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Effects of dietary variation on growth, composition, and maturation of *Manduca sexta* (Sphingidae: Lepidoptera)

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Abstract

Most studies linking dietary variation with insect fitness focus on a single dietary component and late larval growth. We examined the effects of variation in multiple dietary factors over most life stages of the sphingid moth, *Manduca sexta*. Larvae received artificial diets in which protein, sucrose, and water content were varied. The relationship between larval size, growth and consumption rates differed significantly across diets. Larvae on control and low-sucrose diets grew most rapidly and attained the largest pupal and adult sizes. Conversely, larvae on low-water and low-protein diets initially grew slowly, but accelerated in the fifth instar and became pupae and adults comparable to control animals in size. There were no fundamental differences in protein:carbohydrate consumption patterns or strategies among experimental diets and larval instars. However, inadequate dietary water appeared to be more important for early than late instar larvae. Larvae on all artificial diets showed increasing fat content throughout all stages, including wandering and metamorphosis. Compensatory feeding among low-water and low-protein larvae was correlated with significantly higher fat content in larvae, pupae and adults, whereas low-sucrose animals were substantially leaner than those on the control diet. These differences may have strong effects on adult physiology, reproduction, and foraging patterns.

Keywords: Artificial diet; Composition; Fat; Growth; *Manduca sexta*; Nutrition

1. Introduction

A series of studies over the last twenty-five years has established the basic nutritional requirements of most major taxonomic groups of insects, the nutritional ecology of immature forms, and the behavioral and physiological mechanisms by which individuals respond to variation in diet quality (Dadd, 1985; Scriber and Slansky, 1981; Simpson and Simpson, 1990; Slansky and Scriber, 1985; Slansky, 1993), especially with respect to nitrogen nutrition (Mattson, 1980; McNeill and Southwood, 1978; Slansky and Feeny, 1977; White, 1993). Many of the primary studies on which these works drew focused on one or two larval instars and variation in one, and occasionally a few, nutritional variables. More recent work has focused on multi-dimensional aspects of insect nutrition. A fundamental problem faced by feeding insects is how to adjust physiology and behavior to obtain simultaneously all necessary nutrients at reasonable rates (Raubenheimer, 1992; Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1993).

An important, unresolved question for most insect species is whether nutrient requirements change across stages. A number of factors may lead to such shifts, including changes in body size (Reavey, 1993) and proximity to metamorphosis (Slansky and Scriber, 1985). For example, studies focusing on physiological changes in preparation for metamorphosis commonly find that later instars (or individuals at the end of the final instar) switch from protein to lipid or energy accumulation, which sometimes is accomplished by stronger preference for low-protein or carbohydrate-rich foods (Cohen et al., 1987; Stockhoff, 1993; Zucoloto, 1987). These choices may reflect larval tracking of shifting nutritional and intake targets (Raubenheimer and Simpson, 1993), and result in the accumulation of energy then used during pupal (non-feeding) and adult stages. Indeed, the lipid content of insect larvae generally increases from early to late instars (Slansky and Scriber, 1985).

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A related question is how larval nutritional experience affects pupal and adult physiology. Studies of larval nutrition traditionally focus on how dietary variation affects aspects of larval biology—e.g. consumption and growth. But the adult carries out many of the activities (mating, dispersal, oviposition) that are most closely associated with fitness. Evolutionary pressures should therefore shape larval foraging (see Stamp and Casey, 1993) to meet the potentially contradictory requirements for additional larval growth and adult longevity and reproduction. Relatively few studies have examined nutritional effects across metamorphic boundaries—with several notable exceptions (Boggs, 1997; O'Brien et al., 2000; Taylor, 1984). These studies showed that oocyte production may depend on nitrogen availability during the larval period and that substantial portions of the nitrogen and carbon components of eggs can be derived from nutrients obtained in the larval stage.

Here we examine how *Manduca sexta* respond to variation in several important diet components (sugar, protein, and water). We first analyze shifts in larval nutritional requirements during ontogeny by rearing larvae on different diets and measuring whether relative performance on the different diets changes with larval stage. Second, we explore how larval dietary experience influences lipid accumulation by larvae and subsequent lipid use during metamorphosis. Because lipids are an important source of energy during metamorphosis (Downer, 1985) we expected that lipid content would decrease during the pupal period. A number of studies have examined how variation in dietary quality affects lipid accumulation and use (Fernando-Warnakulasuriya et al., 1988; Horie and Nakasone, 1971; Simpson et al., 2002; Thompson and Redak, 2000). These studies show that lipid deposition is positively correlated with the amount of lipid or carbohydrate in the diet. The effects of larval dietary history on adult lipid content have fitness implications for *M. sexta*, because fat reserves acquired by larvae are mobilized to fuel hovering flight in adults (Ziegler, 1991; Ziegler and Schulz, 1986a), at least in the absence of nectar feeding (O'Brien, 1999). Thus, the quantity and quality of larval fat reserves may influence adult success in finding mates and host plants (Ziegler, 1991).

2. Materials and methods

2.1. Animals and diets

Eggs of *M. sexta* were obtained from a laboratory colony at the University of Washington and reared from hatching on a wheat-germ based artificial diet (modified from the diet of Bell and Joachim, 1976, as described in Woods and Chamberlin, 1999). Neonate larvae were placed individually in 1-oz plastic cups and were transferred to 9-oz plastic cups when they reached the fifth instar. Cups were placed into an environmental chamber (26 °C), with a L:D cycle of 16:8. Food was provided ad libitum. Three days after hatching, larvae were randomly assigned to one of four different treatments and were switched onto the experimental diets (Table 1), which consisted of control (standard wheat-germ diet) low-protein, low-sucrose, and low-water artificial diets. The low-protein diet was made by replacing 40% of the casein in the standard diet with α-cellulose; the low-sucrose diet was made by replacing 50% of the sucrose in the standard diet with α-cellulose; and the low-water diet was prepared using 30% less total water. In all treatments, food provided to larvae was changed every 1–2 d. When larvae wandered (appearance of pulsating heart visible on the dorsal side, regurgitation of food, and burrowing behavior), remaining food was removed and replaced with wood bedding material (Aspen Bed I, American Excelsior Co.), and the cups with the larvae were returned to the incubator. Larvae pupated in chambers they constructed beneath the bedding over the subsequent several days.

