

# Macronutrient balance mediates the growth of sexually selected weapons but not genitalia in male broad-horned beetles

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## Summary

1. Condition is defined as the pool of resources available to an individual that can be allocated to fitness-enhancing traits. Consequently, condition could influence developmental trade-offs if any occur. Although many studies have manipulated diet to demonstrate condition-dependent trait expression, few studies have determined the contribution of specific nutrients to condition or trade-offs.
2. We used nutritional geometry to quantify the effects of dietary protein and carbohydrate content on larval performance and the development of adult morphology including body size as well as a primary and secondary sexually selected trait in male broad-horned beetles, *Gnatocerus cornutus*.
3. We found that offspring survival, development rate and morphological traits were highly affected by dietary carbohydrate content and to a lesser extent by protein content and that all traits were maximized at a protein-to-carbohydrate ratio around 1:2. The absolute size of a secondary sexual character, the mandibles, had a heightened response to the increased availability and ratio of both macronutrients. Male genitalia, in contrast, were relatively insensitive to the increased availability of macronutrients.
4. Overall, while nutrition influenced trait expression, the nutritional requirements of development rate and morphological traits were largely the same and resource acquisition seems to implement only weak trade-offs in this species.
5. This finding contrasts with some resource constraint predictions, as beetles seem able to simultaneously meet the nutritional requirements of most traits.

**Key-words:** condition dependence, genitalia, larval diet, nutritional geometry, sexual selection and weapons

## Introduction

Condition can be defined as the pool of resources an organism has available to build a phenotype (Rowe & Houle 1996). As such, resource acquisition and assimilation are important determinants of condition (Tomkins *et al.* 2004), and all organisms face allocation trade-offs as they balance their limited resources between competing demands. Although condition is notoriously difficult to measure

(Blanckenhorn & Hosken 2003; Cotton, Fowler & Pomiankowski 2004; Hunt *et al.* 2004; Tomkins *et al.* 2004), many traits are nonetheless condition-dependent and sexually selected characters are expected to be especially sensitive to variation in condition (Rowe & Houle 1996; Cotton, Fowler & Pomiankowski 2004; Tomkins *et al.* 2004).

Sexually selected traits are likely to be especially sensitive to allocation trade-offs as these characters are costly to produce, play no role in resource acquisition and reduce the resources that can be allocated to other fitness-enhancing traits (Rowe & Houle 1996; Tomkins *et al.* 2004; Wagner Jr *et al.* 2012). However, not all sexually selected traits are sensitive to condition, with genitals being the prime

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example. Male genitalia are primarily subjected to sexual selection (Eberhard 1985; Hosken & Stockley 2004; House *et al.* 2013), but are frequently less sensitive to variation in condition than other sexually selected characters (House & Simmons 2007). This seems to be a general pattern in invertebrates where the plasticity in genital morphology in response to condition is typically limited (Simmons 2014).

To a large extent, traditional tests of condition dependence have assumed that animals maximize the quantity or caloric content of food which is then available to allocate between competing fitness components (*quantitative resource constraints*) (Cotton, Fowler & Pomiankowski 2004; Cotter *et al.* 2011). As a result, manipulation of condition usually involves the calorific content or quantity of food. An alternative possibility is that the nutritional composition of available foods is of paramount importance in building phenotypes (*qualitative resource constraints*) (Morehouse *et al.* 2010; Cotter *et al.* 2011), and indeed, this has been found to be important in a number of invertebrate models. For example, the expression of sexually selected traits in a range of insects (Maklakov *et al.* 2008; South *et al.* 2011; Sentinella, Crean & Bonduriansky 2013) is all affected by the specific balance of nutrients that males ingest. A secondary consequence of the importance of the nutritional composition of food is that trade-offs will usually be fixed at the point of ingestion rather than when resources are allocated to competing traits (Cotter *et al.* 2011). This occurs when the nutritional requirements of different traits vary, so that the optimal diet for one set of traits is not the same as that optimizing the expression of another (Cotter *et al.* 2011). Thus, a more complete understanding of the consequences of dietary intake, and therefore condition, requires estimation of not just the caloric content of food but also the combined effect of specific nutrients for trait expression (Morehouse *et al.* 2010).

Nutritional geometry (NG) provides a powerful approach to manipulate condition by altering the total nutritional content and the ratio of two (or more) vital macronutrients in the diet (South *et al.* 2011). NG varies the ratio of macronutrients along a series of fixed 'nutritional rails' with points along the rail varying in the total caloric content from low to high. These points are connected by isocaloric lines to other rails that have the same total nutrition. Therefore, the extent to which phenotypic changes are driven by the effect of total nutrition vs. the effects of specific macronutrients can be partitioned (Lee *et al.* 2008; Maklakov *et al.* 2008; Archer *et al.* 2009; Simpson & Raubenheimer 2009; South *et al.* 2011). Few studies have used a NG approach to investigate the link between condition and trait elaboration. However, studies that have taken this approach show that total nutrition and specific macronutrient combinations influence trait expression and fitness (Maklakov *et al.* 2008; South *et al.* 2011; Sentinella, Crean & Bonduriansky 2013). In particular, individuals with increased protein in the larval diet can have reduced survival (Cotter *et al.* 2011; Sentinella, Crean & Bonduriansky 2013), but the growth of sexual traits can

increase (Sentinella, Crean & Bonduriansky 2013; Cordes *et al.* 2015). The heightened growth of condition-dependent traits has also been linked to insulin-like pathways that regulate the growth of somatic tissue in response to dietary protein (Shingleton *et al.* 2007; Warren *et al.* 2013). Thus, NG is a valuable tool to explore trait condition dependence, especially for characters like secondary sexual traits that are expected to be particularly sensitive to condition.

