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

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Running title: Macronutrients, weapons and genital traits
Summary

1. Condition is defined as the pool of resources available to an individual and 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 that determine condition and mediate trade-offs. This can occur if life history and morphological traits have different resource demands during juvenile development.

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 primary and secondary sexually selected traits in male broad horned beetles, *Gnatocerus cornutus*.

3. We found that 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. After controlling for body size, the size of the mandibles responded to the increased availability and ratio of both macronutrients with a proportionally greater allocation of resources to the mandibles. Male genitalia, in contrast, were relatively insensitive to the increased availability of macronutrients and varied proportionally with body size.

4. Overall, while nutrition influenced trait expression, the nutritional requirements of development rate and morphological traits were largely complementary and resource acquisition seems unlikely to implement trade-offs in this species.

5. This finding contrasts with some resource constraint predictions, as beetles are 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 that an organism has available to allocate to life history and reproductive traits (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 (Cotton et al. 2004; Tomkins et al. 2004; Rowe & Houle 1996).

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 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 2013). Some evidence suggests that this may arise due to strong stabilizing sexual selection that is imposed by female mate choice for reproductive isolation at one extreme of the continuum or cryptic female at the other end (Eberhard 1985; McPeek et al. 2008; Simmons 2013).

To a large extent, traditional tests of condition-dependent trait expression 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) (Cotter, Simpson & Raubenheimer 2011; Cotton et al. 2003). 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) (Cotter et al. 2011; Morehouse et al. 2010) and indeed, this has been found to be important in a number of invertebrate models. For example, the expression of sexually selected traits in the field cricket, Teleogryllus commodus (Maklakov et al. 2008), the cockroach, Nauphoeta cinerea (South et al. 2011) and the neriid fly, Telostylinus angusticollis (Sentinella, Crean & Bonduriansky 2013), are 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 usually trade-offs will be fixed at the point of ingestion rather than later in the resource gain-use chain (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 optimising 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 alter condition by manipulating the levels of two vital macronutrients in the diet (South et al. 2011). Using this approach, the ratio of macronutrients is fixed along a series of ‘nutritional rails’ but the concentration of macronutrients vary along each rail (i.e. the total calorie content varies from low to high). This allows the effects of total nutrition and the individual effects of specific macronutrients to be partitioned using response surface methodologies and the effects to be visualized as nutritional landscapes (Lee et al. 2008; Maklakov et al. 2008; Simpson & Raubenheimer 2009; South et al. 2011). Research using the NG approach has primarily focussed on investigations to understand the relationship between nutrition, lifespan and reproduction (Archer et al. 2009; Simpson & Raubenheimer 2009). Fewer studies have used a NG approach to investigate the link between condition and trait elaboration. However, those studies that have taken this approach show that specific nutrient combinations influence condition-dependent trait expression and fitness (Maklakov et al. 2008; South et al. 2011; Sentinella et al. 2013). Thus, NG is a valuable tool to explore condition dependent trait expression, especially for characters like secondary sexual traits that are expected to demonstrate heightened condition-dependence.
The horned flour beetle, *Gnatocerus cornutus*, feeds on a variety of grains, flours, yeast and dry animal products and is known to respond plastically to changes in the larval diet (Katsuki, Okada, & Okada 2012). On poor-quality larval diets, development is delayed and the size of a secondary sexual trait, the mandible, is reduced (Katsuki et al. 2012). Male mandibles are sexually selected (Okada, Miyanoshita & 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 the expression of the mandibles trades off with other cephalic characters, such as the head horns, antenna 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 if or how genetic trade-offs between traits are influenced by nutrition. Sexual selection also acts on aedeagus morphology in *G. cornutus* through a male’s ability to engage the female in copula and inseminate and fertilize a female’s ovum following a non-competitive mating (CMH unpublished data). It is currently unknown whether the trade-offs identified between other characters extend to aedeagus morphology in this species.

