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Food fight: sexual conflict over free amino acids in the nuptial gifts of male decorated crickets

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Abstract

In decorated crickets, *Gryllodes sigillatus*, the spermatophore that a male transfers at mating includes a gelatinous spermatophylax that the female consumes, delaying her removal of the sperm-filled ampulla. Male fertilization success increases with the length of time females spend feeding on the spermatophylax, while females may benefit by prematurely discarding the spermatophylaxes of undesirable males. This sexual conflict should favour males that produce increasingly appealing spermatophylaxes, and females that resist this manipulation. To determine the genetic basis of female spermatophylax feeding behaviour, we fed spermatophylaxes to females of nine inbred lines and found that female genotype had a major influence on spermatophylax feeding duration. The amino acid composition of the spermatophylax was also significantly heritable. There was a positive genetic correlation between spermatophylax feeding duration and the gustatory appeal of the spermatophylax. This correlation suggests that genes expressed in males that produce more manipulative spermatophylaxes are positively linked to genes expressed in females that make them more vulnerable to manipulation. Outbred females spent less time feeding on spermatophylaxes than inbred females, and thus showed greater resistance to male manipulation. Further, in a nonspermatophylax producing cricket (*Acheta domesticus*), females were significantly more prone to feeding on spermatophylaxes than outbred female *Gryllodes*. Collectively, these results suggest a history of sexually antagonistic coevolution over the consumption of nuptial food gifts.

Introduction

Sexual conflict occurs when males and females differ in their reproductive interests, and can lead to the evolution of traits that function to enhance the reproductive success of individuals of one sex while causing negative effects on members of the other sex. In some taxa, males attempt to maximize their fitness via sperm competition with structures that assist them in physically grappling for access to females, traumatically inseminating females or producing accessory gland products that improve sperm fertilization success (Chapman *et al.*, 1995; Clutton-Brock & Parker, 1995; Crudgington & Siva-Jothy, 2000). However, sexual conflict can also result in the evolution of male structures that manipulate female mating behaviour in more subtle ways.

Nuptial food gifts are edible items offered by males to females in the course of mating, and are widespread in insects (Thornhill, 1976; Vahed, 1998; Lewis & South, 2012). In crickets and katydids, males synthesize gifts from their own bodies including glandular secretions (Fedorka & Mousseau, 2002; Bussière *et al.*, 2005), complex spermatophores (Gwynne, 1997) and even parts of the males' soma that females consume (Sakaluk *et al.*, 1987; Eggert & Sakaluk, 1994). These gifts constitute a significant reproductive investment on the part of the male, and producing them can lead to morphological damage (Fedorka *et al.*, 2004; Sakaluk *et al.*, 2004) and immunological costs to the male (Leman *et al.*, 2009; Gershman *et al.*, 2010; Kerr *et al.*, 2010).

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Nuptial food gifts can also be a potential source of conflict between males and females. Although in some taxa, gifts benefit both males and females by providing direct nutritional benefits to females and their offspring (Gwynne, 1997), in other taxa, females derive no significant nutritional benefits from nuptial feeding (Will & Sakaluk, 1994; Vahed, 1998, 2007; Ivy & Sakaluk, 2005). Instead, nuptial gifts in these species serve primarily to protect male ejaculates, delaying females from interrupting sperm transfer and increasing male fertilization success (Sakaluk, 1984; Vahed, 1998; Eggert *et al.*, 2003; deCarvalho & Shaw, 2010). Females that accept males' nuptial offerings are consequently deterred from exerting post-copulatory mating preferences, and thereby relinquish some control over the paternity of their offspring. Thus, nuptial food gifts can be viewed as a 'Medea' gift, a vehicle by which males manipulate female behaviour (Arnqvist & Nilsson, 2000; Sakaluk *et al.*, 2006). Indeed, recent studies suggest that males, by offering such gifts, exploit female gustatory biases by offering an appealing gift that is essentially a sham (Sakaluk, 2000; Vahed, 2007; Warwick *et al.*, 2009).

In crickets, copulation is completed with the transfer of the spermatophore, which normally consists of a small, sperm-containing ampulla that remains secured outside the female's body at the base of her ovipositor by a narrow spermatophore tube threaded into her genital opening (Zuk & Simmons, 1997). In decorated crickets, *Gryllodes sigillatus*, the spermatophore includes a large gelatinous mass, the spermatophylax, that envelopes a sperm-containing ampulla. Immediately upon dismounting the male after spermatophore transfer, the female detaches the spermatophylax from the ampulla with her mandibles and begins to feed on it. While the female feeds on this nuptial food gift, sperm are evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female removes and consumes the ampulla, terminating sperm transfer. Females vary in the length of time that they spend consuming the spermatophylax, and the longer that the female delays removing the ampulla, the more sperm are transferred to her sperm storage organ (Sakaluk, 1984, 1985, 1987). Given that females usually mate with many males in a lifetime (Sakaluk *et al.*, 2002), the length of time that females spend consuming the spermatophylax can profoundly influence the outcome of sperm competition (Sakaluk, 1986; Sakaluk & Eggert, 1996; Calos & Sakaluk, 1998; Eggert *et al.*, 2003).

It follows from the description of *Gryllodes* mating behaviour above that males can improve their reproductive success by producing spermatophylaxes that are particularly appealing to females, because it is under these circumstances that sperm transfer is likely to be maximized. Indeed, the spermatophylax of *G. sigillatus* is composed primarily of water and numerous amino acids (Warwick, 1999; Gershman *et al.*, 2012) including alanine, serine, histidine, proline, valine, leucine, methionine, phenylalanine and tryptophan, and threonine, which have phagostimulatory effects in insects (Leckstein & Llewellyn, 1974; Srivastava & Auclair, 1974; Cook, 1977). However, females may benefit by discarding gifts of those males they find undesirable if, upon so doing, they remove the sperm ampulla, thereby terminating sperm transfer. Indeed, it is not widely appreciated that females often discard the spermatophylax by simply dropping it prior to its complete consumption in approximately 25% of all matings (Sakaluk, 1984, 1987; Gershman *et al.*, 2012).

We might expect that food gifts synthesized by different males might vary in their amino acid composition and that females might also vary in their propensity to consume food gifts. In a companion study, we compared the free amino acid profiles of nuptial food gifts that were discarded by females with those that were accepted, and calculated a fitness surface of the free amino acids based on their gustatory appeal (Gershman *et al.*, 2012). In this study, we tested the hypothesis that males vary genetically in the amino acid composition of the spermatophylax and that females differ genetically in their level of resistance to these phagostimulatory components in the spermatophylax. Further, we used the fitness surface describing the optimal combination of amino acids in spermatophylaxes provided in Gershman *et al.* (2012) to determine the genetic covariance between the multivariate attractiveness of the male spermatophylax and female resistance to consuming spermatophylaxes, an indicator of sexual conflict between males and females over the fate of the spermatophylax.

