The gastrointestinal tract as a nutrient-balancing organ

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Failure to provision tissues with an appropriate balance of nutrients engenders fitness costs. Maintaining nutrient balance can be achieved by adjusting the selection and consumption of foods, but this may not be possible when the nutritional environment is limiting. Under such circumstances, rebalancing of an imbalanced nutrient intake requires post-ingestive mechanisms. The first stage at which such post-ingestive rebalancing might occur is within the gastrointestinal tract (GIT), by differential release of digestive enzymes—releasing less of those enzymes for nutrients present in excess while maintaining or boosting levels of enzymes for nutrients in deficit. Here, we use an insect herbivore, the locust, to show for the first time that such compensatory responses occur within the GIT. Furthermore, we show that differential release of proteases and carbohydrases in response to nutritional state translate into differential extraction of macronutrients from host plants. The prevailing view is that physiological and structural plasticity in the GIT serves to maximize the rate of nutrient gain in relation to costs of maintaining the GIT; our findings show that GIT plasticity is integral to the maintenance of nutrient balance.

Keywords: phenotypic plasticity; nutrient balance; digestive enzymes

1. INTRODUCTION

All animals are faced with the challenge of matching the demand for multiple nutrients with the supply of those nutrients from the environment. When food choice is reduced owing to a restricted range of available foods, limited forager mobility or constraining biotic or abiotic factors such as the presence of predators or microclimatic conditions (e.g. Stamp 1997; Lima 1998; Niesenbaum & Kluger 2006; Richards & Coley 2007), many of the sophisticated behavioural mechanisms that animals use to regulate nutritional outcomes in a heterogeneous environment (e.g. Raubenheimer & Simpson 1997; Simpson *et al.* 2004) are unavailable. Animals that are constrained to ingest a nutritionally imbalanced diet face fitness consequences (Simpson *et al.* 2004; Raubenheimer *et al.* 2005) unless they are able to make compensatory post-ingestive adjustments.

There are, broadly speaking, two stages at which such post-ingestive adjustments might be made to rebalance an imbalanced nutrient intake: within the gastrointestinal tract (GIT) through modulation of digestion and absorption; and post-absorptively through metabolism and excretion (e.g. Yang & Joern 1994a; Zanutto *et al.* 1997; Trier & Mattson 2003; Jensen & Hessen 2007). Regarding these possibilities, the prevailing view is that animals maximize the extraction of all macronutrients from food in the GIT and then use post-absorptive mechanisms to regulate retention and use of different nutrients (e.g. Karasov & Diamond 1988; Starck 2005). Hence, control of digestive enzyme secretion is thought to vary positively

with substrate concentration so that the metabolic expense of synthesizing large amounts of enzyme is not wasted when feeding on low-nutrient diets (*sensu* optimal digestion theory; Sibly 1981; Penry & Jumars 1986). Similarly, diet-induced physical remodelling of the GIT (e.g. Yang & Joern 1994b; Raubenheimer & Bassil 2007) and qualitative and quantitative changes in the digestive enzyme system (e.g. Sabat *et al.* 1997, 1999; Bock & Mayer 1999; Caviedes-Vidal *et al.* 2000; Kotkar *et al.* 2009) have been interpreted as maximizing nutrient absorption to allow rapid elimination of surpluses post-absorptively (Raubenheimer & Bassil 2007).

However, not all studies support the view that enzyme secretion is a positive function of substrate concentration. Thus, when foods differ not only in the absolute concentration of nutrients but also in their relative amounts, the relationship between substrate and enzyme activity shows no consistent pattern (e.g. Ishaaya *et al.* 1971; Kotkar *et al.* 2009; Woodring *et al.* 2009), and the untested possibility remains that under circumstances of nutrient imbalance there may be homeostatic secretion of digestive enzymes, with enzymes for nutrients in excess being secreted at lower rates than enzymes for nutrients in deficit. Consistent with such a possibility are results showing that after pharmacological disruption of digestive enzymes by proteinase inhibitors administered in the food, caterpillars responded within 2 h to compensate for the loss of enzymatic function by increasing the production of endogenous enzymes or secreting novel proteases (e.g. Broadway 1997; Lopes *et al.* 2004).