2.2. Growth and consumption rate

We measured growth of 30 larvae per treatment over two time scales, from the beginning of the third instar to the end of the fifth instar and also over 24-h periods within the third, fourth, and fifth instars. For the long-term measures, larvae were weighed after they finished molting to the third instar and again after they began wandering. All larvae were checked once in the morning (0700–0800) and once in the afternoon (1700–1800), so that the observed time of molt would only be a maximum of 12–13 h apart. Overall growth rate was calculated as final minus initial mass divided by number of days, corrected for molting time. For the 24-h measures, larvae were weighed once during the feeding period and again 24-h later. For third instars, the initial weighing took place on the first day following the start of the preceding molt; for fourth instars, the first day after the end of the preceding molt; and for fifths, the second day of feeding. Growth curves were plotted as the average larval mass that each treatment had throughout their larval growth. Relative growth rates (RGR) were calculated by subtracting initial from final mass and dividing by 1 d.

During the 24-h experiments we also measured rates of fresh and dry food consumption. The amount of food given to larvae at the beginning of the 24-h period was adjusted so that at least two-thirds of it was consumed during this interval. Food was weighed before and after the 24-h period. We measured the extent of evaporative water loss from the food by cutting similarly sized pieces of food (at least three per diet) and placing them in cups without larvae, which were placed alongside the others.

Table 1
Composition of the artificial diets (1 l total volume)

Ingredients	Control	Low protein	Low sucrose	Low water
Casein	36 g	12 g	36 g	36 g
Alpha-cellulose	0 g	24 g	22 g	0 g
Wheat germ	80 g	Same	Same	Same
Torula yeast	16 g	Same	Same	Same
Sucrose	32 g	32 g	16 g	32 g
Wesson's salt	12 g	Same	Same	Same
Cholesterol	3.5 g	Same	Same	Same
Ascorbic acid	5 g	Same	Same	Same
Carrageenan	12 g	12 g	6 g	12 g
Water	733 ml	733 ml	733 ml	513 ml

All diets contained in addition (per liter) 2 g sorbic acid, 1 g methyl paraben, 200 mg streptomycin sulphate, 53 mg kanamycin monosulphate, 23.3 ml 10% formalin, 4 ml raw linseed oil, and 10 ml of a vitamin mixture (containing, in mg, 50 nicotinic acid, 25 riboflavin, 11.7 thiamine, 11.7 pyridoxine, 11.7 folic acid, and 1 biotin in 50 ml distilled water).

These blocks of food were weighed before and after the experimental period; the difference in mass indicates water loss by evaporation. We also estimated dry consumption by converting fresh masses of food to dry using a conversion factor determined for each diet. This was done by placing three samples of each diet in a drying oven (60 °C) for three days. The water fraction per diet was calculated as dry mass divided by fresh, and we used these relationships to estimate dry masses of the food before and after consumption. To calculate relative consumption rate (RCR, both fresh and dry), we subtracted final from initial mass of food, divided by 1 d, and divided in both cases by initial fresh larval mass (Martin and Van't Hof, 1988).

2.3. Fat measurements

We also analyzed fat content across developmental stages using Soxhelet extraction. Fifty larvae per diet were reared as described above. Within each treatment, 5–12 individuals were frozen at each of the following developmental stages: the molt from fourth to fifth instar, the feeding period of the fifth instar (1–3 caterpillars per day), the first day of wandering, 10 d after wandering (as pupae), and the first day after adult eclosion. Individuals were then dried (60 °C) for three days, weighed, and ground in a mortar and pestle. Sub-samples (approximately 170 mg) were placed into clean cotton thimbles, which were plugged with cotton, re-dried overnight and weighed afterwards. Fat was extracted with petroleum ether (Inagaki and Yamashita, 1986) in a Soxhelet apparatus for 4 h, after which the thimble units were dried again and weighed. Mass of fat extracted was calculated as the difference between initial and final dry masses of the unit. Fat contents of the individuals were expressed as fat mass divided by total dry mass of the sub-sample.

2.4. Statistical analysis

We analyzed data in terms of both traditional nutritional indices (Waldbauer, 1968) and the more recently developed geometric framework (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1995). For nutritional indices, we used analysis of variance (ANOVA) to examine the effects of instar and diet on rates of growth and consumption. Dependent variables included larval mass gain, overall growth rate, RGR, RCR fresh and dry, number of days taken to reach wandering stage and fat accumulation. If ANOVA detected significant effects of diet, we used Tukey's HSD to conduct pair-wise comparisons of the effects of individual diets testing if they were different. This test adjusts the critical value (α) for each pair-wise comparison such that the Type I experiment-wide error rate is held constant at $\alpha = 0.05$.

Intake data were analyzed by analysis of covariance (ANCOVA) (Raubenheimer and Simpson, 1992), with initial fresh mass as a covariate. In addition, rates of dry food intake were used in conjunction with data on diet composition to calculate rates of protein and carbohydrate intake. Intake rates of protein and carbohydrate were examined as bicoordinate plots (Raubenheimer and Simpson, 1992, 1994) and analyzed as relative intake rates using ANOVA. Growth rates were analyzed by ANCOVA with either initial mass or food consumed as covariates (Raubenheimer and Simpson, 1994). All statistical tests were performed with S-Plus statistical software (version 6.0, Insightful Corp.).