A standard experimental 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 et al. 2003). Here we used NG to determine the effects of protein (P) and carbohydrate (C) as well as the effects of total nutritional content on the survival, development time, body size and the expression of male primary (the aedeagus) and secondary (the mandibles) sexual traits, as well as a non-sexual trait (the eye) in *G. cornutus*. Although male mandibles are condition-dependent it is not clear if this relates to specific nutrients or to total calories (Katsuki et al. 2012). It is also unknown whether the genitalia of *G. cornutus* are impacted by nutrient consumption. First, we test whether the availability of P and C during juvenile development influences survival, development time and body size, as expected if condition is influenced by nutrition. Next, we test whether the expression of condition-dependent mandibles have heightened sensitivity to nutrition whereas the genitalia and a non-sexual trait are relatively insensitive to nutrition. Finally, we test whether trade-offs among life history traits and/or primary and secondary sexual traits are
mediated by nutrition 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 500mL, 120mm OD) and reared on whole meal flour that is enriched with 5% yeast and maintained at 27°C and 60% relative humidity under a 14:10 h light: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**

24 dry, artificial diets that varied in protein and carbohydrate content were produced following the protocol established in Simpson and Abisgold (1985), and represent 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 and the placement of the diets in nutritional space is shown in Figure 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 (1.5cm x 1cm) 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 approximately 125 mg of
whole meal flour (Doves Farms Foods Ltd) that was enriched with 5% brewers yeast (ACROS organics). Between 7 to 15 days post-eclosion, virgin males and females were collected and randomly allocated to one of four mixed sex pots (Thermoscientific Nalgene 500mL, 120mm OD; n = 125 females; n = 125 males) containing wholemeal flour (250 g) that was enriched with 5% brewers yeast. After 7 days, mated females were transferred to one of four new pots (Thermoscientific Nalgene 500mL, 120mm OD; n = 125 females) containing 250 g of plain flour (Doves Farms Foods Ltd) for 24 hrs to oviposit. The duration of oviposition was restricted to a single day to ensure that all resultant offspring were of the same age and plain flour was used as 2nd instar larvae (that are approximately 1mm in length) are easier to see against a white background. 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 125mg of one of the 24 artificial diets. All cells of a given 24 well plate contained a single diet and a total of 96 replicate individuals were allocated at random to one of the 24 diets (i.e. 4 x 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 eclosions 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) that was consistently oriented. A circular coverslip was gently placed on top of the beetle to flatten the body so that the left and right side were symmetrical along the midline of the body. 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), we measured the linear width of the pronotum as a measure of general body size (Figure 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 (Figure S2).
Due to their more complex structure, we used geometric morphometric analysis to measure the size of the male mandibles and genitalia. Since 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. For the left side of the mandible, three homologous landmarks were placed along the outline of the mandible and another 18 semi-landmarks were placed equidistance along the outer and inner curve of the mandible using the TPSUTIL (version 1.46) and TPSDig (version 2.14) programs (Rohlf 2009) (Figure S2). The Cartesian coordinates of these landmarks were extracted using the tpsRELW (version 1.46) program (Rohlf 2008), and we estimated the overall size of the mandible from the centroid size calculated as the square root of the sum of squared distances between landmarks and their centroid (Cardini 2012). To measure the size of the male genitalia, we separated the elytra and wings and removed the aedeagus from the abdominal cavity using fine forceps. The aedeagus was then orientated in a consistent position on a glass slide, mounted in a droplet of Hoyer’s solution, and an image was taken. Using the same procedure and software as for mandible size, three homologous landmarks were placed at the tip and base of the aedeagus, and another 26 semi-landmarks were placed equidistance along its outline to estimate centroid size (Figure S3).

For a subset of 25 experimental males sampled at random across diets, we measured each of our morphological measures twice to assess their repeatability using the R code provided in Wolak et al. (2012). Each of our measurements of adult morphology were 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; 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 = 2227 for our 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 time and adult morphology were based on 20 randomly selected male beetles per diet for 18 out 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

As our response variables (survival, development time and adult morphology) were measured in different units, we standardized each response variable and the P and C content 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 survival to eclosion, have a reduced development time and typically eclose at a larger body size (e.g. Hunt et al. 2004). The consumption of P and C in our experiment is therefore expected to have contrasting effects on these response variables (i.e. cause higher survival and larger body size but shorter development time).

Thus, even though faster development with a high intake of nutrients reflects more efficient growth and development (i.e. increased performance), any statistical comparison of the effects of nutrition on development time, body size and survival will necessarily show significant differences due to the gradients having different signs, even if the absolute magnitude of these effects in similar. We therefore transformed our standardized measures of development time by multiplying the z scores by -1. After this transformation, 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 P and C content of the diet as fixed effects. The parameter estimates from this model were used to estimate the linear effects of these nutrients on our response variables. Next, we ran a second GLMM that contained the same terms but also included the quadratic (P x P and C x C) and cross-product (P x 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. While the parameter estimates (or nutritional gradients) from these models are not biased when the response variable has a binary distribution, this has been shown to bias significance testing of these parameters (Lande & Arnold 1983; Mitchell-Olds & Shaw 1987; Janzen & Stern 1998). Consequently, as survival to eclosion was measured as a binary response (i.e. 0 or 1) 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 of this analysis and accompanying R code are provided in Text S1.