Our aim was to explore the relationship between secretion of proteases and carbohydrases and nutritional state in a model insect herbivore, the locust, *Locusta migratoria*. We initially used chemically defined synthetic foods to determine whether the activity of proteases and

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carbohydrases is plastic and responds to the concentration of substrate *per se* or to the ratio of protein to carbohydrate (P:C) in the diet. Our results indicate that α -chymotrypsin-like and α -amylase-like activities are selectively downregulated when locusts ingest excessive protein or carbohydrate, respectively, indicating a homeostatic role for enzymatic plasticity. We next asked whether these plastic changes in enzyme activity translated into differential digestion and absorption of nutrients from two natural host plants. We found that changes in digestive enzyme activity translated into differential uptake of protein and carbohydrate from two *C₄* grasses.

2. MATERIAL AND METHODS

(a) Locusts and diets

Locusta migratoria came from a long-term culture at the University of Sydney (originally collected from the Central Highlands of Queensland, Australia). Stock locusts were reared at a density of 500–1000 on seedling wheat and wheat germ in large plastic bins (56 × 76 × 60 cm) under a 14L:10D photoperiod in a room kept at 30°C. During the 'light-on' phase, each bin had an additional heat source (250 W heat lamp) mounted on the mesh roof of the bin. Treatment diets consisted of dry granular synthetic foods differing in the ratio and concentration of protein, P (a 3:1:1 mixture of casein, bacteriological peptone and egg albumen) and carbohydrate, C (a 1:1 mixture of sucrose and dextrin). Four treatment diets were used, with the percentage of P and C as follows: 21%P:21%C (PC); 10.5%P:10.5%C (pc); 35%P:7%C (Pc); and 7%P:35%C (pC). All diets contained 4 per cent salts, vitamins and sterols, and all diets contained 54 per cent indigestible cellulose, except pc, which contained 75 per cent cellulose (Simpson & Abisgold 1985). Hence, two diets contained a nutritionally balanced ratio of P and C (1:1, which is close to the ratio that sustains maximal performance; Raubenheimer & Simpson 1993), with one diet (pc) diluted relative to the other (PC), whereas the other two diets were nutritionally imbalanced (pC, Pc). Blades of *Themeda triandra* and *Cynodon dactylon* were harvested locally.

(b) Experimental design

(i) Determination of enzyme activities

Newly moulted fifth-instar nymphs were collected (within 4 h of ecdysis) and weighed (0.01 mg) before being randomly allocated, within a design balanced by sex, time and diet, to a clear plastic box (17(l) × 12(w) × 6(h) cm) containing water, a metal perch and one of the four treatment diets ($n = 120$). All experiments were carried out at $30 \pm 0.5^\circ\text{C}$ under a 14L:10D photophase. On day 3 (where day of ecdysis is day 0), nymphs were observed during *ad libitum* feeding, then removed from their container and promptly killed at the start, upon completion or 30 min after a meal. The start of a meal was defined as feeding for 10 s, with a meal considered complete when a nymph fed for a minimum of 4 min and did not feed within a further 4 min period (Simpson *et al.* 1988). The GIT was removed by pulling the head gently to remove the entire GIT following severing between the last two abdominal sections and the head at the cervical membrane. The GIT was detached from the head at the mouth. The salivary glands (SGs) were then excised via a longitudinal incision through the dorsal surface of the thorax. The foregut (FG), caeca (CA), midgut (MG) and hindgut (HG) regions

were then separated. To prevent flow of the luminal contents, fine forceps were used to clamp the junction of each section of the GIT prior to detachment. Once detached, each section of the GIT was slit longitudinally to release the contents into 250 μl of insect saline (125 mM NaCl, 4 mM KCl, 5 mM CaCl_2 , 2 mM KH_2PO_4 , 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, pH 7.5). Following removal of its contents, each section of GIT was washed in 250 μl insect saline, then blotted dry, weighed (0.01 mg) and placed in 250 μl fresh insect saline. Stock suspensions of tissue and contents in 250 μl insect saline were then stored at -80°C until assayed for enzyme-like activity.

