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The transition from diet to blood: Exploring homeostasis in the insect haemolymph nutrient pool

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Abstract

Nutrition is vital to health, but while the link between diet and body nutritional composition is well explored in humans and other vertebrates, this information is not well understood in insects, despite the vital roles they play in ecosystems, and their increasing use as experimental models. Here we used Nutritional Geometry to explore the rapid physiological response to ingested nutrients in the haemolymph nutritional profile of *Spodoptera littoralis* caterpillars. We ask whether blood nutrients are maintained homeostatically in the face of variable nutritional intake, or if regulation is more flexible for some nutrients than others (allostasis), which allows animals to adapt to stress by responding in a way that prioritises efficiency of responses in the face of trade-offs. Caterpillars were placed on 1 of 20 diets, systematically varying in their nutrient ratios (protein: carbohydrate) and density (calorie content), and their consumption was measured. After 48 h, caterpillars were bled, and the macronutrient (protein, carbohydrates and lipids) and nutrient metabolite (amino acids and simple sugars) content of the haemolymph was measured. Proteins comprised 93% of the haemolymph macronutrient pool on average and their concentration increased with protein eaten. The amino acid (AA) pool was dominated by five AAs, and the total pool increased with total nutrient intake. However, the ratio of essential to non-essential AAs increased as the proportion of protein consumed increased. Carbohydrates were tightly controlled, increasing only on the most extreme carbohydrate intakes. Simple sugars were dominated by glucose and trehalose, and overall, the simple sugar pool showed high levels of homeostasis. Rather than strict homeostasis of blood nutritional properties, an allostatic model seemed to be a better fit for blood nutrient regulation in this generalist herbivore. This flexibility in response to the nutritional composition of the diet may, in part, explain how

this species has evolved to extreme dietary generalism and may play a role in its worldwide pest status. Given the range of fitness-related processes affected by the haemolymph, future studies should examine the physiological impacts of blood nutrient variation on reproduction, growth and response to infection and the trade-offs between them.

KEYWORDS
allostasis, haemolymph nutrients, homeostasis, nutritional ecology, nutritional geometry, *Spodoptera*

INTRODUCTION

The food we consume can have direct and indirect effects on health; nutrients are vital for optimal growth, reproduction and somatic maintenance, and over- and under-consumption can result in negative fitness outcomes (Calder & Jackson, 2000; Tonk-Rügen et al., 2022). However, food is multidimensional, containing a complex mix of macro- and micro-nutrients, secondary chemicals, including potential toxins, indigestible components and water. Animals must navigate this complex mix of elements, in some cases across multiple food sources, to comprise their preferred nutrient intake (Simpson et al., 2004; Simpson & Raubenheimer, 1995). Studies across taxa have shown that animals are capable of integrating multiple signals to achieve their preferred 'intake target', that is, the nutritional composition that maximises fitness (Simpson et al., 2004; Simpson & Raubenheimer, 1995). In insects, the haemolymph is central to this regulation, as it provides a current indication of the animal's nutritional state, with digested nutrients from recent meals and catabolic activity entering the blood, and those being used by somatic maintenance, reproduction, immunity and storage being moved out (Chapman, Douglas, et al., 2013; Simpson & Raubenheimer, 1993, 2012). Understanding the factors that can impact insect health, such as diet, is becoming increasingly important, for both ecosystem health (e.g., pollinator loss) (Garibaldi et al., 2022) and for a better utilisation of insect models of disease (Verheyen, 2022). Despite this, we have a poor understanding of how insect diet translates into nutrient stores in the insect body, which are required for multiple fitness-related process.

In insects, nutrients acquired from the diet may be stored in the fat body as proteins, lipid or glycogen, or in the haemolymph as proteins, carbohydrates, phospholipids, sugars or amino acids (Chapman, Douglas, et al., 2013; Haunerland, 1996). Metabolised nutrients from the gut, and synthesised nutrients from the fat body, are transported into the haemolymph for use by other tissues (Chapman, Simpson, & Douglas, 2013; Simpson & Raubenheimer, 1993). Most arthropod studies measure nutritional status via the haemolymph since it can be a non-destructive sampling method, and the nutritional status of the haemolymph is directly influenced by environmental changes (Simpson & Raubenheimer, 1993). The haemolymph comprises 20%–25% of an organism's water content, rising to about 50% in caterpillars (Chapman, Simpson, & Douglas, 2013). In addition to its role in storing and disseminating nutrients, the haemolymph serves important functions in physical structure, acts as a reservoir for cell hydration, provides oxygen for metabolism and regulates pH and osmolality (Chapman, Simpson, & Douglas, 2013). The open circulatory system of the insect haemocoel necessitates the bathing of the internal organs in haemolymph. The nutritional content of the haemolymph must therefore be kept at an optimal level to provide immediate access for metabolic processes (Thompson, 2003). Defence against parasites can be dependent on haemolymph nutrients. Immune effectors depend on haemolymph transportation and can utilise immediate nutrient stores (Chapman, Douglas, et al., 2013; Haunerland, 1996). However, parasites can be also be directly affected by haemolymph nutritional properties. For example, we have shown that carbohydrate in the diet has little effect on the outcome of infection with a blood-borne bacterium and that dietary protein increases the osmolality of the blood, which is detrimental to bacterial growth in vitro and in vivo (Wilson et al., 2020). As a result, organisms must balance nutrient concentration with the chemical effects of those nutrients on haemolymph fluid dynamics such as volume, osmolality and temperature (reviewed by Beyenbach, 2016). This results in a complex haemolymph nutritional profile containing certain nutrients that are regulated and others that fluctuate freely with dietary intake (Zanotto et al., 1996).

Larvae of the lepidopterans, *Heliothis zea* (Friedman et al., 1991) and *Manduca sexta* (Thompson, 1998), have been shown to allow their blood sugar levels to fluctuate with dietary composition. Previous studies have shown that locusts fed a balanced protein (P): carbohydrate (C) (1:1) diet showed increased blood osmolality and increased concentration of 11 out of the 16 most prominent amino acids compared to those on a low P:C (1:2) diet (Abisgold & Simpson, 1987). A second study compared both blood amino acids and sugars (glucose and trehalose) on a high P:C (3:1) versus a low P:C (1:3) diet (Zanotto et al., 1996). In this case, amino acids were elevated on the high protein diet and glucose and trehalose elevated on the high carbohydrate diet. Similarly, *Drosophila*, which is used as models for hyperglycaemia and type 2 diabetes, shows increased glucose and trehalose on high sugar diets (Meshrif et al., 2022; Morris et al., 2012). It therefore appears that many insects do not tightly regulate their haemolymph nutritional profile in the face of environmental fluctuation (homeostasis). An alternative model of regulation is allostasis, which assumes that the goal of regulation is to maximise fitness and reduce costs, thus allowing parameters to fluctuate under variable conditions, such that trade-offs between functions are minimised (Sterling, 1988).

Despite the fundamental role that nutrients play in the blood, previous studies examining blood composition in response to diet have been done on only two to four diets (Abisgold & Simpson, 1987;

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Friedman et al., 1991; Thompson, 1998; Zanotto et al., 1996). In each case, the diets varied in their P:C ratio, but P and C did not vary independently, such that the effects of each could not be disentangled (Simpson & Raubenheimer, 1995), and in each case, a limited number of blood nutrients was assessed. We do not know, therefore, how the full range of blood nutrients responds to diet composition, and whether dietary protein or carbohydrate act independently or interactively. Understanding how insect physiology responds across a wide range of diets is especially important for extreme generalists who can feed on diets that vary widely in their nutritional composition. Here we attempt to address this gap using the nutritional geometry (NG) framework (Simpson & Raubenheimer, 1995) by rearing caterpillars on 20 diets that vary in their nutrient composition (P:C) and nutrient density, such that protein and carbohydrate levels vary independently across nutrient space. We then take blood samples and analyse the nutrient composition of the blood in terms of macronutrients (proteins, carbohydrates and lipids) and nutrient metabolites (simple sugars and amino acids).

Our model organism is the generalist leaf feeding caterpillar, *Spodoptera littoralis* (Hill, 1987). Several previous studies from our group and others have categorised the preferred dietary composition of this species in terms of P:C and shown that healthy caterpillars perform best on a diet that is approximately 60:40 protein to carbohydrate (61%: Cotter et al., 2011; 65% P: Simpson et al., 1988; 55%: Simpson et al., 2004). However, the species is flexible, with natural dietary composition across known host plants spanning a wide range (Scott Brown et al., 2002; Wilson et al., 2019). So how might blood nutrients respond to short-term fluctuations in the protein and carbohydrate content of the diet? There could be no relationship between nutritional intake and blood nutrients, with the latter being maintained under conditions of complete homeostasis. At the other extreme, blood nutrients could map exactly to diet composition, such that increased carbohydrate consumption increases blood carbohydrates and simple sugars, and increased protein consumption increases blood protein and amino acids. Alternately, nutrients in the blood might fit an allostatic model, which allows fluctuation in the face of environmental perturbation, allowing animals to adapt to stress by responding in a way that prioritises efficiency of responses in the face of trade-offs (Sterling, 1988). We predict that this dietary generalist will have a high tolerance for dietary imbalance, with the ability to maintain blood nutrients across large regions of nutrient intake, particularly blood sugars. We know that dietary carbohydrates have little effect on the outcome of infection in this species and this may be reflected in control of blood sugars (Wilson et al., 2020). Blood proteins may be less well regulated, given our knowledge of their effect on blood osmolality (Wilson et al., 2020). We therefore expect regions of relative homeostasis across nutrient space versus little homeostasis on more extreme diets, with homeostasis being the norm for carbohydrates and simple sugars, but greater fluctuation with diet for proteins and amino acids, reflecting a more allostatic model for blood nutrients as a whole.

