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The complex interplay between macronutrient intake, cuticular hydrocarbon expression and mating success in male decorated crickets

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Abstract

The condition dependence of male sexual traits plays a central role in sexual selection theory. Relatively little, however, is known about the condition dependence of chemical signals used in mate choice and their subsequent effects on male mating success. Furthermore, few studies have isolated the specific nutrients responsible for condition-dependent variation in male sexual traits. Here, we used nutritional geometry to determine the effect of protein (P) and carbohydrate (C) intake on male cuticular hydrocarbon (CHC) expression and mating success in male decorated crickets (*Gryllodes sigillatus*). We show that both traits are maximized at a moderate-to-high intake of nutrients in a P:C ratio of 1 : 1.5. We also show that female precopulatory mate choice exerts a complex pattern of linear and quadratic sexual selection on this condition-dependent variation in male CHC expression. Structural equation modelling revealed that although the effect of nutrient intake on mating success is mediated through condition-dependent CHC expression, it is not exclusively so, suggesting that other traits must also play an important role. Collectively, our results suggest that the complex interplay between nutrient intake, CHC expression and mating success plays an important role in the operation of sexual selection in *G. sigillatus*.

Introduction

Sexual selection is widely acknowledged as a major driving force in the evolution of exaggerated male sexual traits (Andersson, 1994; Andersson & Simmons, 2006). A dominant model in the sexual selection literature, known as the handicap model (Zahavi, 1975, 1977; Johnstone *et al.*, 2009), posits that male sexual traits should serve as reliable indicators of male quality in female mate choice (Johnstone, 1995; Lailvaux & Irschick, 2006) and male–male competition (Zahavi, 1975, 1977; Andersson, 1994; Andersson & Simmons, 2006; Johnstone *et al.*, 2009). According to this model, reliability is maintained by the fact that exaggerated sexual traits are costly to produce: because only males of high quality can afford these costs, exaggerated sexual traits should remain honest because they are impossible to mimic by low-quality males (Zahavi, 1975, 1977; Johnstone *et al.*, 2009). If male sexual traits are costly to produce and/or maintain, they should be sensitive to variation in the acquisition of resources and also be subject to trade-offs resulting from variation in resource allocation (Zahavi, 1975, 1977; Rowe & Houle, 1996; Hunt *et al.*, 2004). Indeed, a central prediction underlying handicap models of sexual selection is that male sexual trait expression should covary positively with condition, which can be conceptually defined as the amount of resources an organism has available for allocation to fitness-

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enhancing traits (Rowe & Houle, 1996; Hunt *et al.*, 2004; Tomkins *et al.*, 2004).

Almost two decades has been spent empirically testing this core prediction, and many examples now exist in a range of species showing that male sexual traits show condition-dependent expression (Cotton *et al.*, 2004; Hunt *et al.*, 2004; Tomkins *et al.*, 2004). The most common approach used in empirical studies to experimentally manipulate condition is to vary diet quantity and/or the caloric content of the diet (Cotton *et al.*, 2004; Tomkins *et al.*, 2004). This approach, however, has two main limitations when studying the condition dependence of male sexual traits. First, typically only a small number of diets is used (i.e. two or three) and these diets are often poorly defined with regard to specific nutrient content (e.g. Holzer *et al.*, 2003; Cotton *et al.*, 2004; Hunt *et al.*, 2004). This makes it difficult (if not impossible) to partition the effects of calories and specific nutrients on male sexual trait expression. Second, most studies do not precisely measure food consumption and therefore ignore any effects of compensatory feeding. Compensatory feeding, the ability of an individual to increase its food consumption to compensate for reduced food quality (Simpson & Raubenheimer, 2012), appears widespread in animals (Behmer, 2009) and means that it is possible for individuals on poor-quality diets to consume as many calories or nutrients as on a good-quality diet. Compensatory feeding therefore has the potential to obscure any differences in condition dependence across dietary treatments.

All of the above limitations can be resolved using chemically defined (holidic) diets of known nutrient composition within the Geometric Framework (GF) for nutrition combined with precise measurements of dietary consumption (Simpson & Raubenheimer, 2012). The GF is a multidimensional nutritional approach, within which the effects of the intake of multiple nutrients (n) can be separated in n-dimensional nutritional space by restricting individuals to a geometric array of diets that differ in known nutrient composition and concentration (i.e. calories) (Simpson & Raubenheimer, 2012). When combined with precise measurements of diet consumption (allowing nutrient intake to be calculated), the GF provides a powerful way to partition the effects of specific nutrient and caloric intake on condition-dependent sexual trait expression (Morehouse *et al.*, 2010). Indeed, empirical studies on a number of insect species have shown that a balanced intake of nutrients is more important to sexual trait expression than the intake of calories *per se* (Maklakov *et al.*, 2008; Sentinella *et al.*, 2013; Harrison *et al.*, 2014; Bunning *et al.*, 2015; Cordes *et al.*, 2015; House *et al.*, 2015).

Most empirical studies on the condition dependence of male sexual traits in insects have focussed on conspicuous male sexual traits, including morphological structures that serve as weapons (e.g. Johns *et al.*, 2014) and ornaments (e.g. Cotton *et al.*, 2004), courtship displays (e.g. Kotiaho, 2002), acoustic signals (e.g. Hunt *et al.*, 2004) and coloration (e.g. Punzalan *et al.*, 2008). In contrast, the condition dependence of chemical signals is relatively poorly studied, which is surprising given that sex pheromones and cuticular hydrocarbons (CHCs) play an important role in the recognition of species, the sexes and kin in insects (Wyatt, 2003; Blomquist & Bagnères, 2010). Male CHCs are also known to be the target of female choice in various cricket (Thomas & Simmons, 2009, 2011; Weddle *et al.*, 2012; Steiger *et al.*, 2013, 2015) and *Drosophila* (Ferveur, 2005; Ingleby *et al.*, 2013, 2014) species. There is also growing evidence that sex pheromone (e.g. Conner *et al.*, 1981; Clark *et al.*, 1997; McGuigan, 2006; Ming & Lewis, 2010) and CHC (e.g. Liang & Silverman, 2000; Hine *et al.*, 2004; Gosden & Chenoweth, 2011; Weddle *et al.*, 2012) expression are sensitive to diet, but the actual nutrients responsible for these effects are poorly understood.

To date, only two studies have used the GF to examine the condition dependence of chemical signals (South *et al.*, 2011; Fedina *et al.*, 2012). Fedina *et al.* (2012) provided female *D. melanogaster* with four diets differing in the percentage of sugar (S) to yeast (Y) in a factorial design and examined CHC expression at various ages. Diet composition was shown to have consistent and significant effects on female CHCs across ages, with dietary S and Y driving changes in CHCs in opposite directions. For example, there was nearly a twofold increase in the total CHCs produced by females with age when consuming a high Y diet, whereas females consuming low Y diets maintained similar levels of CHCs with age. In contrast, the amount of dietary S consumed did not influence total CHC levels or their change with age. This study is limited, however, by the fact that the use of medium-based diets precluded the measurement of dietary intake, and yeast was used as the only source of protein. Although yeast is high in protein, it also contains carbohydrate, lipids, salts and a number of vitamins making it impossible to identify which key nutrients are responsible for the above effects of yeast consumption. In contrast, South *et al.* (2011) used a much larger number of holidic diets and precisely measured the intake of protein (P) and carbohydrate (C) to determine the effects of these macronutrients on the expression of the three male sex pheromones (3-hydroxy-2-butanone, 2-methythiazolidine and 4-ethyl-2-methoxyphenol) in the cockroach *Nauphoeta cinerea*, and the subsequent effects on dominance and attractiveness. All three sex pheromones and male attractiveness increased with the intake of C (being maximized at a P:C ratio of 1 : 8) but were largely unaffected by the intake of P, whereas male dominance was not affected by the intake of either nutrient. Furthermore, when given a choice between alternate diets, males preferentially consumed high C diets to maximize

their attractiveness to females. This work therefore not only illustrates how nutrients can have very different effects on the condition-dependent expression of chemical signals, but also that this can have important consequences for male mating success and the subsequent operation of sexual selection.