The horned flour beetle, *Gnathocerus cornutus*, feeds on a variety of grains, flours, yeast and dry animal products and responds plastically to changes in the larval diet (Katsuki, Okada & Okada 2012). On poor-quality diets, development is delayed and the size of a secondary sexual trait, the mandible, is reduced (Katsuki, Okada & Okada 2012). Male mandibles are sexually selected (Okada, Miyano-shita & Miyatake 2006; Harano *et al.* 2010), with large mandibles conferring an advantage in male–male competition and access to mates (Harano *et al.* 2010; Okada *et al.* 2014). There is also evidence that mandible size trades off with other cephalic characters, such as the head horns, antennae and eyes (Okada & Miyatake 2009). The negative genetic correlations between the mandibles and these traits following artificial selection for increased mandible length may have arisen due to resource competition (Okada & Miyatake 2009), but it is unknown whether or how genetic trade-offs between traits are influenced by nutrition. Sexual selection also acts on aedeagus morphology in *G. cornutus* (C. M. House, unpublished data). It is currently unknown whether the trade-offs extend to the aedeagus. Nonetheless, secondary sexual traits can trade off against genitalia and testes (Moczek & Nijhout 2004; Simmons & Emlen 2006), and across dung beetles, there are negative correlations between horn growth and genitalia (Parzer & Moczek 2008; but see House & Simmons 2012).

A standard approach to assess the condition dependence of a sexual trait is to compare the relative condition dependence of a sexual trait(s) with one or more nonsexual traits (Cotton, Fowler & Pomiankowski 2004). Here, we used NG to determine the effects of total nutritional content, as well as the specific effects of protein (P) and carbohydrate (C) consumption, on larval survival, male development rate, body size and a primary sexual trait (the aedeagus), a secondary sexual trait (the mandibles) and a nonsexual trait (the eye) in *G. cornutus*. Although male mandibles are condition dependent, it is not clear whether this relates to total intake of nutrients or specific key macronutrients (Katsuki, Okada & Okada 2012). It is also unknown whether the genitalia of *G. cornutus* are impacted by nutrient consumption. First, we test whether total nutritional content and the availability of P and C during juvenile development influences larval survival, development rate and body size, as expected if condition is influenced by nutrition. Next, we test whether the condition-dependent mandibles have heightened sensitivity to nutrition, in particular dietary protein, whereas the genitalia and a nonsexual trait are relatively insensitive to nutrition. Finally, we test whether

trade-offs between life-history traits and/or a primary and a secondary sexual trait are mediated by nutrition, especially the availability of dietary P, as these different traits are likely to have different optimal nutritional requirements.

## Materials and methods

### STOCK POPULATIONS

Stock populations of *G. cornutus* were derived from the Japanese National Food Research Institute, which has maintained cultures of *G. cornutus* for more than 50 years. In our laboratory, mixed sex populations ( $n = 50$  individuals per population) have been maintained in pots (Thermoscientific Nalgene 500 mL, 120 mm OD, Leicestershire, UK) and reared on whole meal flour that is enriched with 5% yeast and maintained at 27 °C and 60% relative humidity under a 14-light : 10-h dark cycle for 2 years. At each generation, final instar larvae are randomly removed from each stock population ( $n = 18$ ) and mixed at random with larvae from all other populations to maintain gene flow between the populations. These final instar larvae are then placed in 24-well plates as pupation is inhibited at high larval density (Tsuda & Yoshida 1985). At eclosion, 25 male and 25 female adults (per pot) are randomly selected to form the parents for the next generation.

### EXPERIMENTAL DIETS

Twenty-four dry, artificial diets that varied in protein and carbohydrate content were produced following the protocol established in Simpson & Abisgold (1985), representing the same diets used in South *et al.* (2011) and Bunning *et al.* (2015). The exact composition of each diet is shown in Table S1 (Supporting information), and the placement of the diets in nutritional space is shown in Fig. S1.

### EXPERIMENTAL ANIMALS AND DESIGN

To obtain adults for the present experiment, final instar larvae were randomly collected from 18 stock population pots and individually placed in a single cell (1.5 × 1 cm) of a 24-well plate ( $n = 42$ , 24-well plates). Pupae were checked daily for eclosion, and eclosed adults were separated into single sex, 24-well plates and provided with *c.* 125 mg of whole meal flour (Doves Farms Foods Ltd, Berkshire, UK) that was enriched with 5% brewer's yeast (ACROS Organics, Leicestershire, UK). Between 7 and 15 days posteclosion, virgin males and females were collected and randomly allocated to one of four mixed sex pots (Thermoscientific Nalgene 500 mL, 120 mm OD;  $n = 125$  females;  $n = 125$  males) containing wholemeal flour (250 g) enriched with 5% brewer's yeast. After 7 days, mated females were transferred to one of four new pots (Thermoscientific Nalgene 500 mL, 120 mm OD;  $n = 125$  females) containing 250 g of plain flour (Doves Farms Foods Ltd) for 24 h to oviposit. The duration of oviposition was restricted to a single day to ensure that all resultant offspring were of the same age. On the 14th day, 2nd instar larvae were separated from the flour using a sieve. A fine paint brush was used to transfer the larvae from the sieve to individual cells of a 24-well plate, each containing 125 mg of one of the 24 artificial diets. Second instar larvae were used because eggs and newly hatched larvae are very difficult to manipulate without damage, and as a consequence, all larvae had reduced development time on the experimental diets. All cells of a given 24-well plate contained the same diet, and a total of 96 replicate individuals were allocated at random to one of the 24 diets (i.e. 4 × 24 well plates/per diet).

Larvae were checked daily for evidence of activity (i.e. burrows in the diet) and fed an additional 125 mg of diet every second day

of the experiment. If there was no activity detected or when most of the pupae in a given 24-well plate had eclosed, the diet was removed and sieved to locate any living larvae. Survivors were returned to a clean 24-well plate with the appropriate diet until they either died or eclosed, which we scored as 0 or 1, respectively. The dates of all eclosures were recorded, and development time was calculated as the time between larval being established on experimental diets (14 days of age) and eclosion.

