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Altered dietary nutrient intake maintains metabolic homeostasis in parasitized larvae of the insect *Manduca sexta* L.

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Summary

***Manduca sexta* larvae exhibited altered food selection over a 2- or 3-day feeding period when parasitized by *Cotesia congregata*, and offered a choice of two chemically defined diets, one containing casein without sucrose and a second with sucrose but no casein. While normal larvae consumed the diets in a ratio of approximately 2:1 protein:carbohydrate (w/w), parasitized insects consumed a ratio of approximately 1:1. The altered nutrient ratio consumed by parasitized insects was principally due to a decrease in consumption of the protein diet, and was only partially explained by their lower growth. Conditioning larvae for 1 day to either one of the choice diets had little effect on subsequent dietary intake over a 2-day feeding period. Conditioned larvae, regardless of parasitism, initially fed on the opposite diet immediately after conditioning. Although this suggests that the altered nutrient intake displayed by parasitized insects was not due to any failure in their capacity for dietary selection, these results do not definitively demonstrate an altered nutrient intake target by parasitized larvae. Rather, parasitism may compromise dietary selection, resulting in random feeding. When parasitized larvae were maintained on several isocaloric diets with a varying ratio of casein and sucrose, those larvae feeding on the diet with a ratio of 1:1 of these nutrients supported the largest parasite population. Previous investigation of larvae maintained on a single artificial diet established that parasitized insects display an aberrant induction of gluconeogenesis, so that haemolymph trehalose is maintained at a level equivalent to that of normal insects. In contrast, the present results demonstrated that parasitized larvae offered a choice of diets, and feeding at the altered nutrient ratio above, maintain haemolymph sugar but have the same level of gluconeogenesis as normal larvae given the same dietary choice. These investigations suggest that altered food selection by parasitized *M. sexta* larvae maintains metabolic homeostasis and, moreover, may be adaptive for *C. congregata*, potentially maximizing the number of parasites developing in a single host larva.**

Key words: nutrition, metabolic homeostasis, dietary intake, trehalose, gluconeogenesis, parasite, *Manduca sexta*, *Cotesia congregata*.

Introduction

Many insects when presented with a variety of natural foods will select and feed on one or a combination that results in a balanced intake of nutrients (Waldbauer and Friedman, 1991; Simpson et al., 1995). Laboratory studies of lepidopteran larvae feeding on artificial diets have confirmed these observations and demonstrated that larvae conditioned by feeding on a diet deficient in one or more nutrients, will later compensate by selecting an equally unbalanced diet that is high in those previously deficient nutrients (Waldbauer et al., 1984; Simpson et al., 1988). This selection behaviour is aimed at achieving a nutrient ‘intake target’ and optimal growth under specific physiological and environmental conditions (Schiff et al., 1989; Simpson and Raubenheimer, 1996). One may predict that larvae offered a number of diets, each nutritionally inadequate, will over a long developmental period consume amounts of nutrients as near as possible to the optimal balance (Simpson and Raubenheimer, 1993a; Raubenheimer and Simpson, 1999).

Intake of specific nutrients by insects is regulated through feedback mechanisms (Simpson and Raubenheimer, 1996). In lepidopteran larvae, regulation of nutrient intake involves the central nervous system (Rowell and Simpson, 1992), as well as the action of various biogenic amines, for example, serotonin (Cohen et al., 1988). The chemical composition of the haemolymph, or blood, provides a continuous reading of the insect’s nutritional and metabolic state (Simpson and Raubenheimer, 1993b). The insect fat body, the principal organ for metabolic processing of digestive products following absorption, is also responsible for synthesis of the principal haemolymph sugar, trehalose, a disaccharide of glucose, and for the synthesis and storage of glycogen and fat (Keeley, 1985). Because the fat body lacks a mesoderm-derived lining,

the haemolymph generally contains high levels of metabolites that reflect fat body intermediary metabolism (Mullens, 1985). The level of haemolymph trehalose is important for regulating carbohydrate intake. Friedman et al. (1991) observed dietary selection behavior in relation to trehalose by the lepidopteran insect *Helicoverpa zea* (Boddie). They reported that larvae with low haemolymph trehalose concentrations choose a diet high in carbohydrate rather than a diet high in protein, and demonstrated that this choice could be reversed by injection of trehalose to raise haemolymph sugar.

We have conducted investigations to examine the nature of dietary selection behaviour, and to determine how haemolymph trehalose level alters, or is altered by dietary intake, in fifth-instar larvae of *Manduca sexta* L. (Thompson and Redak, 2000). Commonly called the tobacco hornworm, *M. sexta* larvae are pests of tobacco, as well as many ornamental and garden plants (Reinecke et al., 1980). Larvae maintained on a semi-synthetic artificial diet (Yamamoto, 1969) supplemented with different amounts of sucrose and casein displayed variable haemolymph trehalose levels, depending on the relative amounts of these nutrients. When subsequently presented with a choice of diets containing either sucrose without casein or casein without sucrose, most larvae with low haemolymph trehalose, approximately 20 mmol l^{-1} or less, selected the high carbohydrate diet, while those with high haemolymph trehalose, approximately 30 mmol l^{-1} or more, selected the high protein diet. In the first case, larvae switched over to the high protein diet, presumably having reached a threshold trehalose level of between 20 and 30 mmol l^{-1}. All larvae, regardless of the conditioning treatment, ultimately consumed an average of approximately 2:1 casein:sucrose (w/w) over the entire developmental stadium.

Studies have also been conducted recently to examine the effects of parasitism by *Cotesia congregata* (Say) (= *Apanteles congregatus*) on the metabolism of *M. sexta* larvae (Thompson and Dahlman, 1999). *C. congregata* is a gregarious hymenopteran parasitoid (Fulton, 1940). The adult female oviposits into the haemocoel or body cavity of host larvae, the eggs hatch and the parasite larvae feed on the nutrient-rich host haemolymph. The host continues to feed and develop, but at reduced rates. Fully mature second instar parasite larvae penetrate through the cuticle of the host to the outside. At emergence these parasites moult into third instar larvae that spin cocoons, attach to the external host cuticle and metamorphose to the pupal stage. Parasitized host larvae displayed an equivalent or higher haemolymph trehalose level than normal unparasitized individuals. The trehalose concentration in parasitized insects is maintained by a significant elevation of gluconeogenesis (Thompson and Dahlman, 1998; Thompson, 2000a), the *de novo* or net formation of carbohydrate from amino acid (Mathews and van Holde, 1990). This metabolic alteration may reflect a redirection of nutritional resources from host growth to provide a suitable nutritional balance for the developing parasite larvae.

During these experiments, parasitized and normal larvae were both maintained under identical nutritional conditions. Effects of an altered intake of specific nutrients on metabolism, for example the relative amounts of carbohydrate and protein, were not considered. It may be, for example, that consumption by parasitized larvae of a diet higher in carbohydrate would avoid the costly energetic expenditure of *de novo* haemolymph sugar formation and in that manner be adaptive for parasite development.

Lower growth and decreased total food consumption have been reported for numerous parasitized lepidopteran larvae (Adamo, 1998; Poulin, 1995; Quickie, 1997; Thompson and Hagen, 1999). Studies aimed at determining how nutritional quality affects host and parasite growth and development, however, are lacking. Because nothing is known about possible changes in the intake of specific nutrients in response to parasitism, or how such changes may mediate the physiology of the parasitized host, the present study was conducted to examine the effects of parasitism on nutrient intake and carbohydrate metabolism by fifth-instar *M. sexta*, and to determine the interactions between nutrient intake, metabolism and growth. Moreover, we examined the above interactions in normal and parasitized *M. sexta* larvae conditioned at the beginning of the fifth instar on diets of different nutrient composition, since previous studies by others demonstrated that conditioning or prior dietary experience within a stadium may affect subsequent nutrient intake by lepidopteran larvae (Simpson et al., 1988; Friedman et al., 1991).

Materials and methods

Insect culture and composition of diets

Stock colonies of *Manduca sexta* L. were reared at 28 °C under a 16 h:8 h light:dark photocycle on an artificial diet principally containing Torula yeast and wheat germ (Bell and Joachim, 1976). During the experiments, insects were reared through the fourth stadium on the above rearing diet, and upon moulting to the fifth stadium were transferred onto a chemically defined synthetic diet (Ahmad et al., 1989), modified to contain casein and sucrose as the principal nutrient sources of protein and carbohydrate, respectively. These nutrients were included at 120 g l^{-1}. In addition, the diet contained: Wesson's salts, 15 g l^{-1}; cholesterol, 4 g l^{-1}; ascorbic acid, 6.7 g l^{-1}; cysteine HCl, 1 g l^{-1}; a B vitamin mixture, 215 mg l^{-1}; inositol, 240 mg l^{-1}; choline chloride, 98 mg l^{-1}; β carotene, 240 mg l^{-1} and linseed oil, 3.7 ml l^{-1}. Sorbic acid, 2 g l^{-1}; methyl-*p*-hydroxybenzoate, 2 g l^{-1} and 32 ml l^{-1} of 10 % formalin were included as antimicrobial agents. The nutrients were principally obtained from Nutritional Biochemicals (Cleveland, OH, USA) and Bioserve (Frenchtown, NJ, USA).

Stock colonies of the parasite, *Cotesia congregata* Say, were reared on host larvae parasitized in the second stadium. Adult parasites were maintained in 3 l glass jars closed over the top with muslin fabric. Adults were fed honey spread on small cotton balls placed on the bottom of the jar, and were provided with water in small vials with cotton roll wicks. For the nutritional investigations, pharate, developmentally synchronous fourth-instar host larvae (Beckage et al., 1994)

were placed individually in a jar of parasites and carefully observed. Larvae were parasitized 2–4 times, to ensure that sufficient eggs were deposited in each host for maximum parasite load, i.e. the maximum number of emerged parasites, for fourth-instar *M sexta*, as reported by others (Alleyne and Beckage, 1997a). It was not possible to regulate, or to know precisely the number of times that individual hosts were parasitized. It may be, therefore, that some individuals within a single treatment group received more parasite eggs than others in the same group. However overall, within each experiment, insects for all treatments were parasitized in the same fashion and at the same time, suggesting the likelihood that the distribution of parasite eggs was similar among individuals within all groups. Moreover, after being parasitized, host larvae subsequently used for the dietary selection experiments were returned to the normal wheatgerm rearing diet and, upon moulting to the fifth stadium, were randomly selected and placed on the experimental diets. Following the feeding experiments, parasitized larvae were dissected to observe the presence of developing parasites and confirm that successful parasitism had occurred.

Dietary selection

Unconditioned selection

Newly moulted fifth-instar *M. sexta* larvae were individually offered small blocks, approximately 8 cm^3, of the experimental choice diets, i.e. the protein diet and the sucrose diet, in a disposable plastic Petri plate (14 cm diameter). The two diet blocks were arranged approximately 5 cm apart, each an equal distance from the center of the plate. Individual larvae were then placed between the two blocks, in the center of the plate, and aligned with the blocks to prevent any bias toward either block. 20 larvae were fed for 2 days, then weighed and the amount of each diet consumed determined. Because subsequent experiments on the effects of conditioning larvae on selection involved a total feeding period of 3 days, a second non-conditioning experiment was conducted with larvae feeding for 3 days. Values were recorded as wet mass in both experiments. Dehydration of the choice diets amounted to less than 10 % over the feeding period. This water loss was monitored in Petri plates containing the diets but without larvae, and was accounted for at the end of the experiments in determining the diet consumed by the larvae (Simpson et al., 1988).

