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Biting off more than you can chew: sexual selection on the free amino acid composition of the spermatophylax in decorated crickets

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Nuptial food gifts function to enhance male fertilization success, but their consumption is not always beneficial to females. In decorated crickets, the spermatophore transferred at mating includes a gelatinous mass, the spermatophylax, which is consumed by females after mating. However, females often discard spermatophylaxes shortly after mating, whereupon they terminate sperm transfer. We hypothesized that females discard gifts based on their assessment of the gift itself, and specifically the composition of free amino acids. We tested this hypothesis by comparing spermatophylaxes discarded by females after mating with those that were destined to be fully consumed, and employed multivariate selection analysis to quantify the strength and form of multivariate sexual selection operating on the free amino acid composition of gifts. The analysis yielded a saddle-shaped fitness surface with two local peaks. Different amino acid profiles appear to elicit continued feeding on the spermatophylax either because they offer the same level of gustatory appeal, or because they differentially affect both the gustatory appeal and texture of the spermatophylax. We conclude that the gustatory response of females to males' nuptial food gifts represents an important avenue of post-copulatory mate choice, imposing significant sexual selection on the free amino acid composition of the spermatophylax.

Keywords: *Gryllodes sigillatus*; gustation; multivariate selection analysis; post-copulatory female mate choice; spermatophore

1. INTRODUCTION

In many insects, males are required to provide costly nuptial food gifts to females to entice females to mate or to ensure complete sperm transfer once copulation has commenced [1–3]. Nuptial food gifts probably evolved as a result of a sexual conflict over the fate of the male's ejaculate because, in gift-giving species, females typically exert control over sperm transfer and usage [4,5]. Because it is in the interests of males to have all of their sperm used in fertilizations, such control is inimical to their reproductive interests.

Crickets offer an ideal model with which to examine the sexual conflict over sperm usage: the ejaculate of a male typically remains attached outside the female's genital opening after mating in the form of an externally attached spermatophore, and females are thus well positioned to determine the fate of their mates' gametes through the selective removal of this spermatophore. In decorated crickets, *Gryllodes sigillatus*, the spermatophore includes a large gelatinous mass, the spermatophylax, that envelopes a sperm-containing ampulla. Immediately upon dismounting from the male after spermatophore transfer, the female detaches the spermatophylax from the ampulla with her mandibles and begins to consume it. While the female consumes this nuptial food gift, sperm are evacuated into her reproductive tract from the ampulla. It takes the female about 40 min on average to fully consume the spermatophylax, and normally, within a few minutes of doing so, she removes and eats the sperm ampulla. Smaller spermatophylaxes require less time to consume; consequently, males providing such gifts experience premature ampulla removal and reduced sperm transfer [6–8]. The amount of sperm transferred is critical to the reproductive success of males because it is the principal determinant of a male's fertilization success, particularly when his sperm must compete with those of a female's other mating partners [4,9–11]. Thus, the spermatophylax functions to entice females into relinquishing at least some of their control over the insemination process, thereby furthering the males' own reproductive interests.

Male *G. sigillatus* benefit most when their gifts are fully consumed because it is under these circumstances that sperm transfer is maximized. However, females may benefit by discarding gifts of those males they find

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undesirable if, upon doing so, they remove the sperm ampulla, thereby terminating sperm transfer. Indeed, females discard the spermatophylax by simply dropping it prior to its complete consumption in approximately 25 per cent of all matings [6,8]. Two patterns emerge from this behaviour: (i) if the female discards the spermatophylax, she typically does so 20 min or less after mating, long before complete sperm transfer has occurred; and (ii) in many cases, females remove the sperm ampulla immediately after discarding the spermatophylax.

Why should females discard males' nuptial food gifts? One possibility is that it is simply a non-adaptive consequence of satiation or some other factor intrinsic to females (e.g. age and previous mating experience). An alternative possibility, however, is that it is an adaptive mechanism by which females discriminate against certain males after mating. There are a number of indirect genetic benefits that females could derive by employing such post-copulatory preferences (for documentation of such benefits to polyandry in *G. sigillatus*, see [12]), but the nature of these benefits is not the focus of this study. Instead, we wish to know which features females use in discriminating against males in this fashion. We hypothesized that females discard gifts based on their assessment of the gift itself, its taste or its texture. Specifically, we tested the hypothesis that it is the composition of free amino acids in the spermatophylax that influences whether the female discards the spermatophylax after mating.