### MEASURING MORPHOLOGICAL TRAITS

To measure adult morphology, beetles were placed on a slide on the stage of a dissecting microscope (Leica M125, Leica Microsystems Ltd., Bletchley, UK) that was consistently oriented. Digital images of the head and thorax of each beetle were captured using a mounted digital camera (Leica DFC 295) that was connected to a PC. Using IMAGEJ (version 1.48, National Institutes of Health, USA, <http://rsb.info.nih.gov/ij/>), we measured the linear width of the pronotum as a measure of general body size (Fig. S2). We also used the 'oval tool' in IMAGEJ to measure the area of both eyes, and the average area was used in subsequent analyses (Fig. S2).

Due to their more complex structure, we used geometric morphometric analysis to measure the size of the male mandibles and genitalia. As the mandibles and genitalia have few landmarks that are biologically homologous between specimens, we used a protocol based on a mixture of landmarks (type-two landmarks) and semi-landmarks. A description of the programs used for the digitization of the mandible and genital images and GM analysis is included in Figs S2 and S3.

For a subset of 25 experimental males sampled at random across diets, we mounted individual beetles twice and measured pronotum width, eye area and mandible size twice to assess their repeatability using the R code provided in Wolak, Fairbairne & Paulsen (2012). Each of these measurements was highly repeatable (pronotum width = 0.989, 95% CIs: 0.985, 0.991; mean eye area = 0.943, 95% CIs: 0.910, 0.988; mandible size = 0.998, CIs: 0.998, 0.999). The male genitalia are easily damaged during dissection, so it is not possible to remount specimens. Instead, the repeatability of digitization of two images of the same genitalia was assessed using the same R code (Wolak, Fairbairne & Paulsen 2012) and was found to be highly repeatable (genital size = 0.992, CIs: 0.989, 0.997).

### SAMPLE SIZES

Based on our experimental design (i.e. 96 larvae per diet), a total of 2304 beetle larvae were established on our experimental diets. For our analysis of larval survival to eclosion, however, 76 beetles were not included because plate wells were empty, but dead larva or adults were not found meaning that the fate of the individual was uncertain. Thus, we had a total sample size of  $n = 2228$  for our larval survival analysis. It is important to note that our analysis of larval survival contains both males and females as we were unable to determine the sex of any larvae that died during development. Our analysis of development rate and adult morphology was based on 20 randomly selected male beetles per diet for 18 of the 24 artificial diets. For six of these diets (diets 1, 5, 9, 13, 17 and 21; Table S1, Fig. S1), too few males survived to adulthood. We therefore had a sample size of  $n = 360$  available for the analysis of these traits.

### STATISTICAL ANALYSIS

The square root of eye area was calculated to convert this trait into the same linear scale of our other morphological traits. Next, as all of our response variables (larval survival, development rate

and adult morphology) were measured in different units, we standardized each response variable and the P and C contents of the diet to a mean of zero and standard deviation of one using a Z-transformation prior to analysis. Insects developing on diets of higher nutritional content have a greater probability of larval survival to eclosion, have a reduced development time and typically eclose at a larger body size (e.g. Hunt *et al.* 2004). Therefore, because short development times can be interpreted as beneficial, z-scores of development time were multiplied by  $-1$  to ensure straightforward interpretation compared to other fitness-related traits. Thus, beetle larvae that developed slowly had negative z-scores, whereas those developing faster had positive z-scores.

As beetle larvae developing on a given diet were distributed across four separate 24-well plates, there is the potential for variation across plates to influence our response variables if each beetle in our experiment is treated as independent. We therefore examined the linear and nonlinear effects of P and C consumption on our response variables using generalized linear mixed models (GLMMs) that included 'plate' as a random effect. Following conventional multivariate response surface methodologies (Lande & Arnold 1983), we first ran a GLMM that contained 'plate' as a random effect and the linear terms (P and C content of the diet) as fixed effects. The parameter estimates from this model were used to estimate the linear effects of P and C on our response variables. Next, we ran a second GLMM that contained the same terms (P and C; 'plate') but also included the quadratic ( $P \times P$  and  $C \times C$ ) and cross-product ( $P \times C$ ) terms for these nutrients as fixed effects. The parameter estimates from this model were used to estimate the nonlinear effects of these nutrients on our response variables and were implemented in JMP® (version 8.0.2, SAS Institute Inc., Cary, NC, USA) using restricted maximum likelihood approximation. However, when the response variable has a binary distribution, as for larval survival to eclosion, we ran the above GLMMs to estimate the linear and nonlinear gradients for P and C but used a binary GLMM implemented in the 'MCMCglmm' package of R (version 2.13.0; Hadfield 2010) to test the statistical significance of these gradients. Full details for the rationale for this analysis, the model and accompanying R code are provided in Appendix S1.

When examining the effects of P and C on absolute vs. relative trait size (eye size, mandible size and aedeagus size), we compared two sets of models. As above, we used the above GLMMs to quantify the linear and nonlinear effects of P and C on absolute trait size. Next, we also ran a GLMM for each trait that included body size as a fixed effect to examine the linear and nonlinear effects of P and C on relative trait size. Including body size as a fixed effect enables us to partition the independent responses of our morphological traits to nutrients from those that are due to changes in body size across diets (Sentinella, Crean & Bonduriansky 2013).

Nonparametric thin-plate splines were used to visualize the nutritional landscapes for each of our response variables and were constructed using the *Tps* function in the 'FIELDS' package of R. In all cases, we used the smoothing parameter ( $\lambda$ ) that minimized the generalized cross-validation score when visualizing the nutritional landscapes (Green & Silverman 1994). Even though all analyses were conducted on standardized data, we present our nutritional landscapes on the raw data with the exception of development rate – which is the raw development time data, multiplied by  $-1$  to aid interpretation.

