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Absorption and storage of phosphorus by larval *Manduca sexta*

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Abstract

The role of phosphorus (P) in numerous important biological structures, coupled with the observation that P-content of many insect foods is disproportionately low, suggests that P may be a critical nutrient for growing insects — however, the few studies examining the effects of dietary P on insect performance have generally found only weak relationships. This mismatch may be reconciled by understanding the physiological mechanisms by which insects handle P. Here we describe P processing by larvae of Manduca sexta. When given un-manipulated leaves of a common host plant, Datura wrightii, fifth-instar larvae retained about 85% of P consumed; when given P-enriched leaves larvae retained only 25% of P consumed. Analysis of gut concentrations of P at four sites along the digestive tract, and in leaves and feces, indicates that the rectum is the primary site of P transport between the gut and body and that differences in P retention may be accounted for by differential rates of rectal P transport. Larvae given P-enriched leaves also showed an eightfold increase in the concentration of P in the hemolymph, primarily as α-glycerophosphate — but only a 12% increase in the concentration of P in body tissues, suggesting that hemolymph plays a central role in storage and buffering of P.

Keywords: Phosphorus; Phosphate; Nutrition; Storage; Manduca sexta

1. Introduction

Although insect responses to variation in dietary carbon and nitrogen are well known (Slansky and Feeny, 1977; McNeill and Southwood, 1978; Mattson, 1980; Scriber, 1984; Slansky and Scriber, 1985; Pandian and Vernberg, 1987; Simpson and Simpson, 1990; Simpson and Raubenheimer, 1993; White, 1993; Simpson et al., 1995; Zanotto et al., 1997), insect responses to dietary phosphorus (P) are not. However, data from diverse organisms, including bacteria and zooplankton, indicate that biomass P content is often positively associated with growth rate. This connection appears to reflect fundamental processes linked to the biochemistry of growth (Main et al., 1997; Elser et al., 1996, 2000a, b). In particular, rapidly growing organisms must maintain high concentrations of ribosomes in their cells — and because ribosomes consist of ~50% rRNA which contains significant amounts of P in the sugar phosphate backbone, rapidly growing organisms should have high body P content and thus disproportionately high demands for P in their diets. The connection between growth rate and P content is supported by data from studies of bacteria, phytoplankton, zooplankton, and fish (Elser et al., 2000b) and its ecophysiological significance is now firmly established for zooplankton (Urabe et al., 1997; Elser et al., 2001).

These findings suggest a priori that variation in P intake should affect aspects of insect performance, including growth. Yet most direct studies of the relationship between dietary P availability and performance in insects have demonstrated weak or contradictory effects (Allen and Selman, 1957; Smith, 1960; Dadd et al., 1973; Mattson et al., 1983, 1991; Popp et al., 1989; Boecklen et al., 1991; Clancy and King, 1993; Ayres et al., 2000). Perkins (2001) recently showed that larval *Manduca sexta* given P-enriched leaves or artificial diets showed significantly increased growth rates and body phosphorus contents. These disparate observations may be reconciled to some degree if insects use behavioral or physiological mechanisms to minimize effects of variation in dietary P on growth (Simpson and Simpson, 1990).

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Here we examine physiological mechanisms of P handling by a rapidly growing insect, larval *Manduca sexta*. A useful starting point is the phosphorus mass balance. Net retention of P, compared to retention of other materials, can indicate the relative importance of P in constraining growth (Sterner and Elser, 2002). Retention depends in turn on events occurring in the gut, including the ease with which P-containing compounds are digested and the rates of P transport to and from various gut sections. In most insects, the major site of ion absorption is the rectum (Phillips et al., 1986), and experiments by Andrusiak et al. (1980) showed that everted rectal sacs of the locust *Schistocerca gregaria* could transport phosphate from the lumen to hemolymph side against large concentration differences. The rectum thus may be the site of phosphate absorption in *M. sexta*. We evaluated this possibility by mapping P concentrations in the mid- and hindguts of larval *M. sexta* consuming low- and high-P leaves.

A related issue is phosphorus storage, about which little is known in insects. In bacteria, algae, and yeast, large stores of polyphosphates can accumulate under some environmental conditions, and these stores may buffer cells from subsequent P deprivation (Kulaev, 1979; Schröder et al., 1999). Polyphosphates have been reported in insects, but only in very low concentrations (Kulaev, 1979). Insects also appear to face fundamentally different phosphorus storage problems than do vertebrates, in which approximately 80–85% of total body P occurs as hydroxyapatites in bones and teeth (McDowell, 1992). A candidate location for P storage in insects is the hemolymph, which stores or shuttles a wide variety of compounds. Moreover, several studies (Wyatt et al., 1963; Lenartowicz et al., 1964; Jungreis, 1978) have shown that organic forms of P occur at high concentrations in the hemolymph, especially in Lepidoptera. We therefore examined the concentration and form of hemolymph P in larvae given low- and high-P leaves.