To address these issues, we used nine highly inbred lines developed in the course of previous studies (Ivy *et al.*, 2005; Ivy, 2007), each representing a random subset of genotypes from the larger outbred source population. To determine the genetic basis of amino acid composition, we sampled spermatophylaxes from each of the nine inbred lines. We employed females from the nine inbred lines to determine the genetic basis of female spermatophylax rejection behaviour. To standardize the amino acid composition of the spermatophylaxes fed to females, females were offered spermatophylaxes taken from males of each of the inbred lines. All possible combinations of female and male inbred lines were used in a fully crossed "diallel" design. To examine which inbred line amino acid combinations are most appealing to females, we used multivariate selection analysis presented in Gershman *et al.* (2012) to determine the location of the different combinations in multivariate space.

We also included two additional populations of females in tests of the gustatory appeal of spermatophylaxes: female *G. sigillatus* from the outbred source population and female house crickets, *Acheta domesticus*,

a species lacking a spermatophylax. We predicted that, as a result of a coevolved resistance to the gustatory appeal of the spermatophylax, female *G. sigillatus* would have a lower propensity for consuming spermatophalaxes than female *A. domesticus*, which have no experience with spermatophylax consumption. A similar pattern of coevolved resistance has previously been shown in these two species for female remating time, whereby female *A. domesticus* took longer to remate after consuming a spermatophylax than female *G. sigillatus*, presumably due to chemicals (most likely sex peptides) contained in the spermatophylax (Sakaluk *et al.*, 2006).

Materials and methods

Study animals

G. sigillatus used in this study were the descendants 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) were created by subjecting crickets, randomly selected from the large, panmictic population described above, to 23 generations of full-sib mating followed by 5-6 generations of panmixis within each line (Ivy *et al.*, 2005).

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 Fluker's® cricket chow (Fluker's, 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 the rearing surface area. Moistened peat moss provided in small plastic containers was made available both as an oviposition substrate and as a source of additional water.

Analysis of female spermatophylax feeding behaviour

Female crickets were collected within 48 h of adult eclosion and housed as a group for 5–7 days to ensure their sexual maturity. One spermatophylax was collected from each mature inbred line male by gently squeezing his spermatophore pouch, causing him to extrude the spermatophylax. Spermatophylaxes were immediately sealed in an airtight microcentrifuge vial and stored at −80 °C until they were scheduled to be offered to females, at which time they were thawed to room temperature. It was necessary to freeze spermatophylaxes to ensure that within each bout of mating trials, spermatophylaxes were available from all inbred lines. Each female was used in only one behavioral trial.

Behavioral trials were staged in small Plexiglas arenas (10 × 4 × 7.5 cm) during the dark phase of the light cycle under red light illumination at 31 °C. One female was introduced into each arena and allowed to acclimate for 180 s as a spermatophylax was thawed to room temperature. Using forceps, each female was then presented with a single spermatophylax resting on a small (2 mm in diameter) loop of wire. The wire loop allowed the spermatophylax to be easily handled without deforming or piercing it with the forceps. The spermatophylax was offered to the female by touching the spermatophylax to the female's palps. This process was repeated until either the female accepted the spermatophylax (i.e. took hold of it with her mouthparts) or she withdrew from it. If the female retreated, the spermatophylax and wire loop were placed on the floor of the arena immediately in front of the female's palps. The female was observed for 20 min after the spermatophylax was introduced, and the number of minutes that she fed on the spermatophylax was recorded. If a female carried a spermatophylax in her palps or maxilla, or was actively manipulating a spermatophylax with her palps without passing it to her mandibles, this was also recorded as feeding regardless of whether the female was actively ingesting the spermatophylax. This relaxed definition of feeding was used because in the context of post-copulatory behavior, all of these spermatophylax-handling behaviors effectively deter females from removing the male's sperm-filled ampulla and terminating sperm transfer. Approximately 180 females from each inbred line, the outbred colony and *A. domesticus* were fed spermatophylaxes from each of the nine inbred lines, resulting in 19–20 replicates of each cross, for a total of 1974 behavioral trials.

Amino acid analysis of male spermatophylaxes

We collected a single spermatophylax from each of 22 males per inbred line and immediately placed them in an airtight microcentrifuge vial stored at −80 °C and freeze-dried them using a Labconco Freeze-drier (Labconco, Kansas City, MO, USA). Freeze-dried cricket spermatophylaxes were weighed and then ground using a pestle in an eppendorf, with the addition of 150 μL of ethanol.

Amino acids were extracted from spermatophylaxes using an EZ:Faast™ reagent kit for free amino acid analysis (Phenomenex®, Torrance, CA, USA). One hundred microlitres of sample were pipetted into a sample vial along with 100 μL of internal standard solution (Norvaline 0.2 mM, *N*-propanol 10%). This sample was slowly drawn through a sorbent pipette tip using a 1.5-mL syringe. Two hundred microlitres of washing solution (*N*-Propanol) were added to the sample vial, and also drawn slowly through the sorbent tip. Once all the liquid had passed through the tip into the syringe, air was drawn through to drain the sorbent tip, and the liquid in the syringe was discarded, leaving the sorbent tip in the sample vial. Two hundred microlitres of

eluting medium (a 3 : 2 mix of sodium hydroxide and *N*-Propanol) were added to the sample vial. Using a 0.6-mm syringe with the piston halfway up the barrel, eluting medium was drawn into the tip until the liquid reached the filter at the top of the sorbent particles. The sorbent particles and liquid were then ejected from the tip into the vial. Fifty microlitres of chloroform were then added using a Drummond Dialamatic Microdispenser (Drummond Scientific, Broomall, PA, USA). The liquid in the vial was then emulsified by repeatedly vortexing for 5–8 s. The vial was left for 1 min to allow the reaction to proceed and the liquid to separate into two layers. The sample was then re-emulsified by vortexing for a further 5 s, and the reaction was allowed to proceed for a further minute. One hundred microlitres of iso-octane were then added using the microdispenser, and the sample vortexed for 5 s. The sample was left for a further minute for the reaction to proceed. One hundred microlitres of hydrochloric acid (1 N) were then added using a pipette and the sample vortexed for 5 s. The sample was then allowed to separate, and the top layer was pipetted into an autosampler vial for analysis by GC-MS.