3. Results

3.1. Survivorship and growth

Larval mortality was 0, 3.3, 3.3, and 6.7% on the control, low-sucrose, low-water, and low-protein diets,

respectively. Mean larval masses at the beginning of the third instar (Table 2) were similar for control, low-protein, and low-sucrose treatments and slightly smaller for the low-water treatment. Diet significantly affected larval growth rates from the beginning of the third instar to the end of the fifth (Table 2). Larvae eating control and low-sucrose diets grew the most rapidly and wandered earliest. Those eating low-protein diet grew more slowly, and those given low-water diet grew slowest. Despite growing slowly, larvae in the low-protein treatment reached the same pupal mass as those eating control and low-sucrose diets; larvae in the low-water treatment reached a significantly smaller pupal mass.

In all instars, growth rate over 24 h was strongly related to both initial mass and diet treatment (Fig. 1, Table 4). Additional exploratory analysis of reduced data sets (not shown) indicated that the low-water treatment accounted for essentially all of the significant diet effect in the third instar, but not in the fourth and fifth. Inspection of Fig. 1 suggests that, in the two later instars, the rank order of growth rates was lowest on low-water diet, somewhat higher on low-protein, higher still on control, and highest on low-sucrose. These effects are largely consistent with the overall growth rates (from beginning of third instar to wandering) (Table 3). Interestingly, in the fifth instar, the relationship between initial mass and growth appears for some groups to be slightly negative; this may reflect that some of the larvae used (especially in the control and low-sucrose treatments) were initially larger than 8 g and thus close to wandering.

3.2. Consumption

Larval consumption rates depended significantly on diet, instar, and body size (Fig. 2, Tables 3 and 4). Across instars, larvae given low-protein diet consistently consumed the most diet in 24 h on both fresh and dry bases (Table 3), although this effect was not always significant. Compared to larvae eating control and low-sucrose diets, larvae eating low-protein diets also had higher mass-specific fresh and dry consumption rates, significantly so in instars 3 and 5 (Table 3). This effect also is apparent in Fig. 2, which shows that for their size, larvae in the low-protein treatment in general consumed more than larvae given control or low-sucrose diet. By all measures, larvae consuming control and low-sucrose diets had similar rates of consumption (Fig. 2, Table 3).

Larvae eating low-water diet showed interesting changes in consumption patterns across instars. Total fresh consumption in 24 h by these larvae was always significantly less than by larvae in the other groups (Fig. 2, Table 3). In contrast, total dry consumption, though always lowest, was quite similar to 24-h dry consumption totals in the other groups, differing significantly from all others only in the fifth instar (Table 3). However, a complicating factor in this analysis is that larvae in the low-water group were consistently smaller than larvae in the other groups. Plots of 24-h fresh and dry consumption by larvae given low-water diet (Fig. 2) suggest a qualitatively different view. In the third and fourth instars, low-water larvae ate significantly more diet on a mass-specific dry basis in 24 h than larvae given control or low-sucrose diet (the same mass-specific rate as larvae given low-protein diet). In Fig. 2, this effect is apparent in the position of the cloud of data points representing the low-water larvae: in instars 3 and 4 they ate approximately the same total amount of dry food as larvae given control and low-sucrose diets, but because their initial masses were smaller, their mass-specific rates were significantly higher. On a fresh basis, larvae eating low-water food clearly ate less in each instar than those given other diets (Fig. 2); however, much of this effect is explained by their small body size, such that their mass-specific fresh consumption rates were not always significantly lower (Table 3)

In the ANCOVAs associated with Fig. 2 (Table 4), there were significant interactions in instars 3 and 4 between larval initial mass and both measures of consumption (over 24 h), indicating that the relationship between larval size and consumption rate differs across diets. Inspection of Fig. 2 suggest that these interactions arise because larvae given low-protein and, especially, low-water diets showed steeper relationships between larval size and consumption rate than larvae in the other two groups. These data suggest that larger larvae were relatively better at using behavioral means (i.e. compensatory feeding) to respond to protein- or water-poor diets. The significant interaction effects disappeared in the fifth instar, probably because within each diet the size-dependence of consumption rate was not particularly strong.

Using values of protein and digestible carbohydrate content of each diet, together with total dry consumption rates and larval masses, we calculated mass-specific protein and carbohydrate consumption rates (over 24 h) within each instar (Fig. 3). Although RCRs decreased significantly across instars, the relative patterns of protein and carbohydrate consumption were not particularly different (Fig. 3, Table 5).

3.3. Utilization

Relative growth per unit consumption also varied significantly across diets (Fig. 4, Table 4). Four aspects of these data are notable. First, larvae eating control and low-sucrose diets had broadly overlapping consumption and growth rates; growth is equivalently efficient on these two diets. Second, per unit food consumed, larvae given low-protein diet grew less than larvae given control and low-sucrose diets. Third, in instars 3 and 4, larvae eating low-water diet had the lowest rates of growth

Table 2
Summary of masses of larvae at the beginning of the third instar, at wandering, and in the pupal stage, duration of the growth period, and growth rate (mean ± SE)

	Diet treatment			
	Control	Low protein	Low sucrose	Low water
Mass at beginning of third instar (g)	0.039 ± 0.0009[a]	0.038 ± 0.0011[a]	0.038 ± 0.0008[a]	0.034 ± 0.0007[b]
Mass at wandering (g)	10.41 ± 0.17[a]	10.85 ± 0.14[a]	10.80 ± 0.16[a]	9.75 ± 0.16[b]
Days from hatching to wandering	16.1 ± 0.1[a]	17.4 ± 0.2[b]	15.9 ± 0.1[a]	18.0 ± 0.1[c]
Days from beginning of third instar to wandering	11.7 ± 0.1[a]	12.7 ± 0.1[b]	11.6 ± 0.1[a]	13.4 ± 0.1[c]
Growth rate (g d^{-1})	0.887 ± 0.014[ab]	0.852 ± 0.011[a]	0.932 ± 0.012[b]	0.727 ± 0.015[c]
Pupal mass (g)	6.08 ± 0.09[ab]	6.31 ± 0.07[a]	6.24 ± 0.08[a]	5.92 ± 0.08[b]

Each variable was analyzed in a two-step process. ANOVA was first used to examine whether there were significant differences among treatment groups ($\alpha = 0.05$). If none was found, all treatments for that variable were assigned the letter a. If significant differences were found, Tukey HSD was used to identify which treatments differed. The results of the post hoc tests are denoted with letters; treatments sharing the same letter did not differ significantly.