In the decorated cricket (*Gryllodes sigillatus*), CHCs play an important role in regulating sexual selection. Females have been shown to exert a complex pattern of linear and nonlinear (mainly stabilizing) sexual selection on male CHCs during precopulatory mate choice, and this preference appears independent of the similarity in CHC profile of the male and choosing female (Steiger *et al.*, 2015) suggesting that females do not use CHCs during mate choice to avoid inbreeding (Weddle *et al.*, 2013). CHCs also play an important role in regulating polyandry in *G. sigillatus* (Ivy *et al.*, 2005; Weddle *et al.*, 2013). Female *G. sigillatus* prefer mating with novel males over previous mates and physically imbue males with their own CHCs during mating to enforce this mating preference (Ivy *et al.*, 2005; Weddle *et al.*, 2013; Capodeänu-Nagler *et al.*, 2014). CHC expression in *G. sigillatus* is known to be influenced by diet, but this effect is sex-specific and depends on genotype (Weddle *et al.*, 2012). By varying the quality of the diet fed to juvenile and adult crickets from a series of inbred lines, Weddle *et al.* (2012) found that although the effects of diet and genotype × diet interactions on CHC expression were pronounced in males, dietary effects were small and genotype × diet interactions were absent in females. Unfortunately, the high- and low-quality diets used in this study varied in both nutrient composition and overall energy content, so it is impossible to determine whether the observed dietary effects on male CHCs are due to the intake of specific nutrients or calories *per se*. Furthermore, it is currently not known whether diet also influences male mating success and if so, the role that condition dependent CHC expression plays in mediating this effect.

In this study, we examine the effects of P and C intake on CHC expression in male *G. sigillatus*, as well as the subsequent effects of nutrient intake and CHC expression on mating success. We start by using a GF approach and restricting adult males to one of 24 unique artificial, holidic diets in a geometric array to document the linear and nonlinear effects of nutrient intake on CHC expression and male mating success. Using data from our previous work on *G. sigillatus* (Rapkin *et al.*, 2016), we also map the regulated intake point (RIP) for P and C onto the nutritional landscapes for CHC expression and mating success to determine whether males optimally regulate their intake of nutrients to maximize these traits. This RIP is defined as the point in nutrient space that individuals actively defend when given dietary choice (Simpson & Raubenheimer, 1993) and was calculated as the mean total intake of P and C across all four of the diet pairs used by Rapkin *et al.* (2016) in their dietary choice experiment. Next, we use multivariate selection analysis to determine how this condition-dependent variation in male CHC expression influences male mating success and therefore the strength and form of sexual selection targeting male CHCs. Finally, we use a structural equation modelling (SEM) approach to determine whether the effects of nutrient intake on mating success are mediated exclusively by condition-dependent CHC expression or whether other sexual traits are also involved in mediating this relationship. If nutrient intake has similar effects on male CHC expression and mating success, we predict that the peak (or maximum) on the nutritional landscapes for these traits will occupy the same region in nutrient space. For this to occur, the peaks for the two traits need to be aligned to the same P:C ratio and occur at the same total intake of nutrients. Furthermore, if the condition-dependent expression of CHCs is a key determinant of male mating success, we predict that female mate choice will exert significant sexual selection on male CHC expression and that our SEM model will show that the effect of nutrients on mating success is mediated exclusively through CHC expression.

Materials and methods

Experimental animals

The *G. sigillatus* used in this study were descended from 500 adults crickets collected in Las Cruces, New Mexico, in 2001 which were used to initiate a laboratory culture maintained at a population size of approximately 5000 crickets and allowed to breed panmictically (Ivy & Sakaluk, 2005). Cricket cultures were 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 provided with cat food (Go-Cat Senior®, Purina, St Louis, MO, USA), rat food pellets (SDS Diets, Essex, UK) and 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 in our cultures.

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 cm × 10 cm × 10 cm) and then randomly assigned to a diet.

Artificial diets and measuring dietary intake

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

Each experimental male was randomly assigned to a diet and provided with a single feeding platform of a measured dry weight of diet on their day of eclosion to adulthood. As expected, there was no difference in the pronotum width ($F_{23,744} = 0.70$, $P = 0.85$) or body weight ($F_{23,744} = 0.95$, $P = 0.53$) of males across these 24 diets. This diet was changed every 2 days for a total of 10 days (five feeding sessions) until males were sexually mature and mating behaviour and CHC profile were assessed. 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 (Binder® model FD 115, 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 (Ohaus Explorer Professional model EP214C, USA). Prior to the 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 by multiplying by the percentage of these nutrients in the diet following the procedure outlined in South *et al.* (2011).

Experimental design and measuring male mating success

To determine the effects of P and C intake on the CHC profile of males, as well as their subsequent mating success, 32 males were established at random on each of the 24 diets (total $n = 768$ males) on their day of eclosion to adulthood and fed for 10 days to quantify their intake of P and C. At day 10, each male was placed in a clear plastic arena (20 × 10 × 10 cm), illuminated by red light and maintained in a constant temperature room maintained at 28 ± 1 °C, and allowed to acclimate for 60 s before a virgin, 10-day-old female was introduced. The male was given 20 min to initiate courtship after the female had been introduced into the arena. If the male failed to court during this period, he was excluded from the study, as we could not be certain the females had assessed his quality. A mating was recorded as successful if the male transferred a spermatophore to the female in this 20-min period. Successful males were assigned a score of 1 and those that were unsuccessful were assigned a score of 0. At the end of behavioural observations, each male was immediately placed in an individual microcentrifuge tube and frozen at −80 °C for CHC analysis.

Extraction and chemical analysis of CHCs

CHCs were extracted by whole-body immersion in a 5-ml glass vial containing 3 mL of HPLC-grade hexane (Fisher Scientific, Loughborough, UK) and 10 ppm dodecane as an internal standard for 5 min. The cricket was removed from the vial using metal forceps which were cleaned in methanol between each sample. 50 μL of the extract was then transferred to a glass autosample vial (Chromacol, UK).

CHC extracts were run on an Agilent Technologies 7890 Gas Chromatography with Flame Ionization Detector (GC-FID). A 2 μL volume of each extract was injected using an Agilent G4513A autosampler connected to an Agilent 7692 sample tray chilled to 5 °C onto a DB-1 column (30 m × 0.25 mm internal diameter × 0.25 μm film thickness). Hydrogen was used as the carrier gas. Both inlet lines were set at 325 °C, and the injection was in pulsed splitless mode. Separation of the extract was optimized using a column profile which operated at 50 °C for 30 s and then increased at 20 °C min^{-1} to 320 °C where it then increased at 7.5 °C min^{-1} to 350 °C where it was held for 5 min with a flow rate of 20 mL min^{-1}. The total run time for each extract was therefore 23 min. The area under each CHC peak was quantified using CHEMSTATION software (v. B.04.02.SP1; Agilent Technologies, Stockport, UK).

Prior to statistical analysis, the area under each CHC peak was divided by the area of the internal standard (dodecane) to control for drift in the sensitivity of the GC-FID over time. This proportion was then $\log_{10}$-transformed to ensure normality of each CHC peak in our dataset (Weddle *et al.*, 2012, 2013). Two CHC peaks identified in our previous studies on *G. sigillatus* (5,9-C_{37}diene and 3,9-C_{37}diene, Weddle *et al.*, 2012, 2013; Steiger *et al.*, 2015) were not present in all samples and were therefore excluded from further analysis.

Statistical analysis

Due to the large number of CHC peaks being examined (see Table 1), we extracted principal components (PCs) based on the correlation matrix and retained PCs with eigenvalues exceeding 1 for subsequent analyses (Tabachnick & Fidell, 2001). In total, three PCs were retained for further analysis based on this criterion. We interpret component loadings exceeding |0.30| as biologically important (Tabachnick & Fidell, 2001).

We used a multivariate response surface approach (South *et al.*, 2011) to determine the linear and nonlinear effects of P and C intake on the PCs describing the variation in male CHC expression and mating success. Prior to analysis, we standardized nutrient intake and

Table 1 Principal component (PC) analysis of CHC expression in male *Gryllodes sigillatus*. PCs with eigenvectors exceeding one are presented and used in subsequent analysis. Factor loadings >0.30 (in bold) are interpreted as biological significant (Tabachnick & Fidell, 2001). CHCs are named where known and unnamed CHCs (asterisks) are described by basic chemical structure. CHCs are listed in order of increasing carbon chain length.