We injected 0.2 μL of the extracted amino acid sample into a GC-MS (Agilent 7890A GC coupled with an Agilent 5975B Mass Spectrometer and an Agilent CTC PAL Autosampler chilled to 10 °C, Agilent Technologies, Cheshire, UK) fitted with ZB-AAA column of 10 m × 0.255 mm internal diameter, using helium as a carrier gas. The inlet was set at 325 °C, and the injection was in pulsed splitless mode. Separation of the extract was achieved following the method supplied with the kit, which used a column profile starting at 110 °C, rising at 20 °C/min to 320 °C where it was held for 1 min. The MS transfer line was set at 300 °C. Data were analysed using MSD Chemstation software (version E.02.00.493, Agilent Technologies) and amino acids were quantified based on standard solutions provided in the EZ:Faast™ kit. A range of standard solutions varying in concentration were prepared and calibration curves created for each amino acid, enabling us to measure the absolute quantity of each amino acid (measured in nanomoles per millilitre of internal standard) present in a spermatophylax.

We measured the following 22 free amino acids using the EZ:Faast™ kit: Alanine (ALA), Glycine (GLY), α-aminobutyric acid (AAA), Valine (VAL), Leucine (LEU), Isoleucine (ILE), Threonine (THR), Serine (SER), Proline (PRO), Asparagine (ASN), Aspartic acid (ASP), Methionine (MET), 4-Hydroxyproline (4-HYP), Glutamic acid (GLU), Phenylalanine (PHE), Glutamine (GLN), Orthinine (ORN), Glycyl-proline (GPR), Lysine (LYS), Histidine (HIS), Tyrosine (TYR) and Tryptophan (TRP). Three amino acids (AAA, ORN and GPR) were not present in all spermatophylax samples and were therefore excluded from further analysis. As the quantity of each amino acid was measured in absolute amounts, it was necessary to correct the amount of each amino acid to the weight of the spermatophylax being analysed. Consequently, we divided the amount of each amino acid by the weight of the spermatophylax and therefore our data for each amino acid are expressed in units of nanomoles per millitre of internal standard per gram of spermatophylax (nmol/mL/mg). Data for each amino acid were $\log_{10}$ transformed prior to analysis to ensure normality.

Statistical analysis

Analysis of female spermatophylax feeding behaviour

We used contingency table analysis to examine separately the effects of female line and male line on the proportion of females that accepted the spermatophylax. To evaluate the effects of female line on spermatophylax feeding duration, we used a random effects model including female line, male line (i.e. the inbred line from which the spermatophylax originated) and their interaction as independent variables and spermatophylax feeding duration ($\log_{10}$ transformed to achieve normality) as the dependent variable.

For comparisons between inbred and outbred crickets, it was necessary to correct for the possibility that results could have been confounded by high levels of genetic relatedness between individuals within lines. Individuals within inbred lines share a single genetic origin and so cannot be considered genetically independent, whereas outbred populations are likely to be considerably more genetically heterogeneous in comparison. Thus, spermatophylax feeding duration in inbred and outbred crickets was compared by contrasting average values for each line versus values for each outbred individual using an ANOVA model with female line included as a random effect.

For comparisons between outbred *G. sigillatus* and *A. domesticus*, we used a univariate mixed model ANOVA including female species (fixed effect), male line, and their interaction (random effects) as the independent variables and spermatophylax feeding duration as the dependent variable. As neither male line nor the interaction terms were statistically significant ($P > 0.2$), they were omitted from the final model after model simplification.

Quantitative genetic analysis

Due to the large number of response variables, we examined differences in the free amino acid composition of the spermatophylax across male lines using a discriminant function analysis. We assessed the number of significant discriminant functions using Wilks' Lambda and we interpret factor loadings of the individual free amino acids to these functions as biologically relevant if they were 0.20 or above (Tabachnick & Fidell, 1989). We assessed the adequacy of our discriminant functions to correctly predict group membership

using the cross-validated group classification (Tabachnick & Fidell, 1989).

We estimated the heritabilities of female feeding duration and the quantity of each amino acid in the spermatophylax from our inbred lines by calculating the coefficient of intraclass correlation (t) (Hoffmann & Parsons, 1988; David *et al.*, 2005) as:

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

where n is the number of lines (in our case 9) and V_b and V_w are the between-line and within-line variance components, respectively, estimated directly from an ANOVA including line (male or female) as a random effect. The standard error of the intraclass correlation (SE(t)) was calculated according to Becker (1984) as:

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

where k is the number of individuals sampled within each line (in our case 20 for female feeding behaviour and 22 for the amino acid composition of male spermatophylaxes). The heritability (h^2) of each trait was then estimated according to Hoffmann & Parsons (1988) as:

$$h^2 = \frac{2}{\left(\frac{1}{t} - 0.5\right)} \quad (3)$$

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

$$SE(h^2) = \frac{2}{\left(1 - \frac{t}{2}\right)^2} SE(t) \quad (4)$$

We estimated the genetic correlations (r_A) and their standard errors between the different free amino acids in the male spermatophylax using the jackknife method of Roff & Preziosi (1994). In short, this procedure first estimates the genetic correlation between two traits using inbred line means. A sequence of N (in our case 9) pseudo-values is then computed by dropping, in turn, each of the lines and estimating the resulting correlations using the formula:

$$S_{N,i} = Nr_N - (N-1)r_{N-1,i} \quad (5)$$

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 $r_{N\text{-}1,i}$ is the genetic correlation obtained by dropping the ith inbred line alone (Roff & Preziosi, 1994). The jacknife estimate of the genetic correlation (r_j) is then simply the mean of the pseudo-values and an estimate of the standard error (SE) is given by:

$$SE = \frac{\sum_{i=1}^{i=N} (S_{N,i} - r_j)^2}{N(N-1)} \quad (6)$$

Using simulation models, Roff & Preziosi (1994) showed that this jacknife 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).

Previously, we used a multivariate selection analysis to demonstrate that the free amino acid composition of the male spermatophylax significantly influenced whether females would prematurely discard the spermatophylax after mating (Gershman *et al.*, 2012). Importantly, this study showed that female feeding behaviour exerted significant linear and nonlinear sexual selection on the free amino acid composition of the male spermatophylax (Gershman *et al.*, 2012). Here, we use the results from this analysis to assign "multivariate attractiveness" to spermatophylaxes derived from males from the lines based on their free amino acid composition. Spermatophylaxes with higher multivariate attractiveness have a greater gustatory appeal and are therefore fed upon for a longer duration by females (i.e. not prematurely discarded). As our selection analysis was based on principal component (PC) scores derived from the 19 individual amino acids, it was necessary to first project the amino acid composition of male spermatophylaxes from the inbred lines into the multivariate space described by the selection analysis. This was achieved by substituting the amount of each amino acid present in the spermatophylaxes of males from the inbred lines into the linear equation (i.e. eigenvector) describing each PC in the selection analysis. On the basis of our selection analysis, we determined the equation that best described the effects of amino acid composition on male fitness (w, measured as the acceptance or rejection of the spermatophylax by outbred females) given by the vector of linear selection gradients (Eqn 7):

$$w = (-0.034PC1) + (-0.177PC2) + (-0.181PC3) \quad (7)$$

where PC1, PC2 and PC3 represent the three principal components that describe the variation in amino acid composition of the spermatophylax in our selection analysis (see Table 2 in Gershman *et al.*, 2012). Using this equation, we calculated a multivariate attractiveness value for each spermatophylax produced by an inbred male (see Jia & Greenfield (1997) for an application of this approach to female mating preferences for male acoustic traits). We then estimated the heritability of the multivariate attractiveness of the spermatophylax, as well as the genetic correlation between spermatophylax attractiveness and female feeding duration, using the statistical procedures outlined above.