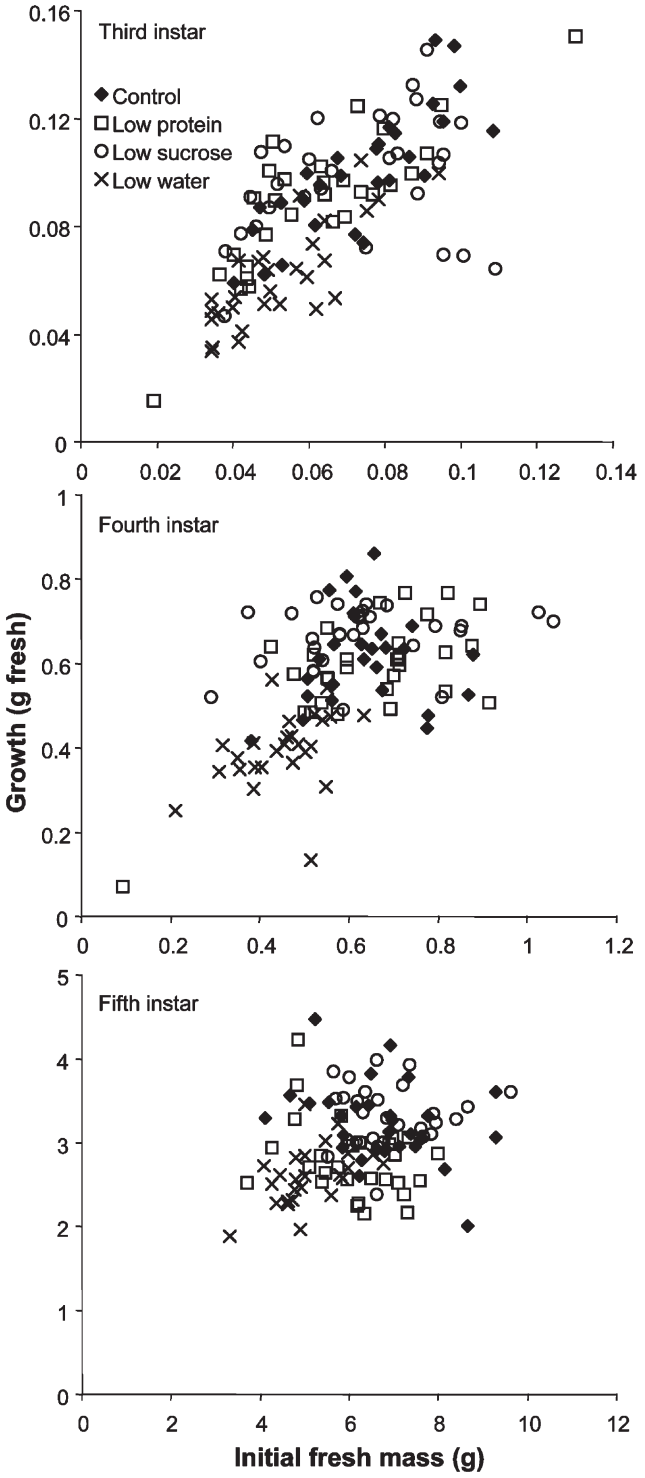

Fig. 1. Fresh growth of larval *M. sexta* on different diets over 24 h in middle of instars 3, 4, and 5.

per unit dry mass consumed. However, as a function of fresh mass consumed, these larvae grew at rates comparable to those achieved by larvae eating control and low-sucrose diets. These data suggest that the primary cost to eating low-water diet is the lack of water *per se*—rather than other possible costs such as interference in protein and carbohydrate processing by poorly hydrated tissues. Fourth, the relative growth efficiencies of larvae on control, low-sucrose, and low-protein diets were similar across instars. Larvae on low-water diet, in contrast, performed relatively better in later instars—that is, they grew relatively more per unit food consumed in later stages, especially the fifth instar. These data indicate that (within the dietary variation imposed in our experiments) the nutrient targets for protein and carbohydrate did not shift across instars but that the nutritional target for water did so. In particular, fifth instars apparently had a substantially reduced need for dietary water compared to earlier instars.

3.4. Fat content

The fat content per unit dry mass of the artificial diets varied between 3.8 and 4.6% (Table 6). Both diet and stage had significant effects on fat content (Fig. 5, Table 7). Fat content increased from 10–20% at the beginning of the fifth instar to between 18 and 26% at wandering—and continued to increase during metamorphosis, reaching 22–35% on the first day after eclosion. In general, individuals eating the low-protein diet had the highest fat content, those eating low-water and control diets had intermediate amounts, and those eating low-sucrose had the lowest. The rank order of fat contents associated with each diet were consistent across growth stages, except during the molt from fourth to fifth instar, when larvae on low-water diet had the highest fraction fat (Fig. 5).

4. Discussion

In this study, the effects of variation in individual dietary factors on growth of larval *M. sexta* were largely consistent with findings of previous studies: the main factors influencing consumption and growth rates were protein and water content (see Karowe and Martin, 1989; Martin and Van't Hof, 1988; Slansky, 1993; Timmins et al., 1988; Van't Hof and Martin, 1989; Woods and Harrison, 2001). Our data, however, provide a unique opportunity to compare, together in a single study, the effects of varying multiple dietary factors over several developmental stages. Such an analysis reveals significant heterogeneity in responses among stages, and suggests that larval dietary experience can strongly affect adult physiology.