Warwick *et al.* [13,14] were the first to propose that free amino acids in the spermatophylax influence its gustatory appeal to females. Although the spermatophylax consists largely of water (approx. 83%) [14,15], chemical analysis of the spermatophylax has revealed that approximately 7 per cent of the solid fraction consists of various free amino acids [13,14]. The free amino acids occurring in the greatest concentration were glycine and proline, whereas those amino acids deemed essential [16] were found only at low concentrations. Free amino acids are known phagostimulants in insects [17–21], and are used by some species to assess nutritional quality of plant tissue [22]. Artificial 'gels' containing the four most abundant free amino acids found in the spermatophylax and fed to female *Gryllodes* resulted in increased feeding time relative to females fed control gels [14].

If, as has been hypothesized, free amino acids in the spermatophylax influence its gustatory appeal to females [13,14], then we would predict a difference in the amino acid profile between those gifts that are discarded by females after mating and those that are fully consumed. To test this prediction, we compared the amino acid profiles of two classes of spermatophylaxes: (i) spermatophylaxes that were discarded by females after mating and (ii) spermatophylaxes that were destined to be fully consumed. To identify the latter class of food gifts, we employed a screening procedure based on a preliminary study that allowed us to predict the fate of the spermatophylax with high certainty. We analysed these data using multivariate selection analysis [23] to estimate the strength and form of linear and nonlinear sexual selection acting on the amino acid composition of the male spermatophylax through female post-copulatory mate choice. We discuss the implications of our findings for the evolution of male nuptial gifts in insects.

2. METHODS

(a) *Cricket maintenance*

Gryllodes sigillatus used in this study were the descendants of approximately 500 adult crickets collected in Las Cruces, NM, USA in 2001, which were used to initiate a laboratory colony maintained at a population size of approximately 5000 individuals and allowed to breed panmictically. The colony has consistently produced at least 150 new adults per week since its inception, and has not experienced any genetic bottlenecks.

Experimental crickets were held in 55-litre plastic containers and maintained in an environmental chamber at 28°C on a 14 L : 10 D photoperiod. Crickets were provisioned with Fluker's cricket chow (Fluker Farms, Baton Rouge, LA) ad libitum, water supplied in 40 ml plastic tissue culture flasks plugged with cotton dental rolls and egg cartons to provide shelter. Moistened peat moss housed in small plastic cups was provided as an oviposition substrate and also served as an additional source of water.

(b) *Experimental design*

Our basic experimental design followed that commonly used for a multivariate selection analysis of phenotypic traits [23]. That is, we related variation in the amino composition of spermatophylaxes to female feeding behaviour using a multiple regression-based approach (see below). Male and female crickets were collected from culture within 24 h of eclosion to adulthood, and housed together for 5 days at an equal sex ratio to ensure that they had mated and that they were sexually competent prior to assessing female spermatophylax feeding behaviour. To increase the frequency of mating during our behavioural trials, males and females were subsequently housed in isolation for 24 h. A single male and female were then paired at random in a clear Plexiglas arena (30 × 16 × 9 cm) and their mating behaviour observed under red lighting. We commenced behavioural recording when the male successfully transferred the spermatophore to the female. Males were removed from the arena immediately after mating to ensure that they did not influence female feeding behaviour [24]. The feeding behaviour of each female was observed for a total of 15 min after the transfer of the spermatophore, and we recorded whether the female discarded the spermatophylax during this period or fed on it continuously. At this point, we collected the spermatophylax (either from the floor of the arena if prematurely discarded or taken from the female with forceps) for analysis of amino acid composition using gas chromatography–mass spectrometry (GC–MS). We measured the amino acid composition and female feeding behaviour for a total of 314 spermatophylaxes.