METHODS

Spodoptera caterpillars

Insect culture

The *S. littoralis* culture was founded in 2002 from eggs collected near Alexandria in Egypt. It was maintained using single pair matings of over 150 pairs per generation. For experiments, larvae were collected in the 2nd instar and reared singly on a semi-artificial wheatgerm-based diet until the start of the final instar (6th). Larvae were kept in 25-mL polypots at 27°C under a LD 12:12 h regime. The final instar sees the largest relative growth rate, the larger caterpillars are easier to handle and collect large blood samples from, allowing for the simultaneous measurement of a range of physiological traits from individual insects, rather than having to pool samples from multiple smaller insects. This also allows for comparisons with previous studies in our own system and others (Cotter et al., 2011; Cotter et al., 2019; Doğan et al., 2021; Thompson, 1998, 2003; Wilson et al., 2020).

Chemically defined diets and haemolymph collection

Within 24 h of moulting to the 6th instar, 200 larvae were transferred to Petri dishes (90 mm diameter), containing 1.5 g of a chemically defined diet (Dadd, 1961a, 1961b; Simpson & Abisgold, 1985). A total of 20 diets were used, which varied in their nutrient density (concentration of digestible nutrients ranged from 17% to 63%) and their protein to carbohydrate ratio (1:5–5:1) (Table 1) (see Cotter et al., 2011; Lee, Raubenheimer, & Simpson, 2004), resulting in a sample size of 10 larvae per diet. This range of diets reflects the broad range of protein to carbohydrate ratios and concentrations present in host plants (Scott Brown et al., 2002; Wilson et al., 2019). Diet blocks were replaced daily, with partially eaten blocks dried and weighed to record consumption. After 48 h, the larvae were weighed, and haemolymph was collected using a sterile hypodermic Microlance™ 3 needle into a sterile ice-cooled 1.5 mL Eppendorf tube, which was then stored at −20°C. Total consumption was the dry weight of the total amount of diet consumed, which included the non-nutritive components. Protein and carbohydrate consumption were calculated from the total consumption based on the proportional protein and carbohydrate content of the diet eaten.

Macronutrients

Protein analysis

Proteins and polypeptides with MW from 3000 to 5000 were assessed using a method previously described for *S. littoralis* haemolymph by Cotter et al. (2011). Briefly, 4 µL haemolymph samples were diluted in 184 µL sodium cacodylate buffer (pH 7.4), mixed under vortex and incubated for 1 h at 25°C. A 5 µL sample was dispensed into

TABLE 1 Twenty diets fed to *Spodoptera littoralis* caterpillars varying in their ratios and concentrations of protein and carbohydrate.

Diet	P:C ratio	Diet concentration (g/100 g)	% protein	Protein (g/100 g)	Carbohydrates (g/100 g)
1	1:5	63	17	10.5	52.5
2	1:5	42	17	7	35
3	1:5	33.6	17	5.6	28
4	1:5	16.8	17	2.8	14
5	1:2	63	33	21	42
6	1:2	42	33	14	28
7	1:2	33.6	33	11.2	22.4
8	1:2	16.8	33	5.6	11.2
9	1:1	63	50	31.5	31.5
10	1:1	42	50	21	21
11	1:1	33.6	50	16.8	16.8
12	1:1	16.8	50	8.4	8.4
13	2:1	63	67	42	21
14	2:1	42	67	28	14
15	2:1	33.6	67	22.4	11.2
16	2:1	16.8	67	11.2	5.6
17	5:1	63	83	52.5	10.5
18	5:1	42	83	35	7
19	5:1	33.6	83	28	5.6
20	5:1	16.8	83	14	2.8

Note: For full details of the diets used, see tab. S1 in Cotter et al. (2019).

a sterile 96-well plate in duplicate with 200 µL Bio-Rad protein reagent. Optical density was measured in a SpectraMax Plus microtiter plate reader (Molecular Devices) at a 360 nm wavelength, and the levels of protein were determined from a standard curve of bovine serum albumin (0–1 mg/mL increasing in steps of 0.1) using SoftMax Pro software.

Carbohydrate and lipids

Total levels of carbohydrates were determined using an anthrone test adapted from Kaufmann and Brown (2008). The sugar portion of the haemolymph was first separated from the lipid components. A 5 µL sample of haemolymph was dispensed into a sterile 5 mL glass sample tube containing 40 µL sodium sulphate solution and mixed under vortex. The salt preparation was diluted in 560 µL of 2:1 chloroform: methanol (Folch et al., 1957) and centrifuged at 13,000 rpm for 3 min to remove cell debris. The two layers were separated by the addition of 400 µL sterile distilled H_2O and mixing under vortex. The top layer containing the sugar–water–methanol component was isolated for carbohydrate analysis by pipetting to a separate glass tube. The bottom layer containing the lipid–chloroform complex was retained for further analysis (see below). Methanol was removed by heating the glass tubes in a heating block at 100°C until all liquid had evaporated, after which 1 mL of anthrone reagent was added. The anthrone–sugar solutions were heated at 100°C for 17 min, cooled and the optical density determined at 625 nm in a SpectraMax Plus microtiter plate reader in duplicate. The concentration of sugar was determined using the SoftMax Pro software using a standard series of glucose (0, 5, 10, 20, 30, 40 µL of 100 mg/100 mL glucose in sterile distilled water).

After separation from sugars, lipid samples were placed in a fume cupboard to evaporate the chloroform. Samples were then transferred to a heating block at 100°C with 40 µL sulphuric acid (95%) for 10 min. A total of 1 mL volume of vanillin reagent was added to each tube and mixed under vortex. The tubes were allowed to cool and the optical density determined at 625 nm with a SpectraMax Plus microtiter plate reader in duplicate. The lipid concentration was calculated using SoftMax Pro software with a standard lipid series (0–80 µL of 100 mg/mL vegetable oil in chloroform).

Nutrient metabolites

Here we define nutrient metabolites as free haemolymph sugars and amino acids. Nutrient metabolites were measured in 100 of the larvae, 5 larvae per diet.

Sugar analysis

Sugars were analysed using high-performance liquid chromatography (HPLC). Haemolymph samples were diluted with nanopure water

(1:10 on average). HPLC was used to measure concentrations of glucose, fructose, sucrose, sorbitol, trehalose, lactose, stachyose and maltose. Analysis was conducted by injecting 20 µL of sample via a Rheodyne valve onto a Carbopac PA-100 column (Dionex, Sunnyvale, CA, USA). Sample components were eluted from the column isocratically using 100 mM NaOH flowing at 1 mL/min. The chromatographic profile was recorded using pulsed amperometric detection (ED40 electrochemical detector, Dionex). Elution profiles were analysed using the Chromeleon software package (Thermofisher Scientific). Daily reference curves were obtained for sugars by injecting calibration standards with concentrations of 10 ppm. for each sugar. Sugars were recorded in nmoles per mL and converted to µg/mL using the molar mass of each sugar.

Amino acids

Amino acids were analysed using ultra-high-performance liquid chromatography (uHPLC). To extract free amino acids, haemolymph samples were diluted in 300 µL HPLC-grade methanol (Sigma-Aldrich, Dorset, UK) and mixed for 60 s in an electrical vortex to extract free amino acids, followed by centrifugation at 13,000 rpm for 5 min. The supernatant was filtered through 0.45 µm syringe-tip filters (Whatman Puradisc 4, nylon 4 mm) to remove particulates and was then analysed for free amino acids. The pellet was analysed for protein-bound amino acids as per (Stabler et al., 2015). Briefly, the pellet was dried down at 70°C; mixed with 30 µL ± 25 of 6 Mol.L-1 hydrochloric acid (HCl) and briefly vortexed. Sealed tubes were placed in plastic microfuge tube boxes, sealed and placed in a domestic 900 W (2450 MHz) microwave oven inside of a fume hood according to Zhong et al., (2005). Samples were irradiated for 15 min on full power, left to cool and then heated at 70°C in a heat block to evaporate the acid. A 300 µL aliquot of de-ionised uHPLC gradient grade water was then added to each dried sample, followed by centrifugation and filtration through 0.45 µm syringe-tip filters (Whatman Puradisc 4, nylon, 4 mm). A 10 µL aliquot of each sample was then quantified by uHPLC. Twenty-one amino acids were quantified in the samples using a Dionex Ultimate 3000 RS system fitted with a 150 × 2.1 mm Accucore RP-MS (Thermo Scientific) column as per Stabler et al. (2015). AA-S-18 amino acid calibration standards supplemented with asparagine, glutamine tryptophan and γ-aminobutyric acid (GABA), diluted to 2.5 µM using HPLC-grade water, were used for comparison. Amino acids were recorded in nmoles per mL and converted to µg/mL using the molar mass of each amino acid.

All technical replicates were measured in duplicate as within-sample variation is typically very low and repeatability consequently high (r >0.82; Cotter et al., 2004).

Data analysis

Data analysis was performed using the R statistical software (4.3.1 'Beagle Scouts'; R Core Team, 2023) in R Studio 2023.06.01. After initial examination of absolute levels of each nutrient, a standardised variable (Z) was produced from each nutrient variable (X) using the mean (μ) and standard deviation (σ) of each nutrient: $(Z = (X - \mu)/\sigma)$, allowing comparisons between nutrient groups (see also Cotter et al., 2019).

