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Determinants of Diet Quality: the Effects of Diet pH, Buffer Concentration and Buffering Capacity on Growth and Food Utilization by Larvae of *Manduca sexta* (Lepidoptera: Sphingidae)

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The growth of fourth-instar *Manduca sexta* larvae on nutrient-rich artificial diets is significantly affected by the characteristics of the buffer system present in the diet. An increase in diet buffer concentration or buffering capacity can cause decreases in total larval weight gain, relative growth rate, net growth efficiency and larval lipid content, and increases in the length of the instar, respiration rate, and the amount of assimilated food allocated to energy metabolism. We conclude that there is a significant metabolic cost associated with processing a diet with a high buffer concentration or buffering capacity. Within the pH range examined in this study (4.4–5.5), pH has a less pronounced effect on herbivore growth parameters, and presumably also on fitness, than do buffer concentration and buffering capacity. These results demonstrate that foliar buffer systems are potentially important determinants of the nutritional value of foliage to insect herbivores.

Diet pH Diet buffer Buffering capacity Herbivory Nutrition *Manduca sexta*

INTRODUCTION

The major determinants of the quality of foliage as a food for herbivores are generally considered to be nitrogen and water content, allelochemical content, and various physical attributes such as toughness and pubescence (Slansky and Scriber, 1985; Bernays and Barbehenn, 1987; Mattson and Scriber, 1987; Tabashnik and Slansky, 1987). The purpose of this study is to explore the additional possibility that pH, buffer concentration and buffering capacity also might affect the nutritional value of foliage for insect herbivores.

The low pH and high buffering capacity typically reported for homogenates of plant tissues are due largely to organic acids, especially malate and citrate, present in the vacuoles, which account for 80–90% of total cell volume (Kurkdjian *et al.*, 1985; Pflanz and Heber, 1986; Kurkdjian and Guern, 1989). Vacuolar pH generally falls in the range 5.0–6.5, although significantly lower values have also been reported (Smith and Raven, 1979; Kurkdjian and Guern, 1989). Schultz and Lechowicz (1986) have reported that the average pH of freshly homogenized leaf tissue from 23 hardwood tree species was 5.1 (range 4.1–6.2), and the average buffering capacity (μmol of hydroxide/g of leaf tissue to raise the pH to 8.75) was 96.9 (range 43.9–247.8).

It is possible to envision several mechanisms by which pH and the properties of the buffering system(s) present in the diet might affect herbivore fitness. First, diet pH might affect palatability, and thus affect consumption. Second, the ingestion of acidic food might alter gut pH and thereby affect the digestion and assimilation of nutrients or the chemical modification and assimilation of allelochemicals. Schultz and Lechowicz (1986) have shown that the midgut pH of late instar gypsy moth (*Lymantria dispar*) larvae is dependent upon diet pH, diet buffering capacity, and time since last feeding. Third, introduction of acidic food into the gut might necessitate a significant expenditure of energy to return gut pH to its normal value, resulting in the diversion of assimilated food from growth and energy storage (e.g. lipid accumulation) to energy metabolism. The metabolic cost of processing acidic foliage might be especially

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high in lepidopteran larvae, which normally maintain highly alkaline midguts (pH 8–12) (Berenbaum, 1980; Dow, 1986). The high pH in caterpillar midguts is believed to be due to the secretion of carbonate, which is accomplished by a coupling of the electrogenic transport of potassium with the electrostatic removal of protons from transported bicarbonate (Dow, 1984, 1986). The metabolic expense of maintaining a high midgut pH must be considerable (Dow, 1986). Fourth, the ingestion of high concentrations of buffer might create osmotic conditions in the gut that require the expenditure of metabolic energy in numerous processes involved in redistributing both ions and water between the midgut lumen, the hemocoel, the Malpighian tubules and the hindgut.