We assumed that the feeding behaviour of newly moulted fifth-instar larvae was unaffected by prior dietary experience in earlier stadia. Other studies, however, have indicated that this may not be the case, and that only newly hatched first instar larvae are truly naïve (Stadler and Hanson, 1978). Newly moulted fifth-instar *M. sexta* larvae reared on plant material subsequently demonstrated strong preferences for the plant or extracts of the plant on which they had been maintained earlier, and this was recently confirmed (Campo et al., 2001). Stadler and Hanson (1978) also reported that larvae reared on a wheatgerm-based artificial rearing diet lacking plant material displayed no preference for the artificial diet or for any other food source tested after moulting to the fifth stadium. Although recent studies with fifth-instar *M. sexta* demonstrate that nutritional experience in earlier stadia affects consumption and growth following moulting regardless of dietary composition (Wood, 1999), all larvae used in the present studies were reared on the same diet until the fifth stadium. In our view, therefore, the assumption of nutritional naivety for newly moulted fifth-instar larvae used in the conditioning experiments was reasonable.

Conditioned selection

Newly moulted fifth-instar larvae were transferred from the rearing diet to one of the two choice diets, containing either casein or sucrose, and were conditioned by feeding on the diet for 1 day in a Petri plate. Following the conditioning period, the larvae were individually given the choice of the two diets presented together as described above, and were allowed to feed for an additional 2 days. Duplicate experiments, each consisting of 20 or 25 larvae, were carried out and data were collected as described above.

Conditioned short-term selection

The short-term effect of conditioning on subsequent feeding was examined in a similar manner to that described (Friedman et al., 1991). Groups of 20 newly moulted fifth-instar larvae were placed on either the sucrose diet or the casein diet for 1 day as above. Following this conditioning period, larvae were transferred to Petri plates containing a small block of each diet and were allowed to self-select for 4 h. The proportion of larvae feeding on each diet was recorded at 10 min intervals for the first 1.5 h, and then every 20 min for an additional 2.5 h. At any one time, 2–5 larvae were not feeding on either diet and these larvae were not included in the calculations. Data were plotted as the percentage of larvae feeding on each diet *versus* time, with the two relationships, carbohydrate and protein, inversely related.

All dietary selection experiments were conducted at room temperature, approximately 21 °C.

Host nutritional status and parasite burden

To examine the effect of the ratio of dietary protein to carbohydrate on parasite growth and development, groups of seven *M. sexta* larvae were parasitized as described above and immediately transferred onto diets with the following ratios (w/w) of casein to sucrose: 0.25:1.75, 0.50:1.50, 1.00:1.00, 1.50:0.50 and 2.00:0. The ratios were based on the amount of casein and sucrose in the stock chemically defined diet, which contained each nutrient at a level of 90 $g\,l^{-1}$. All diets were isocaloric and contained the same total amount of nutrient. During the fifth stadium, as parasite emergence ensued for insects on each diet, the experiment was terminated and the host larvae were dissected. The parasite burden, the total number and mass of parasites emerged as well as mature second-instar parasites that had not yet emerged, was determined. The amount of diet consumed by each host larvae

was also determined. Parasite mass and the amount of casein and sucrose in each diet consumed were expressed as dry mass.

Estimation of gluconeogenesis and haemolymph trehalose level

The net gluconeogenic flux of normal and parasitized *M. sexta* larvae, allowed to self-select between the casein and sucrose diets as described above, was determined by nuclear magnetic resonance spectroscopic (NMR) analysis of pyruvate cycling and the ^{13}C-enrichment of alanine and trehalose following administration of [2-^{13}C]pyruvate, as recently described (Thompson, 2000b). Briefly, alanine was ^{13}C-enriched at C2 and C3 by transamination of [2,3-^{13}C]pyruvate formed following carboxylation of the administered isotopically substituted substrate to oxaloacetate, randomization of the ^{13}C-enrichment at the fumarase-catalyzed step of the tricarboxylic acid cycle (TCA) cycle and formation of [2,3-^{13}C]phosphoenolpyruvate. The alanine C3/C2 ^{13}C-enrichment ratio is a measure of the extent of pyruvate cycling. If randomization of ^{13}C within the TCA cycle is similar between treatment groups then their alanine ratios are directly comparable. The degree of ^{13}C-randomization is indicated from the enrichment of glutamate, glutamine and the glutamyl moiety of glutathione (Gl_x), which are sequential byproducts of TCA cycle metabolism following randomization.

Subsequent to the formation of [2,3-^{13}C]phosphoenolpyruvate, glucose and trehalose are synthesized *via* the gluconeogenic pathway. Phosphoenolpyruvate C3 gives rise to trehalose C1 and C6, while phosphoenolpyruvate C2 gives C2 and C5 of trehalose. The ^{13}C-enrichment of trehalose C1 and C6 relative to that of alanine C3 is a measure of the gluconeogenic flux relative to the glycolytic flux, as both the trehalose and the alanine ^{13}C-enrichments are derived in the same fashion (Thompson, 2000b). This metabolism is summarized in Fig. 8. Due to pentose cycling following gluconeogenesis, however, the ^{13}C-enrichment of trehalose C1 is reduced relative to that of C6 (Thompson, 1999), and the ^{13}C ratio [2trehalose C6/alanine C3] was employed as the measure of gluconeogenic flux, trehalose C6 being unaffected by pentose cycling.

Haemolymph trehalose concentration was estimated by comparing the NMR signal intensities of the individual carbons of trehalose with those of an internal standard, assuming a natural ^{13}C abundance of 1.1 % for trehalose C4. The abundance of ^{13}C in trehalose C2 and C5 was estimated in relation to trehalose C1 and C6 using the Gl_x C2/C3 ^{13}C-enrichment ratio that reflects the non-symmetric ^{13}C-distribution in trehalose. In this case, any contribution of cytoplasmic carboxylation to trehalose formation was not considered. Previous investigation indicated a significant, but very small, cytoplasmic contribution (Thompson and Redak, 2000).

Administration of [2-^{13}C]pyruvate, preparation of haemolymph and NMR analysis

[2-^{13}C]pyruvate (=99 atom %), obtained from Cambridge Isotope Laboratories (Woburn, MA, USA), was administered to normal and parasitized larvae by injection (50 μmol g^{-1} fresh mass) of an aqueous solution (1 mg 5 μl^{-1}) into the dorsal vessel. Haemolymph was collected from incisions in the prolegs 3.5 h post-injection at steady state, as previously outlined (Thompson and Redak, 2000). Haemolymph samples were deproteinized with perchloric acid, neutralized and analyzed by NMR (Thompson, 2000a; Thompson, 2000b). Duplicate experiments were conducted.

NMR analyses were conducted in a GE QE 300 spectrometer at 75.48 MHz as outlined previously (Thompson, 2000a,b)). Quantitation of signal intensities was conducted by the method of Christensen et al. (1974). ^{13}C-enrichment ratios were calculated as described above.

Statistical analyses

Unconditioned dietary selection

To determine the effect of parasitism on growth and consumption of *M. sexta* larvae, one-way analysis of covariance (ANCOVA) was utilized with initial larval mass as the covariate, and parasitism as the main treatment effect (parasitized *versus* normal) (Table 1). Throughout the statistical analyses the initial mass employed was the mass of larvae at the beginning of the fifth stadium and after parasitization. Parasitized and normal larvae have similar masses at this time. Dependent variables estimating growth (final mass including the parasite biomass) and consumption (consumption of protein and carbohydrate diets) were analyzed separately. An ANCOVA was employed to correct for any potential size effect on the dependent variables. Treatments were applied to a replicate group of 20 insects.

Conditioned dietary selection

To determine the effects of parasitism with dietary conditioning on growth and consumption of *M. sexta* larvae, two-way ANCOVA was utilized with initial mass as the covariate. Parasitism (parasitized *versus* normal) and type of conditioning diet (carbohydrate *versus* protein) were considered as main-effect treatments. Dependent variables estimating growth and consumption were analyzed separately, with each treatment applied to a replicate group of 20 insects.

The results from the conditioning experiment indicated that diet consumption, and growth (final mass) were affected by parasitism (Table 2). As with other nutritional studies (Blau et al., 1978; Horton and Redak, 1993), our data showed that the effect of parasitism on consumption was difficult to evaluate due to the known effect of size on consumption, with larger insects consuming more food. Consequently, to distinguish the effects of parasitism and conditioning from size-mediated treatment effects on consumption of protein diet and carbohydrate diet, separate two-way ANCOVAs were utilized with final larval mass as the covariate, and parasitism and dietary conditioning as the main treatment effects. To provide for the most general model, the data from the duplicate experiments were combined.

Table 1. *ANCOVA summary demonstrating the effects of parasitism on diet consumption and growth of the host* Manduca sexta *given a dietary choice (see Fig. 1)*

	d.f.	Mean square	*F*	*P*
Experiment 1				
Protein diet consumption				
Parasitism	1	81.7229	30.94	<0.0001
Initial mass (covariate)	1	2.5012	0.95	=0.3368
Error	37	2.6411		
Carbohydrate diet consumption				
Parasitism	1	0.4742	0.19	=0.6640
Initial mass (covariate)	1	4.8111	1.95	=0.1714
Error	37	2.4732		
Final mass				
Parasitism	1	20.3165	33.05	<0.0001
Initial mass (covariate)	1	2.9188	4.75	=0.0358
Error	37	0.6148		
Experiment 2				
Protein diet consumption				
Parasitism	1	228.4575	43.58	<0.0001
Initial mass (covariate)	1	7.2389	1.38	=0.2474
Error	37	5.2420		
Carbohydrate diet consumption				
Parasitism	1	22.1017	8.90	=0.0050
Initial mass (covariate)	1	1.2931	0.52	=0.4750
Error	37	2.4823		
Final mass				
Parasitism	1	5.8198	4.86	=0.0337
Initial mass (covariate)	1	14.6370	12.23	=0.0012
Error	37	1.1966		

Table 2. *ANCOVA summary demonstrating the effects of dietary conditioning and parasitism on diet consumption and growth of the host* Manduca sexta *given a dietary choice (see Fig. 2)*

	d.f.	Mean square	*F*	*P*
Experiment 1				
Protein diet consumption				
Conditioning	1	0.8976	0.17	=0.6817
Parasitism	1	692.8982	130.84	<0.0001
Interaction – conditioning/ parasitism	1	0.0278	0.01	=0.9425
Initial mass (covariate)	1	7.0081	1.32	=0.2537
Error	75	5.2958		
Carbohydrate diet consumption				
Conditioning	1	0.7004	0.21	=0.6473
Parasitism	1	37.06104	11.16	=0.0013
Interaction – conditioning/ parasitism	1	3.4188	1.03	=0.3135
Initial mass (covariate)	1	1.3227	0.40	=0.5298
Error	75	3.3197		
Final mass				
Conditioning	1	0.6715	0.95	=0.3328
Parasitism	1	34.2878	48.52	<0.0001
Interaction – conditioning/ parasitism	1	0.7386	1.05	=0.3099
Initial mass (covariate)	1	1.3390	1.89	=0.1727
Error	75	0.7067		
Experiment 2				
Protein diet consumption				
Conditioning	1	0.0017	0	=0.9819
Parasitism	1	381.2731	119.1	<0.0001
Interaction – conditioning/ parasitism	1	5.0649	1.58	=0.2117
Initial mass (covariate)	1	45.2194	14.11	=0.0003
Error	95	3.2037		
Carbohydrate diet consumption		24.2545	15.27	=0.0002
Conditioning	1	8.2112	5.17	=0.0253
Parasitism	1	0.0956	0.06	=0.8067
Interaction – conditioning/ parasitism	1	0.2715	0.17	=0.6803
Initial mass (covariate)	1	1.5887		
Error	95			
Final mass				
Conditioning	1	14.4758	20.23	<0.0001
Parasitism	1	49.1065	68.62	<0.0001
Interaction – conditioning/ parasitism	1	2.7825	3.89	=0.0515
Initial mass (covariate)	1	27.3097	38.16	<0.0001
Error	95	0.7156		

Host nutritional status and parasite burden

The data for the total number of parasites in hosts maintained on the various diets were examined by the Kruskall–Wallis non-parametric analysis of variance (ANOVA) with the ratio of dietary casein to sucrose used as a main-effect treatment at five levels (casein:sucrose, 0.25:1.75, 0.50:1.50, 1.00:1.00, 1.50:0.50 and 2.00:0). Treatments were replicated 5–7 times. After establishing a significant difference among ratios, the Nemenyi multiple-range test was applied to determine significant differences between means. In addition, three-dimensional surface plots were generated to visualize the relationship between dietary levels of casein and sucrose with the parasite burden. These plots were created using SAS (version 8, 1999, SAS Institute Inc. Cary, NC, USA). The data were used to create a matrix of 900 points by interpolating a simple linear function describing the relationship between dietary nutrient levels onto a rectangular grid estimating parasite burden (PROC G3GRID). The resulting 900 point data matrix was subsequently plotted in three dimensions (PROC G3D) or as a contoured surface (PROC GCONTOUR).