Generalised additive models

Subsequent analysis was carried out using generalised additive models (GAMs) in the *mgcv* package (v1.8-42; Wood, 2017). GAMs are a nonparametric form of regression analysis that utilise the sum of iterative estimates to calculate a smoothing function, rather than assuming a linear function, as in linear models (Hastie & Tibshirani, 1986). When smoothed models were compared to linear models, the former generally explained more variation (data not shown), making them the preferred choice for analysis. An information theoretic approach was taken to analyse the data (Whittingham et al., 2006), which allows the selection of multiple candidate models accounting for how much variation each explains based on the Akaike information criterion (AIC; Burnham & Anderson, 2004).

Nutrient intake

To assess the effect of diet composition on the amounts of protein, carbohydrate and total food eaten, five models were compared, where X is the assigned number for each series of models: MX.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. MX.P is a model containing total dietary protein, MX.C is a model containing total dietary carbohydrate and MX.PC and MX.PCI are the additive and interactive models for dietary protein and carbohydrate, respectively.

Blood nutrients

The amounts of protein and carbohydrate eaten were strongly associated with the composition of the diets. Therefore, to assess the effect of diet on all blood nutrients or nutrient groups, five models were compared: MX.0, MX.Pe is a model containing total protein eaten, MX.Ce is a model containing total carbohydrate eaten and MX.PeCe and MX.PeCeI are the additive and interactive models for protein and carbohydrate eaten, respectively.

AIC analysis was carried out using the *MuMIn* package (v1.47.5; Barton, 2023) in R, which when combined with the *mgcv* package, ranks models based on the degrees of freedom used to create the smoothed curve. Model fits were analysed using *gam.check* in the *mgcv* package. Where model fits did not conform to Gaussian expectations, the *transformTukey* function in the *Rcompanion* package was used (Mangiafico, 2023). In most cases, transformation improved the fit of the model but did not meaningfully change the results. Where the results were different using transformed data, this is reported below.

Data visualisation

All figures other than Figures 2 and S1 were plotted using thin-plate spline plots with the *fields* package and REML smoothing (Nychka

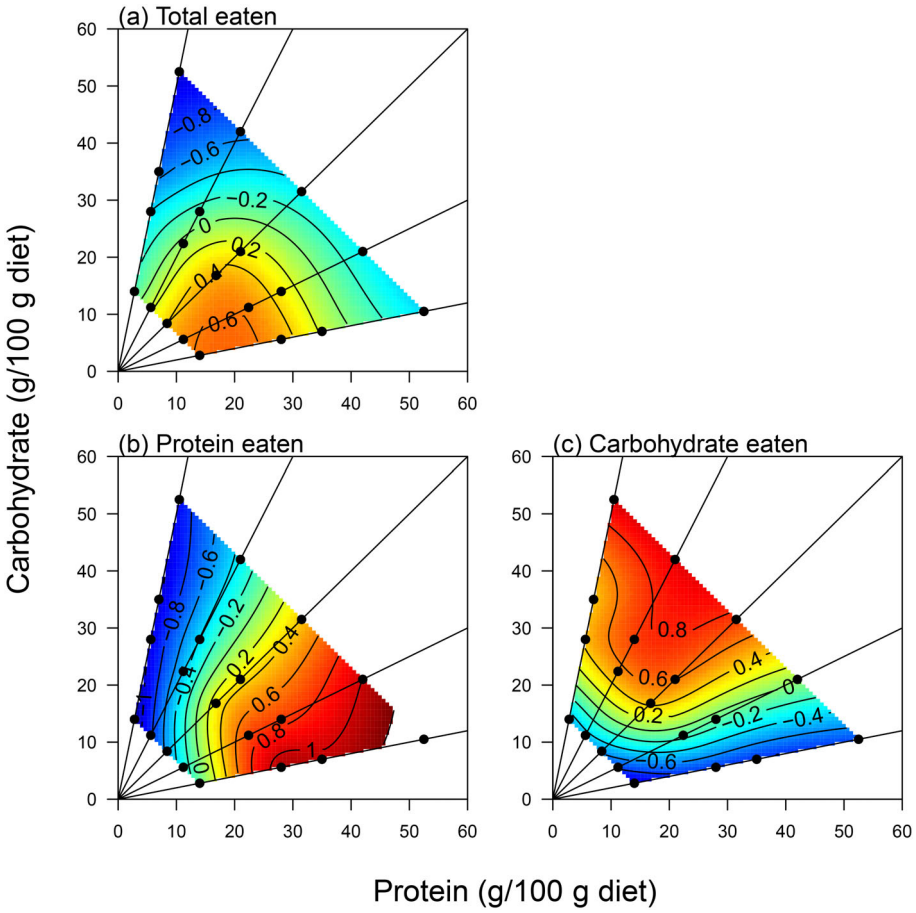

FIGURE 1 The relationship between diet attributes and diet eaten. Various standardised measures of diet eaten (*z*) are plotted against protein content (*x*) and carbohydrate content of the diet (*y*), measured as grams of digestible nutrients per 100 g of diet, simultaneously. *Z* represents (a) total food eaten, (b) protein eaten and (c) carbohydrate eaten. Contour lines represent the type of relationship between *z* and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. *Z* is scaled such that colour is comparable across plots.

et al., 2021), following Cotter et al. (2011). Figure 2 was plotted using ggplot2 (Wickham, 2016). In all main figures, untransformed data were plotted to allow easy comparisons across nutrient groups. Where the transformed data produced markedly different plots, these are presented separately in the Supplementary material. In order to estimate regions of 'homeostasis' for macro and micronutrient variation across nutrient space, each plot was opened in ImageJ (Rueden et al., 2017) and total area of each plot, plus the area that fell within −0.2 and 0.2 SD of the mean was calculated. This allowed the percentage of nutrient space over which blood nutrients were maintained within 0.2 SD of the mean to be reported.

RESULTS

Effect of diet composition on consumption

The best models explaining total consumption, protein consumption and carbohydrate consumption were ones containing dietary protein and carbohydrate content (Table S1). For total consumption, caterpillars generally ingested the most nutrients when on the most diluted diets, but only on the balanced or high protein diets (Figure 1a). On the very low protein (high carbohydrate) diets, consumption remained low, increasing only slightly on the most dilute diets (Figure 1a). Protein intake peaked in a nutrient space containing a high dietary protein content (and low dietary carbohydrate) (Figure 1b). Carbohydrate intake peaked in a nutrient space containing a low dietary protein content (and high dietary carbohydrate) (Figure 1c) and in both cases, intake increased with nutrient density only when corresponding dietary nutrient was predominant. By plotting the consumption of each nutrient against its dietary content, we can see that consumption increased steadily until ~20% protein content (Figure S1a), or carbohydrate content (Figure S1b), before intake slowed (protein) or stabilised (carbohydrate). The total amount of food consumed is fairly stable across dietary protein content (Figure S1c), but decreases with carbohydrate content (Figure S1d). Total consumption also fell as the concentration of nutrients in the diets increased (Figure S1e). The intake array is asymmetrical, with overconsumption of protein-rich diets and underconsumption of protein-poor diets (Figure S1f).

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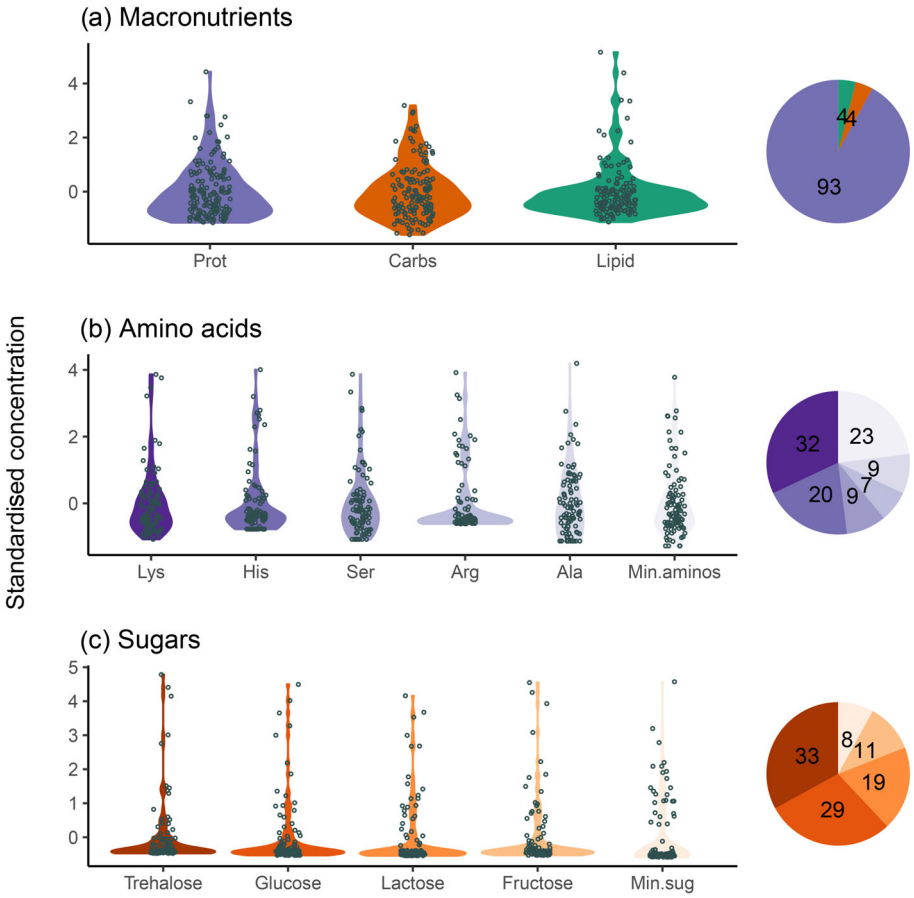

FIGURE 2 Standardised distributions represented as violin plots of (a) macronutrients, (b) amino acids and (c) simple sugars, in blood sampled from larvae that had been restricted to 1 of 20 artificial diets, varying in their protein and carbohydrate content for 2 days. Sugars are separated into the four most abundant sugars, with the remaining sugars pooled. In addition, the absolute variation in each nutrient group is plotted as pie charts, with the percentage of the pool that is made up of each nutrient, displayed in the colours corresponding to the violin plot. Absolute nutrient levels are reported in Table 2.

