J Exp Biol Advance Online Articles. First posted online on 13 November 2015 as doi:10.1242/jeb.126847
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.126847

Dietary phosphate affects food selection, post-ingestive P fate, and performance of a polyphagous herbivore

Arianne J. Cease[1]*, Michelle Fay[2,3], James J. Elser[2], Jon F. Harrison[2]

School of Sustainability, Arizona State University, Tempe, Arizona, 85287, USA[1]
School of Life Sciences, Arizona State University, Tempe, Arizona, 85287, USA[2]
Orange Coast College, Costa Mesa, California, 92626, USA[3]

*To whom correspondence should be addressed.
E-mail: acease@asu.edu

Abstract

Comparisons of the carbon, nitrogen, and phosphorus contents of plants and insect herbivores suggest that phosphorus (P) limitation and herbivore foraging to balance P intake could be common. However, lack of synthetic diets for testing the effects of lower ranges of dietary P has been a major impediment to experimental assessment of the ecological importance of, and physiological responses to, P limitation for terrestrial herbivores. We manipulated dietary P content (% P) over its observed range in terrestrial foliage using artificial diets containing near-optimal contents of other nutrients for the grasshopper *Schistocerca americana*. Over much of the ecologically relevant range, when consuming single diets over a lifetime, higher P stimulated growth rates and increased survival, with an optimal dietary % P of 0.25-0.50% when measured throughout development. Excessive dietary P (1%) reduced growth and survival. However, with only short-term (3 day) confinement to single diets, dietary P had no effect on food consumption or growth rates. During these short exposures, fifth (but not third) instar hoppers increased the proportion of P excreted relative to P assimilated as dietary P increased. Target experiments demonstrated that, when given a choice, grasshoppers select among foods to attain a P intake target of 0.6%. These data suggest that P-limitation could be common for terrestrial insect herbivores and that they can exhibit ingestive and post-ingestive mechanisms to attain sufficient but not excessive P.

Keywords: ecological stoichiometry, geometric framework, grasshopper, phosphorus, synthetic diet, diet choice

Introduction

The relative availability of various nutrients strongly affects the growth and fitness of herbivores, whose biomass generally contains much greater concentrations and different ratios of elements such as nitrogen (N) and phosphorus (P) relative to plants (Boswell et al, 2008; Sterner and Elser, 2002). The relative availabilities of carbohydrate and protein (or carbon (C) and N) have been shown to be particularly important, and many recent studies have shown that feeding rates, choices, and performance of a diversity of animals can be explained by the dietary carbohydrate:protein ratio (Behmer, 2009; Simpson and Raubenheimer, 2012). It is also well recognized that the nutritional landscape and requirements of animals are multi-dimensional, with striking examples of the importance of particular nutrients (e.g. changes in sodium availability influence reproduction, growth rates, and abundances of insects (Joern et al., 2012; Kaspari et al., 2014; Smedley and Eisner, 1996)). Nevertheless, we still have a relatively incomplete picture of the relationships between nutrient concentrations and herbivore performance, providing a major impediment to predictive ecology.

In freshwater herbivores, many studies have shown that certain species (especially the fast-growing crustacean *Daphnia*) grow more slowly, show reduced survival, and reproduce at lower rates when diets are relatively depleted in phosphate (high C:P ratio) (DeMott et al., 1998; Elser et al., 2001; Hessen et al., 2013). These studies suggest that in many aquatic ecosystems, P is a key nutrient that limits growth and fitness not only of primary producers but also of their consumers, as P-limited producers develop high biomass C:P ratios, and pass P limitation on to their consumers. Thus, variation in environmental conditions due to natural or anthropogenic impacts, such as changes in nutrient loading or shifts in climate, can have strong effects on community structure by altering the C:P stoichiometry of phytoplankton at the base of the food web (Elser et al., 2000b). Indeed, these impacts appear to be mediated by the fact that P, in the form of phosphate (PO_4), is an important constituent that is essential for synthesis of RNA, DNA, and other cellular constituents (Sterner and Elser, 2002). A key physiological link between P intake and growth is provided by the "growth rate hypothesis," which points out that, at least in small, fast-growing organisms, most body P is in ribosomal RNA, the

cellular levels of which are tightly linked to the maximal growth rate attainable by an animal under ideal conditions (Elser et al., 2000b; Hessen et al., 2013).

Comparisons of the P contents (%P, as percentage of dry mass and generally dominant in determining C:P ratio) of the plants and herbivores of freshwater and terrestrial systems suggest that P-limitation should be as common in terrestrial ecosystems as it is in aquatic systems (Boswell et al., 2008; Elser et al., 2000a; Joern et al., 2012; Lemoine et al., 2014; Boswell et al., 2008). This contention is supported by experimental tests of plant nutrient limitation (Bishop et al., 2010; Elser et al., 2007) and correlations between host plant P content and grasshopper abundances (Joern et al. 2012). Indeed, P levels in terrestrial foliage range widely. The mean global foliar % P is 0.12%; a few plants have P concentrations as high as 1.00%, but most leaves (87%) have values between 0.05 and 0.45% P (Elser et al., 2000a). Joern et al. (2012) compared elemental compositions of grasses and forbs collected from central Nebraskan grasslands and found a phosphorus range from about 1500-3600 ppm (0.15-0.36% P). Boswell et al. (2008) showed that *Schistocerca americana* grasshoppers maintained a body P content of about 1% throughout development, which is at least three fold higher than the values for host plants reported by Joern et al. (2012). Surprisingly, as yet there are few studies of terrestrial herbivores that have measured the effect of dietary P level below 0.45% P and across the ecologically observed range on consumer growth and survival. Thus, our understanding of the relative importance of natural and human-induced variation in P availability on terrestrial herbivore performance, abundance, and community composition remains elementary.

The few existing studies of the effect of variation in dietary phosphate on performance of insect herbivores have yielded mixed results. Several studies showed positive effects of increased dietary % P. The caterpillar, *Manduca sexta*, exhibited faster growth and improved survival on artificial diets enriched in phosphate in the natural range, and also performed better on natural diets spiked with phosphate (Perkins et al., 2004). Similarly, higher leaf P levels were correlated with faster growth in spruce budworm caterpillars (Clancy and King, 1993), and the growth and survival of several Lepidopteran larvae was better on wild and greenhouse-grown plants with higher % P, independent of % N (Apple et al., 2009). House crickets, *Acheta domestica*, grew and

developed faster on artificial diets with higher phosphate contents (Visanuvimol and Bertram, 2011). Schade *et al.* (2003) linked population dynamics of the weevil *Sabinia setosa* to precipitation patterns as mediated by effects of rainfall on soil P cycling and the P content of its food, mesquite leaves. Littoral mayfly larvae (*Caenis* sp. and *Ephemerella* sp.) grew faster on diets enriched in P (Frost and Elser, 2002). However, the development rate of the grasshopper, *Melanoplus bivittatus*, was unaffected by dietary % P of artificial diets, except for negative effects at the highest levels (Loaiza et al., 2008). Smith (1960) found similar results for *M. bivittatus* using fertilized wheat, as did Harrison et al (2014) who found no effects of dietary P intake on most fitness parameters of crickets (*Gryllus veletis*) except for some negative effects of high dietary P on a male chirping rate. Finally, fertilization of plots with phosphate increased survival, body size and development rate of one planthopper species but not another (Huberty and Denno, 2006).

