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Macronutrient intake regulates sexual conflict in decorated crickets

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Abstract

Sexual conflict results in a diversity of sex-specific adaptations, including chemical additions to ejaculates. Male decorated crickets (*Gryllodes sigillatus*) produce a gelatinous nuptial gift (the spermatophylax) that varies in size and free amino acid composition, which influences a female's willingness to fully consume this gift. Complete consumption of this gift maximizes sperm transfer through increased retention of the sperm-containing ampulla, but hinders post-copulatory mate choice. Here, we examine the effects of protein (P) and carbohydrate (C) intake on the weight and amino acid composition of the spermatophylax that describes its gustatory appeal to the female, as well as the ability of this gift to regulate sexual conflict via ampulla attachment time. Nutrient intake had similar effects on the expression of these traits with each maximized at a high intake of nutrients with a P : C ratio of 1 : 1.3. Under dietary choice, males actively regulated their nutrient intake but this regulation did not coincide with the peak of the nutritional landscape for any trait. Our results therefore demonstrate that a balanced intake of nutrients is central to regulating sexual conflict in *G. sigillatus*, but males are constrained from reaching the optima needed to bias the outcome of this conflict in their favour.

Introduction

Sexual conflict arises whenever the reproductive interests of males and female do not perfectly coincide (Parker, 1979) and is known to promote the evolution of adaptations that enhance the fitness of individuals of one sex at the expense of the other (Parker, 1979; Arnqvist & Rowe, 2005). These adaptations range from mating behaviours, such as persistent harassment (Córdoba-Aguilar, 2009) and reluctance to mate (Rowe *et al.*, 1994), to morphological structures that make it harder for males to force matings (Arnqvist & Rowe, 1995) or that cause damage to the female during mating (Crudgington & Siva-Jothy, 2000; Stutt & Siva-Jothy, 2001). Conflict between the sexes, however, does not always end at copulation as males may continue to manipulate females long after mating has finished (Arnqvist & Rowe, 2005). A classic example of this occurs in *Drosophila melanogaster* where males transfer seminal fluid proteins (SFPs) in their ejaculate during mating that are known to have a wide range of physiological effects on females including altering sperm storage, decreasing receptivity to additional matings, increasing ovulation and egg production and reducing lifespan (Wolfner, 2002). Although SFPs and other chemicals in the ejaculate appear to be taxonomically widespread (Poiani, 2006; Perry *et al.*, 2013), surprisingly little is known about their effects on females in systems other than *D. melanogaster*. Consequently, chemical manipulation continues to be one of the least well-understood aspects of sexual conflict (Arnqvist & Rowe, 2005).

Nuptial gifts refer to any material beyond obligatory gametes that is provided by a donor (typically the male)

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to a recipient (typically the female) during courtship or copulation that acts to improve the fitness of the donor (Lewis *et al.*, 2014) and are taxonomically widespread in insects and spiders (Vahed, 1998, 2007; Gwynne, 2008; Lewis & South, 2012). Males of numerous field cricket and katydid species synthesize their own gifts, including complex spermatophores (e.g. Gwynne, 1997), glandular secretions (e.g. Bussière *et al.*, 2005) and even part of the male's own body (e.g. Eggert & Sakaluk, 1994). Collectively referred to as endogenous oral gifts (Lewis & South, 2012; Lewis *et al.*, 2014), these gifts constitute a major form of reproductive investment and their production has been shown to be costly to the male (e.g. Sakaluk *et al.*, 2004; Leman *et al.*, 2009). In many species, males provision gifts with nutrients or defensive compounds that are otherwise absent or limited in the female's diet (Lewis & South, 2012) and there are many examples where the female benefits directly and/or indirectly through the fitness of her offspring by consuming these gifts (e.g. Gwynne, 1997, 2008). In other species, however, no such benefits have been detected and nuptial gifts predominantly serve to protect the male ejaculate and cause the female to relinquish some of her control over sperm transfer and eventual paternity (Vahed, 1998, 2007). Consequently, it has been argued that sexual conflict has played a key role in shaping the evolution of nuptial gifts (Vahed, 1998, 2007; Gwynne, 2008; Lewis & South, 2012). For gifts that are produced endogenously, there is the potential for the male to add manipulative substances during production, although it has been noted that such substances may be infrequent in oral gifts because they would be degraded as they pass through the female digestive system (Gwynne, 2008).

In decorated crickets (*Gryllodes sigillatus*), males produce an externally attached spermatophore that is transferred to the female during mating. This spermatophore consists of two discrete components: the sperm-containing ampulla and the much larger gelatinous spermatophylax. Immediately upon dismounting the male after spermatophore transfer, the female detaches the spermatophylax from the ampulla with her mandibles and commences feeding on it. While the female feeds on this nuptial gift, sperm are evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female immediately removes and consumes the ampulla, thereby terminating sperm transfer. Females vary considerably in the length of time that they feed on the spermatophylax, and the longer that the female is delayed from removing the ampulla, the more sperm are transferred to her sperm storage organ (Sakaluk, 1984, 1985, 1987). The size of the spermatophylax plays an important role in this process, as it takes a female longer to fully consume a larger spermatophylax (Sakaluk, 1985), a pattern that also appears common across bushcricket species (e.g. Wedell & Arak, 1989; Reinhold & Heller, 1993). As females are highly polyandrous, the length of time that a female spends consuming the spermatophylax has profound consequences for the outcome of sperm competition (Sakaluk, 1986; Sakaluk & Eggert, 1996; Eggert *et al.*, 2003). Thus, although producing a spermatophylax comes at a direct cost to the immune function of males (Gershman *et al.*, 2010; Kerr *et al.*, 2010), consumption of this gift by the female clearly enhances male fitness. In contrast, there appears to be little direct benefit to the female in consuming a spermatophylax (Will & Sakaluk, 1994; Kasuya & Sato, 1998; Ivy & Sakaluk, 2005), the one notable exception being a hydration benefit to female lifespan under conditions of limited water availability (Ivy *et al.*, 1999). In fact, females that accept a nuptial gift from the male are actively prevented from exerting post-copulatory mate choice, which is known to reinforce precopulatory mating biases in this species (Ivy & Sakaluk, 2007). Thus, by consuming the spermatophylax, a female relinquishes some of the control over the paternity of her offspring which is unlikely to be in her best 'evolutionary' interests. Consequently, it has been argued that sexual conflict has been a major driver of nuptial gift evolution in *G. sigillatus* (Sakaluk, 2000; Sakaluk *et al.*, 2006; Gershman *et al.*, 2012, 2013).

