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Dietary Phosphorus Affects the Growth of Larval *Manduca sexta*

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Although phosphorus has long been considered an important factor in the growth of diverse biota such as bacteria, algae, and zooplankton, insect nutrition has classically focused on dietary protein and energy content. However, research in elemental stoichiometry has suggested that primary producer biomass has similar N:P ratios in aquatic and terrestrial systems, and phosphorus-rich herbivores in freshwater systems frequently face phosphorus-limited nutritional conditions. Therefore, herbivorous insects should also be prone to phosphorus limitation. We tested this prediction by rearing *Manduca sexta* larvae on artificial and natural (*Datura wrightii* leaves) diets containing varying levels of phosphorus (approximately 0.20, 0.55, or 1.2% phosphorus by dry weight). For both artificial and natural diets, increased dietary phosphorus significantly increased growth rates and body phosphorus contents, and shortened the time to the final instar molt. Caterpillars did not consistently exhibit compensatory feeding for phosphorus on either type of diet. The growth and body phosphorus responses were not explicable by changes in amounts of potassium or calcium, which co-varied with phosphorus in the diets. Concentrations of phosphorus in *D. wrightii* leaves collected in the field varied over a range in which leaf phosphorus is predicted to affect *M. sexta*'s growth rates. These results suggest that natural variation in dietary phosphorus is likely to affect the growth rate and population dynamics of *M. sexta*, and perhaps larval insects more generally. Arch. Insect Biochem. Physiol. 55:153–168, 2004.

KEYWORDS: phosphorus; insect; growth; nutrition; *Manduca sexta*; *Datura wrightii*; diet

INTRODUCTION

Many ecological processes are limited by the availability of certain elements, particularly nutrient elements such as nitrogen, phosphorous, or iron (Stiling, 1996; Schlesinger, 1997). Primary production in terrestrial ecosystems is generally considered to be nitrogen limited (Vitousek and Howarth, 1991; Vitousek et al., 1993). Studies of terrestrial insect nutrition have correspondingly concentrated on protein and energy content, with little investigation of other elements such as phosphorus (Scriber and Slansky, 1981; Simpson and Simpson, 1990; Chapman, 1998). Insect responses to variation in dietary protein and energy content have been well-characterized, the two primary organismal effects being growth rate changes and compensatory feeding (McNeill and Southwood, 1978; Simpson and Simpson, 1990; Raubenheimer and Simpson, 1993; Isaacs et al., 1998; Woods, 1999).

Phosphorus has been considered as a limiting element in lake (Schindler, 1977; Hecky and Kilham, 1988), ocean (Redfield, 1958; Karl et al., 1995), and terrestrial ecosystems (Tanner et al., 1998). Phosphorus is a growth-limiting nutrient for many organisms, including bacteria (Vadstein, 2000), algae (Rothhaupt, 1992), and zooplankton

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(DeMott et al., 1998; Elser et al., 2001). A comparison of the aquatic and terrestrial carbon, nitrogen, and phosphorus elemental ratios of plants and herbivores implies that, based on the imbalances in elemental ratios, terrestrial invertebrate herbivores are as likely to show phosphorus limitation as aquatic herbivores (Elser et al., 2000a). However, observed effects of dietary phosphorus variation on insect herbivore growth in studies to date have not been consistent. Increasing dietary phosphorus has been shown to increase growth rates in caterpillars (Clancy and King, 1993) and crickets (McFarlane, 1991). Growth rates decreased in grasshoppers (Smith, 1960) and remained unchanged in mosquitoes (Dadd et al., 1973) in response to fluctuations in the amount of dietary phosphorus. The range of dietary phosphorus in three of these studies was not compared with the variation in phosphorus of the insects' natural diets (Smith, 1960; Dadd et al., 1973; McFarlane, 1991), and in the sole exception the minimum dietary phosphorus concentration was almost double the minimum observed foliar phosphorus concentration (Clancy and King, 1993). All but one of the studies were performed using only artificial diets. The one study employing natural diets (Smith, 1960) used plants reared from seed on media varying in potassium phosphate, potentially altering leaf characteristics, such as metabolic organization, structure, or content of secondary compounds (Crafts-Brandner et al., 1990). None of these studies controlled for possible confounding effects of cations (potassium, calcium, or sodium) added with the phosphate salts. Thus, we consider the current state of experimental evidence regarding the ecological importance of phosphorus as a nutrient for larval insects to be, at best, uncertain.

In this study we investigate the effect of variation in dietary phosphorus on the growth, consumption, and body phosphorous content of an herbivorous insect, the tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), and assess the potential for low phosphorus intake to limit growth of *M. sexta* on both an artificial diet and its natural diet of jimsonweed *(Datura wrightii*, Solanaceae). *M. sexta* larvae feed primarily on plants in the *Solanaceae* family, such as tobacco and *D. wrightii*, and are common pests on tobacco and related plants (Rothschild and Jordan, 1903; Madden and Chamberlin, 1945; Yamamoto and Fraenkel, 1960; Mechaber and Hildebrand, 2000). *M. sexta* spends an average of 20 days in the larval stage in the wild and can have multiple generations in a single season, up to three or four in some areas, depending on conditions (Madden and Chamberlin, 1945; Rabb, 1969); therefore dietary effects on growth rates are likely to affect population dynamics.

METHODS

Animal Care

M. sexta eggs were obtained from either a colony maintained at the University of Washington by L. Riddiford (natural diet experiments) or Carolina Biological Supply (Burlington, NC; artificial diet experiments). Eggs were kept at room temperature in small plastic cups until hatching. Within 11 h of hatching, larvae were weighed (± 2 μg, Cahn C-33, Cerritos, CA) and then placed individually onto the experimental diets in 37-ml plastic cups. At the terminal instar molt, caterpillars were transferred to 266-ml cups. Larvae were maintained at 23–27°C with a light cycle of 16:8 light:dark. Cups were organized in a 6 × 5 modified Latin square design to control for bias from possible environmental gradients. Fresh batches of the artificial diets were made every 4–6 days and the food in each cup was changed at least every two days.

Natural Diet Experimental Design

We manipulated the phosphorus content of field-grown *Datura wrightii* leaves by soaking cut stems in various solutions. *D. wrightii* plants (0.5–1.5 years old) were grown pesticide-free in a field at the Arizona State University Horticultural Center in Tempe, AZ. The cut ends of cuttings from the plants, ∼0.75 m long containing numerous leaves, were placed in a beaker of distilled water for approximately 30 min to rehydrate them, and

then set in flasks containing one of the following experimental solutions: 10 mM KH_2PO_4, 20 mM KH_2PO_4, 10 mM KCl, 20 mM KCl, or distilled water (all pH 6.0). The cuttings remained in the solutions in the lab (~25°C) for 48 h, and then all leaves were trimmed from the cuttings and immediately placed into cups with the caterpillars. Six leaves from each treatment were sampled on days 8 and 18 after the larvae hatched and were dried and analyzed for phosphorus content. Day 18 leaves were also analyzed for C and N content (see below for chemical analysis methods).

In experiment ND1, 30 larvae were initially set up for each of the five treatments. In experiment ND2, 50 additional larvae from the experiment ND1 egg hatch were maintained for 4 days on freshly picked *D. wrightii* leaves (not from manipulated cuttings) before being placed, 8 per treatment, onto manipulated leaves.