Multiple explanations are plausible for the diversity of effects of P on insect performance. Based on the growth rate hypothesis, faster growing species with high body P contents should exhibit a greater sensitivity to dietary P during development (Sterner and Elser, 2002). For experiments in which % P is varied in natural foliage by fertilization of soils or "spiking" plants by placing clipped stems in a phosphate-rich aqueous solution, the effect is expected to vary depending on the levels of other nutrients in the diet (Simpson and Raubenheimer, 2012), as well as potential effects of fertilization on plant structure or content of allelochemicals (Behmer, 2009). Even in synthetic diets with optimal concentrations of other nutrients, the effect of dietary P on performance is likely to be nonlinear. That is, there are likely species-specific positive effects over some low range of P content, a plateau region in which selective absorption/excretion allows sufficient P assimilation and growth to be independent of P intake, and a higher region in which higher P content has negative effects. The negative effects of high dietary P have been shown in a variety of aquatic taxa and perhaps reflects inhibition of feeding or elevated costs of excretion (Boersma and Elser, 2006).

A significant technical reason for the diversity of responses of terrestrial herbivores to P could be lack of a synthetic diet with sufficiently low P due to commonly used phosphate-rich proteins. A second problem with most prior synthetic diets that have

examined responses to P is a lack of control for co-variation in cations. To address these difficulties, we developed a new synthetic diet for grasshoppers that enables lowering of %P to 0.02%. We maintained constant macronutrient concentrations in these diets and diets contained an estimated N:P ratio of 6 to 460 (atomic), which encompasses the N:P ratios found in terrestrial plant foliage (Elser et al., 2000a). We tested the effect of % P on the growth and survival of a polyphagous grasshopper, *Schistocerca americana* (Drury), using artificial diets with near-optimal contents of other nutrients. We ran two experiments where grasshoppers were confined to a single % P diet. The first was for their entire nymphal development to determine long-term developmental effects of dietary P on growth and performance parameters. The second experiment was conducted within the 3rd and final nymphal instars so we could carefully monitor feeding rate and P assimilation and excretion. In addition, we conducted a test to investigate the presence of a P intake target (sensu Simpson and Raubenheimer, 1993). Harrison et. al. (2014) recently reported that adult *G. veletis* crickets do not have a P target; however their minimal dietary P was 0.20% (% of dry diet), with an estimated N:P ratio of 40 (atomic) which may not have captured a broad enough range of dietary P. Previously, grasshoppers (and many other animals) have been shown to exhibit intake targets for protein to carbohydrate ratios and NaCl levels (Simpson and Raubenheimer, 2012). In such tests, animals are provided with pairs of diets with high or low levels of the nutrients of interest. If the animal consistently consumes from such pairs in such a way that it obtains the same amount of the nutrient, the animal can be considered to be behaviourally adjusting its food selection to obtain a target quantity of the nutrient. Because numerous prior studies have demonstrated that intake targets (at least for carbohydrate:protein ratio) closely match the diet composition that maximizes fitness (Roeder and Behmer, 2014), these behavioural tests allow the animal itself “tell us” its optimal dietary phosphate level.

Materials and methods

Animals, artificial diets, and general conditions.

Schistocerca americana is a large polyphagous grasshopper species distributed throughout much of North America that feeds on both forbs and grasses. The animals used in these experiments were obtained from a culture maintained at Arizona State University for twenty years as previously described (Harrison and Kennedy, 1994). Male and female grasshoppers were distributed equally among experiments. To manipulate % P, we used dry, granular, chemically-defined foods, replacing the normally used phosphate-rich casein with soy protein and essential amino acids (see methods for 'Development of a P-flexible synthetic diet' below). Phosphorus levels were manipulated between 0.02% and 1.50% by adding phosphate salts, with the cation concentrations kept constant within an experiment. The particular cations and anions used to balance the phosphate varied with experiment (see below). In all cases, food and water were provided *ad libitum*.

Experiment 1: Phosphate effects on growth rate, development time, and survival throughout juvenile development.

Animals were reared on the standard lettuce bran diet (Harrison and Kennedy, 1994) for one week before initiation of experiments because all animals died when attempts were made to rear grasshoppers from hatching on artificial diets. All animals from each treatment were held communally in separate aluminium 96 l cages (n=20 individuals per treatment). Air temperature was 31ºC±1ºC. Light was provided by individual fluorescent tubes beside each cage and set to 14:10 L:D schedule. Water was supplied by gravity-feed into a dish with gravel-lined base to slow evaporation and allow insects to stand on the gravel and drink water. Food was held in plastic petri dishes and changed at least once every three days (once daily for lettuce-fed animals). Phosphate levels were increased by adding greater amounts of an equimolar mix of calcium- and potassium- phosphate and included 0.02%, 0.05%, 0.10%, 0.25%, 0.50%, or 1.00% P by dry mass. Calcium and potassium levels were kept constant across all diets by reciprocal additions of a mixture of calcium- and potassium- salts of chloride, carbonate and sulfate. We used a diversity of anions to reduce the likelihood of specific negative effects.

Grasshoppers were weighed and survival and developmental stage noted approximately weekly for 60 days. Specific growth rates (μ) were calculated as $\mu = \ln(M_2/M_1)/dt$, where M_1 and M_2 are the grasshopper body masses at the start and end of the experiment, respectively, and dt is the time between weighings in days. Because grasshoppers were not tracked individually, we estimated initial body mass by taking the average of all grasshoppers at the beginning of the experiment.

Experiment 2: Effects of dietary P on consumption, excretion, assimilation, and growth for third and final instar grasshoppers.

As in Experiment 1, grasshoppers were reared for one week after hatching on the standard lettuce-bran diet. Then animals were switched to the 0.50% P artificial diet until the test instar (3rd or final juvenile instar). On day one of the target instar, animals were weighed and then placed in individual 0.5 l plastic cages and provided *ad libitum* access to a dish containing a weighed amount of one of seven artificial diets that ranged from 0.02 to 1.50% P. In these experiments, P was increased by addition of sodium- and potassium- phosphate salts (as opposed to calcium- and potassium- salts as was done in Experiment 1), with cations kept constant across diets by reciprocal addition of sodium- and potassium- chloride, carbonate and sulfate. For the 1.00% P diet, we also tested a diet in which % P was increased using calcium- and potassium- phosphate (as in Experiment 1), to test whether these specific cations affected the results. After three days (instar duration was about five days), the grasshopper and food were weighed; consumption rate and growth rate were calculated from the changes in mass and the time between the initial and final weighings. P ingestion rates were calculated by multiplying food consumption times the P content of the diet. Third instar treatment groups started with 10 animals; final instar groups started with 8. Animals that died or moulted during the experiment were excluded. Eight grasshoppers moulted and 14 died out of 144. Mortality and moulting were distributed across treatment groups and no treatment group had more than two mortalities or moults.

All faecal pellets were collected, dried, and weighed after which the P contained in the pooled sample of all faecal pellets from an individual animal was measured in duplicate after persulfate digestion as described in Perkins et. al (2004). Data were then

expressed as a percentage of dry mass (% P). We calculated phosphate excretion rates of individuals (µg/day) by multiplying the total faecal mass by the average faecal P proportion and dividing by the duration of the experiment. Phosphate assimilation rates (µg/day) were calculated by subtracting the rate of P excretion from the rate of P ingestion.

Experiment 3: Testing for a phosphorus intake target.

We collected grasshoppers from the colony population within six hours of moult to the final juvenile instar. All individuals collected had moulted after 12 PM on a given day and food and water were withheld for that evening. Trials were started the following morning at 8 AM. Individuals were given a choice of two artificial diets for a total of six days, with food dishes being replaced at day two. We weighed the amount of each diet consumed and used that to determine the total P ingested by each individual. In this experiment, P was increased by addition of sodium-, potassium-, and calcium- phosphate salts, with cations kept constant across diets by reciprocal addition of sodium-, potassium-, and calcium- chloride, carbonate and sulfate. We made the following diets expressed as % P dry mass: 1.40, 1.20, 0.15, and 0.05% P. The diets were paired such that one high and one low % P diet was present in every cage, giving the following four combinations: 1.40+0.15; 1.40+0.05; 1.20+0.15; 1.20+0.05. There were 10 grasshoppers each in pairs A and B and 11 grasshoppers each in pairs C and D, with a total N=42. The four treatment groups were blocked within the environmental chamber to avoid any bias caused by light, temperature, or disturbance.