(c) *Amino acid analysis of spermatophylaxes*

(i) *Extraction of amino acids from the spermatophylax*

Immediately after behavioural trials, spermatophylaxes were stored in an airtight microcentrifuge vial, frozen at −80°C in a freezer and then freeze-dried using a Labconco freeze-drier (Labconco, Kansas City, MO). Freeze-dried cricket spermatophylax samples were weighed and then ground using a pestle in an Eppendorf tube, 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). A sample (100 µl) was 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. Washing solution (200 µl; *N*-propanol) was 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. Eluting medium (200 µl; a 3 : 2 mix of sodium hydroxide and *N*-propanol) was 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. Chloroform (200 µl) was then added using a Drummond dialamatic microdispenser. 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 allowed to proceed for a further minute. Iso-octane (100 µl) was 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. Hydrochloric acid (100 µl; 1 M) was 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.

(ii) *Quantification of amino acid composition in the spermatophylax*

We injected 0.2 µl of the extracted amino acid sample into a GC–MS (Agilent 7890A gas chromatograph coupled with an Agilent 5975B mass spectrometer and an Agilent CTC PAL autosampler chilled to 10°C) fitted with a 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° per minute 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 (v. 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 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 (HYP), glutamic acid (GLU), phenylalanine (PHE), glutamine (GLN), orthinine (ORN), glycyl-proline (GPR), lysine (LYS), histidine (HIS), tyrosine (TYR), and tryptophan (TRP). Amino acid peaks (including the internal standard) were labelled by peak number (1–23), which corresponds to their retention time on the gas chromatograph (electronic supplementary material, table S1 and figure S1), and the qualitative ion used to quantify each amino acid is provided in the electronic supplementary material, table S1. Three amino acids (α-aminobutyric acid, orthinine and glycyl-proline) were not present in all spermatophylax samples and were therefore excluded from further analysis.

(d) *Statistical analysis*

Female feeding behaviour significantly altered the mass of the spermatophylax (mean ± s.e.: consumed for 15 min = 0.63 ± 0.02 mg, prematurely discarded = 0.92 ± 0.02 mg; ANOVA: $F_{1,313} = 73.98$, $p = 0.0001$). It was therefore necessary to express the actual amount of each amino acid relative 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 millilitre of internal standard per gram of spermatophylax. Data for each amino acid were $\log_{10}$-transformed prior to analysis to ensure normality. Owing to the large number of amino acids examined, we used principal component analysis to reduce the variation in amino acids into a smaller number of dimensions. We extracted principal components (PCs) based on the correlation matrix, and retained PCs with eigenvalues exceeding 1 for further analysis [25]. We interpret factor loadings that exceed 0.35 as biologically important [25].

(i) *Multivariate selection analysis*

Each spermatophylax was assigned a score of 1 if the female fed on it continuously for 15 min or 0 if the spermatophylax was prematurely discarded before this time. As recommended by Lande & Arnold [23], this absolute score was transformed to relative fitness by dividing by the mean absolute fitness of the population. We then fitted a linear regression including the PCs describing the amino acid composition of the spermatophylax to estimate the vector of standardized linear selection gradients, $\boldsymbol{\beta}$. A quadratic regression model including all linear, quadratic and cross-product terms was then used to estimate the matrix of nonlinear selection gradients, $\boldsymbol{\gamma}$ [23]. Quadratic regression coefficients are known to underestimate the stabilizing and disruptive selection gradients by a factor of 0.5, and therefore we doubled the quadratic selection gradients, as recommended by Stinchcombe *et al.* [26].

It is likely that the strength of nonlinear selection will be underestimated if the size and significance of the individual $\boldsymbol{\gamma}$ terms are interpreted individually [27]. We therefore explored the extent of nonlinear selection by conducting a canonical analysis to locate the major axes of the fitness surface [28]. The strength of linear selection along each of the eigenvectors ($\boldsymbol{m_i}$) is given by theta ($\boldsymbol{\theta_i}$) and the strength of nonlinear selection is given by their eigenvalues ($\boldsymbol{\lambda_i}$). We estimated $\boldsymbol{\theta_i}$ and $\boldsymbol{\lambda_i}$ for each eigenvector using the double regression method of Bisgaard & Ankenman [29].