When examining the effects of P and C on adult morphology (eye size, mandible size and adeagus size), we compared two sets of models. First, we used the above GLMMs to quantify the linear and nonlinear effects of P and C on absolute trait size. Second, 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 et al. 2013). With the exception of the binary GLMM, all GLMMs were implemented in JMP®(version 8.0.2) using Restricted Maximum Likelihood (REML) approximation.

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 (λ) that minimized the generalized cross-validation (GCV) score when visualizing the nutritional landscapes (Green & Silverman 1994). Even though all analyses were conducted on standardized data, and in the case of development time on transformed data, we present our nutritional landscapes on the raw data 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 (Chenoweth & Blows 2005; Draper & John 1988; South et al. 2011; Bunning et al. 2015). In short, this sequential approach tests the difference in the sign and magnitude of the linear, quadratic and correlational nutritional gradients across the different response variables by comparing the change in variance explained by a GLMM that fits a single relationship through the two response variables being compared (model 1) to a GLMM that fits a separate relationship for each response variable (model 2). If model 2 explains significantly more variance than model 1, as determined by a partial F test, this demonstrates that the nutritional gradients differ across response variables. Full details of this analysis, including linear equations, are included in Text 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 as $\theta = \cos^{-1}(a \cdot b / \| a \| \| b \|)$ (Equation 1) where $a$ is the linear effects of P and C consumption on the first response variable being compared, $b$ is the linear effects of these nutrients for the second response variable, $\| a \| = \sqrt{a \cdot a}$ and $\| b \| = \sqrt{b \cdot b}$. 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 Text S3.

Results

SURVIVAL, DEVELOPMENT TIME and BODY SIZE

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 survival (Table 1). The significant negative quadratic terms
indicate a peak for all three traits with the consumption of C and a peak for 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 approximately 1:2 on the nutritional landscape (Fig. 1). For survival, the significant negative correlational gradient suggests that longevity is highest at a high C and low P consumption (Table 1, Fig. 1A).

A sequential model building approach showed significant differences in the linear, quadratic and correlational effects of P and C consumption on survival and development time (Table S2). The difference in the linear gradients for survival and development time was due to the fact that survival was more responsive to P consumption than development time, whereas the difference in quadratic gradients occurred because curvature of the peak for P consumption was significant for survival and not for development time (Table S2). The difference in the correlational gradient for survival and development time was due to the fact that the negative gradient was significant for 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 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 survival, whereas the difference in quadratic gradients was due to the curvature of the peak for P consumption being stronger for 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). 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 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 angle between the linear vectors for survival and development time (θ = 8.83°, 95% CIs: 1.96°, 14.80°), between survival and body size (θ = 2.85°, 95% CIs: 1.72°, 3.99°) and between development time and body size (θ = 11.74°, 95% CIs: 2.97°, 18.99°).

THE ABSOLUTE SIZE OF MORPHOLOGICAL TRAITS

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 2). In contrast, mean
eye area only increased linearly with the consumption of C (Table 2). There were significant negative quadratic terms for the consumption of C for all three traits but not for the consumption of P (Table 2). Inspection of the nutritional landscapes shows that these peaks occur at a P:C ratio of approximately 1:2 for each trait (Fig 2). None of the correlational gradients were statistically significant (Table 2).

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 S3). In both cases, this was because mandible size was more responsive to the consumption of P and C (Table S3). 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 S3). Not surprisingly, the maximum size of these three morphological traits occurred in the same region on the nutritional landscape (Fig. 2), as evidenced by the small angle between the linear vectors for mandible and genital size ($\theta = 9.74^\circ$, 95% Cls: 2.41°, 18.25°), mandible and eye size ($\theta = 3.64^\circ$, 95% Cls: 0.00°, 11.22°) and genital and eye size ($\theta = 13.32^\circ$, 95% Cls: 5.51°, 22.89°).

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 3). After controlling for the effects of body size on these traits (Sentinella et al. 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 3). There were also significant linear and quadratic effects of C consumption on relative mandible size (Table 3). 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 3). In contrast, the relative size of the genitalia was relatively insensitive to the consumption of P and C (Table 3).