Development of P-flexible synthetic diet.

To determine that our P-flexible diet would yield similar growth and performance as the normal colony diet and the standard Dadd diet, we compared insect performance when fed the normal colony diet of romaine lettuce with the wheat bran/vitamin mix supplement, the standard Dadd diet, a new synthetic diet in which casein/peptone were replaced with just amisoy (recipe not shown), and another new synthetic diet in which the essential amino acid mix was added to the amisoy diet (Table 4). All treatment groups started with n=20. The P content of the amisoy and amisoy plus essential amino acid

diets was set to 0.5% (similar to the Dadd diet, which is 0.54 % P) using salt mixes as indicated in Table 5. We changed the P content of diets by altering the ratio of phosphate salts (salt mix 1) to a mixture of chloride, carbonate, and sulfate salts (salt mix 2), while maintaining equal contents of sodium (Na), calcium (Ca), and potassium (K) across all diets.

To calculate the amount of each salt mix to add to the base diet, we first set the desired highest proportional dietary P (P_{max}) and calculated the grams of salt mix 1 (M_{S1}) needed to achieve this upper limit, given the desired amount of base diet used (M_{base}; a constant across diets, see Table 7 for definitions of terms used in the diet calculations).

At P_{max},

(1) $M_{S1} = (M_{base} * (P_{max} - P_{base}))/(P_{S1} - P_{max})$.

We then calculated the value for M_K, which we kept constant across diets:

At P_{max} (where only salt mix 1 is added to the base diet),

(2) $M_K = (K_{S1} * M_{S1})$

Keeping M_K (the mass of K added to the base diet) constant will also keep the masses of Na and Ca constant across diets because the ratios of Na:Ca:K are the same in salt mixes 1 and 2 (see Table 6). Knowing the value for M_{S1} at P_{max} also gives us the value for M_{mix} because M_{S1} at P_{max} is the maximum mass of any set of mixes that will be added to the base diet. Therefore,

At P_{max}, (3) $M_{S1} = M_{mix}$

We then used the following matrix and vectors to calculate the amount of each salt mix to add to a given diet to achieve a given dietary P level (P_{test}). We also calculated the amount of cellulose to add to keep the total diet mass constant. Here, [A][B]=[C]

$$[A] = \begin{vmatrix} 1 & 1 & 1 \\ P_c & P_{S1} & P_{S2} \\ K_c & K_{S1} & K_{S2} \end{vmatrix}$$

$$[B] = \begin{vmatrix} M_C \\ M_{S1} \\ M_{S2} \end{vmatrix}$$

$$[C] = \begin{vmatrix} M_{mix} \\ M_P \\ M_K \end{vmatrix}$$

The first row in [A] represents the proportion of cellulose in cellulose, S_1 in S_1, and S_2 in S_2, respectively, thus the values are all equal to 1. The second two rows in [A] contain the proportions of P and K in the cellulose, salt mix 1, and salt mix 2 diet components (the three columns, left to right). [B] contains the grams of cellulose (M_C), salt mix 1 (M_{S1}), and salt mix 2 (M_{S2}). [C] contains the grams of P (M_P), grams of K (M_K), and total grams of all the mixes ($M_C+M_{S1}+M_{S2}=M_{mix}$) added to the base diet.

We next calculated the required mass of phosphate to add to the base diet (M_P) to obtain a given P content for each test diet (P_{test}). P_{test} takes into account the amount of P added to the base diet (M_P) as well as the amount of P already present in the base diet. The amount of P in the base diet is calculated by $P_{base}* M_{base}$. To calculate M_P:

(4) $M_P=P_{test}(M_{base}+ M_{mix})-(P_{base}* M_{base})$.

Knowing [A] and [C], we solved for the vector [B], using [B]=[A]'[C] to obtain the grams of cellulose, salt mix 1, and salt mix 2 to add to each test diet to achieve different levels of dietary P while maintaining constant K, Ca, and Na, and similar total salt contents across all diets.

Statistics

All data were tested for assumptions of normality and homoscedasticity implicit in parametric tests. Analyses were performed using Statistica 10 (2011).

Results

Experiment 1: Phosphate effects on growth rate, development time, and survival throughout juvenile development.

Dietary P level significantly and nonlinearly affected grasshopper body mass, survival, and performance, measured on the final collection point on day 57 (Figs 1 and 2). We removed the 0.05% P diet treatment from the statistical analysis for body mass because only one individual survived until the end of the experiment. Body mass increased with % P from 0.02% to 0.50%, and then decreased for the 1.00% P diet ($ANOVA_{(4,48)}$ F = 30.29 $P < 0.001$; Fig. 1A). There was a significant difference in survival rate among the treatment groups ($\chi^2_{(5)}$ Survival test = 47.64 $P < 0.001$, followed by pairwise Cox F tests corrected for multiple comparisons; Fig. 1B). Again, the effect was nonlinear, with low survival below 0.10% P, but no significant differences in survival among the treatment groups fed diets with greater than 0.10% P. In three of six diet treatment groups, no grasshoppers moulted to adults during the experiment (0.02%, 0.05%, and 0.10% P; Fig. 1C). There were no differences in the rates at which grasshoppers moulted to adults among the remaining three treatment groups (0.25%, 0.50%, 1.00% P) ($\chi^2_{(2)}$ Survival test = 4.54 $P = 0.10$). To compare overall performance of the groups, we multiplied the specific growth rate (μ) of an individual by the survival rate of its treatment group. Performance increased from 0.02 % to 0.25% P where it plateaued through 0.50% P and then decreased at 1.00% P ($ANOVA_{(4,48)}$ F = 156.67 $P < 0.001$; Fig. 2).

Experiment 2: Effects of dietary P on consumption, excretion, assimilation, and growth for third and final instar grasshoppers.

There were some significant instar-diet interaction terms (see below), however, when instars were analysed separately for the effect of dietary P on food consumption, P consumption, P assimilation and P excretion the statistical significance of the results were the same as when instars were pooled. Therefore, we concentrated our interpretations on pooled results for both instars, using ANOVAs on mass specific data (Table 1). Grasshoppers maintained similar total food consumption rates on all dietary P treatments, with third instars eating more on a mass specific basis than final instar juvenile nymphs

(Table 1). Third instar grasshoppers had higher specific growth rates than final instar grasshoppers, but diet did not affect growth rate over this 3-day period (Table 1).

We analysed mass specific P excreted and assimilated using ANOVAs (Table 1). Both instars consumed, excreted, and assimilated more P when consuming higher P diets. However, there was an interactive effect of diet*instar on amount of P consumed and assimilated (Table 1). Visual inspections suggest that this significant interaction term arose from third instar grasshoppers consuming and assimilating relatively more P than final instar grasshoppers on a mass specific basis when eating higher P diets (dietary P > 1.00%).

We tested for the possibility that grasshoppers were increasing excretion relative to assimilation when eating diets with higher P contents by plotting P excreted and P assimilated vs. P consumed (Fig. 3). We transformed mass-specific data ($\mu g*day^{-1}*g^{-1}$) by adding 100 to remove negative values and then taking the log. For third instars, slopes of P excreted and P assimilated on P consumed were statistically identical ($t_{(128)} = 0.8$; $P = 0.42$), indicating no physiological modulation. However, for final instar grasshoppers, the slope of the excretion line was significantly steeper than the slope of the assimilation line ($t_{(128)} = 2.69$; $P = 0.008$), indicating that final instar nymphs excreted more and assimilated less P as P consumption increased due to eating diets with higher P concentrations.