Relative fitness was not normally distributed, and while this does not influence the sign or magnitude of selection gradients [23], it does create problems for testing the significance of these gradients [30,31]. Thus, to assess the significance of our linear and nonlinear selection gradients, we used a resampling procedure where relative fitness values were randomly shuffled across individuals in the dataset to obtain a null distribution for each selection gradient where there is no relationship between trait and fitness. Probabilities are the number of times (out of 9999 permutations) in which the gradient pseudo-estimate was equal to or less than the original estimated gradient. We conducted separate randomization tests for the multiple regression models for directional selection (i.e. model containing only linear terms) and for the full quadratic model (i.e. model containing linear, quadratic and correlational terms). We used the same resampling procedure to assess the

significance of $\boldsymbol{\theta_i}$ and $\boldsymbol{\lambda_i}$ for each eigenvector after the canonical rotation of $\boldsymbol{\gamma}$.

We used thin-plate splines [32] to visualize the major axes of the fitness surface extracted from the canonical rotation of $\boldsymbol{\gamma}$. Thin-plate splines are a non-parametric approach that provides a less-constrained view of the fitness surface than is provided by the best quadratic approximation [33]. We used the *Tps* function in the FIELDS package in R (v. 2.12.2, www.r-project.org) to fit the thin-plate splines and to visualize them in both perspective and contour map views. We used the value of the smoothing parameter ($\boldsymbol{\lambda} = 0.04$) that minimized the generalized cross-validation score when fitting the thin-plate splines.

(e) *Validating our experimental approach*

Our ability to accurately relate the amino acid composition of the spermatophylax to female feeding behaviour using the experimental approach we outline above is based on two important assumptions. First, our approach assumes that our assessment of female feeding behaviour for the first 15 min after mating accurately predicts longer-term feeding behaviour. More specifically, it assumes that if a female fed on the spermatophylax continuously for our 15 min observation period, then she would be unlikely to discard the spermatophylax after this time period. To test this assumption, we observed the behaviour of 45 females allowed to feed on a spermatophylax without interruption. We found that females who continuously fed on a spermatophylax for 15 min after mating always went on to completely consume the spermatophylax, taking on average 28.02 ± 3.27 ($\pm$s.e.) minutes to do so. This finding agrees with a previous study [8] that was conducted on a much larger number of females ($n = 105$), showing that only 13.3 per cent (14 out 105) of females prematurely discarded the spermatophylax after 15 min, with the average time to discard a spermatophylax being 9.42 ± 1.66 min. Together, these findings suggest that observing female feeding behaviour for 15 min after mating allows us to predict the fate of the spermatophylax with a high degree of accuracy, while still allowing us to collect a sufficient sample of the spermatophylax to quantify amino acid composition.

Second, our approach assumes that differences in the feeding behaviour of females (i.e. prematurely discarding versus continuous feeding) did not alter the amino acid composition of the spermatophylax. This may occur if, for example, digestive enzymes or the mechanical effects of chewing alter the quantity or structural integrity of amino acids in the spermatophylax. To test this assumption, we conducted two preliminary experiments. In the first, we used a single highly inbred line of crickets (F_{29}) that are known to show low levels of variation in the amino acid composition of the spermatophylax (S. N. Gershman, J. Hunt & S. K. Sakaluk 2011, unpublished data). We removed spermatophylaxes from 24 different males of the inbred line and allowed females to feed on half of them for 15 min, leaving the other half unchewed. The spermatophylaxes were freeze-dried and the amino acid composition quantified using the GC–MS protocol outlined above. Discriminant function analysis was unable to significantly distinguish between the amino acid composition of spermatophylaxes that had been partially eaten from those that had been left intact (Wilks's lambda $= 0.303$, $\chi^2 = 16.721$, $p = 0.404$). Moreover, a series of one-way ANOVAs showed that none of 19 individual amino acids measured in the spermatophylax differed significantly between these two treatment groups (range of $F_{1,23}$ values $= 0.022$–1.831, $p = 0.190$–0.875).

In the second preliminary experiment, we obtained spermatophylaxes from 20 outbred males and divided them in half, feeding one half to a female for a short period of time before recovering it, and leaving the other half unchewed. Each spermatophylax was cut in half using a sterile scalpel blade: one half was held at room temperature for 15 min and then freeze-dried, while the remaining half was given to the female to feed on for 15 min. After this time, the remaining portion of the spermatophylax was taken from the female, freeze-dried and the amino acid composition of both samples from each spermatophylax determined using GC–MS. Paired *t*-tests revealed that none of the 19 individual amino acids measured differed in abundance owing to female feeding (range of t_{19}-values $=$ 0.725–1.870, $p = 0.077$–0.477). On the basis of findings of these two preliminary experiments, we are confident that any difference in female feeding behaviour does not alter the amino acid composition of the male spermatophylax.