2. Methods

2.1. Animals and rearing

M. sexta Johansson (Lepidoptera: Sphingidae) obtained as eggs from Carolina Biological (Burlington, NC) were reared from hatching on leaves of *Datura wrightii* Regel, a natural host of *M. sexta* in desert grasslands of North America. Leaves available to larvae were replaced every 2nd or 3rd day with freshly picked leaves from an outdoor plot at Arizona State University's horticultural center. Hatchling larvae were reared in the laboratory (L : D 14: 10, 24–25°C) in 1-oz plastic cups (Solo) until they reached the molt preceding the fifth (final) instar, at which point they were switched to 9-oz plastic cups. A subset of larvae was reared analogously on a wheat-germ based artificial diet, modified from that of Bell and Joaquim (1976); see Woods and Chamberlin (1999) for a description of the modifications).

2.2. Phosphorus assays

Dried and ground samples (0.5–4 mg) were weighed on an electronic balance (Mettler MT5, ± 0.1 μg), and their total P contents were assayed by persulfate digestion and ascorbic acid colorimetry, as described by Clesceri et al. (1998). For some samples, we measured inorganic P rather than total P. For these samples, we proceeded with the color reaction without first oxidizing them with persulfate. Total organic P was calculated as the difference between total P and inorganic P. In each assay, we included duplicate P standards (0, 60, 125, 250, 500 nmol KH_2PO_4 added as different volumes of a 500 μmol l^{-1} stock solution), with P concentrations chosen so that they would fall in the linear range of absorbance at 880 nm (absorbance <0.6 units). 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), adult *Drosophila melanogaster* (1.00% P by dry mass), and fifth-instar *M. sexta* excluding gut contents (0.81% P by dry mass).

2.3. Experiment 1: Phosphorus mass balance

We measured the P mass balance of 14 fifth-instar *M. sexta* that had been reared on *D. wrightii*. On day 2 or 3 of the fifth instar, larvae were weighed (mean initial mass 3.01 ± 0.24 g, range 1.77–4.71 g) and placed on a known mass of *Datura* leaves (mean initial mass 6.57 ± 0.74 g, range 3.36–13.32 g). After approximately 24 h (22–25 h), larvae were reweighed. Larvae (killed by decapitation), leaves, and feces were separated, dried in a drying oven (60°C) for several days, and reweighed. All samples were then ground to a fine powder in a mortar and pestle and were placed back in the drying oven until subsamples were taken for P analysis.

Several values for the mass balance calculations were available directly from the measurements described above, including dry mass and P content of feces and of larvae at the end of the 24-h period. Other values had to be estimated — in particular, leaf fraction dry matter, leaf P content, and initial larval dry mass. We estimated the first two values by sampling leaves (N=36) from the same branches that supplied leaves for larval consumption. Leaves were weighed fresh, dried for several days (60°C), reweighed, and ground for P analysis. Larval dry consumption was calculated as initial dry mass of leaves (initial fresh mass multiplied by a wet-to-dry conversion factor, which varied between batches from 0.15 to 0.20) minus final dry mass (measured directly). Larval P intake was estimated as dry intake multiplied by the measured leaf P content.

Estimation of initial larval dry mass was complicated by an apparent relationship between fresh mass and fraction dry: smaller larvae had lower fraction dry mass than larger larvae. We corrected for this difference by fitting a line (least squares regression) to the data on fresh mass and fraction dry of larvae at the end of the 24-h period (final fraction dry=0.0912+0.0065×final fresh mass (g), r^2=0.47) and then using this relationship to estimate initial fraction dry from initial fresh mass.

2.4. Experiment 2: Body phosphorus content of larvae consuming low- and high-phosphorus leaves

We gave larvae leaves of *D. wrightii* manipulated so that they contained either normal (control) or high amounts of P. Cuttings were obtained from a plot at Arizona State University's horticultural center and returned to the laboratory. Cut ends were trimmed and placed into either distilled water (control) or 20 mmol l^{-1} KH_2PO_4 that had been adjusted to pH 6 with NaOH. Cuttings were then left in the laboratory for 48 h (room temperature and ambient lighting), after which leaves were picked and stored in Ziploc bags at 4°C until used. Most leaves were used within 2 days of picking.

Larvae were reared on unmanipulated leaves of *D. wrightii* through the end of the third instar, as described above. During the molt to the fourth instar, larvae were transferred to control or P-enriched leaves. Leaves were changed every 2 days until the 2nd day of the fifth instar (approx. 1 week total exposure to experimental leaves), when all measurements were made. During a 24-h period early in the fifth instar, we measured leaf P concentration from both groups and consumption and excretion of P over 24 h, as described above. We also measured concentrations of organic and inorganic P in the various gut compartments and in the hemolymph (described below).