We used a sequential model building approach to test whether the linear and nonlinear effects of P and C consumption differed across our response variables (Draper & John 1988; Chenoweth & Blows 2005; South *et al.* 2011; Bunning *et al.* 2015). A description of the test and full details of this analysis, including linear equations, are included in Appendix S2. A potential limitation of this approach is that the optimal expression of these traits may reside in a similar location in nutrient space even though the magnitudes

of these gradients differ. Therefore, in addition to using a sequential model approach, we also calculated the angle ( $\theta$ ) between the linear vectors for pairs of response variables being compared. In brief, when the angle between the vectors is small (i.e.  $\theta = 0^\circ$ ), the optima for the two response variables reside in the same location in nutrient space, whereas when the angle between the vectors is large (i.e.  $\theta = 180^\circ$ ), the optima for the two response variables are maximally divergent. To estimate and determine the statistical significance of  $\theta$ , we used a Bayesian approach implemented in the 'MCMCglmm' package of R. Full details of this analysis and accompanying R code are provided in Appendix S3.

## Results

### LARVAL SURVIVAL, DEVELOPMENT TIME AND BODY SIZE

Larval survival, development rate and body size all increased linearly with the content of P and C in the diet (Table 1). However, development rate and body size were over twice as responsive (i.e. steeper gradient) to the consumption of C than P, whereas the consumption of P and C had roughly equal effects on larval survival (Table 1). The significant negative quadratic terms indicate a peak for all three traits with the consumption of C and a peak for larval survival (but not development time or body size) with the consumption of P (Table 1). For each trait, this peak was centred around a P : C ratio of *c.* 1 : 2 on the nutritional landscape (Fig. 1). For larval survival, the significant negative correlational gradient suggests that longevity is highest at a high C and low P consumption (Table 1, Fig. 1).

A sequential model building approach showed significant differences in the linear, quadratic and correlational effects of P and C consumption on larval survival and development time (Table S2). The difference in the linear gradients for larval survival and development time was due to the fact that larval survival was more responsive to P consumption than development time, whereas the difference in quadratic gradients occurred because the curvature of the peak for P consumption was significant for larval survival and not for development time (Table S2). The difference in the correlational gradient for larval survival and development time was due to the fact that the negative gradient was significant for larval survival, whereas it was not for development time (Table S2). There were significant differences in the linear and quadratic effects of P and C consumption on larval survival and body size but not in the correlational effect of these nutrients (Table S2). The difference in linear gradients was due to the fact that body size was more responsive to C consumption than larval survival, whereas the difference in quadratic gradients was due to the curvature of the peak for P consumption being stronger for larval survival than body size (Table S2). Finally, there were significant differences in the linear effects of P and C consumption on development time and body size due to the fact that body size was more responsive to C consumption than development time (Table S2).

**Table 1.** Mean trait values ( $\pm$ SE) pooled across all diets and linear and nonlinear effects of the protein (P) and carbohydrate (C) contents of the diet on larval survival of beetle larvae (both sexes), development rate, adult body size, and the absolute size of a nonsexual trait [eye area – linearized by square root (SQRT) transformation], a primary sexual trait (genitalia) and a secondary sexual trait (mandible) in male *G. cornutus*. We used GLMMs, including plate as a random effect and the linear and nonlinear effects of nutrients, to determine the gradients and used a binary GLMM to test the significance of these gradients for larval survival (*pMCMC*)

Response variable	Mean trait values ( $\pm$ SE)	Linear effects		Nonlinear effects		
		P	C	P $\times$ P	C $\times$ C	P $\times$ C
<b>Larval survival</b>						
Gradient $\pm$ SE	0.72 $\pm$ 0.01	0.49 $\pm$ 0.06	0.58 $\pm$ 0.06	-0.26 $\pm$ 0.04	-0.45 $\pm$ 0.04	-0.63 $\pm$ 0.07
<i>t</i>		7.98	9.49	7.25	11.79	9.00
d.f.		93.08	93.14	86.94	87.08	90.31
<i>pMCMC</i>		<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>	<b>0.0001</b>
<b>Development rate</b>						
Gradient $\pm$ SE	44.40 $\pm$ 0.71	0.29 $\pm$ 0.10	0.65 $\pm$ 0.10	0.00 $\pm$ 0.11	-0.31 $\pm$ 0.14	0.19 $\pm$ 0.26
<i>t</i>		3.07	6.37	0.02	2.18	0.72
d.f.		65.26	66.11	61.56	62.23	61.21
<i>P</i>		<b>0.003</b>	<b>0.0001</b>	0.99	<b>0.033</b>	0.47
<b>Body size</b>						
Gradient $\pm$ SE	1.08 $\pm$ 0.005	0.38 $\pm$ 0.07	0.95 $\pm$ 0.07	-0.09 $\pm$ 0.06	-0.52 $\pm$ 0.07	-0.17 $\pm$ 0.13
<i>t</i>		5.44	13.08	1.41	7.15	1.29
d.f.		67.44	58.32	64.94	65.35	63.56
<i>P</i>		<b>0.0001</b>	<b>0.0001</b>	0.16	<b>0.0001</b>	0.20
<b>Mean SQRT eye area</b>						
Gradient $\pm$ SE	0.15 $\pm$ 0.001	0.12 $\pm$ 0.07	0.74 $\pm$ 0.07	0.05 $\pm$ 0.07	-0.39 $\pm$ 0.08	0.01 $\pm$ 0.15
<i>t</i>		1.59	9.92	0.69	4.85	0.07
d.f.		65.47	66.28	62.50	62.21	59.97
<i>P</i>		0.12	<b>0.0001</b>	0.49	<b>0.0001</b>	0.95
<b>Mandible size</b>						
Gradient $\pm$ SE	348.69 $\pm$ 4.61	0.35 $\pm$ 0.07	0.89 $\pm$ 0.07	-0.02 $\pm$ 0.06	-0.52 $\pm$ 0.07	-0.17 $\pm$ 0.13
<i>t</i>		4.83	12.09	0.32	7.78	1.34
d.f.		67.61	68.45	65.82	66.11	64.44
<i>P</i>		<b>0.0001</b>	<b>0.0001</b>	0.75	<b>0.0001</b>	0.19
<b>Genital size</b>						
Gradient $\pm$ SE	1072.55 $\pm$ 2.36	0.22 $\pm$ 0.07	0.65 $\pm$ 0.07	-0.02 $\pm$ 0.08	-0.35 $\pm$ 0.08	0.00 $\pm$ 0.16
<i>t</i>		3.07	9.01	0.22	4.17	0.01
d.f.		63.36	63.57	63.03	61.32	57.68
<i>P</i>		<b>0.003</b>	<b>0.0001</b>	0.83	<b>0.0001</b>	0.99