Field *D. wrightii* Phosphorus Content

To gain an initial estimate of the natural range of *D. wrightii* leaf phosphorus content, we collected *D. wrightii* leaves in May and June of 2000 from both the ASU Horticultural Center in Tempe, AZ (33.39 °N, 111.93 °W) and five field sites in California. Three of the field sites in California were located within 5 miles of the city of Red Mountain (35.54 °N, 117.29 °W), one was at the White Mountain Research Station in Bishop (37.37 °N, 118.40 °W), and one was near Cabazon (33.91 °N, 116.77 °W).

Artificial Diet Experimental Design

To test for the effects of phosphorus on growth under more controlled conditions, caterpillars were also reared on artificial diets based on a previous method (Ahmad et al., 1989) containing different levels of phosphorus. The major ingredients of the artificial diet were water, sucrose, casein, cellulose, agar, egg albumin, and Wesson's salts (Ahmad et al., 1989). The major sources of phosphorus in this diet were casein (~0.6% P), albumin (~0.1% P), and Wesson's salts (10.2% P; Wesson, 1932). Soybean β-sitosterol (0.149 g, Sigma Chemical, St. Louis, MO) was added to the diet, as preliminary experiments demonstrated slightly increased growth rates. In addition, most dry non-vitamin ingredients were premixed and stored in the freezer before use, and vitamins were added as a liquid mix (containing calcium pantothenate, niacinamide, riboflavin, pyridoxine HCl, thiamine HCl, biotin, vitamin B_{12}, and inositol). Attempts to lower the phosphorus content by removing all the phosphorus from the Wesson's salts and either replacing casein with albumin or increasing the albumin and sugar content without increasing casein resulted in 100% mortality, regardless of the phosphorus level of the diet.

Diet phosphorus content was manipulated by varying the phosphorus salts contained in the Wesson's salt mixture (Table 1). In experiment AD1, 25–26 animals were reared on each of five diets: standard, low PKCa, low P, high KP, and high K. The low PKCa diet had low phosphorus, potas-

TABLE 1. Experimental Treatments in Both the Artificial and Natural Diet Experiments

Treatment name	%P[a]	n[b]	Manipulation[c]	% Cellulose removed[d]
Natural diets[e]				
Distilled water	0.209	8,6		
10 mM KH_2PO_4	0.559	12,8		
20 mM KH_2PO_4	1.178	9,8		
10 mM KCl	0.180	3,5		
20 mM KCl	0.186	18,7		
Experiment AD1				
Standard	0.605	14	—	—
High KP	1.145	14	Added KH_2PO_4	14
Low PKCa	0.135	10	Removed KH_2PO_4 and $Ca_5OH(PO_4)_3$	(14)
Low P	0.137	5	Removed KH_2PO_4 and $Ca_5OH(PO_4)_3$ Added KCl and $CaCl_2$	(2)
High K	0.622	4	Added KCl	8
Experiment AD2				
Standard	0.524	22	—	—
High NaP	1.252	14	Added Na_2HPO_4	32
Low P	0.149	20	Removed K_2HPO_4 and $Ca_5OH(PO_4)_3$ Added KCl and $CaCl_2$	1

[a]Mean diet percent phosphorus by dry weight.
[b]Sample size for each treatment for the days 0–16 mass data. Sample sizes for the terminal instar data are typically 1 or 2 animals fewer. Natural diet sample sizes are for experiment ND1 (first) and experiment ND2 (second).
[c]Manipulations compared to the standard artificial diet.
[d]Percent of cellulose removed from the artificial diets. Values in parentheses are additions to the diet.
[e]Natural diet treatments were the same for both experiments ND1 and ND2. Cut ends of *D. wrightii* cuttings were soaked in the indicated solution for 48 h.

sium, and calcium relative to the standard diet and was prepared by withholding all the potassium phosphate and calcium phosphate normally added to the diet in the Wesson's salts. The low P diet was low in phosphorus compared to the standard diet and was prepared by adding potassium chloride and calcium chloride to the low PKCa diet, restoring the concentrations of potassium and calcium to those found in the standard diet. In the high KP diet, the amount of potassium phosphate was increased by 152%, so both potassium and phosphorus were elevated relative to the standard diet. The high K diet (also high in chloride) was prepared by adding potassium chloride to the standard diet to provide an equimolar concentration of potassium as in the high KP diet.

In experiment AD2, we replicated the basic diets of experiment AD1, varying the cation used in the creation of the highest phosphorous diet. In experiment AD2, 24 larvae were reared on each of three diets, termed: standard, high NaP, and low P (Table 1). The high NaP diet was created by adding sodium phosphate to the standard diet (5.3% by dry weight). The low P diet was created identically to the low P diet in experiment AD1 except that 7% of the sucrose was removed and replaced with equal masses of glucose and fructose. All manipulations in experiments AD1 and AD2 were accompanied by changes in the amount of cellulose (Table 1), an inert, non-digestible diet component, to maintain a constant mass of dry ingredients added to the diet. Diets from experiments AD1 and AD2 were analyzed for phosphorus content using the method described below. All batches of all diets in experiment AD1 were analyzed for phosphorus content and little variation was observed; the maximum and minimum phosphorous content of the various days never varied more than 21% from the mean diet phosphorus content of all days, and in all but one case were less than 12% from the mean diet value.

Measurements

Each caterpillar was weighed on days 0, 4, 8, 12, and 16 in experiment AD1 and experiment ND1, on days 0, 5, 10, and 15 in experiment AD2, and on days 4, 8, 12, 14, and 16 in experiment ND2. After day 12, caterpillars in experiments AD1, ND1, and ND2 were checked daily for the molt to their terminal instar (defined as molting mass > 0.8 g).

Growth and consumption rates were also measured for each animal over a period of approximately 24 h (range 20.3–26.1 h) in its terminal instar, starting 2 or 3 days into the instar, a time frame that has been used in prior studies of *M. sexta* (Woods, 1999). At the beginning of the 24-h period, the caterpillar was weighed (range 2.0–4.2 g) and given a weighed portion of fresh diet (range 3.9–9.6 g). After the 24-h measurement period, the caterpillar and uneaten food were weighed again (caterpillar masses at the end of the experiments ranged from 2.35–6.5 g). The uneaten diet was dried at 60°C for 2 or 3 days and then reweighed to obtain a dry mass. Initial dry mass of the diets was calculated using a wet:dry relationship determined for each treatment on each day for the leaves and the experimental mean wet:dry relationship for the artificial diets.

Caterpillars were sacrificed less than 12 h after the termination of the 24-h measurement period. Immediately after beheading, an incision was made on their dorsal side and their gut and gut contents were removed. In experiments AD2, ND1, and ND2, the gut-free carcass was then ground in liquid nitrogen using a mortar and pestle (Lehoux and Fournier, 1999). In experiment AD1, the gut contents were washed from the gut and the gut tissue was ground with the carcass; this procedural change had no effect on the body P contents as they were not significantly different from animals reared in experiment AD2 (see statistical analyses below for details). Approximately half of the ground sample was placed in an aluminum weigh boat, dried for 2 days at 60°C, and reground using a mortar and pestle. Dried samples were stored in a desiccator until analysis for phosphorus content. All animals in experiments AD1, ND1, and ND2 were analyzed for body P content, while seven animals were randomly selected from each treatment to be analyzed for body P content in experiment AD2.