Profile of types of macronutrients and nutrient metabolites in haemolymph

Protein comprised the bulk of the haemolymph macronutrient profile (93%) with carbohydrates and lipids comprising equal amounts of the remainder (Figure 2a; Table 2a). Lysine was the most abundant amino acid in *S. littoralis* haemolymph (32% relative abundance) (Figure 2b; Table 2b). This was followed by histidine (20%), serine (9%), arginine (9%) and alanine (7%), with the remaining 16 amino acids found in much lower abundances (≤5%), which together comprised 25% of the circulating haemolymph amino acid pool. Of the 10 essential amino acids (EAAs) for animals (Karowe & Martin, 1989; Rock & King, 1967), only lysine, histidine and arginine appeared among the most abundant amino acids. The other EAAs comprised 15% of the haemolymph amino acid pool, with only methionine and tryptophan present at less than a 1% concentration.

Trehalose was the most abundant haemolymph sugar (33%) followed by glucose (29%), lactose (19%) and fructose (11%) (Figure 2c; Table 2c). Together, the remaining sugars (sorbitol, sucrose, stachyose and maltose) comprised 8% of the total haemolymph sugar pool, 58% were reducing sugars (RSs; glucose, fructose, maltose and lactose) and 42% were non-reducing sugars (NRSs; trehalose, sucrose, sorbitol and stachyose).

Effect of diet on haemolymph macronutrients

Haemolymph protein and carbohydrate levels were both best explained by the model containing the amount of protein and carbohydrate eaten (Table S2; Figure 3a,b). Haemolymph protein increased strongly with protein eaten and very slightly with carbohydrate eaten ($r^2 = 0.47$; Figure 3a). In contrast, the surface for haemolymph carbohydrates was flat across most of the nutrient space, increasing only on the very high carbohydrate (low protein) diets and decreasing only on the very low carbohydrate (high protein) diets ($r^2 = 0.25$; Figure 3b). In contrast, lipids were invariable across diets, none of the models performed better than the null model ($r^2 = 0.01$; Figure 3c; Table S2). In terms of homeostasis, approximately 19% of nutrient space was within 0.2 SD of the mean for blood proteins (Figure 3a), 42% of carbohydrates (Figure 3b) and 85% of lipids (Figure 3c).

TABLE 2 Mean, non-standardised concentrations of haemolymph macro (a) and nutrient metabolites: (b) amino acids and (c) simple sugars, after the removal of extreme outliers; summaries for these nutrient groups is shown in (d).

	Nutrient	Mean	SD	Proportion of total abundance
(a) Macronutrients (mg/mL)	Protein	29.38	15.06	0.93
	Carbohydrate	1.17	0.73	0.04
	Lipid	1.17	1.04	0.04
(b) AAs (μg/mL)	*Lysine*	53.01	47.00	0.32
	Histidine	34.14	45.64	0.20
	Serine	15.56	15.37	0.09
	Arginine	15.34	25.08	0.09
	Alanine	11.26	10.67	0.07
	Threonine	7.87	12.88	0.05
	Leucine	5.89	5.53	0.04
	Valine	5.60	5.15	0.03
	Cysteine	5.06	4.76	0.03
	Glycine	4.96	5.37	0.03
	Isoleucine	2.07	1.88	0.01
	Phenylalanine	2.01	1.59	0.01
	Tyrosine	1.67	2.88	0.01
	Methionine	0.79	1.10	0.00
	Glutamate	0.66	0.75	0.00
	Proline	0.63	0.66	0.00
	Aspartate	0.49	0.70	0.00
	Glutamine	0.02	0.02	0.00
	Asparagine	0.00	0.00	0.00
	Tryptophan	0.00	0.00	0.00
(c) Simple sugars (μg/mL)	Trehalose	202.13	434.61	0.33
	Glucose	173.93	329.70	0.29
	Lactose	115.82	215.24	0.19
	Fructose	64.89	123.53	0.11
	Stachyose	20.63	49.01	0.03
	Sucrose	17.15	42.22	0.03
	Sorbitol	15.15	33.34	0.02
	Maltose	0.21	1.65	0.00
(d) Nutrient groups (μg/mL)	*Essential AA*	124.69	82.33	0.75
	Non-essential AA	42.31	29.60	0.25
	Reducing sugars	354.85	631.42	0.58
	Non-reducing	255.06	500.46	0.42

Note: Essential amino acids and reducing sugars are in italics (Rock & Hodgson, 1971).
Abbreviation: AA, amino acid.

Effect of diet on haemolymph nutrient metabolites

Physiologically important metabolites groups

First, we looked at whether physiologically important metabolite groups varied with the consumption of diet by examining RSs and NRSs, total simple sugars (RS + NRS) and the balance between them (RS/(RS + NRS)) and EAA and non-essential amino acids (NEAAs) total amino acids (NEAA + EAA) and the balance between them (EAA/(EAA + NEAA)) (Figures 4 and 5; Table S3). For haemolymph RSs, none of the models was better than the null model ($r^2 = 0.06$; Table S3; Figure 4a). For NRSs, all models containing protein eaten were best supported ($r^2 = 0.20$), but only when data were transformed (Table S3; Figure 4b, Figure S2). NRSs were highest on the highest and lowest P:C diets (Figure S2). For total simple sugars, the model containing protein and carbohydrate eaten was best

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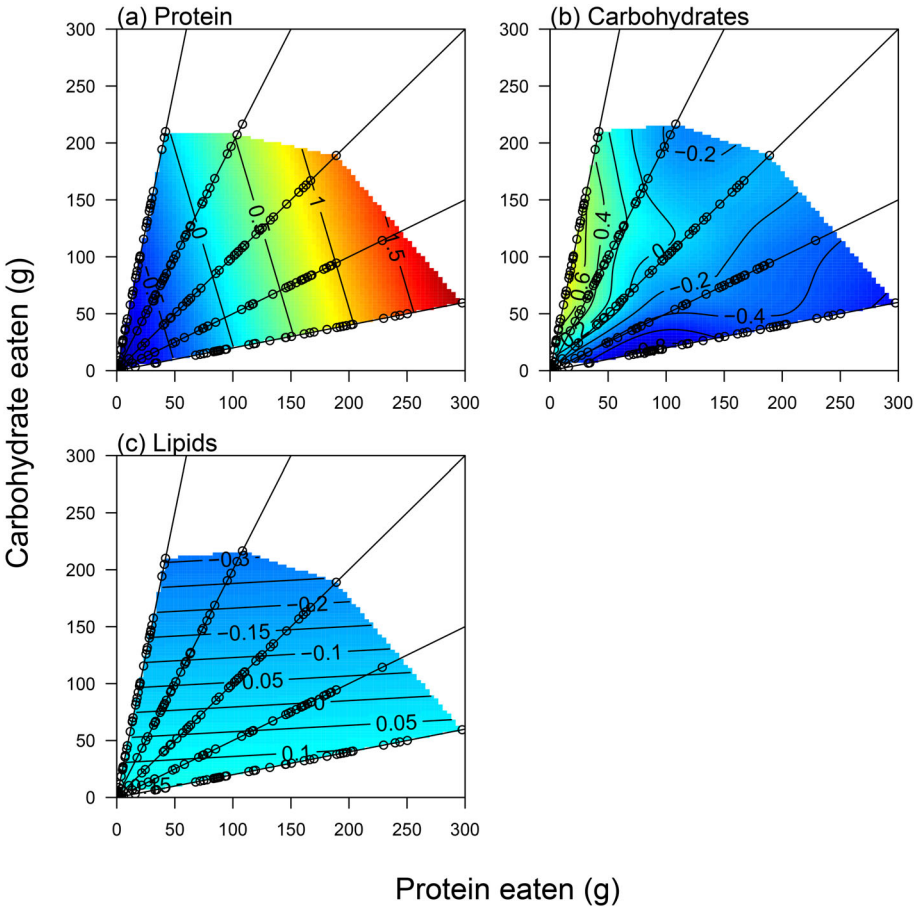

FIGURE 3 The relationship between diet eaten and haemolymph macronutrients. 2D contour plot comparing various standardised dietary macronutrients (*z*) with protein (*x*) and carbohydrate (*y*) eaten, simultaneously. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by macronutrient intake. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. *Z* is scaled such that colour is comparable across plots.

supported, and the patterns were very similar to those of NRSs ($r^2 = 0.12$; Table S3; Figure 4c).

For EAAs, the models containing protein eaten were equally well supported ($r^2 = 0.10$–0.15; Table S4), with EAA concentration increasing with dietary protein consumption, but peaking at high energy consumption, irrespective of nutrient ratios (Figure 5a). Diet was poorer at explaining variation in NEAAs; the best models only explained 4% of the variation, but showed that NEEAs increased with the amount of carbohydrate eaten (Table S4; Figure 5b). Total amino acids were best explained by the interaction between carbohydrates and proteins and increased with the amount of energy consumed ($r^2 = 0.13$; Table S4).

When considering the balance between reducing and NRSs, and EAAs and NEAAs, the best supported models all contained dietary protein eaten (Table S4), and the pattern across nutrient space was remarkably consistent (compare Figures 4d and 5d). In both cases, the proportion was lowest on the diets that had the lowest protein to carbohydrate ratio irrespective of the total amount of nutrients consumed. Proportions peaked on low carbohydrate/high protein ratio diets, increasing strongly as more nutrients were consumed.