In this study we have assessed the extent to which pH, buffer concentration and buffering capacity (a composite property determined by both pH and buffer concentration) affect growth, efficiency of food utilization, and energy metabolism in the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae). We have measured growth rate, food utilization, respiration rate, and lipid accumulation by larvae reared on artificial diets that differ in pH, buffer concentration and buffering capacity. Our experiments address the following questions: (1) Can growth and food utilization be affected significantly by diet pH and the buffer system present in the diet? (2) Is there a metabolic cost associated with processing a diet with low pH and/or high buffer concentration and/or buffering capacity? (3) Can such metabolic costs, if they exist, cause reduced growth? (4) Can diet pH, buffer concentration or buffering capacity affect larval lipid content? Only by answering these questions will it be possible to ascertain the extent to which foliage buffering systems are important determinants of the nutritive value of foliage.

MATERIALS AND METHODS

Insects and artificial diets

Eggs of *M. sexta* were obtained from Carolina Biological Supply Co. (Burlington, NC). Seven different artificial diets were prepared by combining salt-free Tobacco Hornworm Diet (15.43 g, BioServ Inc., Frenchtown, NJ), agar (2.42 g), and a salt mixture (1.5 g) in 100 ml of water or one of six different buffers. The salt mixture consisted of cupric sulfate (0.59 mg), ferric phosphate (22.1 mg), manganous sulfate (0.3 mg), potassium aluminum sulfate (0.14 mg), magnesium sulfate (135.0 mg) potassium iodide (0.08 mg), sodium chloride (157.5 mg), sodium fluoride (0.86 mg), calcium sulfate (516.0 mg), calcium phosphate (22.5 mg), potassium chloride (600.0 mg) and potassium dihydrogen phosphate (45.0 mg). The buffers were 0.05, 0.10 or 0.25 M succinate, adjusted either to pH 4.19 or 5.57 with sodium hydroxide. The final pH of each diet was determined using a Metrohm/Brinkmann pH-103 model pH meter.

Buffering capacity, defined as the number of millimoles of hydroxide required to bring 1 gram of fresh diet to pH 8.75, was determined by slowly titrating a sample of freshly prepared diet with 0.05 M sodium hydroxide solution (Schultz and Lechowicz, 1986). Thus, buffering capacity in this experiment is equivalent to "diet titer" in Schultz and Lechowicz (1986).

Immediately upon hatching, larvae were placed into 25-ml polystyrene cups (10 larvae per cup) containing one of the seven artificial diets, and placed in an incubator (25°C, 16 h light–8 h dark). Larval density was reduced to 2 per cup at the onset of the second instar. Fresh diet was provided every second day.

Quantitative nutritional studies

Immediately upon molting into the fourth instar, 23–30 larvae from each diet were weighed and placed individually into 25-ml polystyrene cups containing a preweighed amount of the same diet upon which they had fed during the first three instars. The cups containing the larvae and their food were placed in an incubator (25°C, 16 h light–8 h dark). Fresh food was provided every second day or more often if necessary. In order to maximize the accuracy of nutritional indices, the quantity of food supplied was such that at least 75% was consumed (Schmidt and Reese, 1986). Upon molting to the fifth instar, larvae were frozen, and larvae, frass and uneaten food were dried for 72 h at 60°C and reweighed. To provide a conversion factor for calculating the initial dry masses of the larvae, 16–28 freshly molted larvae from each diet were weighed, frozen, dried for 72 h at 60°C, and reweighed. There were no significant differences in mean initial weight of larvae assigned to the seven diets. The dry mass of food provided to larvae was estimated in analogous fashion, using twenty-five 1–2 g samples of each diet.