Chemical Analyses

Phosphorus content of dry, ground samples was determined by colorimetric analysis after persulfate digestion using the molybdate method (Jasco spectrophotometer model 7800, Tokyo, Japan; Clesceri et al., 1998). We included duplicate P standards in each assay, choosing P concentrations so they fell in the linear range of absorbance at 880 nm (absorbance < 0.6 U). We also included a set of internal standards consisting of duplicate samples of finely ground apple leaves (NIST standard no. 1515, 0.154% P by dry mass) and adult *Drosophila melanogaster* (1.00% P by dry mass). Leaf C and N content were measured for pre-weighed dry, ground samples using a Perkin Elmer Elemental analyzer (model 2400).

Statistical Analyses

All statistics were performed using SAS v8.1. The mass data (days 0–16) for all experiments were log-transformed and analyzed by repeated-measures analysis of variance (using a Greenhouse-Geisser correction for violation of sphericity for all comparisons), with comparisons between treatments run as separate analyses with only those groups included. All other data (day to final instar molt, 24-h growth and consumption rates, body phosphorus content) were pooled within both natural diet experiments (ND1 and ND2) and both artificial diet experiments (AD1 and AD2). Day to final instar molt was not measured in experiment AD2 and thus artificial diet day to final instar molt data was not pooled. Two-factor ANOVAs were run for all pooled data, but because the main effect of experiment was never significant ($P > 0.05$ for all seven analyses) and the experiment by treatment interaction was significant only for the artificial diet hourly consumption rate ($F_{2,59} = 3.39$, $P = 0.0404$; $P > 0.05$ for all six other analyses), the pooled results were all analyzed as a one-factor ANOVA for all data except the artificial diet hourly consumption rate. Twenty-four-hour growth rates and 24-h consumption rates were analyzed (as rates per hour) by analysis of covariance using initial fresh mass as the covariate (Packard and Boardman, 1988). There were no significant interactions of initial mass with the treatment groups for any of the 24-h growth or consumption rate ANCOVAs ($P > 0.05$). For ease of presentation, the growth and consumption rates are presented as hourly mass-specific rates. Natural diet leaf phosphorus contents in experiments ND1 and ND2 were analyzed using a two-factor ANOVA testing date and treatment. For all non-repeated-measures ANOVAs, all pairwise comparisons were tested for significance, using a Tukey correction to hold experimentwise type I error rates at 0.05. Caterpillars that died before the terminal instar molt were removed from all data analysis, and caterpillars that died or exhibited problems after the terminal instar molt (the most common of which was incomplete shedding of the cuticle) were not included in the body P or 24-h growth and consumption measures.

RESULTS

Natural Diet

Effect of soaking solution on D. wrightii leaves. Leaf phosphorus content varied significantly with treatment (Fig. 1, Table 1, $F_{4,49} = 52$, $P < 0.0001$) but showed no significant variation with date (date * treatment $F_{4,49} = 0.49$, $P = 0.74$; date $F_{1,49} = 0.08$, = 0.78). Post hoc analyses showed that the DI, 10 mM KCl, and 20 mM KCl treatments were not significantly different from each other, but were significantly lower than either the 10 or 20 mM KH_2PO_4 treatments, and that the 20 mM KH_2PO_4 treatment had a significantly higher phosphorus content than the 10 mM KH_2PO_4 treatment. Day 18 leaves showed no significant variation in molar C:N ratio for any treatments (mean = 12.2, $F_{4,25} = 1.24$, $P = 0.32$). The average carbon content of the leaves was 43.8% and the average nitrogen content was 4.5%.

Effect of dietary variation in P on caterpillar growth. Caterpillars reared from hatching on the 10 and 20 mM KH_2PO_4 treated leaves were on average 18 and 59% heavier on day 16 than those reared on distilled water treated leaves (Fig. 2A). Growth on the two high phosphorus treatments (10 and 20

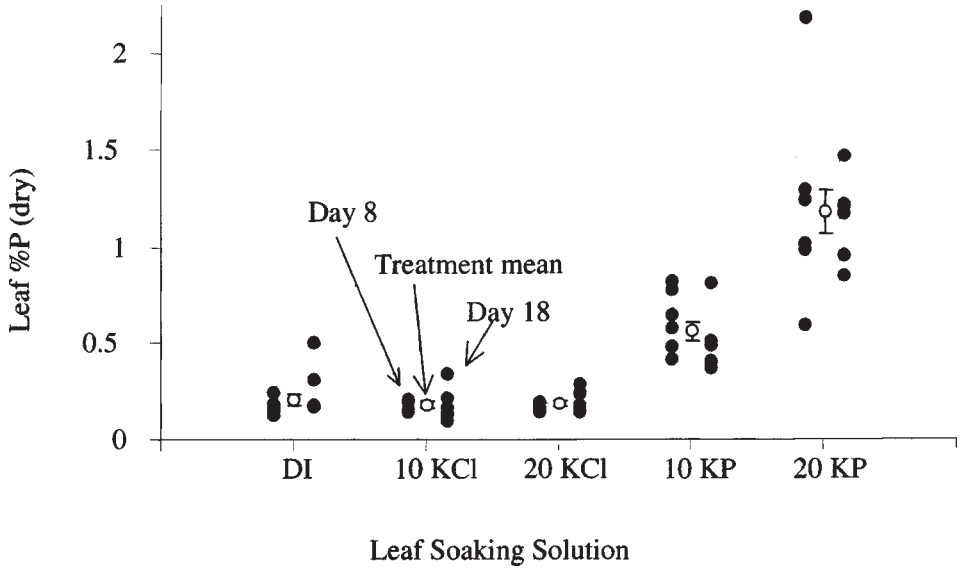

Fig. 1. Phosphorus content of leaves in the five treatments used for the natural diet experiments. For each treatment, the left-most circles indicate data for leaves used on day 8 of the experiment and the right-most circles indicate data for leaves used on day 18 of the experiment. The open circle is the mean for both days. For this and all subsequent figures, the mean is plotted ± one standard error.