4.1. Stage by diet interactions

A priori, we expect that shifting nutritional requirements and capabilities are a general feature of lepidopteran larval development, as most caterpillars undergo substantial changes in body size, ecology, and physiology across larval instars (Reavey, 1993). Functional

Table 3
Summary of growth and feeding data for 24-h test periods in instars 3 through 5

	Initial larval mass (g)	Final larval mass (g)	Growth rate (g d^{-1})	Mass-specific growth rate (g g^{-1} d^{-1})	Fresh consumption (g)	Mass-specific fresh consumption (g g^{-1} d^{-1})	Dry consumption (g)	Mass-specific dry consumption (g g^{-1} d^{-1})
Third instar								
Control	0.073 ± 0.003^{a}	0.172 ± 0.007^{a}	0.099 ± 0.004^{a}	1.40 ± 0.04^{ac}	0.188 ± 0.007^{a}	2.66 ± 0.08^{a}	0.0464 ± 0.0017^{ab}	0.656 ± 0.020^{a}
Low protein	0.062 ± 0.004^{ab}	0.152 ± 0.009^{a}	0.089 ± 0.005^{a}	1.47 ± 0.06^{a}	0.207 ± 0.012^{a}	3.35 ± 0.13^{b}	0.0515 ± 0.0029^{a}	0.835 ± 0.031^{b}
Low sucrose	0.072 ± 0.004^{a}	0.171 ± 0.007^{a}	0.098 ± 0.004^{a}	1.46 ± 0.076^{a}	0.187 ± 0.008^{a}	2.74 ± 0.12^{a}	0.0443 ± 0.0018^{ab}	0.650 ± 0.029^{a}
Low water	0.052 ± 0.003^{b}	0.114 ± 0.006^{b}	0.062 ± 0.004^{b}	1.19 ± 0.04^{c}	0.128 ± 0.007^{b}	2.50 ± 0.12^{a}	0.0423 ± 0.0024^{b}	0.823 ± 0.040^{b}
Fourth instar								
Control	0.635 ± 0.022^{a}	1.25 ± 0.032^{a}	0.617 ± 0.022^{ab}	1.00 ± 0.04^{ab}	1.086 ± 0.043^{a}	1.75 ± 0.08^{ab}	0.247 ± 0.010^{a}	0.400 ± 0.019^{a}
Low protein	0.646 ± 0.032^{a}	1.23 ± 0.051^{a}	0.586 ± 0.024^{a}	0.93 ± 0.04^{a}	1.251 ± 0.051^{a}	1.98 ± 0.06^{a}	0.290 ± 0.012b	0.459 ± 0.014^{ab}
Low sucrose	0.629 ± 0.032^{a}	1.294 ± 0.038^{a}	0.665 ± 0.013^{b}	1.13 ± 0.06^{b}	1.155 ± 0.056^{a}	1.97 ± 0.10^{ab}	0.257 ± 0.013^{ab}	0.436 ± 0.021^{a}
Low-water	0.449 ± 0.018^{b}	0.844 ± 0.030^{b}	0.395 ± 0.017^{c}	0.91 ± 0.04^{a}	0.729 ± 0.027^{b}	1.68 ± 0.06^{b}	0.222 ± 0.008^{a}	0.512 ± 0.018^{b}
Fifth instar								
Control	6.72 ± 0.22^{ab}	9.95 ± 0.21^{a}	3.23 ± 0.09^{ab}	0.50 ± 0.03^{a}	7.62 ± 0.15^{a}	1.16 ± 0.03^{a}	1.74 ± 0.04^{a}	0.265 ± 0.007^{a}
Low protein	6.11 ± 0.20^{a}	8.90 ± 0.19^{b}	2.79 ± 0.09^{a}	0.48 ± 0.03^{a}	8.19 ± 0.17^{a}	1.38 ± 0.06^{b}	1.91 ± 0.04^{b}	0.322 ± 0.013^{b}
Low sucrose	6.89 ± 0.18^{b}	10.21 ± 0.20^{a}	3.32 ± 0.07^{b}	0.49 ± 0.02^{a}	8.07 ± 0.15^{a}	1.19 ± 0.04^{a}	1.80 ± 0.03^{a}	0.266 ± 0.008^{a}
Low-water	5.09 ± 0.16^{c}	7.70 ± 0.20^{c}	2.60 ± 0.07^{c}	0.52 ± 0.01^{a}	4.42 ± 0.21^{b}	0.90 ± 0.03^{c}	1.36 ± 0.07^{c}	0.278 ± 0.008^{a}

Within instars, each variable was analyzed in a two-step process. First, ANOVA was used to examine whether there were significant differences among treatment groups ($\alpha = 0.05$). If none was found, all treatments for that variable (within an instar) were assigned the letter a. If significant differences were found, Tukey HSD was used to identify which treatments differed. The results of the post hoc tests are denoted with letters; treatments sharing the same letter did not differ significantly.

Table 4
F and *P* values for ANCOVAs of 24-h performance data

Source (df)[a]	Dry consumption		Fresh consumption		Growth versus initial mass		Growth versus dry consumption		Growth versus fresh consumption	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Third instar										
Covariate[b] (1)	115.7	0.000	188.6	0.000	176.0	0.000	209.0	0.000	322.4	0.000
Diet (3)	10.3	0.000	19.7	0.000	11.0	0.000	37.9	0.000	12.9	0.000
Cov × diet (3)	5.3	0.002	4.9	0.003	5.1	0.002	4.8	0.004	1.8	0.157
Fourth instar										
Covariate[b] (1)	49.2	0.000	85.7	0.000	79.3	0.000	569.2	0.000	790.1	0.000
Diet (3)	3.6	0.016	12.1	0.000	22.1	0.000	119.7	0.000	47.4	0.000
Cov × diet (3)	3.5	0.018	3.1	0.030	3.1	0.031	1.2	0.315	0.6	0.589
Fifth instar										
Covariate[b] (1)	2.9	0.090	159.2	0.000	8.9	0.004	49.5	0.000	59.4	0.000
Diet (3)	7.9	0.001	78.1	0.000	17.8	0.000	18.3	0.000	14.6	0.000
Cov × diet (3)	1.1	0.322	2.7	0.052	3.6	0.017	0.9	0.467	1.3	0.294

[a] Error degrees of freedom for all tests are between 99 and 109.
[b] Covariate for dry and fresh consumption is larval fresh mass at the beginning of the 24-h period. Covariate for growth is either initial mass, dry consumption, or fresh consumption.