Standard gravimetric techniques (Waldbauer, 1968) were used to measure relative growth rate (RGR), relative consumption rate (RCR), approximate digestibility (AD), and net growth efficiency or efficiency of conversion of digested food (ECD). All indices were calculated on a dry weight basis, using the following definitions and formulae: average larval weight = arithmetic mean of initial and final weights; weight of food assimilated = weight of food ingested minus weight of frass; weight of food respired = weight of food assimilated minus larval weight gain.

$$\text{RGR} = \frac{\text{larval weight gained}}{\text{average larval weight} \times \text{days}}$$

$$\text{RCR} = \frac{\text{weight of food ingested}}{\text{average larval weight} \times \text{days}}$$

$$\text{AD} = \frac{\text{weight of food assimilated}}{\text{weight of food ingested}}$$

$$\text{ECD} = \frac{\text{larval weight gained}}{\text{weight of food assimilated}}$$

Lipid analyses

Lipid analyses were conducted on material prepared by grinding whole frozen larvae under liquid nitrogen with a mortar and pestle and drying for 24 h at 70°C. Lipid content was determined by weighing dried larval powder (28–32 mg) before and after extraction for 3 min with two portions (3 ml each) of a mixture of chloroform:methanol (2:1 v/v) in a motor-driven tissue grinder. The defatted larval powder was separated from the extract by centrifugation (5 min, 2200 rpm). Analyses were conducted on 9–10 larvae from each diet. In order to provide a representative sample of larvae from each diet, the third, sixth, ninth, etc. larvae to molt to the fourth instar were selected for analysis.

Respiration rates

Third-instar larvae (24 h old), selected to have very similar weights, were placed individually into 22-ml respirometer flasks containing 0.5 ml of deionized distilled water in the sidearm and 0.4 ml of 10% potassium hydroxide in the center well, and the flasks were attached to the respirometer (Gilson Model GR14). After a 15-min equilibration period, oxygen consumption at 25°C was measured over a period of 1 h. Respiration rate was calculated as microliters of oxygen consumed per hour per mg larva.

Statistical analyses

The relative importance of diet pH, buffer concentration and buffering capacity for all performance parameters was determined by stepwise regression analysis. In addition, for those parameters that met the requirement of homogeneity of variance (food ingested, food assimilated, food respired, weight gain, final weight, respiration rate, and lipid content) means were compared by ANOVA, and the significance of pairwise differences was determined by LSD. Before conducting the ANOVA on respiration rate, it was established by ANCOVA that respiration rate was independent of larval weight, which varied over a very narrow range. For those parameters that did not fulfill the requirements of an ANOVA (instar duration, RGR, RCR, AD and ECD), means were compared by Kruskal–Wallis tests, and the significance of pairwise differences was determined by Mann–Whitney U tests with α adjusted to 0.01 to correct for multiple comparisons.

TABLE 1. Buffer concentration, pH and buffering capacity (mean ± SD) of diets fed to fourth-instar *M. sexta* larvae

Diet	Buffer concentration (M)	pH	Titer (μmol OH^-/g)
1	0.00	4.82 ± 0.05	46.3 ± 5.69
2	0.05	5.22 ± 0.08	54.2 ± 1.76
3	0.10	5.29 ± 0.12	62.8 ± 2.75
4	0.25	5.49 ± 0.08	90.0 ± 3.61
5	0.05	4.58 ± 0.12	76.0 ± 1.80
6	0.10	4.54 ± 0.16	100.7 ± 3.79
7	0.25	4.40 ± 0.11	187.0 ± 3.46

RESULTS

The pHs and buffering capacities of the artificial diets used in this study (Table 1) represent a range of values likely to be encountered by insect herbivores consuming natural foliage. Diets 1–4 had pHs in the range 4.8–5.5, which is a range that brackets the average values reported for vacuoles (5.4) (Kurkdjian and Guern, 1989) and homogenates of natural foliage (5.1) (Schultz and Lechowicz, 1986). Diets 5–7 were more acidic, with pHs in the range 4.4–4.6, but were still well within the range of reported values. The buffering capacities of diets 1–6 ranged from 46 to 101, below or near the mean value reported for natural foliage, whereas the buffering capacity of diet 7 was considerably higher, but still within the normal range. Thus, in terms of acidity and buffering capacity, diets 2–6 appear to be representative of much of the foliage normally encountered by insect herbivores, whereas diet 1 is representative of foliage that is less highly buffered than most and diet 7 is representative of foliage that is both more acidic and more highly buffered than most.