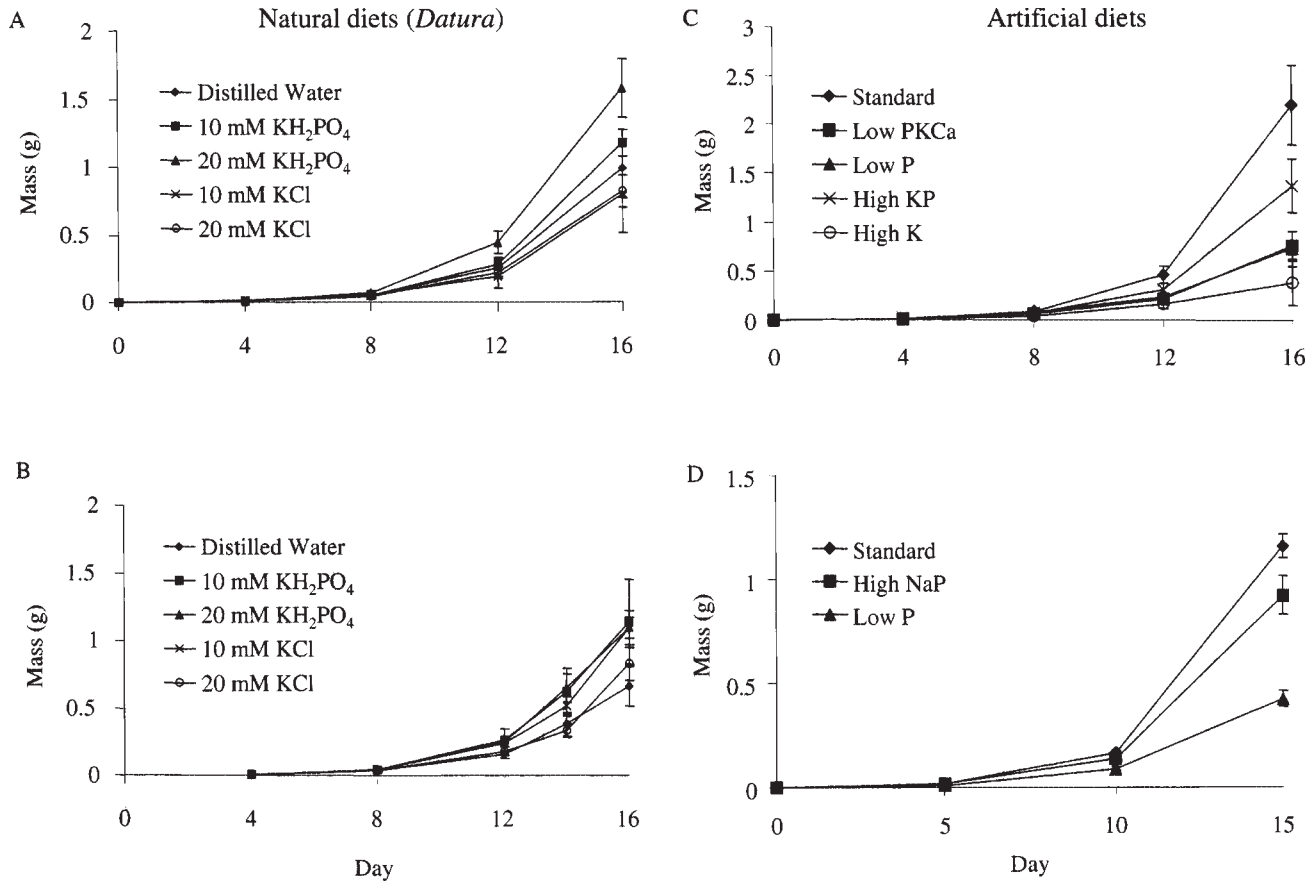

Fig. 2. Caterpillar masses for *M. sexta* reared on manipulated *D. wrightii* leaves in experiment ND1 (**A**) and ND2 (**B**), and on artificial diets for both experiment AD1 (**C**) and AD2 (**D**).

mM KH_2PO_4) did not differ significantly, but when pooled together the 10 and 20 mM KH_2PO_4 treatment animals grew significantly faster than the three pooled control treatments (Table 2). Larvae in the 20 mM KH_2PO_4 treatment, but not in the 10 mM KH_2PO_4, grew significantly faster than larvae from the pooled control treatments (Table 2). Caterpillars reared on manipulated leaves from day 4 showed no significant growth differences for any treatments, though both the 10 and 20 mM KH_2PO_4 treated animals were approximately 70% heavier on day 16 than the distilled water treatment animals (Fig. 2B, Table 2). This lack of overall growth effect from days 4–16 in the animals reared from day 4 may be due to either the importance of early exposure to phosphorus or the lack of power in this experiment.

Day of molt to the final instar was significantly affected by leaf treatment (Fig. 3; $F_{4,80} = 3.7$, $P = 0.0086$). Animals reared on the 20 mM KH_2PO_4 treated leaves molted significantly earlier (average of 2 days or 11% sooner) than those reared on either distilled water or 20 mM KCl (Fig. 3). Ten mM KH_2PO_4 reared animals' molt day did not significantly differ from any of the other treatments.

There was no significant effect of leaf treatment on the caterpillar's hourly growth rate in the final instar (Fig. 4; $F_{4,77} = 2.21$, $P = 0.075$), or the hourly consumption rates of the caterpillars in the final instar (Fig. 5; $F_{4,77} = 1.50$, $P = 0.21$).

TABLE 2. Summary of Repeated-Measures ANOVAs Performed on Mass Data Collected From Days 0–16 for the Four Individual Experiments*

Treatment groups[a]	df[b]	F	*P*
Experiment ND1 (natural diet) overall analysis	16,176	2.19	0.0362
DI, 10 and 20 mM KCl	8,100	0.77	0.6341
10 and 20 mM KH_2PO_4	4,76	2.73	0.0719
[10 and 20 mM KH_2PO_4] and [DI, 10 and 20 mM KCl]	4,188	5.14	0.0076
20 mM KH_2PO_4 and [DI, 10 and 20 mM KCl]	4,140	5.35	0.0074
10 mM KH_2PO_4 and [DI, 10 and 20 mM KCl]	4,152	1.60	0.2093
Experiment ND2 (natural diet) overall analysis	16,116	1.55	0.1487
Experiment AD1 (artificial diet) overall analysis	16,168	4.20	0.0006
Standard and Low P	4,68	5.66	0.0089
High KP and Standard	4,104	1.79	0.1801
High KP and Low P	4,68	2.93	0.0734
High KP and High K	4,64	5.05	0.0187
High K and Standard	4,64	8.79	0.0015
Low P and Low PKCa	4,52	0.83	0.4084
Experiment AD2 (artificial diet) overall analysis	6,159	11.90	<0.0001
Standard and Low P	3,120	21.30	<0.0001
High NaP and Standard	3,102	1.16	0.3266
High NaP and Low P	3,96	13.36	<0.0001

*F-ratios and *P* values are listed for the treatment*day interaction.
[a]The first row (non-indented in each group) is the overall analysis, which includes all treatment groups used in that experiment. Subsequent rows (indented) are post-hoc comparisons including only the groups listed. Brackets indicate pooling.
[b]Degrees of freedom are listed for the stated comparison and the within-groups error term.

Effect of Dietary Cations on Growth

Since the high phosphorus leaves were also high in potassium, we tested whether effects of potassium might confound the effects of phosphorus in these manipulations by rearing larvae on 10 and 20 mM KCl treated leaves. In experiment ND1, the growth rate of animals fed 10 and 20 mM KCl treated leaves did not significantly differ from those fed the distilled water treatment (Fig. 2, Table 2), indicating there was no effect of potassium. In experiment ND2, there was no significant diet*day interaction effect in the overall analysis, meaning there was no effect of KCl-treated leaves (Fig. 2, Table 2). Additionally, the 10 and 20 mM KCl caterpillars were not significantly different from the distilled water treatment caterpillars for day of molt to the terminal instar, body phosphorus content, 24-h terminal instar growth rate, and 24-h terminal instar consumption rate (Figs. 3–6). Thus, the positive effects of phosphorus enrichment seen in these experiments were not due to confounding effects of altered K intake.

Effect of Dietary Variation in Phosphorus on Body Phosphorus Measurements

Caterpillars reared on the different leaf treatments had significantly different gut-content-free body phosphorus concentrations (Fig. 6; $F_{4,82} = 100$, $P < 0.0001$). The phosphorus contents of animals given 10 and 20 mM KH_2PO_4 treated leaves did not significantly differ from each other, but were significantly higher than those of animals given any of the three control diets. Caterpillars fed 10 and 20 mM KH_2PO_4 treated leaves had on average 42 and 48% more phosphorus in their bodies than caterpillars fed distilled water treatment leaves (1.10 and 1.14% vs. 0.78% P).

