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Macronutrient balance mediates trade-offs between immune function and life history traits

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Summary

1. Diet and health are intimately linked and recent studies have found that caloric restriction can affect immune function. However, when given a choice between diets that differ in their macronutrient composition, pathogen–infected individuals can select a diet that improves their survival, suggesting that the nutritional composition of the diet, as well as its calorie content, can play a role in defence against disease. Moreover, as individuals change their diet when infected, it suggests that a diet that is optimal for growth is not optimal for immunity, leading to trade-offs.

2. Currently, our knowledge of the effects of diet on immunity is limited because previous experiments have manipulated either single nutrients or the calorie content of the diet without considering their interactive effects. By simultaneously manipulating both the diet composition (*quality*) and its caloric density (*quantity*), in both naive and immune-challenged insects, we asked how do diet quality and quantity influence an individual's ability to mount an immune response? And to what extent are allocation trade-offs driven by quantity- versus quality-based constraints?

3. We restricted individuals to 20 diets varying in their protein and carbohydrate content and used 3D response surfaces to visualize dietary effects on larval growth and immune traits. Our results show that both constitutive and induced immune responses are not limited by the total *quantity* of nutrients consumed, but rather different traits respond differently to variation in the ratios of macronutrients (diet *quality*), and peak in different regions of macronutrient space. The preferred dietary composition therefore represents a compromise between the nutritional requirements of growth and immune responses. We also show that a non-pathogenic immune challenge does not affect diet choice, rather immune-challenged insects modify their allocation of nutrients to improve their immune response.

4. Our results indicate that immune traits are affected by the macronutrient content of the diet and that no diet can simultaneously optimize all components of the immune system. To date the emphasis has been on the effects of micronutrients in improving immunity, our findings indicate that this must be widened to include the neglected impact of macronutrients on defence against disease.

Key-words: bacteria, caloric restriction, life-history, nutritional ecology, parasite, self medication, *Spodoptera*, trade-offs

Introduction

Resource availability is a powerful driver of evolution by natural selection (Grant & Grant 2002), and competing demands among organismal traits for resources generate allocation trade-offs that are fundamental to life history theory (Stearns 1992; Zera & Harshman 2001). Evolutionary ecology, likewise, is underpinned substantially by the concept of nutrition-dependent condition, with nutritional state influencing numerous traits, from reproduction (Joern & Behmer 1997; Fricke, Bretman & Chapman 2008), and longevity (Punzalan *et al.* 2008) to defence against parasites

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or pathogens (Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002). It is thus critical that these sciences are guided by models in nutritional biology that best represent the acquisition and allocation of resources by animals.

To a large extent, nutritional ecology has been dominated by the *quantitative resource constraints* paradigm, which assumes that animals forage to maximize intake of a single nutritional resource, usually either energy or nitrogen, a lack of which can lead to resource allocation trade-offs as animals are prevented from optimally investing in all functional traits simultaneously. It is this idea that has been most frequently addressed when considering the role of nutrition in immune responses, by testing the effects of starvation (e.g. Moret & Schmid-Hempel 2000; Siva-Jothy & Thompson 2002) or caloric restriction (e.g. Murray & Murray 1979; Kristan 2007; Ayres & Schneider 2009) on defence. However, recent developments in nutritional biology have demonstrated that in many cases this single-currency approach provides, at best, a crude tool for understanding the responses of animals to their nutritional environments, compared with an approach which takes into account the animal's concurrent needs for multiple nutrients (Sterner & Elser 2002; Simpson *et al.* 2004).

An alternative possibility is to view traits as co-existing within an organismal 'ecology', each with its own specific nutritional requirements. If these requirements are complementary then such traits can coexist in a kind of *intra-organismal niche partitioning*. For other pairs of traits, these requirements will be non-complementary, such that no single blend of ingested nutrients can optimally satisfy all. In this case, investment trade-offs will be decided at the point of ingestion – rather than allocation – because the blend of nutrients that is ingested will determine the relative performance of competing traits. This *qualitative resource constraints* hypothesis has been slower to develop than the question of whether a one-dimensional approach adequately represents animal nutrition, in part because testing it requires a robust framework for modelling nutrition as a multi-dimensional phenomenon.

The development in recent years of such a framework, the geometric approach to nutrition, enables these issues to be systematically explored (Simpson & Raubenheimer 1995). Here we use this approach to address the question of whether the relationship between diet and immunity is simply driven by energy consumption or whether the blend of nutrients is key in determining an individual's immune response. In addition, we examine, for the first time, the extent to which allocation trade-offs within and among immune function and life-history traits are driven by quantity- *versus* quality-based constraints, or whether these trade-offs are averted by complementary nutrient allocation.

Insects provide excellent models for addressing these questions. The insect immune system comprises cellular and humoral components which work together to overcome invaders. Haemocytes phagocytose smaller pathogens, form nodules around clumps of bacteria or encapsulate larger organisms (Gupta 1991), whilst the phenoloxidase (PO) enzyme reaction melanises capsules and provides toxic intermediates to help kill parasites (Sugumaran & Kanost 1993). In addition, lysozymes and other antimicrobial peptides are up-regulated upon recognition of microbial cell wall components (Briese 1981). Despite its relative simplicity, previous studies have found evidence for trade-offs within the insect immune system, with PO activity showing negative genetic and phenotypic correlations with antibacterial activity (Moret & Schmid-Hempel 2001; Cotter, Kruuk & Wilson 2004), thus, with an insect model it is possible to examine nutrient-based trade-offs both within the immune system and between immune traits and other life-history traits.

Our chosen system is the caterpillar *Spodoptera littoralis* (See Fig. S1 in supporting information), in which we examine macronutrient allocation to somatic growth and simultaneously test the allocation dynamics for constitutive components of the immune response: haemolymph-based lysozyme-like activity, phenoloxidase activity (PO) and the degree of cuticular melanism, which is indicative of defence against fungae and parasitoids in this species (Wilson *et al.* 2001). We then challenge the immune system with an elicitor to measure the effect of diet on induced immune responses, and on the diet caterpillars choose to eat when allowed to self-select. If immune traits peak in different regions to the diet choice of naive insects then infected insects could respond in one of three ways: they could alter their diet choice to fall in the region of peak activity for a particular immune response, they could modify their internal allocation of ingested nutrients for a given diet such that the response surfaces for immune traits would differ in challenged insects, or they could use a combination of the two mechanisms to improve immune responses.

Specifically, we tested three predictions: (1) traits will map onto different regions of nutrient space, as predicted by the qualitative resource constraints hypothesis; (2) PO and lysozyme will map onto different regions of nutrient space, providing a basis for the observed trade-off in this species; and (3) immune-challenged insects will shift their diet-choice to one that maximizes an appropriate immune response, or they will modify their internal allocation of nutrients such that response surfaces differ for naive and challenged insects.

Materials and methods

SPODOPTERA LITTORALIS CULTURE

The *Spodoptera littoralis* culture was established from eggs collected near Alexandria in Egypt in 2002 and high numbers were maintained at each generation to reduce inbreeding. The colony had been reared using single pair matings for 40 generations, with over 150 pairs established each generation. Larvae were reared singly from the 2nd instar on a semi-artificial wheatgerm-based diet in 25 ml polypots until the start of the penultimate larval instar (5th) in experiment 1, or final larval instar (6th) experiment 2, at which point they were used in the experiments described below. *Spodoptera littoralis* spend *c.* 2 weeks in the larval stage, about 8 days of which are spent in the 5th and 6th instars. Insects were maintained at 25 °C under a 12 : 12 light : dark photoregime.

DIET TREATMENTS

For each of the experiments larvae were restricted to, or given a choice between chemically defined diets containing precisely controlled amounts of protein and carbohydrate, hereafter referred to as P and C respectively. In the experiments where larvae were restricted to a single diet, foods contained one of five ratios of protein (P) (a 3 : 1 : 1 mix of casein, peptone and albumen) to digestible carbohydrate (C) (sucrose): 17, 33, 50, 67 or 83% protein as a proportion of the total digestible nutrients (P/(P + C)). Foods also differed in their total concentration of protein and carbohydrate through the addition of indigestible cellulose. For each of the five P : C ratios there were four such dilutions: P + C = 63, 42, 34, or 17% by dry mass giving 20 diets in total. See Table S1 in supporting information for a summary of the precise protein and carbohydrate content of each diet. As protein and carbohydrate are similar in caloric density (*c*. 4 calories per gram (Merrill & Watt 1973)), the different P : C ratio diets within each dilution were isocaloric. This allowed us to separate the effects of the calorie content of the diet from its composition.