At the conclusion of the 24-h period, larvae were cooled on ice for 20–40 min and 0.2 – 1 ml hemolymph was collected from a cut proleg. Most hemolymph samples were centrifuged gently (1000×g, 5 min, 4°C) to remove hemocytes (checked with a hemacytometer). To estimate the P content of hemocytes, we compared (for a subset of samples) total P contents of centrifuged versus uncentrifuged aliquots from the same individuals. Hemolymph samples were frozen in liquid nitrogen and stored at −70°C until used. We determined both total and inorganic P contents of each hemolymph sample by assaying 15–μl subsamples (actual amount determined gravimetrically, ± 0.1 mg). We also determined concentrations of α-glycerophosphate following the method of Michal and Lang (1974). Subsamples (15–100 μl) of uncentrifuged hemolymph were deproteinized by perchloric acid precipitation (6 N) and neutralized with potassium carbonate. Neutralized extract (1 ml) was incubated for 20 min with 1 ml hydrazine/glycine buffer (pH 9.5), 100 μl 2.3 mM β-NAD, and 30 units α-glycerophosphate dehydrogenase. The absorbance (1 cm pathlength, 340 nm) was read on a spectrophotometer (Novaspec II, Pharmacia Biotech) before and after addition of enzyme. Standards were prepared from L-α-glycerophosphate (Sigma).

Following hemolymph collection, the gut was exposed by a dorsal incision and the body cavity was held open with insect pins. To avoid contamination of the gut contents with hemolymph, we washed away remaining hemolymph with approximately 5 ml of a P-free saline (in mmol l^{-1}: 10 KCl, 10 $MgCl_2$, 1 $CaCl_2$, 10 NaCl, 240 sucrose, 10 piperazine-N,N′-bis[2-ethanesulfonic acid] (PIPES), pH 6.7). Before sampling gut contents, we gently tied a thread around the midgut at the junction between the anterior and middle portions of the midgut and a second thread between the middle and posterior portions (Cioffi, 1979) to prevent the contents of one section from contaminating those of another. In each of the three midgut sections and the rectum we cut through the gut wall and collected the contents (including peritrophic envelope). The contents from the midgut sections were centrifuged (2 min, room temperature, 10,000×g). Approximately 50–100 μl of each supernatant was collected and frozen (−70°C) until assayed (15 μl subsamples) for total and reactive P. The remaining pelleted material was dried (60°C), ground, and assayed for total P. The material from the rectal lumen was dry enough that supernatants generally did not form, so the complete rectal lumen contents were assayed. At the conclusion of each dissection, the threads were removed and the carcass was washed extensively with P-free saline to remove remaining gut contents and as much as possible of the remaining hemolymph. Carcasses were dried, ground, and assayed as described above.

2.4.1. Determination of hemolymph volume and residual hemolymph in carcasses

For another set of larvae (reared on artificial diet) we used radioisotope dilution (Levenbook, 1958; Buckner and Caldwell, 1980) to estimate hemolymph volume and the amount of residual hemolymph remaining in the carcass after washing. To estimate the relationship between fresh mass and hemolymph volume, we cooled fifth-instar larvae (N=14, mass 2.3–4.2 g) on ice for 40 min and injected 10 μl (0.01 μCi) [methoxy-^{3}H] methoxyinulin (Sigma) dissolved in P-free saline (composition described above) along the dorsal midline between the second and third abdominal segments. Larvae were allowed to recover at room temperature for 45 min (preliminary experiments showed that this time was adequate for equilibration of inulin within the hemocoel; see also Buckner and Caldwell (1980)) and then were cooled again on ice for 10 min and hemolymph was collected from a cut proleg. To avoid extensive oxidation of hemolymph, duplicate 20-μl samples were immediately

mixed with 5 ml scintillation cocktail (Fisher ScintiSafe Plus 50%).

To estimate the amount of residual hemolymph in the carcass after washing, we injected a separate set of larvae (N=6, mass 2.9–4.0 g) with 10 μl (0.1 μCi) [methoxy-^{3}H] methoxy-inulin. After hemolymph was collected as described above, larvae were dissected and washed as they were during collection of gut contents. Carcasses were dried for 3 days at 60°C and ground in a mortar and pestle. Duplicate samples weighing 20–40 mg were incubated in 500 μl 6% perchloric acid for 2 h and then centrifuged (400×g, 4 min, 4°C). Supernatant samples (300 μl) were neutralized with 3 mmol l^{-1} NaOH and samples were mixed with 5 ml scintillation cocktail (Fisher ScintiSafe Plus 50%). Radioactivity of all samples was assayed in a Beckman LS 6500 scintillation counter and corrected for quenching.