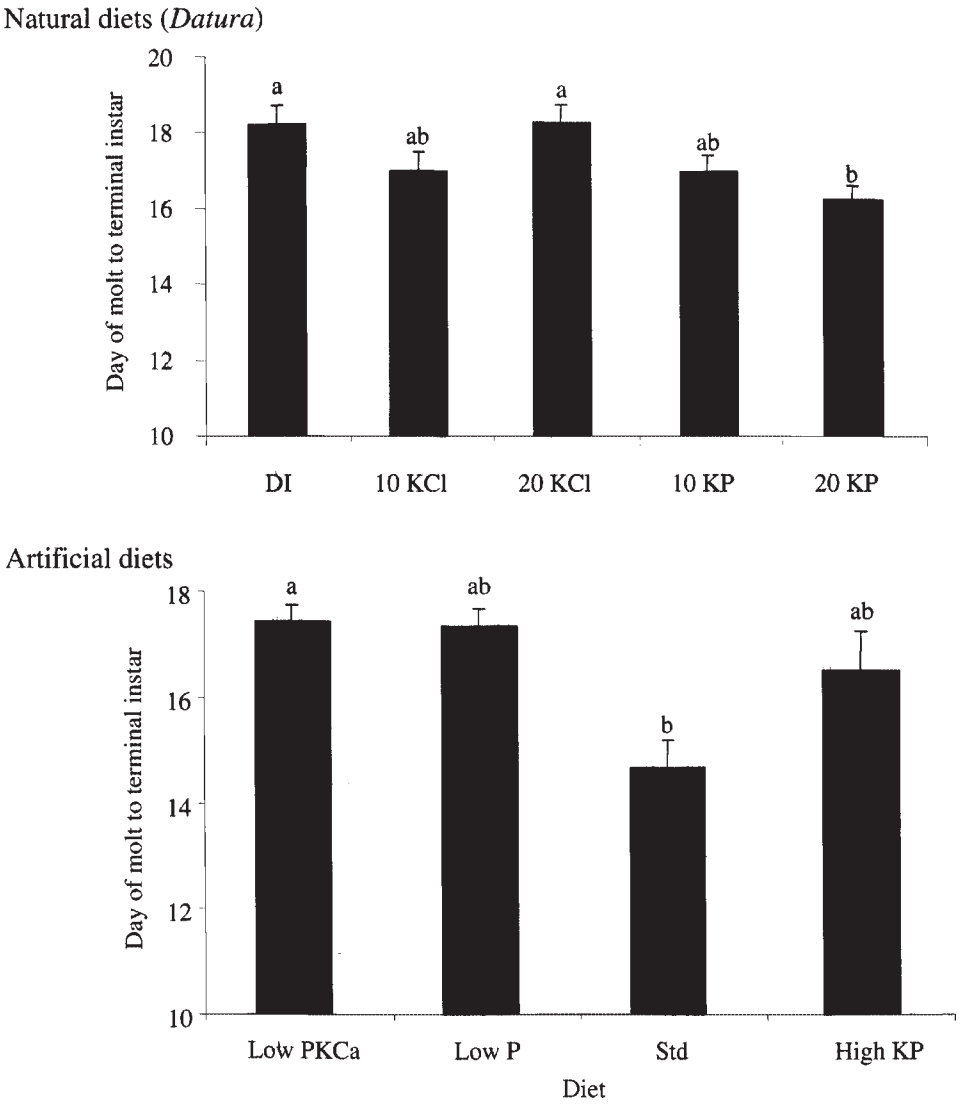

Fig. 3. Day of molt to the final instar for *M. sexta* reared on *D. wrightii* in both experiments ND1 and ND2 (**top**), and on artificial diets in experiment AD1 (**bottom**). In this and subsequent figures, treatments with the same letters above them are not significantly different from each other based on pairwise comparisons using a Tukey correction to hold the experimentwise type 1 error rate at 0.05.

Phosphorus Variation in Natural *D. wrightii*

Phosphorus content in field-collected *D. wrightii* leaves varied more than threefold (Fig. 7). The average leaf phosphorus concentration of all the leaves analyzed was 0.24% (range 0.14–0.51, N = 57). There was no significant difference between the phosphorus contents of the leaves at the ASU Horticultural center (range 0.15–0.43% P, N = 24) and those collected in California (range 0.14–0.51% P, N = 33; *t*-test,$P = 0.11$).

Artificial Diets

Effect of dietary variation in P on caterpillar growth. There were significant effects of dietary phosphorus on larval growth rate for both experiments AD1 and AD2 (Fig. 2, Table 2). Caterpillars reared on the standard diet and high-P diet were on average 203 and 88% heavier than those reared on the low P diet on day 16 in experiment AD1 and 174 and 118% heavier than those reared on the low P diet on day 15 in experiment AD2. In both experiments AD1 and AD2, there was no significant difference between the growth rates of larvae fed standard or high P diets (Fig. 2, Table 2). However, *M. sexta* reared on the standard diet grew significantly faster than those reared on the low P diet for both experiments AD1 and AD2 (Fig. 2, Table 2). In experiment AD1, the growth rate of animals fed the high KP diet did not significantly differ from

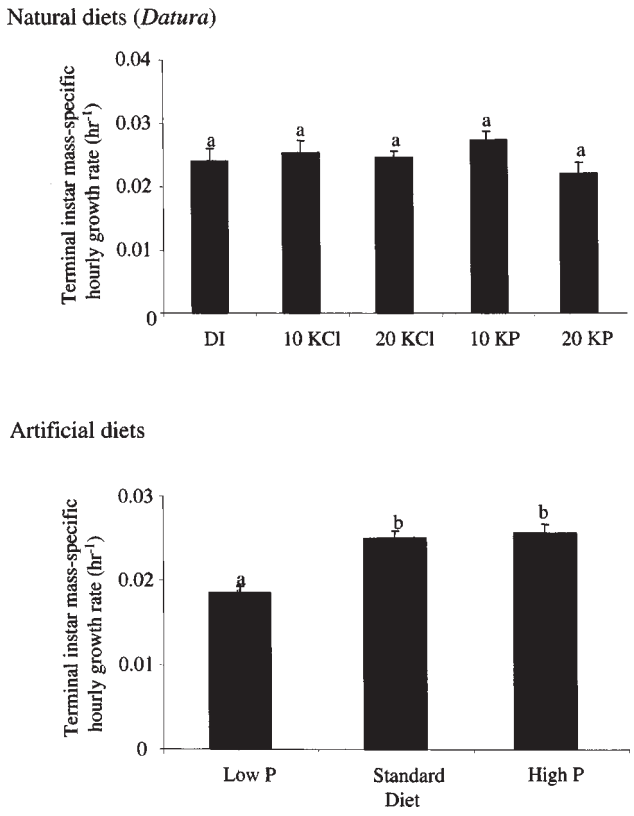

Fig. 4. Mass-specific hourly growth rate in the terminal instar for *M. sexta* reared on manipulated *D. wrightii* in both experiments ND1 and ND2 (**top**), and on artificial diets in both experiments AD1 and AD2 (**bottom**).