In the self selecting treatments, larvae were given a choice between complementary pairs of diets. In each case, a balanced food block (50% P) at a concentration of 42% digestible nutrients (i.e. the dry diet contained 21% protein and 21% carbohydrate – Table S1) was paired with a protein rich food block (83% P), which varied in its concentration between treatments such that: (1) P + C = 42%; (2) P + C = 33%; or (3) P + C = 25%. For all of the diets, the remaining dietary ingredients (salts, vitamins, cholesterol and linoleic acid) totalled 4% and the dry ingredients were suspended at a 1 to 6 ratio w/v in 1% agar solution. In each experiment, larvae were provided with food blocks weighing *c*. 1·5 g. In both experiments, larvae were restricted to their assigned diets for a single instar. It is not feasible to restrict larvae for longer periods as survival on the more extreme diets can be very low (SCC pers obs).

EXPERIMENT 1: THE EFFECTS OF NUTRIENT COMPOSITION ON THE RESPONSE SURFACES OF CONSTITUTIVE IMMUNE TRAITS, HAEMOLYMPH PROTEIN LEVELS AND LARVAL PERFORMANCE

Upon moulting, 5th instar larvae from 56 full-sibling families were weighed to the nearest 0·1 mg and each was placed in its own 9-cm-diameter Petri dish with pre-weighed blocks (weighing *c*. 1·5 g) of one of the 20 chemically-defined diets described above. The experiment was repeated twice with 200 larvae per replicate, giving 400 larvae in total. Food was replaced each day and uneaten food was removed and dried to a constant mass. Consumption was calculated as the difference between initial dry mass (estimated from initial wet mass) and final dry mass of food. Although some previous studies have not measured individual consumption rates (e.g. Carey *et al*. 2008; Fricke, Bretman & Chapman 2008) compensatory feeding can alter the relationship between the diet offered and the nutrients ingested (Lee, Raubenheimer & Simpson 2004), we therefore measured daily consumption so that the amount of protein and carbohydrate ingested by each individual could be calculated.

As the larval cuticle in the final instar is laid down during the previous instar, insects were maintained on their assigned diet for an entire instar including a moult (5·3 ± 0·7 days). allowing us to measure the effects of the diet treatment on cuticular melanism. At this point larvae were weighed and haemolymph was sampled by piercing the cuticle between the final pair of prolegs using a fine needle. Haemolymph was collected in Eppendorf tubes and frozen at −80 °C until needed. Larvae were then sacrificed and their cuticles dissected for melanism scoring. Larval performance was measured as the change in body mass over the instar multiplied by survival for each diet treatment. Those insects that died prior to haemolymph sampling were recorded but removed from analyses of intake and larval performance.

EXPERIMENT 2: THE EFFECTS OF IMMUNE CHALLENGE ON RESPONSE SURFACES AND DIET CHOICE IN CHALLENGED AND NON-CHALLENGED LARVAE

For the dietary restriction treatments, newly-moulted 6th – instar larvae from 10 full-sibling families were weighed and provided with one of the 20 chemically-defined diets described above. The experiment was repeated twice with 200 larvae per replicate, giving 400 larvae in total. In the self-selecting treatments, 60 newly-moulted, final-instar larvae were each provided with one of three pairs of nutritionally complementary food blocks. Larvae were allowed to self-select between the foods to establish whether, and to which point, they would regulate their intake of protein and carbohydrate. Each of the paired foods differed in their concentration of total digestible nutrients so that larvae would have to consume different amounts of food in each treatment to converge at the same point in intake space.

In both the no-choice and self-selecting treatments, on the second day of the experiment half of the larvae had their immune systems challenged by piercing the cuticle with a needle dipped in a 10 mg mL^{-1} solution of *Micrococcus lysodeikticus* lyophilised cells in phosphate buffered saline (PBS, pH 7·4). The expectation was that this challenge would up-regulate antibacterial immune activity. The immune system can recognize bacterial cell wall components as non-self and respond as if to an infection, however as this is not an actively replicating parasite we can separate the resource costs of mounting an immune response from the resources required by the parasite. On day3, haemolymph was sampled by piercing the cuticle between the final pair of prolegs using a fine needle. Haemolymph was collected in Eppendorf tubes and frozen at −80 °C until needed. As for experiment 1, food was replaced daily and consumption was calculated for each caterpillar. Caterpillars spent 3·96 (+0·06) days on the experimental diets before pupating. In this experiment, larvae were maintained on their diets until pupation, so that larvae had been feeding on their assigned diets for a whole instar. Those insects that died were recorded but removed from analyses of intake and growth. Insect performance was measured as growth rate over the final instar multiplied by survival for each diet treatment; a measure which combines two variables known to contribute substantially to fitness in caterpillars (Simpson *et al*. 2004).

Phenoloxidase assay

Haemolymph PO activity was assayed spectrophotometrically with dopamine as a substrate (Cotter, Beveridge & Simmons 2008). 8 µl of haemolymph was added to 400 µl of ice-cold PBS in a plastic Eppendorf tube and vortexed. 100 µl of 4 mM dopamine was added to 100 µl of the buffered haemolymph and duplicate samples of the mixture were incubated on a temperature-controlled *VERSAmax* tuneable microplate reader (Molecular Devices, Sunnyvale, CA) at 490 nm for 10 minutes at 25 °C. PO activity was expressed as the slope of the line over 10 minutes, which is in the linear phase of the reaction.

Protein assay

Protein was measured using the *BioRad* protein assay kit (BioRad, Hercules, CA, USA) with BSA as the protein standard. This method detects large proteins (> 3 KD in size) and does not detect free amino acids or smaller peptides. Two replicates of 5 μl of the haemolymph/PBS mixtures were used to measure the protein in each sample. Absorption was measured at 25 °C on a temperature-controlled *VERSAmax* tuneable microplate reader (Molecular Devices, Sunnyvale, CA) at 600 nm.

Lysozyme-like antibacterial activity

Lytic activity against *M. lysodeikticus* was determined using a lytic zone assay. Agar plates containing 10 ml of 1% agar with 5 mg per ml freeze-dried *M. lysodeikticus* were prepared. For each plate, 20 holes with a diameter of 2 mm were punched in the agar and 1 μl of haemolymph was placed in each well, two replicates per sample. The plates were incubated at 33 °C for 18 hours then photographed using a *Polaroid DMC* digital camera (Polaroid, Bethesda, MD, USA) and the diameter of the clear zones calculated using *Image Pro Plus* software (Media Cybernetics). Standard curves were obtained using a serial dilution of hen egg white lysozyme. Concentration of 'hen egg white lysozyme equivalents' was then calculated.

Melanism scoring

The degree of melanism in the dissected cuticles was quantified using an Avaspec-2048 fibre optic spectrometer with an AvaLight-HAL tungsten halogen light source (Avantes, Eerbeek, The Netherlands) as described in (Lee & Wilson 2006b). Briefly, measurements were taken using a 2 mm diameter bifurcated fibre optic probe that was positioned at a 90° angle to the cuticle. The relative paleness of a sample was expressed as an absorbance value (%), where 0% was equivalent to the white standard and 100% was equivalent to the dark standard. Triplicate absorbance values were recorded at 575 nm wavelength for each larva along the dorsal midline of the cuticle. The repeatability of this technique was high ($r = 0{\cdot}86$; Cotter *et al.* 2008).

STATISTICAL ANALYSES

All data were standardized using the mean (μ) and standard deviation (σ) of each trait ($Z = (X-\mu)/\sigma$) prior to analysis so that the response surfaces for the different traits could be compared using partial *F*-tests (Chenoweth & Blows 2005). The effects of P and C consumption on each trait were analysed using linear mixed models (REML) in Genstat 10, including the family from which each larva originated as a random effect. The amount of protein eaten (P), carbohydrate eaten (C), both squared terms (P^2 and C^2), and the interaction between protein and carbohydrate eaten (P×C), were included as fixed explanatory terms (Lande & Arnold 1983). The effect of replicate was also included for both experiments but in each case the effects were non-significant and so it was removed from the final models. The shape of the response surface for each trait was then visualized using non-parametric thin-plate splines in *R* (v2.6.1), a powerful technique that does not constrain the shape of the surface (Blows & Brooks 2003). However, it should be noted that these are an aid to visualizing the surfaces and are not a direct output from the statistical models used to test the significance of the diet components. Mean values ± SE are reported throughout.

Results

EXPERIMENT 1: THE EFFECT OF NUTRIENT COMPOSITION ON CONSTITUTIVE IMMUNE TRAITS, HAEMOLYMPH PROTEIN AND LARVAL PERFORMANCE

The concentration and percentage protein composition (%P) of the diet affected the total amount of food consumed by the larvae, providing clear evidence for compensatory feeding when restricted to suboptimal diets (diet concentration: $F_{3,327} = 31{\cdot}27$, $P < 0{\cdot}001$; %P: $F_{1,334} = 11{\cdot}09$, $P < 0{\cdot}001$; $\%P^2$: $F_{3,334} = 16{\cdot}66$, $P < 0{\cdot}001$). For any given %P, the highest consumption was on the lowest total nutrient concentration diet, with consumption decreasing as the concentration of the diet increased (Fig. S2a). Across the range of %P within a given total nutrient concentration, food consumption increased with increasing percentage protein up to around 42% protein, then fell sharply (Fig. S2b). Despite these clear compensatory responses to the concentration of nutrients in the diet, when the actual amounts of protein and carbohydrate consumed were calculated and the resulting intake array plotted, it can be seen that the diet treatments were successful in causing larvae to consume protein and carbohydrate levels that covered a large area of intake space (Fig. S2b). Thus, despite much higher levels of consumption on the lower concentration diets (Fig. S2a), larvae were unable to consume enough to match the protein and carbohydrate intake of larvae on the more concentrated diets (Fig. S2b).