	PC1	PC2	PC3
Eigenvalue	7.24	3.04	1.36
% Variance	45.25	18.97	8.50
Loadings			
7-MeC_{33}	**0.93**	0.06	−0.15
5-MeC_{33}	**0.84**	0.14	−0.27
3-MeC_{33}	**0.85**	0.03	−0.27
3,7-diMeC_{33}	**0.85**	0.12	−0.12
7-C_{35}ene	**0.40**	0.28	−0.23
3,13-diMeC_{36}	**0.64**	0.28	−0.03
5,9-diMeC_{36}	**0.57**	**0.62**	−0.05
9,31-C_{37}diene	**0.39**	**0.82**	0.16
7,31-C_{37}diene	**0.40**	**0.77**	0.12
9,31-C_{38}diene	**0.74**	−0.20	0.17
Alkatriene ($C_{39}H_{74}$)*	**0.62**	−0.19	**0.60**
Alkatriene ($C_{39}H_{74}$)*	**0.57**	**−0.41**	**0.58**
9,31-C_{39}diene	**0.89**	−0.18	0.18
7,31-C_{39}diene	**0.71**	**−0.46**	0.15
Alkatriene ($C_{41}H_{78}$)*	**0.50**	**−0.66**	−0.28
9,31-C_{41}diene	**0.49**	**−0.51**	**−0.48**

our response variables to a mean of zero and a standard deviation of one using a *Z* transformation so that our nutritional gradients were in comparable units and to ensure that any differences between nutritional gradients were not driven by scale alone. Our measure of male mating success (0 or 1) did not conform to a normal distribution and although this does not influence the estimation of gradients from a response surface (Lande & Arnold, 1983), it can bias tests of their statistical significance (Mitchell-Olds & Shaw, 1987). We therefore used a resampling procedure to assess the significance of our nutritional gradients (Mitchell-Olds & Shaw, 1987). We randomly shuffled mating success across males in our data set to create a null distribution for each nutritional gradient where there is no relationship between the intake of nutrients and mating success. We then used a Monte Carlo simulation to determine the number of times (out of 10 000 iterations) that each nutritional pseudo-gradient (β_{rand}) was greater than or equal to the original nutritional gradient (β_{real}) and this was used to calculate a two-tailed probability value following the protocol outlined in Manly (1997). We used nonparametric thin-plate splines (Green & Silverman, 1994) to visualize the nutritional landscapes for each of our response variables. Thin-plate splines were constructed using the *Tps* function in the FIELDS package of R (version 2.15.1, www.r-project.org) and were visualized as contour maps using the value of the smoothing parameter (λ) that minimized the generalized cross-validation score (Green & Silverman, 1994). Although analyses were conducted on standardized data, we visualize our nutritional landscapes on raw data for ease of interpretation.

We used a sequential model building approach (Draper & John, 1988) to determine whether the linear and nonlinear effects of P and C intake differed across our response variables (South *et al.*, 2011). Full details of this approach are provided elsewhere (South *et al.*, 2011; Bunning *et al.*, 2015; Rapkin *et al.*, 2016). In brief, we started by fitting a linear model to the data, including a dummy variable (response type = PC1, PC2, PC3 or mating success) as a fixed effect, P and C intake as covariates and the actual measures associated with the dummy variable as the response variable. From this reduced model, we extracted the residual sums of squares (SS_r). We then ran a second linear model that included all the interactions between the dummy variable and the covariates and again extracted the residual sums of squares for this complete model (SS_c). A partial *F*-test was then used to statistically compare SS_r and SS_c, whereby a significant reduction in SS_c compared to SS_r indicates that the complete model significantly increases the amount of variance explained and therefore demonstrates that the nutritional gradients differ significantly across the dummy variable. This model was repeated by sequentially adding the quadratic terms for nutrient intake (P × P and C × C) and then the correlational term (P × C). In cases where an overall significant difference was detected, univariate interaction terms from the complete model were used to determine which nutrients contributed to this effect. Importantly, this approach only statistically compares the magnitude of linear and nonlinear nutritional gradients between the response variables and does not provide any information of the direction of this difference in nutritional space. Therefore, it is possible for two response variables to differ in the magnitude of their nutritional gradients, but be optimized in similar regions on the nutritional landscape. Consequently, we also calculated the angle (θ) between the linear vectors for the two response variables being compared using trigonometry and the 95% confidence interval for θ using a Bayesian approach implemented in the MCMCglmm package of R. When $\theta = 0°$, the vectors are perfectly aligned, whereas $\theta = 180°$ represents the maximum possible divergence between these vectors. Full details of these calculations and associated R code are presented in Data S1. The above statistical comparisons were only conducted between response variables that showed a statistically significant effect of nutrient intake.

We used standard multivariate selection analysis (Lande & Arnold, 1983) to evaluate the strength and form of linear and nonlinear sexual selection acting on male CHCs through female mate choice. Following convention (Lande & Arnold, 1983), we transformed our absolute measure of male mating success (i.e.

1 = successful, 0 = unsuccessful) to relative mating success by dividing by the mean absolute mating success of the population. We use this measure of relative mating success as a proxy for relative fitness in the population (e.g. Steiger *et al.*, 2015). To estimate the standardized linear selection gradients (β), a first-order linear multiple regression model was fitted using the three PCs describing the variation in male CHC expression as the predictor variables and relative fitness as the response variable (Lande & Arnold, 1983). We then used a second-order quadratic multiple regression model that included all linear, quadratic and cross-product terms to estimate the matrix of standardized nonlinear selection gradients ($\boldsymbol{\gamma}$) that describes the curvature of the fitness surface (Lande & Arnold, 1983). As multiple regression analysis is known to underestimate the quadratic regression coefficients by a factor of 0.5, we doubled the standardized quadratic selection gradients derived from this model (Stinchcombe *et al.*, 2008). As relative fitness does not conform to a normal distribution, we used the Monte Carlo procedure outlined above to test the significance of our standardized selection gradients.

The strength of nonlinear selection gradients is known to be underestimated by interpreting the size and significance of individual $\boldsymbol{\gamma}$ gradients (Blows & Brooks, 2003). We therefore used canonical analysis of the $\boldsymbol{\gamma}$ matrix to locate the major eigenvectors of the fitness surface (Phillips & Arnold, 1989). We used the double regression method (Bisgaard & Ankenman, 1996) to estimate the strength of linear selection (θ_i) operating along each eigenvector ($\mathbf{m}_i$). This approach, however, is known to inflate type I error when estimating the strength of nonlinear selection ($\boldsymbol{\lambda}_i$) operating along $\mathbf{m}_i$. We therefore used the permutation procedure outlined in Reynolds *et al.* (2010) to determine the strength and significance of nonlinear selection operating along $\boldsymbol{\lambda}_i$. We used thin-plate splines (Green & Silverman, 1994) to visualize the major eigenvectors of the fitness surface following the procedure outlined above for the visualization of nutritional landscapes.

We used SEM to partition the direct effects of nutrient intake on mating success from the indirect effects of nutrient intake on mating success that are mediated through CHC expression. We evaluated two competing models. In the first, we modelled mating success as influenced directly by the standardized linear and quadratic effects of nutrient intake and indirectly through their influence on CHCs (Fig. 1). We refer to this model as our *partial mediation model*, as it estimates the residual influence of nutrient intake on mating success after controlling for the influence of CHC expression on mating success. In contrast, our second model constrained the residual influences (i.e. the slopes) of the standardized linear and quadratic effects of nutrient intake on mating success to zero (Fig. 1). In other words, we fit a model – which we refer to as our *full mediation model* – in which the influence of nutrient intake was forced to impact mating success exclusively through changes in CHC expression, as opposed to allowing nutrient intake to directly influence mating success through other, unaccounted for, pathways. As the interaction between nutrients in our response surface analysis (i.e. P × C) and between the PCs in our multivariate selection analysis (i.e. standardized correlational selection terms) were not statistically significant (see Results), and would require more demanding forms of model estimation given their nonlinearity, we omitted these terms from our SEM models. We analysed our SEM models using the LAVAAN package in R (Rosseel, 2012). As mating success was measured as a categorical variable, we used the diagonal-weighted least-squares estimator when fitting all models. We then evaluated each of our models using two descriptive fit indexes – a relative index and an absolute index (Hu & Bentler, 1999) – and carrying out nested model comparisons using competing models' chi-square fit statistics. The relative index we used was the comparative fit index (CFI), which compares the fit of the estimated model to the fit of a null model in which all observed variables are uncorrelated; a CFI value >0.90 indicates an acceptable fit (Hu & Bentler, 1999; Little, 2013). The absolute index we used was the root-mean-square error of approximation (RMSEA), which indicates the amount of misfit in the model per degree of freedom; a RMSEA value <0.08 indicates an acceptable fit, and a 90% confidence interval can be estimated to perform a test of 'close fit' of the data (i.e. RMSEA ≤ 0.05).