More recently, research has focused on the chemical composition of the spermatophylax and the potential role this plays in mediating sexual conflict in *G. sigillatus* (Warwick *et al.*, 2009; Gershman *et al.*, 2012, 2013). The spermatophylax in this species consists mostly of water, as well as a mixture of 19 different free amino acids (Warwick *et al.*, 2009). Many of these amino acids are known phagostimulants in insects (e.g. Cook, 1977). This explains the propensity of females to accept and feed on a gift after mating in *G. sigillatus*, as well as females from numerous other nongift-giving cricket species (Sakaluk, 2000; Sakaluk *et al.*, 2006). It is important to highlight, however, that up to 25% of female *G. sigillatus* prematurely discard the spermatophylax before it is fully consumed (Sakaluk, 1984, 1987) and there is some evidence to suggest that this behaviour is linked to the amino acid composition of the spermatophylax (Warwick *et al.*, 2009; Gershman *et al.*, 2012). By feeding artificial, gelatin-based gels containing the four most abundant amino acids in the spermatophylax (proline, glycine, arginine and alanine) in varying concentrations, Warwick *et al.* (2009) showed that female feeding time increased with amino acid concentration, peaking at approximately 14% gelatin dry mass. More recently, Gershman *et al.* (2012) used a multivariate selection analysis to show that specific combinations of amino acids decreased the likelihood that a female would prematurely discard a spermatophylax, most likely by influencing the gustatory appeal of this gift (Warwick *et al.*, 2009). Importantly, there is a positive genetic correlation between this combination of amino acids in the spermatophylax and female feeding duration which

indicates that genes expressed in males to produce more manipulative spermatophylaxes are positively linked to genes expressed in females that make them more vulnerable to being manipulated (Gershman *et al.*, 2013). This finding is consistent with an evolutionary history of sexual antagonistic coevolution over the consumption of this nuptial gift in *G. sigillatus* (Gershman *et al.*, 2013). However, although these studies provide strong evidence that free amino acids are important in mediating sexual conflict in *G. sigillatus*, we currently do not know how these substances are regulated in the spermatophylax.

In general, very little is known about the regulation of chemicals used in sexual conflict, although diet appears to be a strong candidate. In *D. melanogaster*, both the larval and adult diets of males have a significant effect on female remating behaviour, most likely by altering SFP expression in the ejaculate (McGraw *et al.*, 2007; Fricke *et al.*, 2008). For example, males fed a diet containing higher levels of protein as a larva (provided in the form of yeast) were more effective at preventing remating in their partner as an adult and had a higher relative transcript abundance of at least one known SFP (*Acp36DE*) (McGraw *et al.*, 2007). Furthermore, males fed a high-protein diet as an adult (after being reared on a standard medium diet) were also able to better inhibit remating in their partner (Fricke *et al.*, 2008). Qualitatively similar effects on female remating behaviour have also been shown for male Mediterranean fruit flies (*Ceratitis capitata*) fed a high-protein diet (provided as protein hydrolysate)(Blay & Yuval, 1997), a species where SFP expression is known to share many homologies with *D. melanogaster* (Davies & Chapman, 2006). Although there is growing evidence showing that the consumption of a nuptial gift influences female remating behaviour (e.g. Ortiz-Jimenez & Cueva del Castillo, 2015), surprisingly little is known about the effect of male nutrition on the addition of manipulative chemicals to nuptial gifts.

A limitation shared by all of the above studies examining the relationship between male nutrition and the production of manipulative chemicals is that diet was not manipulated in a controlled manner. For example, although yeast consists mainly of protein and is therefore used to manipulate this nutrient in experimental diets (McGraw *et al.*, 2007; Fricke *et al.*, 2008), it also contains carbohydrates, lipids, salts and a range of different vitamins. Thus, it is not possible to identify the key nutrient(s) responsible for any observed effects when using such manipulations, or to partition the effects of specific nutrients from the total intake of calories. Consequently, an explicit nutritional framework is needed when examining the effects of nutrition, preferably using chemically defined (holidic) diets. Here, we use a multidimensional nutritional framework, known as nutritional geometry (NG; Simpson & Raubenheimer, 2012), to determine the effects of protein (P) and carbohydrate (C) intake on the regulation of sexual conflict in the decorated cricket, *G. sigillatus*. To test this, we conducted three separate experiments using holidic diets with precise chemical composition. In our first experiment, we restricted male crickets to feed on one of 24 artificial diets during sexual maturation to quantify the effects of P and C intake on the size and amino acid composition of the spermatophylax that describes its gustatory appeal to females (Experiment 1). Next, we restricted a second group of males to feed on these diets during sexual maturation and then mated them to a randomly allocated virgin female. These females were then observed, and the time taken for the female to remove the ampulla (and terminate sperm transfer) was recorded (Experiment 2). In our final experiment, we gave males the choice between alternate diets in four diet pairings to determine whether they regulate their intake of P and C to optimize the size and chemical composition of the spermatophylax, as well as ampulla attachment time (Experiment 3). If nutrient intake regulates sexual conflict in *G. sigillatus*, we predict that the intake of P and/or C will influence the size and the amino acid composition that describes the gustatory appeal of the spermatophylax and this will have a similar effect on ampulla attachment time. This should result in the nutritional landscapes for these traits being closely aligned. Moreover, if males are biasing the outcome of this conflict through their intake of nutrients, we predict that the regulated intake of nutrients under dietary choice will coincide with the peak in ampulla attachment time on the nutritional landscape.