Response variables were Z-transformed prior to analysis so that the response of different traits to nutrition was standardized and therefore comparable. The linear gradients describe the sign and magnitude of the relationship between nutrients and the response variable. The quadratic gradients (P  $\times$  P and C  $\times$  C) describe the curvature of the nutritional landscape with peaks indicated with a negative slope and a troughs by a positive slope. The correlational gradient (P  $\times$  C) describes how the covariance between P and C influences the response variable, with a positive gradient indicating that the response variable is greater when both the P and C contents of the diet are high, whereas a negative gradient indicates that the response variable is greatest when one nutrient is high and the other low. Values provided in bold are statistically significant ( $< 0.05$ ).

The quadratic and correlational effects for these two traits did not differ significantly (Table S2).

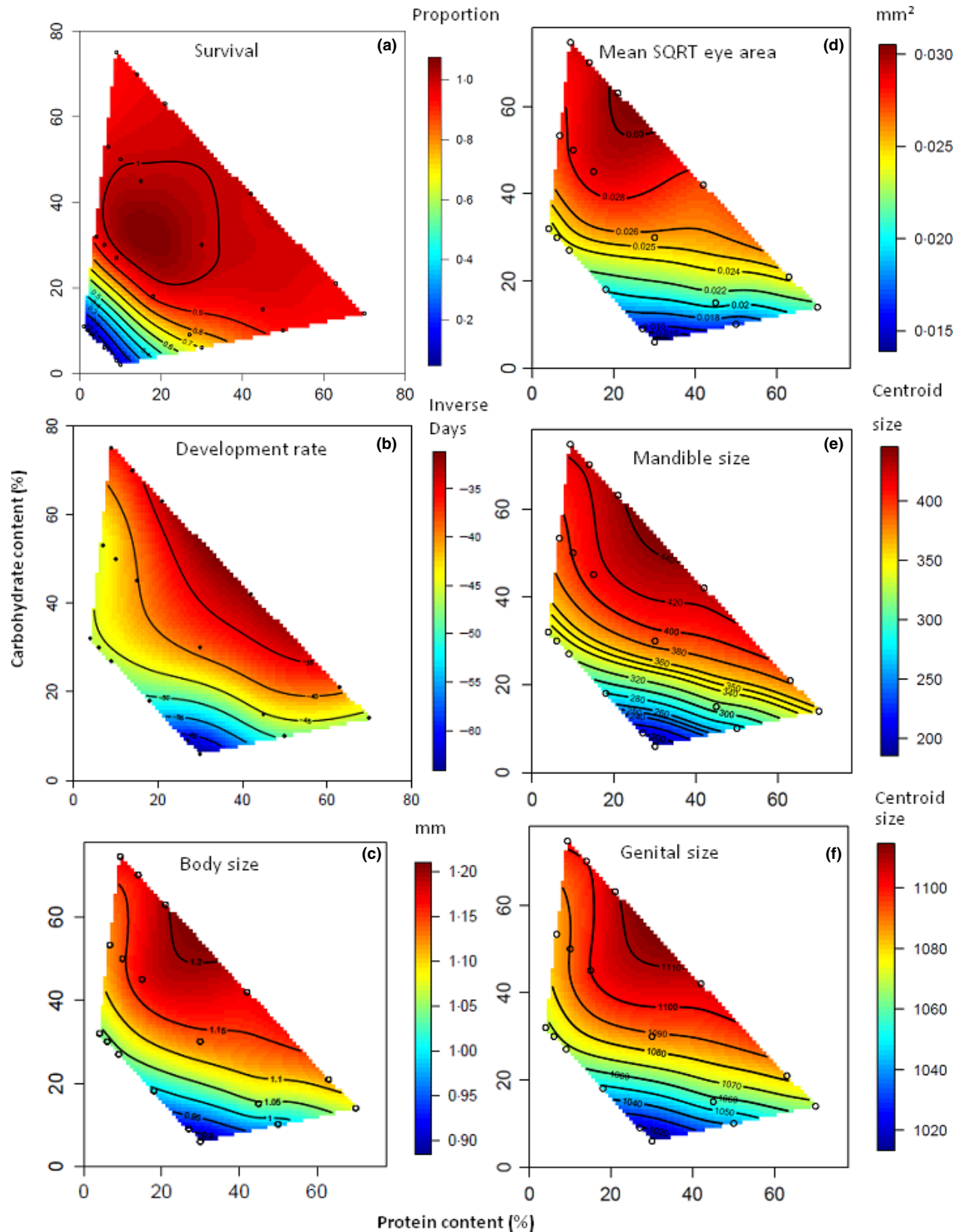
Despite the differential effects of P and C consumption on larval survival, development time and body size, the peaks for these traits occupy the same region on the nutritional landscape (Fig. 1). This is evidenced by the small angles between the linear vectors for larval survival and development time ( $\theta = 8.83^\circ$ , 95% CIs: 1.96°, 14.80°), larval survival and body size ( $\theta = 2.85^\circ$ , 95% CIs: 1.72°, 3.99°) and development time and body size ( $\theta = 11.74^\circ$ , 95% CIs: 2.97°, 18.99°).