After enzyme assay trials had been conducted to determine the conditions required for linearity, stock suspensions of tissue and content samples were diluted 25- and 250-fold, respectively, with insect saline containing 3.2 mM *N*-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (*N*-succ-AAPF-*p*NA), 2.3 mM *N* α -benzoyl-DL-arginine 4-nitroanilide (BAPNA) or 3.3 mM 4-nitrophenyl α -D-glucopyranoside (α -PNG) for the α -chymotrypsin, trypsin and α -glucosidase assays, respectively (Sigma Aldrich, Castle Hill, New South Wales, Australia). For these three enzymes, the changes in absorbance at 405 nm were determined over a 30 min period and converted to nanomoles of *p*-nitroanilide produced per GIT sample ($\text{nmol } p\text{NA min}^{-1}$) using extinction coefficients (ϵ , $\mu\text{mol}^{-1} \text{ ml}$) as follows: for *N*-succ-AAPF-*p*NA, 0.8; BAPNA, 0.7; and α -PNG, 0.95, estimated from maximal absorbance readings obtained from reactions that had gone to completion over a 30 min period. α -Amylase-like activity was determined differently from the other enzymes studied. We used the EnzChek Ultra Amylase assay kit (E33651, Invitrogen) in a 96-well plate fluorimeter according to the manufacturer's instructions and referenced the activities obtained after 30 min incubations in insect saline at 25°C to a recommended α -amylase standard (*Bacillus* sp. Sigma A6380) in U ml^{-1} .

(ii) Feeding trials

To test whether the differential release of proteases and carbohydrases found in the synthetic diet experiments resulted in differences in absorption of nutrients from host plants, nymphs were confined to either of the unbalanced diets, Pc or pC, as described above, until day 3. On day 3, nymphs were fed for 3 h on blades of either *C. dactylon* or *T. triandra*. Grass blades were detached at the ligule and offered to the locusts. A known amount of grass was provided, and at the end of the 3 h feeding period, all remaining grass was removed and nymphs were allowed to feed on the initial conditioning diet (i.e. Pc or pC) until the meals of grass had passed through the GIT. At this point, the nymphs were sacrificed, all faeces were removed from the container, and faeces derived from grass were easily identified by colour and texture, and separated from the synthetic diet faeces. Intake of total dry matter was estimated from the ratio of fresh mass to dry mass (following lyophilization) determined from subsets of blades of grass from both species that were put aside, less the dry weight of the remaining grass. Protein and non-structural carbohydrates were determined from lyophilized samples of the grasses and faeces that had been finely ground in a Spex freezer/mill prior to analysis. Protein was extracted from replicate 10 mg samples with 0.1 M NaOH and determined using the Bio-Rad protein microassay for microtiter plates based on the Bradford assay. This assay was chosen as the Bradford reagent only responds to

protein and not amino acids or other nitrogenous waste products that are present in locust faeces. Although faecal protein from post-absorptive origin such as peritrophic membrane and digestive enzymes will be determined, this makes an extremely small contribution to total faecal nitrogen, which does not vary with the amount of protein absorbed (Zanotto *et al.* 1993); thus, differing amounts of protein in the faeces should reflect differences in absorption within the GIT. Total non-structural carbohydrate was determined colorimetrically from 10 mg replicate samples following extraction with 0.1 M H₂SO₄ (Smith *et al.* 1964) using the phenol–sulphuric assay (Dubois *et al.* 1956).

(c) Statistical analysis

The concentration of each enzyme in both locations (tissues and contents) was modelled separately (repeated-measures ANOVA) using a partly nested design (the GIT region within an insect) with diet treatment and time as ‘between’ factors. Time was removed from all subsequent analyses as no differences or interactions were observed for diet treatment or the GIT region ($p > 0.05$) for any enzyme or location. Where a significant effect of the diet treatment or interaction was found, enzyme concentration between diet treatments was investigated for each GIT region using ANCOVA with the initial wet weight of the insect as the covariate. Effects of dietary treatment on wet weight of GIT regions were also tested using ANCOVA with initial wet weight as a covariate. Sex was not treated as a separate factor as no differences in enzyme production or GIT weight were observed with sex other than that which can be explained by differences in body weight (tissues $F_{1,473} = 0.548$, $p = 0.459$; contents $F_{1,473} = 0.191$, $p = 0.662$). The chemical properties of the two grasses were compared using ANOVA with replicates being individual blades of grass ($n = 6$). Estimated median food retention time (mean duration between subsequent meals), intake and absorption were compared using ANOVA and ANCOVA where appropriate to avoid the pitfalls of ratio-based analyses (Raubenheimer 1995). Prior to all analyses, box plots were used to check for normality and homogeneity of variances across the treatments. ANOVA and ANCOVA were performed following the techniques outlined in Quinn & Keough (2002).