Materials and methods

Experimental animals

The *G. sigillatus* used in this study are descended from 500 adult crickets collected in Las Cruces, New Mexico, in 2001 used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (Ivy & Sakaluk, 2005). Culture crickets are housed in ten 15-L plastic containers in an environmental chamber (Percival I-66VL) maintained at 32 ± 1 °C on a 14-h:10-h light/dark cycle and are provided with cat food (Go-Cat Senior®, Purina, St Louis, MO, USA) and rat food (SDS Diets, Essex, UK) pellets, water *ad libitum* in 60-mL glass test tubes plugged with cotton wool and an abundance of cardboard egg cartons to provide shelter. Each generation, nymphs were collected at hatching and randomly allocated across culture containers to enforce gene flow.

Experimental crickets were collected from our culture as newly hatched nymphs and housed individually in a plastic container (5 cm × 5 cm × 5 cm). Each container was provided with a piece of cardboard egg carton for shelter and water in a 2.5-mL test tube plugged

with cotton wool. Nymphs were fed ground cat food pellets for the first 2 weeks post-hatching and thereafter solid pellets until eclosion to adulthood. Containers were cleaned and food and water replaced weekly. Experimental animals were checked daily for eclosion to adulthood. At eclosion, males were transferred to a larger individual plastic container (20 × 10 × 10 cm) and then randomly allocated to an experiment (experiments 1, 2 or 3).

Artificial diets and measuring dietary intake

We made 24 artificial, dry diets that varied in P and C, as well as overall nutrition, based on the established protocol outlined in Simpson & Abisgold (1985). The distribution of these diets in nutritional space can be seen in Fig. S1 and the composition of these diets in Table S1 (Appendix S1).

Each experimental male was given either one (experiments 1 and 2) or two (Experiment 3) dishes of diet of measured dry weight on their day of eclosion to adulthood and diet was changed every 2 days for a total of 10 days until males were sexually mature. Food and water were provided in feeding platforms constructed by gluing a vial lid (1.6 cm diameter, 1.6 cm deep) upside down onto a Petri dish (5.5 cm diameter). This design allowed any diet spilled during feeding to be collected in the Petri dish. Diet was kept in a drying oven (model FD 115; Binder, Tuttlingen, Germany) at 30 °C for 48 h to remove any moisture prior to weighing. Feeding platforms containing diet were weighed before and after each feeding period, using an electronic balance (model EP214C; Ohaus Explorer Professional, Ohaus, Pine Brook, NJ, USA). Prior to final weighing, any faeces were removed from the feeding platform using a pair of fine forceps. Diet consumption was calculated as the difference in dry weight of diet before and after feeding. This was converted to a P and C intake following the procedure outlined in South *et al.* (2011).

Experiment 1: The effects of nutrient intake on spermatophylax weight and amino acid composition

To determine the effects of P and C intake on the size and amino acid composition of the spermatophylax, 16 males were established at random on each of the 24 diets (total $n = 384$ males) on their day of eclosion to adulthood and fed for 10 days to quantify their intake of P and C (as described above). Each male was then mated to a virgin female of the same age (on day 10), and the spermatophylax removed from the female using a pair of fine forceps and stored in an airtight microcentrifuge vial. Spermatophylaxes were dried using a freeze dryer (Heto PowerDry LL3000, ThermoFisher Scientific, Waltham, MA, USA) and then weighed using an electronic balance (UMX2, Mettler Toledo, Columbus, OH, USA). A total of 22 free amino acids were extracted from these dried samples and quantified using an EZ:-faast reagent kit (Phenomenex, Torrance, CA, USA) optimized for gas chromatography–mass spectrometry (GCMS) following the protocol outlined in Gershman *et al.* (2012). Full details of this chemical analysis are provided in the Appendix S2.

Previously, we have used multivariate selection analysis to show that the free amino acid composition of the spermatophylax is a significant predictor of whether females prematurely discard the spermatophylax after mating (Gershman *et al.*, 2012). It is clear from this work that multivariate combinations of amino acids in the spermatophylax are a far better predictor of this female behaviour than the amount of specific amino acids. We therefore used the results of this selection analysis to assign a multivariate score of gustatory appeal to each spermatophylax based on its amino acid composition, following the approach used by Gershman *et al.* (2013). In short, this score was calculated by substituting the amount of each amino acid measured in the spermatophylax into the vector of standardized linear selection gradients derived by Gershman *et al.* (2012). This produces a unique score for each spermatophylax, whereby higher scores represent spermatophylaxes with greater gustatory appeal to females (i.e. not prematurely discarded) and therefore promote greater sperm transfer (Gershman *et al.*, 2013). Full details of how these multivariate scores of gustatory appeal were calculated are presented in Appendix S3. We quantified the effect of P and C intake on this multivariate score rather than focussing on the amount of specific amino acids present in the spermatophylax.

Experiment 2: The effects of nutrient intake on ampulla attachment time

To determine the effects of P and C intake on ampulla attachment time, 16 males were established at random on each of the 24 diets (total $n = 384$ males) on their day of eclosion to adulthood and fed for 10 days to quantify the intake of P and C measured (as described above). Each male was then mated to a virgin female of the same age, and the time taken for the female to remove the ampulla (and therefore terminate sperm transfer) was recorded under red lighting. The male was removed immediately after spermatophore transfer using fine forceps to ensure that he did not influence female behaviour.