#### THE ABSOLUTE SIZE OF MORPHOLOGICAL TRAITS

A correlation matrix for the linear relationship between male traits indicates that all traits were positively and

significantly correlated (Table S3). Mandible size and genital size both increased linearly with the consumption of P and C and were over twice as responsive to the consumption of C than P (Table 1). In contrast, mean eye area only increased linearly with the consumption of C (Table 1). There were significant negative quadratic terms for the consumption of C for all three traits but not for the consumption of P (Table 1). Inspection of the nutritional landscapes shows that these peaks occur at a P : C ratio of *c.* 1 : 2 for each trait (Fig. 1). None of the correlational gradients were statistically significant (Table 1).

Formal statistical comparison showed that the linear effects of P and C consumption on mandible size differed significantly from the effects on mean eye area and genital size (Table S4). In both cases, this was because mandible size was more responsive to the consumption of P and C



**Fig. 1.** Nutritional landscapes illustrating the effects of the protein and carbohydrate contents of the diet on (a) larval survival to eclosion, (b) developmental rate (i.e. raw development time \* -1), (c) body size, (d) mean eye area [linearized by square root (SQRT) transformation], (e) mandible size and (f) genital size of male *Gnatocerus cornutus*. Hotter colours (red) represent areas of increased larval survival, a faster development rate and larger body size, and colder colours (blue) represent areas of reduced larval survival, slower development rate and smaller body size. The circles show the nutritional compositions of our artificial diets.

(Table S4). There was no difference in the linear effects of P and C on mean eye area and genital size, and none of the traits differed in the quadratic or correlational effects of these nutrients (Table S4). Not surprisingly, the maximum size of these three morphological traits occurred in the same region on the nutritional landscape (Fig. 1), as evidenced by the small angles between the linear vectors for mandible and genital size ( $\theta = 9.74^\circ$ , 95% CIs:  $2.41^\circ$ ,  $18.25^\circ$ ), mandible and eye size ( $\theta = 3.64^\circ$ , 95% CIs:  $0.00^\circ$ ,  $11.22^\circ$ ) and genital and eye size ( $\theta = 13.32^\circ$ , 95% CIs:  $5.51^\circ$ ,  $22.89^\circ$ ).

#### THE RELATIVE SIZE OF MORPHOLOGICAL TRAITS

Mean eye area, mandible size and genital size all scaled positively and significantly with body size, although the linear gradient for mandible size was steeper than for mean eye area and genital size (Table 2). After controlling for the effects of body size on these traits (Sentinella, Crean & Bonduriansky 2013), the consumption of P had a linear effect on mean eye area with this trait decreasing in relative size as the consumption of P increased (Table 2, Fig. S4a). There were also significant linear and quadratic effects of C consumption on relative mandible size (Table 2). Relative mandible size increased with the consumption of C, and there was also a negative quadratic gradient indicative of a peak in relative mandible size with C consumption (Table 2, Fig. S4b). Importantly, the relative size of both traits peaked in the same region in nutritional space at c. 4% P and 32% C (Fig. S4) providing little evidence for a trade-off. In contrast, the relative size of the genitalia was relatively insensitive to the consumption of P and C (Table 2).

#### Discussion

Variation in condition can be influenced by both the abundance of resources and the ease in which an individual may acquire these resources from their environment (e.g. Hunt *et al.* 2004). Here, we show that individuals that consumed higher calorie diets were more likely to survive, developed faster and eclosed at a larger body size. Thus, as predicted by theory (Andersson 1982; Rowe & Houle 1996), beetles that acquired more resources were in better 'condition' and able to allocate more resources to their naturally and sexually selected traits. However, our work also shows that it is not simply the total intake of calories that is important for maximal trait expression as the highest trait values did not occur universally on the most concentrated diets. Rather, beyond a minimal caloric concentration, the ratio of ingested nutrients was critical for trait expression, with increases in expression with the consumption of both nutrients; the only exception being the effect of P consumption on mean eye area that was not significant. Furthermore, despite variation in the responsiveness of larval survival, development time and adult morphology to the consumption of P and C, the P : C ratio that maximized the expression of these traits was the same (i.e. P : C = 1 : 2), resulting in nutritional landscapes that were closely aligned across all traits. We therefore demonstrate that the predicted trade-offs between life-history and a primary and secondary sexual trait are only weakly determined by the consumption of at least two important macronutrients (P and C). A possible exception is between larval survival and adult morphological traits, where the former peaks at a lower total nutrition than morphological traits. While this could, in theory, suggest a

**Table 2.** The linear and nonlinear effects of the protein (P) and carbohydrate (C) content of the diet on mean eye size [linearized by square root (SQRT) transformation], mandible size and genital size in male *G. cornutus* when controlling for the effects of body size on these traits

Response variable	Linear effects			Nonlinear effects		
	P	C	Body size	P × P	C × C	P × C
<b>Mean SQRT eye area</b>						
Gradient ± SE	-0.16 ± 0.05	0.09 ± 0.07	0.70 ± 0.05	0.10 ± 0.06	-0.09 ± 0.07	0.11 ± 0.12
<i>t</i>	3.21	1.29	12.83	1.78	1.24	0.92
d.f.	70.27	108.60	193.10	62.33	81.16	57.50
<i>P</i>	<b>0.002</b>	0.20	<b>0.0001</b>	0.08	0.22	0.36
<b>Mandible size</b>						
Gradient ± SE	0.02 ± 0.03	0.08 ± 0.04	0.87 ± 0.03	0.05 ± 0.03	-0.12 ± 0.03	-0.03 ± 0.06
<i>t</i>	0.81	2.31	29.81	1.62	3.60	0.49
d.f.	77.72	121.40	223.60	70.37	90.41	63.38
<i>P</i>	0.42	<b>0.02</b>	<b>0.0001</b>	0.11	<b>0.0005</b>	0.63
<b>Genital size</b>						
Gradient ± SE	-0.05 ± 0.05	-0.01 ± 0.07	0.70 ± 0.06	0.05 ± 0.06	-0.01 ± 0.07	0.14 ± 0.12
<i>t</i>	1.06	0.10	11.82	0.80	0.19	1.24
d.f.	77.93	106.7	130.70	65.70	78.09	50.48
<i>P</i>	0.29	0.92	<b>0.0001</b>	0.43	0.85	0.22