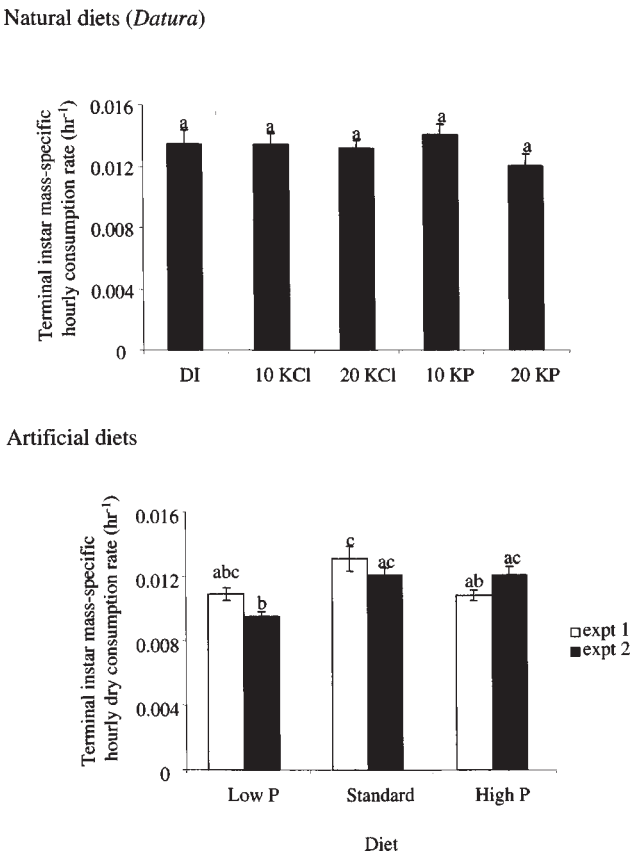

Fig. 5. Mass-specific hourly consumption rate in the terminal instar for *M. sexta* reared on manipulated *D. wrightii* in both experiments ND1 and ND2 (**top**), and on artificial diets in both experiments AD1 and AD2 (**bottom**).

animals fed the low P diet. However, in experiment AD2, the high NaP fed animals grew significantly faster than the low P fed animals (Fig. 2, Table 2).

In experiment AD1, the day of final instar molt was significantly affected by diet (Fig. 3, $F_{3,33} = 3.8$, $P = 0.02$). The only significant difference between the treatments was that caterpillars reared on the standard diet molted significantly sooner (average of 2.75 days or 15% sooner) than those reared on the low PKCa diet.

The caterpillars' hourly growth rate in the final instar was significantly affected by diet (Fig 4, $F_{2,62} = 14.6$, $P < 0.0001$). Hourly growth rates of animals fed the high P and standard diets were not significantly different from each other, but animals given both high P and standard diets grew significantly faster than the low P fed animals (38 and 35% respectively).

Effect of dietary variation in P on diet consumption. There was a significant interaction of diet and experiment on dry consumption rate (Fig. 5, $F_{1,59} = 3.39$, $P = 0.04$) as well as a significant effect of diet ($F_{2,59} = 6.9$, $P = 0.002$). Animals fed the high KP diet from experiment AD1 consumed significantly less than those fed the standard diet from experiment AD1. Additionally, animals fed the low P diet from experiment AD2 consumed significantly less than animals receiving the high NaP diet from experiment AD2, the standard diet from experiment AD1, and the standard diet from experiment AD2.

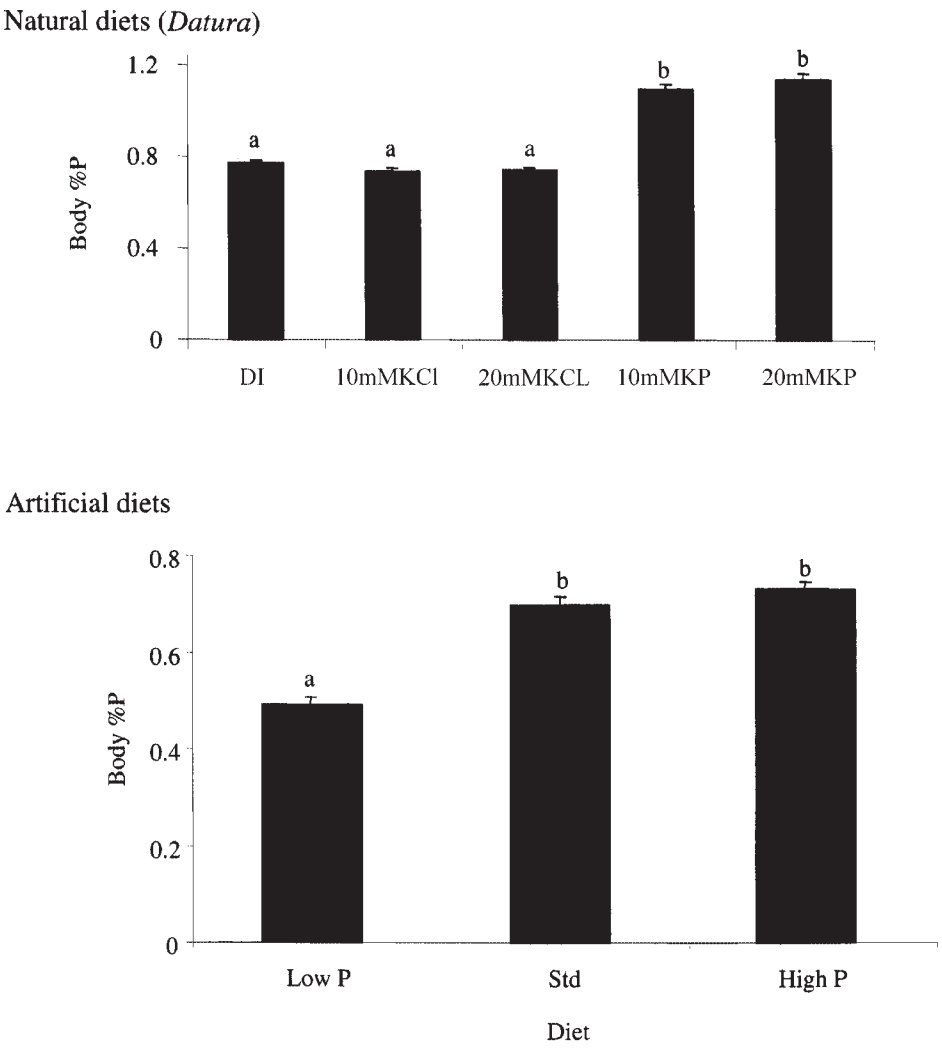

Fig. 6. Body phosphorus content of *M. sexta* reared on manipulated *D. wrightii* in both experiments ND1 and ND2 (**top**), and on artificial diets in both experiments AD1 and AD2 (**bottom**).

Effect of cations on caterpillar growth. Addition of potassium without phosphate to the diet did not affect growth, since animals reared on the low P diet did not grow significantly differently (from days 0–16) than those fed the low PKCa diet (Fig. 2, Table 2). The low PKCa and low P diet fed animals also did not differ significantly on their molt day, their hourly growth rates ($F_{1,3} = 1.40$, $P = 0.32$), their hourly consumption rates ($F_{1,3} = 0.08$, $P = 0.79$), or their body phosphorus content.

Animals fed the high K diet experienced unusually high mortality, as only four survived to the final instar molt and only one survived to the middle of the final instar without molt problems. The surviving high K animals grew significantly slower (from days 0–16) than the standard and high phosphorus diet–fed animals in experiment AD1 (Fig. 2, Table 2).