Experiment 3: Measuring nutrient intake under dietary choice

To determine whether males actively regulate their intake of nutrients when given dietary choice, a total of 160 males were assigned at random on their day of eclosion to adulthood to one of four possible diet pairings ($n = 40$ per diet pair). These diet pairs differ in

both the P to C ratio, as well as total nutritional content (P : C ratio(total nutrition): Pair 1: 5 : 1(36%) vs. 1 : 8(36%), Pair 2: 5 : 1(36%) vs. 1 : 8(84%), Pair 3: 5 : 1(84%) vs. 1 : 8(36%) and Pair 4: 5 : 1(84%) vs. 1 : 8(84%). This corresponds to diets 2, 4, 22 and 24 in Table S1 and is marked by red symbols in Fig. S1. Diet consumption and the intake of P and C were measured every 2 days for a total of 10 days for each cricket using the protocol outlined above.

Statistical analysis

In experiments 1 and 2, we used a multivariate response-surface approach (South *et al.*, 2011) to estimate the linear and nonlinear effects of P and C intake on our response variables (spermatophylax weight, multivariate attractiveness of the spermatophylax and ampulla attachment time). Nonparametric thin-plate splines were used to visualize the nutritional landscapes for each response variable and were constructed using the 'Tps' function in the 'FIELDS' package of R (R Core Team, version 2.15.1, Vienna, Austria).

We used a sequential model building approach (South *et al.*, 2011) to determine whether the linear and nonlinear (quadratic and correlational) effects of P and C intake differed across our response variables (South *et al.*, 2011; Appendix S4). Inspection of the individual interaction terms in these models were used to determine which nutrient(s) contributed to any overall effects (South *et al.*, 2011). As our response variables were measured in different units, we standardized each response variable to a mean of zero and a standard deviation of one using a Z transformation prior to analysis. Although this approach statistically tests for differences in the magnitude of linear and nonlinear gradients between the response variables, it does not provide information on the direction of this difference in nutritional space. It is therefore possible that the response variables show differences in the magnitude of gradients but are optimized in similar regions on the nutritional landscape. We therefore also calculated the angle (θ) between the linear vectors for the two response variables being compared using trigonometry and the 95% confidence intervals for θ using a Bayesian approach implemented in the 'MCMCglmm' package of R. When $\theta = 0°$ the vectors are perfectly aligned and the optima for the two response variables reside in the same location in nutrient space, whereas $\theta = 180°$ represents the maximum possible divergence between vectors. Full details of these calculations and accompanying R code are provided in Appendix S5.

We used paired t-tests to compare the consumption of diets in each diet pair and a multivariate analysis of variance (MANOVA) to compare the total intake of P and C across diet pairs (Experiment 3). Univariate ANOVAS were used to determine which nutrients contributed to the overall multivariate difference across diet pairs and Tukey HSD tests to contrast the total intake of nutrients across each of the diet pairs. We calculated the regulated intake point, defined as the point in nutrient space that individuals actively defend when given dietary choice, as the mean intake of P and C across diet pairs (Simpson & Raubenheimer, 1993).

Results

The intake of both P and C had clear linear effects on the weight and gustatory appeal of the spermatophylax, as well as ampulla attachment time (Table 1, Fig. 1). All traits increased with the intake of these nutrients, with the intake of P and C having roughly similar effects on the expression of each trait (Table 1, Fig. 1). There were significant quadratic effects of P intake on the weight and gustatory appeal of the spermatophylax and ampulla attachment time (Table 1)

Table 1 The effect of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of the SPHYLAX and ampulla (AMP) attachment time in *Gryllodes sigillatus*.

	Linear effects		Nonlinear effects		
Response variable	P	C	P × P	C × C	P × C
SPHYLAX weight					
Coefficient ± SE	0.22 ± 0.05	0.17 ± 0.05	−0.12 ± 0.04	−0.01 ± 0.04	0.09 ± 0.07
t_{378}	4.50	3.50	2.75	0.25	1.24
P	0.0001	0.001	0.006	0.81	0.22
SPHYLAX appeal					
Coefficient ± SE	0.11 ± 0.05	0.11 ± 0.05	−0.10 ± 0.04	−0.02 ± 0.04	0.17 ± 0.08
t_{378}	2.13	2.06	2.25	0.52	2.24
P	0.03	0.04	0.03	0.60	0.03
AMP attachment time					
Coefficient ± SE	0.32 ± 0.05	0.26 ± 0.05	−0.14 ± 0.05	−0.00 ± 0.04	0.20 ± 0.08
t_{381}	6.63	5.42	3.05	0.02	2.66
P	0.0001	0.0001	0.002	0.98	0.008

and inspection of the nutritional landscapes (Fig. 1a–c) reveals peaks centred around a P : C ratio of approximately 1 : 1.3 for each trait. There were also significant positive correlational gradients for the gustatory appeal of the spermatophylax and ampulla attachment time (Table 1), providing further evidence that the expression of these traits increases with the intake of both nutrients.

Formal statistical comparisons showed that the linear and nonlinear effects of P and C intake on spermatophylax weight did not differ significantly from the effects of these nutrients on the gustatory appeal of the spermatophylax and ampulla attachment time (Table 2). Consequently, the angle (θ) between the linear vectors for the weight and gustatory appeal of the spermatophylax and between spermatophylax weight and ampulla attachment time were small, being 15.47° (95% CIs: 7.25°, 26.57°) and 8.06° (3.92°, 13.69°), respectively. The linear effect of P and C intake on the gustatory appeal of the spermatophylax and ampulla attachment time differed significantly but the quadratic and correlational effects of these nutrients did not (Table 2). The difference in linear effects was due to the fact that ampulla attachment time was more responsive to the intake of P and C than the gustatory appeal of the spermatophylax rather than P and C intake having contrasting effects on these traits (Table 1). As a result, θ was also small between these two traits at 14.27° (6.65°, 24.38°). Collectively, these analyses demonstrate that there is little divergence in the effects of P and C intake on the weight and gustatory appeal of the spermatophylax and ampulla attachment time in *G. sigillatus*.