Discussion
One important source of variation in condition are environmental factors that influence 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 (Hunt et al. 2004; Tomkins et al. 2004; 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 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 the expression of life history traits, primary and secondary sexual traits, as well as non-sexual traits, with linear increases in trait 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 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 our traits. We therefore demonstrate that the predicted trade-offs between life history and primary and secondary sexual traits is not determined by the consumption of at least two important macronutrients (P and C).

Secondary sexual traits typically show a high degree of condition-dependent expression as shown in a diversity of animal taxa (Cotton et al. 2003). In some instances, secondary sexual trait exaggeration may be an extension of individuals’ body size (Cotton et al. 2003), in which case changes in the secondary sexual trait occurs because of changes in other components of the phenotype – for example as body size increases other traits may also increase in size (i.e. 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 et al. 2003). We demonstrate that the secondary sexual trait, the male mandibles, responded indirectly and directly 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 mandibles are significantly more
responsive to the intake of P and especially C in the larval diet of *G. cornutus* compared to a primary sexual trait and a non-sexual trait, as predicted for a condition-dependent exaggerated trait. This heightened sensitivity of the mandibles to nutrition is consistent with previous work (Katsuki *et al.* 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 *Telostylinus angusticollis* is essential for the expression of characters that appear to be strongly targeted by sexual selection. However there, C concentrations in the larval diet had little effect, leading the authors to suggest that a P-rich larval diet may be a general requirement of sexually-selected condition-dependent traits (Sentinella *et al.* 2013). Similar findings were also found in the lesser wax moth, *Achroia grisella*, where the expression of pre- and post-copulatory traits appeared to be increased in individuals that were reared on high P larval diets (Cordes *et al.* 2015). The fact that we found contrasting effects in *G. cornutus* is likely to reflect the evolutionary history of the species which feeds exclusively on plant derived products (such as flour) that typically contain limited amounts of P (for example, flour contains approximately 13% P and between 60 - 85 % C).

In contrast to the mandibles, the eyes were less responsive to the intake of dietary P. This reflects the slower growth of the eye relative to the rest of the body with increasing intake of P. Previously, it has been found that 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 1996). Evidence suggestive of resource allocation trade-offs between a secondary sexual and a non-sexual trait has been found in *Onthophagine* dung beetles; between horn length and eye size (*O. acuminatus* and *O. taurus*; Nijhout & Emlen 1998) and relative horn length and wing length (*O. taurus*; Tomkins *et al.* 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 amount of resources that were available for a smaller number of structures (Nijhout & Emlen 1998). In this study, the effect of dietary protein may reinforce underlying resource allocation trade-offs between
neighbouring traits as dietary protein that is 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 growth of the genitalia only responded indirectly and relatively weakly to variation in the consumption of P and C. 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 the hemipteran treehopper, *Enchenopa binotata* (Rodriguez & Al-Wathiqi 2011, 2012), fly, *Drosophila melanogaster* (Shingleton et al. 2009, Dryer & Shingleton 2011) and the dung beetle, *O. taurus* (House & Simmons 2007). However, this is not always the case for genitalia, with evidence of environmentally induced plasticity in the expression of the genitalia in the water strider, *Gerris incognitos*, albeit less than non-genital traits (Arnqvist & Thornhill 1998), and fly *D. mediopunctata* (Andrade et al. 2005).

Canalization of the genitalia should reduce trait size variation and contribute to the observed negative allometry of the male genitalia that is observed in many arthropod species (Eberhard et al. 1998; Hosken et al. 2005; Dryer & Shingleton 2011; Simmons, 2013). The adaptive significance of this pattern has been the subject of considerable discussion (Bertin & Fairbairn 2007; Dryer & Shingleton 2011; Eberhard et al. 1998; Eberhard, Rodriguez, & Polihronakis 2009; Simmons 2013), but a general explanation is that selection favours intermediate male genital size (stabilising selection), which selects for canalized growth (Eberhard et al. 2009; Dryer & Shingleton 2011; Simmons 2013). In *G. cornutus*, we have found significant directional selection on aedeagus size such that males with a small aedeagus are more effective in inseminating a female and consequently produce more offspring following a non-competitive mating – although this pattern of selection may alter when males compete to fertilize a females ovum (CMH unpublished data). This pattern of apparent directional selection and the insensitivity to nutrition 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 aedeagus size (i.e. large size) that confers lower fitness.