Effect of dietary variation in phosphorus on body phosphorus measurements. Body phosphorus content was significantly affected by rearing diet (Fig. 6, $F_{2,36} = 50$, $P < 0.0001$). The body phosphorus contents of animals fed the high P diets did not significantly differ from standard diet–fed animals. Animals given both high P and standard diets had significantly higher body phosphorus contents (46 and 52%, respectively) than the low P diet–fed animals. The body phosphorus concentrations varied between the artificial and natural diets, with caterpillars reared on the natural diets consistently having between 55 and 60% more phosphorus than their artificial diet counterparts.

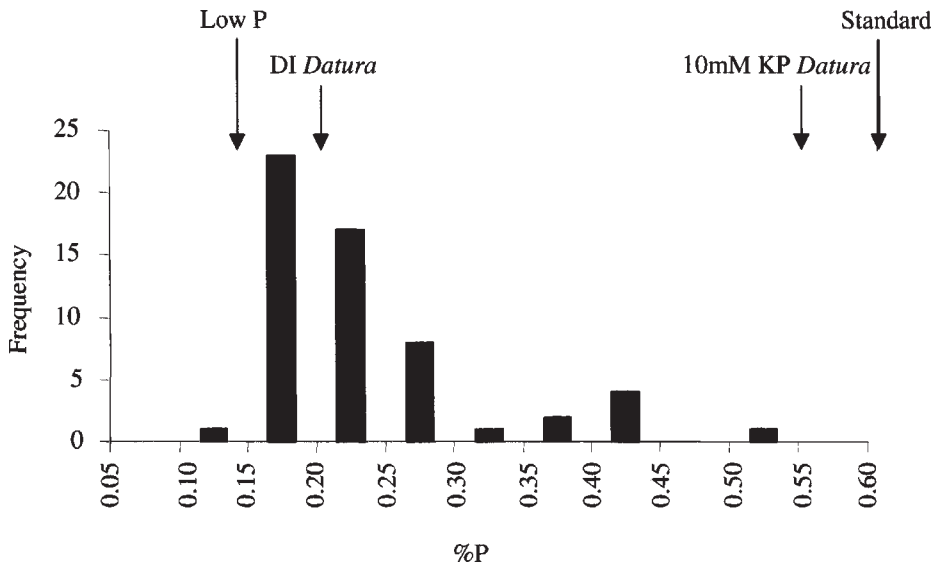

Fig. 7. Variation in leaf phosphorus content in field-collected *D. wrightii*. Arrows indicate the phosphorus concentration of the selected diets written above them; the low P and standard artificial diet values (longer arrows) are from experiment AD1.

DISCUSSION

Dietary Phosphorus May Limit *M. sexta* Growth in the Field

Our laboratory experiments show that dietary phosphorus content significantly affects the growth of larval *M. sexta*. On both artificial and natural diets, caterpillars given high-phosphorus diets grew significantly faster than those given low-phosphorus diets. These results likely are relevant to wild larvae. Fifth instar caterpillars retain most P if consuming less than 3 mg P day^{-1}, while excreting most P if consuming more than 3 mg P day^{-1} (Woods et al., 2002). This suggests that a P intake of 3 mg day^{-1} is required for optimal growth at this stage. Since fifth instar caterpillars consume approximately 0.94 dry g *D. wrightii* leaf day^{-1} (Woods et al., 2002), *D. wrightii* leaves containing less than 0.32% P will not provide the optimal 3 mg P day^{-1}. Approximately 85% of field *D. wrightii* leaves have a % P less than this (Fig. 7), suggesting that P limitation of growth may be common in the field for this insect. Caterpillars fed on artificial diets containing P levels in the range of the lower 10% of *D. wrightii* leaves in the field (Fig. 7) grew at only 25–50% of the rate of caterpillars containing diets with P contents in the upper range of that available in the field (Figs. 2, 7). Thus, unless eggs are laid primarily on high-P leaves, or larvae behaviorally select higher P leaves or leaf portions, these data strongly suggest P-limitation of *M. sexta* growth in the field. Clearly, further field and lab experiments are warranted to test this exciting possibility.

These results are consistent with some previous work and provide grounds for clarifying contradictions between a number of published studies. The quadratic response of survival to phosphorus variation observed with other caterpillars (Clancy and King, 1993) appears to be echoed in our measures of growth rate in this study, as caterpillars on the standard diet grew significantly faster than those on the low-phosphorus diet, and those on the high-phosphorus diet appeared to have slightly lower growth rates than those on the standard diet in both artificial diet experiments (though this was not significant). Thus, it appears that our low phosphorus and standard artificial diets were in the ascending portion of the quadratic curve, while the high-phosphorus artificial diets were at or beyond the crest in the curve. McFarlane (1991) intended to alter only dietary calcium and potassium levels, but did so by varying phosphate-containing salts, and a decrease in growth was observed when the phosphorus concentration of the diet decreased by more than 16% (our calculations). These observa-

tions suggest that McFarlane's (1991) initial dietary phosphorus concentrations were on the ascending portion of the quadratic curve. The finding of Dadd et al. (1973) that additional potassium phosphate did not increase the growth rate of larval mosquitoes may have been due to the fact that the concentration of phosphate was already high in the base diet (8.6 mM P, not including the phosphorus in the RNA added to the diet), and thus was parallel to the standard and high-phosphorus artificial diets in our experiments and may have been on the descending portion of the quadratic curve. Smith (1960) showed that increased plant phosphorus led to lower grasshopper growth rates, a finding inconsistent with this study. However, the phosphorus contents of the plants used by Smith (1960) were increased from 0.17 or 0.25% P to 1.86 or 1.81%P (between 620 and 990%), while the phosphorus content of leaves in this study were increased from 0.21 to 1.18% P (490% increase; distilled water to 20 mM KH_2PO_4). Thus, it is possible that the very high levels of leaf phosphorus in the Smith study might have decreased performance by going over the crest of the quadratic curve, as was observed in artificial diets (Clancy and King, 1993). Additionally, because the plants used in Smith (1960) were grown from seed under different phosphorus conditions, there may have been significant changes in variables other than leaf phosphorus (e.g., Crafts-Brandner et al., 1990). Therefore, the widespread existence of quadratic responses to diet phosphorus content may partially explain the diversity of responses to diet phosphorus variation reported in the literature.

Our data indicate that variation in growth rates among treatment groups was not caused by changes in dietary cation content. With the sole exception of the high K artificial diet, cations did not significantly affect growth rate, consumption rate, or body phosphorus contents of caterpillars in either the artificial or natural diet experiments. The high K artificial diet, designed to test whether any differences in growth between the standard and high KP diet in experiment AD1 were due to increased levels of potassium, had high mortality and significantly slower growth from days 0–16 than the high KP and standard diet reared animals. This observed decrease in growth may have been due to either increased potassium or chloride levels, or an interaction of the two. The levels of potassium in the high K diet (110 mM based on the salts added, 52 mM higher than the standard diet) are similar to the amount of potassium in natural diets for *M. sexta* (137 mM in tobacco; Dow and Harvey, 1988). Additionally, *M. sexta* midguts typically contain between 180 and 210 mM potassium (Dow and Harvey, 1988), much more potassium than was in the high K diet. Thus, potassium toxicity seems unlikely. However, the level of chloride in the high K diet (103 mM based on the salts added, 52 mM higher than the standard diet) is more than quadruple that seen in *M. sexta* midguts (23 mM; Harvey et al., 1983), and almost triple that seen in other plant tissues (e.g., lettuce leaves contain 35 mM chloride; Phillips, 1981). Thus, the most likely explanation for the decreased performance on the high K diet is that increased chloride levels caused the caterpillar's hemolymph chloride concentrations to increase (normally 28 mM; Harvey et al., 1983), potentially affecting cell membrane potentials and the function of physiological systems.