In terms of homeostasis, sugars were more tightly controlled than amino acids. Approximately 81% and 71% of nutrient space were within 0.2 SD of the mean for blood reducing and NRSs, respectively (Figure 4a,b), and 65% for total sugars (Figure 4c). These values were 26% and 48% for EAAs and NEAAs and 34% for total amino acids (Figure 5a–c). Estimates of homeostasis differed less when proportions were considered. For sugars, the proportion of sugars that were RSs was between −0.2 and 0.2 SD over 50% of nutrient space, while the proportion of AAs that were essential was within these limits over 39% of nutrient space.

Single nutrient metabolites

We also considered whether diet affected the predominant nutrient metabolites individually. This included glucose and trehalose as the major reducing and non-reducing blood sugars, respectively, and lysine, histidine, serine, alanine and arginine as the most abundant AAs. As for the grouped RSs, variation in glucose was poorly explained by diet, with none of the models performing better than the null

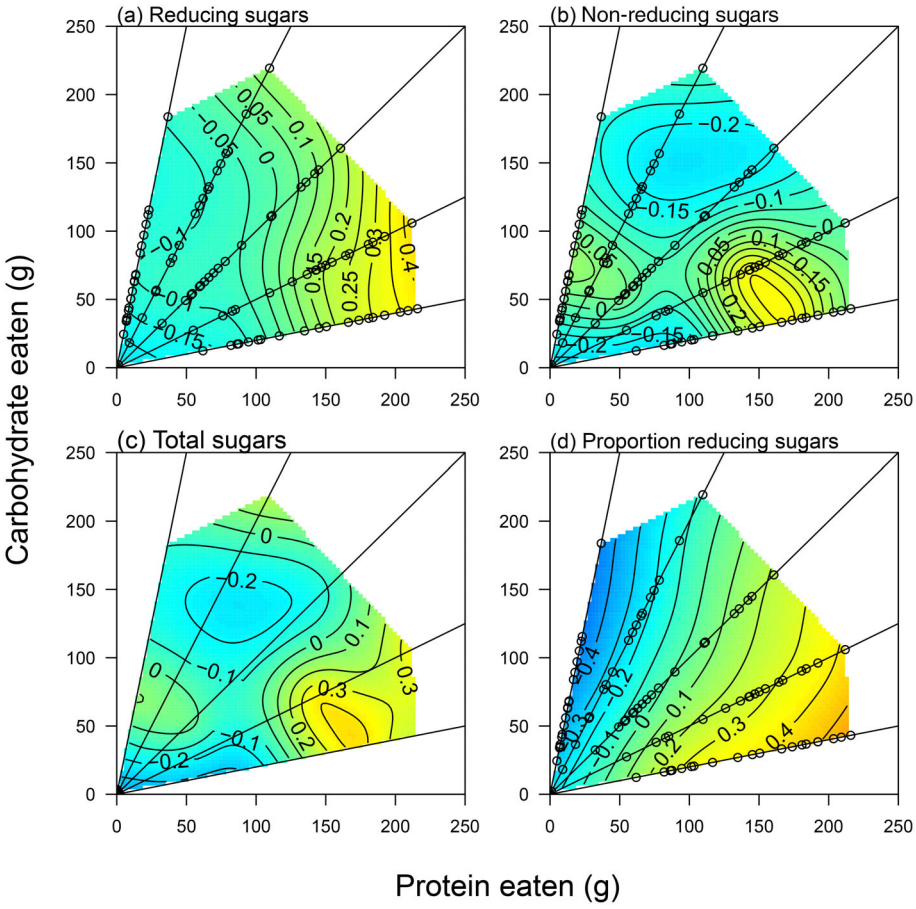

FIGURE 4 The relationship between diet eaten and haemolymph simple sugars grouped by function. 2D contour plot comparing various standardised simple sugars (*z*) with protein (*x*) and carbohydrate (*y*) eaten, simultaneously. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by macronutrient intake. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. *Z* is scaled such that colour is comparable across plots. Plots for sugars using transformed data are in Figure S1.

model (Table S5; Figure S3a,b). Trehalose mirrored the variation seen in the RSs, peaking on the most extreme nutrient ratio diets, but only when data were transformed (Table S5; Figure S3c,d). For the major amino acids, the r^2 of the best models varied from 0.02 to 0.12 (Table S6). Variation in lysine was best explained by models containing protein and carbohydrate eaten (Table S6), increasing with the energy consumed (additive effects of protein and carbohydrate eaten) (Figure 6a). Serine was also best explained by models containing protein and carbohydrate eaten (Table S6), but tended to increase strongly with carbohydrate and decrease weakly with protein eaten (Figure 6c). Alanine was best explained by the models containing carbohydrate eaten, increasing strongly with carbohydrate eaten (Figure 6d). Variation in neither histidine nor arginine was explained by nutrient consumption (Table S6; Figure 6b,e).

DISCUSSION

Here we characterised the blood nutrient pool of the leaf feeding caterpillar, *S. littoralis*, across a broad swathe of nutrient space. By feeding caterpillars on 1 of 20 diets that varied in both nutrient density (17%–63% digestible nutrients) and percentage protein (17%–83%), we were able to force caterpillars to feed across a wide range of nutrient intakes. Although compensatory feeding does occur, it was incomplete (Figure S1), hence protein and carbohydrate intake increased as the levels of these nutrients increased in the diet (Figure 1), with the rate of increase slowing (protein: Figure S1a) or plateauing (carbohydrate: Figure S1b) as the intake target was reached (~120 mg P: 100 mg C). For blood nutrients, our key findings were that most of the macronutrients in the blood were proteins (93%; Figure 2), and the protein content of the blood increased almost linearly with the amount of protein consumed (Figure 3). In contrast, carbohydrates comprised just 4% of the blood nutrient pool (Figure 2) and increased only when the ratio of carbohydrates to protein eaten was very high (Figure 3). The amino acid pool was dominated (75%) by five AAs (lysine, histidine, serine, alanine and arginine), while the sugars were dominated by trehalose, glucose, lactose and fructose (93%; Figure 2). Total and EAAs increased with the energy content of the diet while sugars were not well explained by diet (Figures 4 and 5). This was reflected in homeostasis, with sugars showing stability

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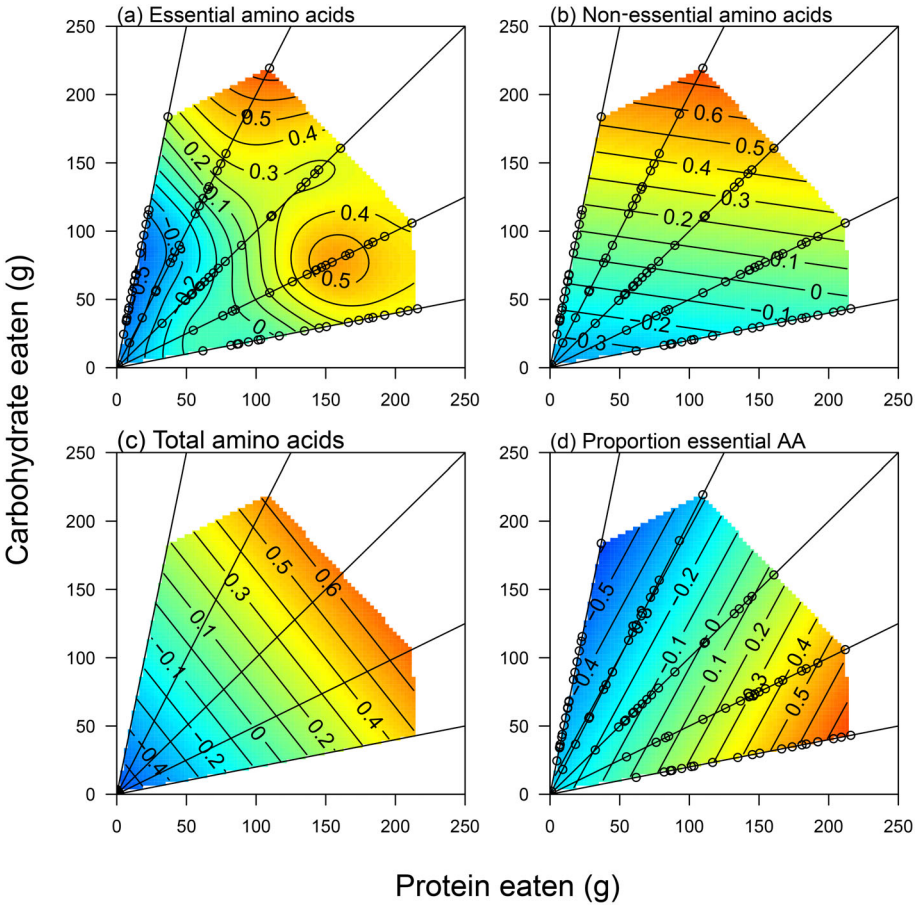

FIGURE 5 The relationship between diet eaten and haemolymph amino acids grouped by function. 2D contour plot comparing standardised amino acids (*z*) with protein (*x*) and carbohydrate (*y*) eaten, simultaneously. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by macronutrient intake. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. *Z* is scaled such that colour is comparable across plots.

over 71%–81% of nutrient space, while amino acids were stable over 26%–48% of nutrient space. The relative proportion of EAA to NEAAs and the proportion of reducing to NRSs responded to diet in remarkably similar ways, increasing consistently as the ratio of protein to carbohydrate in the diet increased (Figures 4 and 5). Homeostasis varied much less when considering these proportions with 50% stability for sugars and 39% for amino acids.