Body size was included in the model to partition out the independent responses of traits to nutrients from those that were due to changes in body size across diets. Values provided in bold are statistically significant ( $< 0.05$ ).

trade-off between these traits, it is important to note that the broad peak for larval survival means that any region in nutritional space where expression of an adult morphological trait is high and larval survival is low (or vice versa) is actually quite small, further highlighting that any trade-offs between these traits are likely to be weak. However, larvae were already 14 days old when exposed to the experimental diets. This may have diminished the effects of total nutritional content as well as the effects of P and C on beetle traits and may have decreased our power to detect trade-offs between traits. However, we suggest that this effect is small as we detected significant linear and nonlinear effects of P and C on the traits that we measured.

Secondary sexual traits typically show heightened condition dependence (Cotton, Fowler & Pomiankowski 2004). In some instances, secondary sexual trait exaggeration may be an extension of individuals' body size (Cotton, Fowler & Pomiankowski 2004), in which case changes in them occur because of changes in overall size (indirect response). Alternatively, secondary sexual trait exaggeration may reflect a wider response to condition, in which case changes in the trait occur independently of changes in body size (i.e. direct response) (Cotton, Fowler & Pomiankowski 2004). We demonstrate that mandibles, a secondary sexual trait, responded directly and indirectly to variation in nutrition after controlling for body size, although the indirect response was much larger, suggesting that the mandibles are an exaggerated representation of body size. We also show that the absolute response of the mandibles to the intake of P and C in the larval diet of *G. cornutus* was stronger compared to a primary sexual trait and a nonsexual trait, as predicted for an exaggerated trait that is condition dependent. This heightened sensitivity of the mandibles to nutrition is consistent with previous work (Katsuki, Okada & Okada 2012) and shows that a C-rich larval diet is likely to enhance male fitness. In a contrasting invertebrate system, abundant P in the larval diet of the neriid fly *Telostylinus angusticollis* is essential for the expression of sexually selected characters. However there, dietary C had little effect, leading to suggestions that a P-rich diet may be a general requirement of sexually selected condition-dependent traits (Sentinella, Crean & Bonduriansky 2013). Similar findings were also found in the lesser wax moth *Achroia grisella* where traits important for pre- and postcopulatory success increased on high P larval diets (Cordes *et al.* 2015). The composition of the standard laboratory larval/adult diet of *G. cornutus* is ~P : C = 1 : 4 which is within the range of the diets used here, but is off-centre from where life-history and morphological traits were found to peak. Thus, like the aforementioned studies, increasing protein in the larval diet appears to increase fitness, but nonetheless, we did not find a disproportionate effect of dietary protein on a secondary sexual trait unlike previous findings.

In contrast to the mandibles, the absolute response of the eyes to the intake of dietary P was weaker. This reflects

the slower relative growth of the eye compared to the rest of the body with increasing intake of P. Previously, artificial selection for mandible length was negatively genetically correlated with the head horn, antenna and eye area, which was suggested to have arisen due to resource allocation trade-offs (Okada & Miyatake 2009). Resource allocation trade-offs are predicted to occur when the precursors of the adult body structures (i.e. the imaginal discs) undergo a period of explosive growth after larval feeding has stopped and therefore compete for limited resources (Nijhout & Emlen 1998). Evidence suggestive of resource allocation trade-offs between a secondary sexual and a nonsexual trait has been found in dung beetles (*Onthophagus acuminatus* and *Onthophagus taurus*; Nijhout & Emlen 1998; Tomkins, Kotiaho & LeBas 2005). Similarly, in the butterfly *Precis coenia*, the removal of the hind wing imaginal discs from larvae gave rise to enlarged forewings, thorax and forelegs, presumably because the manipulation increased the resources available for a smaller number of structures (Nijhout & Emlen 1998). In our study, the effect of dietary protein may reinforce underlying resource allocation trade-offs between neighbouring traits as dietary protein required to promote the growth of the body and mandibles actually slows the growth of at least one neighbouring nonsexual trait – the eye. Thus, in some instances, structures may compete for resources, but in addition, the macronutrients that they acquire may have negative effects for the growth of neighbouring structures.

Unlike all other morphological traits, the genitalia only responded indirectly and relatively weakly to variation in dietary composition. Thus, although the genitalia of *G. cornutus* are subject to sexual selection, it is clear that the size of the aedeagus is not particularly sensitive to nutrition and is an unlikely signal of a male's underlying genetic quality. Similar patterns of environmentally canalized growth of genital traits have been found in other insects (House & Simmons 2007; Shingleton *et al.* 2009; Dryer & Shingleton 2011; Rodríguez & Al-Aathiqui 2011, 2012). However, there is evidence of environmentally induced genital plasticity in the water strider *Gerris incognitos*, albeit less than nongenital traits (Arnqvist & Thornhill 1998), and fly *Drosophila mediopunctata* (Andrade, Hatadani & Klaczko 2005).

Canalization of the genitalia should reduce size variation and contribute to the observed negative allometry of the male genitalia that is observed in most insects (Eberhard *et al.* 1998; Dryer & Shingleton 2011; Simmons 2014). The significance of this has been widely debated (Eberhard *et al.* 1998; Bertin & Fairbairn 2007; Eberhard, Rodríguez & Polihronakis 2009; Dryer & Shingleton 2011; Simmons 2014), but a general explanation is that stabilizing selection favours intermediate male genital size which selects for canalized growth (Eberhard, Rodríguez & Polihronakis 2009; Dryer & Shingleton 2011; Simmons 2014). In *G. cornutus*, there is directional selection for smaller aedeagus size in noncompetitive matings – although this pattern of selection may alter when females mate multiply (C. M.