3. Results

3.1. Phosphorus mass balance

Larval tissues contained substantially higher concentrations of phosphorus (0.74 ± 0.01% P) than did leaves (0.21 ± 0.01% P) or feces (0.05 ± 0.006% P), indicating that P is concentrated by the larvae. P content (as % of dry mass) did not vary significantly with larval mass within the fifth-instar animals examined (data not shown). High retention was not due to highly asymmetrical rates of dry consumption and excretion — larvae consumed on average 0.94 ± 0.10 g day^{-1} dry leaf material and excreted 0.56 ± 0.06 g day^{-1} (dry mass assimilation 40.4%). Larvae in this experiment consumed 2.00 ± 0.83 mg P day^{-1} and excreted 0.30 ± 0.07 mg P day^{-1} (Fig. 1), thus retaining 85% of the P consumed. The difference between rates of P consumption and excretion (1.70 mg P day^{-1}) is one estimate of the rate at which larvae obtain P. This value can be calculated independently by multiplying mean larval dry growth rate (0.57 ± 0.05 g day^{-1}) by larval fraction P (0.74%), giving 1.75 ± 0.19 mg P day^{-1}. These two values differ by less than 3%, indicating that we accounted for virtually all P in the system.

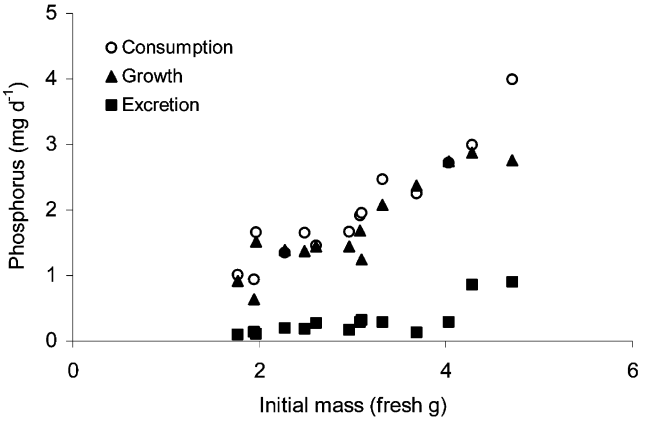

Fig. 1. Phosphorus mass balance over 24 h for fifth-instar *M. sexta* fed un-manipulated leaves of *D. wrightii* (0.%% P). P growth was calculated by multiplying mean larval dry growth rate by larval fraction P. On average, larvae excreted 15% of phosphorus consumed.

3.1.1. Experiment 2: Body phosphorus content of larvae consuming low- and high-phosphorus leaves

P-enriched leaves (N=18) contained on average five times the P (1.01 ± 0.06% by dry mass) of control leaves (0.22 ± 0.01% P, N=24, 1-tailed t-test: $P<0.001$). For a subset of six leaves in both treatment groups, we analyzed total and inorganic P. In control leaves, inorganic P made up ~35% of total P. Leaves in the control and P-enriched groups had organic P contents of 0.16 ± 0.02% and 0.15 ± 0.05% (t-test: $P>0.1$), respectively, indicating that the P manipulation affected only levels of inorganic P. Leaf water content in the two groups (72%) did not differ (data not shown).

Larvae eating high-P and control leaves consumed dry material at similar rates (0.86 ± 0.05 and 0.78 ± 0.07 g day^{-1}, respectively (t-test: $P>0.1$) — thus, larvae eating P-enriched leaves consumed and excreted P at much higher rates. The efficiency of P retention also differed between groups — larvae eating control leaves retained 83% of the P they consumed, whereas those on P-enriched leaves retained only 25%. We examined these effects further using analysis of covariance (Raubenheimer and Simpson, 1992), with amount of P consumed as the covariate (Table 1 and Fig. 2). For these analyses, we combined data from Experiments 1 and 2. The primary difference between the groups is that larvae in Experiment 2 (given either control or P-enriched leaves) had their guts removed prior to analysis of body P content, which should not have affected consumption or excretion of P. ANCOVA indicates that P excretion was strongly and positively related to P consumption. Importantly, there was a significant interaction between covariate and treatment (Table 1 and Fig. 2). Inspection

Table 1
Summary of F ratios from ANCOVA using amount of P consumed as a covariate and leaf treatments[a] as factors for fifth-instar Manduca sexta

Source of variation	d*f*	P excretion
P consumed (P)	1	628∗∗∗[b]
Treatment (T)	2	1.3
T×P	2	3.4∗[b]
Residuals	29	

[a] Treatments are control leaves from Experiment 1, control leaves from Experiment 2, and P-enriched leaves from Experiment 2 (see Fig. 2).

[b] ∗∗∗P<0.001; ∗ P<0.05.