The mechanisms that generate nutritional reaction norms for body size and trait size, such as we document here, have not yet been elucidated in *G. cornutus*. Past studies show that the signalling pathways that regulate cell growth and proliferation are responsive to the nutritional status of most animals (Mirth & Riddiford 2007; Shingleton et al. 2007, 2008). 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 (Emlen et al. 2012). Although, the imaginal discs themselves have been found to vary in their sensitivity to insulin-like peptides (ILPs) which can lead to varied nutritional reaction norms (Shingleton et al. 2007; Warren et al. 2013).

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), *A. grisella* and *T. angusticollis* although the precise signalling pathways are yet to be confirmed in *A. grisella* and *T. angusticollis* (Cordes et al. 2015; Sentinella et al. 2013). 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). Our work illustrates the utility of NG to begin to pin point the dominant macronutrients that stimulate growth and give rise to the nutrient reaction norms for different traits. 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 no evidence that the optimal nutritional requirements of one trait constrain the dietary optimum of other traits. We show that life history traits (survival, development time and body size), primary and secondary sexual traits, and a non-sexual
trait 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 those of male T. angusticollis flies and A. grisella wax moths that show that the positive effects of P for secondary sexual trait expression is countered by the severe negative effects of P during the developmental stages and therefore dietary P is likely to mediate trade-offs (Cordes et al. 2015; Sentinella et al. 2013). Similarly, in male N. cinerea varied requirements for dietary C across different episodes of male reproductive effort is likely to mediate trade-offs. During pre-copulatory sexual selection, male-male competition and female choice is mediated by three pheromones, the expression of which is optimized at a P:C ratio of 1:8 (South et al. 2011), whereas sperm production is optimized at a P:C ratio of 1:2 (Bunning et al. 2015). Consequently, the dietary optimum that enhances pre-copulatory performance is likely to trade-off with the dietary optimum that enhances post-copulatory performance (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 trade-offs in G. cornutus, our results show that nutrition is likely to have long lasting effects on male non-sexual and sexual 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 did so, but at a cost to their nonsexual fitness as they developed more slowly. Prolonged development time is a plastic response which allows the animal to achieve its required caloric and nutritional consumption in poor nutrient environments (Chown & Nicolson 2004). For example, larvae of the African armyworm Spodoptera exempta that were reared on poor quality grasses and larvae of the seed beetle, Stator limbatus that competed with siblings for resources were both 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 larval diet and also were smaller adults. The fitness cost of delayed development, small body size and reduced secondary sexual characters 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 (Adler et al. 2013; Boggs & Freeman
Given the probable fitness consequences of a poor quality larval environment it would be of interest to determine whether *G. cornutus* can feed selectively to reach an intake target that maximises the expression of life history traits and male secondary sexual traits as has been reported for some other insects, e.g. *Spodoptera littoralis* (Simpson *et al*. 2004), *Chortoicetes terminifera* (Simpson *et al*. 2015) and *N. cinerea* (South *et al*. 2011).

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 complementary nutritional requirements and therefore trade-offs are unlikely to occur at the point of ingestion. Coupled with this, our estimates of sexual selection on male primary (CMH *unpublished data*) and secondary sexual traits (Okada *et al*. 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 condition-dependent weapons but also maintain a small aedeagus.

**Acknowledgments**

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**Data accessibility**

**References**


**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.

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

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

**Table S3.** Comparison of the effects of P and C on eye area, mandible size and genital size.

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

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

**Text S3.** Calculating the angle (θ) and 95% CIs between linear nutritional gradients.
Table 1. Linear and nonlinear effects of the protein (P) and carbohydrate (C) content of
the diet on survival of beetle larvae (both sexes), as well as the development time and
adult body size of 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 survival (pMCMC).

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Linear effects</th>
<th>Nonlinear effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>C</td>
</tr>
<tr>
<td><strong>Survival</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.49 ± 0.06</td>
<td>0.58 ± 0.06</td>
</tr>
<tr>
<td>t</td>
<td>7.98</td>
<td>9.49</td>
</tr>
<tr>
<td>df</td>
<td>93.08</td>
<td>93.14</td>
</tr>
<tr>
<td>pMCMC</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Development time</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.29 ± 0.10</td>
<td>0.65 ± 0.10</td>
</tr>
<tr>
<td>t</td>
<td>3.07</td>
<td>6.37</td>
</tr>
<tr>
<td>df</td>
<td>65.26</td>
<td>66.11</td>
</tr>
<tr>
<td>P</td>
<td>0.003</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Body size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.38 ± 0.07</td>
<td>0.95 ± 0.07</td>
</tr>
<tr>
<td>t</td>
<td>5.44</td>
<td>13.08</td>
</tr>
<tr>
<td>df</td>
<td>67.44</td>
<td>58.32</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