Variation in each of the traits with respect to diet consumption can be visualized as a response surface. If any of the traits were constrained by a single currency (*quantitative resource constraint*) then we could predict what the landscape might look like. As protein and carbohydrate are near isocaloric, a trait constrained by energy alone would increase with increasing calorie intake, irrespective of whether those calories came from a protein or carbohydrate source (Fig. 1a). Conversely, if a trait was constrained by nitrogen, which is available in protein but not carbohydrate, then we would expect performance to increase with increasing protein only (Fig. 1b). If a trait was constrained by both over- and under-ingesting proteins and carbohydrates, then the landscape would exhibit a peak in some part of nutrient space (Fig. 1c). Deviation from these hypothetical landscapes would be expected if trait performance was affected by the blend of protein and carbohydrate consumed (*qualitative resource constraint*).

To examine the nutritional dependency of each of the measured traits, we first considered whether a single response surface could explain variation in all of the measured traits by comparing a statistical model including the interactions between the diet intake variables (protein (P), carbohydrate (C), their squared terms (P^2 and $C^{2)}$) and the interaction between the two (P×C) and trait type (larval performance, haemolymph protein, lysozyme activity, PO activity or cuticular melanism), with a model without any of these interactions. The interactions between trait type and the diet variables did explain significant variation in the data (Partial

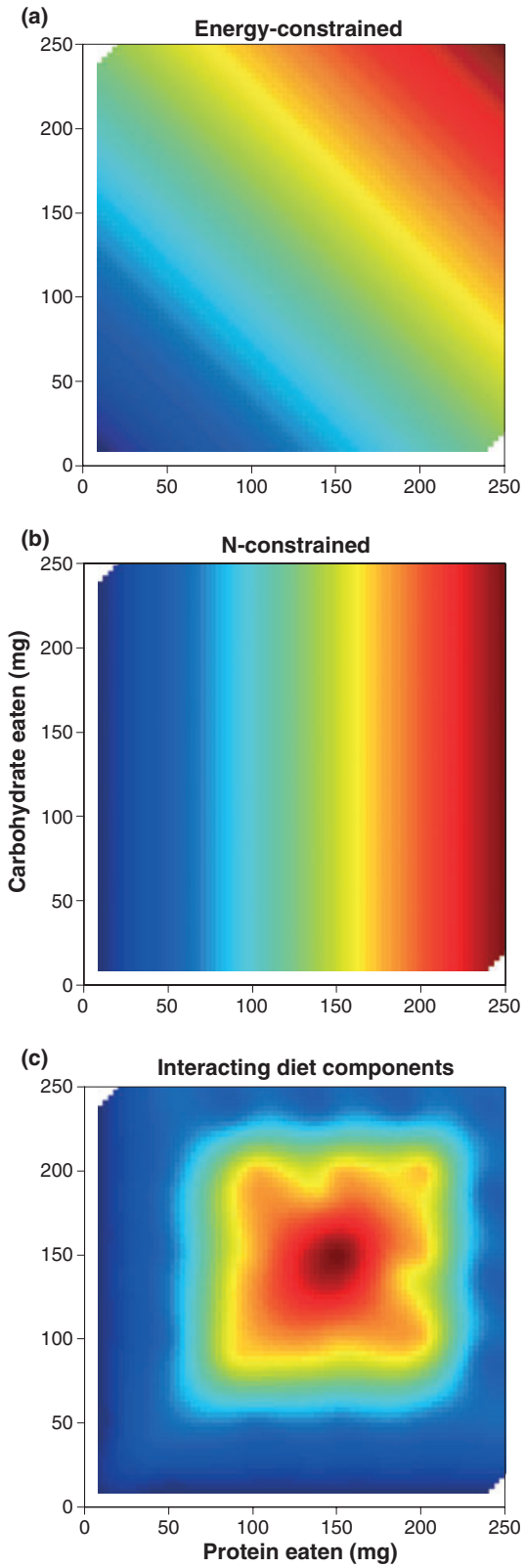

Fig. 1. Hypothetical surfaces showing how a trait might be expected to vary with carbohydrate and protein intake under either the qualitative or quantitative resource constraints paradigms. Surfaces depict variation if the trait was constrained by a single currency such as (a) energy or (b) nitrogen, or if it was affected by the composition of the diet (c) such that there was an interaction between protein and carbohydrate.

$F_{20} = 6{\cdot}68$, $P < 0{\cdot}001$); therefore, individual surfaces were produced for each trait for further analysis. When compared pair-wise, all of the surfaces, with the exception of PO and cuticular melanism, were significantly different from each other (Table 1).

If any of the traits were constrained by a single currency, as predicted by the quantitative constraints model, we would expect linear effects of P and C combined (energy-constrained) or of P alone (N-constrained). With the exception of melanism, the measured traits did not conform to either of these hypothetical landscapes, lending support to the qualitative constraints model. Larval performance showed strong effects of P, C and P^2 (Table 2, see Table S2 for estimated surface gradients). Larval performance was maximized over a broad range of carbohydrate consumption (10–110 mg C) but a rather narrow range of protein consumption (90–130 mg P), suggesting that both under- and over-ingestion of protein was detrimental to performance (Fig. 2a). The significant negative P^2 term confirms this, as it means that there is an optimal level of protein for growth. Haemolymph protein levels were also affected by P, C and P^2 (Tables 2 and S2). The landscape shows haemolymph protein levels increasing mostly in response to increasing protein consumption, with the highest levels occurring at the highest protein consumption, above 220 mg P, and between 40 and 150 mg C (Fig. 2b). However, the negative P^2 term again suggests that over-ingesting protein leads to a reduction in the levels of P in the haemolymph. Moreover, the marginally significant negative coefficient for C in this model indicates that the haemolymph protein pool decreases as carbohydrate consumption increases.

Lysozyme activity was affected by P, C, P^2 and C^2 (Tables 2 and S2), but again, the strongest effect was for protein consumption. Similar to haemolymph protein levels, lysozyme activity tended to increase with increasing protein consumption, with the change in carbohydrate levels having relatively little effect (Fig. 2c). The range of highest activity occurred between 100 and 250 mg C, and above 220 mg P. Again, the significant squared terms indicate optimal levels of P and C for lytic activity, rather than levels increasing linearly with the availability of either nutrient.

The landscape for cuticular melanism was similar to that for haemolymph protein and lysozyme, with melanism increasing with the protein content of the diet, peaking above 220 mg P, and between 40 and 100 mg C (Fig. 2e). As neither of the squared terms was significant, this suggests, that at least within the region of nutrient space we covered, melanism does not have an optimal level of P but increases linearly with P availability. Whilst this would be statistically consistent with a trait that is N-constrained, the figure produced by the spline model shows quite a different pattern to the hypothetical N-constrained landscape (cf. Figs 1b and 2e). This is because the spline model is non-parametric and not constrained in the same way as the parametric REML model (Blows & Brooks 2003). In contrast, there were no significant effects of any of the diet variables on PO activity (Tables 2 and S2), though the spline plot predicts a peak in the region of 50–100 mg P and 50–100 mg C (Fig. 2d).

Table 1. Pairwise comparisons between response surfaces for experiment 1

	Haemolymph protein	Lysozyme activity	PO activity	Melanism
Larval performance	**$F_5 = 8{\cdot}76$ $P < 0{\cdot}001$**	**$F_5 = 11{\cdot}02$ $P < 0{\cdot}001$**	**$F_5 = 6{\cdot}16$ $P < 0{\cdot}001$**	**$F_5 = 3{\cdot}45$ $P = 0{\cdot}004$**
Haemolymph protein	–	**$F_5 = 3{\cdot}14$ $P = 0{\cdot}008$**	**$F_5 = 32{\cdot}42$ $P < 0{\cdot}001$**	**$F_5 = 8{\cdot}08$ $P < 0{\cdot}001$**
Lysozyme activity	–	–	**$F_5 = 20{\cdot}97$ $P < 0{\cdot}001$**	**$F_5 = 6{\cdot}00$ $P < 0{\cdot}001$**
PO activity	–	–	–	$F_5 = 1{\cdot}55$ $P = 0{\cdot}173$

Results of Partial *F*-tests comparing models with and without the interactions between each trait type (larval performance, haemolymph protein, lysozyme activity, PO activity and cuticular melanism) and the diet variables. Surfaces that are significantly different are highlighted in bold type.