Table 1. Principal component (PC) analysis of the 19 free amino acids examined in the spermatophylax of *Gryllodes sigillatus*. We have retained PCs with an eigenvalue greater than 1 in our multivariate selection analysis and we interpret factor loadings greater than 0.35 as biologically significant (in bold).

	PC1	PC2	PC3
eigenvalue	12.011	1.480	1.016
% variance	63.216	7.788	5.346
amino acid			
ALA	**0.856**	−0.270	0.131
GLY	**0.898**	−0.076	0.108
VAL	**0.690**	0.035	**−0.373**
LEU	**0.761**	**−0.546**	0.063
ILE	**0.868**	−0.249	−0.139
THR	**0.813**	0.077	−0.228
SER	**0.805**	0.145	0.118
PRO	**0.876**	−0.074	0.135
ASN	**0.854**	−0.106	−0.271
ASP	**0.815**	0.200	−0.052
MET	**0.674**	0.017	0.289
HYP	**0.463**	−0.048	**0.728**
GLU	**0.830**	0.254	−0.024
PHE	**0.727**	**−0.538**	−0.134
GLN	**0.750**	**0.509**	−0.052
LYS	**0.793**	**0.426**	0.029
HIS	**0.769**	**0.357**	−0.005
TYR	**0.851**	0.071	0.104
TRP	**0.895**	−0.159	−0.131

3. RESULTS

PC analysis on the free amino acid composition of the male spermatophylax yielded three PCs with eigenvalues exceeding 1, which collectively explain 76.26 per cent of the total variation in amino acid composition (table 1). PC1 accounts for 63.22 per cent of the variance in the amino acid content of the spermatophylax and is positively loaded to each amino acid (table 1). Consequently, this vector describes the absolute amount of amino acids present in the male spermatophylax. PC2 explains a further 7.79 per cent of the variance in amino acid content and

Table 2. The vector of standardized linear selection gradients ($\boldsymbol{\beta}$) and the matrix of standardized quadratic and correlational selection gradients ($\boldsymbol{\gamma}$) for the free amino acid composition of the spermatophylax of *Gryllodes sigillatus*. Asterisks indicate randomization tests.

		$\boldsymbol{\gamma}$		
	$\boldsymbol{\beta}$	PC1	PC2	PC3
PC1	−0.034	0.166*		
PC2	−0.177*	0.016	−0.120*	
PC3	−0.181**	0.112*	−0.090*	−0.096

Randomization tests: *$p < 0.05$, **$p < 0.01$.

Table 3. The $\boldsymbol{M}$ matrix of eigenvectors from the canonical analysis of $\boldsymbol{\gamma}$ for the amino acid composition of the spermatophylax of *Gryllodes sigillatus*. The linear ($\boldsymbol{\theta_i}$) and quadratic ($\boldsymbol{\lambda_i}$) gradients of selection along each eigenvector are given in the last two columns. The quadratic selection gradient ($\boldsymbol{\lambda_i}$) of each eigenvector ($\boldsymbol{m_i}$) is equivalent to the eigenvalue. Asterisks represent randomization tests.

	$\boldsymbol{M}$			selection	
	PC1	PC2	PC3	$\boldsymbol{\theta_i}$	$\boldsymbol{\lambda_i}$
$\boldsymbol{m}_1$	0.776	−0.116	0.620	−0.060	0.127**
$\boldsymbol{m}_2$	−0.605	−0.414	0.680	0.017	0.025
$\boldsymbol{m}_3$	−0.178	0.903	0.392	−0.127*	−0.081*

Randomization tests: *$p < 0.05$, **$p < 0.01$.

describes the trade-off between GLN, LYS and HIS (positive loadings) and LEU and PHE (negative loadings; table 1). PC3 accounts for 5.35 per cent of the variance in amino acid composition, and describes the trade-off between HYP (positive loading) and VAL (negative loading; table 1).