Stepwise multiple regression analysis suggests that among the characteristics examined, buffering capacity is the most important determinant of larval performance (Table 2), followed by buffer concentration and then diet pH. Because buffering capacity and buffer concentration are themselves correlated, the absence of either one from the stepwise multiple regression model is not evidence of its unimportance. In fact, it is possible that buffering capacity and buffer concentration may, in some instances, exert their influence on larval growth through a common mechanism. Diet buffering capacity accounts for 27, 8, 8, 43, 27 and 41% of the variation in instar duration, weight gain, final weight, RGR, RCR and ECD, respectively. For each of these performance parameters, the effect of increased buffering capacity is one that would be expected to reduce fitness. An increase in buffering capacity results in a decrease in weight gained, final larval weight, relative growth and consumption rates, and efficiency of conversion of assimilated food and an increase in the duration of the fourth instar (Table 2).

Of the diet characteristics evaluated in this study, buffer concentration has the greatest effect on performance parameters related to the allocation of assimilated food to energy metabolism and storage. Diet buffer concentration explains 19, 18 and 20% of the variation in food respired, respiration rate and larval lipid content, respectively. Like the effects of an increase in buffering capacity, the effects of an increase in buffer concentration are also ones that reduce larval fitness. An increase in buffer concentration is correlated with an increase in respiration rate and the amount of assimilated food allocated to energy metabolism, and with a decrease in the amount of energy stored as lipid reserve.

We cannot absolutely rule out the possibility that the adverse effects of high buffer concentrations are due in part to specific toxic effects of succinate, rather than to

TABLE 2. Stepwise multiple regression of performance parameters against diet pH, buffer concentration and buffering capacity

Performance paremeter	Step	Variable	Cumulative r^2	SE	Partial	P
Instar duration	1	Buffering capacity	0.27	1.17	0.52	<0.0001
Food ingested	1	pH	0.09	102.0	0.30	<0.0001
	2	Buffer concentration	0.11	101.1	0.15	0.0454
Food assimilated	1	pH	0.03	49.0	0.17	0.0225
Food respired	1	Buffer concentration	0.19	24.6	0.44	<0.0001
Weight gained	1	Buffering capacity	0.08	26.3	−0.29	<0.0001
Final weight	1	Buffering capacity	0.08	27.9	−0.28	<0.0001
RGR	1	Buffering capacity	0.43	0.05	−0.63	<0.0001
	2	pH	0.45	0.05	−0.19	0.0115
RCR	1	Buffering capacity	0.27	0.17	−0.52	<0.0001
	2	Buffer concentration	0.34	0.17	0.33	<0.0001
	3	pH	0.36	0.16	−0.16	0.0370
AD	1	pH	0.25	0.02	−0.50	<0.0001
ECD	1	Buffering capacity	0.41	0.04	−0.69	<0.0001
	2	pH	0.47	0.04	−0.31	<0.0001
Respiration rate	1	Buffer concentration	0.18	1.88	0.42	0.0004
	2	pH	0.27	1.78	0.34	0.0054
Larval lipid content	1	Buffer concentration	0.20	0.02	−0.44	0.0002

Sample size is 57 for lipid content, 127 for respiration rate, and 156 for all other measures.
For abbreviations see Materials and Methods.

general characteristics of the buffering system, such as buffering capacity or ionic strength. However, we consider that possibility to be very remote. Even in diets 2 and 5, the diets with the lowest buffer concentrations, succinate concentrations are at least 2–3 orders of magnitude higher than in most plant tissues (Beutler, 1985). Thus, if elevated concentrations of succinate had an adverse impact on *M. sexta* larvae, we would expect larval performance on diets 2 and 5 to be significantly poorer than on control diet 1. No adverse effects are evident (Table 3).