In locusts that were fed on diets with the same amino acid profile as used in this study (protein content comprising casein, peptone and albumen 3:1:1), the most abundant blood amino acids were glycine and proline, followed by glutamine, histidine, lysine, serine, methionine and isoleucine (high protein diet) or alanine (low protein diet) (Abisgold & Simpson, 1987). In contrast, the most abundant blood amino acid here was lysine, followed by histidine, serine, alanine and arginine. Glycine was the 6th most abundant (5% of pool) followed by threonine and valine. Proline was one of the minor amino acids, making up less than 1% of the protein pool. The fact that lysine was the most abundant amino acid here is interesting. In locusts, lysine did not vary consistently across diets (Abisgold & Simpson, 1987), while we found that it increased with the amount of food consumed (Table S6; Figure 5a). In locusts, lysine is selectively egested on high protein diets to allow continued consumption to gain limiting carbohydrates (Zanotto et al., 1996). This is because lysine is the most potent of the amino acids in locust blood for determining protein appetite (Simpson et al., 1991; Zanotto et al., 1994). We did not measure frass amino acids here, but given the abundance of lysine in the blood, it is unlikely that lysine plays the same role in *S. littoralis*. Our results closely match those of Jeschke et al. (2016) who quantified amino acids in *S. littoralis* haemolymph on an artificial white bean flour diet (see Table S3 in Jeschke et al., 2016). Despite the very different diets, the only major difference in the amino acid profiles between our two studies was that Jeschke et al. (2016) found that lysine was more abundant (48% vs. 29%) and histidine much less abundant (3% vs. 17% of the AA pool). Jeschke et al. (2016) did look at amino acids in the frass and found that lysine was considerably under-excreted (48% in blood vs. 2% in frass), while glutamine, glycine, proline and arginine were over-represented, suggesting that any of these amino acids could play the same role as lysine in locusts—

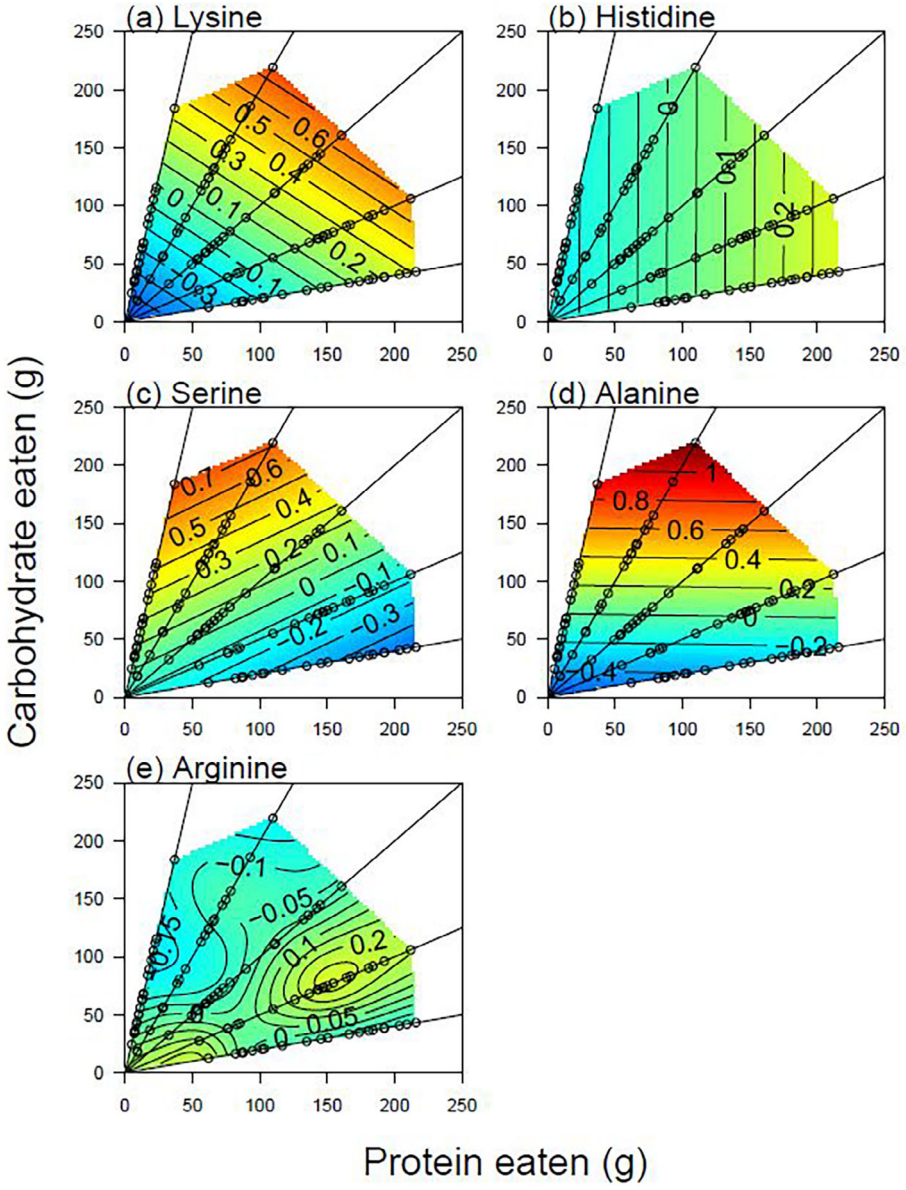

FIGURE 6 The relationship between diet eaten and the five most abundant amino acids using standardised data. 2D contour plot comparing various amino acids (*z*) with protein (*x*) and carbohydrate (*y*) eaten simultaneously. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by macronutrient intake. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. *Z* is scaled such that colour is comparable across plots. The plots using the transformed data can be found in Figure S4.

unjamming the protein satiation mechanism to allow continued protein consumption.

As predicted, proteins and amino acids varied much more consistently with diet than carbohydrates and simple sugars. Proteins in the blood increased predominantly with the amount of protein eaten, while total amino acids increased with both protein and carbohydrate eaten—that is, energy consumed. However, the make-up of the amino acid pool depended strongly on the ratio of proteins to carbohydrates consumed: as the protein proportion of the diet consumed increased, so did the proportion of the amino acid pool that was made up of EAAs. The EAAs are derived entirely from dietary protein, whereas the non-EAAs are derived from various sources, including sugars, though nitrogen must be scavenged from other amino acids (O'Brien et al., 2002). For example, serine can be synthesised in the fat body using glucose or glycogen (Chapman, Simpson, & Douglas, 2013; Karowe & Martin, 1989; O'Brien et al., 2002). When considered alone, we also found that serine predominantly increased with the carbohydrate content of the diet, especially when protein content was low. This might explain why diet intake corresponded to a significant amount of variation in EAA concentration, but not the concentration of non-EAAs. The positive correlation observed between EAAs and protein ingested was not surprising, since the haemolymph amino acid

pool is crucial for protein synthesis (Kerkut & Gilbert, 1985). Moreover, certain individual EAAs perform important roles in feeding regulation, as noted above (Simpson et al., 1991; Zanotto et al., 1994). Our findings reflect previous studies on this species, showing that there is a high conversion of ingested protein into haemolymph protein resources (Cotter et al., 2011; Lee et al., 2002). The prioritisation of amino acids can be ascribed to the need for larvae to accumulate protein (Chown & Nicolson, 2004; Lee et al., 2002; O'Brien et al., 2002; Zanotto et al., 1996); *S. littoralis* adults are nectar feeders restricted to carbohydrate-dominated diets (Chapman & de Boer, 1995), and so must build protein reserves at the larval stage, and the haemolymph is major location for the storage of these proteins (Haunerland, 1996).

Haemolymph carbohydrate levels, in contrast to proteins and amino acids, varied little over much of nutrient space, suggesting homeostasis, except when dietary extremes are encountered, especially on very high carbohydrate diets. This may be due to the tight regulation of metabolite levels by insects to modulate their effects on homeostatic factors, such as haemolymph pH and osmolality (Abisgold & Simpson, 1988; Thompson, 2003; Wyatt, 1961). RSs, such as glucose, should be regulated due to their toxicity at high concentrations (Boctor, 1974; Kerkut & Gilbert 1985), but trehalose, the primary NRS, is stored in the haemolymph as a crucial carbohydrate reserve, utilised in periods of stress such as flight or starvation (Saito, 1963; Thompson, 2003), and is not normally regulated (Raubenheimer et al., 2012), but this might be stage specific. For example, Doğan et al. (2021) showed increased trehalose on a high sugar diet in 3rd instar, but not in 5th instar *Spodoptera* caterpillars (closer to the final instar caterpillars used here). This stage variation could be due to insects storing more dietary carbohydrates and lipids in the fat body in preparation for pupation in late-stage caterpillars (Warbrick-Smith et al., 2006). However, while other studies, including those on *S. littoralis*, have found trehalose in substantially higher concentrations than glucose (Boctor, 1974; Jeschke et al., 2016; Thompson, 2003), here glucose was almost as abundant on average. It is worth noting, that on many individual diets, trehalose levels were substantially higher than glucose, while on others, glucose was higher (compare diets 1 and 12 vs. diet 6; Figure S5). The majority of studies measure blood glucose and trehalose across very few diets, typically 2–4, and so this variation in glucose levels is likely to be missed (Abisgold & Simpson, 1987; Friedman et al., 1991; Thompson, 1998; Zanotto et al., 1996).

We have previously shown that carbohydrate in the diet has little effect on the outcome of infection with a blood-borne bacterium and that dietary protein increases the osmolality of the blood, which is detrimental to bacterial growth in vitro and in vivo (Wilson et al., 2020). So what might our results mean for caterpillars when faced with infection? As sugars are tightly controlled, except on extreme diets, any benefits of increased blood sugar could only be realised on extremely high carbohydrate diets. The toxicity of high levels of glucose (Boctor, 1974; Kerkut, 2013) mean that utilising dietary sugars to moderate blood properties might harm the insect as much as the parasite. In contrast, as predicted, proteins, total amino acids and EAAs can all be changed in the blood via consumption of protein. This lack of apparent homeostasis over much of nutrient space means that consumption of proteins can be used to change the haemolymph protein pool in response to parasitism (Wilson et al., 2020).