Experiment 3: Testing for a phosphorus intake target.

In the intake target experiments, all treatment groups consumed similar amounts of P and total food, despite their differing diet combinations (Table 2). Groups B, C, and D differed from the expected ratio of dry mass consumed from each diet if each food dish was eaten from randomly; however, group A did not (Table 3). We also compared the amount of phosphate ($PO_4^{=}$) versus the amount of replacement anions (Cl^-, $CO_3^{=}$, and $SO_4^{=}$) consumed. These anions were used to maintain calcium (Ca^{2+}), potassium (K^+), and sodium (Na^+) concentrations and were the only ingredients (other than small amounts of cellulose) that differed among any of the diets (see 'Development of P-flexible synthetic diet' in methods for details). There was no effect of diet pairing on the amount of $PO_4^{=}$ or Cl^-, $CO_3^{=}$, and $SO_4^{=}$ consumed ($MANCOVA_{(6, 72)}$ F = 1.6, $P = 0.17$) (Fig. 4).

We calculated the average % P eaten by individuals in all diet treatment groups (A-D) to estimate that grasshoppers selected for a diet with 0.6% P ± 0.04 SE by balancing feeding among the low and high P diets.

Development of P-flexible synthetic diet.

There were no significant differences in survival rates among the diet treatment groups (Figure 5A). However, grasshoppers fed the amisoy diet *without* the essential amino acids added attained lower body masses than the other three diet treatments (Figure 5B). Adding essential amino acids to the amisoy diet (amisoy+EAA in Figure 5) increased body masses to levels similar to those of grasshoppers fed the standard Dadd diet (Dadd 1960; 1961) and grasshoppers fed the standard colony diet (lettuce, bran, and vitamins).

How high can P be raised with this synthetic diet? At some point, we expected high salt content to serve as a deterrent to feeding. We conducted an initial test of whether *S. americana* would feed on 3% and 1.4% P diets, with 20 animals tested per treatment. All grasshoppers fed, survived, and grew well on the 1.4% P diet, while 100% of grasshoppers on the 3% P diet did not feed and died within one week. Thus, this synthetic diet allows testing of the behavioral and growth responses of grasshoppers from 0.02-1.4% P, a range that includes most of the ecologically realistic possibilities for terrestrial herbivores.

To test whether the use of different cations affected the results, we used two treatment groups for the 1.00% P diet in Experiment 2: one diet manipulated with sodium- and potassium- phosphate and the other with calcium- and potassium-phosphate. We found no differences between the two diets for growth rate or for the amounts of food consumed, P consumed, or P assimilated (t-tests $P > 0.60$), so we combined these groups for all further analyses for Experiment 2.

Discussion

Our results indicate that, in the context of artificial diets optimal for growth in other regards, *S. americana* grasshoppers respond nonlinearly to dietary phosphate levels. Long-term consumption of diets of 0.10% P and below suppressed growth, development, and survival (Figs 1, 2). The mean for terrestrial foliage is 0.12% P and the median is 0.14% P (Elser et al., 2000a), indicating that P-limitation may occur for *S. americana* and likely other terrestrial herbivores if unable to feed selectively on P-rich plants or plant-parts. Grasshoppers self-selected a diet of 0.60% P, which is likely in the range of a plateau of peak performance (Figs. 2 and 4). However, diets with P contents of 0.1% and below or 1% and above could support maximal growth during 3-day time periods when animals had been fed on the optimal (0.5 %) diets for most of their lives, suggesting insects have a considerable capacity to buffer effects of non-optimal P consumption. Long-term consumption of diets with a P content of 1.00% or higher suppressed growth, consistent with the "intake target" behaviour of *S. americana* and with observations of reduced performance of various aquatic consumers (Boersma and Elser, 2006) and some insect species when provided with diets well above the mean P values for terrestrial leaves (Harrison et al., 2014; Loaiza et al., 2008; Smith, 1960). Together these results demonstrate that ecologically relevant variation in plant P content affects the behaviour and performance of this grasshopper.

Effects of dietary P on growth, development and survival.

When confined to single artificial diets over their lifetime, the growth of *S. americana* during early development was positively related to dietary % P up to 1.00% P; however, later in the juvenile period, growth rates decreased for grasshoppers feeding on 1.00% P and maximal growth over the entire juvenile period was observed for hoppers consuming the 0.50% P diet (Fig. 1). Grasshoppers did not attain adulthood on diets with % P of 0.10% or lower, leading to zero fitness. Phosphorus is needed to produce RNA, DNA, membranes and proteins; our data suggest that long-term consumption of diets with less than 0.1% P do not yield sufficient P to grow an adult *S. americana*, even with all other diet components near optimal. The mechanisms by which increases in dietary P from 0.1 to 0.5% increased growth rate are unclear, but may reflect the P demands

needed to maintain higher body levels of P-rich RNA, as found in *Daphnia* and *Drosophila* (Elser et al., 2003; Watts et al., 2006). The lower growth rates of grasshoppers on 1.00% P diets (Fig. 1A) also support the conclusion that 1.00% P diets contain excessive P that imposes a fitness cost on grasshoppers. It is not clear why elevated P might reduce survival; possibly this could be associated with the costs of excreting large amounts of phosphate (Fig. 3) and associated cations.

Our results for *S. americana* are consistent with those for another polyphagous grasshopper, *Melanoplus bivittatus*. Loaiza et al (2008) found negative effects of high-P synthetic diets (1.74-2.07% P) on the development rate of the *M. bivittatus*. An earlier study reported decreased survival and growth when *M. bivittatus* nymphs were fed P-fertilized wheat (1.86% P) (Smith, 1960) and suggested that, perhaps counter-intuitively, P fertilizer could be a way to improve wheat crops and decrease pests. These results fit with a growing body of literature showing cases in nature where herbivores may not be N or P limited and that high levels of these nutrients can decrease growth and survival (Boersma and Elser, 2006; Cease et al., 2012; Simpson and Raubenheimer, 2012). On the lower end of the P diets, Smith (1960) reported that the low-P wheat (0.17% P) decreased survival relative to control wheat. Loaiza et al (2008) found no negative effect of low dietary P; however, the lowest level of dietary P tested in their experiment was 0.34%, and these tests were run only for one instar (the 5^{th}). The 0.34% P diet was within the plateau-range where dietary P had no effect on performance throughout juvenile development (Fig. 2).

In contrast to the strong effects of dietary P when grasshoppers consumed a single diet over their lifetime, there were no effects of dietary P on growth when grasshoppers were tested over three days within a single instar after feeding for multiple instars on diets with 0.50% P. Possibly, body P stores compensated for short-term shortages of dietary P. Insects can increase body P in response to higher dietary P; *Manduca sexta* caterpillars and *M. bivittatus* grasshoppers had 40-50 % higher body P contents on higher-P foods (Perkins et al., 2004; Smith, 1960), while house crickets increased body P by 10-15% when raised on higher P diets (Visanuvimol and Bertram, 2011). A similar temporal decoupling of dietary P effects has been reported for *Daphnia* (Sterner and

Schwalbach, 2001). Our results demonstrate that single-instar feeding trials, commonly used for insects, can misrepresent long-term requirements for some nutrients.

Effects of dietary P on food consumption.