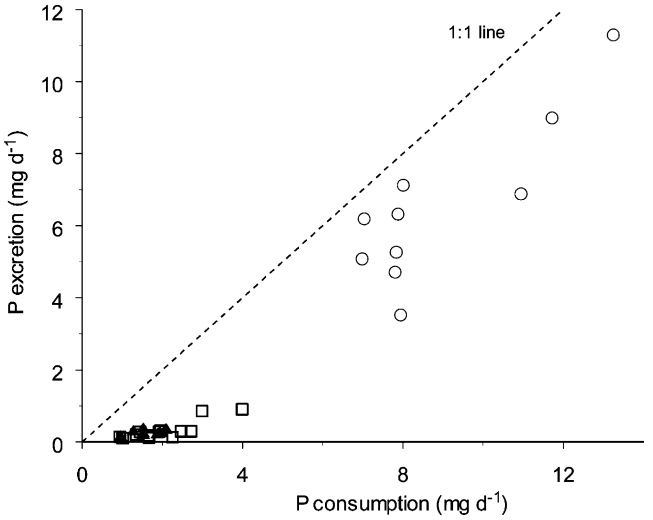

Fig. 2. Consumption and excretion of phosphorus over 24 h by fifth-instar Manduca sexta from Experiment 1 (□, eating control Datura) and from Experiment 2 (▲, eating control Datura; ○, eating P-enriched leaves).

of Fig. 2 shows that this interaction arises because the slope is shallow for larvae eating control leaves (combined slope=0.23) but steep for larvae eating P-enriched leaves (slope=0.87). Thus, larvae eating less than about 3 mg day^{-1} P retained almost all of ingested P whereas those eating more excreted most of the excess.

Larvae eating control *Datura* showed large changes in P concentration of lumen contents along the digestive tract (Fig. 3; repeated-measures ANOVA, $P<0.001$). From the food to the anterior midgut, the concentration of P tripled from about 0.2 to 0.6% P; and from the posterior midgut to the rectum the concentration of P fell by 90% from 0.53 to 0.052% (both changes significant by Tukey HSD). From the anterior to posterior midgut, the concentration of P remained fairly constant. Larvae eating P-enriched *Datura* did not show either of these step-changes. Although P appeared to be slightly

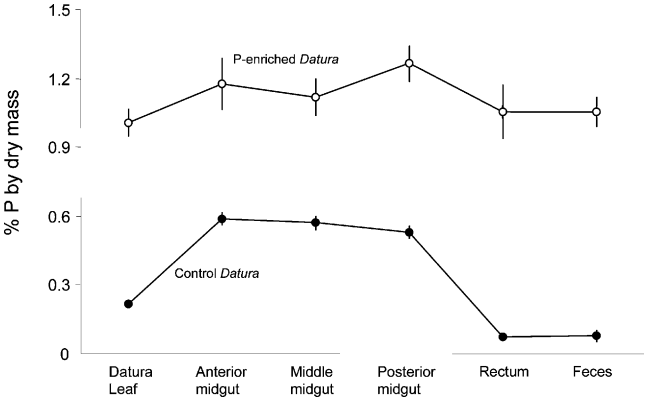

Fig. 3. Phosphorus content of the food, digestive tract, and feces of fifth-instar M. sexta eating control (N=10) or P-enriched leaves (N=10). Error bars are SEM.

more concentrated in the midgut than elsewhere, this difference was not significant (repeated-measures ANOVA, $P>0.1$).

For larvae eating control *Datura* (Table 2), the concentration of inorganic P increased significantly from the anterior to posterior midgut (repeated-measures ANOVA, $P<0.001$). The concentration of organic P was two to fivefold higher than the concentration of inorganic P and did not vary significantly among midgut regions. For larvae eating P-enriched *Datura* (Table 2), the concentrations of neither inorganic nor organic P varied significantly among midgut regions, but the concentration of inorganic P in the midguts of these larvae was sixfold higher than in control larvae. Using additional published data on pH variation along the midgut (Dow, 1984) together with the Henderson-Hasselbalch equation, we calculated ratios of di- to monovalent forms of phosphate (Table 2). Subsequently, we used the Nernst equation to calculate equilibrium potentials, which together with the transepithelial potential (assumed to be 90 mV; Chamberlin et al. (1997) allowed calculation of electrochemical gradients (ECG) across the midgut. These gradients generally were large and lumen-positive, especially for the monovalent form (200–300 mV). Uncertainty about transepithelial potentials and P concentrations in the fluid of the rectal lumen precluded similar calculations of ECGs for phosphate across the hindgut.