CONCLUSION

Although some blood nutrients were tightly controlled in this species, there is little evidence that the blood nutrient profile as a whole is tightly regulated, suggesting that the homeostatic model is not well supported. Rather than homeostasis of blood nutritional properties, allostatic models (Sterling, 1988) seem to be a better fit for blood nutrient regulation in this generalist herbivore. This flexibility in response to the nutritional composition of the diet may, in part, explain how this species has evolved to extreme dietary generalism and may play a role in its worldwide pest status (Hill, 1987). Given the range of fitness-related processes affected by the haemolymph, future studies should examine the physiological impacts of blood nutrient variation on reproduction, growth and response to infection and the trade-offs between them.

AUTHOR CONTRIBUTIONS

Robert Holdbrook: Investigation; writing – original draft; methodology; writing – review and editing; data curation. **Awawing A. Andongma:** Writing – review and editing; validation. **Joanna L. Randall:** Investigation; methodology; validation; writing – review and editing. **Catherine E. Reavey:** Investigation; methodology; validation; writing – review and editing. **Yamini Tummala:** Methodology; validation; writing – review and editing. **Geraldine A. Wright:** Methodology; validation; writing – review and editing. **Stephen J. Simpson:** Writing – review and editing; conceptualization; funding acquisition. **Judith A. Smith:** Conceptualization; writing – review and editing; funding acquisition. **Kenneth Wilson:** Conceptualization; funding acquisition; writing – review and editing; project administration; supervision; investigation. **Sheena C. Cotter:** Conceptualization; writing – original draft; writing – review and editing; formal analysis; visualization; supervision.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in figshare at https://doi.org/10.24385/lincoln.24774114.v1.

ETHICS STATEMENT

Ethical approval was not required for this work as insects are not covered under the Animals (Scientific Procedures) Act 1986. Nonetheless, all insects were kept under appropriate rearing conditions throughout their lives, with handling minimised. Animals used in experiments were provided with food and shelter, handled with care and euthanised by rapid freezing where appropriate to minimise distress.

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REFERENCES

Abisgold, J.D. & Simpson, S.J. (1987) The physiology of compensation by locusts for changes in dietary protein. *The Journal of Experimental Biology*, 129, 329–346.

Abisgold, J.D. & Simpson, S.J. (1988) The effect of dietary protein levels and haemolymph composition on the sensitivity of the maxillary palp chemoreceptors of locusts. *The Journal of Experimental Biology*, 135, 215–229.

Barton, K. (2023) MuMIn: multi-model inference. *Rpackage version*, 1(47), 5. https://CRAN.R-project.org/package=MuMIn

Beyenbach, K.W. (2016) The plasticity of extracellular fluid homeostasis in insects. *The Journal of Experimental Biology*, 219, 2596–2607.

Boctor, I.Z. (1974) Carbohydrates in the haemolymph of the prepupal and pupal stages of *Spodoptera littoralis* Boisduval. *Journal of Comparative Physiology*, 94, 353–356.

Burnham, K.P. & Anderson, D.R. (2004) Multimodel inference: understanding AIC and BIC in model selection. *Sociological Methods & Research*, 33, 261–304.

Calder, P.C. & Jackson, A.A. (2000) Undernutrition, infection and immune function. *Nutrition Research Reviews*, 13, 3–29.

Chapman, R.F. & de Boer, G. (1995) *Regulatory mechanisms in insect feeding*. New York, NY: Springer Science & Business Media.

Chapman, R.F., Douglas, A.E., Siva-Jothy, M.T. & Simpson, S.J. (2013) Circulatory system, blood and the immune system. In: Chapman, R.F., Simpson, S.J. & Douglas, A.E., (Eds.) *The insects: structure and function*. Cambridge, UK: Cambridge University Press, pp. 107–131.

Chapman, R.F., Simpson, S.J. & Douglas, A.E. (2013) *The insects: structure and function*. Cambridge, UK: Cambridge University Press.

Chown, S.L. & Nicolson, S. (2004) *Insect physiological ecology: mechanisms and patterns*. Oxford, UK: OUP Oxford.

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., Reavey, C.E., Tummala, Y., Randall, J.L., Holdbrook, R., Ponton, F. et al. (2019) Diet modulates the relationship between immune gene expression and functional immune responses. *Insect Biochemistry and Molecular Biology*, 109, 128–141.

Cotter, S.C., Simpson, S.J., Raubenheimer, D. & Wilson, K. (2011) Macronutrient balance mediates trade-offs between immune function and life history traits. *Functional Ecology*, 25, 186–198.

Dadd, R.H. (1961a) The nutritional requirements oflocusts—IV. Requirements for vitamins of the B complex. *Journal of Insect Physiology*, 6, 1–12.

Dadd, R.H. (1961b) The nutritional requirements oflocusts—V: observations on essential fatty acids, chlorophyll, nutritional saltmixtures, and the protein or amino acid components of synthetic diets. *Journal of Insect Physiology*, 6, 126–145.

Doğan, C., Güney, G., Güzel, K.K., Can, A., Hegedus, D.D. & Toprak, U. (2021) What you eat matters: nutrient inputs Alter the metabolism and neuropeptide expression in Egyptian cotton leaf worm, *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Frontiers in Physiology*, 12, 773688. Available from: https://doi.org/10.3389/fphys.2021.773688

Folch, J., Lees, M. & Sloane Stanley, G.H. (1957) A simple method for the isolation and purification of total lipides from animal tissues. *The Journal of Biological Chemistry*, 226, 497–509.

Friedman, S., Waldbauer, G.P., Eertmoed, J.E., Naeem, M. & Ghent, A.W. (1991) Blood trehalose levels have a role in the control of dietary self-selection by *Heliothis zea* larvae. *Journal of Insect Physiology*, 37, 919–928.

Garibaldi, L.A., Gomez Carella, D.S., Nabaes Jodar, D.N., Smith, M.R., Timberlake, T.P. & Myers, S.S. (2022) Exploring connectionsbetween pollinator health and human health. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, 377, 20210158.

Hastie, T. & Tibshirani, R. (1986) Generalized additive models. *Statistical Science*, 1, 297–310.

Haunerland, N.H. (1996) Insect storage proteins: gene families and receptors. *Insect Biochemistry and Molecular Biology*, 26, 755–765.

Hill, D.S. (1987) *Agricultural insect pests of temperate regions and their control*. Cambridge, UK: CUP Archive.

Jeschke, V., Gershenzon, J. & Vassão, D.G. (2016) A mode of action of glucosinolate-derived isothiocyanates: detoxification depletes glutathione and cysteine levels with ramifications on protein metabolism in *Spodoptera littoralis*. *Insect Biochemistry and Molecular Biology*, 71, 37–48.

Karowe, D.N. & Martin, M.M. (1989) The effects of quantity and quality of diet nitrogen on the growth, efficiency of food utilization, nitrogen budget, and metabolic rate of fifth-instar *Spodoptera eridania* larvae (Lepidoptera: Noctuidae). *Journal of Insect Physiology*, 35, 699–708.

Kaufmann, C. & Brown, M.R. (2008) Regulation of carbohydrate metabolism and flight performance by a hypertrehalosaemic hormone in the mosquito *Anopheles gambiae*. *Journal of Insect Physiology*, 54, 367–377.

Kerkut, G.A. & Gilbert, L.I. (Eds.). (1985) *Comprehensive insect physiology, volume 4: regulation: digestion, nutrition, excretion*. Oxford: Pergamon Press.

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

Mangiafico, S.S. (2023) rcompanion: functions to support extension education program evaluation.

Meshrif, W.S., El Husseiny, I.M. & Elbrense, H. (2022) Drosophila melanogaster as a low-cost and valuable model for studying type 2diabetes. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, 337, 457–466.

Morris, S.N.S., Coogan, C., Chamseddin, K., Fernandez-Kim, S.O., Kolli, S., Keller, J.N. et al. (2012) Development ofdiet-induced insulin resistance in adult Drosophila melanogaster. *Biochimica et Biophysica Acta*, 1822, 1230–1237.

Nychka, D., Furrer, R., Paige, J. & Sain, S. (2021) fields: tools for spatial data. R package version 14.1.

O'Brien, D.M., Fogel, M.L. & Boggs, C.L. (2002) Renewable and nonrenewable resources: amino acid turnover and allocation to reproduction in Lepidoptera. *Proceedings of the National Academy of Sciences of the United States of America*, 99, 4413–4418.

Raubenheimer, D., Simpson, S.J. & Tait, A.H. (2012) Match and mismatch: conservation physiology, nutritional ecology and the timescales of biological adaptation. *Philosophical Transactions of the Royal Society of London Series B Biological Sciences*, 367, 1628–1646.

R Core Team. (2023) *R: a language and environment for statistical computing*. Vienna, Austria: Foundation for Statistical Computing.