Table 2. The effects of protein and carbohydrate consumption on each trait – experiment 1

Fixed term	Larval performance	Protein	Lysozyme activity	PO activity	Melanism
P	**$F_{1,332} = 44{\cdot}75$ $P < 0{\cdot}001$**	**$F_{1,334} = 47{\cdot}84$ $P < 0{\cdot}001$**	**$F_{1,336} = 21{\cdot}34$ $P < 0{\cdot}001$**	$F_{1,320} = 0{\cdot}16$ $P = 0{\cdot}685$	**$F_{1,342} = 6{\cdot}77$ $P = 0{\cdot}010$**
C	**$F_{1,327} = 34{\cdot}61$ $P < 0{\cdot}001$**	**$F_{1,343} = 4{\cdot}07$ $P = 0{\cdot}045$**	**$F_{1,331} = 10{\cdot}00$ $P = 0{\cdot}002$**	$F_{1,316} = 1{\cdot}68$ $P = 0{\cdot}195$	$F_{1,339} = 1{\cdot}52$ $P = 0{\cdot}219$
P^2	**$F_{1,332} = 37{\cdot}76$ $P < 0{\cdot}001$**	**$F_{1,334} = 13{\cdot}24$ $P < 0{\cdot}001$**	**$F_{1,336} = 4{\cdot}32$ $P = 0{\cdot}038$**	$F_{1,318} = 0{\cdot}21$ $P = 0{\cdot}650$	$F_{1,334} = 2{\cdot}74$ $P = 0{\cdot}099$
C^2	**$F_{1,326} = 6{\cdot}00$ $P = 0{\cdot}015$**	**$F_{1,325} = 2{\cdot}78$** $P = 0{\cdot}096$	**$F_{1,329} = 5{\cdot}98$ $P = 0{\cdot}015$**	$F_{1,314} = 1{\cdot}23$ $P = 0{\cdot}268$	$F_{1,321} = 1{\cdot}59$ $P = 0{\cdot}208$
P×C	$F_{1,326} = 3{\cdot}15$ $P = 0{\cdot}077$	$F_{1,325} = 0{\cdot}88$ $P = 0{\cdot}349$	$F_{1,329} = 1{\cdot}24$ $P = 0{\cdot}265$	$F_{1,316} = 1{\cdot}24$ $P = 0{\cdot}265$	$F_{1,322} = 1{\cdot}12$ $P = 0{\cdot}073$

Results from the linear mixed models examining the effects of protein (P) and carbohydrate (C) consumption on larval performance, haemolymph protein, lysozyme and PO activity. Significant terms are highlighted in bold type.

It is clear from the figures that PO activity and larval performance both peak at lower %P, than haemolymph protein, lysozyme and cuticular melanism suggesting that no dietary choice could maximize performance of all traits (cf. Fig. 2a,d and 2b,c,e). So what diet do larvae select if given a free choice, and is this choice affected by their health status?

EXPERIMENT 2: THE EFFECT OF AN IMMUNE SYSTEM CHALLENGE ON DIET CHOICE AND THE RESPONSE OF IMMUNE TRAITS AND LARVAL PERFORMANCE TO NUTRIENT COMPOSITION

Self-selecting treatment

The purpose of the challenge treatment was to stimulate an antibacterial response, and this was successful, as larvae that were challenged with lyophilised bacterial cells exhibited an up-regulation of lysozyme-like antibacterial activity relative to control larvae (control: −0·572 ± 0·225; challenged: 0·573 ± 0·225, $F_{1,49} = 65{\cdot}14$, $P < 0{\cdot}001$). However, there was no effect of challenge treatment on PO activity ($F_{1,48} = 0{\cdot}79$, $P = 0{\cdot}38$), haemolymph protein levels ($F_{1,48} = 0{\cdot}07$, $P = 0{\cdot}79$) or larval performance ($F_{1,40} = 0{\cdot}47$, $P = 0{\cdot}50$). Nutrient intake targets were calculated for control and challenged larvae using consumption data. The challenge treatment did not alter diet choice as there was no effect on the amount of protein ($F_{1,56} = 0{\cdot}206$, $P = 0{\cdot}65$) or carbohydrate ($F_{1,56} = 0{\cdot}005$, $P = 0{\cdot}94$) consumed. Therefore, a single intake target for both treatment groups was used with $P = 123{\cdot}4$ (± 4·62) mg and C = 79·1 (±2·85) mg, giving a percentage protein of 61% (±0·8%) (Fig. S3), which falls between the two previous estimates for this species of 65% P (Simpson, Simmonds & Blaney 1988a) and 55% P (Simpson *et al*. 2004).

Carbohydrate was more tightly regulated than protein; there was no effect of the diets offered on the amount of carbohydrate consumed in either treatment group ($F_{2,57} = 0{\cdot}21$, $P = 0{\cdot}81$), but there was an effect on the protein consumed ($F_{2,57} = 6{\cdot}09$, $P = 0{\cdot}004$). More protein was eaten when the protein-rich diet block was at its most concentrated compared to the amount consumed with the other two diets (amount of protein eaten on each diet choice: 1 = 0·23 g ± 0·01; 2 = 0·19 g ± 0·01; 3 = 0·20 g ± 0·01; Fig. S3).

Dietary restriction treatment

As before, we considered whether a single response surface could explain all of the variation in the traits in both naive and immune-challenged larvae. To test this, the standardized data were analysed including all measures of intake (P, C, P^2, C^2 and P×C), treatment (challenged or control), trait type (larval performance, haemolymph protein, lysozyme or PO

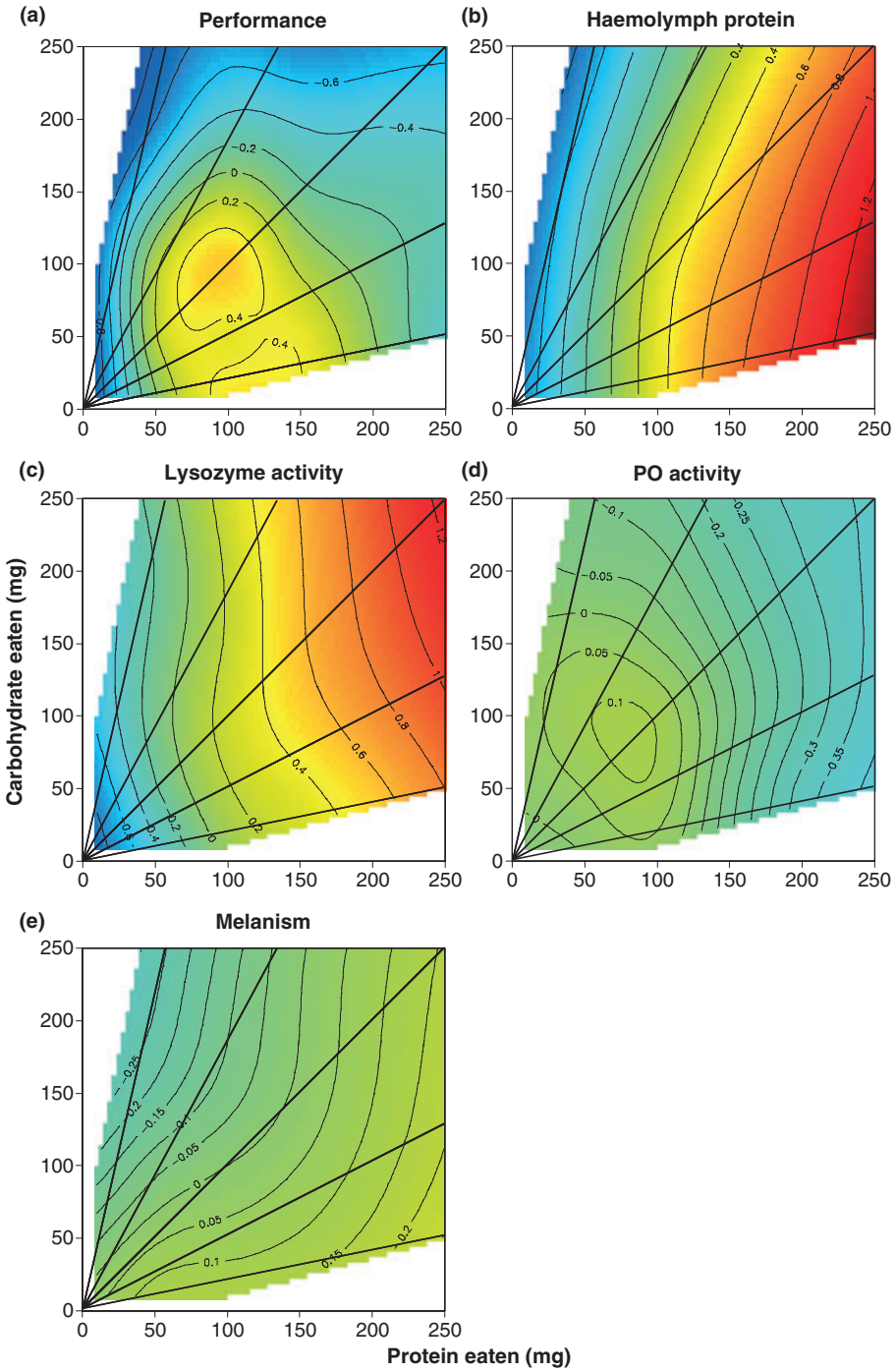

Fig. 2. Response surfaces showing the effects of protein (P) and carbohydrate (C) intake on the measured traits in experiment 1. (a) larval performance, (b) haemolymph protein levels, (c) lysozyme activity, (d) PO activity and (e) Cuticular melanism. Consumption was recorded for individual caterpillars confined to 1 of 20 diets varying in both the %P and the total amount of P and C. The solid lines indicate the %P rails that the larvae were restricted to (17, 34, 50 and 67 or 83%). The colour scale represents standard deviations from the mean with dark blue below the mean and dark red above the mean.