In the case of parasite mass, the data were analyzed by one-way ANOVA after determining that there was no relationship between parasite mass and initial host mass. Following a significant treatment effect, data were subjected to the Ryan–Einot–Gabriel–Welch multiple-range test.

Pyruvate cycling and gluconeogenesis

To evaluate the effects of parasitism, one-way ANOVA was conducted on the data for estimated percentage ^{13}C content in

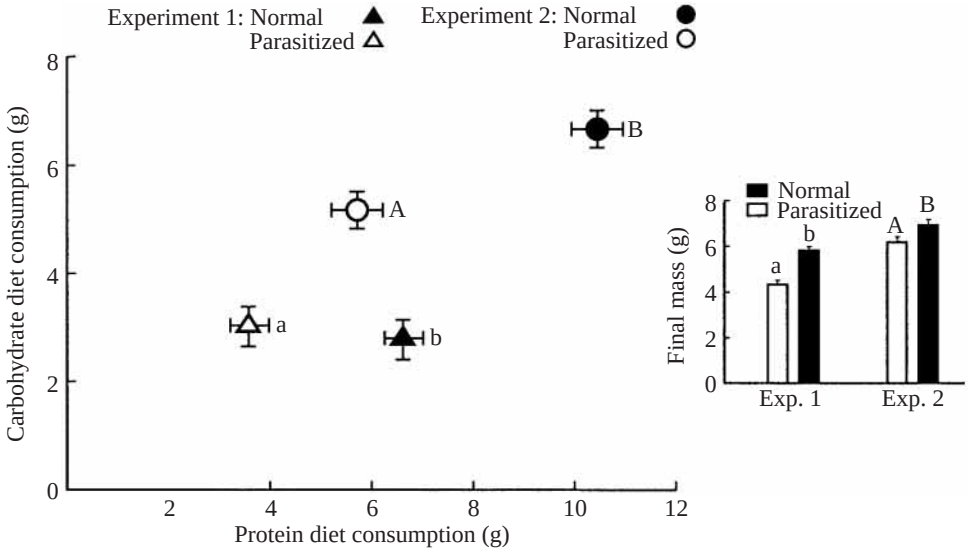

Fig. 1. Effects of parasitism by *Cotesia congregata* on diet consumption and growth of the host *Manduca sexta* given a dietary choice. Nutrient consumption is shown as a bivariate plot of protein and carbohydrate consumption. Values are means ± least-squares S.E.M.; horizontal bars refer to protein diet and vertical bars to carbohydrate diet. Values followed by different letters are statistically different for protein diet or carbohydrate diet, indicated by the directional error bar. Triangles and lower case letters refer to Experiment 1, where larvae were fed for 2 days. Circles and upper case letters refer to Experiment 2, where larvae were fed for 3 days. Growth for the two experiments is shown in the accompanying bar graph. Newly moulted normal (filled bars) and parasitized (open bars) fifth-instar larvae were given a choice of two synthetic artificial diets, one containing sucrose without casein, and the other, casein without sucrose, both nutrients at 120 g l^{-1}. All values are g wet mass. Data were analyzed by ANCOVA with initial mass as the covariate. For a statistical summary, see Table 1.

trehalose. Data for the ^{13}C-enrichment ratios, Gl_x C2/C3, alanine C3/C2 and [2trehalose C6/alanine C3], as well as the haemolymph trehalose level, were analyzed by one-way ANCOVA, using initial mass as the covariate; parasitism was considered to be the main-effect treatment.

Fulfilment of statistical assumptions

Data from the feeding experiments that were analyzed by ANCOVA or ANOVA met the assumptions of normality and homogeneity of variance based on the Shapiro–Wilk 'W' test and assessment with a normal probability plot. Data for parasite biomass as well as the biochemical data were also normally distributed. Data for parasite burden, however, were not normal, and non-parametric analyses were conducted as described above.

Results

Dietary selection

Unconditioned selection

After newly moulted fifth-instar *M. sexta* larvae were presented with a choice of casein or sucrose diets immediately upon moulting to the fifth stadium, normal larvae consumed significantly more of the protein diet than did parasitized larvae over both the 2-day and 3-day feeding periods (Fig. 1, Table 1). Consumption of the sucrose diet during the 3-day experiment was significantly different between normal and parasitized insects, with parasitized insects consuming less. The difference in carbohydrate consumption, however, was much smaller than was observed with protein consumption in both experiments. The mean ratios of protein to carbohydrate diets consumed in the 48 h experiment were 3.33±0.54 (mean ± S.E.M.) for normal and 1.64±0.054 for parasitized larvae. During the 3-day experiment the ratios were 1.68±0.13 (mean ± S.E.M.) and 1.14±0.13 for normal and parasitized animals, respectively. In both experiments, the difference between the ratios of normal and parasitized insects was statistically significant (Experiment 1, $F_{1,37}$=4.53, P=0.0400; Experiment 2, $F_{1,37}$=7.91, P=0.0078). The final masses of normal insects were also significantly greater than those of parasitized insects (Fig. 1, Table 1).

Conditioned selection

Conditioning newly moulted fifth-instar *M. sexta* larvae on either the casein diet or the sucrose diet had little effect on subsequent dietary choice, but differences were evident between the duplicate experiments (Fig. 2, Table 2). In Experiment 2, conditioning on the protein diet resulted in a proportionately small but significant increase in carbohydrate consumption that was not evident in the first experiment. Similarly, in Experiment 2, there was a significant increase in the final mass of larvae conditioned on the protein diet.

In both experiments, normal larvae consumed significantly more protein diet, approximately twofold more, than did parasitized insects. In the first experiment, the mean ratios of protein to carbohydrate diet consumed by normal and parasitized insects were 1.81±0.08 (mean ± S.E.M.) and 1.19±0.08, respectively ($F_{1,95}$=25.46, P<0.0001), and in the second experiment, 1.82±0.11 (mean ± S.E.M.) for normal larvae and 1.13±0.11 for parasitized insects ($F_{1,75}$=19.37, P<0.0001). The mean final mass of the parasitized larvae was significantly less than that of normal insects following conditioning on the casein or sucrose diets (Fig. 2, Table 2).

When the above data for the conditioning experiments were evaluated to determine the effects of conditioning and parasitism, independently of the relationship between consumption and final mass or size (Table 3), conditioning was found to have a significant effect on subsequent carbohydrate diet consumption

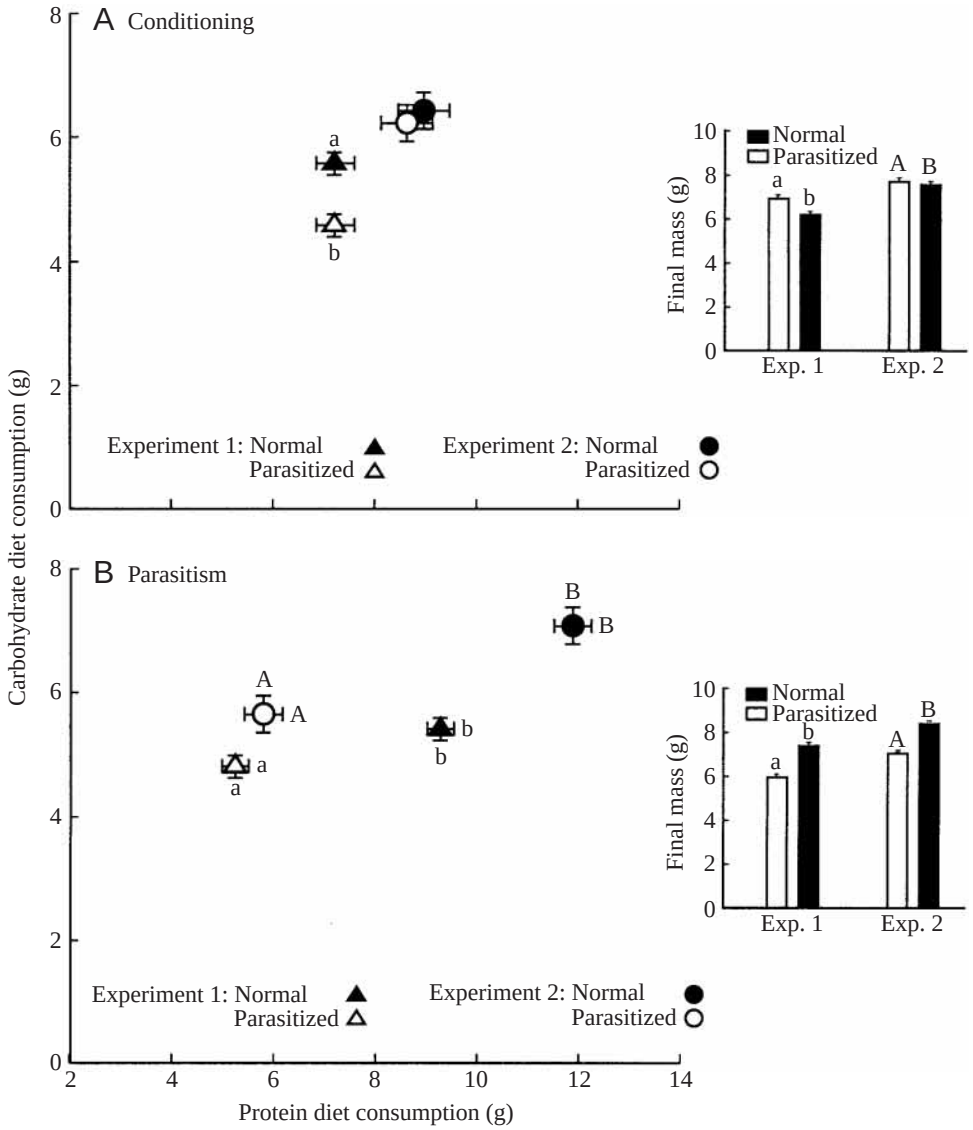

Fig. 2. Effects of dietary conditioning (A) and parasitism (B) by *Cotesia congregata* on diet consumption and growth of the host *Manduca sexta*, when given a dietary choice. Nutrient consumption is shown as a bivariate plot of protein diet and carbohydrate diet consumption. Values are indicated by mean ± least-squares S.E.M.; horizontal bars refer to protein diet and vertical bars to carbohydrate diet. Values followed by different letters are statistically different for the protein diet or carbohydrate diet, indicated by the directional error bar. Triangles and lower case letters, and circles and upper case letters, refer to the duplicate Experiments (Exp.) 1 and 2, respectively. Newly moulted normal and parasitized fifth-instar larvae were conditioned for 1 day on a synthetic artificial diet containing sucrose without casein, or casein without sucrose (both nutrients at 120 g l^{-1}), and subsequently given a choice of the two diets for an additional 2 days. The effects of conditioning and parasitism on growth are shown in the insets. All values are g wet mass. Data were analyzed by two-way ANCOVA with initial mass as the covariate. No significant interaction between conditioning and parasitism was evident. For a statistical summary, see Table 2.