Results

PC analysis of the 16 individual CHC peaks yielded three PCs with eigenvalues exceeding 1, which collectively explain 72.72% of the total variation in male CHC expression (Table 1). PC1 accounts for 45.25% of the total variation in CHC expression and is positively loaded to each CHC peak (Table 1). Consequently, this vector represents the absolute amount of CHCs possessed by males. PC2 explains a further 18.97% of the total variation in male CHC expression and is positively loaded to shorter-chained CHCs (less than C_{37}) and negatively loaded to longer-chained CHCs (greater than C_{37})(Table 1). This vector therefore describes the trade-off between long- and short-chained CHCs. PC3 explains the remaining 8.50% of the total variation in male CHC expression and is positively loaded to the two unidentified alkatrienes ($C_{39}H_{74}$) and negatively loaded to 9,31-C_{41}diene (Table 1). This vector therefore describes the trade-off between these specific CHCs.

The effects of nutrition on CHCs and male mating success

The intake of P and C had clear linear and nonlinear effects on the variation in CHC expression described by

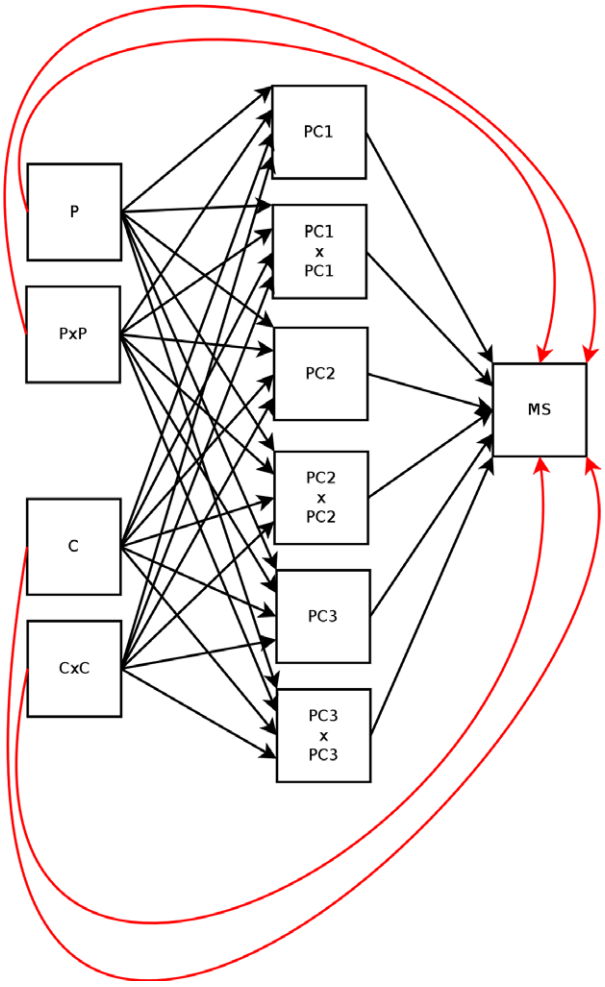

Fig. 1 Path diagram illustrating the alternate models used to predict male mating success (MS) from the standardized linear and quadratic effects of protein (P) and carbohydrate (C) intake and the three principal components describing the variation in CHC expression (PC1, PC2, PC3). In the *partial mediation model*, we model the effects of nutrient intake as directly influencing MS (red pathways) and indirectly influencing MS through their effects on CHC expression. In the *full mediation model*, we model the effects of nutrient intake as influencing MS exclusively through their effects on CHC expression (i.e. the red pathways are constrained to zero).

PC1 and PC3 (Table 2, Fig. 2a,b). PC1 and PC3 increased linearly with the intake of P and C, and these traits were equally responsive to the intake of both nutrients (Table 2). The significant negative quadratic terms indicate a peak in PC1 and PC3 with the intake of both nutrients (Table 2), and inspection of the nutritional landscapes shows that these peaks occur at high intakes of P and C, centred around a P:C ratio of approximately 1 : 1.5 for PC1 (Fig. 2a) and 1 : 1 for PC3 (Fig. 2b). There were no significant correlational effects of nutrients on PC1 or PC3 (Table 2). Formal statistical comparison using a sequential model building approach showed that the linear, quadratic and correlational effects of P and C intake on PC1 and PC3 did not differ significantly (Table 3). Furthermore, the angle between the linear nutritional gradients for PC1 and PC3 was small (9.74°) indicating that the peaks for these traits are closely aligned (Table 3) and inspection of the landscapes showed they occupy similar regions in nutrient space (Fig. 2a,b). In contrast to PC1 and PC3, there was no linear or nonlinear effect of P and C intake on PC2 (Table 2).

Male mating success also increased linearly with the intake of P and C, and this trait was equally responsive to the intake of both nutrients (Table 2). There was also significant negative quadratic terms indicating a peak in mating success with the intake of both nutrients (Table 2), and inspection of the nutritional landscape shows that this peak occurs at a high intake of P and C at a P:C ratio of approximately 1 : 1.5 (Fig. 2c). The correlational effect of P and C intake on mating success was not significant (Table 2). Formal comparison showed that the linear effects of nutrient intake on mating success differed significantly from the linear effects on PC1 and PC3 (Table 3). In the case of PC1, this difference was due to the fact that PC1 was more responsive to the intake of C than mating success, whereas for PC3 was due to the fact that PC3 was more responsive to the intake of both nutrients than mating success (Table 3). Despite these differences, the angles between the linear nutritional gradients for PC1 and mating success (16.07°) and between PC3 and mating success (16.11°) were small indicating that all these traits are closely aligned in nutrient space (Table 3). Inspection of the nutritional landscapes, however, showed that mating success peaked at a lower total intake of nutrients than PC1 and PC3 (Fig. 2a–c). There were no significant differences in the quadratic or correlational effects of nutrient intake on PC1, PC3 and mating success (Table 3).

In our previous work examining the nutritional regulation of sexual conflict in male *G. sigillatus* (Rapkin *et al.*, 2016), males were given the choice between alternate pairs of diets to determine how they regulate their intake of P and C. We found that males regulated their intake of nutrients to a mean (±SE) P and C intake of 26.39 ± 1.16 mg and 45.79 ± 1.70 mg, respectively, which equates to a P:C ratio of 1 : 1.74 (Rapkin *et al.*, 2016). Importantly, the RIP was not well aligned with any of the traits that regulate sexual conflict in *G. sigillatus* (spermatophylax weight, the gustatory appeal of the spermatophylax and ampulla attachment time) suggesting that males are not regulating their intake of nutrients to optimize these traits (Rapkin *et al.*, 2016). To determine whether males regulate their intake of nutrients to optimize CHC expression and/or mating success, we similarly mapped the RIP estimated in Rapkin *et al.* (2016) onto the

Table 2 The linear and nonlinear (quadratic and correlational) effects of protein (P) and carbohydrate (C) intake on the three PCs describing CHC expression, as well as on mating success, in male *G. sigillatus*.