activity) and their interactions. A number of the interactions between trait type or treatment and the diet components were significant (Trait type×P×C: $F_{3,1110} = 5{\cdot}79$, $P < 0{\cdot}001$; Trait type×P^2×C^2: $F_{3,1110} = 3{\cdot}45$, $P = 0{\cdot}016$; Treatment × Trait type: $F_{3,1110} = 51{\cdot}17$, $P < 0{\cdot}001$), suggesting that the surfaces for each trait type were different. A partial F test, comparing a model including all of the interaction terms with one without, determined that the interactions between treatment, trait type and the diet components did explain significant variation in the data (Partial $F_{45} = 10{\cdot}15$, $P < 0{\cdot}001$), therefore, as before, individual landscapes were produced for each trait. When compared pairwise, all of the landscapes within each treatment, with the exception of lysozyme and PO activity in challenged larvae were significantly

different from each other (Table 3). There were no significant interactive effects of treatment with the diet variables for larval performance (Partial $F_5 = 0{\cdot}208$, $P = 0{\cdot}96$), haemolymph protein (Partial $F_5 = 0{\cdot}254$, $P = 0{\cdot}93$) or lysozyme ($F_8 = 1{\cdot}056$, $P = 0{\cdot}39$). However, the effects of treatment alone were highly significant for lysozyme activity, indicating that lysozyme activity was up-regulated in response to the bacterial challenge (Table 4). The predicted coefficients for each of the variables in the models are reported in Table S3.

The effects of P and C on larval performance in final instar larvae was very similar to the effects seen in 5th instar larvae in the previous experiment, with larval performance showing strong effects of P, C, P^2 and C^2 (Tables 4 and S3). As might be expected, larval performance was higher at a higher absolute intake of nutrients in the older larvae, and it appears that there are fewer costs of overconsumption (cf. Figs 2a and 3a). However, the squared terms indicate that there is an optimal level of both P and C for growth. Haemolymph protein levels responded differently in final instar larvae than in 5th instar larvae (cf. Figs 2b and 3b), in that there was an interactive effect of P and C intake. The response surface again shows haemolymph protein levels increasing with P but peaking at intermediate levels of C (Fig. 3b). Both larval performance and haemolymph protein levels peaked at slightly higher absolute intakes than the intake target.

Results for the immune traits were again different to the patterns found for larval performance and haemolymph protein. Lysozyme activity was not affected by carbohydrate consumption, but was significantly affected by both P and P^2

Table 3. Pairwise comparisons between response surfaces for experiment 2

	Larval performance	Protein	Lysozyme activity	PO activity
Larval performance	–	**$F_5 = 2{\cdot}97$** **$P = 0{\cdot}012$**	**$F_5 = 4{\cdot}85$** **$P < 0{\cdot}001$**	**$F_5 = 7{\cdot}47$** **$P < 0{\cdot}001$**
Protein	**$F_5 = 4{\cdot}05$** **$P = 0{\cdot}001$**	–	**$F_5 = 7{\cdot}88$** **$P < 0{\cdot}001$**	**$F_5 = 12{\cdot}14$** **$P < 0{\cdot}001$**
Lysozyme activity	**$F_5 = 3{\cdot}33$** **$P < 0{\cdot}001$**	**$F_5 = 10{\cdot}25$** **$P < 0{\cdot}001$**	–	**$F_5 = 2{\cdot}69$** **$P = 0{\cdot}02$**
PO activity	**$F_5 = 4{\cdot}51$** **$P < 0{\cdot}001$**	**$F_5 = 10{\cdot}21$** **$P < 0{\cdot}001$**	$F_5 = 1{\cdot}90$ $P = 0{\cdot}09$	–

Results of Partial *F*-tests comparing models with and without the interactions between each trait type (Performance, haemolymph protein, lysozyme and PO) and the diet variables for control landscapes (above the diagonal) and for challenged landscapes (below the diagonal). Surfaces that are significantly different are highlighted in bold type.

Table 4. The effects of protein and carbohydrate consumption on each trait – experiment 2

Fixed term	Larval performance	Protein	Lysozyme activity	PO activity
P	**$F_{1,367} = 56{\cdot}20$** **$P < 0{\cdot}001$**	**$F_{1,366} = 160{\cdot}0$** **$P < 0{\cdot}001$**	**$F_{1,366} = 52{\cdot}39$** **$P < 0{\cdot}001$**	**$F_{1,365} = 3{\cdot}39$** **$P = 0{\cdot}066$**
C	**$F_{1,370} = 74{\cdot}73$** **$P < 0{\cdot}001$**	**$F_{1,369} = 101{\cdot}9$** **$P < 0{\cdot}001$**	$F_{1,291} = 1{\cdot}17$ $P = 0{\cdot}280$	$F_{1,364} = 2{\cdot}75$ $P = 0{\cdot}098$
P^2	**$F_{1,370} = 19{\cdot}49$** **$P < 0{\cdot}001$**	**$F_{1,371} = 28{\cdot}25$** **$P < 0{\cdot}001$**	**$F_{1,366} = 13{\cdot}81$** **$P = 0{\cdot}003$**	**$F_{1,366} = 3{\cdot}60$** **$P = 0{\cdot}058$**
C^2	**$F_{1,368} = 48{\cdot}97$** **$P < 0{\cdot}001$**	**$F_{1,370} = 36{\cdot}28$** **$P < 0{\cdot}001$**	$F_{1,364} = 0{\cdot}59$ $P = 0{\cdot}444$	$F_{1,364} = 1{\cdot}97$ $P = 0{\cdot}162$
Treatment	$F_{1,363} = 1{\cdot}27$ $P = 0{\cdot}260$	$F_{1,362} = 0{\cdot}05$ $P = 0{\cdot}821$	**$F_{1,365} = 324{\cdot}0$** **$P < 0{\cdot}001$**	$F_{1,363} = 0{\cdot}88$ $P = 0{\cdot}350$
P×C	$F_{1,365} = 0{\cdot}06$ $P = 0{\cdot}809$	**$F_{1,369} = 30{\cdot}63$** **$P < 0{\cdot}001$**	$F_{1,359} = 0{\cdot}50$ $P = 0{\cdot}482$	$F_{1,362} = 2{\cdot}06$ $P = 0{\cdot}153$
P×Treatment	$F_{1,359} = 0{\cdot}18$ $P = 0{\cdot}668$	$F_{1,359} = 0{\cdot}02$ $P = 0{\cdot}883$	$F_{1,358} = 0{\cdot}34$ $P = 0{\cdot}559$	**$F_{1,362} = 4{\cdot}21$** **$P = 0{\cdot}041$**
C×Treatment	$F_{1,359} = 0{\cdot}11$ $P = 0{\cdot}742$	$F_{1,359} = 0{\cdot}01$ $P = 0{\cdot}923$	$F_{1,358} = 1{\cdot}17$ $P = 0{\cdot}280$	$F_{1,361} = 3{\cdot}42$ $P = 0{\cdot}065$
P^2×Treatment	$F_{1,360} = 0{\cdot}13$ $P = 0{\cdot}715$	$F_{1,360} = 0{\cdot}00$ $P = 0{\cdot}990$	$F_{1,358} = 0{\cdot}46$ $P = 0{\cdot}499$	**$F_{1,364} = 5{\cdot}58$** **$P = 0{\cdot}019$**
C^2×Treatment	$F_{1,360} = 0{\cdot}26$ $P = 0{\cdot}608$	$F_{1,360} = 0{\cdot}01$ $P = 0{\cdot}909$	$F_{1,358} = 1{\cdot}00$ $P = 0{\cdot}317$	$F_{1,359} = 1{\cdot}70$ $P = 0{\cdot}193$
P×C×Treatment	$F_{1,359} = 0{\cdot}30$ $P = 0{\cdot}582$	$F_{1,359} = 0{\cdot}78$ $P = 0{\cdot}378$	$F_{1,357} = 0{\cdot}64$ $P = 0{\cdot}423$	$F_{1,358} = 2{\cdot}38$ $P = 0{\cdot}124$

Results of the linear mixed models examining the effects of protein (P) and carbohydrate (C) consumption and challenge treatment on larval performance, haemolymph protein, lysozyme and PO activity. Significant terms are highlighted in bold.