Hemolymph total P concentrations increased eightfold (from 5.5 to 44.7 mmol l^{-1}) in larvae switched onto P-enriched leaves (Fig. 4), and the increase was distributed unequally between inorganic and organic pools. Inorganic P increased 4.9-fold, from 1.4 to 6.9 mmol l^{-1}, whereas organic P increased 9.2-fold, from 4.1 to 37.8 mmol l^{-1} (Fig. 4, Table 2). Most of the increase in the organic pool represented increases in α-glycerophosphate: the mean concentration of α-glycerophosphate in hemolymph from larvae eating control leaves was 2.5 mmol l^{-1} (61% of total organic) and increased ten fold to 25.3 mmol l^{-1} (67% of total organic) in larvae eating P-enriched leaves (Fig. 4). Centrifugation had little effect on hemolymph P concentrations. The ratio of total P in centrifuged versus non-centrifuged hemolymph was 0.98 in larvae eating control *Datura* and 1.03 in those eating P-enriched *Datura*, indicating that hemocytes contained little P.

Dilution of radioactive methoxy-inulin indicated that larval hemolymph volume increased with larval fresh mass, with the relationship described by the equation: ml hemolymph=0.133 + 0.331×larval fresh mass in g (r^2=0.71). Larvae injected with radioactivity and then dissected and washed contained 19.8 ± 0.5% of the counts initially injected. Using this relationship and the data on concentrations of total P in the hemolymph (Fig. 4), we calculated that the total% P (excluding gut contents) of larvae (1.44 ± 0.05%, dry mass basis) eating P-enriched *Datura* was about 70% higher (relatively)

Table 2.
Concentrations of organic and inorganic phosphorus in midgut lumen and hemolymph, and calculated electrochemical gradients for phosphate of fifth-instar *Manduca sexta* consuming control or P-enriched *Datura*

	Organic phosphate (mmol l^{-1})	Inorganic phosphate (mmol l^{-1})	pH[a]	Ratio[b] of HPO_4^{2-} to $H_2PO_4^-$	HPO_4^{2} (mmol l^{-1})	ECG[c] for HPO_4^{2-} (mV)	$H_2PO_4^-$ mmol l^{-1})	ECG[c] for $H_2PO_4^-$ (mV)
Larvae eating control *Datura*								
Anterior midgut	9.96±0.76	1.19±0.20	9.5	200	1.18	76	0.006	219
Middle midgut	9.47±0.80	1.74±0.38	11	6340	1.74	72	0.0003	297
Posterior midgut	8.13±1.03	3.38±0.30	9.5	200	3.36	63	0.02	193
Hemolymph	4.10±0.93	1.41±0.23	6.8	0.40	0.40		1.01	
Larvae eating high-P *Datura*								
Anterior midgut	10.94±0.52	11.16±1.19	9.5	200	11.10	68	0.055	203
Middle midgut	11.75±1.34	11.99±1.27	11	6340	11.99	67	0.002	288
Posterior midgut	13.02±0.90	14.39±1.93	9.5	200	14.32	65	0.071	197
Hemolymph	37.82±0.97	6.89±0.75	6.8	0.40	1.97		4.92	

[a] Values from Dow (1984)

[b] Calculated using the Henderson-Hasselbalch equation; additional assumptions were an activity coefficient of 1 and pK_2 of 7.20 (Robinson and Stokes, 1965)

[c] Electrochemical gradients (ECG; mV lumen positive) calculated as the Nerst equilibrium potential subtracted from the transepithelial potential (assumed to be 90 mV lumen positive; Chamberlin et al. (1997).

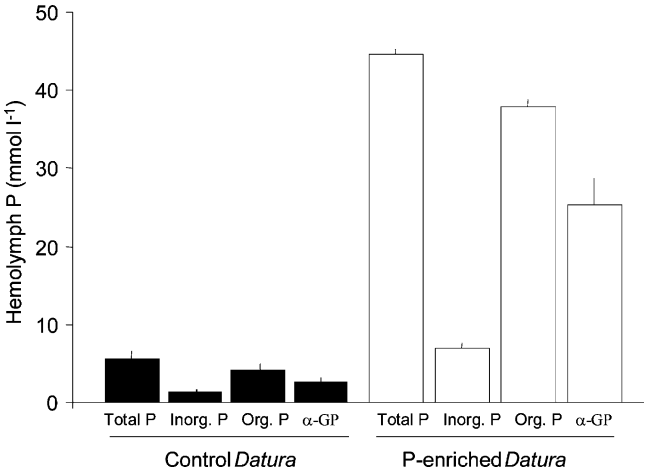

Fig. 4. Concentration of phosphorus (total, inorganic, organic) and of α-glycerophosphate (α-GP) in hemolymph of fifth-instar M. sexta eating control (0.%% P, N=11) or P-enriched leaves (1.0% P, N=10) of D. wrightii. Total and inorganic P were measured directly; organic was calculated as their difference. α-glycerophosphate was measured in samples of deproteinized hemolymph using an enzyme assay (N=7 or 8). Error bars are SEM.

than total% P of larvae (0.84 ± 0.03) eating control *Datura* (two-tailed *t*-test, $P<0.001$). We also estimated the% P associated with tissues alone (excluding contributions from the gut lumen and hemolymph) by subtracting the P associated with the hemolymph remaining in each carcass. This calculation showed that in larvae eating P-enriched *Datura* tissue concentrations of P (0.86 ± 0.03%) were significantly higher than tissue% P of larvae (0.77 ± 0.02) eating control *Datura* (two-tailed *t*-test, $P<0.01$). Variation in P content of the tissues (12%) was much less pronounced than variation in P content of the blood (eightfold).