	Linear effects		Nonlinear effects		
Response variable	P	C	P × P	C × C	P × C
PC1					
Gradient ± SE	0.16 ± 0.04	0.20 ± 0.04	−0.12 ± 0.03	−0.10 ± 0.03	0.07 ± 0.05
t_{765}	4.51	5.71	4.03	3.28	1.26
P	0.0001	0.0001	0.0001	0.001	0.21
PC2					
Gradient ± SE	−0.02 ± 0.04	−0.03 ± 0.04	0.01 ± 0.03	−0.04 ± 0.03	−0.02 ± 0.06
t_{765}	0.62	0.87	0.32	1.20	0.27
P	0.54	0.39	0.75	0.23	0.79
PC3					
Gradient ± SE	0.19 ± 0.04	0.20 ± 0.04	−0.07 ± 0.03	−0.16 ± 0.03	−0.06 ± 0.05
t_{765}	5.44	5.59	2.33	5.42	1.02
P	0.0001	0.0001	0.02	0.0001	0.31
Mating success					
Gradient ± SE	0.08 ± 0.04	0.09 ± 0.04	−0.09 ± 0.03	−0.12 ± 0.03	−0.08 ± 0.06
$\beta_{rand} \geq \beta_{real}$	210	68	9953	9999	9197
P	0.04	0.01	0.009	0.0002	0.16

nutritional landscapes for PC1, PC3 and mating success (Fig. 2). In the case of PC1 and PC3, the RIP did not occupy the same nutritional space as the peaks on the nutritional landscapes (Fig. 2a,b). Specifically, even though the peaks for PC1 and PC3 and the RIP are aligned on a similar P:C ratio, the RIP was at a much lower intake of nutrients than the peak for either trait (Fig. 2a,b). In contrast, the RIP was well aligned with the peak for mating success on the nutritional landscape (Fig. 2c) suggesting that when given dietary choice, males regulate their intake of nutrients to optimize this trait.

The effects of CHCs on male mating success

Standardized linear and nonlinear selection gradients for the PCs describing the variation in male CHCs are presented in Table 4. There was significant linear sexual selection favouring higher values of PC1 and lower values of PC2 (Table 4). There was also significant stabilizing selection operating on PC1 and PC3 (Table 4). There was, however, no significant correlational selection targeting the covariance between PC scores (Table 4).

Canonical analysis of the γ matrix resulted in two eigenvectors ($\mathbf{m_2}$ and $\mathbf{m_3}$) with significant nonlinear sexual selection, and in both cases, the associated eigenvalues were negative, indicative of multivariate stabilizing selection (Table 5, Fig. 3). The dominant eigenvector of stabilizing selection ($\mathbf{m_3}$) is negatively weighted to PC3 and positively weighted to PC1, whereas $\mathbf{m_2}$ is positively weighted to both PC1 and PC3 (Table 5). There was also significant linear selection favouring high values of $\mathbf{m_3}$, which equates to higher values of PC1, and higher values of $\mathbf{m_2}$, which equates to higher values of PC1 and PC3 (Table 5).

Does condition-dependent CHC expression exclusively mediate the effect of nutrient intake on mating success?

The partial mediation model provided a good fit to the data (χ^2_{12} = 37.57, $P < 0.001$, CFI = 0.96, RMSEA = 0.05, 90% CIs = 0.03, 0.07) and accounted for approximately 15% of the observed variation in male mating success. In comparison, the full mediation model did not appear to fit the data as well, (χ^2_{16} = 70.86, $P < 0.001$, CFI = 0.91, RMSEA = 0.06, 90% CIs = 0.05, 0.08), accounting for only 14% of the observed variation in male mating success. Indeed, a nested model comparison confirmed that the partial mediation model provided a significantly better fit to the data than the full mediation model ($\Delta\chi^2_{2.71}$ = 24.68, $P < 0.001$). This formally demonstrates that the effect of nutrient intake on mating success is not mediated exclusively through CHCs meaning that other traits must also play an important role in mediating this relationship.

SEM parameter estimates for the partial mediation model are presented in Table 6. These parameter estimates are largely consistent with our previous response surface and multivariate selection analyses. Consistent with our response surface analysis, the intake of P and C was both positively associated with PC1 and PC3, although the significant negative quadratic terms indicate that these relationships plateaued at higher intakes of both nutrients (Table 6). In contrast, PC2 was relatively unaffected by the intake of nutrients (Table 6). Consistent with our multivariate selection analysis,

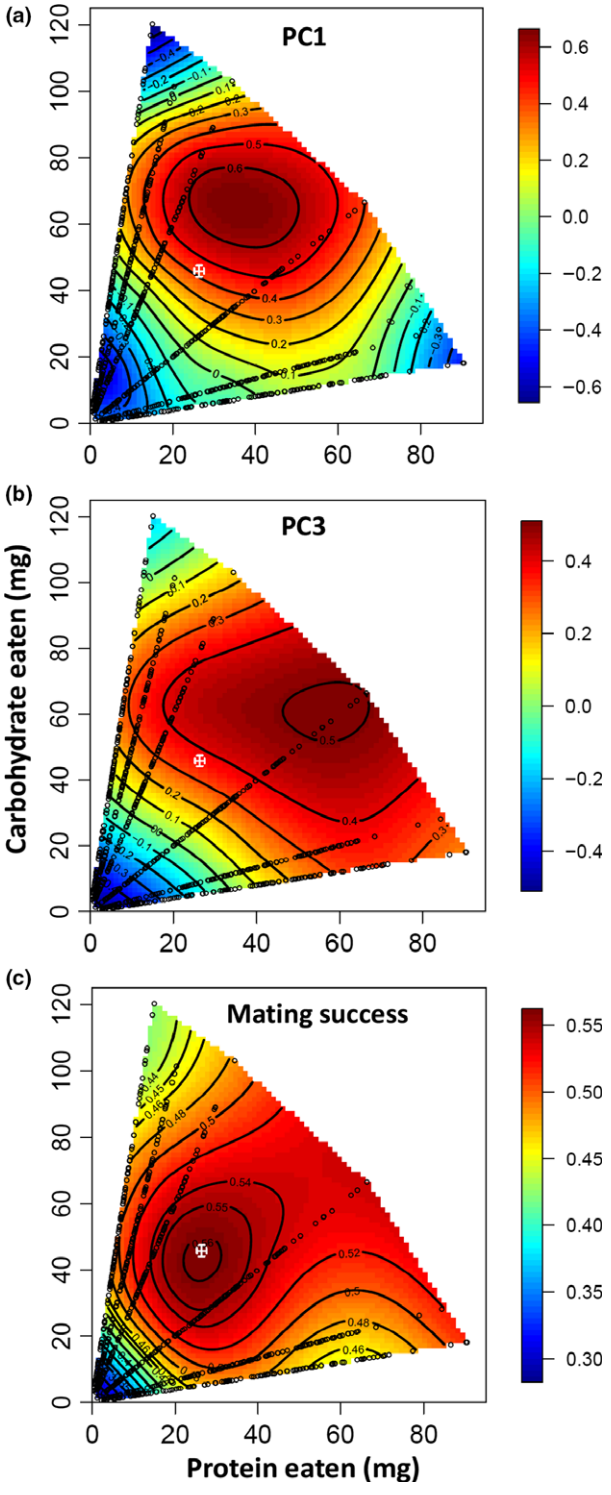

Fig. 2 Nutritional landscapes illustrating the effects of protein and carbohydrate intake on (a) PC1 and (b) PC3 that describe the variation in CHC expression and (c) mating success in male *Gryllodes sigillatus*. High values of these response variables are given in red and low values in blue. The black dots represent the actual nutrient intake for each male cricket in our experiment, and the white cross on each landscape represents the regulated intake point (±SE) estimated in Rapkin *et al.* (2016) for male *G. sigillatus* when given dietary choice.

mating success increased with higher values of PC1 and the significant negative quadratic term indicates that this relationship plateaued at higher PC1 values (Table 6). In contrast, mating success increased at lower values of PC2 and the significant negative quadratic term for PC3 indicates that mating success peaks at intermediate values of this vector (Table 6). The residual linear effects of P and C intake on mating success, after controlling for the variation in CHC expression, were positive and significant (Table 6). Furthermore, the residual quadratic effects of P and C intake on mating success were significant and negative indicating that these relationships plateaued at higher intake of both nutrients (Table 6). A formal test of the linear mediated effects of P and C intake on mating success through changes in CHC expression using the Monte Carlo method for assessing mediation (Preacher & Selig, 2012) indicated that only PC1 significantly mediated the effects of both nutrients on mating success (Table 7). However, given the significant negative quadratic relationships between the intake of both nutrients and PC1, and between PC1 and mating success, it is highly likely that the strength of this mediated effect would also plateau at higher nutrient intakes and values of PC1 (Table 7).