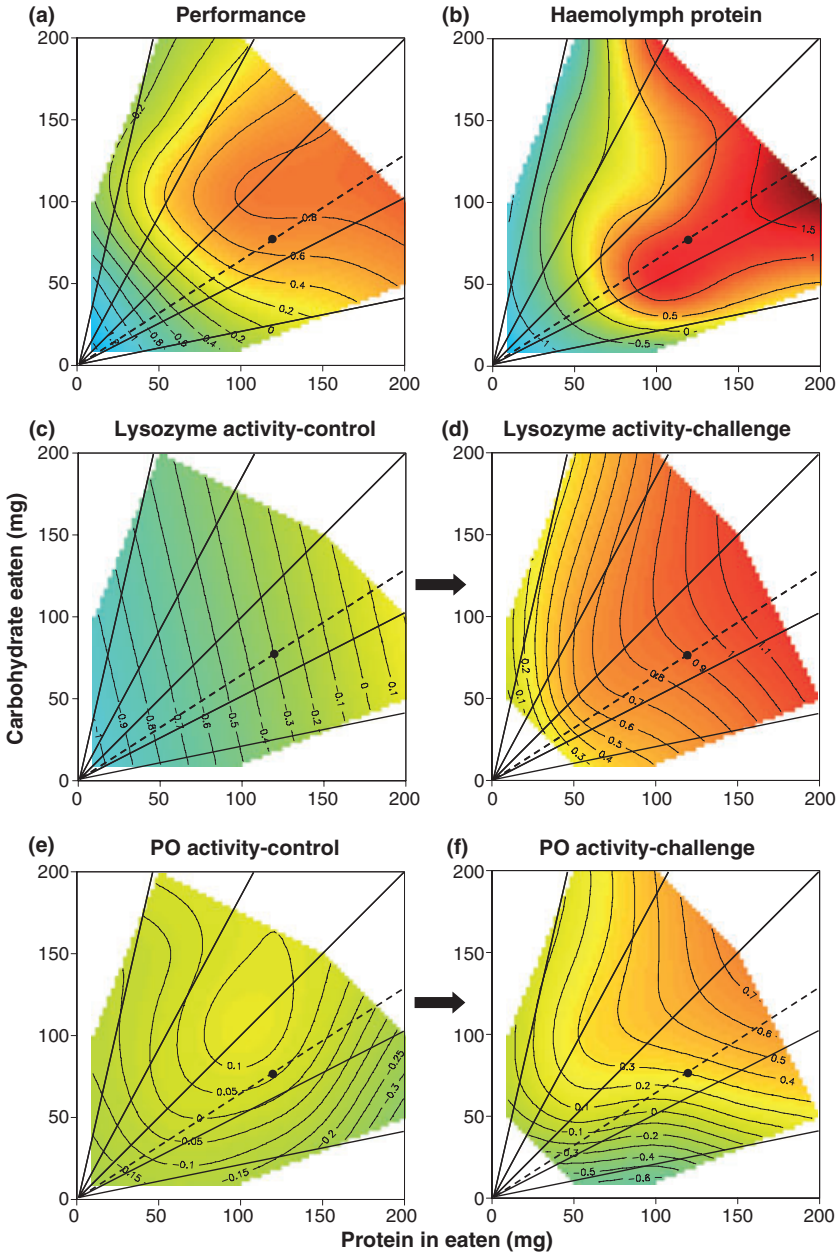

Fig. 3. Response surfaces showing the effects of protein (P) and carbohydrate (C) intake on the measured traits in experiment 2. (a) larval performance, (b) haemolymph protein levels, (c,d) lysozyme activity and (e,f) PO activity. Based on statistical analyses, for performance and haemolymph protein levels, a single landscape was fitted for control and challenged larvae. For lysozyme and PO activity, separate landscapes were fitted for control (c,e) and challenged (d,f) larvae (solid arrows link the two landscapes for each trait). Consumption was recorded for individual caterpillars confined to 1 of 20 diets varying in both the %P and the total amount of P and C. The dot indicates the intake target and the dashed line the %P selected by larvae in the choice treatment. The solid lines indicate the %P rails that the larvae were restricted to (17, 34, 50 and 67 or 83%). The colour scale represents standard deviations from the mean with dark blue below the mean and dark red above the mean.

(Table 4). The predicted surface was remarkably similar for both 5th and 6th instar larvae (cf. Figs 2c and 3c,d). Although the shapes of the response surfaces for control and challenged larvae were not significantly different, the absolute amounts of lysozyme activity did differ, as indicated by the highly significant treatment term ($P < 0{\cdot}001$, Table 4). Moreover, in pairwise comparisons, PO and lysozyme surfaces for control larvae were significantly different, but those for immune-challenged larvae were not; both response surfaces are plotted for comparison (cf. Fig. 3c–f). It can be seen from the surface for control larvae that lysozyme levels peaked at a higher protein intake (both amounts and ratio relative to carbohydrate) than were chosen by larvae in the choice experiment (Fig. 3c). However, when challenged, larvae did not modify their diet choice to increase lysozyme activity, nonetheless activity at the intake target increased from −0·3 units to +0·9 units (Fig. 3d), suggesting that larvae instead modified their internal allocation of the available nutrients.

In contrast, the PO activity response surfaces differed significantly between the treatment groups (Partial $F_5 = 2{\cdot}673$, $P = 0{\cdot}02$), as reflected in significant interactions between treatment and both the amount of protein consumed and the squared protein term (Table 4). It seems that PO activity in final instar larvae is more strongly influenced by diet than in 5th instar larvae, though in both cases the surfaces show peak activity in a more carbohydrate-rich region of nutrient space than for the other measured traits (cf. Figs 2 and 3e,f). The PO data were analysed for the control and challenged groups separately. In the control larvae, PO activity was strongly affected by both P ($F_{1,181} = 7{\cdot}35$; $P = 0{\cdot}007$) and P^2 ($F_{1,182} = 8{\cdot}97$, $P = 0{\cdot}003$), whilst the immune-challenged larvae showed significant effects for C ($F_{1,177} = 7{\cdot}64$, $P = 0{\cdot}006$) and C^2 only ($F_{1,177} = 5{\cdot}78$, $P = 0{\cdot}017$). Predictions from the response surfaces showed that the peak of PO activity shifted after immune-challenge from 101 mg P and 104 mg C, to 150 mg P and 150 mg C, though both peaks fell along the 50% protein rail (cf. Fig. 2e,f), which is in a more carbohydrate-rich region of nutrient space than the intake target (61% protein, 39% carbohydrate). However, it should also be noted that, similar to the effects found in 5th instar larvae, variation in PO activity with respect to nutrient intake was still quite low compared with the other traits, as indicated by the reduced colour range of the figures.

Discussion

In this study we used the geometric approach to nutrition analysis to address the effects of concurrent ingestion of two key macronutrients on larval performance and immune traits in naive and immune challenged insects. Specifically, we addressed three predictions:

TRAITS WILL MAP ONTO DIFFERENT REGIONS OF NUTRIENT SPACE, AS PREDICTED BY THE QUALITATIVE RESOURCE CONSTRAINTS HYPOTHESIS

Our results show that the relationship between larval performance, haemolymph protein levels, and immune traits is more complex than suggested by models which assume that a single dietary resource is limiting. Each of the measured traits showed different responses to nutrient intake, and all were differentially affected by the amount of protein and carbohydrate ingested. Thus, each trait was affected by the specific *blend* of nutrients ingested (qualitative resource constraints) rather than the amount available of any one predominant resource, such as energy (quantitative resource constraints). Previous studies have shown that calorie restriction, or the restriction of a specific nutrient, such as protein, can either decrease (Peck, Babcock & Alexander 1992; Siva-Jothy & Thompson 2002; Ayres & Schneider 2009) or increase (Ayres & Schneider 2009; Oarada *et al.* 2009) resistance to parasitism. These effects are not always linear, for example, a study examining the effects of host dietary carbohydrate on tapeworm infection in rats found that individual worms were heavier at an intermediate concentration of mannose, suggesting that parasites also have an optimal supply of nutrients (Keymer, Crompton & Singhvi 1983) also see (Smith 2007) for recent review of the effects of nutrient supply on pathogenic infection.

Smith & Holt (1996) presented a surface plot of the effects of protein and carbohydrate on mouse mortality after infection with *Salmonella*, but were restricted by having just eight treatment groups that did not cover a large area of nutrient intake space (see Fig. 3 in (Smith & Holt 1996), data taken from (Peck, Babcock & Alexander 1992)). Similarly, the two previous studies examining the effect of diet on *Spodoptera* immunity used five P : C ratios but only one concentration, giving a single slice across the nutrient landscape (Lee *et al.* 2006a; Povey *et al.* 2009). Here, we highlight the limitations of such as approach, we reanalysed the lysozyme data for each diet concentration separately to see how our conclusions might have differed had we not covered a large region of nutrient space. The relationship between the protein content of the diet and lysozyme activity differed markedly between each concentration (Fig. S4). We have shown that the measured immune traits vary across nutrient space, such that slices across the landscape could show immunity decreasing or increasing as the nutrient content of the diet changes (Fig. S4). It is only by describing the entire surface that we have been able to detect patterns and non-linearities that previous studies have missed.