In each diet pair, males consumed significantly more of the high-C diet than the high-P diet (Fig. S3, Appendix S6). Not surprisingly, there was a significant difference across the diet pairs in the intake of nutrients (MANOVA: Pillai's Trace = 0.97, $F_{6,312} = 48.61$, $P = 0.0001$) and univariate ANOVAS showed that this effect was driven by the intake of both P ($F_{3,156} = 34.43$, $P = 0.0001$) and C ($F_{3,156} = 66.83$, $P = 0.0001$). Tukey HSD tests (at $P < 0.05$) showed that the order of diets pairs for P intake was $1 = 2 < 4 < 3$ and for C intake was $3 < 1 < 2 = 4$ (Fig. 2). The regulated intake point was estimated at an intake of 26.39 ± 1.16 mg of P and 45.79 ± 1.70 mg of C, which corresponds to a P : C ratio of 1 : 1.74 (Fig. 2). Importantly, this regulated intake point does not correspond with the peaks for the weight and gustatory appeal of the spermatophylax or ampulla attachment time (Fig. 1) and therefore demonstrates that the regulation of P and C intake is not optimal for the expression of these traits.

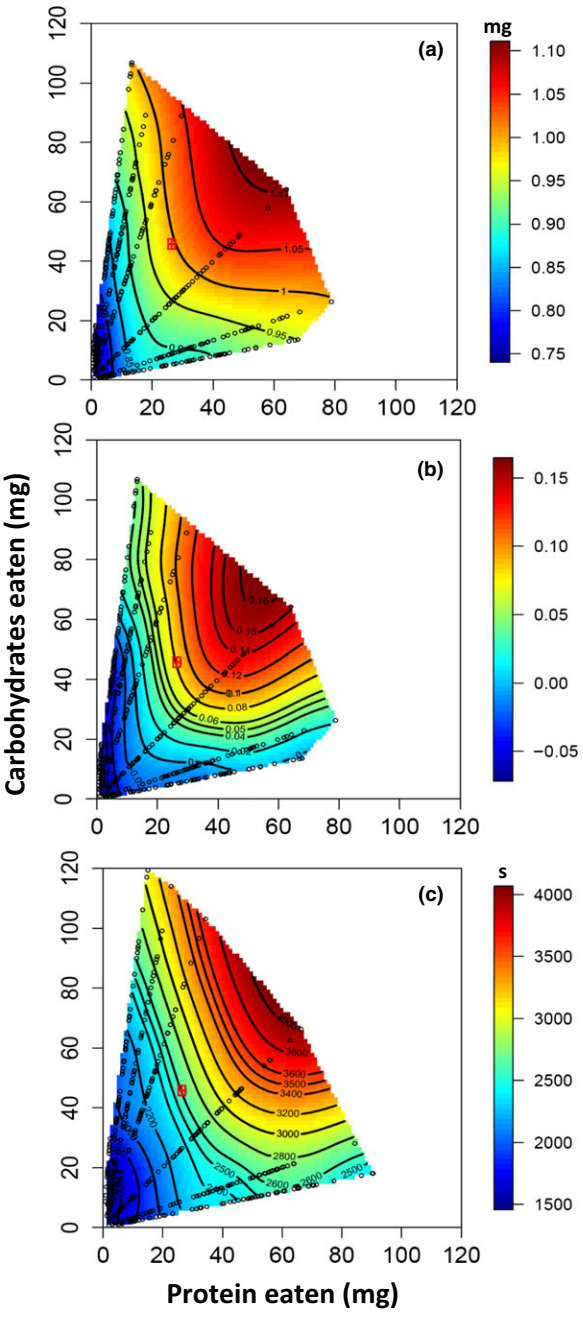

Fig. 1 Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (a) spermatophylax weight, (b) the gustatory appeal of the spermatophylax and (c) ampulla attachment time in *Gryllodes sigillatus*. High values of these traits are given in red and low values in blue. The black dots represent the actual nutrient intake data for each individual cricket and the red cross on each landscape represents the regulated intake point (± SE) that is presented in Fig. 2 and derived from our choice experiment (Experiment 2).

Discussion

In this study, we used the NG to examine the effects of P and C intake on the weight and gustatory appeal of the male spermatophylax in *G. sigillatus*, as well as the role this plays in mediating sexual conflict via ampulla attachment time. If the intake of these nutrients regu-

Table 2 Sequential model comparing the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on spermatophylax (SPHYLAX) weight, the gustatory appeal of SPHYLAX and ampulla (AMP) attachment time in *Gryllodes sigillatus*.

	SS_R	SS_C	DF_1	DF_2	F	P	θ (95% CI)
SPHYLAX weight vs. appeal							
Linear	731.08	727.79	2	762	1.72	0.18	15.47° (7.25°, 26.57°)
Quadratic	718.42	718.20	2	758	0.12	0.89	
Correlational	712.46	711.94	1	756	0.56	0.46	
SPHYLAX weight vs. AMP attachment time							
Linear	683.10	680.17	2	762	1.64	0.19	8.06° (3.92°, 13.69°)
Quadratic	665.80	665.44	2	758	0.21	0.81	
Correlational	659.17	658.20	1	756	1.12	0.29	
SPHYLAX appeal vs. AMP attachment time							
Linear	713.29	701.33	2	762	6.50	0.002*	14.27° (6.65°, 24.38°)
Quadratic	690.22	689.14	2	758	0.59	0.55	
Correlational	678.51	678.42	1	756	0.10	0.75	

Univariate test: *P: $F_{1,762} = 8.96$, $P = 0.003$; C: $F_{1,762} = 4.91$, $P = 0.027$.