3. RESULTS

(a) Enzyme activities

Activities of enzymes in the tissues and GIT contents were not explained by the weight of the GIT, total food intake or by the amount of protein or carbohydrate ingested *per se* (for statistical outcomes, see electronic supplementary material, table S1 and fig. S1; figure 1). Instead the balance of nutrients affected enzyme-like activity for α -chymotrypsin and α -amylase in both the tissues and GIT contents (electronic supplementary material, tables S2 and S3). In the FG contents, where the activities of all enzymes were highest (electronic supplementary material, fig. S2), significantly less α -chymotrypsin-like activity was observed when excessive P was ingested relative to C (diet Pc, imbalanced), but no difference was found when P and C intake were balanced in their ratio (1:1) but varied in total concentration owing to dilution (diet PC versus pc; figure 1a,c). In contrast to the FG contents, locusts consuming pC had significantly more α -chymotrypsin-like activity in

the tissues of the FG and CA than those consuming any of the other diets (electronic supplementary material, table S3). Similarly, α -amylase-like activity was reduced in the contents of the FG when excessive C was ingested relative to P (pC, imbalanced), but did not vary with dilution of a near-optimal P to C ratio in the diet (PC versus pc) (figure 1b,d). Reduced α -amylase-like activity was found in the lumen contents from all regions of the GIT where locusts consumed pC (electronic supplementary material, table S3). This reduction was associated with less α -amylase-like activity being found in the SGs and was accompanied by a reduction in the mass of the SGs (figure 2). Neither trypsin-like activity nor α -glycosidase-like activity varied in the tissues or lumen contents in any of the diets for any region of the GIT (figure 1e,f; electronic supplementary material, table S3). In addition, no changes in weight were recorded for any other section of the GIT (electronic supplementary material, table S4).

The activity of all measured enzymes was approximately 40 times higher in the contents of the lumen than in the tissues, with time of sampling relative to feeding having no effect (electronic supplementary material, table S2 and fig. S2). In the GIT tissues, for all enzymes, activity varied along the GIT, with the highest levels of activity found in the CA followed by MG (approx. 50% of that for the CA). Negligible activities were found in tissues from other GIT regions for all enzymes except α -amylase, where similar levels of activity were found for all regions of the GIT as found in the MG (electronic supplementary material, fig. S2a–d). Within the GIT lumen, the highest activities were found in the FG followed by the MG and CA, which both displayed similar levels, approximately 50 per cent of that found in the FG, with negligible levels in the HG (electronic supplementary material, figure S2e–h). For the proteases, chymotrypsin and trypsin, α -chymotrypsin-like activity was 10 times greater than that of trypsin-like activity in both the tissues and contents.

(b) Feeding trials and nutrient absorption

Since results from the synthetic diet studies showed that a 3-day pretreatment on the high-protein, low-carbohydrate diet (Pc) and the low-protein, high-carbohydrate (pC) synthetic diet resulted in downregulation of α -chymotrypsin-like and α -amylase-like activity, respectively, we next tested whether these changes in enzyme activities affected nutrient absorption from two host grasses. Locusts that had previously consumed protein in excess of carbohydrate (Pc pretreated) absorbed less protein from the grasses per unit of protein intake ($F_{1,96} = 5.76$, $p = 0.018$) than locusts that had consumed excessive carbohydrate (pC pretreated; figure 3a). In an analogous fashion, less carbohydrate was absorbed per unit carbohydrate intake (Pc pretreated; $F_{1,96} = 4.01$, $p = 0.048$) by locusts that had consumed carbohydrate in excess of protein (pC pretreated) than locusts that were in carbohydrate debt (figure 3b). As a result of downregulation of digestive enzyme activities, the P:C ratio assimilated from the two grasses varied by around 25 per cent (figure 4a). This differential absorption of protein and carbohydrate allowed nymphs to shift their vector in nutrient space towards the ratio of nutrients where growth and development are optimized (figure 4b).