Discussion

In this study, we used the GF to examine the effects of P and C intake on CHC expression in *G. sigillatus*, as well as the relative importance of nutrient intake and condition-dependent CHC expression to male mating success. If P and C intake have similar effects on male CHC expression and mating success, we predicted that these traits would occupy a similar region in nutrient space. Consistent with this prediction, we found that the nutritional landscapes for these traits occupied similar regions in nutrient space with the dominant vector of CHC expression (PC1) and mating success both being maximized at a high intake of nutrients in a P:C ratio of 1 : 1.5, whereas the third vector (PC3) describing CHC variation was maximized at a high nutrient intake with a P:C ratio of 1 : 1. Furthermore, if the condition-dependent expression of CHCs is a key determinant of male mating success, we predicted that female precopulatory mate choice will exert significant sexual selection on male CHC expression and that our SEM modelling approach would show that the effect of nutrient intake on male mating success is significantly mediated

	SS_R	SS_C	DF_1	DF_2	F	P	θ (95% CI)
PC1 vs. PC3							
Linear	1435.15	1434.72	2	1530	0.23	0.80	9.74° (0.00°, 23.48°)
Quadratic	1383.53	1379.94	2	1526	1.98	0.14	
Correlational	1379.91	1377.57	1	1524	2.59	0.11	
PC1 vs. mating success							
Linear	1480.40	1473.34	2	1530	3.67	0.03[A]	16.07° (0.00°, 40.83°)
Quadratic	1428.31	1427.16	2	1526	0.61	0.54	
Correlational	1427.14	1423.82	1	1524	3.56	0.06	
PC3 vs. mating success							
Linear	1485.29	1476.19	2	1530	4.71	0.009[B]	16.11° (0.00°, 40.59°)
Quadratic	1422.29	1421.52	2	1526	0.41	0.66	
Correlational	1418.75	1418.66	1	1524	0.09	0.76	

[A]P: $F_{1,1530} = 2.71$, $P = 0.10$, C: $F_{1,1530} = 5.03$, $P = 0.03$; [B]P: $F_{1,1530} = 5.29$, $P = 0.02$, C: $F_{1,1530} = 4.69$, $P = 0.03$.

Table 3 Sequential model comparing the linear and nonlinear effects of protein (P) and carbohydrate (C) intake on PC1 and PC3 that describe the variation in CHC expression and mating success in male *Gryllodes sigillatus*. The angle (θ) and 95% confidence intervals (CI) between the linear nutritional vectors for these traits are also provided.

	β	γ		
		PC1	PC2	PC3
PC1	0.21 ± 0.04***	−0.16 ± 0.06**		
PC2	−0.09 ± 0.04*	0.04 ± 0.04	0.06 ± 0.06	
PC3	−0.02 ± 0.04	0.06 ± 0.04	0.01 ± 0.04	−0.21 ± 0.06***

Table 4 Vector of standardized linear selection gradients (β) and the γ matrix of standardized nonlinear selection gradients for the three PCs that describe the variation in male CHCs. Significance testing with permutation test: ***$P < 0.0001$, **$P < 0.01$, *$P < 0.05$.

	M			Selection	
	PC1	PC2	PC3	θ_i	λ_i
m_1	−0.187	−0.980	−0.070	0.046	0.067
m_2	0.800	−0.193	0.568	0.174***	−0.126**
m_3	0.570	−0.050	−0.820	0.138**	−0.254**

Table 5 Canonical analysis to locate the major dimensions of the γ matrix given in Table 4. θ_i and λ_i are the linear and nonlinear selection acting on each vector, respectively. Significance tested via permutation test: ***$P < 0.0001$, **$P < 0.001$.

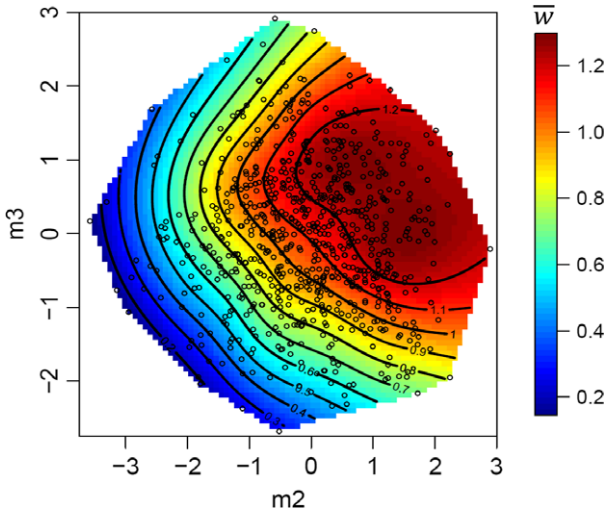

Fig. 3 Thin-plate spline contour view visualization of the fitness surface on the two major axes of significant nonlinear sexual selection, $\mathbf{m_2}$ and $\mathbf{m_3}$. The open symbols represent individual data points for each male in our experiment. Colours represent the relative mating success of males ($\overline{w}$), with red representing the highest relative fitness and blue representing the lowest relative fitness.

through CHC expression. Consistent with our prediction, we found significant linear and quadratic sexual selection acting on the condition-dependent variation in male CHC expression. We also found, however, that the effect of nutrient intake on mating success was not mediated exclusively through CHC expression. This demonstrates that sexual traits other than CHCs must also contribute to the relationship between nutrient intake and mating success. This finding may also explain why the RIP of P and C (P:C 1 : 1.74) calculated in previous work for male *G. sigillatus* did not occupy the same nutrient space as the maxima for PC1 and PC3 but almost perfectly did with the peak for mating success. This suggests that when given dietary choice, males regulate their intake of nutrients to optimize mating success but not CHC expression. Collectively, our findings show that there is a complex interplay between nutrient intake, CHC expression and mating success in male *G. sigillatus* and that this is likely to have important consequences for the operation of sexual selection in this species.

Table 6 Structural equation model parameter estimates for protein (P) and carbohydrate (C) intake on the three principal components describing variation in CHCs (PC1, PC2, PC3) and male mating success (MS) taken from the partial mediation model. Significance values: $*P < 0.05$. $**P < 0.01$. $***P < 0.001$.

Predictor	PC1	PC1 × PC1	PC2	PC2 × PC2	PC3	PC3 × PC3	MS
P	0.30***	−0.10	−0.04	0.08	0.26***	0.06	0.18**
P × P	−0.12**	0.03	0.01	−0.04	−0.07**	0.06	−0.14*
C	0.28***	−0.21**	0.01	0.16*	0.36***	−0.03	0.22***
C × C	−0.09***	0.14**	−0.04	−0.05	−0.16***	0.03	−0.23***
PC1	–	0.41***	–	–	–	–	0.14**
PC1 × PC1	−0.41***	–	–	–	–	–	−0.12*
PC2	–	–	–	0.40***	–	–	−0.10*
PC2 × PC2	–	–	0.40***	–	–	–	0.03
PC3	–	–	–	–	–	0.05**	−0.09
PC3 × PC3	–	–	–	–	0.05**	–	−0.15**

Table 7 The linear effects of protein (P) and carbohydrate (C) intake on male mating success mediated through the three principal components (PC1, PC2 and PC3) that describe the variation in CHCs. Confidence intervals for the mediated effects have been simulated using the Monte Carlo method described by Preacher & Selig (2012). Values in bold are considered statistically significant (i.e. the 95% CI does not overlap zero).

Predictor	Mediator	Mediated effect (95% CI)
P	**PC1**	**0.01, 0.08**
P	PC2	−0.01, 0.02
P	PC3	−0.05, 0.001
C	**PC1**	**0.01, 0.08**
C	PC2	−0.01, 0.01
C	PC3	−0.07, 0.002

Our work combining the GF with a large number of holidic diets shows that both the intake of calories and specific nutrients (P and C) are key to CHC expression in male *G. sigillatus*. Both PC1 and PC3 peaked at a high intake of nutrients, indicating that caloric intake is important to CHC expression. This reliance on a high caloric intake suggests that CHCs are costly produce, a finding that is supported in a number of *Drosophila* species (Blows, 2002; Ferveur, 2005). This cost of CHC production, as well as the fact that this sexual trait positively covaries with condition, is in general agreement with the handicap model of sexual selection and suggests that CHCs have the potential to function as reliable indicators of male quality in *G. sigillatus* (Zahavi, 1975, 1977; Johnstone *et al.*, 2009). However, the fact that PC1 and PC3 in our study both peak at a specific P:C ratio demonstrates that it matters what nutrients these calories are coming from. The small angle ($\theta = 9.74°$) between the linear nutritional vectors, the lack of difference in the nutritional gradients from our sequential model and the similar optimal P:C ratio for PC1 and PC3 (1 : 1.5 and 1 : 1, respectively) indicate that both vectors of CHC expression are maximized at an almost equal intake of P and C. Our work therefore adds to the growing list of studies showing that the balanced intake of specific nutrients are key to the condition-dependent expression of male sexual traits (Maklakov *et al.*, 2008; South *et al.*, 2011; Fedina *et al.*, 2012; Sentinella *et al.*, 2013; Harrison *et al.*, 2014; Cordes *et al.*, 2015; House *et al.*, 2015).