Physiological and Behavioral Mechanisms

A number of physiological processes may link higher dietary P to more rapid growth. Phosphorus is an important component of numerous cellular structures, including membrane phospholipids, ATP, phosphocreatine, DNA, and RNA. If synthesis of any of these structures limits growth rate, additional dietary P may relieve the constraint. The last component, RNA, is especially interesting in light of recent work on the biochemistry of growth. Elser et al. (1996, 2000b) have proposed direct links between growth rate and cellular contents of ribosomes and rRNA. Ribosomes, the protein synthesis centers of the cell, contain more than 50% RNA and are required in large numbers for fast protein synthesis and growth (Lewin, 1994). RNA is almost 10% phosphorus, meaning that ribosomes are the most phosphorus-rich major cellu-

lar component (Lewin, 1994; Elser et al., 1996). Thus, it is likely that the observed changes in body phosphorus content are partially caused by increases in ribosome content, which could have resulted in an increase in growth rate (Elser et al., 1996, 2000b). Testing these possibilities will require finer partitioning of body P among pools. Our results indicate only that body P content increased with dietary P level (Fig. 6). Although the chemical bases of the increase are unclear, recent work in *M. sexta* has shown that fluctuation in dietary P availability can lead to large changes in hemolymph levels of organic phosphorus compounds, mainly α-glycerophosphate (Woods et al., 2002).

If P is naturally limiting in *M. sexta* (or other insects), a reasonable expectation is that mechanisms of P regulation will have evolved. One possibility is that larvae alter feeding decisions as a function of leaf P content, either by making decisions about what to eat when choices are available or deciding how rapidly to eat particular items. Both kinds of responses are widely observed in feeding insects given foods containing different levels of carbohydrate or protein (e.g., McNeill and Southwood, 1978; Simpson and Simpson, 1990). Our data, however, gave no evidence that caterpillars engaged in compensatory feeding for P. Across all experimental diets, the only significant effect of feeding that was consistent with compensatory feeding predictions was that caterpillars reared on the high KP artificial diet in experiment AD1 ate significantly less food than those on the standard diet in experiment AD1, though caterpillars on similar diets in experiment AD2 did not differ in their consumption rates. It is possible that the caterpillars overall lack of response to dietary variation in phosphorus was due to an inability to taste phosphate, as observed in cattle (Blair-West et al., 1992), or to sense internal levels of phosphate. It is also possible that the primary effects on feeding occur earlier during ontogeny, or were too subtle for detection during one 24-hour trial. In this study, we did not examine food choice by caterpillars.

Alternatively, caterpillars may use physiological mechanisms to respond to variation in dietary P availability. In our study, body phosphorus levels were similar on the two highest phosphorus content diets in both natural and artificial diet experiments, even though the phosphorus content of these two diets varied approximately 2-fold. Fifth instar *M. sexta* excrete the majority of consumed P when fed on 1.01% P *D. wrightii* leaves, but retain most consumed P when fed leaves with 0.22% P, indicating that caterpillars treat P as an important nutrient at low leaf P concentrations, and as a excess nutrient when leaf P is high (Woods et al., 2002). The renal complex is primarily responsible for variation in P excretion (Woods et al., 2002). Together, these results suggest that caterpillar responses to dietary P involve primarily physiological, rather than behavioral, mechanisms.

Ecological Implications

The observed decrease in time to the terminal instar molt on both the artificial and natural diets indicates that caterpillars eating high-phosphorus leaves in the field may pupate sooner and thus reduce their exposure to predation and parasitism. Because field populations of *M. sexta* achieve a variable number of generations in a single season (3 or 4 in the southeastern United States; Madden and Chamberlin, 1945), even small decreases in development time may increase the chances of producing an additional generation in one season.

Consideration of the elemental ratios of herbivores and their hosts suggests that phosphorus limitation of growth is likely to be widespread among insects. The mean atomic C:P ratio of terrestrial autotroph foliage is 968 (Elser et al., 2000a), while the mean atomic C:P of *D. wrightii* is 628, indicating that *D. wrightii* is phosphorus rich relative to most other plant taxa studied (*D. wrightii* C:P obtained from the three control treatments). In addition, the mean atomic C:P of terrestrial invertebrate herbivores is 116 (Elser et al., 2000a), while the estimated C:P of *D. wrightii*-reared *M. sexta* is 160, indicating that *M. sexta* is a relatively phosphorous poor insect (*M. sexta* C:P based on mean C of 47.7% from measures of animals reared on artificial diets and P of 0.77% from the *D. wrightii* distilled water treatment animals).

Thus, in our experiments, phosphorus-limitation was evident in a phosphorus-poor herbivore consuming a phosphorus-rich host, suggesting that, with respect to C:P, other insect herbivore/host systems are likely to show greater phosphorus limitation than *M. sexta* consuming *D. wrightii*.

However, taking both nitrogen and phosphorus into account, it appears that *M. sexta* may be somewhat more likely to be phosphorus limited than the average terrestrial insect folivorous herbivore. The mean atomic N:P ratio of terrestrial autotroph foliage is 28 (Elser et al., 2000a), while the mean atomic N:P of *D. wrightii* is 54 (*D. wrightii* N:P obtained from the three control treatments), meaning that, from an N:P perspective, *D. wrightii* is phosphorus poor relative to most other plant taxa studied. *Manduca sexta*'s estimated atomic N:P is similar to the mean atomic N:P of terrestrial herbivores (33 and 26, respectively; *M. sexta* N:P based on mean N content of 11.76% from measures of animals reared on artificial diets and P of 0.77%; Elser et al., 2000a). Thus, from an N:P perspective, our experiment involved an average-phosphorus-content herbivore consuming a phosphorus-poor host, meaning that the *M. sexta*/*D. wrightii* system is more likely to show phosphorus limitation than many herbivore/host systems. Nevertheless, even from an N:P perspective, a number of terrestrial plant species have N:P values that are close to *D. wrightii*'s N:P. Thus, regardless of whether N:P or C:P is considered, since *M. sexta* consuming *D. wrightii* exhibit phosphorus limitation, and this system does not seem extraordinarily likely to show phosphorus limitation based on its C:P and N:P ratios, a number of other insect herbivore/host systems are likely to exhibit phosphorus limitation as well.

More work should be undertaken to further test the ecological significance of phosphorous, even in *M. sexta*. While our study exposes caterpillars to tightly controlled variations in *D. wrightii* phosphorus, it is clear that plants in the field would have significant time to physiologically respond to soil phosphate levels. It is likely that plants that vary in P content in the field may differ in many other characteristics, including the chemical form of P in the plants. Thus, feeding caterpillars leaves from plants containing different phosphorous levels, both field and greenhouse grown, should be undertaken. Additionally, larval growth rates under field conditions on plants varying in phosphorous levels should be measured. Examining larval growth rates in fertilized fields could be a method of examining larval growth rate under varying plant tissue phosphorous concentrations.

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