4. Discussion

Our data provide the first integrated view of organismal P balance in any insect. Larvae eating control leaves of a common host plant retained a high fraction (85%) of dietary P. In a second experiment, the fraction of dietary P retained by larvae varied inversely with diet P concentration. Patterns of P concentration along the digestive tract indicate that variation in retention is likely due to the actions of the renal complex (Malpighian tubules and hindgut) rather than to differential absorption by the midgut. In addition, larvae fed high-P leaves had eightfold higher concentrations of P in the hemolymph, primarily in the form of α-glycerophosphate. This pool of organic P may play a role in protecting larval tissues from excessively high concentrations of inorganic phosphate in the hemolymph and may buffer larvae from natural variation in P content of the diet.

4.1. Phosphorus mass balance: patterns and mechanisms

Assimilation of P by larvae eating control leaves was substantially higher (85%) than overall dry mass assimilation (40%) and appears to have been higher than reported values of P assimilation in other studies (22–47%; Larsson and Tenow (1979); Slansky and Fogal (1985)). Retention of P also varied with dietary P availability — larvae eating control leaves retained on average 85% of ingested P whereas those eating high-P leaves retained about 25% (Fig. 2). This pattern suggests that larvae use physiological mechanisms to alter P retention, as do crustacean zooplankton consuming foods with contrasting P content (Urabe and Watanabe, 1992; DeMott et al., 1998).

The map of P concentrations along the gut (Fig. 3) gives some indication of where and how differential transport occurs along the gut. Larvae eating control *Datura* contained concentrations of P (as% of dry mass) in their anterior midguts that were almost three times higher than in their food. Because larvae absorbed only about 40% of total dry material consumed, high% P in the midgut lumen cannot be explained simply by disproportionate absorption of other dry components. Two more likely explanations are passive movement or active secretion of P from the hemolymph into the midgut lumen, which may be driven by large, lumen-positive electrochemical gradients (Table 2). A similarly large electrochemical gradient for α-glycerophosphate (negatively charged at physiological pH: $pK_1<2$, pK_2~6.65; Serjeant and Dempsey (1979) may exist, although its magnitude is unknowable without measures of α-glycerophosphate concentrations in the midgut lumen. Such movement may explain why soluble organic P comprised 85–90% of total soluble P in the anterior and middle midgut lumen but only 65% in control *Datura* leaves. *Manduca* midgut has in addition been shown to excrete a variety of injected anionic dyes (Nijhout, 1975) and may have a generalized affinity for organic anions.

The tenfold drop in P concentration between the mid- and hindguts of larvae eating control *Datura* (Fig. 3) strongly implicates the hindgut as the principal location of P absorption. In insects generally, the hindgut is specialized for ion and water transport (Phillips et al., 1986), and Andrusiak et al. (1980) showed that everted rectal sacs of the locust *Schistocerca gregaria* could transport phosphate from lumen to hemolymph against large concentration differences. A key difference between the two experimental groups of *Manduca* is that larvae eating high P *did not* show a sharp drop in P concentration in the hindgut. Thus, differential net retention of P (Fig. 2) probably results from differential action of the renal complex (Malpighian tubules and hindgut). The simplest explanation is differential rates of P absorption by the

hindgut — high when dietary P is scarce and low when it is plentiful. But the pattern could also arise by the combined actions of rectal P absorption and differential rates of P secretion in the urine (Ramsey, 1956; Berridge, 1969; Maddrell and Klunsuwan, 1973; Maddrell et al., 1974).

We point out that our data are consistent with Hoskins and Harrison (1934) proposed 'phosphate cycle' in honeybees. They showed that honeybees secrete P (mostly in inorganic form) into the ventriculus, reaching concentrations of 45 mmol l^{-1}. Low levels of P in the excreta (5 mmol l^{-1}) suggested that most of the ventricular P was subsequently absorbed downstream in the gut. These data led the authors to propose a 'phosphate cycle,' in which P is secreted anteriorly into the gut, absorbed posteriorly, and shuttled in between by the hemolymph. Our data on P concentrations in the gut and hemolymph of larval *M. sexta* support this model, with the principal modification being the secretion of organic, rather than inorganic, P into the gut lumen.