Standardized linear, quadratic and correlational selection gradients are presented in table 2. There was significant directional selection favouring lower values for PC2 and PC3. There was also significant disruptive selection operating on PC1 and stabilizing selection operating on PC2. There was significant positive correlational selection operating on the covariance between PC1 and PC3, and significant negative correlational selection operating on the covariance between PC2 and PC3.

Canonical analysis of the $\boldsymbol{\gamma}$ matrix resulted in two eigenvectors ($\boldsymbol{m}_1$ and $\boldsymbol{m}_3$) with significant nonlinear sexual selection (table 3). There was significant disruptive selection operating along the major axis of nonlinear selection ($\boldsymbol{m}_1$) that was heavily weighted by PC1 and PC3 (table 3). There was also significant stabilizing selection operating on the second strongest vector of nonlinear selection ($\boldsymbol{m}_3$) that was heavily loaded to PC2 (table 3). Linear selection also favoured decreased values of $\boldsymbol{m}_3$, which involves decreased levels of PC2 (table 3). The combination of positive ($\boldsymbol{m}_1$) and negative ($\boldsymbol{m}_3$) eigenvalues (table 3) formally indicates the presence of a multivariate saddle on the fitness surface (figure 1*a*,*b*).

4. DISCUSSION

The results of this study show that the free amino acid composition of the spermatophylax of *G. sigillatus* influences its gustatory appeal, and hence the probability that the female will discard it before its complete consumption. This has important fitness consequences for males because females frequently remove the male's sperm ampulla shortly after discarding the spermatophylax, terminating sperm transfer and decreasing a male's fertilization success [4,6,8,11]. Thus, the gustatory response of females to males' spermatophylaxes represents an important avenue of post-copulatory female choice, leading to significant sexual selection on the amino acid composition of the spermatophylax.

Other aspects of nuptial gifts—most notably, their size—have been linked to reproductive performance in other gift-giving taxa. In mecopterans and empidid flies, for example, males provide females with nuptial prey or salivary masses, the size and palatability of which are directly correlated with sperm transfer and male fertilization success. Male tree and ground crickets prolong sperm transfer by allowing females to consume external glandular secretions. Similar to *G. sigillatus*, males of many bushcricket species produce spermatophylaxes that are consumed by females and increase the transfer of male ejaculate [2]. Although the chemical composition of spermatophylaxes has been investigated in *G. sigillatus* [13,14], as well as other orthopteran species [2], our results add to a growing body of evidence that sexual selection has influenced the evolution of the chemical composition of a nuptial food gift synthesized by males [14].

The composition of free amino acids in the spermatophylax varied along three PCs, all of which affected the gustatory appeal of the spermatophylax to some extent. PC1 had strong positive loadings on all free amino acids in the spermatophylax, and thus represents the total concentration of free amino acids in the spermatophylax. In an earlier study in which artificial gels containing the four most common amino acids in the spermatophylax (PRO, GLY, ARG and ALA) were fed to female *G. sigillatus*, feeding duration increased with increasing amino acid concentration up to intermediate values, and then decreased slightly thereafter [14]. In contrast to this result, we found that females were less likely to discard the spermatophylax at the lowest and highest total concentrations of free amino acids, and more likely to discard it at intermediate values, indicative of disruptive selection acting on this PC (table 2 and figure 1). Furthermore, canonical analysis revealed significant disruptive selection along the major axis of nonlinear selection ($\boldsymbol{m}_1$), heavily weighted by both PC1 and PC3. PC3 describes a trade-off between 4-hydroxyproline (positive loading) and valine (negative loading), which may relate to the texture of the spermatophylax. Valine is a branched-chain amino acid that is highly hydrophobic and can inhibit tight protein folding: when valine substitutes for glutamic acid in haemoglobin, it causes the haemoglobin to fail to fold properly and results in sickle cell disease [34]. Conversely, 4-hydroxyproline promotes tight molecular folding, as in the formation of a flexible, springy molecule such as collagen [35]. Thus, it is possible that the presence of 4-hydroxyproline promotes increased spermatophylax chewiness, whereas valine inhibits this physical property. It is therefore tempting to speculate that the disruptive selection occurring along $\boldsymbol{m}_1$ arises out of a complex interaction between the gustatory appeal of the spermatophylax and its texture.