The only performance parameters influenced primarily by pH are AD, food ingested and food assimilated. Diet pH explains 25 and 9% of the variation in AD and food ingested, respectively. As diet pH increases, larvae ingest a greater total amount of food but digest it less

TABLE 3. Dry matter budgets, nutritional indices, respiration rates and lipid content (mean and SD) of *M. sexta* larvae on artificial diets differing in pH and buffering capacity

Performance parameter	Diet 1	2	3	4	5	6	7
Instar duration (days)	3.81^{a}	3.84^{a}	3.84^{a}	4.22^{b}	3.75^{a}	3.97^{ab}	6.06^{c}
	(0.43)	(0.47)	(0.44)	(0.42)	(0.37)	(0.40)	(2.92)
Food ingested (mg)	519^{a}	560^{ab}	578^{bc}	627^{c}	536^{ab}	529^{ab}	525^{ab}
	(73)	(112)	(109)	(109)	(93)	(83)	(128)
Food assimilated (mg)	262^{a}	280^{ab}	283^{ab}	299^{b}	274^{ab}	271^{a}	273^{ab}
	(38)	(55)	(50)	(52)	(43)	(40)	(63)
Food respired (mg)	128^{a}	138^{a}	139^{a}	164^{b}	134^{a}	137^{a}	158^{b}
	(19)	(28)	(25)	(29)	(19)	(20)	(32)
Weight gain (mg)	134^{a}	142^{bc}	144^{c}	135^{a}	140^{bd}	136^{ad}	114^{e}
	(19)	(29)	(27)	(24)	(25)	(22)	(26)
Final weight (mg)	161^{a}	171^{a}	171^{a}	163^{a}	167^{a}	162^{a}	141^{b}
	(20)	(32)	(29)	(27)	(25)	(24)	(37)
RGR (mg/day/mg)	0.38^{ab}	0.38^{ab}	0.38^{ab}	0.34^{c}	0.39^{a}	0.37^{b}	0.26^{d}
	(0.031)	(0.042)	(0.032)	(0.026)	(0.027)	(0.028)	(0.087)
RCR (mg/day/mg)	1.46^{ab}	1.48^{ab}	1.52^{ab}	1.57^{a}	1.47^{ab}	1.44^{b}	1.15^{c}
	(0.12)	(0.16)	(0.13)	(0.14)	(0.11)	(0.11)	(0.29)
AD (%)	50.5^{ac}	50.3^{ac}	$49.^{ab}$	47.7^{b}	51.3^{ac}	51.4^{c}	52.1^{c}
	(1.6)	(2.9)	(1.9)	(1.7)	(1.6)	(1.6)	(3.9)
ECD (%)	51.1^{a}	50.7^{a}	50.8^{a}	45.3^{b}	51.0^{a}	49.5^{a}	40.6^{c}
	(1.6)	(3.2)	(2.2)	(1.7)	(2.5)	(2.1)	(8.3)
Respiration rate	0.057^{a}	0.057^{a}	0.075^{b}	0.071^{b}	0.065^{ab}	0.075^{b}	0.074^{b}
(μl O_2/h/mg)	(0.002)	(0.002)	(0.002)	(0.002)	(0.002)	(0.003)	(0.002)
Larval lipid content (%)	26.9^{ab}	27.1^{b}	25.6^{ac}	24.1^{d}	26.8^{ab}	25.4^{c}	25.0^{cd}
	(1.4)	(2.7)	(1.5)	(2.5)	(1.9)	(1.4)	(2.0)