4.2. Phosphorus variation in hemolymph and tissues

Inorganic P in *Manduca* hemolymph varied between 1 and 7 mmol l^{-1} and organic P between 4 and 40 mmol l^{-1}. Inorganic P thus made up a small fraction (15-25%) of total hemolymph P, consistent with prior work on Lepidoptera (including *M. sexta*) and Hymenoptera (Bishop et al., 1925; Hoskins and Harrison, 1934; Wyatt et al., 1963; Lenartowicz et al., 1964; Jungreis, 1978; Terra et al., 1982). In addition, Wyatt et al. (1963) and Mueller et al. (1981) showed that most of the organic P in *M. sexta* occurs as α-glycerophosphate, and our data are consistent with this result (Fig. 4). In general, α-glycerophosphate plays important roles in gluconeogenesis and is the backbone for acylation reactions producing phospholipids and triacylglycerols (Horton et al., 1996). It also is used in the glycerol phosphate shuttle prominent in insect flight muscle (Chapman, 1998). Perhaps this mechanism is redirected in larval tissues to store phosphorus under conditions of P excess.

Two additional patterns are apparent in the data. First, P concentrations in the hemolymph varied much more (eightfold) with environmental availability than did P concentrations in the tissues (12%), suggesting that larvae guard tissue concentrations more closely. This result is consistent with studies of vertebrates, which show that concentrations of P in plasma and bone vary more with dietary availability than do concentrations of P in soft tissues (Day and McCollum, 1939; Freeman and McLean, 1941; Williams et al., 1991; McDowell, 1992). The comparatively small changes in tissue P of *Manduca* and other animals may be related to phosphate's structural and regulatory roles. Extra free phosphate could drive phosphorylation reactions toward attaching phosphate, disturb calcium or magnesium regulation, alter energy (ATP, ADP) metabolism, or affect intracellular signaling and regulation (Williams and Fraústo da Silva, 1996).

Second, variation in hemolymph P reflected changes primarily in the organic, rather than inorganic, component. Possibly, larvae attempted to minimize damage from excess inorganic P by attaching it to organic molecules in the hemolymph. As argued above, high levels of hemolymph phosphate could disturb reactions taking place in the hemolymph or could drive phosphate into cells at unacceptably high rates. Very high levels of orthophosphate could also lead to hyperosmotic hemolymph (see Jungreis (1978). Alternatively, larvae eating high-P *Datura* may have stored excess phosphate resulting from 'luxury consumption' (Stevenson and Stoermer, 1982). In this scenario, animals convert inorganic phosphate to α-glycerophosphate for use if dietary availability later decreases. How long could observed increases in this pool sustain growth if a larva suddenly had access only to P-free food? A 4-g larva contains about 1.46 ml blood. If the larva were eating P-enriched leaves for the period we examined (about 1 week), that blood would contain 44.7 mmol l^{-1} P or 65.3 μmol total P (2 mg P). P at 2 mg is well over 1/3 of total body P and is over 2/3 the P used in growth per day by a 4-g larva (Fig. 1). By drawing on this pool, a larva should be able to grow by about 50% even if dietary P availability were nil. Hemolymph stores of P thus appear sufficient to buffer growth against short-term fluctuations (on the order of 1/2 –1 day) in P availability. Analogous mechanisms operate in other organisms: yeast can draw on stored polyphosphates to support growth for 2–3 generations under conditions of phosphate starvation (Shirahama et al., 1996), and plants can maintain constant levels of phosphate in cytoplasm by transport to and from vacuoles (Mimura, 1995).

4.3. Phosphorus storage and the stoichiometry of growth

Elser et al. (1996, 2000b) have described a 'growth rate hypothesis,' which proposes a positive association between body P-content and mass-specific growth rates by the following set of connections: (i) growth rate (especially maximal growth rate) depends proximately on overall biosynthetic capacity; (ii) biosynthetic capacity depends on concentrations of ribosomes; (iii) ribosomes contain about 50% ribosomal RNA, which is approximately 10% P by mass (Alberts et al., 1983). Thus, rapidly growing animals should have high contents of both rRNA and P (Elser et al., 1996, 2000b), and *variation* in concentration of total P should reflect variation in concentration of rRNA. These predictions are supported for a number of taxa, especially freshwater zooplankton (Elser et al., 2000b). Our results for *M. sexta* show, by contrast, that concentrations of total body

P and rRNA can be uncoupled by hemolymph storage of organic P. Whether this finding is generally applicable to other Insecta is unclear. Bishop et al. (1925) and Wyatt et al. (1963) demonstrated large shifts in body P in Hymenoptera and Lepidoptera. Markow et al., (1999), in contrast, showed that body P contents of several species of *Drosophila* increased only moderately when larvae were reared on P-rich laboratory medium. Future work should examine whether hemolymph P storage occurs broadly across the insects and, if so, whether it uncouples contents of P and RNA.

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