Heritability estimates and genetic correlations were considered statistically significant if the estimates divided by their standard errors were greater than 1.96, rejecting the null hypothesis of no correlation with a two tailed t-distribution and infinite degrees of freedom (Zar, 1999). It is important to note that estimates of

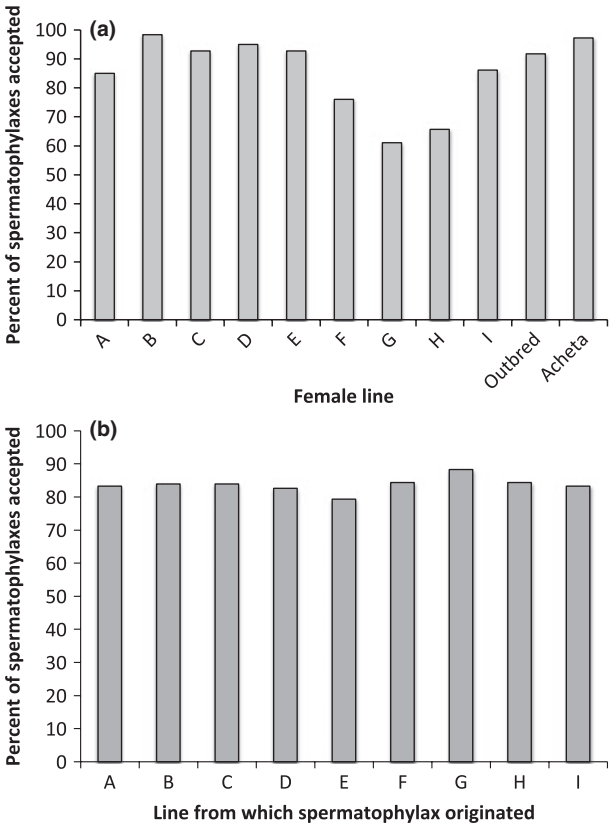

Figure 1 (a) Percent of spermatophylaxes accepted by inbred female *G. sigillatus* (lines A-I), outbred female *G. sigillatus*, and female *A. domesticus*, and (b) per cent of spermatophylaxes accepted by female *G. sigillatus* as a function of the line from which the spermatophylax originated.

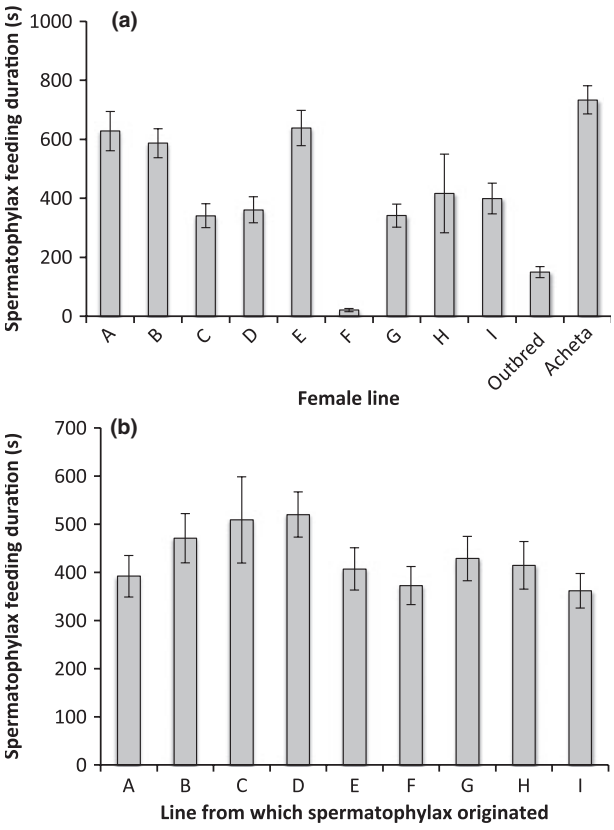

Figure 2 (a) Mean (± SE) spermatophylax feeding duration of female *G. sigillatus* from inbred lines (A-I), outbred female *G. sigillatus*, and female *A. domesticus*, and (b) Mean (± SE) duration of feeding by female *G. sigillatus* on spermatophylaxes from inbred lines (A-I).

genetic (co)variance from inbred lines contain variance due to dominance and/or epistasis and therefore represent broad-sense estimates (Falconer & Mackay, 1996).

Results

There was a significant effect of female line (Fig. 1a; $\chi^2_8 = 184.1$, $P < 0.0001$) but not male line (Fig. 1b; $\chi^2_8 = 5.6$, $P = 0.69$) on the willingness of females to initially accept a spermatophylax. There was a significant effect of both female (Fig. 2a; $F_{8,65.20} = 95.61$, $P = 0.0001$) and male line (Fig. 2b; $F_{8,68.68} = 2.35$, $P = 0.027$) on the feeding duration of inbred females, but there was no significant interaction between these main effects ($F_{64,1244} = 1.10$, $P = 0.28$). In agreement with this finding, we found that the duration of spermatophylax feeding by females was highly heritable ($h^2 = 0.98 \pm 0.007$).

Outbred *G. sigillatus* females were more likely to completely reject spermatophylaxes than were *A. domesticus* females (Fig. 1a; $\chi^2_1 = 5.29$; $P = 0.0187$). Further, outbred *G. sigillatus* females fed on spermatophylaxes for significantly less time than female *A. domesticus* (Fig. 2a; $F_{1,338} = 124.57$, $P < 0.001$). For outbred female *G. sigillatus* and *A. domesticus*, male line had little effect on whether females accepted spermatophylaxes (outbred *G. sigillatus*: $\chi^2_8 = 9.21$, $P = 0.33$; *A. domesticus*: $\chi^2_8 = 8.9$, $P = 0.35$). Male spermatophylax line also had little effect on spermatophylax feeding duration (outbred *G. sigillatus*: $F_{8,156} = 0.66$, $P = 0.73$; *A. domesticus*: $F_{8,166} = 1.52$, $P = 0.14$).