Many animals forced to consume only a diet deficient in protein or carbohydrate overconsume that diet (Raubenheimer and Simpson, 1997). For example, such compensatory feeding has been found for salt. NaCl content serves as a phagostimulant in *Locusta migratoria* over a range of NaCl concentrations below the NaCl concentration that supports maximal growth (Trumper and Simpson, 1993). Similarly, juvenile house crickets (*Acheta domestica*) consumed more of diets with % P values that maximized growth (0.6% or higher) than diets with lower P contents (Visanuvimol and Bertram, 2011). There was no evidence for such compensatory feeding for phosphorus in this study when measured over three days (Table 1). However, we also did not find an effect of % P diet treatment on growth rate over this short duration, suggesting that grasshoppers have compensatory mechanisms beyond increasing total food consumption to cope with intermittent P shortage. Similarly, adult field crickets, *Gryllus veletis*, did not preferentially consume high P foods when given pairings ranging from 0.20 to 1.1% P of dry diet (Harrison et al., 2014). These crickets were fed a P-rich diet as juveniles (1.1% P) and so may have fulfilled their P requirements before reaching adulthood. Possibly, longer restriction to low P diets and/or exposure to a broader range of dietary P may lead to compensatory feeding for P.

Our "intake target" study suggests that the grasshopper *S. americana* can regulate P intake to about 0.6%, likely within a plateau that maximizes growth and performance (Figs 2 and 4). However, as one of the diet pairings did not result in consumption significantly different from random, the target behaviour for P may not be as tightly regulated as for carbohydrate and protein (reviewed in Simpson and Raubenheimer, 2012). For our diets, 0.6% P is equivalent to an N:P ratio of ~15 N:P (atomic), which is the most frequent value Elser et al (2000a) found for body N:P ratio of invertebrate terrestrial herbivores. Boswell et al. (2008) found similar values for *S. americana* grasshoppers, ranging from about 15 to 9 N:P throughout development. In contrast to our target experiment, Harrison et al. (2014) showed that adult *G. veletis* crickets did not

exhibit an intake target for P when given pairings of 0.20, 0.66, and 1.1% P of dry diet and that crickets prioritized balancing protein and carbohydrate over P intake in the range of 0.07-1.87% P. At present, it is not clear whether the differences between *S. americana* and *G. veletis* represent effects of species, developmental stage, or the range of P diets tested. The mechanisms by which *S. americana* determine P content of food are not known. Indeed, it is unknown whether grasshoppers or any other insect can directly sense the phosphate content of their diets. Grasshoppers might also sense dietary P via sensation of haemolymph phosphate levels, or via sensation of the effect of the diet on growth. *Schistocerca americana* is highly polyphagous in the field, and the results of the target experiment suggest that dietary P should affect feeding behaviour in the field. Clearly, new studies of the sensory and behavioural mechanisms associated with insect response to dietary P content are needed.

Assimilation and excretion of P.

Post-ingestive regulation of P also occurs. Fifth but not third instar grasshoppers modulated P excretion relative to P assimilation when consuming higher P diets (Fig. 3). This response suggests that, when *S. americana* are consuming diets with high P content, they down-regulate processes that promote P digestion and absorption and/or increase processes that promote P excretion. The rectum of *Schistocerca* can actively transport phosphate from lumen to haemolymph, suggesting a possible site of the regulation of phosphate absorption/excretion (Andrusiak et al., 1980). These general patterns of P excretion and assimilation have been previously shown for the caterpillar *Manduca sexta* (Woods et al., 2002) and in field studies of the locust *Oedaleus asiaticus* (Zhang et al., 2014).

The growth rate hypothesis predicts that animals with higher growth rates should have higher P requirements. Third instar grasshoppers consumed and assimilated more P per body mass when eating high P diets than final instar grasshoppers (Table 1). This finding is consistent with the growth rate hypothesis as smaller, faster-growing third instars may have higher mass-specific demands for P.

Relevance for field conditions.

Our results, and those of others, suggest that P-limitation might be an important factor affecting the behaviour and possibly abundances of grasshoppers and other polyphagous insects in the field. For polyphagous insects, the ability to select among plant species, individuals, and leaves could allow the herbivore to attain adequate nutrients despite low levels of the nutrient in the majority of the foliage available. Furthermore, the carbohydrate and protein needs (targets) of grasshoppers are approximately 40 times higher, by mass, than those for phosphate. Thus, grasshoppers might be able to attain their P target by including small amounts of a high-P plant in their diet while primarily foraging for carbohydrate or protein. Plausibly occasional foraging on plants high in phosphate (or other nutrients) may be a key factor driving polyphagous behaviour in insects such as grasshoppers.

The ability of grasshoppers to obtain adequate P by foraging occasionally on high-P plants, and the negative effects of high P diets, may explain why N but not P fertilization has been shown to affect grasshopper population densities in central North American grasslands, despite the observation that average foliar P concentrations available were in the range of those shown to be limiting to growth in this study (Loaiza et al., 2011). On the other hand, Joern et al. (2012) found that plant P content was the most important element predicting overall grasshopper abundances in the central Nebraskan grasslands. Moreover, fertilization of natural plants with P has directly stimulated growth and population density of a variety of insects, including Orthopterans on Mount St. Helens volcano (Bishop et al., 2010). For monophagous or limited-mobility insects, limitations by dietary P may be more likely and prevalent (Apple et al., 2009; Perkins et al., 2004). We suggest that limitation by dietary P may be an under-appreciated factor affecting insect herbivores, and we predict that P-limitation of insect herbivores in the field may be most likely to occur in fast-growing (high RNA content) monophagous or oligophagous species.

Acknowledgements

Thanks to M. Weed for help carrying out experiment 3; J.H. Fewell for use of lab space; A.L. Peace and E.P. Fenichel for helpful discussion on presenting the math equations; and S.J. Simpson for helpful discussion of this study.

Competing interests

The authors declare no competing financial interests.

Author contributions

AJC, JJE, MF, and JFH designed the study; AJC and MF carried out the experiments; AJC and JFH performed data analyses; and AJC, JJE, and JFH interpreted the findings and drafted the article.

Funding

This research was supported by the National Science Foundation [IBN-9977047 to JJE and JFH; DMS-0920744 to JJE; DEB-1313693 to AJC, JJE, and JFH; and CHE-1313958 to AJC].

References

Andrusiak, E., Phillips, J. and Speight, J. (1980). Phosphate transport by locust rectum in vitro. *Can. J. Zool.* **58**, 1518-1523.

Apple, J. L., Wink, M., Wills, S. E. and Bishop, J. G. (2009). Successional change in phosphorus stoichiometry explains the inverse relationship between herbivory and lupin density on Mount St. Helens. *PLoS ONE* **4**, e7807.

Behmer, S. (2009). Insect herbivore nutrient regulation. *Annu. Rev. Entomol.* **54**, 165-187.

Bhanja, S., Reddy, V., Panda, A., Rao, S. R. and Sharma, R. (2007). Requirement of non-phytate phosphorus in synthetic broiler breeder diet. *Asian-Australas. J. Anim. Sci.* **20**, 933-938.

Bishop, J. G., O'Hara, N. B., Titus, J. H., Apple, J. L., Gill, R. A. and Wynn, L. (2010). NP co-limitation of primary production and response of arthropods to N and P in early primary succession on Mount St. Helens volcano. *PLoS ONE* **5**, e13598.

Boersma, M. and Elser, J. (2006). Too much of a good thing: on stoichiometrically balanced diets and maximal growth. *Ecology* **87**, 1325-1330.

Boswell, A. M., Provin, T., and Behmer, S. T. (2008). The relationship between body mass and elemental composition in nymphs of the grasshopper Schistocerca americana. *Journal of Orthoptera Research* **17**, 307-313.

Cease, A. J., Elser, J. J., Ford, C. F., Hao, S., Kang, L. and Harrison, J. F. (2012). Heavy livestock grazing promotes locust outbreaks by lowering plant nitrogen content. *Science* **335**, 467-469.

Chaudhary, K. D. and Lemonde, A. (1962). Phosphorus in the nutrition of *Tribolium confusum* duval. *Can. J. Zool.* **40**, 375-380.