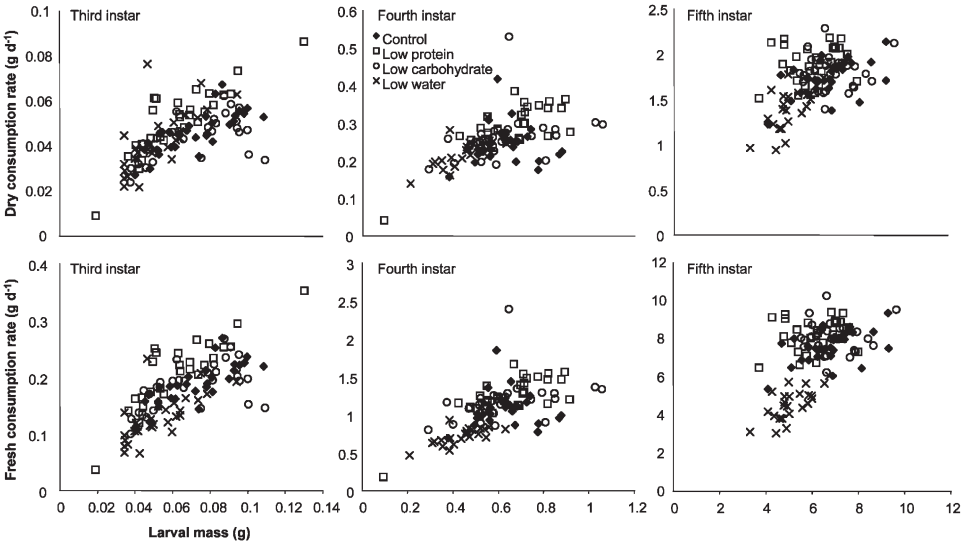

Fig. 2. Fresh and dry consumption of diets by *M. sexta* over 24 h in instars 3, 4, and 5.

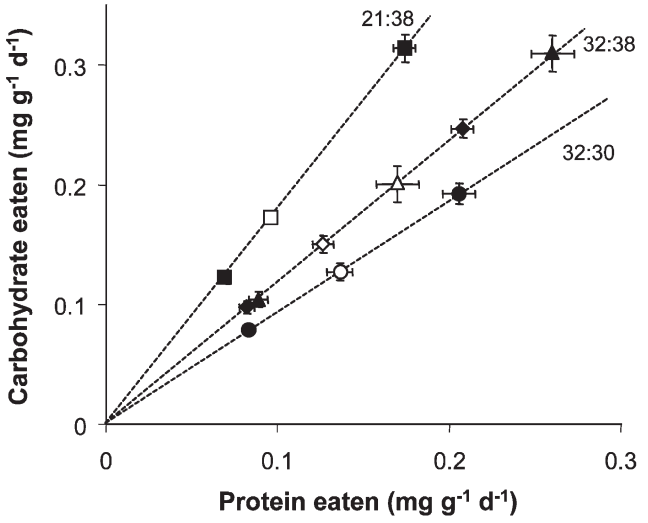

Fig. 3. Bicoordinate plot of protein and carbohydrate eaten over 24 h in instars 3, 4, and 5. Error bars are SEM. Dashed lines represent nutritional rails whose slopes are given as ratios of protein:carbohydrate. Artificial diet symbols: control (diamonds), low protein (squares), low sucrose (circles), low water (triangles). Third instars are the filled outer symbols, fourth instars the unfilled symbols, and fifth instars the filled inner symbols.

nutritional goals should change from (in early instars) acquiring nutrients necessary for additional growth to (in later instars) acquiring nutrients necessary for metamorphosis and adulthood. Indeed, our analyses revealed significant stage by diet interactions, however, this conclusion requires careful qualification.

First, the overall pattern of protein and carbohydrate consumption did not change substantially over the three instars examined (Fig. 3), suggesting that larval intake targets did not change over development. On the other hand, our data also show that larval responses to variation in both water and protein varied across instars. In particular, larvae given low-water diet grew relatively slowly in the third instar but accelerated in later instars. This effect did not reflect instar-specific patterns of consumption (Fig. 2); rather, larvae in the fifth instar were able to use low-water diet particularly efficiently for growth (Fig. 4). Apparently water becomes less growth limiting with age or size. Our data also show instar-specific differences in protein consumption and use. Compensatory feeding for protein was strongest in the third instar and became less pronounced in instars 4 and 5 (Fig. 2). In addition, the relative efficiency of turning ingested low-protein diet into new biomass was lowest in the fifth instar (Fig. 4). As a result, individuals on low-protein diet grew slowest (relative to larvae on other diets) in the fifth instar. This result was unexpected: early instars should have had the highest demand for protein to support growth and therefore the greatest susceptibility to protein restriction. Also, other studies (Cohen et al., 1987; Stockhoff, 1993; Zucoloto, 1987) have suggested that larvae prior to wandering prefer diets weighted away from protein and toward carbohydrate. Perhaps in our study, individuals on the low-protein diet had difficulty processing the relatively high amounts of associated carbohydrate (see subsequently).