Table 3. *ANCOVA summary demonstrating the size-mediated effects of dietary conditioning and parasitism on diet consumption by the host* Manduca sexta *given a dietary choice (see Fig. 3)*

Dependent variable	Effect	d.f.	Mean square	*F*	*P*
Protein diet consumption	Conditioning	1	9.3798	3.63	0.0585
	Parasitism	1	11.5521	4.47	0.0360
	Interaction-conditioning/parasitism	1	0.7108	0.27	0.6008
	Final mass (covariate)	1	448.9223	173.61	<0.0001
	Interaction-final mass/conditioning	1	6.9308	2.68	0.1034
	Interaction-final mass/parasitism	1	37.2785	14.42	0.0002
	Interaction-final mass/parasitism/conditioning	1	0.2719	0.11	0.7461
Carbohydrate diet consumption	Conditioning	1	15.0154	7.62	0.0064
	Parasitism	1	30.9839	15.73	0.001
	Interaction-conditioning/parasitism	1	0.7018	0.36	0.5514
	Final mass (covariate)	1	127.2502	64.59	<0.0001
	Interaction-final mass/conditioning	1	13.8378	7.02	0.0088
	Interaction-final mass/parasitism	1	29.3795	14.91	0.0002
	Interaction-final mass/parasitism/conditioning	1	0.3723	0.19	0.6643

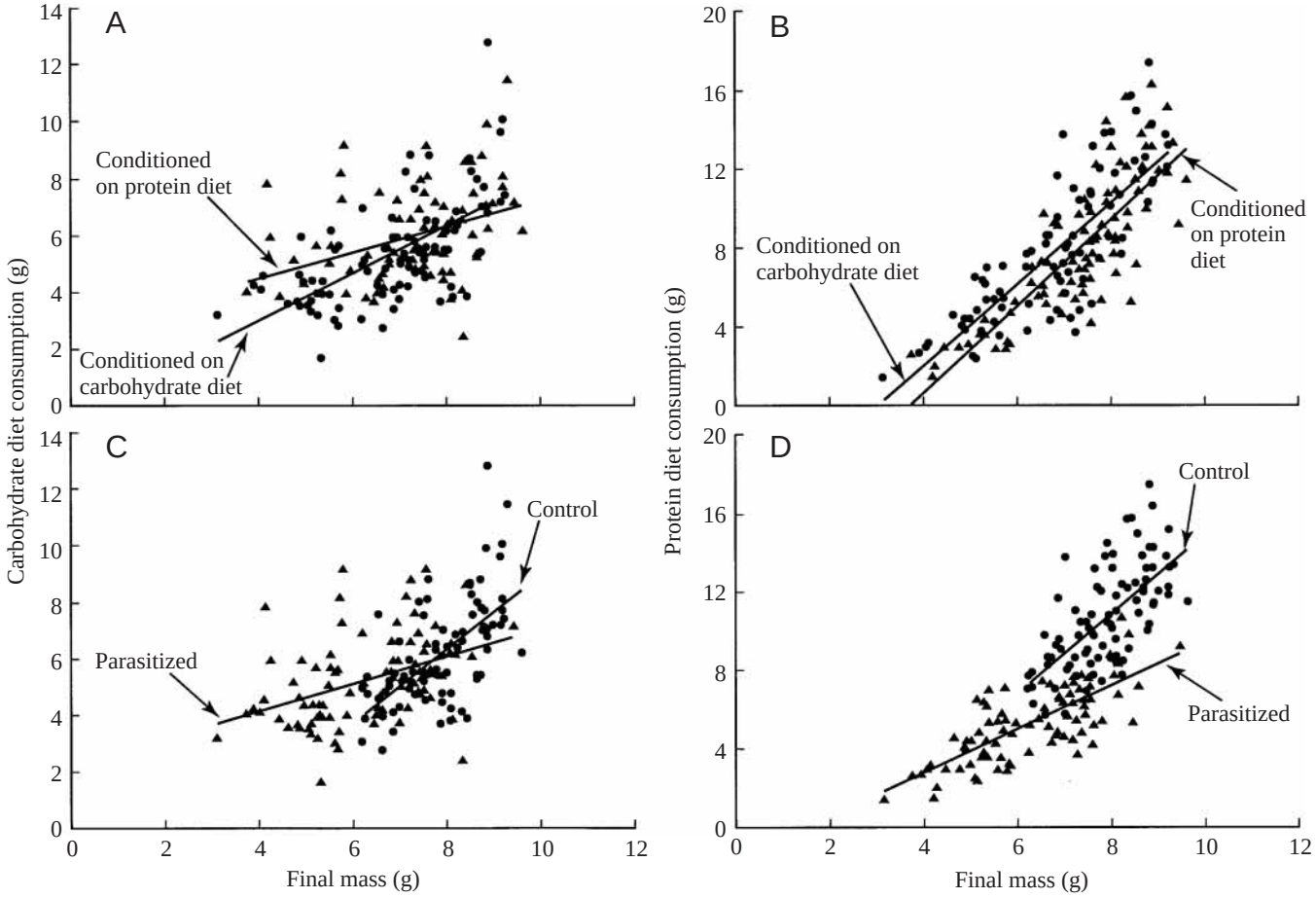

Fig. 3. Size-mediated effects of dietary conditioning and parasitism by *Cotesia congregata* on consumption of a protein diet and a carbohydrate diet by the host *Manduca sexta* given a dietary choice. Results for individual larvae are shown with regression lines depicting the relationships between size and consumption for each treatment level. (A) Effects of conditioning on carbohydrate consumption. For larvae conditioned on the protein diet (triangles), consumption=0.2091×final mass; for larvae conditioned on the carbohydrate diet (circles), consumption=0.6820×final mass. (B) Effects of conditioning on protein consumption. For larvae conditioned on the protein diet (triangles), consumption=1.3072×final mass; for larvae conditioned on the carbohydrate diet (circles), consumption=0.9861×final mass. (C) Effects of parasitism on carbohydrate consumption. For parasitized animals (triangles), consumption=0.2091×final mass; for normal animals (circles), consumption=0.9405×final mass. (D) Effects of parasitism on protein consumption. For parasitized animals (triangles), consumption=1.3072×final mass; for normal animals (circles), consumption=2.3149 (final mass). The results of the ANCOVAs are shown in Table 3.

(Fig. 3A), but not on protein diet consumption (Fig. 3B). In the former case, the slopes of the regression lines for the relationships between conditioning and consumption were significantly different, while in the latter case, the slopes were not different. Both carbohydrate and protein diet consumption were significantly affected by insect size (significant covariate, Table 3). Larger insects consumed more of both diets (Fig. 3A,B), but the effect of insect size on consumption of the carbohydrate diet was mediated by conditioning (a significant treatment by covariate interaction was detected, Table 3). The effect of conditioning was stronger for smaller larvae (approximately 5 g or less), than for larger insects (approximately 7.5 g or more). In the case of smaller larvae, consumption of the carbohydrate diet was significantly greater for larvae conditioned on the protein diet, than for insects that were conditioned on the carbohydrate diet (Fig. 3A). There was a significant interaction between conditioning and final mass (Table 3). As larval size increased, the difference in carbohydrate diet consumption between conditioning treatments became negligible.

The effect of larval mass on carbohydrate and protein diet consumption was mediated by parasitism (Table 3). The increase in consumption of carbohydrate diet (Fig. 3C) and protein diet (Fig. 3D) with increased larval size was not as great for parasitized larvae as was the case for normal insects. The slopes of the regression lines for the relationships between carbohydrate and protein diet consumption with final mass of parasitized and normal larvae were significantly different. In this study, the effects of parasitism on consumption of the carbohydrate diet were most pronounced for individuals at the extremes of the size distribution. With smaller larvae, parasitism resulted in higher carbohydrate diet consumption than was observed by normal insects of similar mass, while for

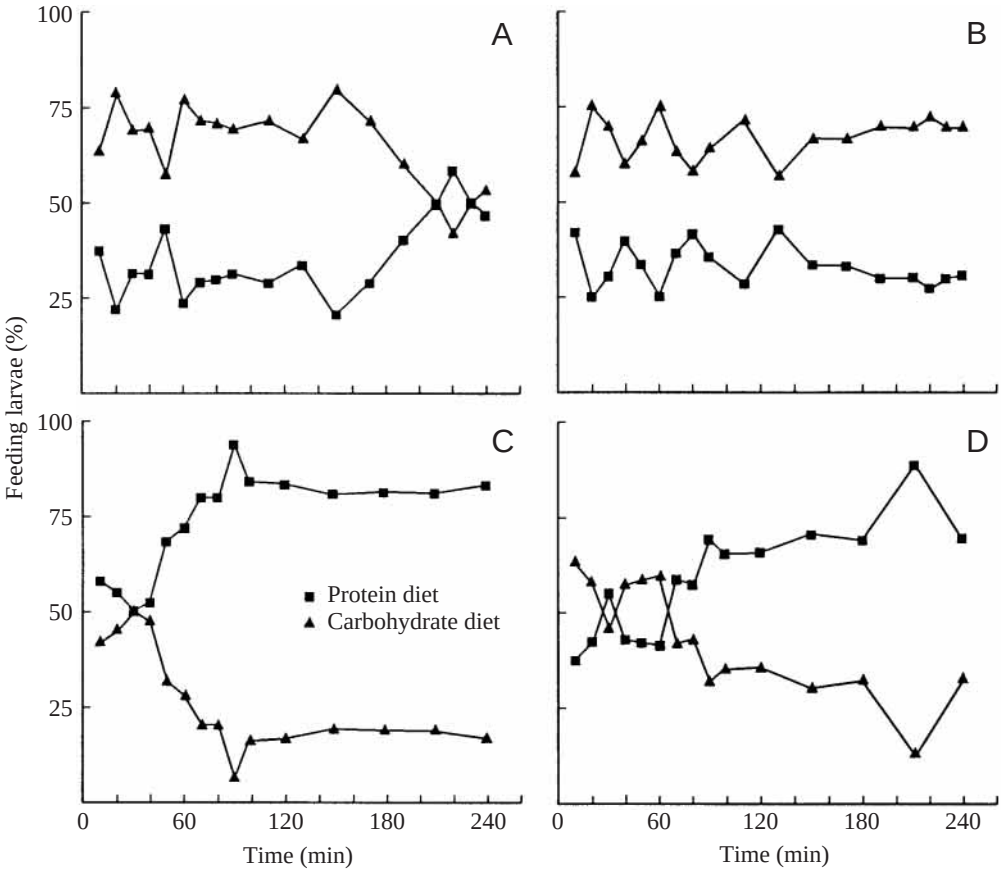

Fig. 4. Short-term dietary selection by the host *Manduca sexta*. Newly moulted fifth-instar larvae were conditioned for 1 day on a synthetic artificial diet containing sucrose without casein, or casein without sucrose (both nutrients at 120 g l^{-1}), and subsequently given a choice of the two diets. (A,B) The percentage of normal larvae (A) and parasitized larvae (B) choosing the protein (squares) and carbohydrate (triangles) diets following conditioning on the protein diet. (C,D) The percentage of normal (C) and parasitized (D) larvae choosing the protein and carbohydrate diets following conditioning on the carbohydrate diet. Each graph shows the distribution of 20 larvae.

larger larvae, parasitism resulted in lower carbohydrate diet consumption (Fig. 3C). The effect of parasitism on consumption of protein diet was most pronounced for larger larvae and was minimal for smaller insects (Fig. 3D). Larger parasitized insects consumed significantly less protein diet than normal larvae. For both carbohydrate and protein diet consumption there were no main-effect interactions between parasitism and conditioning (Table 3).