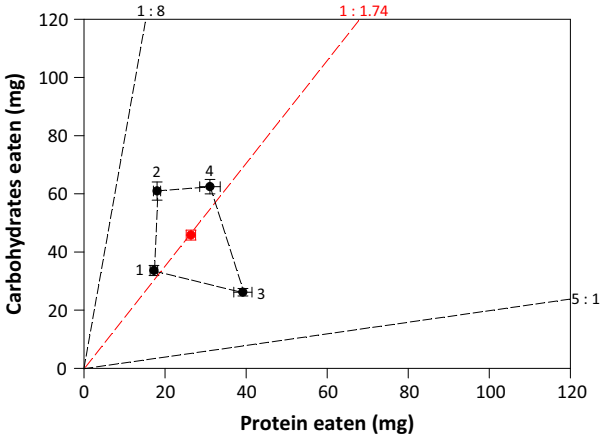

Fig. 2 The mean (± SE) intake of protein (P) and carbohydrates (C) for the 4 diet pairs (black symbols, labelled by number) and the regulated intake point (± SE, red symbol), calculated as the mean P and C intake across diet pairs. The black dashed lines represent the outer boundaries of our choice experiment design (P : C ratios of 5 : 1 and 1 : 8), and therefore, crickets are able to feed to any point in nutritional space within these rails. The red dashed line represents the P : C ratio that passes through the regulated intake point, estimated at a P : C ratio of 1 : 1.74. This is the P : C ratio that crickets actively defend when given dietary choice.

lates sexual conflict in *G. sigillatus*, we predicted that nutrient intake would have similar effects on the weight and gustatory appeal of the spermatophylax, as well as ampulla attachment time. Consistent with this prediction, we found that the nutritional landscapes for these traits were all closely aligned, with each trait being maximized at a high intake of nutrients in a P : C ratio of 1 : 1.3. Furthermore, if males are able to regulate their intake of nutrients to bias the outcome of sexual conflict in their favour, we predicted that when given dietary choice, males would regulate their intake of nutrients to coincide with the peak in ampulla attachment time on the nutritional landscape. In agreement with this prediction, we found that males regulated their intake of nutrients at a P : C ratio of 1 : 1.74, which is close (but not identical) to the P : C ratio maximizing ampulla attachment time and the weight and gustatory appeal of the spermatophylax. This regulated intake point, however, did not fall on the optima for ampulla attachment time or for the weight and gustatory appeal of the spermatophylax. Collectively, our findings show that a balanced intake of P and C plays a crucial role in regulating sexual conflict in *G. sigillatus* but males are unable to completely bias this process in their favour by regulating the intake of these nutrients.

The intake of P and C had clear effects on both the weight and gustatory appeal of the spermatophylax and the nature of these nutritional effects provide two important insights into how this endogenous gift is produced in *G. sigillatus*. First, we show that the weight of the spermatophylax and the amino acid composition that increases the likelihood that a female will fully consume this gift increased with the overall intake of nutrients (and therefore caloric or energy intake). This finding is consistent with the weight and gustatory appeal of the spermatophylax being energetically costly to produce, as only males with the highest intake of P and C have sufficient nutritional resources available to allocate to maximizing these traits. Supporting this view are studies on *G. sigillatus* showing that immune function is traded against spermatophylax weight (Gershman *et al.*, 2010) and males that have their immune function challenged with lipopolysaccharides (Kerr *et al.*, 2010) or sexually transmitted nematodes (Luong & Kaya, 2004) produce smaller spermatophylaxes. We currently do not know whether similar trade-offs exist for the combination of amino acids that enhances the gustatory appeal of the spermatophylax. Second, we

show that the weight and gustatory appeal of the spermatophylax was maximized at a P : C ratio of 1 : 1.3. Thus, it is not only a high intake of nutrients that is important for maximizing these traits in *G. sigillatus,* but the intake of P and C must also be balanced. This P : C ratio is strikingly similar to the ratio known to optimize egg production in a range of female insect species (e.g. P : C = 1 : 2 in *D. melanogaster* (Lee *et al.*, 2008; Reddiex *et al.*, 2013; Jensen *et al.*, 2015); 1 : 2.3 and 1 : 1 in Queensland fruit flies, (Fanson *et al.*, 2009; Fanson & Taylor, 2012); 1 : 1 and 1 : 3 in field crickets, (Maklakov *et al.*, 2008; Harrison *et al.*, 2014), as well as a number of male traits, including sperm production in cockroaches (P : C = 1 : 2, (Bunning *et al.*, 2015) and competitive ability in *D. melanogaster* (P : C = 1 : 2, (Reddiex *et al.*, 2013; Jensen *et al.*, 2015). It contrasts, however, the P : C ratio commonly found to maximize male traits used in precopulatory sexual selection, including calling effort in crickets (P : C = 1 : 8, (Maklakov *et al.*, 2008) and pheromone production in cockroaches (P : C = 1 : 8, (South *et al.*, 2011), where a relatively higher intake of C is needed to fuel these energetically costly traits. Just as female insects require a higher intake of P than males to manufacture eggs (Maklakov *et al.*, 2008; Jensen *et al.*, 2015), there is good reason to expect that producing a heavy spermatophylax with an enhanced gustatory appeal also has a relatively high demand for P. Over 90% of the dry mass of the spermatophylax in *G. sigillatus* consists of P with only 7% of this representing the free amino acids that influence gustatory appeal (Warwick *et al.*, 2009). Consequently, our measure of dry spermatophylax weight largely reflects sources of P in the gift other than free amino acids and it has been speculated that this may represent elastin-like structural proteins that contribute to the gummy consistency of the spermatophylax (Heller *et al.*, 1998). Casein (bovine milk) and albumin (egg yolk) which comprise 80% of the P used in our artificial diets contain high quantities of essential amino acids, including lysine which plays a key role in elastin synthesis (Cohen, 2015). Furthermore, these sources of P are also known to be abundant in many of the essential (e.g. lysine, phenylalanine and valine), and some nonessential (e.g. glycine, histidine, leucine and 4-hydroxyproline) amino acids (Cohen, 2015) known to contribute heavily to the gustatory appeal of the spermatophylax (Gershman *et al.*, 2012). More work is needed, however, to determine how these sources of P are degraded to free amino acids during digestion in *G. sigillatus* and how these amino acids are absorbed and possibly converted before they are incorporated into the spermatophylax.