4.2. Ratio of protein to carbohydrate

Many insects when given a choice of diets will select a combination that gives balanced nutrient intake (Simpson et al., 1995; Waldbauer and Friedman, 1991). The quantities of different nutrients consumed in such tests define an ‘intake target’ (sensu Raubenheimer and Simpson, 1993). We used only no-choice diets and therefore were unable to quantify the intake target for protein and carbohydrate. Nonetheless, the bicoordinate plot of protein and carbohydrate consumption (Fig. 3)—despite

Table 5
Summary of F and P from repeated-measures ANOVA of consumption data shown in Fig. 2

	Relative protein eaten		Relative carbohydrate eaten	
Source of variation (df)	F	P	F	P
Within individuals				
Instar (1)	834.2	0.000	847.4	0.000
Instar × diet (3)	9.6	0.000	14.4	0.000
Residuals (210)				
Between individuals				
Instar (1)	23.1	0.000	34.0	0.000
Diet (3)	35.9	0.000	45.9	0.000
Instar × diet (3)	0.45	0.715	0.20	0.894
Residuals (111)				

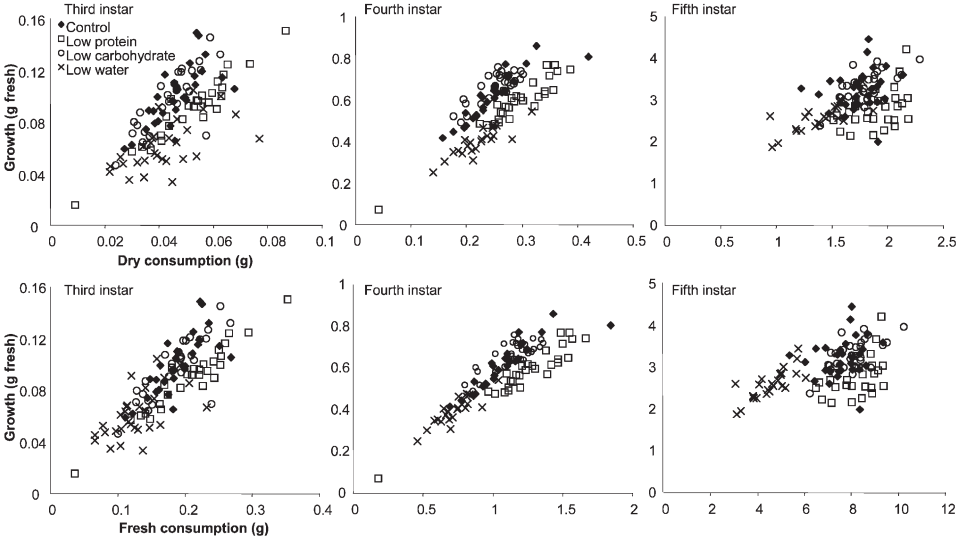

Fig. 4. Fresh growth over 24 h as a function of fresh and dry consumption (utilization plots).

Table 6
Summary of mean ± standard error values of water and fat content for the different diets

Diet	N	Water fraction	Fat content
Control	3	0.774 ± 0.001	0.046 ± 0.006
Low protein	3	0.771 ± 0.001	0.041 ± 0.002
Low sucrose	3	0.780 ± 0.002	0.039 ± 0.002
Low water	3	0.698 ± 0.002	0.038 ± 0.002

the small number of different dietary 'rails' (Raubenheimer and Simpson, 1993)—suggests that larvae did not use functional rules such as 'eat until a particular amount of protein is ingested' or 'eat until a particular amount of carbohydrate is ingested.' Rather, the arc-shaped pattern (excluding the low-water diet) in all instars is consistent with the rule, 'eat until geometrically closest to the intake target' (Raubenheimer and Simpson, 1993; Simpson and Raubenheimer, 1995). Under such an interpretation, the intake target would lie on or near the 32:38 protein:carbohydrate rail.

Other published data, however, suggest another interpretation. Thompson et al. (2001) recently demonstrated that fifth-instar *M. sexta* (over the first two or three days of the instar), given a choice between diets containing 120 g/l sucrose and no casein or 120 g/l casein and no sucrose, selected diets such that they consumed protein:carbohydrate in a ratio of approximately 2:1. This ratio is more protein-rich than any of the diets in our experiment (the low-sucrose diet contained these components in a ratio of 32:30), implying that the intake target lies below all three rails as shown in Fig. 3. In

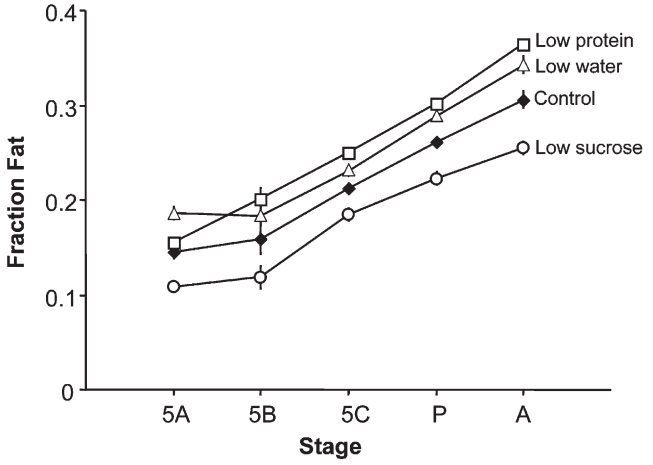

Fig. 5. Mean (± SEM) fat content (g g^{-1} dry mass) of larvae molting from fourth to fifth instar (5A), middle of fifth instar (5B), and when wandering (5C), pupae (P), and adults (A) on the four experimental diets. Where error bars are not visible, SEM was smaller than the symbol.

addition, Thompson and Redak (2000) measured consumption of casein and sucrose by larvae given no-choice, semi-synthetic diets containing casein:sucrose ratios of 12.5:17, 25:17, 50:17, or 75:17. On the three latter diets larvae appeared to consume food primarily to reach a particular level of casein intake, rather than consuming for a particular level of sucrose intake or minimizing error around an intake target; on the 12.5:17 diet, by contrast, consumption of casein fell off sharply. These data suggest that two concurrent processes may shape larval consumption patterns: low sucrose (or carbohydrate) levels allow larvae to feed primarily to reach protein intake targets, whereas high sucrose levels (low ratios of protein:carbohydrate) provide excess carbohydrates that are increasingly difficult to process or use. Thus, in our experiment, larvae given diet 20:38 may have eaten less protein because of the simultaneous load of excess carbohydrate. Clearly additional experiments exploring a broader range of diets, especially those with higher protein and lower sucrose contents, would be of interest.