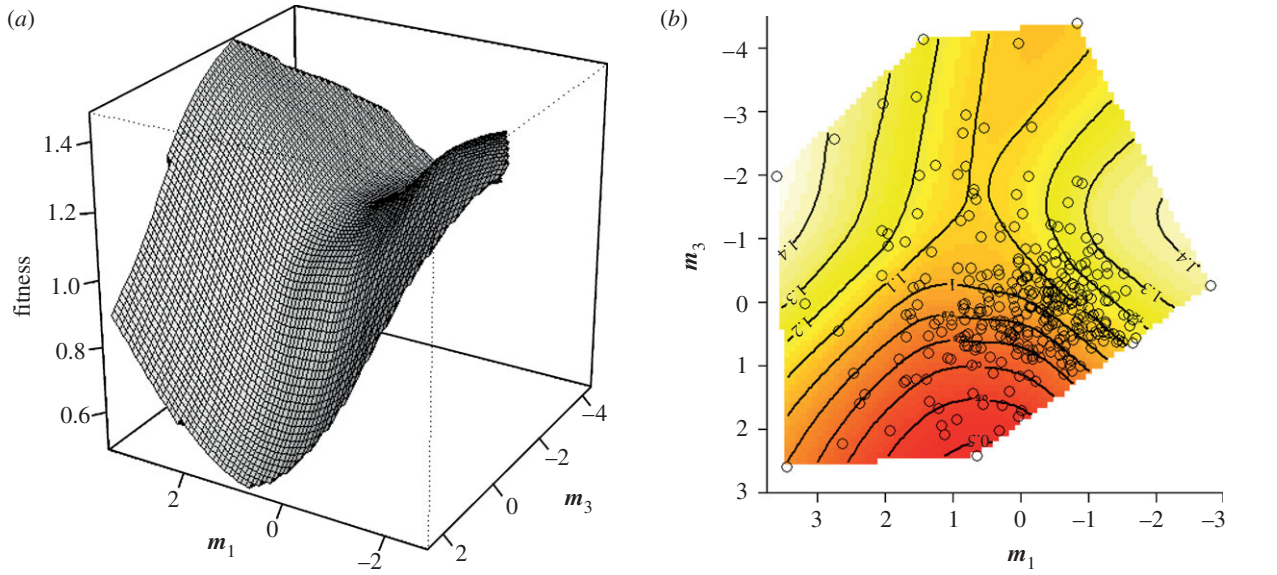

Figure 1. Thin-plate spline (*a*) perspective view and (*b*) contour map visualizations of the two major axes of nonlinear selection ($\boldsymbol{m}_1$ and $\boldsymbol{m}_3$) operating on the amino acid composition of the spermatophylax produced by *Gryllodes sigillatus*. Actual amino acid profiles are overlaid on the contour map graph (open circles).

Directional selection favoured lower values for both PC2 and PC3. Five amino acids contributed significantly to PC2, with leucine and phenylalanine having opposing loadings to glutamine, lysine and histidine. Thus, differences in the relative concentrations of these amino acids influenced the gustatory appeal of the spermatophylax, with relatively higher concentrations of leucine and phenylalanine resulting in increased appeal, and higher concentrations of glutamine, lysine and histidine leading to increased aversion and a greater likelihood of the female discarding the spermatophylax. Glutamine, lysine and histidine all have polar side chains with attached amines, whereas leucine and phenylalanine have non-polar R-groups [36], but whether this difference contributes to their differential effect on the gustatory appeal of the spermatophylax remains unknown. Four of the five amino acids (excluding glutamine) are essential amino acids for insects [37], and must be acquired from dietary sources because they cannot be synthesized, yet are essential for growth and reproduction. Extensive research has revealed that insects respond to the phagostimulatory effects of a variety of amino acids, including alanine, serine, histidine, proline, valine, leucine, methionine, phenylalanine, tryptophan and threonine [17–19]. However, the effects of amino acids are frequently context-dependent, and the multivariate effect of different amino acids on feeding has received little attention. Furthermore, the ability to taste different amino acids and the perception of these tastes varies widely among taxa: amino acids that may be appealing in one species may be undetectable or unpleasant in another [38,39]. Thus, it is likely that the amino acids that contributed significantly to PC2 in this study did so because: (i) female *G. sigillatus* have receptors that allow the detection of these amino acids, but not others; or (ii) females have a direct preference for the specific taste profile created by this combination of amino acids.