Outbred females were more likely to accept spermatophylaxes than inbred females (Fig. 1a; $\chi^2_1 = 7.84$; $P = 0.0051$). However, outbred female *G. sigillatus* fed on spermatophylaxes for significantly less time than inbred female *G. sigillatus* (Fig. 2a; $F_{1,188} = 11.90$, $P = 0.001$). Contrasts between each line and the outbred group (applying the sequential Bonferroni correction) revealed that outbred females had significantly shorter spermatophylax feeding durations than females from every inbred line with the exception of females from line F, which fed on spermatophylaxes for a significantly shorter time than outbred females (Fig. 2a). Variance in spermatophylax feeding duration

also differed between inbred and outbred *G. sigillatus*: inbred females (pooled across all lines) had a greater variance in spermatophylax feeding duration than outbred females (Levene's test for unequal variance, $F_{1, 1792} = 61.40$, $P < 0.0001$).

Our discriminant function analysis revealed that males could be successfully classified to their genetic lines based on the free amino acid composition of the spermatophylax in 92.4% of cross-validated cases. A total of eight significant functions (Wilks' Lambda = 0.001, d.f. = 152, $P = 0.0001$) were extracted from our discriminant function analysis and together they explained all of the variation in free amino acids in the spermatophylax across male lines (Table 1). With the exception of ASN, all free amino acids contributed significantly to at least one of the discriminant functions (Table 1). The first discriminant function (DF1) is positively loaded by eight free amino acids (VAL, ILE, SER, PRO, ASP, GLN, LYS, HIS) and negatively loaded by one amino acid (PHE), and therefore describes the trade-off between these amino acids, whereas DF2 is negatively loaded by SER, GLN and TYR and therefore describes a decrease in these three amino acids (Fig. 3, Table 1). DF3 describes an increase in five amino acids (ILE, THR, MET, PHE, TRP), while DF4 describes a decrease in two amino acids (MET, TRP) (Table 1). DF5 describes an increase in five amino acids (LEU, THR, MET, 4-HYP, TYR) and DF6 describes the trade-off between ASP and GLN (negatively loaded) and TYR (positively loaded) (Table 1). DF7 describes an increase in 12 of the 19 free amino acids (ALA, GLY, VAL, LEU, PRO, MET, GLU, PHE, GLN, LYS, TYR, TRP), whereas DF8 describes an increase in PRO and 4-HYP (Table 1).

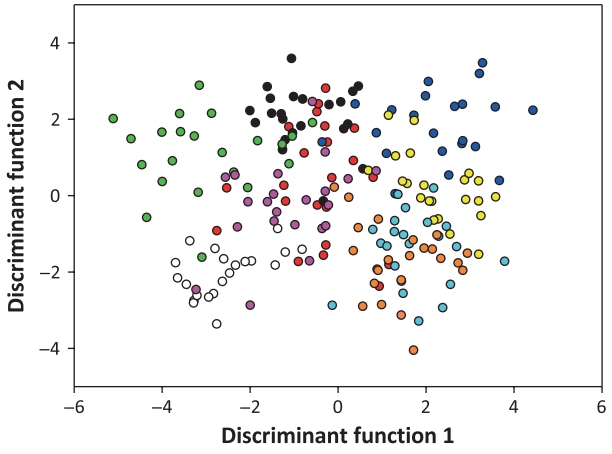

Figure 3 The first two discriminant functions (DF1 and DF2) showing the separation between inbred lines in the free amino acid of the male spermatophylax. DF1 explains 30.40% of the variation in free amino acids, while DF2 explains a further 17.12% of this variation. The different male lines are colour coded: line A = red, line B = black, line C = green, line D = dark blue, line E = white, line F = yellow, line G = cyan, line H = orange and line I = pink.

Table 2 presents the line means (± SE) for the different free amino acids, as well as the univariate GLMs examining the differences in these individual amino acids across lines. With the exception of ASN, all free amino acids differed significantly across lines after Bonferroni correction for multiple comparisons ($\alpha < 0.0026$) (Table 2). Consistent with these line differences, we found that all amino acids were significantly heritable with the lowest heritability estimate being 0.60 ± 0.13 for ASN (Table 3). Moreover, there was a high degree of genetic covariation between the different amino acids (Table 3). In most cases, the different amino acids were positively genetically correlated, the most notable exception being PHE, which was negatively genetically correlated with 12 of the 18 amino acids (Table 3). Using Eqn 7, we calculated the multivariate attractiveness of each spermatophylax collected from the male line and found that this measure was highly heritable (0.925 ± 0.036). Furthermore, the genetic correlation between this attractiveness measure of the male spermatophylax and female feeding time was significantly positive (0.623 ± 0.049) indicating that the genes expressed in males to produce spermatophylaxes that manipulate females to feed for longer durations (i.e. high multivariate attractiveness) are linked to genes that make females more susceptible to feeding on spermatophylaxes for longer durations (i.e. females are less able to exert post-copulatory choice).

Discussion

The chase-away model of sexual conflict posits that males and females are locked in a cycle of antagonistic coevolution in which males evolve increasingly persuasive display traits to induce females to mate, while females resist this pressure by decreasing their responsiveness to these traits (Holland & Rice, 1998). An underlying assumption of this model is that females vary in their resistance to the male trait and that this variation is significantly heritable. In *Gryllodes sigillatus*, males offer females nuptial food gifts that confer few, if any, direct benefits to females (Will & Sakaluk, 1994; Ivy & Sakaluk, 2005; but see Ivy *et al.*, 1999), but which increase male fertilization success, thereby furthering males' interests. This should often lead to a sexual conflict, particularly in those situations where the female would benefit by discarding the gift of an undesirable mate, enabling her to terminate sperm transfer. This sexual conflict should, in turn, select for increased resistance in females to the gustatory appeal of the spermatophylax generating the cycle of antagonistic coevolution captured in Holland & Rice's (1998) model. Here, we found that inbred females from different lines differed greatly in their willingness to accept the spermatophylax and the time they spent feeding on it, and that this variation was significantly heritable. Thus, it appears that females vary in their resistance to the gustatory appeal of males' nuptial food gifts, leav-

ing open the potential for further antagonistic coevolution between the composition of nuptial food gifts and females' responses to them.