Figure 1. The amount of (a) protein and (b) carbohydrate consumed in the meal prior to determining enzyme activity. The four treatment diets contained combinations of high or low concentrations of protein and carbohydrate (both high, PC; both low, pc; high protein, low carbohydrate, Pc; low protein, high carbohydrate, pC). (c–e) Total enzyme-like activity of four digestive enzymes (α -chymotrypsin, trypsin, α -amylase, α -glucosidase) is shown in the FG contents, where activity levels for all enzymes were greatest and where the majority of digestion occurs in locusts. (c) α -Chymotrypsin-like activity and (d) α -amylase-like activity were strongly downregulated only when their nutrient substrate was present in the diet in excess relative to the other macronutrient (i.e. on Pc and pC for α -chymotrypsin and α -amylase, respectively). Treatment diet did not affect the activity of (e) trypsin-like activity or (f) α -glucosidase-like activity. The down arrows indicate a significant difference ($p < 0.05$) and bars with a line across the top are not statistically different. (c–f) Values are ANCOVA-adjusted (GIT weight) means $\pm$ s.e. (c) $p = 0.010$; (d) $p = 0.039$; (e) $p = 0.989$; (f) $p = 0.308$.

The differential uptake of protein and carbohydrate from the GIT occurred for both species of grass tested, although they differed chemically and were retained in the GIT for differing periods of time. *Cynodon dactylon* had a higher P:C ratio than *T. triandra* ($F_{1,10} = 16.66$, $p = 0.003$) because of differences in percentage of protein (9.8 ± 0.5 versus $7.2 \pm 0.5\%$, respectively; $F_{1,10} = 15.51$, $p = 0.003$), but not carbohydrate (22.6 ± 0.3 versus $21.8 \pm 0.4\%$, respectively; $F_{1,10} = 2.22$, $p = 0.173$) per

unit dry matter. Nymphs ingested more *T. triandra* than *C. dactylon* ($F_{1,89} = 39.05$, $p < 0.001$). Over the 3 h when locusts were feeding on the grasses, nymphs that had previously ingested excessive carbohydrate (pC pre-treated), where α -amylase-like activity but not α -chymotrypsin-like activity was downregulated, ingested ($F_{1,96} = 7.15$, $p = 0.009$) and assimilated ($F_{1,96} = 10.80$, $p = 0.001$) more nutrients irrespective of grass eaten ($F_{1,96} = 0.01$, $p = 0.93$), as meals were ingested

Figure 2. Effect of dietary treatments on the mass of the SGs. When carbohydrate was ingested in excess of protein (pC-fed insects), associated with the downregulation of α -amylase-like activity seen in figure 1*d* was a decrease of approximately 50 per cent in the mass of the SGs. The down arrow signifies a significant difference ($p < 0.05$) and symbols with a line across the top are not statistically different. Values are ANCOVA-adjusted (initial wet weight of the insect) means $\pm$ s.e.

more frequently ($F_{1,96} = 21.27$, $p < 0.001$). Owing to differences in intake, carbohydrate-deprived locusts (Pc pretreated) and protein-deprived locusts (pC pretreated) assimilated similar amounts of carbohydrate ($F_{1,96} = 0.40$, $p = 0.733$), but carbohydrate-deprived locusts (Pc) absorbed a lower ratio of P:C as less protein was assimilated over the 3 h the locusts were feeding on the grasses ($F_{1,96} = 7.20$, $p = 0.009$; figure 4*a*).

4. DISCUSSION

In contrast to the commonly accepted view that physiological and structural plasticity in the GIT serves to maximize absorption of all macronutrients, our results demonstrate that plasticity of digestive enzyme activity plays a role in regulating the balance of nutrients absorbed. Locusts were found to sacrifice maximal nutrient absorption rate for supplying a ratio of protein to carbohydrate that more closely approximated an optimal diet. Demonstrating the existence of regulatory digestion required precise manipulation of dietary composition to take account of both macronutrient concentration and ratio, as well as measuring enzyme activities in GIT tissues and lumen content. Additionally, we were able to show that plasticity in enzyme activity is functionally relevant by altering absorption of macronutrients from host plants (figures 3 and 4).