The reliance of male CHCs on nutrient intake is also broadly consistent with our earlier work on *G. sigillatus* that found males consuming a high-quality diet produced a greater total abundance of CHCs (equivalent to PC1 in our current study), but there was little effect of diet on the trade-off between long- and short-chained CHCs (equivalent to PC2 in our study) (Weddle *et al.*, 2012). This latter finding is expected as the relative abundance of long- and short-chained CHCs is known to play an important role in preventing evaporative water loss in insects (e.g. Frentiu & Chenoweth, 2010; Foley & Telonis-Scott, 2011; Ingleby *et al.*, 2013), and this vector of CHC expression is known to be under strong stabilizing natural selection in male *G. sigillatus* (J. Hunt, unpublished data). Our studies differ, however, in the effect of diet on PC3. In Weddle *et al.* (2012), males consuming a high-quality diet produced more positive PC3 scores which reflects more short-chained alkanes and less unnamed alkadienes ($C_{39}H_{76}$), whereas in our current study, an increase in PC3 reflects more of two unnamed alkatrienes ($C_{39}H_{74}$) and less 9,31-C_{41}diene. Despite this broad overlap in studies, our current work provides two important advances. First, as the high- and low-quality diets used by Weddle *et al.* (2012) varied in both nutrient composition and overall caloric content, it is impossible to determine their relative influences on male CHC expression. In contrast, our work unambiguously shows that a large portion of the dietary effects observed in Weddle *et al.* (2012) are due to a balanced intake of P and C. Second, Weddle *et al.* (2012) manipulated the quality of diet provided to males through juvenile development and adulthood. Males reared on a high-quality diet were larger at eclosion and produced a greater total

abundance of CHCs, which is expected as larger crickets have a greater cuticular surface area covered in CHCs. In contrast, our current experiment only examined the effect of diet on male CHCs after being randomly allocated to diets at eclosion to adulthood when growth is complete. The effects of nutrient intake on male CHCs that we observe are therefore unlikely to be driven by the confounding effect of diet on body size shown in Weddle *et al.* (2012). Indeed, statistically controlling for male pronotum width in our response surface analyses did little to alter the relationship between nutrient intake and male CHC expression (Table S2). Consequently, our work shows that the intake of P and C during early adulthood and sexual maturation is sufficient to generate size-independent changes in male CHC expression.

Although it is likely that CHCs first evolved to reduce evaporative water loss in terrestrial arthropods (Hadley, 1981), it is now well documented that male CHCs are also the focus of female mate choice decisions in a diversity of insect species (Wyatt, 2003; Blomquist & Bagnères, 2010). In this regard, crickets and *Drosophila* have become particularly useful insect models for understanding how sexual selection has shaped the evolution of male CHC expression. Even though empirical studies applying formal multivariate selection analysis to male CHC expression are still quite rare, the handful that exist have shown that precopulatory mate choice often exerts a complex pattern of linear and nonlinear sexual selection on male CHC expression, but that the exact strength and form of selection appears to be species-specific. For example, sexual selection on male CHCs is predominantly linear in *D. serrata* (Blows *et al.*, 2004; Chenoweth & Blows, 2005; Gosden & Chenoweth, 2011; Delcourt *et al.*, 2012) and *D. bunnanda* (Van Homrigh *et al.*, 2007; McGuigan, 2009), nonlinear in the Pacific field cricket (*Teleogryllus commodus*, Thomas & Simmons, 2009; Simmons *et al.*, 2013) and a mixture of linear and nonlinear in the sagebrush cricket (*Cyphoderris strepitans*, Steiger *et al.*, 2013) and *D. simulans* (Ingleby *et al.*, 2014). Recently, we used multivariate selection analysis to show that precopulatory female choice in *G. sigillatus* exerts multivariate stabilizing selection on male CHCs (Steiger *et al.*, 2015). This stabilizing selection was restricted to the two lowest vectors of male CHC expression: PC3 which is positively loaded to two alkadienes (5,9-C_{37}diene and 3,9-C_{37}diene) and two unnamed alkatrienes ($C_{39}H_{74}$) and PC4 which is positively loaded to 7-C_{35}ene and 3,13-diMeC_{36} and negatively loaded to two alkadienes (9,31-C_{38}diene and 9,31-C_{39}diene (alkadienes). There was also significant (albeit weak) negative linear selection on PC4 indicating that the curvature of the fitness surface along this dimension is not perfectly symmetrical (see figure 1 in Steiger *et al.* (2015)). In agreement with this earlier work, we found that stabilizing selection was also the most dominant form of nonlinear sexual selection acting on the condition-dependent variation in male CHC expression, which is expected if the female sensory system is optimally tuned to detect male CHCs (e.g. Baker *et al.*, 1998). In contrast, however, in our current study, stabilizing selection targeted PC1 and PC3. Furthermore, linear selection was the dominant form of sexual selection in our current study, favouring higher values of PC1 and, to a lesser degree, lower values of PC2. Linear selection is generated whenever the mean phenotype in the population does not reside on the peak of the fitness surface (Lande, 1979; Lande & Arnold, 1983). Consequently, the stronger linear selection observed in our current study suggests that condition dependence has shifted the population mean CHC expression in males away from the peak of the fitness surface (with the peak remaining stationary) and/or the location of the fitness peak itself (with mean CHC expression remaining stationary). Clearly more work is needed, however, to test between these alternatives.

Life-history models have shown that if the variation in total resources acquired is larger than the variation in how these resources are allocated, the expected negative phenotypic (Van Noordwijk & de Jong, 1986) and genetic (Houle, 1991; de Jong & Van Noordwijk, 1992) covariance between traits competing for these resources (indicating a trade-off) will become positive. That is, rather than traits directly competing for a common pool of resources and being subject to a trade-off, fitness can be optimized by increasing the allocation of resources to both traits simultaneously (Roff & Fairbairn, 2007). Consequently, the fact that males in higher condition have a larger pool of resources to allocate to competing traits (Rowe & Houle, 1996) predicts that they should have a higher fitness (Hunt *et al.*, 2004). Indeed, numerous empirical studies have shown positive effects of diet on important components of male fitness, including mating (Blay & Yuval, 1997; Aluja *et al.*, 2001; Shelly & Kennelly, 2002; Dukas & Mooers, 2003; Engqvist & Sauer, 2003; Holzer *et al.*, 2003; McGuigan, 2009; South *et al.*, 2011) and reproductive (Fedina & Lewis, 2006; McGraw *et al.*, 2007; Bunning *et al.*, 2015; Jensen *et al.*, 2015) success. Few of these studies, however, have examined the specific nutrients responsible for these effects (South *et al.*, 2011; Bunning *et al.*, 2015; Jensen *et al.*, 2015). A notable exception is the work on male cockroaches (*N. cinerea*) showing that pheromone production and attractiveness to females were maximized at a high intake of nutrients in a P:C ratio of 1 : 8 (South *et al.*, 2011), whereas the number of sperm produced and subsequent fertility (measured as the number of offspring produced by the male's single mating partner) were maximized at a high intake of nutrients in a P:C ratio of 1 : 2 (Bunning *et al.*, 2015). In addition, the lifetime number of offspring sired by male *D. melanogaster* in a competitive situation is maximized at a P:C ratio of 1 : 16 (Jensen *et al.*, 2015). In our study, we found that mating success in male