Warwick and his colleagues (Warwick, 1999; Warwick *et al.*, 2009) were the first to propose that free amino acids in the spermatophylax influence its gustatory appeal to females based on several lines of evidence: (1) free amino acids constitute a significant component of the solid fraction of the spermatophylax (Warwick, 1999; Warwick *et al.*, 2009), (2) free amino acids are known phagostimulants in a diversity of insect species (Calatayud *et al.*, 2002; Kugimiya *et al.*, 2003) and (3) artificial 'gels' containing the four most abundant free amino acids found in the spermatophylax and fed to female *G. sigillatus* resulted in increased feeding time relative to females fed control gels (Warwick *et al.*, 2009). Here, we show that the amino acid composition of the spermatophylax is heritable, both in terms of each individual amino acid and the overall multivariate attractiveness of the spermatophylax. These results suggest that there is the potential for sexual selection to act on the amino acid composition of the spermatophylax as well as female resistance to consuming the spermatophylax, leading to conflict between males and females over the fate of the spermatophylax. Further, in a companion study (Gershman *et al.*, 2012), we performed selection analysis on the amino acid composition of spermatophylaxes that were preferentially consumed by females. The finding of a significant male line effect in our feeding experiment further corroborates this finding and illustrates that the composition of amino acids that a male allocates to his spermatophylax significantly alters the feeding behaviour of his mating partner and influences his eventual reproductive success. Using an index of spermatophylax attractiveness based on the fitness surface, we were able to demonstrate that the genetic correlation between spermatophylax attractiveness and female feeding duration in the present study was positive. This positive genetic correlation suggests that genes expressed in males that make more manipulative spermatophylaxes (i.e. amino acid combinations that stimulate females to feed for longer periods) are positively linked to genes expressed in females that make them more vulnerable to being manipulated (i.e. feed for longer rather than discarding the spermatophylax prematurely and exerting post-copulatory choice). Other components of the spermatophylax in addition to amino acids may also vary (e.g., protein content), and these attributes too might influence females' acceptance of food gifts.

Although suggestive, the genetic correlation between male manipulation and female resistance shown here cannot be taken as diagnostic of sexual conflict. As Rowe & Day (2006) illustrate, sexual conflict is a complex, continually evolving process during which the sign of the genetic correlation between female resistance and

Table 1 Discriminant function analysis to identify the major dimensions of free amino acids that differ between the different genetic lines. Trait loadings $\geq$ 0.20 are considered biologically significant and are shown in bold.

	Discriminant Functions							
	1	2	3	4	5	6	7	8
Eigenvalue	3.73	2.10	1.90	1.56	1.17	0.98	0.50	0.32
% variance	30.40	17.12	15.53	12.74	9.63	8.10	4.13	2.35
Canonical correlation	0.89	0.82	0.81	0.78	0.73	0.70	0.58	0.49
Amino Acids								
ALA	0.10	−0.09	−0.11	0.02	0.15	−0.03	**0.24**	0.17
GLY	0.14	−0.10	0.03	−0.13	0.07	0.01	**0.29**	0.17
VAL	**0.41**	−0.11	0.17	−0.06	0.06	0.02	**0.24**	0.08
LEU	−0.01	−0.08	0.12	0.04	**0.21**	0.18	**0.34**	0.04
ILE	**0.28**	−0.12	**0.34**	−0.08	0.02	0.00	0.14	0.18
THR	0.12	−0.07	**0.36**	0.17	**0.21**	0.07	0.01	0.08
SER	**0.20**	**−0.20**	−0.13	−0.15	0.04	0.05	0.16	0.17
PRO	**0.20**	0.01	0.00	−0.19	0.04	0.02	**0.31**	**0.20**
ASN	0.03	−0.15	0.14	−0.01	0.00	0.02	0.18	−0.13
ASP	**0.34**	−0.04	−0.05	−0.11	−0.01	**−0.25**	0.15	0.12
MET	0.08	0.03	**0.25**	**−0.25**	**0.21**	−0.03	**0.28**	0.08
4-HYP	0.17	−0.07	−0.04	0.06	**0.20**	0.00	0.14	**0.40**
GLU	0.13	−0.19	−0.00	−0.19	−0.14	−0.18	**0.32**	0.15
PHE	**−0.21**	0.12	**0.24**	0.04	0.05	0.10	**0.37**	−0.03
GLN	**0.36**	**−0.23**	−0.06	0.01	0.13	**−0.20**	**0.40**	0.01
LYS	**0.44**	0.02	0.11	−0.02	−0.15	0.17	**0.32**	−0.03
HIS	**0.33**	−0.06	0.10	−0.17	−0.01	0.00	0.16	−0.04
TYR	0.08	**−0.21**	0.08	−0.19	**0.22**	**0.23**	**0.26**	−0.07
TRP	−0.11	0.05	**0.32**	**−0.22**	0.12	0.03	**0.24**	0.04

Table 2 Mean mass (± standard error (μg)) of each amino acid contained in the male spermatophylax for each inbred line. *P*-values in bold are statistically significant with a Bonferroni correction for multiple comparisons ($P < 0.0026$).