Short-term selection

Larvae conditioned for 1 day on the casein diet fed predominantly on the sucrose diet when given a choice of the casein and the sucrose diets (Fig. 4A,B). The results with parasitized and normal larvae were comparable, although normal larvae began to switch to the protein diet after approximately 2.5 h. Similarly, both normal and parasitized larvae conditioned on the sucrose diet selected the casein diet when given the dietary choice (Fig. 4C,D).

Host nutritional status and parasite burden

There were significant differences in the numbers of parasites developing on hosts maintained on isocaloric diets with varying ratios of dietary casein and sucrose (χ^2=22.2080, d.f.=4, P=0.0002) (Fig. 5A). The number of parasites increased with increasing protein and reached a maximum when the protein to carbohydrate ratio was 1:1 (w/w), and thereafter decreased. A further Kruskal–Wallis analysis confirmed that the 1:1 diet supported the highest parasite burden, expressed as numbers of parasites g^{-1} host mass, after adjusting for final host mass (χ^2=19.8499, d.f.=4, P=0.0005).

There were no significant differences among the masses of individual parasites from host larvae reared on any of the diets, except for the 0.25:1.75 diet with the lowest level of casein ($F_{4,25}$=25.50, P<0.0001, Fig. 5B). The total parasite mass, however, increased with increasing protein and was maximal at the 1:1 protein/carbohydrate ratio ($F_{4,25}$=17.56, P<0.0001, Fig. 5B). At higher relative protein levels the total mass decreased. The results for the effects of dietary nutrient ratio on the total mass of parasites, therefore, generally reflected the total parasite numbers.

The three-dimensional view showing the relationship between consumption of sucrose and casein diets with parasite burden suggests a complex relationship, but clearly demonstrates that increases in both dietary casein and sucrose increase parasite burden (Fig. 6A). The effect of protein consumption, however, was significantly greater than that of carbohydrate consumption. The large peak in the upper left quadrant was due to the exceptionally high number of parasites, 670, in an individual host larva on the 1.50:0.5 diet. Without this contribution the relationship would have shown a continuous upward slope. The mean number of parasites in hosts on the 1.5:0.5 diet was 398±77 (mean ± S.E.M.), which includes the high value. In contrast, the number of parasites in host larvae on the 1:1 diet, the diet supporting the greatest

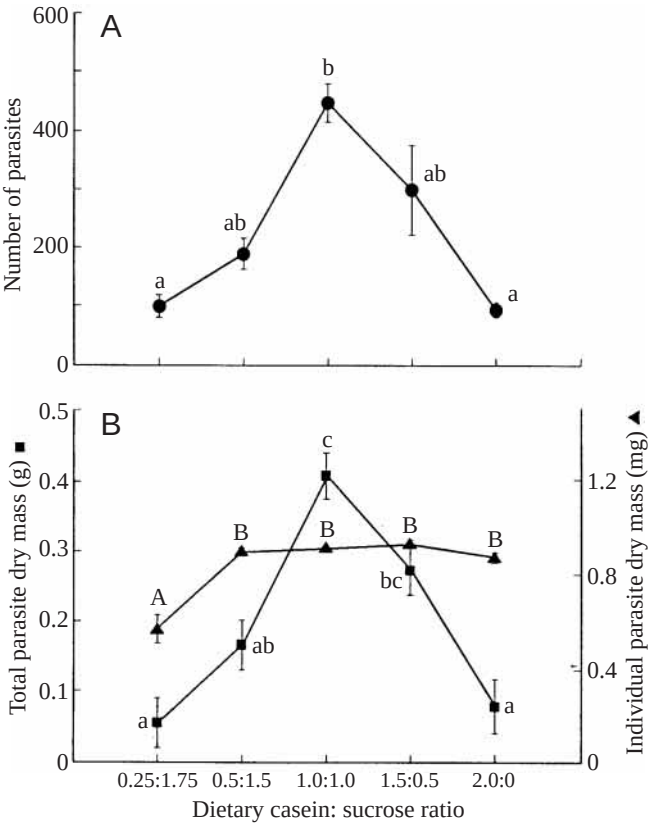

Fig. 5. Effect of dietary protein/carbohydrate ratio on the numbers and mass of *Cotesia congregata* developing in the host *Manduca sexta*. (A) Effect of dietary nutrient ratio on total parasite number. Values are means ± S.E.M. Values followed by different letters are statistically different at α=0.05 as determined by the Nemenyi multiple-range test. (B) Effect of dietary nutrient ratio on total (squares) and individual (triangles) parasite biomass. Values are means ± S.E.M. Values followed by different letters are statistically different at α=0.05 from those in the same data set, as determined by the Ryan–Einot–Gabriel–Welsch multiple-range test. All diets were isocaloric and contained an equivalent total amount of casein and sucrose.

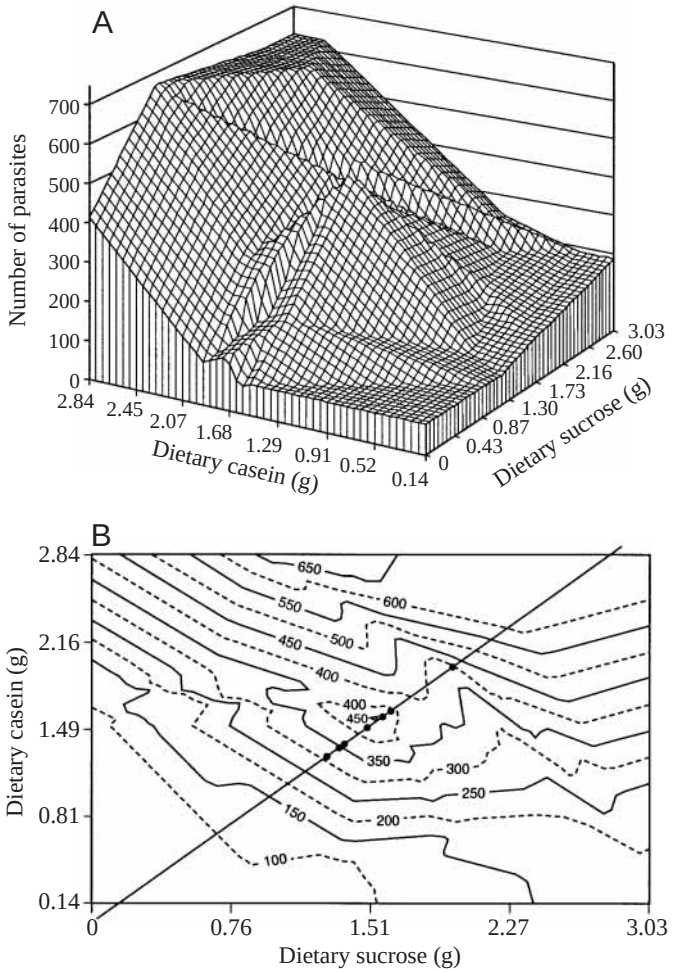

Fig. 6. The relationship between dietary protein and carbohydrate consumption and the numbers of *Cotesia congregata* developing in the host *Manduca sexta*. (A) Three-dimensional representation. (B) Topographical representation of A. The diagonal line shows the predicted relationship for the 1:1 diet containing an equivalent amount of casein and sucrose, based on actual experimental values as shown.

parasite burden, was 450±32. Fig. 6B, a two-dimensional topographical representation of these same data, shows the predicted relationship between dietary casein and sucrose consumption and parasite burden. The relationship for the 1:1 dietary ratio and parasite burden is indicated on the figure, with the actual experimental data for that ratio shown. The results suggest that the dietary ratio supporting the greatest number of parasites may be slightly more than 1:1.

Gluconeogenic formation of haemolymph trehalose

All normal and parasitized *M. sexta* larvae were glucogenic and displayed net trehalose formation *via* gluconeogenesis (Tables 4–6). The selective and paired enrichment of trehalose C1 and C6, and C2 and C5, whiah are typical of glucogenesis from [2-^{13}C]pyruvate (Thompson, 2000a,b), were clearly evident in the ^{13}C NMR spectra of haemolymph extracts (Fig. 7). The degree of randomization of ^{13}C by the TCA cycle was indicated by the ^{13}C-enrichment in C2 and C3 of Gl_x produced as byproducts of TCA cycle metabolism (Table 5, Fig. 8). The Gl_x C2/C3 ^{13}C-enrichment ratio was not significantly different in either experiment, confirming that the alanine C3/C2 ratio in an accurate measure of pyruvate cycling. The measurements of pyruvate cycling, alanine C3/C2 and glucogenic flux, [2trehalose C6/ alanine C3], were also not significantly different between normal and parasitized larvae. ^{13}C-enriched metabolites were mixtures of singly enriched isotopomers. No mutiply enriched species were detected in any of the experiments.

Generally, parasitized and normal larvae had similar ranges of haemolymph trehalose concentration, despite the difference in the ratio of protein/carbohydrate diets that the larvae consumed (Table 5). In experiment 2, parasitized larvae displayed a significantly higher mean haemolymph sugar level, but this, in part, reflected the contribution of a single insect with an unusually high trehalose concentration

Table 4. *Estimated ^{13}C in the individual carbons of trehalose in haemolymph extracts of normal and parasitized* Manduca sexta *larvae, given a dietary choice*

	^{13}C in trehalose (%)					
State of host	C1	C2	C3	C4	C5	C6
Experiment 1						
Normal	3.14±0.67	6.93±1.42	1.10±0.13	1.1	9.45±1.77	4.20±0.77
Parasitized	2.57±0.75	4.91±1.58	1.12±0.14	1.1	5.46±1.98	2.87±0.86
	F=0.32, P=0.5902	F=0.91, P=0.3724	F=0.01, P=0.9192		F=2.26, P=0.1767	F=1.32, P=0.2886
Experiment 2						
Normal	1.85±0.19	2.84±0.30	0.94±0.57	1.1	4.24±0.30	2.74±0.08
Parasitized	1.35±0.19	2.24±0.30	0.91±0.57	1.1	3.08±0.38	1.83±0.21
	F=3.36, P=0.1043	F=1.96, P=0.1989	F=0.10, P=0.7551		F=4.76, P=0.0607	F=9.26, P=0.0160

Values are means ± S.E.M. and were estimated from the integrated intensities of the individual carbons for trehalose in the ^{13}C NMR spectrum assuming a natural abundance of 1.1% for C4.

Table 5. *Effects of parasitism on the ratio of protein and carbohydrate diets consumed and glucogenic formation of trehalose and haemolymph sugar level by the host* Manduca sexta *given a dietary choice*

	Consumption ratio	^{13}C-enrichment ratio			
State of host	Protein diet/ carbohydrate diet	$G1_x$ C2/C3	Alanine C3/C2	2trehalose C6/alanine C3	[Trehalose] (mmol l^{-1})
Experiment 1					
Normal	2.34±0.19*	1.05±0.23	0.29±0.04	5.67±1.25	35.71±7.94
Parasitized	1.32±0.21*	1.35±0.26	0.22±0.04	6.51±1.40	34.88±8.88
Experiment 2					
Normal	2.21±0.32*	0.63±0.13	0.32±0.08	2.36±0.74	23.24±5.11*
Parasitized	0.68±0.36*	0.66±0.13	0.22±0.08	4.56±0.74	52.21±5.11*

Newly molted fifth-instar larvae were given a choice of two synthetic artificial diets, one containing sucrose without casein, and the other, casein without sucrose. Both nutrients were at 120 g l^{-1}.