When locusts were fed diets that were imbalanced in the ratio of protein to carbohydrate, the activity of the enzyme for the nutrient consumed in excess was downregulated within the FG, where the activities of all enzymes were highest and where the majority of digestion is thought to occur (Evans & Payne 1964). However, this pattern was not reflected in either the tissues or contents for other regions of the GIT, which might help to explain some of the inconsistencies in previous studies. Other studies have reported what often seem to be arbitrary differences in the relative activities of proteases and/or carbohydrases in the GIT in response to variations in

Figure 3. (a) Protein and (b) carbohydrate absorbed from grass after a period of pretreatment on one of the two synthetic diets (Pc or pC) that caused downregulation of α -chymotrypsin-like activity (figure 1*c*) and α -amylase-like activity (figure 1*d*), respectively. Significantly less protein was absorbed per unit protein intake when α -chymotrypsin-like activity was downregulated (in Pc-pretreated locusts; $p = 0.018$), whereas when α -amylase-like activity was downregulated (pC-pretreated insects) significantly less carbohydrate was absorbed ($p = 0.048$). For reference, the grey bars and grey arrow on each graph show the relative differences in (a) α -chymotrypsin-like activity and (b) α -amylase-like activity, derived from figure 1. The black arrows show the relative decrease in (a) protein and (b) carbohydrate absorbed between the two treatments (i.e. downregulating α -amylase-like activity resulted in a bigger reduction in carbohydrate absorption than downregulation of α -chymotrypsin-like activity on protein absorption). Data from the two grass species, *T. triandra* and *C. dactylon*, have been combined.

diet composition (e.g. Sabat *et al.* 1999; Caviedes-Vidal *et al.* 2000; Kotkar *et al.* 2009). It may not be possible to interpret the effect of variable enzyme activities in the tissues of the GIT on subsequent nutrient absorption, as high enzyme levels in the tissues may result from increased synthesis or synthesized enzymes not being released, with starved insects often exhibiting high enzyme levels in the tissues of the GIT (e.g. Woodring *et al.* 2007). Inconsistencies reported in previous studies might also have arisen from the use of natural foods,

Figure 4. (a) The bi-coordinate trajectories in P–C space showing the absorption of protein and carbohydrate by locusts feeding on either *T. triandra* or *C. dactylon* over 3 h following confinement to Pc (dashed line) or pC (solid line) from day 0 (ecdysis) to day 3. The P : C ratio of each grass is shown, with *C. dactylon* having a lower ratio of P : C than *T. triandra*. On both grasses, locusts treated on Pc, in which α -chymotrypsin-like activity was downregulated, absorbed significantly less protein than locusts treated on pC. Although locusts treated on pC, in which α -amylase-like activity was downregulated, absorbed less carbohydrate per unit intake (figure 2), over the 3 h feeding period the amount of carbohydrate absorbed was the same from both grasses owing to increased rates of intake for pC locusts. (b) As a result of differential release of protease and amylase, nymphs were able to redress their nutrient imbalances, as demonstrated by the bolder arrows in this cartoon representation. The locusts have a specific protein to carbohydrate (P : C) requirement (the nutrient target, indicated as a bullseye symbol on the long-dashed grey line), which, if ingested, optimizes growth and development. This is known to lie close to a P : C ratio of 1 : 1 (Chambers *et al.* 1995). When restricted to eating either Pc or pC (indicated by the short-dashed lines), locusts are diverted away from the nutrient target. The two thin black arrows represent the change in nutrient absorption trajectory resulting from the transfer from either pC or Pc synthetic diets onto grass (an average of the two grass species is used). The thick black arrows indicate the extra advantage in terms of shifting the trajectory towards the target provided by plasticity in enzyme activity.

imposing confounds where variations both in the type and amount of enzyme can result from the presence of enzyme inhibitors (e.g. Broadway 1997; Jongsma & Bolter 1997), variations in the monomer composition of complex polymers (e.g. Lopes *et al.* 2004; Sato *et al.* 2008), and where the concentrations and ratio of nutrients absorbed from natural foods are unknown (Clissold *et al.* 2006).