Importantly, we found that the intake of P and C that maximized the weight and gustatory appeal of the spermatophylax were closely aligned with the intake of nutrients needed to maximize ampulla attachment time. Indeed, the largest angle between the vectors describing the linear effects of nutrient intake on these traits was only 15.47°. This close alignment provides support for our proposed causal relationship linking the properties of the spermatophylax to ampulla attachment time in *G. sigillatus*. That is, a high intake of nutrients with a P : C ratio of 1 : 1.3 enables males to produce a heavy spermatophylax with high gustatory appeal, both of which are known to prolong female feeding on the spermatophylax and increase subsequent ampulla attachment time (Sakaluk, 1984; Gershman *et al.*, 2012). As the number of sperm transferred to a female increases linearly with ampulla attachment time (up to a maximum of 55 min, (Sakaluk, 1984) and sperm competition conforms to a simple lottery system (Eggert *et al.*, 2003), this intake of nutrients is likely to enhance the number of offspring sired by a male *G. sigillatus* when mating in competitive situations. This argument assumes, however, that the intake of nutrients that maximize the weight and gustatory appeal of the spermatophylax also enables males to produce a large number of viable sperm. Although the intake of nutrients that maximizes sperm number and viability is currently not known for *G. sigillatus*, a recent study on the cockroach *Nauphoeta cinerea* found that sperm number was maximized at a high intake of nutrients in a P : C ratio of 1 : 2, whereas the intake of these nutrients did not influence sperm viability (Bunning *et al.*, 2015). Therefore, although more work is needed to confirm this, it appears likely that a male is able to capitalize on producing a heavy and appealing spermatophylax by also producing a large number of viable sperm.

Although the peaks for ampulla attachment time and the gustatory appeal of the spermatophylax were closely aligned, with an angle of only 14.27° between the linear effects of nutrient intake, there was a significant difference in the magnitude of the linear effects of both P and C intake on these traits. More specifically, ampulla attachment time was more responsive to the intake of these nutrients than the gustatory appeal of the spermatophylax. This suggests that factors other than the appeal of the spermatophylax may also influence a female's decision to terminate mating by removing the ampulla. It is possible that females prolong attachment time as a form of post-copulatory mate choice because males with this intake of nutrients are more attractive. This is unlikely, however, as post-copulatory mate choice is known to reinforce precopulatory mate choice in *G. sigillatus* (Ivy & Sakaluk, 2007) and male traits known to be attractive to females before mating, such as calling effort and cuticular hydrocarbon expression, are optimized at a much higher intake of C (P : C ratio of 1 : 8, J. Rapkin, unpublished). It is also possible that substances (other than free amino acids) transferred in the spermatophylax and/or ejaculate influence this female behaviour, especially if substances are influenced by the intake of P and C. A prime candi-

date is SFPs which have been detected in both the ejaculate of field crickets (e.g. Andres *et al.*, 2008; Simmons *et al.*, 2013) and the spermatophylax of bushcrickets (Marchini *et al.*, 2009) and our recent work (Pauchet *et al.*, 2015) has shown that SFPs are also present in the spermatophylax of *G. sigillatus*. SFPs in insects are known to influence many aspects of female reproduction and behaviour, with examples of the latter including an increase in feeding and general activity levels (Avila *et al.*, 2011). Given that most identified insect SFPs represent numerous classes of proteins (e.g. peptides, proteases) (Avila *et al.*, 2011), it is not surprising that their expression is influenced by the amount of dietary P consumed by the male (McGraw *et al.*, 2007). It is more difficult to envisage how the intake of C would influence SFP expression, although it is important to note that seminal fluid in insects also contains a variety of nonprotein molecules (i.e. steroids, prostaglandins) that also influence female behaviour (Avila *et al.*, 2011) and may be influenced by the intake of this nutrient. More work is clearly needed, however, to determine whether the intake of P and C influences SFP expression in the spermatophylax of male *G. sigillatus* and whether this influences the ampulla removal behaviour of females.

Our work shows that males can clearly optimize the size and gustatory appeal of the spermatophylax, as well as subsequent effects on ampulla attachment time, by consuming a high intake of nutrients in a P : C ratio of 1 : 1.3. Yet, when provided with dietary choice, males regulated their intake of nutrients towards a slightly higher intake of C than is optimal (a P : C ratio of 1 : 1.74) and at a much lower overall intake of nutrients. Consequently, this regulated intake point did not coincide with the optima for ampulla attachment time or for the weight and gustatory appeal of the spermatophylax (Fig. 1a–c) and therefore provides clear evidence that male *G. sigillatus* do not optimally regulate their intake of these nutrients to bias the outcome of sexual conflict in their favour. Indeed, inspection of the nutritional landscape in Fig. 1c shows that this observed pattern of nutrient regulation reduces ampulla attachment time by approximately 23 min (or 35%). This reduction in ampulla attachment time is biologically important as comparison to the sperm transfer curve for this species (see fig. 2 in Sakaluk, 1984) reveals that 4×10^{-3} fewer sperm would be transferred to the female compared to the optimal intake of P and C. Given the substantial costs to the suboptimal regulation of nutrient intake shown here, it is surprising that optimality does not appear to be the norm more generally: NG studies on *D. melanogaster* (Jensen *et al.*, 2015), field crickets (Maklakov *et al.*, 2008; Harrison *et al.*, 2014) and cockroaches (South *et al.*, 2011; Bunning *et al.*, 2015) all show that males do not optimally regulate the intake of P and C even though this comes at a substantial cost to trait expression.