PO AND LYSOZYME WILL MAP ONTO DIFFERENT REGIONS OF NUTRIENT SPACE, PROVIDING A BASIS FOR THE OBSERVED TRADE-OFF BETWEEN THESE TWO TRAITS

Whilst cuticular melanism, haemolymph protein and lysozyme levels were most affected by dietary protein, peaking in regions of high protein intake, larval performance peaked at intermediate protein levels and was also strongly affected by carbohydrate intake. In contrast, the third immune trait, PO activity, was relatively unaffected by dietary composition, though the highest activity did occur at a more carbohydrate-biased intake than the other traits. This confirmed our second prediction that PO and lysozyme would map onto different regions of nutrient space, providing a basis for the observed putative trade-off in this and other insect species (Moret & Schmid-Hempel 2001; Cotter, Kruuk & Wilson 2004). This suggests that the nutrient requirements of PO and lysozyme are non-complementary, such that the trade-off is determined at the point of ingestion.

IMMUNE-CHALLENGED INSECTS WILL SHIFT THEIR DIET-CHOICE TO ONE THAT MAXIMIZES AN APPROPRIATE IMMUNE RESPONSE, OR THEY WILL MODIFY THEIR INTERNAL ALLOCATION OF NUTRIENTS SUCH THAT RESPONSE SURFACES FOR NAIVE AND CHALLENGED INSECTS DIFFER

In contrast to some other studies using *Spodoptera* larvae (e.g. Lee *et al.* 2006a; Povey *et al.* 2009), we found that naive

and immune-challenged insects chose a similar diet but the response surfaces for the immune traits differed between the groups. In fact, in the challenged group, lysozyme activity peaked at the same intake ratio (*c*. 60%) as the intake target. This suggests that rather than modifying their acquisition of nutrients, larvae modified their allocation of the available nutrients to the immune traits. The intake targets for the two groups were very similar but both groups also ate more protein when given access to the most concentrated, protein-rich food block. The tight regulation of nutrient intake can fail when insects are faced with an exceptionally rich food source (see Lee *et al*. 2002). In this case it was protein, however, it is worth noting that the diet options presented allowed an overconsumption of protein but not carbohydrate. Had we given larvae the choice of an extremely carbohydrate-biased diet we may have seen a similar failure of regulation of carbohydrate intake.

The fact that no single blend of ingested nutrients can optimally satisfy all of the measured traits suggests that the composition of the diet ingested by the caterpillars represents a trade-off between optimizing different traits. Whilst lysozyme, in particular, performs well on a high protein diet, larval performance does not.The important point for this trade-off is that larval performance would be compromised in the *absence* of infection on a high protein diet. Therefore, individuals that choose a high protein diet when uninfected to maintain certain immune responses at high levels would grow more slowly than individuals that choose a lower protein diet. Therefore, there must be some compromise between the competing needs of different traits in an individual's diet choice. Which pattern of compromises does diet selection by uninfected caterpillars support? When given a wide range of complementary food choices, we found that larvae selected a 61 : 39 protein : carbohydrate ratio, close to the intake targets measured in previous studies with this species (Simpson, Simmonds & Blaney, 1988b, Simpson *et al*. 2004). Whilst it appears that it is not possible for a larva to choose a diet that maximizes all responses, were one to obtain a diet close in composition to that which it would select (and regulate to) under free-choice conditions, then it would perform well on all measures. The intake target P : C ratio aligns relatively closely with the peak in all measures, albeit PO activity peaks at a lower P : C ratio than other measures. The intake target therefore represents a compromise point whereby all traits perform well. However, were the insect to be constrained by its nutritional environment to diets differing from the intake target ratio of protein to carbohydrate, then this relatively close coupling of traits would fall apart. In line with our prediction, PO and lysozyme activity peak in different regions of nutrient space, hence, an increase in dietary protein from the intake target would favour lysozyme levels but see a decline in PO activity, whereas a decrease in P : C would produce the opposite effect. This result adds an important new aspect to the evidence for a trade-off within the immune system between PO activity and antibacterial activity reported from this and a number of other insect species, as differing dietary requirements for each trait would preclude the possibility of simultaneously maximizing both. However, this suggests that rather than traits coexisting in a form of intra-organismal 'niche partitioning', they do in fact compete for ingested resources, leading to trade-offs.

An extension of this logic is that the composition of the optimal diet should change if the relative contribution of different immune traits to overall fitness shifts in response to parasitic infection. Lysozymes are constitutively expressed in most insects but are also up-regulated upon recognition of microbial cell wall components (Briese 1981), helping to kill microbial pathogens. Hence, bacterial infection might be expected to promote the role and benefit of lysozymes, and the importance of sustaining high levels of protein in the haemolymph, shifting the optimal diet to one with higher protein content. In fact, we found that simulating a bacterial infection and thus causing larvae to up-regulate lysozyme activity had no effect on their diet selection. The diet choice of both challenged and non-challenged larvae was 61 : 39 protein : carbohydrate. This is in contrast to two previous studies using *Spodoptera* caterpillars infected with live pathogens, where in both cases infected individuals chose a diet richer in protein than their healthy counterparts (Lee *et al*. 2006a; Povey *et al*. 2009). The lack of a diet-choice shift in challenged larvae in our case may be because whilst the antibacterial response requires protein, it is not as draining of protein reserves as a real infection, and a simple reallocation of resources may be adequate to up-regulate an antibacterial response. With a live pathogen, for example, with a baculovirus infection, larvae will slough off infected midgut cells and may replace them with immune cells (Keddie, Aponte & Volkman 1989), and in a bacterial infection, replicating bacteria will use protein resources, in addition to the extra protein required to produce antibacterial peptides and blood cells to phagocytose and nodulate bacterial cells (Tanada & Kaya 1993). We may have observed a dietary shift in challenged larvae had we stimulated the immune system more than once, or with a larger challenge, causing larvae to maintain a heightened response for longer and depleting protein reserves further. It is interesting to note that it was the antibacterial response that peaked at the intake target in challenged insects, and that we chose to use a bacterium as the challenging agent. It is possible that if we had chosen to challenge the larvae with a macroparasite requiring encapsulation and melanisation that we might have either seen a shift in diet choice to a more carbohydrate-rich region of nutrient space, or PO activity may have increased at the intake target.

Of all the traits we measured, PO alone peaked in a carbohydrate rich region of nutrient space. The PO response may require more sugars than an antibacterial response as it requires blood cells to burst open to release PO into the haemolymph (Ashida & Brey 1997), the subsequent haematopoiesis would require sugars as well as proteins, both for cell composition and for energy. A previous study with *Anopheles stephensi* found that the melanisation of sephedex beads increased with the sugar concentration of food after a blood meal, adding further weight to the finding that the

PO response requires carbohydrates (Koella & Sorensen 2002).

A recent study using *S. littoralis* compared melanism, PO and lysozyme activity levels in larvae that were provided with diets that differed in their protein quality (based on amino acid composition), and found that whilst melanism and lysozyme activity levels were affected by protein quality, PO activity levels were not (Lee, Simpson & Wilson 2008). The current study also finds that PO activity is less variable with respect to diet than lysozyme activity. This could be due to the different physiological functions performed by PO. In addition to their role in the immune system, phenoloxidases are also involved in cuticular melanization after moulting (Hiruma & Riddiford 1988) and so may be maintained in favour of other functions when protein levels are limiting. This mechanistic relationship between PO and melanism is reflected in their similar performance landscapes and relative stability with respect to dietary intake when compared to the amount of variation seen in the other traits. In addition, excess levels of PO could be dangerous; uncontrolled activation of PO in the haemocoel would result in the production of toxic quinones and dangerous reactive oxygen species which could harm self-tissue (Nappi & Vass 1993). As such, it may be necessary for the insect to maintain PO at a moderate level rather than allow it to fluctuate in response to dietary variation. It would be interesting to discover whether infection by a macroparasite that promotes an encapsulation response alters the nutritional landscape in relation to PO.

In conclusion, we have demonstrated that the response of individual life-history and immune traits to variation in diet composition cannot be explained by a single currency 'quantitative constraints' model. Rather, it is the blend of nutrients that determines performance and the optimal blend is different for each trait. The dietary composition chosen by this species appears to represent a compromise that allows each of the traits to perform well simultaneously, albeit none maximally. Accordingly, future studies should consider the quality rather than just the quantity of resources available when considering allocation to life-history and functional traits.

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References

Ashida, M. & Brey, P.T.(1997) Recent advances in research on the insect prophenoloxidase cascade. *Molecular Mechanisms of Immune Responses in Insects* (eds D. Hultmark & P.T. Brey), pp. 135–172. Chapman and Hall, London.

Ayres, J.S. & Schneider, D.S. (2009) The role of anorexia in resistance and tolerance to infections in *Drosophila*. *PLoS Biology*, **7**, e1000150.