pH and titer of each diet are given in Table 1. Respiration rates were measured on third instars; all other measurements were on fourth instars. For respiration rate measurements, N was 20, 20, 24, 22, 26, 19 and 16 for diets 1, 2, 3, 4, 5, 6 and 7 respectively; for larval lipid content, N was 10, 10, 9, 10, 9, 10 and 9; for all other measurements, N was 30, 26, 24, 27, 25, 28 and 26. Means were compared by ANOVA and the significance of pairwise differences was determined by LSD analysis for those parameters that met the requirement for homogeneity of variance; for those that did not, means were compared by Kruskal–Wallis tests and the significance of pairwise differences was determined by Mann–Whitney U tests with α adjusted to 0.01 to correct for multiple comparisons. For abbreviations see Materials and Methods.

efficiently. The relationship between diet pH and food assimilated is positive but only marginally significant, with diet pH explaining only 3% of the variation in food assimilated.

The overriding importance of diet buffering capacity and buffer concentration are also evident when means are compared among diets of similar pH (Table 3). pH values for diets 2–4 are similar and slightly above the reported average for natural foliage (5.1), while pH values for diets 5–7 are similar and substantially below the reported average for natural foliage. Within each group, larvae fed the most highly buffered diet displayed significantly reduced RGR (by 12–34%) and ECD (by 11–20%) and significantly increased instar duration (by 10–62%) and total food respired (by 15–19%).

DISCUSSION

From the relationships revealed in Tables 2 and 3 we conclude that the effect of the diet buffer system on larval growth is mediated largely by effects on energy metabolism. Increased buffering capacity results in increased instar duration, which, in turn, mandates the allocation of a larger amount of assimilated food to maintenance metabolism, and less to growth and lipid synthesis. A similar increase in the amount of assimilated food allocated to energy metabolism has been observed when instar duration has been increased by food deprivation (Schroeder, 1976) and by intoxication (Appel and Martin, 1991). An increase in buffer concentration also causes an increase in respiration rate. However, despite a slightly higher respiration rate, larvae on diet 3 displayed a 12% higher RGR than larvae on diet 4 (Table 3). A similar pattern obtains for larvae on diets 6 and 7. Thus, we conclude that the higher respiration rate caused by a higher buffer concentration contributes less to the reduced larval growth rate than does the greater allocation of assimilated food to maintenance metabolism during the extended instar.

The effect of diet buffering capacity on energy metabolism is further evidenced by the significant negative influence of buffering capacity on larval lipid content (Tables 2 and 3). As larvae on more highly buffered diets allocate more assimilated food to energy metabolism, less is available for conversion to and storage as lipid. Moreover, it should be emphasized that we have detected these effects among larvae fed energy-rich artificial diets. It seems most likely that high buffering capacity and buffer concentration would be even greater impediments to growth and energy storage in larvae consuming natural foliage, which is considerably less energy-rich than the diets used in this study.

Our findings provide definitive answers to the questions posed in the Introduction. Growth, and presumably also fitness, can be affected strongly by diet buffer concentration and buffering capacity. Diet pH has a much smaller effect on growth than either buffer concentration or buffering capacity, at least within the pH range explored in this study. When pH has an effect on larval performance, it is through effects on ingestion and assimilation. Buffering capacity and buffer concentration affect energy metabolism, resulting in reduced larval growth on more highly buffered diets. Thus, while diet acidity alone does not impose a metabolic cost, there is a metabolic cost associated with processing a diet with a high buffer concentration and high buffering capacity. Metabolic costs imposed by the processing of a highly buffered diet can cause reduced growth and reduced accumulation of lipid reserves. We conclude, therefore, that buffer concentration and buffering capacity are potentially important determinants of the nutritional value of foliage to insect herbivores.

In order to evaluate the ecological and evolutionary significance of the variation in foliage pH and buffering capacity observed by Schultz and Lechowicz (1986), it will be necessary to determine the extent to which these foliar characteristics affect host plant selection and use, and to identify the suite of traits that enable some herbivores to exploit highly buffered or highly acidic foliage.

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