Directional selection also favoured lower values of PC3, and thus spermatophylaxes with high valine : 4-hydroxyproline ratios were less likely to be discarded. If, as we surmised earlier, the valine : 4-hydroxyproline ratio affects the texture of the spermatophylax, this may influence the ability of the female to discard the spermatophylax should she choose to do so. Partially consumed spermatophylaxes are occasionally 'sticky' and appear difficult to drop, and in such cases, females attempting to discard the spermatophylax often attempt to smear it against the substrate to dislodge it from their mouthparts (S. N. Gershman 2011, personal observation; see also [8]).

In addition to the directional selection acting on PC2, there was also strong multivariate stabilizing selection acting on the second strongest vector of nonlinear selection ($\boldsymbol{m}_3$), which was heavily loaded on PC2. If the combination of amino acids represented by PC2 influences the gustatory appeal of the spermatophylax, then such selection may not be entirely surprising. We might expect that the receptors associated with females' gustatory responses should be acutely tuned to the demands of optimal food selection, and thus subject to strong stabilizing natural selection arising through nutritional needs unrelated to mating. Selection would thus favour males that synthesize spermatophylaxes with an amino acid profile most likely to elicit a response from these receptors. In support of this hypothesis, Sakaluk [40] offered spermatophylaxes derived from male *G. sigillatus* to mated females of several non-spermatophylax-producing cricket species, and found that females readily consumed these novel food gifts. Thus, the amino acid profile of the spermatophylax may be subject to stabilizing selection imposed, in part, by the intrinsic properties of the receptors and neurosensory pathways mediating gustation in females (for a similar example concerning the influence of female sensory audition on cricket call properties, see [41]).

The combination of disruptive selection on $\boldsymbol{m}_1$ and stabilizing selection on $\boldsymbol{m}_3$ yielded the saddle-shaped fitness surface depicted in figure 1. There were two local fitness peaks corresponding to low and high values of $\boldsymbol{m}_1$, respectively. Thus, different amino acid profiles are, for different reasons, likely to elicit continued female

feeding on the spermatophylax and, hence, increased retention of the sperm-containing ampulla. Why might this be so? First, different amino acid compositions may offer the same level of gustatory appeal, particularly if they are linked functionally to disparate dietary requirements. Second, a spermatophylax may vary simultaneously in both its gustatory appeal and texture, which may independently affect female feeding duration. Thus, even a spermatophylax of low gustatory appeal may be favoured if its texture makes it difficult to physically discard. In support of the latter possibility, we found significant negative correlational selection operating on the covariance between PC2 and PC3 ($\gamma = -0.090$; table 2), which suggests that selection favours an increase in one of these dimensions but a decrease in the other. This pattern of selection would be expected if it is difficult for males to synthesize a spermatophylax with an amino acid profile that simultaneously optimizes both its taste and texture. However, the proximate mechanisms generating the pattern of multivariate selection observed here must remain speculative, until such time as manipulative experiments altering the amino acid composition of food gifts, in either real or artificial spermatophylaxes [14], have been conducted.

The 'Candymaker' hypothesis predicts that if the spermatophylax functions, in part, as a sensory trap, then selection will favour males that produce spermatophylaxes (which are appealing to females), but that do not necessarily provide any nutritional benefits [14]. As *G. sigillatus* females gain little material benefit from consuming spermatophylaxes [42], but have demonstrable preferences for specific combinations of amino acids, it seems likely that the chemical composition of the spermatophylax has evolved partly as a form of sensory trap. However, we cannot rule out the possibility that in discarding the gifts of some males while consuming those of others, females selectively fertilize their eggs with the sperm of high-quality males, and thereby secure indirect genetic benefits. This avenue of mate choice would necessarily lead to sexual conflict, because females might benefit from this form of post-copulatory assessment, whereas males benefit most when they transfer their full complement of sperm. Thus, sexual conflict may also be an important source of the sexual selection driving the evolution of the chemical composition of the spermatophylax [5].

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