decorated crickets is maximized at an intermediate nutrient intake in a P:C ratio of 1 : 1.5, which is more P biased than mating in male *N. cinerea* (South *et al.*, 2011) and reproductive success in male *D. melanogaster* (Jensen *et al.*, 2015). Furthermore, although the linear nutritional gradients for CHC expression are steeper than for mating success, the optima for these traits are closely aligned in nutritional space (~16°) suggesting the potential for the condition dependence of CHCs to be an important determinant of male mating success. It is important to note that our measure of male mating success, quantified as the ability of a male to gain a mating in a 'no-choice' trial, represents a proxy of fitness (Hunt & Hodgson, 2010). Recent work, however, suggests that this measure of mating success is likely to correlate well with male fitness in *G. sigillatus*. Male decorated crickets transfer an externally attached spermatophore, consisting of a sperm-containing ampulla and a gelatinous spermatophylax, to the female at mating. Immediately after successful transfer of the spermatophore, the female removes the spermatophylax and begins feeding on it, as sperm is evacuated into her reproductive tract from the ampulla. After consuming the spermatophylax, the female immediately removes and consumes the ampulla, thereby terminating sperm transfer (Sakaluk, 1984). Both the size (Sakaluk, 1985) and free-amino acid composition (Gershman *et al.*, 2012) of the spermatophylax have been shown to extend the time the ampulla remains attached to the female and as sperm competition in *G. sigillatus* conforms to a simple 'lottery' (Sakaluk and Eggert, 1996; Eggert *et al.*, 2003), greater sperm transfer is expected to increase the number of offspring sired by a given male. We have recently used GF to show that the size and free-amino composition of the spermatophylax, as well as ampulla attachment time, all peak at a P:C ratio of 1 : 1.3 (Rapkin *et al.*, 2016). The similarity in the nutritional optima of these traits to that shown for mating success suggests that males consuming an optimal diet will not only be likely to gain more matings but also transfer more sperm at each mating. It remains to be shown, however, whether this diet will increase the number of offspring sired over the lifetime of a male, especially under the more competitive conditions that mating typically occurs.

Our work clearly shows that the complex interplay between diet- and condition-dependent CHC expression is an important determinant of mating success in male *G. sigillatus*. That is, nutrient intake influenced both CHC expression and mating success in a similar way, and our multivariate selection analysis showed that the condition-dependent expression of CHCs has important linear and nonlinear effects on mating success. This raises the obvious question of whether the effect of nutrient intake on mating success is mediated primarily through condition-dependent CHC expression or whether other traits play an equally important role in mediating this relationship? The results of our SEM model comparison found that our partial mediation model provided a significantly better fit to our data, thereby demonstrating that condition-dependent CHC expression is not the primary trait mediating the observed relationship between nutrient intake and male mating success. It is important to note, however, that this does not mean that condition-dependent CHC expression is not an important mediator of this relationship *per se*, just that CHC expression does not influence male attractiveness independently and must work in combination with other traits. This point is further illustrated by the fact that within our partial mediation model, PC1 (but not PC2 or PC3) significantly mediated the effects of P and C intake on mating success. The finding that traits other than CHC expression must also mediate the relationship between nutrient intake and mating success also explains why the RIP for male *G. sigillatus* (P:C = 1 : 1.74, Rapkin *et al.*, 2016) was aligned almost perfectly with the nutritional optima for mating success but was less well aligned with the optima for PC1 and PC3. This suggests that under dietary choice, males regulate their intake of P and C to optimize mating success rather than CHC expression, an outcome that would not be expected if CHC expression was the sole mediator of the relationship between nutrient intake and mating success. Our finding that CHCs are not the only trait mediating the relationship between nutrient intake and mating success in male *G. sigillatus* is perhaps not altogether unexpected given that a large range of condition-dependent traits are known to influence mating success in insects (e.g. weapons: Johns *et al.*, 2014; ornaments: Cotton *et al.*, 2004; courtship displays: Kotiaho, 2002; acoustic signals: Hunt *et al.*, 2004; coloration: Punzalan *et al.*, 2008; sex pheromones and CHCs: Blomquist & Bagnères, 2010) and that females regularly use multiple male signals in their mate choice decisions (e.g. Candolin, 2003; Scheuber *et al.*, 2004; Simmons *et al.*, 2013).

The next logical question is what other traits are likely to mediate the relationship between nutrient intake and mating success in *G. sigillatus*? When in close proximity, male crickets use a series of stereotypical courtship behaviours to elicit a mating, including contacting the female with his antennae, positioning his body to allow mounting and the production of a courtship call. Although successful mating can occur without the first two behaviours, the production of a courtship call is typically essential for mating and studies on a range of cricket species have shown that various properties of the courtship call are targeted by females in precopulatory mate choice decisions (e.g. Wagner & Reiser, 2000; Hall *et al.*, 2008; Rebar *et al.*, 2009), including in *G. sigillatus* (Ketola *et al.*, 2007). Furthermore, both auditory (courtship song) and chemical signals (CHCs) in males have been shown to be under significant female selection preferences in the cricket *T. oceanicus,* with both traits

being shown to convey different signals of male quality to females (Simmons *et al.*, 2013). However, the condition dependence of the courtship call in crickets has also received little attention and existing studies have only used a low number of poorly defined diets in their manipulations. There is little effect of diet on the structure of the courtship call in *Gryllus texensis* (Gray & Eckhardt, 2001) and *G. lineaticeps* (Wagner & Reiser, 2000), but work on *G. sigillatus* (Mallard & Barnard, 2004) shows that males increase the stridulatory rate of their courtship call when consuming a high-quality diet. Thus, the courtship call is a prime candidate to act in conjunction with CHCs to mediate the relationship between nutrient intake and male mating success in *G. sigillatus*, although more work is needed to determine the exact effect that P and C intake has on this male sexual trait. Collectively, our work demonstrates the important insights into condition dependence that can be gained through measuring both sexual trait expression and mating success in the same experimental design (as advocated by McGuigan, 2009), but also highlights the additional benefits that can come through measuring nutrient intake.

In conclusion, we show that male CHC expression and mating success are contingent on the balanced intake of P and C and that there are complex linear and nonlinear effects of condition-dependent CHC expression on mating success driven by female precopulatory mate choice. However, despite the close alignment of the nutritional landscapes for CHC expression and mating success, we found that CHC expression was not the only factor mediating the effects of nutrient intake on mating success. It is therefore likely that other sexual traits are also condition-dependent and play an important role in mediating this relationship, and we propose the male courtship call as a potential candidate. Collectively our work shows that the complex interplay between nutrient intake, condition-dependent CHC expression and mating success in male decorated crickets is likely to have an important effect on the operation of sexual selection in this species. For example, the condition dependence of male CHC expression that we demonstrate in our study provides an important mechanism promoting the maintenance of genetic variation in this phenotypic trait that we show is the target of strong sexual selection (see also Steiger *et al.*, 2015). Furthermore, our work shows that the intake of P and C is subjected to indirect sexual selection, via their effects of sexual trait expression and mating success, and that males are able to actively influence this process by regulating their intake of nutrients through dietary choice. However, even though males were able to optimally regulate their intake of nutrients to maximize mating success, males consumed fewer calories than optimal for maximal CHC expression under dietary choice. This demonstrates that not all males in the population are able to meet the high nutritional demands of CHC production and therefore the potential for this chemical signal to act as a 'handicap' that reliably signals male quality during male–male competition and female mate choice. It also suggests that honest signalling and condition-dependent CHC expression in *G. sigillatus* are likely to be dynamic processes, depending critically on the caloric content and nutrient composition of diets that are available during feeding. This highlights that feeding behaviour is likely to be key components of condition dependence and reliable signalling of male quality and therefore sends a clear message that it should be better integrated with sexual selection theory (Morehouse *et al.*, 2010).

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Supporting information

Additional Supporting Information may be found online in the supporting information tab for this article:

Table S1 Protein (P) and carbohydrate (C) composition of the 24 artificial diets used in our no-choice feeding experiment.

Table S2 The effects of protein (P) and carbohydrate (C) intake on male CHC expression (described by PC1, PC2 and PC3) in *G. sigillatus* when correcting for variation in male body size (pronotum width, PW).

Figure S1 The distribution of the 24 artificial diets used in our no-choice feeding experiment.

Data S1 R code used to calculate the angle (θ) 95% CI between linear nutritional vectors.

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