4.3. Fat accumulation

Larvae on all diets deposited fat during the fifth instar. Increasing fat content from early to late instars is a general feature of insect development (Slansky and Scriber, 1985) and has been demonstrated previously in *M. sexta* (Fernando-Warnakulasuriya et al., 1988). We did not measure the location of fat deposition or its chemical form, but previous work suggests that it is stored in the fat body as triacylglycerol. The fat body is analogous to adipose tissue and liver in vertebrates, serving as a depot and also playing a central role in mobilizing fat (usually as diacylglycerol) into circulation during muscular effort (Dean et al., 1985; Van der Horst, 1990). Triacylglycerols in particular tend to be the major source of metabolic energy in insects during non-feeding stages of development (Downer, 1985; Soulages and Wells, 1994). The biochemical mechanisms of fat deposition and mobilization are increasingly well known (Arrese et al., 2001; Downer, 1985; Fernando-Warnakulasuriya et al., 1988; Siegert, 1987; Siegert et al., 1993; Ziegler and Schulz, 1986a), although the digestion and uptake of dietary lipids remains poorly understood (Turunen and Chippendale, 1989).

Diet strongly affected overall fat content. First, larvae reared on low-sucrose diet were leaner than larvae from other artificial diet treatments. Insect larvae in general are capable of transforming dietary carbohydrate into storage forms of fat (Downer, 1985; Horie and Nakasone, 1971), and a number of studies have demonstrated positive correlations between diet carbohydrate content and fat deposition (e.g. Zanotto et al., 1993), including studies of *M. sexta* (Thompson and Redak, 2000). Presumably, in this study, larvae given low-sucrose diet converted a greater fraction of ingested carbohydrate into energy, leaving less left over for conversion to fat. Second, larvae eating low-water and low-protein diets had significantly higher fat contents than control larvae (see also Karowe and Martin, 1989; Lii et al., 1975; Simpson et al., 2002; Thompson and Redak, 2000). It is likely that larvae on both diets were unable to use assimilated carbohydrate because of shortages of other nutrients (protein or water, Martin and Van't Hof, 1988), and

Table 7
Summary of ANOVA of the effects of stage and diet on fat accumulation. Values of fraction lipid were arcsin-square root transformed prior to analysis

	df	MS	*F*	*P*
Stage	4	0.246	338.2	0.000
Diet	3	0.077	105.6	0.000
Stage × diet	12	0.002	2.3	0.011
Residuals	147	0.001		

that extra carbohydrate was deposited as fat (Downer, 1985; Thompson and Redak, 2000). Such an effect may be amplified by compensatory consumption for protein.

Unexpectedly, fat content increased from wandering to mid-pupal stages, and showed further increases on the first day after eclosion (Fig. 5). This effect must result from one of two processes, and possibly both. Increasing fat content may be due to differential loss of non-fat dry mass during metamorphosis and eclosion. Alternatively, during pupation and metamorphosis, larvae may have transformed non-extractable carbohydrates into fat.

Differences in larval dietary experience resulted in adults with substantially different fat contents (Fig. 5), likely with strong effects on adult physiology, reproduction, and foraging patterns. Ziegler (1991) observed only one in four adult male *M. sexta* reared on artificial diet to feed even once during a week of captivity. Diet-reared *M. sexta* males show a similar reluctance to feed in wind-tunnel assays (Raguso and Willis, 2003) and Pavlovian conditioning trials (Daly and Smith, 2000). However, most captive *M. sexta* males respond to sex pheromone and mate successfully within two days after eclosing (Willis and Arbas, 1991) and thus are likely to fuel mate-searching flights with fat derived from larval food consumption. In contrast, 83% of female *M. sexta* in Ziegler's (1991) experiments fed avidly from sucrose solution in artificial flowers, and laid 10-fold more eggs than did starved females. O'Brien et al.'s (2000) elegant studies of another hawkmoth species, *Amphion floridensis*, offer a potential explanation; adult nectar meals provide up to 60% of carbon allocated to eggs, while the remainder is derived from larval diet. Interestingly, starved *A. floridensis* adults utilize fat body as flight fuel, while those that feed upon nectar utilize carbohydrate only (O'Brien, 1999). Such physiological flexibility may also occur in *M. sexta*, which use fat as flight fuel when starved (Ziegler and Schulz, 1986a) but carbohydrate during warmup (Joos, 1987; Ziegler and Schulz, 1986b) and perhaps after nectar feeding.

All diets in this study, regardless of composition, were rich sources of energy—indeed, artificial diets were developed with this express goal (Bell and Joachim, 1976; Yamamoto, 1969). Data on fat contents of leaf-fed individuals would be of considerable interest. In this study, we intended to include a group of larvae reared on tobacco leaves (*Nicotiana tabacum*). However, several problems with this group (related to dietary induction) precluded our using them in the formal analyses above. Nonetheless, we did measure fat contents of individuals in this group, and the results suggest that leaf-fed individuals are much leaner than those given artificial diet. In larvae eating tobacco leaves, fat increased from 6–10% during the fifth instar and subsequently fell during metamorphosis, such that eclosing adults were about 8% fat (one-half to one-third the fat content of those given artificial diet). In addition, nine larval *M. sexta* (middle of fifth instar) collected from natural populations on tobacco and *Datura wrightii* (North Carolina and Arizona, respectively) had a mean fat content of 7.7%. These observations suggest that wild individuals store only limited quantities of fat. Additional work should examine fat accumulation by wild larvae under natural feeding conditions and the consequences of fat content for adult foraging and reproductive success.

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