Rock, G.C. & Hodgson, E. (1971) Dietary amino requirementsfor Heliothis zea determined by dietary deletion and radiometrictechniques. *J. Insect Physiol.*, 17, 1087–1097.
Rock, G.C. & King, K.W. (1967) Qualitative amino acidrequirements of the red-banded leaf roller, Argyrotaenia velutinana(Lepidoptera: Tortricidae). *J. Insect Physiol.*, 13, 59–68.
Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T. et al. (2017) ImageJ2: ImageJ for the next generation of scientific image data. *BMC Bioinformatics*, 18, 529.
Saito, S. (1963) Trehalose in the body fluid of the silkworm, *Bombyx mori* L. *Journal of Insect Physiology*, 9, 509–519.
Scott Brown, A.S., Simmonds, M.S.J. & Blaney, W.M. (2002) Relationship between nutritional composition of plant species and infestation levels of thrips. *Journal of Chemical Ecology*, 28, 2399–2409.
Simpson, S.J., James, S., Simmonds, M.S. & Blaney, W.M. (1991) Variation in chemosensitivity and the control of dietary selection behaviour in the locust. *Appetite*, 17, 141–154.
Simpson, S.J. & Raubenheimer, D. (1993) The central role of the haemolymph in the regulation of nutrient intake in insects. *Physiological Entomology*, 18, 395–403.
Simpson, S.J. & Raubenheimer, D. (1995) The geometric analysis of feeding and nutrition: a user's guide. *Journal of Insect Physiology*, 41, 545–553.
Simpson, S.J. & Raubenheimer, D. (2012) *The nature of nutrition: a unifying framework from animal adaptation to human obesity*. Princeton and Oxford: Princeton University Press.
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.
Simpson, S.J. & Abisgold, J.D. (1985) Compensation bylocusts for changes in dietary nutrients: behavioural mechanisms. *Physiological Entomology*, 10, 443–452.
Simpson, S.J., Simmonds, M.S.J. & Blaney, W.M. (1988) A comparison of dietary selection behaviour in larval *Locusta migratoria* and *Spodoptera littoralis*. *Physiological Entomology*, 13, 225–238.
Stabler, D., Paoli, P.P., Nicolson, S.W. & Wright, G.A. (2015) Nutrient balancing of the adult worker bumblebee (*Bombus terrestris*) depends on the dietary source of essential amino acids. *The Journal of Experimental Biology*, 218, 793–802.
Sterling, P. (1988) Allostasis: a new paradigm to explainarousal pathology. In: Fisher, S. & Reason, J.T. (Eds.) *Handbook of life stress, cognition and health*. Chichester and New York: John Wiley & Sons.
Thompson, S.N. (1998) Long-term regulation of glucogenesis by dietary carbohydrate and relevance to blood sugar level in an insect *Manduca sexta* L. *The International Journal of Biochemistry & Cell Biology*, 30, 987–999.
Thompson, S.N. (2003) Trehalose—the insect 'blood' sugar. *Advances in Insect Physiology*, 31, 205–285.
Tonk-Rügen, M., Vilcinskas, A. & Wagner, A.E. (2022) Insect models in nutrition research. *Biomolecules*, 12, 1668. Available from: https://doi.org/10.3390/biom12111668
Verheyen, E.M. (2022) The power of Drosophila in modeling human disease mechanisms. *Disease Models & Mechanisms*, 15. Available from: https://doi.org/10.1242/dmm.049549
Warbrick-Smith, J., Behmer, S.T., Lee, K.P., Raubenheimer, D. & Simpson, S.J. (2006) Evolving resistance to obesity in aninsect. *Proceedings of the National Academy of Sciences*, 103, 14045–14049.
Whittingham, M.J., Stephens, P.A., Bradbury, R.B. & Freckleton, R.P. (2006) Why do we still use stepwise modelling in ecology and behaviour? *The Journal of Animal Ecology*, 75, 1182–1189.
Wickham, H. (2016) ggplot2: elegant graphics for data analysis.
Wilson, J.K., Ruiz, L., Duarte, J. & Davidowitz, G. (2019) The nutritional landscape of host plants for a specialist insect herbivore. *Ecology and Evolution*, 9, 13104–13113.
Wilson, K., Holdbrook, R., Reavey, C.E., Randall, J.L., Tummala, Y., Ponton, F. et al. (2020) Osmolality as a novel mechanism explaining diet effects on the outcome of infection with a blood parasite. *Current Biology*, 30, 2459–2467.e3.
Wood, S.N. (2017) *Generalized additive models: an introduction with R*, 2nd edition. New York: CRC Press.
Wyatt, G.R. (1961) The biochemistry of insect hemolymph. *Annual Review of Entomology*, 6, 75–102.
Zanotto, F.P., Raubenheimer, D. & Simpson, S.J. (1994) Selective egestion of lysine by locusts fed nutritionally unbalanced foods. *Journal of Insect Physiology*, 40, 259–265.
Zanotto, F.P., Raubenheimer, D. & Simpson, S.J. (1996) Haemolymph amino acid and sugar levels in locusts fed nutritionally unbalanced diets. *Journal of Comparative Physiology B*, 166, 223–229.
Zhong, H., Marcus, S.L. & Li, L. (2005) Microwave-assisted acid hydrolysis of proteins combined with liquid chromatography MALDI MS/MS for protein identification. *Journal of the American Society for Mass Spectrometry*, 16, 471–481.

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Figure S1. Amount of diet consumed over 48 h. The amount of (a) protein or (b) carbohydrate eaten as a function of the dietary protein or carbohydrate content of the diet, and the total amount of food consumed as a function of the dietary (c) protein or (d) carbohydrate or (e) total nutrient content of the diet. Dots are raw data and lines are loess smoothed fits from GAM models. The intake array (f) shows average consumption ± SE on each of the dietary ratios. The blue line indicates where intake would be expected if caterpillars were following the 'The closest distance rule' where animals eat until they reach the point in nutrient space that minimises the distance to the intake target (Raubenheimer & Simpson, 1997).

Figure S2. The relationship between diet eaten and (a) standardised non-reducing sugars and (b) total simple sugars. 2D contour plot comparing sugars (z) with protein (x) and carbohydrate (y) eaten using transformed data. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by dietary macronutrient composition. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in z, with dark blue indicating the lowest values going up to red which represents the highest values. Colour is unscaled as the transformations mean that the variables are not on a comparable scale.

Figure S3. The relationship between diet eaten and the 2 major haemolymph sugars. 2D contour plots compare each standardised sugar (z) with protein (x) and carbohydrate (y) eaten, simultaneously, using untransformed (a, c) and transformed (b, d) data. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by dietary macronutrient composition. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in z, with dark blue indicating the lowest values going up to red which represents the highest values. Colour is unscaled for transformed data as the transformations mean that the variables are not on a comparable scale.

Figure S4. The relationship between diet eaten and the five most abundant amino acids using transformed data. 2D contour plot comparing various standardised amino acids (*z*) with protein (*x*) and carbohydrate (*y*) simultaneously. Straight lines originating at 0 represent nutrient rails with grey dots showing the nutrient space created by macronutrient intake. Contour lines represent the type of relationship between the dependent variable and the two independent variables. Colour represents the variation in *z*, with dark blue indicating the lowest values going up to red which represents the highest values. Colour is unscaled as the transformations mean that the variables are not on a comparable scale.

Figure S5. Variation in (a) Trehalose and (b) Glucose concentration in μg per mL of haemolymph, from caterpillars reared on one of 20 diets that varied in their nutrient density (concentration of digestible nutrients ranged from 17% to 63%) and their protein to carbohydrate ratio (1:5–5:1) (Table 1).

Table S1. Consumption model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.P is a model containing total dietary protein, Mx.C is a model containing total dietary carbohydrate and Mx.PC and Mx.PCI are the additive and interactive models for dietary protein and carbohydrate respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect. Tukey transformation improved model fit and so results from the transformed data are reported.

Table S2. Haemolymph macronutrient model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.Pe is a model containing total protein eaten, Mx.Ce is a model containing total carbohydrate eaten and Mx.PeCe and Mx.PeCeI are the additive and interactive models for protein and carbohydrate eaten respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect. Tukey transformation improved model fit and so results from the transformed data are reported for protein and lipids.

Table S3. Haemolymph sugars model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.Pe is a model containing total protein eaten, Mx.Ce is a model containing total carbohydrate eaten and Mx.PeCe and Mx.PeCeI are the additive and interactive models for protein and carbohydrate eaten respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect. Tukey transformation improved model fit for reducing, non-reducing and total sugars, and so results from the transformed data are reported.

Table S4. Haemolymph amino acids model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.Pe is a model containing total protein eaten, Mx.Ce is a model containing total carbohydrate eaten and Mx.PeCe and Mx.PeCeI are the additive and interactive models for protein and carbohydrate eaten respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect.

Table S5. Haemolymph individual sugars model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.Pe is a model containing total protein eaten, Mx.Ce is a model containing total carbohydrate eaten and Mx.PeCe and Mx.PeCeI are the additive and interactive models for protein and carbohydrate eaten respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and

the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect. Tukey transformation improved model fit and so results from the transformed data are reported.

Table S6. Haemolymph individual amino acids model summary tables. (a) AIC model comparison table. Mx.0 (Null) is a model with no explanatory terms included, providing a baseline measure of variation. Mx.Pe is a model containing total protein eaten, Mx.Ce is a model containing total carbohydrate eaten and Mx.PeCe and Mx.PeCeI are the additive and interactive models for protein and carbohydrate eaten respectively. df is the degrees of freedom used by the model to produce a fit and not the degrees of freedom based on the number of explanatory variables. K is the number of terms in the model. AICc is the model Akaike values. Delta represents the difference between a model and

the model explaining the most variation. Weight is determined by the amount of variation a model explains penalized for the degrees of freedom used by the model. (b) Summary information for each model in the AIC comparison table where delta <2. The edf provides information about the shape of the curve, relating to the basis dimensions used by the model to fit the curve; for example, an edf close to 1 represents a linear effect, whilst an edf close to 2 represents a quadratic effect. Tukey transformation improved model fit and so results from the transformed data are reported.

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