Chavez-Sanchez, C., Martinez-Palacios, C. A., Martinez-Perez, G. and Ross, L. G. (2000). Phosphorus and calcium requirements in the diet of the American cichlid *Cichlasoma urophthalmus* (Gunther). *Aquacult. Nutr.* **6**, 1-10.

Clancy, K. M. and King, R. M. (1993). Defining the western spruce budworm's nutritional niche with response surface methodology. *Ecology* **74**, 442-454.

Dadd, R. H. (1960). Observations on the palatability and utilisation of food by locusts, with particular reference to the interpretation of performances in growth trials using synthetic diets. *Entomol. Exp. Appl.* **3**, 283-304.

Dadd, R. H. (1961). The nutritional requirements of locusts 4. Requirements for vitamins of the b-complex. *J. Insect Physiol.* **6**, 1-12.

DeMott, W. R., Gulati, R. D. and Siewertsen, K. (1998). Effects of phosphorus-deficient diets on the carbon and phosphorus balance of *Daphnia magna*. *Limnol. Oceanogr.* **43**, 1147-1161.

Elser, J. J., Acharya, K., Kyle, M., Cotner, J., Makino, W., Markow, T., Watts, T., Hobbie, S., Fagan, W., Schade, J. et al. (2003). Growth rate-stoichiometry couplings in diverse biota. *Ecol. Lett.* **6**, 936-943.

Elser, J. J., Bracken, M. E. S., Cleland, E. E., Gruner, D. S., Harpole, W. S., Hillebrand, H., Ngai, J. T., Seabloom, E. W., Shurin, J. B. and Smith., J. E. (2007). Global analysis of nitrogen and phosphorus limitation of primary producers in freshwater, marine, and terrestrial ecosystems. *Ecol. Lett.* **10**, 1135-1142.

Elser, J. J., Fagan, W. F., Denno, R. F., Dobberfuhl, D. R., Folarin, A., Huberty, A., Interlandi, S., Kilham, S. S., McCauley, E., Schulz, K. L. et al. (2000a). Nutritional constraints in terrestrial and freshwater food webs. *Nature* **408**, 578-580.

Elser, J. J., Hayakawa, K. and Urabe, J. (2001). Nutrient limitation reduces food quality for zooplankton: *Daphnia* response to seston phosphorus enrichment. *Ecology* **82**, 898-903.

Elser, J. J., Sterner, R. W., Gorokhova, E., Fagan, W. F., Markow, T. A., Cotner, J. B., Harrison, J. F., Hobbie, S. E., Odell, G. M. and Weider, L. J. (2000b). Biological stoichiometry from genes to ecosystems. *Ecol. Lett.* **3**, 540-550.

Frost, P. and Elser, J. (2002). Growth responses of littoral mayflies to the phosphorus content of their food. *Ecol. Lett.* **5**, 232-240.

Harrison, J. F. and Kennedy, M. J. (1994). In-vivo studies of the acid-base physiology of grasshoppers: The effect of feeding state on acid-base and nitrogen-excretion. *Physiol. Zool.* **67**, 120-141.

Harrison, S. J., Raubenheimer, D., Simpson, S. J., Godin, J.-G. J. and Bertram, S. M. (2014). Towards a synthesis of frameworks in nutritional ecology: interacting effects of protein, carbohydrate and phosphorus on field cricket fitness. *Proc. R. Soc. B* **281**, 20140539.

Hessen, D. O., Elser, J. J., Sterner, R. W. and Urabe, J. (2013). Ecological stoichiometry: An elementary approach using basic principles. *Limnol. Oceanogr* **58**, 2219-2236.

Huberty, A. F. and Denno, R. F. (2006). Consequences of nitrogen and phosphorus limitation for the performance of two planthoppers with divergent life-history strategies. *Oecologia* **149**, 444-455.

Joern, A., Provin, T., Behmer, S.T. (2012). Not just the usual suspects: Insect herbivore populations and communities are associated with multiple plant nutrients. *Ecology* **93**, 1002-1015.

Kaspari, M., Clay, N. A., Donoso, D. A. and Yanoviak, S. P. (2014). Sodium fertilization increases termites and enhances decomposition in an Amazonian forest. *Ecology* **95**, 795–800.

Khalaji, S., H. Naderi, A., N. Mousavi, S., Zaghari, M. and Malakzadegan, A. (2013). Determination of Phosphorus Requirement for Post Molted Laying Hens using Nonlinear and Multivariate Nonlinear Mixed Effects Models. *J. Poult. Sci.* **51**, 22-28.

Lemoine, N., Giery, S. and Burkepile, D. (2014). Differing nutritional constraints of consumers across ecosystems. *Oecologia* **174**, 1367-1376.

Loaiza, V., Jonas, J. L. and Joern, A. (2008). Does dietary P affect feeding and performance in the mixed-feeding grasshopper, (Acrididae) *Melanoplus bivitattus*? *Environ. Entomol.* **37**, 333-339.

Loaiza, V., Jonas, J. L. and Joern, A. (2011). Grasshoppers (Orthoptera: Acrididae) select vegetation patches in local-scale responses to foliar nitrogen but not phosphorus in native grassland. *Insect Sci.* **18**, 533-540.

Ögren, G., Holtenius, K. and Jansson, A. (2013). Phosphorus balance and fecal losses in growing Standardbred horses in training fed forage-only diets. *J. Anim. Sci.* **91**, 2749-2755.

Perkins, M. C., Woods, H. A., Harrison, J. F. and Elser, J. J. (2004). Dietary phosphorus affects the growth of larval *Manduca sexta*. *Arch. Insect Biochem. Physiol.* **55**, 153-168.

Puggaard, L., Lund, P., Liesegang, A. and Sehested, J. (2014). Long term effect of reduced dietary phosphorus on feed intake and milk yield in dry and lactating dairy cows. *Livest. Sci.* **159**, 18-28.

Raubenheimer, D. and Simpson, S. (1997). Integrative models of nutrient balancing: application to insects and vertebrates. *Nutr. Res. Rev.* **10**, 151-179.

Roeder, K. A. and Behmer, S. T. (2014). Lifetime consequences of food protein-carbohydrate content for an insect herbivore. *Funct. Ecol.* **28**, 1135-1143.

Schade, J. D., Kyle, M., Hobbie, S. E., Fagan, W. F. and Elser, J. J. (2003). Stoichiometric tracking of soil nutrients by a desert insect herbivore. *Ecol. Lett.* **6**, 96-101.

Simpson, S. and Raubenheimer, D. (1993). A multi-level analysis of feeding behaviour: the geometry of nutritional decisions. *Philos. Trans. R. Soc. London Ser. B* **342**, 381-402.

Simpson, S. J. and Raubenheimer, D. (2012). The nature of nutrition: a unifying framework from animal adaptation to human obesity: Princeton University Press.

Smedley, S. R. and Eisner, T. (1996). Sodium: a male moth's gift to its offspring. *Proc. Natl. Acad. Sci. U.S.A.* **93**, 809-813.

Smith, D. (1960). Effects of changing the phosphorus content of the food plant on the migratory grasshopper, *Melanoplus bilituratus* (Walker)(Orthoptera: Acrididae). *Can. Entomol.* **92**, 103-107.

Sterner, R. W. and Elser, J. J. (2002). Ecological stoichiometry: the biology of elements from molecules to the biosphere: Princeton University Press.

Sterner, R. W. and Schwalbach, M. S. (2001). Diel integration of food quality by *Daphnia*: luxury consumption by a freshwater planktonic herbivore. *Limnol. Oceanogr.* **46**, 410-416.

Trumper, S. and Simpson, S. J. (1993). Regulation of salt intake by nymphs of *Locusta migratoria*. *J. Insect Physiol.* **39**, 857-864.