There are a number of possible explanations for why male *G. sigillatus* do not optimally regulate their intake of P and C. First, males may regulate their intake of nutrients to maximize the expression of other, more heavily prioritized traits. The regulated intake point shown here for *G. sigillatus* is similar to that observed in male *D. melanogaster* (P : C = 1 : 4; Lee *et al.*, 2013; Jensen *et al.*, 2015), field crickets (P : C = 1.3, Maklakov *et al.*, 2008); 1 : 4.1, (Harrison *et al.*, 2014) and cockroaches (P : C = 1 : 3.2, South *et al.*, 2011; 1 : 4.95, Bunning *et al.*, 2015), where a C biased intake is also preferred. In all of these species, important fitness-related traits in males, such as pheromone expression, competitive ability, calling effort and lifespan, are also maximized at a high intake of C biased diets. Similarly, cuticular hydrocarbon expression, calling effort and lifespan in male *G. sigillatus* are all maximized at a high intake of nutrients in a P : C ratio of approximately 1 : 8 (J. Rapkin, unpublished). It is therefore possible that males bias their relative C intake for the expression of these traits, although given the proximity of the regulated intake point to the optima for traits used in sexual conflict, this appears unlikely to have a large impact on the regulated intake point. Alternatively, males may be regulating their nutrient intake to balance the expression of multiple, competing traits. For example, male cockroaches regulate their intake of nutrients at a P : C ratio of 1 : 4.95 which is midway between the ratio maximizing sperm production (P : C = 1 : 2, Bunning *et al.*, 2015) and pheromone production (P : C = 1 : 8, South *et al.*, 2011). However, it is currently unclear exactly what traits male *G. sigillatus* may be balancing, although we have recently shown that immune function in males is maximized at a P : C ratio of approximately 5 : 1 (J. Rapkin, unpublished). If males are balancing immunity against calling effort, this would produce a regulated intake of nutrients close to our observation in the present study. Second, males may not optimally regulate the intake of P and C because they are constrained from doing so. One constraint that is likely to explain the reduced intake of nutrients at the regulated intake point compared to the optima for traits involved in sexual conflict are physiological constraints on feeding behaviour. It is well known that dietary assimilation, digestion, absorption and utilization can all constrain feeding behaviour in animals (Henson & Hallam, 1995) and that the efficiency of these processes is often contingent on gut morphology (e.g. Penry & Jumars, 1990; McWhorter & del Rio, 2000). If the choice between complex diets limits the efficiency of one or more of these processes and/or is limited by gut morphology, it may prevent males from regulating their intake of nutrients optimally. Another possibility is that male nutrient intake under dietary choice is genetically constrained. Males and females share most of their genome, including the genes that underlie nutrient regulation and this

generates strong positive genetic correlations between the intakes of nutrients across the sexes (r_{MF}). For example, there is a strong positive r_{MF} for C intake across the sexes in *D. melanogaster,* but this relationship is much weaker for P intake (Reddiex *et al*., 2013). In *G. sigillatus*, these positive r_{MFs} are much stronger for the intake of both nutrients (J. Hunt, unpublished). In theory, positive r_{MFs} can prevent nutrient regulation from evolving independently in the sexes, thereby constraining the evolution of sexual dimorphism in this trait (Bonduriansky & Chenoweth, 2009). Thus, if the sexes have different optima for nutrient intake, as is known to occur for lifespan and reproduction in *G. sigillatus* (J. Hunt, unpublished), intralocus conflict over nutrient regulation can occur and one or both sexes can be displaced from their optimal nutrient intake. Although the evidence for intralocus conflict over optimal nutrient intake is not strong in *D. melanogaster* (Reddiex *et al*., 2013), our work suggests that it plays a more significant role in the evolution of the regulated intake point in male *G. sigillatus* (J. Hunt, unpublished). In general, understanding the constraints that shape the evolution of nutrient regulation remains one of the major unanswered questions in nutritional ecology and is clearly a topic that deserves more attention.

In conclusion, we show that nutrition is a key determinant of the weight and gustatory appeal of the spermatophylax, as well as the subsequent effects of consuming this gift on ampulla attachment time, in male *G. sigillatus*. This relationship was complex, however, with a high intake of P and C in a specific balance being required to maximize these traits. Although well-defined nutritional optima for these traits illustrates that males have the potential to bias the outcome of sexual conflict in their favour by regulating their intake of nutrients, males did not do so despite coming at a substantial cost to sperm transfer. Collectively our work shows that sexual conflict is regulated by the intake of these important macronutrients in *G. sigillatus* and highlights the value of using a standardized framework, such as NG, to reveal the complexity of this process.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1. Composition of artificial diets.

Appendix S2. Extraction and quantification of free amino acids in the spermatophylax.

Appendix S3. Estimating the gustatory appeal of the spermatophylax.

Appendix S4. Sequential model building approach to compare the nutritional landscapes for the weight and gustatory appeal of the spermatophylax and ampulla attachment time.

Appendix S5. Calculating the angle (θ) between nutritional vectors and 95% credible intervals.

Appendix S6. Dietary preference in male decorated crickets.

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