed, our results show that within-line variation for male lytic activity, which is predominantly environmental in origin, was as variable as the variation we observed between lines, explaining the low broad-sense heritability for this immune component. For this reason, it has been argued that only genetically based trade-offs have direct evolutionary consequences and should be preferentially targeted in empirical studies (Reznick, 1985). It is important to note, however, that this is true only if selection targets one of the traits involved in the trade-off in isolation (Roff, 2002). Whenever selection acts simultaneously on both traits involved in a trade-off, as is likely to be more biologically realistic, both the phenotypic and genetic covariance between these traits are important to how these traits evolve (Roff, 2002). In fact, selection on one trait can theoretically influence the evolutionary response of the other trait even if there is no genetic covariance between them (Roff, 2002). Consequently, examining both the phenotypic and genetic trade-offs is likely to provide a much more complete view of how the traits, as well as the trade-off itself, will evolve.

Using analysis of inbred lines, we found that body mass, ampulla mass, PO activity and implant darkness were all significantly heritable. The strongest genetic correlation was between spermatophylax and ampulla mass. Both components of the spermatophore were positively genetically correlated with body mass, which is not altogether surprising as larger crickets are known to produce larger ejaculates in this species (Schaus & Sakaluk, 2001). However, the relationship between spermatophylax and ampulla mass was not driven solely by their relationships with body mass, as the analysis of residuals to remove this interdependence with body mass also yielded a strong positive genetic correlation between these spermatophore components. This relationship may be driven by developmental constraints (Arnold, 1992), if, for example, both traits are influenced by male accessory gland size or by the size of the morphological structure used to mould the spermatophore. It is also possible that selection for successful sperm transfer has shaped the genetic architecture of these spermatophore components (Lande, 1980). Spermatophylax size is likely to be under stabilizing selection in *G. sigillatus*, needing to be sufficiently large to allow the transfer of all sperm contained in the ampulla but not so large as to waste resources that could be used to produce subsequent spermatophylaxes or be allocated to somatic maintenance. Furthermore, ampulla mass is highly correlated

with sperm number; and thus, larger ampullae require larger spermatophylaxes to ensure successful sperm transfer (Schaus & Sakaluk, 2001). As a result, this pattern of selection could have shaped the genetic covariance between spermatophylax and ampulla mass so that these traits are co-inherited to conserve their functional integrity (Cheverud, 1996).

We also found a strong positive genetic correlation between both spermatophore components and implant darkness. As both spermatophore components and implant darkness are positively genetically correlated with body size, it suggests that the larger amount of circulating haemolymph in larger crickets allows more melanizing enzymes and hemocytes to encapsulate the implant. However, spermatophylax and ampulla mass were still both positively genetically correlated with implant darkness when the effects of body mass were removed statistically. This suggests that the positive genetic relationship between spermatophore production and the ability to encapsulate a foreign body is not determined by body mass alone. Further, although it is possible for variation in overall viability of inbred individuals to create spurious positive correlations between traits (Roff, 2002), all lines used in this study show evidence of past purging selection. Although lines show inbreeding depression in some traits, they also demonstrate improved performance in other traits. Interestingly, a recent genomic study in bumblebees (*Bombus terrestris*) also found a positive genetic association between a major quantitative trait locus for sperm number and one for antibacterial activity of the haemolymph against the insect pathogen *Paenibacillus alvei* (Wilfert *et al.*, 2007). Thus, our results, and those of Wilfert *et al.* (2007), suggest that some male genotypes in the population are likely to be successful at both sperm competition and at least one component of immunity. However, additional studies examining how immune function in *G. sigillatus* is related to other traits important to sperm competition, such as mating rate, are needed before the generality of this pattern can be tested.

We found neither phenotypic nor genetic correlations between lytic activity, PO activity and implant darkness. In the current literature, there is little consensus with respect to the strength and direction of correlations between measures of immunity. In some studies, there are trade-offs between components of immunity (Cotter *et al.*, 2008), whereas in others, measures of immunity are positively correlated (Adamo, 2004a; Rantala & Roff, 2005) or uncorrelated (Rantala & Roff, 2006). However, immunity does not represent a single unified response: components of immunity are specialized to respond to different challenges (Adamo, 2004b). Lytic activity is primarily a defence against bacterial invaders, in contrast to PO production and encapsulation, which are both involved in defence against macroparasites. Because PO is essential to the production of melanin, which in turn is needed to encapsulate foreign bodies, it would appear that PO activity and implant darkness should be positively correlated (Cotter & Wilson, 2002). However, intervening factors may prevent this positive correlation. Our assays of PO represent the total PO an individual could potentially mobilize; nevertheless, individuals may differ in their ability to actually mobilize PO. Further, implant darkness is also influenced by variation in the accumulation of hemocytes surrounding the foreign body. Clearly, relationships between components of immunity are incompletely understood (Leclerc *et al.*, 2006) and present many opportunities for future research.

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