Blows, M.W. & Brooks, R. (2003) Measuring nonlinear selection. *American Naturalist*, **162**, 815–820.

Briese, D.T. (1981) Resistance of insect species to microbial pathogens. *Pathogenesis of Invertebrate Microbial Diseases* (ed. E.W. Davison), pp. 511–545. Allanheld, Osmun Publishers, Totowa, NJ.

Carey, J.R., Harshman, L.G., Liedo, P., Muller, H.G., Wang, J.L. & Zhang, Z. (2008) Longevity-fertility trade-offs in the tephritid fruit fly, *Anastrepha ludens*, across dietary-restriction gradients. *Aging Cell*, **7**, 470–477.

Chenoweth, S.F. & Blows, M.W. (2005) Contrasting mutual sexual selection on homologous signal traits in *Drosophila serrata*. *American Naturalist*, **165**, 281–289.

Cotter, S.C., Beveridge, M. & Simmons, L.W. (2008) Male morph predicts investment in larval immune function in the dung beetle, *Onthophagus taurus*. *Behavioral Ecology*, **19**, 331–337.

Cotter, S.C., Kruuk, L.E.B. & Wilson, K. (2004) Costs of resistance: genetic correlations and potential trade-offs in an insect immune system. *Journal of Evolutionary Biology*, **17**, 421–429.

Cotter, S.C., Myatt, J.P., Benskin, C.M.H. & Wilson, K. (2008) Selection for cuticular melanism reveals immune function and life-history trade-offs in *Spodoptera littoralis*. *Journal of Evolutionary Biology*, **21**, 1744–1754.

Fricke, C., Bretman, A. & Chapman, T. (2008) Adult male nutrition and reproductive success in *Drosophila melanogaster*. *Evolution*, **62**, 3170–3177.

Grant, P.R. & Grant, B.R. (2002) Unpredictable evolution in a 30-year study of Darwin's finches. *Science*, **296**, 707–711.

Gupta, A.P. (ed) (1991) *Immunology of Insects and Other Arthropods*. CRC Press, Boca Raton, FL.

Hiruma, K. & Riddiford, L.M. (1988) Granular phenoloxidase involved in cuticular melanisation in the tobacco hornworm: regulation of its synthesis in the epidermis by juvenile hormone. *Developmental Biology*, **130**, 87–97.

Joern, A. & Behmer, S.T. (1997) Importance of dietary nitrogen and carbohydrates to survival, growth, and reproduction in adults of the grasshopper *Ageneotettix deorum* (Orthoptera: Acrididae). *Oecologia*, **112**, 201–208.

Keddie, B.A., Aponte, G.W. & Volkman, L.E. (1989) The pathway of infection of *Autographa californica* Nucleopolyhedrovirus in an insect host. *Science*, **243**, 1728–1730.

Keymer, A., Crompton, D.W.T. & Singhvi, A. (1983) Mannose and the crowding effect of hymenolepis in rats. *International Journal for Parasitology*, **13**, 561–570.

Koella, J.C. & Sorensen, F.L. (2002) Effect of adult nutrition on the melanization immune response of the malaria vector *Anopheles stephensi*. *Medical and Veterinary Entomology*, **16**, 316–320.

Kristan, D.M. (2007) Chronic calorie restriction increases susceptibility of laboratory mice (*Mus musculus*) to a primary intestinal parasite infection. *Aging Cell*, **6**, 817–825.

Lande, R. & Arnold, S.J. (1983) The measurement of selection on correlated characters. *Evolution*, **37**, 1210–1226.

Lee, K.P., Raubenheimer, D. & Simpson, S.J. (2004) The effects of nutritional imbalance on compensatory feeding for cellulose-mediated dietary dilution in a generalist caterpillar. *Physiological Entomology*, **29**, 108–117.

Lee, K.P., Simpson, S.J. & Wilson, K. (2008) Dietary protein-quality influences melanization and immune function in an insect. *Functional Ecology*, **22**, 1052–1061.

Lee, K.P. & Wilson, K. (2006b) Melanism in a larval Lepidoptera: repeatability and heritability of a dynamic trait. *Ecological Entomology*, **31**, 196–205.

Lee, K.P., Behmer, S.T., Simpson, S.J. & Raubenheimer, D. (2002) A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). *Journal of Insect Physiology*, **48**, 655–665.

Lee, K.P., Cory, J.S., Wilson, K., Raubenheimer, D. & Simpson, S.J. (2006a) Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. *Proceedings of the Royal Society B-Biological Sciences*, **273**, 823–829.

Merrill, A.L. & Watt, B.K. (1973) Energy value of foods: basis and derivation. USDA agricultural report no. 74, pp. 105.

Moret, Y. & Schmid-Hempel, P. (2000) Survival for immunity: the price of immune system activation for bumblebee workers. *Science*, **290**, 1166–1168.

Moret, Y. & Schmid-Hempel, P. (2001) Entomology – Immune defence in bumble-bee offspring. *Nature*, **414**, 506–506.

Murray, M.J. & Murray, A.B. (1979) Anorexia of infection as a mechanism of host defense. *American Journal of Clinical Nutrition*, **32**, 593–596.

Nappi, A.J. & Vass, E. (1993) Melanogenesis and the generation of cytotoxic molecules during insect cellular immune reactions. *Pigment Cell Research*, **6**, 117–126.

Oarada, M., Kamei, K., Gonoi, T., Tsuzuki, T., Toyotome, T., Hirasaka, K., Nikawa, T., Sato, A. & Kurita, N. (2009) Beneficial effects of a low-protein

diet on host resistance to *Paracoccidioides brasiliensis* in mice. *Nutrition*, **25**, 954–963.
Peck, M.D., Babcock, G.F. & Alexander, J.W. (1992) The role of protein and calorie restriction in outcome from *Salmonella* infection in mice. *Journal of Parenteral and Enteral Nutrition*, **16**, 561–565.
Povey, S.R., Cotter, S.C., Simpson, S.J., Lee, K. & Wilson, K. (2009) Can the protein costs of bacterial resistance be offset by altered feeding behaviour? *Journal of Animal Ecology*, **78**, 437–446.
Punzalan, D., Cooray, M., Rodd, F.H. & Rowe, L. (2008) Condition dependence of sexually dimorphic colouration and longevity in the ambush bug *Phymata americana*. *Journal of Evolutionary Biology*, **21**, 1297–1306.
Simpson, S.J. & Raubenheimer, D. (1995) The geometric analysis of feeding and nutrition – a users' guide. *Journal of Insect Physiology*, **41**, 545–553.
Simpson, S.J., Simmonds, M.S.J. & Blaney, W.M. (1988a) A comparison of dietary selection behavior in larval *Locusta migratoria* and *Spodoptera littoralis*. *Physiological Entomology*, **13**, 225–238.
Simpson, S.J., Sibly, R.M., Lee, K.P., Behmer, S.T. & Raubenheimer, D. (2004) Optimal foraging when regulating intake of multiple nutrients. *Animal Behaviour*, **68**, 1299–1311.
Siva-Jothy, M.T. & Thompson, J.J.W. (2002) Short-term nutrient deprivation affects immune function. *Physiological Entomology*, **27**, 206–212.
Smith, V. (2007) Host resource supplies influence the dynamics and outcome of infectious disease. *Integrative and Comparative Biology*, **47**, 310–316.
Smith, V.H. & Holt, R.D. (1996) Resource competition and within-host disease dynamics. *Trends in Ecology & Evolution*, **11**, 386–389.
Stearns, S.C. (1992) *The Evolution of Life Histories*. Oxford University Press, Oxford.
Sterner, R.W. & Elser, J.J.(2002) *Ecological Stoichiometry: The Biology of Elements from Molecules to the Biosphere*. Princeton University Press, Princeton.
Sugumaran, M. & Kanost, M.R. (1993) Regulation of insect hemolymph phenoloxidases. *Parasites and Pathogens of Insects* (eds N.E. Beckage, S.N. Thompon & B.A. Federici), pp. 317–342. Academic Press Limited, London.
Tanada, Y. & Kaya, H.(1993) *Insect Pathology*. Academic Press Limited, London.
Wilson, K., Cotter, S.C., Reeson, A.F. & Pell, J.K. (2001) Melanism and disease resistance in insects. *Ecology Letters*, **4**, 637–649.
Zera, A.J. & Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics*, **32**, 95–126.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1. The protein and carbohydrate composition of each diet.

Table S2. Estimated coefficients from the parametric response surfaces – experiment 1.

Table S3. Estimated coefficients from the parametric response surfaces – experiment 2.

Fig. S1. Spodoptera littoralis: Left – a male moth, right – a final instar larva.

Fig. S2. Variation in consumption on the 20 diets differing in their protein and carbohydrate composition.

Fig. S3. The total amount of protein (P) and carbohydrate (C) consumed by larvae in the self-selecting diet treatment.

Fig. S4. The effect of protein, as a percentage of the total digestible nutrients in the diet, on lysozyme activity.

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