	Male Inbred Lines									ANOVA	
Amino Acid	*A*	*B*	*C*	*D*	*E*	*F*	*G*	*H*	*I*	$F_{8,197}$	*P*
ALA	276.33 (32.27)	185.76 (23.68)	265.30 (44.33)	336.55 (27.91)	266.24 (35.63)	317.87 (34.27)	352.61 (37.48)	342.91 (53.52)	407.43 (38.78)	3.34	**0.001**
GLY	758.90 (87.07)	566.04 (73.32)	780.55 (114.34)	935.01 (76.73)	842.39 (85.81)	1352.20 (130.17)	1241.16 (124.11)	939.32 (155.38)	913.17 (93.63)	4.28	**0.0001**
VAL	23.63 (3.84)	24.04 (3.37)	19.07 (3.00)	63.03 (19.82)	23.88 (4.12)	96.90 (8.96)	78.68 (10.14)	59.97 (9.86)	29.25 (3.96)	17.80	**0.0001**
LEU	6.52 (0.83)	11.01 (1.34)	14.03 (2.46)	9.59 (0.87)	17.33 (4.21)	16.48 (1.87)	14.40 (1.74)	12.80 (2.59)	26.56 (4.95)	4.39	**0.0001**
ILE	6.07 (0.61)	8.13 (1.01)	8.34 (1.53)	8.14 (0.69)	6.92 (0.75)	25.14 (2.43)	17.34 (2.03)	14.72 (2.35)	6.87 (0.74)	13.63	**0.0001**
THR	6.34 (1.06)	18.85 (2.56)	17.80 (3.14)	10.41 (1.84)	9.96 (1.29)	40.13 (6.24)	20.34 (4.06)	38.91 (7.13)	17.50 (2.28)	9.79	**0.0001**
SER	223.43 (29.19)	104.37 (14.20)	110.57 (20.45)	241.01 (24.18)	236.83 (33.08)	286.27 (29.29)	339.56 (47.75)	256.26 (50.61)	205.68 (21.72)	7.57	**0.0001**
PRO	860.52 (68.20)	674.99 (87.89)	768.44 (107.75)	1196.73 (71.01)	769.23 (86.51)	1503.25 (106.65)	1283.09 (131.24)	862.71 (124.45)	896.57 (94.74)	6.24	**0.0001**
ASN	8.69 (0.94)	11.27 (1.35)	16.45 (3.74)	10.62 (1.18)	15.96 (1.64)	16.30 (1.97)	19.70 (2.55)	18.19 (3.22)	12.76 (1.46)	2.62	0.01
ASP	34.12 (3.90)	16.83 (3.54)	21.41 (3.39)	60.04 (6.58)	18.15 (3.37)	53.16 (5.60)	55.52 (6.87)	52.77 (9.63)	20.47 (3.02)	12.43	**0.0001**
MET	2.60 (0.32)	3.17 (0.48)	6.05 (1.35)	4.13 (0.35)	4.07 (0.39)	9.05 (0.83)	4.10 (0.54)	3.31 (0.58)	3.72 (0.43)	8.03	**0.0001**
4-HYP	3.55 (0.58)	2.01 (0.40)	2.63 (0.71)	4.14 (0.69)	1.96 (0.33)	5.85 (0.85)	4.37 (0.56)	6.47 (1.44)	6.28 (1.02)	5.52	**0.0001**
GLU	184.52 (23.28)	80.88 (14.99)	172.55 (32.33)	161.62 (16.30)	179.57 (23.63)	207.66 (20.19)	357.81 (44.20)	178.94 (32.83)	114.94 (14.35)	7.16	**0.0001**
PHE	1.08 (0.18)	3.07 (0.44)	5.06 (1.12)	1.23 (0.14)	2.30 (0.36)	2.07 (0.28)	1.99 (0.34)	1.10 (0.21)	2.92 (0.46)	9.19	**0.0001**
GLN	78.87 (12.27)	33.23 (7.05)	57.74 (10.80)	226.14 (28.09)	76.49 (14.62)	191.61 (23.49)	323.01 (48.47)	283.53 (52.43)	125.66 (21.69)	17.61	**0.0001**
LYS	8.07 (1.58)	13.09 (2.07)	4.67 (1.21)	23.43 (2.65)	4.91 (0.83)	29.70 (3.96)	41.00 (6.40)	16.22 (3.12)	8.63 (1.71)	19.94	**0.0001**
HIS	14.54 (2.36)	13.11 (2.07)	9.27 (2.04)	33.76 (4.49)	14.73 (2.47)	65.50 (9.86)	46.55 (8.83)	29.55 (6.15)	11.16 (2.48)	11.46	**0.0001**
TYR	1.02 (0.12)	1.26 (0.25)	1.23 (0.23)	1.72 (0.17)	3.46 (0.63)	3.47 (0.42)	2.34 (0.34)	1.97 (0.41)	2.92 (0.52)	7.09	**0.0001**
TRP	0.21 (0.03)	0.37 (0.05)	0.61 (0.11)	0.24 (0.02)	0.45 (0.05)	0.62 (0.08)	0.30 (0.04)	0.19 (0.03)	0.33 (0.04)	8.80	**0.0001**

male manipulation can vary in strength and sign, making it an unreliable signal of sexual conflict. Nevertheless, the positive genetic correlation we show suggests that as males are selected to become more manipulative, females should coevolve to become less resistant. Although this finding is interesting, it is not new: a large number of studies on *Drosophila melanogaster* have used experimental evolution to show that female resistance and male manipulation coevolve (Holland & Rice, 1999; Wigby & Chapman, 2004), which requires significant genetic covariance between these traits, and that both male manipulation (Linder & Rice, 2005) and female resistance (Civetta & Clarke, 2000) are heritable. However, fewer studies have directly quantified the genetic correlation between female resistance and male manipulation. To our knowledge, only one other study involving seed beetles (*Callosobruchus maculatus*) has provided such an estimate, and this too revealed a significant positive genetic correlation between male manipulation and female susceptibility to manipulation (Gay *et al.*, 2011).

The fact that females of the nonspermatophylax-producing *A. domesticus* readily accept and consume the spermatophylax, despite its novelty and the absence of nutritional benefits, suggests that the spermatophylax functions as a form of sensory trap designed to elicit a feeding response in females (Sakaluk, 2000). If this were indeed the case, we would predict that *A. domesticus* females should be more responsive to this gustatory trap compared with female *G. sigillatus* females that have had the opportunity to evolve resistance to the gustatory

Table 3 Heritabilities (along diagonal) and genetic correlations (below diagonal) for the 19 free amino acids in the male spermatophylax of *Grylloides sigillatus*. Standard errors for these estimates are provided in parenthesis. Estimates in bold are statistically significant at $P < 0.05$.