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 x P and C x 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 x 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 content of the diet is high, whereas a negative gradient indicates that the response variable is
greatest when one nutrient is high and the other low.
Table 2. Linear and nonlinear effects of the protein (P) and carbohydrate (C) content of the diet on the absolute size of a non-sexual trait (eye area), primary sexual trait (mandibles), and secondary sexual trait (genitalia) in male G. cornutus.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Linear effects</th>
<th>Nonlinear effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>C</td>
</tr>
<tr>
<td><strong>Mean Eye Area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.12 ± 0.07</td>
<td>0.75 ± 0.06</td>
</tr>
<tr>
<td>t</td>
<td>1.66</td>
<td>10.54</td>
</tr>
<tr>
<td>df</td>
<td>65.62</td>
<td>66.34</td>
</tr>
<tr>
<td>P</td>
<td>0.10</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Mandible Size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.35 ± 0.07</td>
<td>0.89 ± 0.07</td>
</tr>
<tr>
<td>t</td>
<td>4.83</td>
<td>12.09</td>
</tr>
<tr>
<td>df</td>
<td>67.61</td>
<td>68.45</td>
</tr>
<tr>
<td>P</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
<tr>
<td><strong>Genital Size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.22 ± 0.07</td>
<td>0.65 ± 0.07</td>
</tr>
<tr>
<td>t</td>
<td>3.07</td>
<td>9.01</td>
</tr>
<tr>
<td>df</td>
<td>63.36</td>
<td>63.57</td>
</tr>
<tr>
<td>P</td>
<td>0.003</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
Table 3. The linear and nonlinear effects of the protein (P) and carbohydrate (C) content of the diet on mean eye size, mandible size and genital size in male *G. cornutus* when controlling for the effects of body size on these traits.

<table>
<thead>
<tr>
<th>Response variable</th>
<th>Linear effects</th>
<th>Nonlinear effects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>P</td>
<td>C</td>
</tr>
<tr>
<td><strong>Mean eye area</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>-0.15 ± 0.05</td>
<td>0.12 ± 0.07</td>
</tr>
<tr>
<td><em>t</em></td>
<td>3.04</td>
<td>1.78</td>
</tr>
<tr>
<td><em>df</em></td>
<td>71.34</td>
<td>108.20</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.003</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Mandible size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>0.02 ± 0.03</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td><em>t</em></td>
<td>0.81</td>
<td>2.31</td>
</tr>
<tr>
<td><em>df</em></td>
<td>77.72</td>
<td>121.40</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.42</td>
<td>0.02</td>
</tr>
<tr>
<td><strong>Genital size</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gradient ± SE</td>
<td>-0.05 ± 0.05</td>
<td>-0.01 ± 0.07</td>
</tr>
<tr>
<td><em>t</em></td>
<td>1.06</td>
<td>0.10</td>
</tr>
<tr>
<td><em>df</em></td>
<td>77.93</td>
<td>106.7</td>
</tr>
<tr>
<td><em>P</em></td>
<td>0.29</td>
<td>0.92</td>
</tr>
</tbody>
</table>

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.
Fig. 1. Nutritional landscapes illustrating the effects of the protein and carbohydrate content of the diet on (A) larval survival to eclosion, as well as the (B) developmental rate and (C) body size of male *G. cornutus*. Hotter colours (red) represent areas of increased survival, a slower development time and larger body size and colder colours (blue) represent areas of reduced survival, faster development time and smaller body size. The circles show the nutritional compositions of our artificial diets.

Fig. 2. Nutritional landscapes illustrating the effects of the protein and carbohydrate content of the diet on (A) mean eye area, (B) mandible size and (C) genital size of male *G. cornutus*. Hotter colours represent areas of larger trait size and colder colours represent areas of smaller trait size. Note that no beetles survived at extremely nutrient poor diets (near the origin) and hence adult morphology could not be measured for these animals. The circles show the nutritional compositions of our artificial diets.
Figure 1

- **A**. Survival proportion over carbohydrate and protein content (%).
- **B**. Development time in days over carbohydrate and protein content (%).
- **C**. Body size in mm over carbohydrate and protein content (%).
Figure 2

Mean Eye Area

Mandible Size

Genital Size

Carbohydrate content (%)

Protein content (%)

mm²

Centroid size

Centroid size

Centroid size

Centroid size