Values are least-squares means ± S.E.M. For experiment 1, N=5 (normal), N=4 (parasitized); for experiment 2, N=5 (normal and parasitized).

*Values are significantly different between normal and parasitized groups within each experiment.

See Table 6 for statistical analysis.

of approximately 71 mmol l^{-1}. Haemolymph sugar level in the present experiments was much more variable than was observed during previous studies where insects were provided with a single diet, rather than being offered a choice of diets.

Discussion

The present study demonstrated dietary selection by fifth-instar *M. sexta* larvae when insects were given a choice between two nutritionally inadequate diets, one containing casein without carbohydrate, and the other sucrose without protein. Normal larvae consistently consumed more of the casein diet relative to the sucrose diet, which in most cases meant approximately twofold more protein. This result is in agreement with the dietary ratios of protein and carbohydrate consumed by many lepidoperan insects feeding on natural foods as well as on artificial diets (Simpson and Raubenheimer, 1993a; Raubenheimer and Simpson, 1999). Parasitism by *C. congregata* significantly altered the relative amounts of the carbohydrate and protein diets consumed by host larvae.

Conditioning newly moulted parasitized and normal larvae on either the carbohydrate diet or the protein diet before offering the dietary choice had limited long-term effects on subsequent diet consumption. The results of these experiments, however, must be interpreted with care, as both conditioning and parasitism independently affected the allometric relationship between larval size and consumption. We know of no other investigations demonstrating this type of size-related phenomenon regarding conditioning and parasitism.

A short-term effect of conditioning clearly was evident in larvae conditioned on the protein diet. When offered a choice between the diets, larvae selected and fed on the carbohydrate diet for several hours rather than on the protein diet. These results are consistent with those of Friedman et al. (1991), who also observed short-term feeding on a sucrose diet selected by *H. zea* conditioned on a high casein diet. Our results were also consistent, in part, with those reported by Simpson et al. (1988)

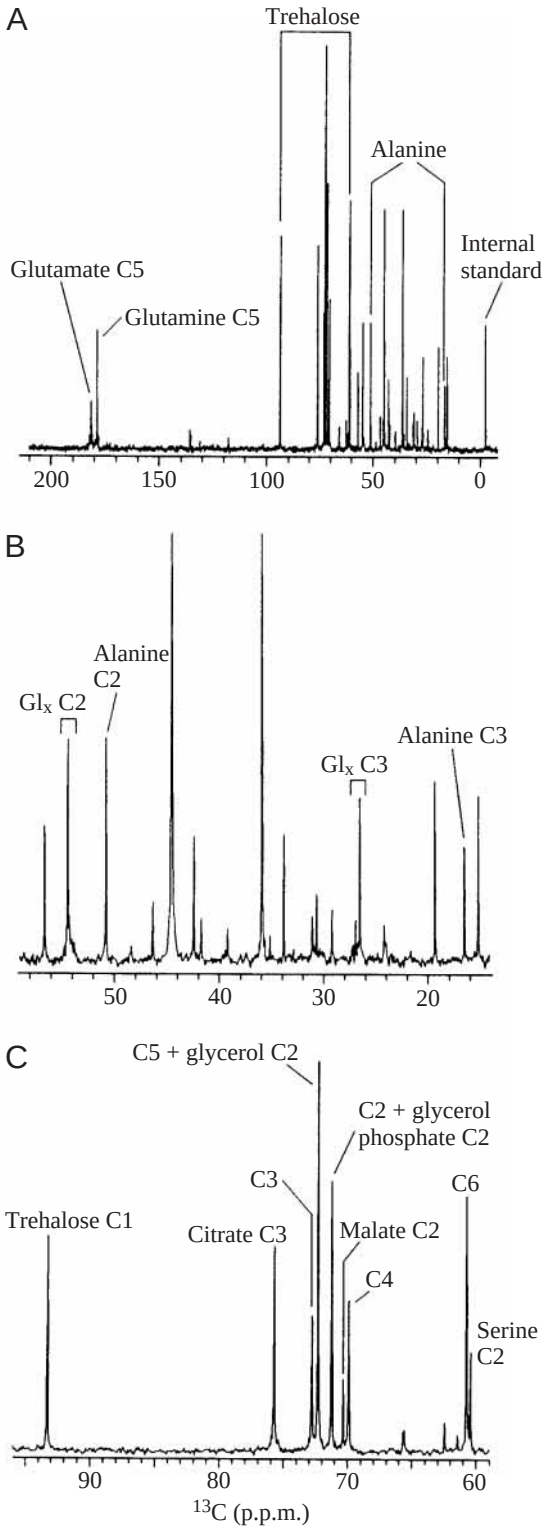

Fig. 7. Typical ^{13}C NMR spectrum of perchloric acid extracts of haemolymph from fifth-instar *M. sexta* larvae given a dietary choice for 48 h and administered [2-^{13}C]pyruvate. (A) Spectrum in the region –5.0 p.p.m. to 210 p.p.m. showing enrichments for alanine, trehalose and C5 of glutamate and glutamine. (B) Partial spectrum in the region 14–96 p.p.m. showing ^{13}C-enrichments for C2 and C3 of alanine and Gl_x. (C) Partial spectrum in the region 59–96 p.p.m. showing the selective ^{13}C-enrichment in trehalose, as well as other metabolites, including glycerol and glycerol phosphate with the same or similar chemical shifts. Haemolymph was collected 3.5 h post-injection. The spectrum was generated from 4000 data acquisitions and is shown with 3 Hz line broadening. See text for explanation of the metabolic derivation of ^{13}C-enrichments.

Table 6. *ANCOVA summary demonstrating the effects of parasitism on the haemolymph trehalose level and glucogenic formation of trehalose by the host* Manduca sexta, *given a dietary choice (see Table 5)*

	d.f.	Mean square	*F*	*P*
Experiment 1				
Protein/carbohydrate diet consumption ratio				
Parasitism	1	2.959	12.89	=0.0115
Initial mass (covariate)	1	0.0287	0.16	=0.7021
Error	6	0.1781		
$G1_x$ C2/C3 ^{13}C-enrichment ratio				
Parasitism	1	0.1957	0.72	=0.4278
Initial mass (covariate)	1	0.3295	1.22	=0.3122
Error	6	0.2707		
Alanine C3/C2 ^{13}C-enrichment ratio				
Parasitism	1	0.0124	1.98	=0.2080
Initial mass (covariate)	1	0.0001	0.01	=0.9073
Error	6	0.0063		
2trehalose C6/alanine C3 ^{13}C-enrichment ratio				
Parasitism	1	1.5882	0.20	=0.6679
Initial mass (covariate)	1	0.7331	0.09	=0.7697
Error	6	7.8099		
[Trehalose] (mmol l^{-1})				
Parasitism	1	1.5001	0.00	=0.9472
Initial mass (covariate)	1	182.9278	0.58	=0.4743
Error	6	314.1513		
Experiment 2				
Protein/carbohydrate diet consumption ratio				
Parasitism	1	1.3841		=0.0510
Initial mass (covariate)	1	0.1667		=0.4415
Error	7	0.2505		
$G1_x$ C2/C3 ^{13}C-enrichment ratio				
Parasitism	1	0.0006	0.02	=0.8986
Initial mass (covariate)	1	0.0033	0.10	=0.7602
Error	7	0.0326		
Alanine C3/C2 ^{13}C-enrichment ratio				
Parasitism	1	0.0067	0.61	=0.4600
Initial mass (covariate)	1	0.0075	0.68	=0.4352
Error	7	0.0109		
2trehalose C6/alanine C3 ^{13}C-enrichment ratio				
Parasitism	1	2.8740	2.76	=0.1408
Initial mass (covariate)	1	8.3012	7.96	=0.0257
Error	7	1.0424		
[Trehalose] (mmol l^{-1})				
Parasitism	1	498.5463	9.95	=0.0161
Initial mass (covariate)	1	367.6446	7.34	=0.0303
Error	7			

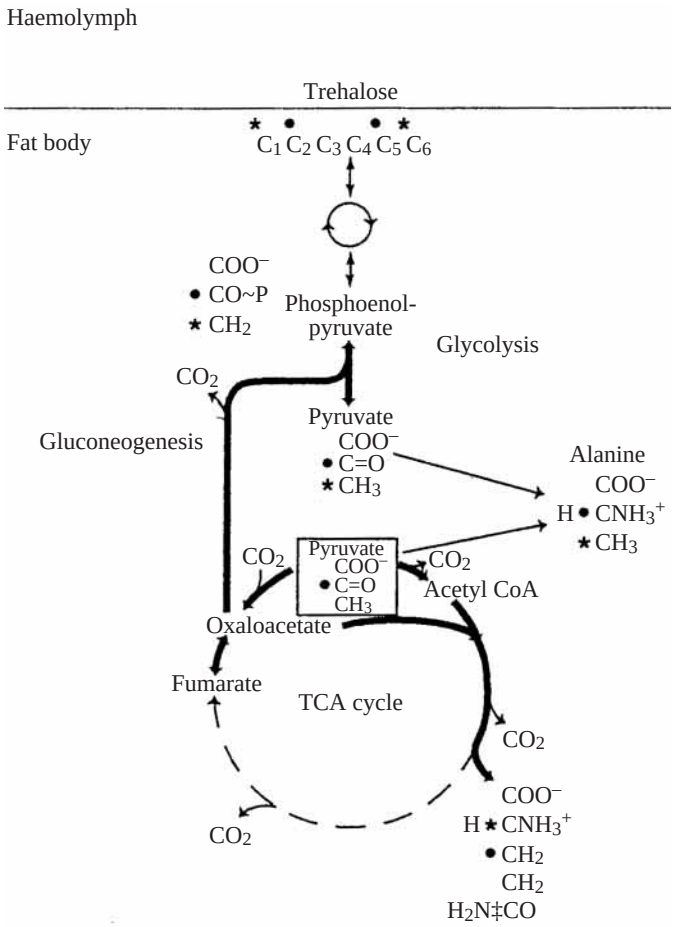

Fig. 8. Metabolism of [2-^{13}C]pyruvate by fifth-instar *M. sexta* larvae, illustrating the derivation of ^{13}C-enrichment in glycolytic and gluconeogenic intermediates, and in haemolymph trehalose. Principal metabolic derivations are shown with different symbols. Circles, ^{13}C enrichments derived directly following carboxylation of the original ^{13}C in [2-^{13}C]pyruvate, as administered to larvae; *^{13}C-enrichments derived from [2-^{13}C]pyruvate, but following carboxylation and randomization at the fumarase-catalyzed step of the TCA cycle; ‡^{13}C-enrichments also derived from [2-^{13}C]pyruvate following decarboxylation to acetyl-coenzyme A, condensation with oxaloacetate and subsequent synthesis of Gl_x. The metabolic effects of pentose cycling are not shown.

from short-term experiments with larvae of *Spodoptera exigua* (Hubner), another lepidopteran insect. In that study, insects were conditioned for 12 h on a casein diet, and subsequently given the choice of a casein or a sucrose diet. Larvae consumed significantly more of the carbohydrate diet over a 12 h feeding period. When *S. exigua* larvae were conditioned in the same manner, but on the sucrose diet, and were then presented with a choice of the diets, they not only fed more on the protein diet, but consumed significantly less of the carbohydrate diet than did larvae that were conditioned on a nutritionally complete diet containing both casein and sucrose. Although the amount of diet consumed by *M. sexta* during the present short-term selection experiments was not determined, our observations on the proportion of larvae choosing each diet following the conditioning period indicate that larvae conditioned on the sucrose diet selected in a manner similar to larvae that were not conditioned, a nutritional state that may be comparable to that of larvae conditioned on a nutritionally complete diet.