It is striking that, in the current study, downregulation of enzyme activity only occurred when the substrate nutrient (protein or carbohydrate) was present in excess relative to the other macronutrient. Thus, increasing the concentration of protein and carbohydrate in unison (PC) did not evoke a change in enzyme activity relative to that evoked by a diet containing the same ratio of nutrients but diluted with cellulose (pc). This has been previously reported in relation to other post-ingestive components of nutrient regulation. Facultative diet-induced thermogenesis as a means of burning off excess ingested carbohydrate was elevated only when carbohydrate was in excess and protein in deficit (Zanotto *et al.* 1997; Jensen & Hessen 2007; see also Jensen & Hessen 2007 for another example in *Daphnia*). Similarly, elevated rates of nitrogen excretion occur mainly when locusts are fed high-protein, low-carbohydrate diets (Zanotto *et al.* 1993). The reason for all these responses is clear: provided that the ratio of protein to carbohydrate in the diet is close to balanced (a 1 : 1 P : C ratio is close to the intake target for locusts; e.g. Chambers *et al.* 1995), then animals can maintain an optimal rate of nutrient

supply by changes in rates of intake; but when the ratio of nutrients is imbalanced, the only means of achieving nutritional balance is post-ingestively (Raubenheimer & Simpson 1993; Simpson & Raubenheimer 1993).

The relatively low levels of tryptic activity when compared with chymotryptic activity in the present study were surprising. Tryptic activity was tenfold less than that found for α -chymotrypsin-like activity and even when downregulated, α -chymotryptic activity was still seven to eight times higher than tryptic activities (figure 1). Both trypsin and α -chymotrypsin occur as multiple isoforms in *L. migratoria* and other insects (Lam *et al.* 1999, 2000; Lopes *et al.* 2006; Sato *et al.* 2008). While the use of substrates with poor affinity for the predominant isoforms has been responsible for underestimating levels of enzyme activity, especially that of chymotrypsin (Baumann 1990; Johnston *et al.* 1991, 1995; Christeller *et al.* 1992; Peterson *et al.* 1995), the substrates we used in this study have previously been shown to be suitable for trypsin-like and α -chymotrypsin-like activity from *L. migratoria* GITs (Lam *et al.* 1999, 2000). Chymotrypsins in insects appear to have broader substrate specificities than vertebrate chymotrypsin (e.g. Sato *et al.* 2008) and, in consequence, significant tryptic activity may not be required for efficient digestion of proteins.

Downregulation of digestive enzyme activity in the FG in response to nutrient substrate excess had a more pronounced effect on the absorption of carbohydrate than

protein from host grasses (figure 3), perhaps owing to factors regulating food throughput. In locusts, the rate of GIT emptying is influenced by blood-borne metabolic feedbacks for protein (i.e. amino acids; Simpson & Simpson 1992; Simpson & Raubenheimer 2000). Consequently, decreased activity of carbohydrases should decrease the amount of carbohydrate assimilated per unit protein as the GIT will empty before all the carbohydrate can be assimilated. However, decreased protease activity is likely to lead to longer intermeal intervals (as seen in locusts consuming high-protein diets; Simpson & Abisgold 1985), allowing additional time for carbohydrate to be assimilated. Consistent with such an explanation, locusts in which α -chymotrypsin-like activity was downregulated in the FG (those pretreated on Pc) passed food through the GIT more slowly than locusts with higher levels of α -chymotrypsin-like activity (pC-pretreated locusts).

When post-ingestive regulatory responses were first described for carbohydrate and protein in locusts, the suggestion was made that differential digestive responses may play a role through release of fixed amounts and ratios of digestive enzymes, such that nutrients in excess in the food would be voided from the GIT undigested, whereas scarce nutrients would be digested and absorbed (Raubenheimer & Simpson 1993). However, the majority of studies appeared to indicate secretagogue enzyme responses to substrate concentrations. Because regulation of intake is based on systemic responses to the products of digestion, amino acids and monosaccharides (Simpson & Raubenheimer 1993, 1996), it was reasoned that perhaps animals maximize uptake of all nutrients from the GIT and that post-ingestive regulatory responses occur once nutrients enter the blood (Simpson *et al.* 1995). The current findings indicate that post-ingestive nutrient regulation does occur at the level of the GIT, in a more active way than originally suggested (Raubenheimer & Simpson 1993).