	ALA	GLY	VAL	LEU	ILE	THR	SER	PRO	ASN	ASP	MET	4-HYP	GLU	PHE	GLN	LYS	HIS	TYR	TRP
ALA	**0.69**																		
	(0.11)																		
GLY	**0.75**	**0.76**																	
	(0.03)	**(0.09)**																	
VAL	**0.52**	**0.85**	**0.94**																
	(0.03)	**(0.01)**	**(0.03)**																
LEU	**0.41**	**0.43**	**0.31**	**0.77**															
	(0.08)	**(0.02)**	**(0.08)**	**(0.09)**															
ILE	**0.27**	**0.80**	**0.92**	**0.39**	**0.93**														
	(0.05)	**(0.04)**	**(0.00)**	**(0.13)**	**(0.04)**														
THR	**0.10**	**0.37**	**0.62**	**0.69**	**0.78**	**0.90**													
	(0.03)	**(0.13)**	**(0.06)**	**(0.09)**	**(0.02)**	**(0.05)**													
SER	**0.79**	**0.85**	**0.73**	**0.12**	**0.51**	0.03	**0.87**												
	(0.02)	**(0.01)**	**(0.01)**	**(0.04)**	**(0.02)**	(0.06)	**(0.06)**												
PRO	**0.61**	**0.92**	**0.86**	**0.23**	**0.79**	0.27	**0.76**	**0.84**											
	(0.03)	**(0.00)**	**(0.02)**	**(0.06)**	**(0.08)**	(0.21)	**(0.01)**	**(0.07)**											
ASN	**0.20**	**0.55**	**0.51**	**0.66**	**0.63**	**0.67**	**0.29**	**0.26**	**0.60**										
	(0.06)	**(0.03)**	**(0.06)**	**(0.12)**	**(0.03)**	**(0.08)**	**(0.08)**	**(0.09)**	**(0.13)**										
ASP	**0.63**	**0.76**	**0.84**	**−0.14**	**0.65**	**0.16**	**0.76**	**0.83**	0.15	**0.92**									
	(0.10)	**(0.02)**	**(0.00)**	**(0.07)**	**(0.03)**	**(0.10)**	**(0.02)**	**(0.01)**	(0.11)	**(0.04)**									
MET	**0.24**	**0.78**	**0.74**	**0.50**	**0.99**	**0.71**	**0.33**	**0.81**	**0.47**	**0.46**	**0.87**								
	(0.04)	**(0.10)**	**(0.23)**	**(0.05)**	**(0.29)**	**(0.20)**	**(0.15)**	**(0.14)**	**(0.04)**	**(0.11)**	**(0.06)**								
4-HYP	**0.87**	**0.74**	**0.73**	**0.39**	**0.58**	**0.43**	**0.72**	**0.68**	**0.15**	**0.68**	**0.47**	**0.82**							
	(0.00)	**(0.02)**	**(0.03)**	**(0.07)**	**(0.06)**	**(0.05)**	**(0.03)**	**(0.04)**	**(0.07)**	**(0.06)**	**(0.19)**	**(0.08)**							
GLU	**0.63**	**0.82**	**0.61**	0.01	**0.53**	−0.00	**0.76**	**0.67**	**0.52**	**0.73**	**0.36**	**0.44**	**0.86**						
	(0.22)	**(0.02)**	**(0.02)**	(0.08)	**(0.03)**	(0.05)	**(0.04)**	**(0.01)**	**(0.10)**	**(0.02)**	**(0.05)**	**(0.11)**	**(0.06)**						
PHE	**−0.43**	**−0.30**	**−0.42**	**0.50**	**−0.13**	0.24	**−0.74**	**−0.32**	**0.25**	**−0.65**	**0.19**	**−0.46**	**−0.36**	**0.89**					
	(0.07)	**(0.07)**	**(0.08)**	**(0.06)**	**(0.06)**	(0.14)	**(0.05)**	**(0.08)**	**(0.11)**	**(0.01)**	**(0.09)**	**(0.06)**	**(0.03)**	**(0.05)**					
GLN	**0.84**	**0.82**	**0.83**	**0.23**	**0.60**	**0.30**	**0.84**	**0.73**	**0.45**	**0.89**	**0.35**	**0.79**	**0.79**	**−0.57**	**0.94**				
	(0.03)	**(0.02)**	**(0.01)**	**(0.03)**	**(0.02)**	**(0.06)**	**(0.01)**	**(0.03)**	**(0.07)**	**(0.00)**	**(0.06)**	**(0.01)**	**(0.05)**	**(0.04)**	**(0.03)**				
LYS	**0.36**	**0.69**	**0.92**	**0.17**	**0.77**	**0.42**	**0.65**	**0.80**	**0.37**	**0.78**	**0.48**	**0.57**	**0.51**	**−0.43**	**0.70**	**0.91**			
	(0.07)	**(0.04)**	**(0.00)**	**(0.08)**	**(0.01)**	**(0.06)**	**(0.06)**	**(0.01)**	**(0.12)**	**(0.02)**	**(0.16)**	**(0.05)**	**(0.09)**	**(0.10)**	**(0.04)**	**(0.04)**			
HIS	**0.43**	**0.85**	**0.97**	0.14	**0.89**	**0.45**	**0.75**	**0.90**	**0.44**	**0.87**	**0.76**	**0.60**	**0.65**	**−0.48**	**0.79**	**0.91**	**0.87**		
	(0.08)	**(0.02)**	**(0.00)**	(0.13)	**(0.02)**	**(0.11)**	**(0.01)**	**(0.01)**	**(0.06)**	**(0.00)**	**(0.21)**	**(0.07)**	**(0.01)**	**(0.07)**	**(0.01)**	**(0.00)**	**(0.06)**		
TYR	**0.52**	**0.77**	**0.57**	**0.71**	**0.61**	**0.46**	**0.65**	**0.58**	**0.69**	0.25	**0.70**	**0.43**	**0.45**	−0.07	**0.49**	**0.35**	**0.56**	**0.89**	
	(0.05)	**(0.02)**	**(0.11)**	**(0.01)**	**(0.12)**	**(0.14)**	**(0.03)**	**(0.09)**	**(0.02)**	(0.13)	**(0.08)**	**(0.16)**	**(0.05)**	(0.16)	**(0.06)**	**(0.12)**	**(0.09)**	**(0.05)**	
TRP	**−0.26**	0.24	0.06	**0.49**	0.46	**0.43**	−0.32	0.21	**0.44**	−0.24	**0.79**	−0.20	0.01	**0.76**	**−0.26**	−0.14	0.08	**0.40**	**0.63**
	(0.05)	(0.21)	(0.31)	**(0.06)**	(0.29)	**(0.17)**	(0.18)	(0.28)	**(0.07)**	(0.14)	**(0.01)**	(0.17)	(0.05)	**(0.03)**	**(0.10)**	(0.20)	(0.31)	**(0.17)**	**(0.12)**

appeal of the spermatophylax. In support of this prediction, we found that female *G. sigillatus* were significantly more resistant to consuming spermatophylaxes than female *A. domesticus*. This result parallels earlier work exploring the differential effect of refractory-inducing substances contained in the spermatophylax (Sakaluk *et al.*, 2006; Gordon *et al.*, 2012). Female *A. domesticus* allowed to consume food gifts of male *G. sigillatus* took significantly longer to remate than females given no such opportunity. In contrast, the consumption of food gifts has no comparable effect on the propensity to remate in female *G. sigillatus* (Sakaluk *et al.*, 2006). Collectively, these results provide support to the idea that female physiology and behaviour in regards to the ingestion of the spermatophylax have coevolved antagonistically with the chemical composition of the spermatophylax.

We found that outbred female crickets spent less time consuming spermatophylaxes than did females from eight of the nine inbred lines and exhibited a lower variance in spermatophylax consumption duration compared with inbred females. Female resistance to the amino acid composition of the male spermatophylax is likely to be a complex process that involves numerous physiological, morphological and behavioural traits that enable the female to detect combinations of amino acids, as well as chew and discard the spermatophylax. We posit that females from eight of our nine inbred lines (the exception being females from line F) are deficient in one or more of these traits, leading to variable degrees of decreased resistance to feeding on spermatophylaxes relative to outbred females.

In conclusion, we were able to find intra- and interspecific support for the occurrence of sexually antagonistic coevolution over the consumption of nuptial food gifts. Although sexually antagonistic coevolution has been demonstrated in other arenas of conflict, the genetic underpinnings of the conflict over nuptial feeding have not previously been addressed.

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