Findings from the short-term dietary selection experiments with *M. sexta* larvae suggest an explanation for the difference in the results observed between the duplicate long-term selection conditioned experiments. In Experiment 2, normal larvae conditioned on the protein diet consumed a small but significantly greater amount of the carbohydrate diet than larvae conditioned on the carbohydrate diet (Fig. 2, Table 2). Moreover, these larvae displayed a significantly greater final mass. The length of time that larvae initially feed on the sucrose diet after being conditioned on the casein diet may affect the total amount of carbohydrate and protein consumed over the entire 2-day feeding period. The total amount of carbohydrate consumed may be significantly reduced, while the amount of the protein diet consumed may increase. The latter effect could reasonably result in increased growth. Previous studies suggest that this initial feeding period is highly variable and may range from a few to several hours (Friedman et al., 1991; Simpson et al., 1988; Thompson and Redak, 2000). In the present short-term experiments, for example, the majority of normal larvae that fed on the sucrose diet after being conditioned on the casein diet began to switch and feed on the protein diet after approximately 2.5 h (Fig. 4A). Most parasitized larvae did not change diets during the 4 h observation period (Fig. 4B). Studies by Friedman et al. (1991) indicate that with *H. zea* the length of this period is related to haemolymph trehalose level. After conditioning on a casein diet lacking sucrose, larvae fed on a sucrose diet for approximately 3 h, and then they began to change over and feed on the casein diet. Analysis of haemolymph trehalose demonstrated that larvae have a very low trehalose level after conditioning on the casein diet, and upon switching to the carbohydrate diet trehalose levels increase and normal levels are reached in approximately 3 h.

The altered ratio of protein/carbohydrate diet consumed by parasitized *M. sexta* larvae resulted principally from a decrease in protein, rather than a change in carbohydrate consumption. As parasitized larvae consumed a ratio of protein/carbohydrate approaching 1:1 in most of the experiments, it is important to consider whether the results reflect random unregulated feeding or an alteration in the intake target. The present study is equivocal in this regard. While the short-term experiments clearly indicated the ability of parasitized insects to select between diets of different nutritional content following the conditioning period, this result does not definitively establish an altered food preference by parasitized larvae. To clearly establish whether parasitism induces an altered intake target, larvae must be nutritionally challenged by being provided with choices of diets having a variety of nutrient ratios, and documented to defend the same intake target (Raubenheimer and Simpson, 1999). A lack in the defense of an intake target by larvae would indicate random feeding. In the case of parasitized *M. sexta*, random feeding could reflect a

pathophysiological effect on the basic mechanism(s) responsible for regulating dietary selection. Although, as indicated above, these mechanisms are not well understood, biogenic amines in the central nervous system may be involved. Recent studies with *M. sexta* parasitized by *C. congregata* have demonstrated changes in the octopamine content (Adamo and Shoemaker, 2000) and peptide distribution (Zitnan et al., 1995) in the central nervous system. The significance of these findings is currently unresolved, although octopamine levels were correlated with behavioural events immediately before parasite emergence and eclosion. Regardless of the basis for the altered nutrient intake observed here, the change may be adaptive for the parasite, providing optimal nutrition and ensuring the continued survival of the host.

To evaluate the potential significance of altered dietary nutrient intake on developing parasite larvae, experiments were conducted with parasitized *M. sexta* maintained on single diets having a range of sucrose and casein ratios. These experiments demonstrated that significantly more parasites develop in host larvae fed on a diet with a protein/carbohydrate ratio of 1:1 than on diets with unequal ratios of these nutrients. Although studies by others established an upper limit to the number of parasites that actually emerge from an individual host, and that many apparently mature second-instar larvae fail to emerge (Allyene and Beckage, 1997a), we included all mature larvae in this investigation as the most accurate indicator of the effects of host nutrition on parasite growth and development. The results suggest that the alteration in dietary nutrient intake observed in parasitized host larvae is an adaptive response to parasitism that benefits the parasite.

Possible changes in nutrient intake by parasitized *M. sexta* larvae may have significant nutritional and ecological consequences. Investigations on field populations of *M. sexta* and *C. congregata* in tobacco, *Nicotiana tobacum*, demonstrate that the parasite is most active late in the season when host populations are highest (Fulton, 1940; Gilmore, 1938). By this time, often as late as October, the tobacco plants are mature and and starting to become senescent. Maturation begins with the bottom leaves and progresses upward. Observations of host feeding behaviour of *M. sexta* demonstrate that inter- and intra-plant movement depends upon larval density, but at normal densities larvae generally remain and complete development on an individual plant (Madden and Chamberlin, 1945; McFadden, 1968). Larvae feeding on the maturing lower leaves quickly move up the plant to fresh foliage as the leaves are consumed. Moreover, they do not consume senescent leaves until the rest of the plant has been very heavily fed upon, or is completely denuded. The chemistry of the tobacco plant is complex. Maturation and senescence result in significant changes in chemical composition (Akehurst, 1981; Leffingwell, 1999). Parasitized larvae feeding on mature senescing leaves may remain there because of different nutrient requirements or an inability to select a specific intake target, thus avoiding competition with normal individuals that move to fresher leaves. Following maturation and during senescence, a decrease in photosynthetic rate is accompanied by a rapid degradation of starches, which elevates the levels of reducing sugars. The latter serve as the principal substrate for plant cellular respiration in the absence of carbon fixation. Starches, which typically comprise 30 % of fresh leaf dry mass, are reduced to less than half this amount during senescence, while reducing sugar may nearly triple in content to more than 15 % of dry mass. While protein is also degraded, and soluble protein degradation products are reduced by translocation, the relative change is slight compared with that of carbohydrate. Overall, however, there is a significant quantitative and qualitative shift in the carbohydrate/protein balance in the leaf. Also of interest, is the finding that as leaf maturity progresses and senescence begins, there is a dramatic decrease in the concentration of nicotine. Nicotine is the major alkaloid of tobacco and has been shown by others to have a negative impact on the development of *C. congregata* at concentrations that do not affect the host larvae (Barbosa et al., 1991). How the chemical differences within the plant may differentially affect feeding, behaviour and development of normal and parasitized *M. sexta* remains a matter of conjecture at this time. Further investigations are required to assess this, to determine the significance of dietary selection in the nutrition of *M. sexta*, and to understand the short- and long-term effects of dietary conditioning on subsequent dietary choice.

The present investigation also suggests that the altered dietary intake of *M. sexta* larvae during parasitism by *C. congregata*, and the resultant change in nutrient balance, play a role in maintaining metabolic homeostasis in the parasitized host. Previous studies examined the effects of parasitism on carbohydrate metabolism in *M. sexta* when parasitized and normal larvae were maintained on the same diet. In those investigations, parasitized insects displayed increased synthesis of glycogen and trehalose from dietary sugar, and aberrant regulation over *de novo* trehalose formation (Thompson et al., 1990; Thompson and Dahlman, 1999). When maintained on low sucrose diets both parasitized and normal larvae were glucogenic, but parasitized larvae displayed significantly higher levels of net trehalose formation (Thompson and Dahlman, 1999). On diets high in sucrose, parasitized larvae remained glucogenic while normal larvae were non-glucogenic (Thompson, 2000a,b). It was concluded that the increased glucogenesis was adaptive and enabled the host larvae to maintain the concentration of trehalose in the haemolymph, on which the developing parasites feed. Altered nutrient intake by parasitized insects indicates that the above metabolic effects may be mediated by dietary selection. In the present study, parasitized insects, when allowed to regulate their intake of carbohydrate and protein by choosing between diets of different nutritional quality, displayed equivalent glucogenic flux and similar haemolymph trehalose levels to those of normal larvae.

Our finding that all insects fed the choice diets were glucogenic is of interest, because the larvae displayed relatively high haemolymph sugar levels, generally in the range of 15 to 45 mmol l^{-1}. This range was equivalent to that

of larvae maintained on individual, nutritionally complete diets with near optimal levels of casein and sucrose (Thompson and Redak, 2000). When nutritionally complete diets were consumed, larvae were glucogenic only at very low dietary sucrose levels, where carbohydrate intake was insufficient to maintain normal haemolymph sugar. Under those dietary conditions, haemolymph sugar was also low, less than 15 mmol l^{-1}, and was maintained principally by gluconeogenesis. Those results indicated a relationship between the ratio of dietary casein and sucrose levels and gluconeogenesis, with haemolymph sugar level decreasing as the proportion of dietary protein increased. Such a relationship is consistent with the present finding, where the decreased protein intake of parasitized larvae exceeded that expected by the lower growth of these insects. One-half of the decrease in protein consumption, approximately 2.5 g of the approximate 4–6 g total (Fig. 2), was due to parasitism. With decreased growth, amino acids from dietary protein are available as glucogenic substrate. Moreover, the present results, demonstrating glucogenesis at relatively high haemolymph trehalose levels, indicate that regulation over gluconeogenesis is not directly linked to haemolymph trehalose levels. This supports an earlier investigation that failed to demonstrate the inhibition of *de novo* trehalose formation in glucogenic larvae whose haemolymph sugar level was artificially elevated by injection of glucose or short-term feeding on a high glucose diet (Thompson, 1997a).

To further investigate the basis of the variable haemolymph trehalose level in *M. sexta* larvae, a correlation analysis of the ^{13}C-enrichment ratio [2trehalose C6/alanine C3] and haemolymph trehalose level was conducted. The data for all insect groups were combined and a non-significant partial correlation coefficient of 0.48 was obtained, demonstrating that the two parameters were not directly related. Because the ^{13}C ratio reflects the ratio of gluconeogenesis/glycolysis, and is only a measure of net glucogenic flux, the above finding indicates that the absolute gluconeogenic and/or glycolytic rates are highly variable. This conclusion is consistent with a previous study that demonstrated that the rate of gluconeogenesis from glycerol in glucogenic *M. sexta* varied between larvae by as much as an order of magnitude (Thompson, 1997b). Thus, although the relative level of gluconeogenesis between larvae maintained under the same nutritional conditions may be similar, the absolute rate may differ, explaining, in part, the highly variable haemolymph sugar level. In addition, this conclusion supports the results of Alleyne and Beckage (1997b) who reported variable metabolic rates, determined by gas exchange patterns, in *M. sexta* larvae, due partly to sporadic locomotion.

Precisely how the altered nutrient intake by parasitized *M. sexta* larvae, and the resultant metabolic homeostasis, affect the nourishment and development of the parasite *C. congregata* remains unexplained at this time. The results demonstrated that the altered intake is principally due to a decrease in protein, and thus that the intake of carbohydrate has increased relative to that of protein. The data showing an increased parasite burden supported by host larvae reared on the 1:1 protein/carbohydrate diet clearly suggests that the change in intake is nutritionally beneficial for the parasite. Whether a decreased protein intake of the magnitude observed here may limit the availability of haemolymph protein or amino acids for parasite consumption is unknown. We have yet to conduct comprehensive haemolymph analyses to determine the effect of host diet on haemolymph content and the difference between normal and parasitized larvae. Certainly, the developing parasites require protein for growth, and the biomass of the developing parasites is not insignificant, reaching approximately 30 % of the total host biomass in host larvae with the heaviest parasite burdens examined in the present experiment. Earlier studies by others have examined the effects of parasitism by a variety of hymenopteran species on the haemolymph protein and amino acids compositions of their insect hosts. Significant qualitative and quantitative changes have been reported (Vinson and Iwantsch, 1980), but these were not considered in relation to nutrition. Haemolymph amino acids, as well as sugars, are known to play important roles in regulating dietary selection (Simpson and Raubenheimer, 1993b; Simpson et al., 1995), thus having potentially important implications for host food selection and hence indirectly the nutrition of developing endoparasites.