House, unpublished data). Apparent directional selection and nutritional insensitivity appear to be incompatible unless directional selection for smaller genital size is stronger and more negative in large males than it is in small males (Dryer & Shingleton 2011). In this context, environmental canalization may protect the morphology of the aedeagus from environmental conditions that increase body size and other traits via an indirect response to body size changes that would otherwise lead to increased aedeagus size that confers lower fitness.

The mechanisms that generate nutritional reaction norms for body size and trait size have not yet been elucidated in *G. cornutus*. Past studies show that signalling pathways, in particular the highly conserved insulin receptor signalling pathway (IIS) and insulin-like peptides (ILPs) that are responsive to nutrition, have been implicated in the regulation of the growth rate of extreme animal structures (Mirth & Riddiford 2007; Shingleton *et al.* 2007; Shingleton, Mirth & Bates 2008; Emlen *et al.* 2012). For example, in the rhinoceros beetle *Trypoxylus dichotomus*, interference of the transcription of the insulin receptor (InR) gene in developing larvae significantly reduced the size of nutrition-dependent male horns, while wing size was relatively insensitive and genitalia size was not affected at all (Emlen *et al.* 2012). Furthermore, it has been shown that the insulin receptor signalling pathway (IIS) is primarily regulated by dietary P in *Drosophila* (Britton & Edgar 1998; Britton *et al.* 2002). In contrast, we find that the secondary sexual traits of *G. cornutus* responded directly to dietary C. This may arise if the levels of circulating carbohydrates such as glucose have a role in the regulation of the insulin receptor signalling pathway (IIS) as has been shown in the silkworm *Bombyx mori* and insects more broadly (Masumura *et al.* 2000). Further study is required to confirm the involvement of specific pathways that mediate metabolic changes and differences in cell growth and proliferation in different tissues in *G. cornutus*.

Interestingly, we found weak evidence that the optimal nutritional requirements of one trait constrain the dietary optimum of other traits. We show that life-history traits, sexual traits and a nonsexual trait all share a common requirement for P and C (despite their varied sensitivity) and optimize their trait expression at a P : C ratio of 1 : 2. These findings contrast with male neriid flies and wax moths, where the positive effects of P for secondary sexual trait expression are countered by the severe negative effects of P during development, and therefore, dietary P is likely to mediate trade-offs (Sentinella, Crean & Bonduriansky 2013; Cordes *et al.* 2015). Similarly, in male cockroaches, requirements for dietary C vary across different episodes of reproductive effort. Consequently, the dietary optimum that enhances cockroach precopulatory performance is likely to trade-off with the dietary optimum that enhances postcopulatory performance (South *et al.* 2011; Bunning *et al.* 2015). Whether nutrition influences a putative trade-off between male morphology and ejaculate traits in *G. cornutus* remains to be investigated.

Although nutrition seems unlikely to mediate strong trade-offs in *G. cornutus*, it probably has lasting effects on male fitness. Extreme nutrient restriction during development was fatal at the lowest concentrations of protein and carbohydrate, presumably because larvae were unable to consume enough calories from these diets. Larvae that survived and developed on suboptimal or unbalanced diets suffered costs to their nonsexual fitness as they developed more slowly. Prolonged development time allows animals to achieve required caloric and nutritional consumption in poor nutrient environments (Chown & Nicolson 2004). For example, many insects are able to attain the same final size as those reared on good quality diets by prolonging development time (Chown & Nicolson 2004). Here, larvae that developed more slowly were unable to compensate for their poor diet and became smaller adults. The fitness cost of delayed development, small body size and a reduced secondary sexual character for males is likely to be considerable in holometabolous species such as *G. cornutus*, as they are unable to overcome these deficits in any adult environment (Boggs & Freeman 2005; Adler *et al.* 2013).

In conclusion, we show that specific nutrients are important for the expression of a condition-dependent, secondary sexual trait, but less so for a primary sexual trait. Furthermore, among the life-history and morphological traits that we measured, we find that they have the same nutritional requirements, and therefore, only weak trade-offs are likely to occur at the point of ingestion. Coupled with this, our estimates of sexual selection on genitalia (C. M. House, unpublished data) and a secondary sexual trait (Okada, Miyano-shita & Miyatake 2006; Harano *et al.* 2010) suggest that development in an enriched C and weakly supplemented P larval environment would confer high fitness as males can then invest in large body size and a condition-dependent weapon but also maintain a small aedeagus.

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## Data accessibility

Condition data, survival and development and morphological data are deposited in the Dryad Digital Repository: <http://dx.doi.org/10.5061/dryad.4gg88> (House *et al.* 2015).

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## Supporting Information

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

**Fig. S1.** The distribution of artificial, holidic diets in nutrient space.

**Fig. S2.** Measurements of male *G. cornutus* external morphology.

**Fig. S3.** Measurement of the aedeagus of *G. cornutus*.

**Fig. S4.** The effect of P and C intake on residual (a) mean (SQRT) eye area and (b) mandible size in *G. cornutus*.

**Table S1.** The protein (P) and carbohydrate (C) content of artificial, holidic diets.

**Table S2.** Comparison of the effects of P and C on larval survival, development rate and body size.

**Table S3.** Pearson's correlation matrix ( $r$ 's) for the linear relationships between male traits.

**Table S4.** Comparison of the effects of P and C on eye area, mandible size and genital size.

**Appendix S1.** Estimating the effects of P and C on larval survival.

**Appendix S2.** Sequential model building approach for comparing nutritional landscapes.

**Appendix S3.** Calculating the angle ( $\theta$ ) and 95% CIs between linear nutritional gradients.