Visanuvimol, L. and Bertram, S. M. (2011). How dietary phosphorus availability during development influences condition and life history traits of the cricket, *Acheta domesticas*. *J. Insect Sci.* **11**, 1-17.

Watts, T., Woods, H. A., Hargand, S., Elser, J. J. and Markow, T. A. (2006). Biological stoichiometry of growth in *Drosophila melanogaster*. *J. Insect Physiol.* **52**, 187-193.

Woods, H. A., Perkins, M. C., Elser, J. J. and Harrison, J. F. (2002). Absorption and storage of phosphorus by larval *Manduca sexta*. *J. Insect Physiol.* **48**, 555-564.

Yu, H. R., Zhang, Q., Xiong, D. M., Huang, G. Q., Li, W. Z. and Liu, S. W. (2013). Dietary available phosphorus requirement of juvenile walking catfish, *Clarias leather*. *Aquacult. Nutr.* **19**, 483-490.

Zhang, S., Gillihan, R., He, N., Fields, T., Liu, S., Green, T. and Stubbs, J. R. (2013). Dietary phosphate restriction suppresses phosphaturia but does not prevent FGF23 elevation in a mouse model of chronic kidney disease. *Kidney Int.* **84**, 713-721.

Zhang, Z. J., Elser, J. J., Cease, A. J., Zhang, X. M., Yu, Q., Han, X. G. and Zhang, G. M. (2014). Grasshoppers regulate N:P stoichiometric homeostasis by changing phosphorus contents in their frass. *PLoS ONE* **9**, e103697.

Figures

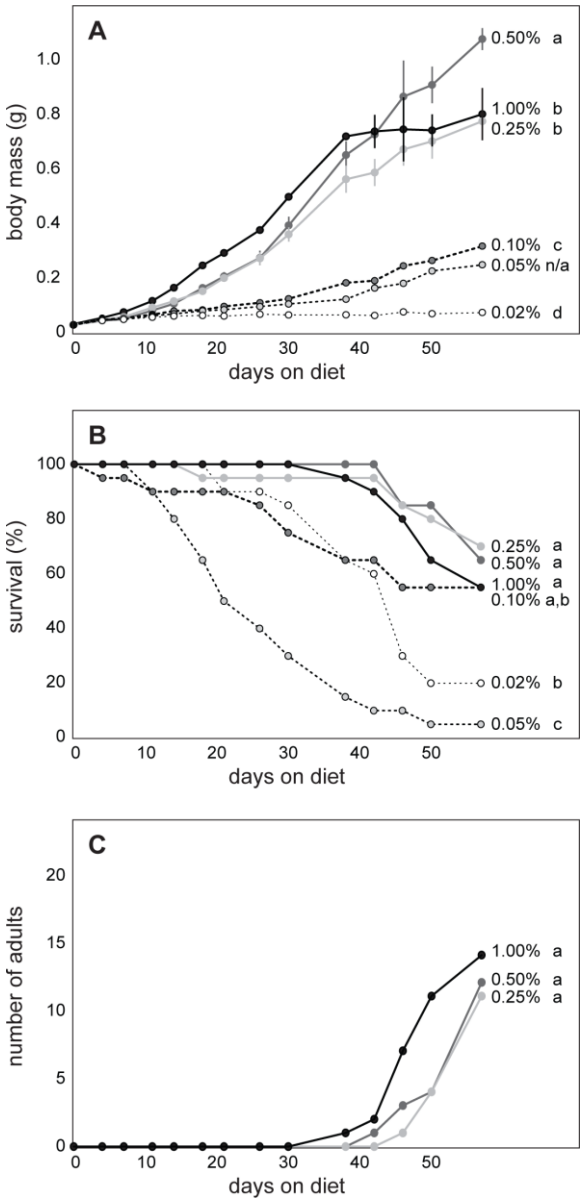

Figure 1. Experiment 1: Change in body mass (A), survival rate (B), and number developed into the adult stage (C) of grasshoppers reared for 60 days on different % P diets. n=20 individuals per treatment. Here and throughout unless otherwise indicated, values are mean ± s.e.m. and letters indicate statistically significant differences among groups using Tukey post-hoc analyses.

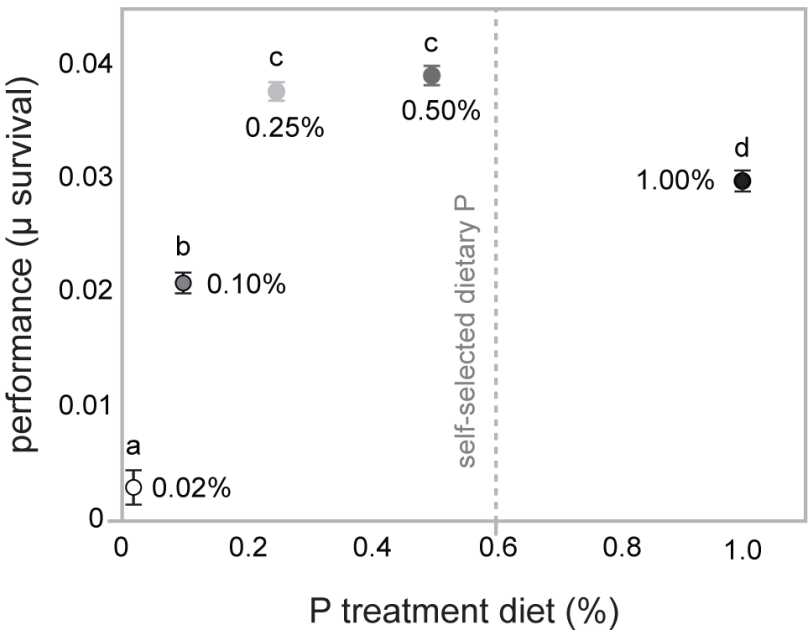

Figure 2. Experiment 1: Long-term grasshopper performance on different % P diets. Performance responses of grasshoppers reared for 60 days on different % P diets and their self-selected %P intake target. To calculate performance, we multiplied the specific growth rate (μ) of an individual by the survival rate of its treatment group. n=20 individuals per treatment.

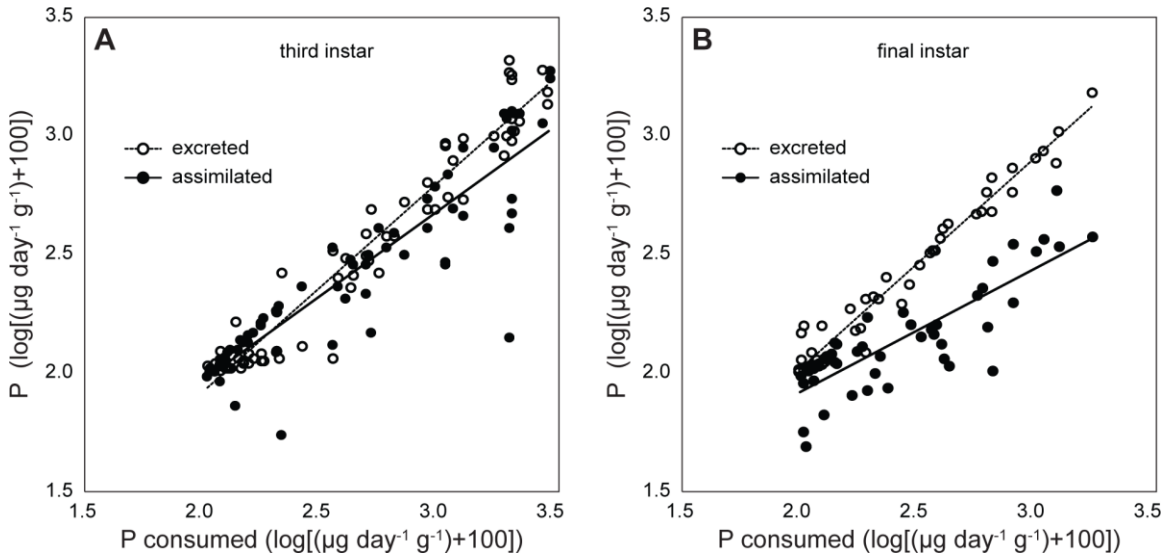

Figure 3. Experiment 2: P assimilation and excretion vs. P consumption for grasshoppers fed single diets for three days as either third instar (panel A) or final instar (panel B) nymphs. We transformed mass-specific data ($\mu g*day^{-1}*g^{-1}$) by adding 100 to remove negative values and then taking the log. The slopes of the assimilated and excreted lines were statistically identical. However, for final instars, the slope of the excretion line was significantly steeper ($t_{(128)} = 2.69$; $P = 0.008$), indicating that final instar nymphs excreted more and assimilated less P as P consumption increased. See methods for treatment groups and sample sizes.