The altered nutrient intake observed here in parasitized host larvae may partially reflect qualitative and/or quantitative changes in haemolymph amino acid composition. Our earlier investigation with normal *M. sexta* larvae demonstrated that a decrease in the level of dietary protein intake relative to carbohydrate increased the haemolymph trehalose level, and this was derived principally from dietary sucrose. Gluconeogenesis was only induced at relatively high dietary protein levels when actual sucrose consumption was low (Thompson and Redak, 2000). In the present study, the haemolymph trehalose level was maintained in parasitized larvae despite the decrease in protein intake. This may reflect sugar uptake by the developing parasites. Although parasitized as well normal insects were glucogenic, their relative rates of gluconeogenesis were similar despite the significantly lower protein consumption by parasitized larvae. Unfortunately, however, is not possible to determine the actual rate of trehalose synthesis in the present investigation.

References

Adamo, S. A. (1998). Feeding suppression in the tobacco hornworm, *Manduca sexta*: costs and benefits to the parasitic wasp *Cotesia congregata*. *Can. J. Zool.* **76**, 1634–1640.

Adamo, S. A. and Shoemaker, K. L. (2000). Effects of parasitism on the octopamine content of the central nervous system of *Manduca sexta*; a possible mechanism underlying host behavioural change. *Can. J. Zool.* **78**, 1580–1587.

Ahmad, I. M., Waldbauer, G. P. and Friedman, S. (1989). A defined artificial diet for the larvae of *Manduca sexta*. *Entomol. Exp. Appl.* **53**, 189–191.

Akehurst, B. C. (1981). *Tobacco*. New York: Longman.

Alleyne, M. and Beckage, N. E. (1997a). Parasitism-induced effects on host growth and metabolic efficiency in tobacco hornworm larvae parasitized by *Cotesia congregata*. *J. Insect Physiol.* **43**, 407–424.

Alleyne, M. and Beckage, N. E. (1997b). Effect of parasitism by the braconid wasp *Cotesia congregata* on metabolic rate in host larvae of the Tobacco Hornworm, *Manduca sexta*. *J. Insect Physiol.* **43**, 143–154.

Barbosa, P., Gross, P. and Kemper, J. (1991). Influence of plant alelochemicals on Tobacco Hornworm and its parasitoid, *Cotesia congregata*. *Ecology* **72**, 1567–1575.

Beckage, N. E., Tan, F. F., Schleifer, K. W., Lane, R. D. and Cherubin, L. L. (1994). Characterization and biological effects of *Cotesia congregata* polydnavirus on host larvae of the tobacco hornworm *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **26**, 165–195.

Bell, R. A. and Joachim, R. G. (1976). Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. *Ann. Entomol. Soc. Amer.* **69**, 365–373.

Blau, P. A., Feeney, P., Contardo, L. and Robson, D. S. (1978). Allylglucosinolate and herbivorous caterpillars: contrast in toxicity and tolerance. *Science* **200**, 1296–1298.

Campo, M. L. Del, Miles, C. I., Schroeder, F. C., Mueller, C., Booker, R. and Renwick, J. (2001). Host recognition by the tobacco hornworm is mediated by a host plant compound. *Nature* **411**, 186–189.

Christensen, K. A., Grant, D. M., Schulman, E. M. and Walling, C. (1974). Optimal determination of relaxation times for Fourier transform nuclear magnetic resonance. Determination of spin-lattice relaxation times in chemically polarized species. *J. Phys. Chem.* **78**, 1971–1977.

Cohen, R. W., Friedman, S. and Waldbauer, G. P. (1988). Physiological control of nutrient self-selection in *Heliothis zea* larvae: the role of serotonin. *J. Insect Physiol.* **34**, 935–940.

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

Fulton, B. B. (1940). The hornworm parasite, *Apanteles congregatus* Say and the hyperparasite, *Hypopteromalus tabacum* (Fitch). *Ann. Entomol. Soc. Amer.* **33**, 231–243.

Gilmore, J. U. (1938). Observations on the Hornworms attacking tobacco in Tennessee and Kentucky. *J. Econ. Entomol.* **31**, 706–712.

Horton, D. R. and Redak, R. A. (1993). Further comments on analysis of covariance in insect dietary studies. *Entomol. Exp. Appl.* **69**, 263–275.

Keeley, L. L. (1985). Physiology and biochemistry of the fat body. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 211–248. London: Pergamon.

Leffingwell, J. C. (1999). Basic chemical constituents of Tobacco leaf and differences among Tobacco types. *Tobacco, Production, Chemistry and Technology* (ed. D. L. Davis and M. T. Nielson), pp. 265–284. Oxford: Blackwell Science.

McFadden, M. W. (1968). Observations on feeding and movement of Tobacco Hornworm larvae. *J. Econ. Entomol.* **61**, 352–356.

Madden, A. H. and Chamberlin, F. S. (1945). Biology of the tobacco hornworm in the southern cigar tobacco district. *USDA Tech. Bull.* **896**, 51.

Mathews, C. K. and van Holde, K. E. (1990). *Biochemistry*. New York: Benjamin/Cummings.

Mullens, D. E. (1985). Chemistry and physiology of the hemolymph. In *Comprehensive Insect Physiology, Biochemistry and Pharmacology*, vol. 3 (ed. G. A. Kerkut and L. I. Gilbert), pp. 356–399. London: Pergamon.

Poulin, R. (1995). 'Adaptive' changes in the behaviour of parasitized animals: a critical review. *Int. J. Parasitol.* **25**, 1371–1383.

Quickie, D. J. L. (1997). *Parasitic Wasps*. New York: Chapman and Hall.

Raubenheimer, D. and Simpson, S. J. (1999). Integrating nutrition: a geometrical approach. *Entomol. Exp. Appl.* **91**, 67–82.

Reinecke, J. P., Buckner, J. S. and Grugel, S. R. (1980). Life cycle of laboratory-reared tobacco hornworms, *Manduca sexta*, a study of development and behavior, using time-lapse cinematography. *Biol. Bull.* **158**, 129–140.

Rowell, C. H. F. and Simpson, S. J. (1992). A peripheral input of thoracic origin inhibits chewing movements in the larvae of *Manduca sexta*. *J. Insect Physiol.* **38**, 475–483.

Schiff, N. M., Waldbauer, G. P. and Friedman, S. (1989). Dietary self-selection by *Heliothis zea* larvae: roles of metabolic feedback and chemosensory stimuli. *Entomol. Exp. Appl.* **52**, 261–270.

Simpson, S. J. and Raubenheimer, D. (1993a). A multi-level analysis of feeding behaviour: the geometry of nutritional decisions. *Phil. Trans. R. Soc.* **342**, 381–402.

Simpson, S. J. and Raubenheimer, D. (1993b). The central role of the haemolymph in the regulation of nutrient intake in insects. *Physiol. Entomol.* **18**, 395–403.

Simpson, S. J. and Raubenheimer, D. (1996). Feeding behaviour, sensory physiology and nutrient feedback: a unifying model. *Entomol. Exp. Appl.* **80**, 55–64.

Simpson, S. J., Raubenheimer, D. and Chambers, P. G. (1995). The mechanisms of nutritional homeostasis. In *Regulatory Mechanisms of Insect Feeding* (ed. R. F. Chapman and J. De Boer), pp. 251–276. New York: Chapman and Hall.

Simpson, S. J., Simmons, M. S. J. and Blaney, W. M. (1988). A comparison of dietary selection behaviour in larval *Locusta migratoria* and *Spodoptera littoralis*. *Physiol. Entomol.* **13**, 225–238.

Stadler, E. and Hanson, F. E. (1978). Food discrimination and induction of preference for artificial diets in the tobacco hornworm, *Manduca sexta*. *Physiol. Entomol.* **3**, 121–133.

Thompson, S. N. (1997a). Absence of short-term regulation over gluconeogenesis by glucose in the insect *Manduca sexta* L. *Biochem. Biophys. Res. Comm.* **237**, 702–706.

Thompson, S. N. (1997b). Glucogenesis in an insect, *Manduca sexta* L., estimated from the ^{13}C isotopomer distribution in trehalose synthesized from [1,3-$^{13}C_2$]glycerol. *Biochim. Biophys. Acta* **1336**, 110–116.

Thompson, S. N. (1999). Blood sugar formation from dietary carbohydrate is facilitated by the pentose phosphate pathway in an insect *Manduca sexta* Linnaeus. *Biochim. Biophys. Acta* **1472**, 565–575.

Thompson, S. N. (2000a). Parasitism enhances the induction of glucogenesis by the insect *Manduca sexta* L. *Int. J. Biochem. Cell Biol.* **33**, 163–173.

Thompson, S. N. (2000b). Pyruvate cycling and implications for regulation of gluconeogenesis in the insect, *Manduca sexta* L. *Biochem. Biophys. Res. Comm.* **274**, 787–793.

Thompson, S. N. and Dahlman, D. L. (1998). Aberrant nutritional regulation of carbohydrate synthesis by parasitized *Manduca sexta* L. *J. Insect Physiol.* **44**, 745–753.

Thompson, S. N. and Dahlman, D. L. (1999). Blood sugar formation due to abnormally elevated gluconeogenesis: aberrant regulation in a parasitized insect, *Manduca sexta* Linnaeus. *Biochim. Biophys. Acta* **1454**, 133–142.

Thompson, S. N. and Hagen, K. S. (1999). Nutrition of entomophagous insects and other arthropods. In *Handbook of Biological Control* (ed. T. S. Bellows and T. W. Fisher), pp. 594–652. New York: Academic Press.

Thompson, S. N. and Redak, R. A. (2000). Interactions of dietary protein and carbohydrate determine blood sugar level and regulate nutrient selection in the insect *Manduca sexta* L. *Biochim. Biophys. Acta* **1523**, 91–102.

Thompson, S. N., Lee, R. W.-K. and Beckage, N. E. (1990). Metabolism of parasitized *Manduca sexta* examined by nuclear magnetic resonance. *Arch. Insect Biochem. Physiol.* **13**, 127–143.

Vinson, S. B. and Iwantsch, G. F. (1980). Host regulation by insect parasitoids. *Q. Rev. Biol.* **55**, 143–165.

Waldbauer, G. P. and Friedman, S. (1991). Self-selection of optimal diets by insects. *Ann. Rev. Entomol.* **36**, 43–63.

Waldbauer, G. P., Cohen, R. W. and Friedman, S. (1984). Self-selection of an optimal nutrient mix from defined diets by larvae of the corn earworm, *Heliothis zea* (Boddie). *Physiol. Zool.* **57**, 590–597.

Wood, H. A. (1999). Patterns and mechanisms of growth of fifth-instar *Manduca sexta* caterpillars following exposure to low- or high-protein food during early instars. *Physiol. Biochem. Zool.* **72**, 445–454.

Yamamoto, R. T. (1969). Mass rearing of the tobacco hornworm. II. Larval rearing and pupation. *J. Econ. Entomol.* **62**, 1427–1431.

Zitnan, D., Kingan, T. G., Kramer, S. J. and Beckage, N. E. (1995). Accumulation of neuropeptides in the cerebral neurosecretory system of *Manduca sexta* larvae parasitized by the braconid wasp *Cotesia congregata*. *J. Comp. Neurol.* **356**, 83–100.