How might regulatory enzyme secretion be controlled? In both mammals and invertebrates, rapid (less than 4 h) changes in enzyme activities in response to diet have been reported (e.g. insect, Broadway 1997; crustacean, Pan *et al.* 2005; mammal, Reisenauer & Gray 1985). Endocrine cells in the insect MG monitor the nutritional status of ingested food and of the haemolymph, and modulate enzyme activity accordingly (Fuse *et al.* 1999; Bede *et al.* 2007). The diffuse endocrine system of the insect MG is considered to be analogous to the gastroenteropancreatic system of vertebrates (Montuenga *et al.* 1989). In *L. migratoria*, one key cell type of the diffuse endocrine system that is ideally placed to play a coordinating role in linking nutritional state to GIT function are the cells located in the ampullae of the malpighian tubules. These cells have processes that make contact with the GIT contents, the haemolymph and the primary urine, and release FMRFamides as a function of stage in the feeding cycle and the nutritional quality (protein and carbohydrate ratio and concentration) of the food (Zudaire *et al.* 1998). Dietary quality also affects DNA synthesis and may thus impact structural as well as enzymatic changes in the GIT with diet composition (Zudaire *et al.* 2004). Structural remodelling of the GIT over longer time scales (e.g. Yang & Joern 1994b; Raubenheimer & Bassil 2007; Sorensen *et al.* 2010) may alter rates of

absorption via increases in nutrient transporters (Karasov 1992). Consequently, the GIT plays an important role in regulating balanced nutrient absorption in both the short and the longer term, the mechanisms of which are only beginning to be understood.

We did not observe changes in the mass of the GIT under the conditions of the present study, presumably owing to the relatively short period of dietary pretreatment compared with previous studies (Raubenheimer & Bassil 2007). However, when carbohydrate was over-ingested relative to protein, the mass of the SGs was reduced, consistent with the reduction in α -amylase-like activity. On the other hand, α -glucosidase activity was unchanged. Amylase hydrolyses starch to disaccharide units, which are reduced to the absorbable monosaccharides by glucosidases (Terra & Ferreira 1994). Locusts can only use non-structural carbohydrates (Clissold *et al.* 2004), and in the tillers of *C₄* grasses starch typically represents 60 to 90 per cent of non-structural carbohydrates (Gibson 2009). Thus, downregulation of α -amylase activity would provide effective control of carbohydrate absorption by limiting hydrolysis of polysaccharides so that they pass through the GIT intact. A similar situation may exist for protein. Proteases, such as chymotrypsin, cleave proteins to oligopeptides, which are acted on by aminopeptidases, carboxypeptidases and/or dipeptidases to produce single amino acids that are absorbed (Terra & Ferreira 1994). This requires further investigation.

Finally, we hypothesize that animals constrained to a particular food source should show greater compensatory plasticity in the activity of digestive enzymes than animals that can use food selection to achieve an optimal nutrient balance. Dietary restriction might arise from host specialization, low mobility, presence of natural enemies or microclimatic conditions (e.g. Stamp 1997; Lima 1998; Niesenbaum & Kluger 2006; Richards & Coley 2007). An extreme example of an animal with a restricted diet is the web-building desert spider, *Stegodyphus lineatus*, which compensates for nutritional imbalances by the selective extraction of nitrogen relative to carbon from individual prey items (Mayntz *et al.* 2005), presumably through the differential injection of digestive enzymes into their prey during feeding. By contrast, the host plant generalist lepidopteran, *Spodoptera exigua*, showed no such compensatory plasticity in relation to one salivary component, glucose oxidase (GOX). GOX is released prior to ingestion and converts glucose to gluconate, which the caterpillar is unable to absorb. Babic *et al.* (2008) proposed that caterpillars could use GOX to adjust the ratio of ingested nutrients in foods containing excessive carbohydrate. However, neither increased GOX activity nor gluconate in the faeces was recorded when diets were consumed that contained excessive carbohydrate relative to protein (Babic *et al.* 2008).

As a result of the current study, the digestive enzyme system of the GIT can now be considered a key member of the arsenal of regulatory mechanisms employed by animals to achieve nutrient balance. Together, behavioural, digestive, absorptive, metabolic and excretory responses offer an exquisitely coordinated suite of mechanisms. Understanding how these components are deployed by animals in relation to their ecology, life history and phylogeny would appear to be a fertile area for future research.

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