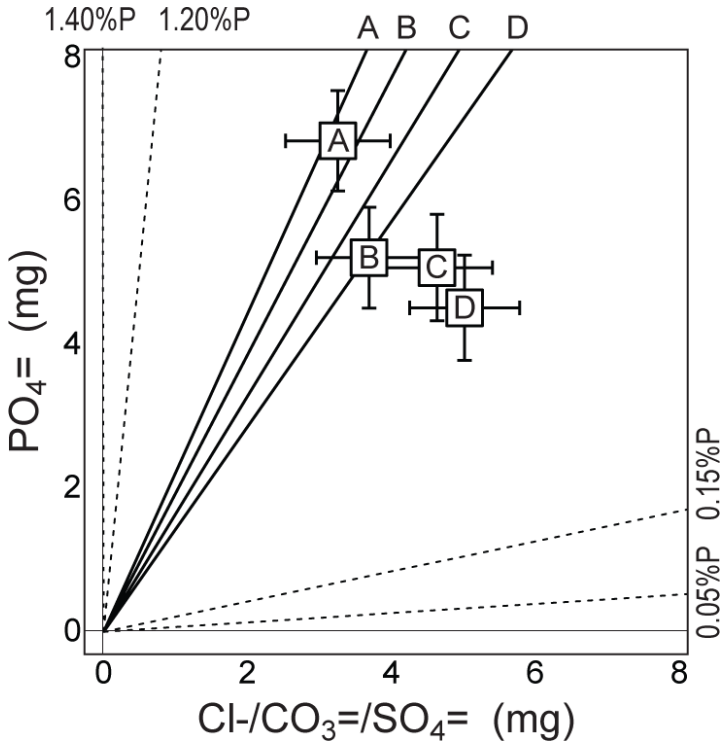

Figure 4. Experiment 3: Mean phosphorus vs. alternate anion intake by grasshoppers given one of four pairings of synthetic diets. Dashed lines show composition of the four diets and solid lines show expected intake if equal quantities of each diet in a given pair were eaten. Letters indicate the diet pairing (see Tables 2 and 3 for statistics). Note that phosphate concentrations are higher than phosphorus concentrations due to differences in molecular weight. For diet pairings A and B, n=11; for diet pairings C and D, n=10.

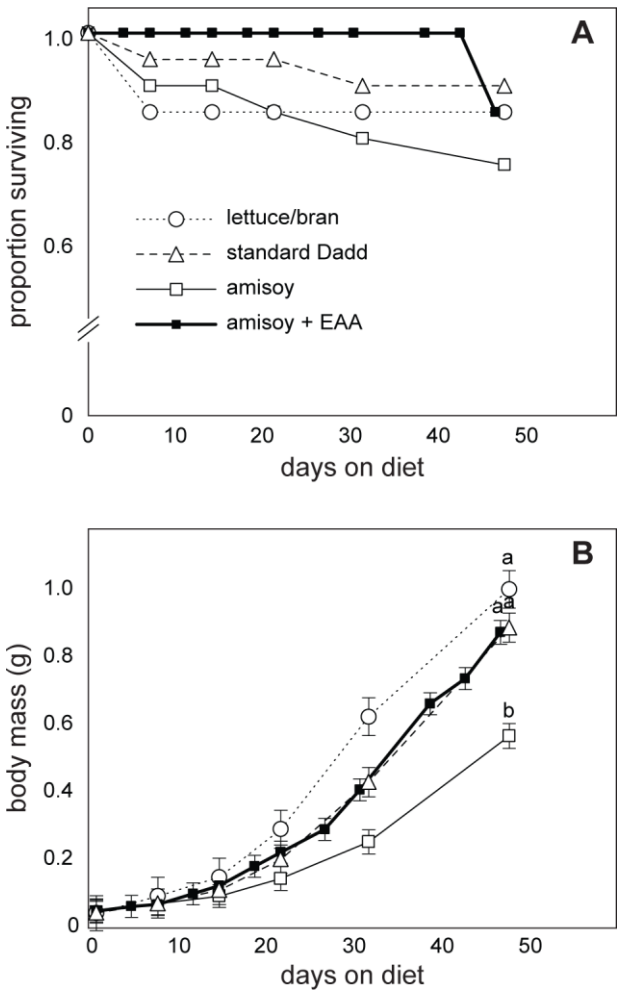

Figure 5. Development of P-flexible synthetic diet. Survival rate (A) and grasshopper body mass (B) over the duration of the feeding experiment. Panel A: There were no differences in survival rate among the treatment groups ($\chi^2_{(3)}$ Survival test = 1.03 P = 0.79). Panel B: There were significant differences in body mass (mean ± SE) on the final day of the experiment ($ANOVA_{(3,63)}$ = 7.01, P = 0.0004). Tukey post-hoc analyses revealed that grasshoppers fed the amisoy diet *without* essential amino acids added had lower body masses than the other three groups, as indicated by lower case letters in the figure. n=20 individuals per treatment.

Tables

Table 1. Experiment 2: three-day feeding experiment in the third and final instars. Summary of ANCOVA and ANOVAs testing the effects of dietary P content on specific growth rate (mass gain (initial body mass^{-1}), unit-less) and on body-mass corrected food consumption, P consumption, P assimilation, and P excretion. Significant effects are in bold.

Treatment	Specific growth rates (μ)	Food consumption (mg*day^{-1}*g)	P consumption (μg*day^{-1}*g)	P excreted (μg*day^{-1}*g)	P assimilated (μg*day^{-1}*g)
	ANOVA	ANCOVA	ANOVA	ANCOVA	ANCOVA
Instar	**$F_{(1, 106)}$ = 65.21, *P* < 0.001**	**$F_{(1, 106)}$ = 59.38, *P* < 0.001**	**$F_{(1, 106)}$ = 21.42, *P* < 0.001**	**$F_{(1, 106)}$ = 7.28, *P* = 0.008**	**$F_{(1, 106)}$ = 25.01, *P* < 0.001**
P diet	$F_{(6, 106)}$ = 1.30, *P* = 0.26	$F_{(6, 106)}$ = 1.59, *P* = 0.16	**$F_{(6, 106)}$ = 24.92, *P* < 0.001**	**$F_{(6, 106)}$ = 21.22, *P* < 0.001**	**$F_{(6, 106)}$ = 11.42, *P* < 0.001**
Instar x P diet	$F_{(6, 106)}$ = 1.01, *P* = 0.42	$F_{(6, 106)}$ = 0.54, *P* = 0.78	**$F_{(6, 106)}$ = 4.01, *P* = 0.001**	$F_{(6, 106)}$ = 1.74, *P* = 0.12	**$F_{(6, 106)}$ = 4.45, *P* = 0.0004**

Table 2. Experiment 3: phosphorus and total food consumption by grasshoppers in target experiment. Means ± SE from ANCOVA's for total food consumed ($F_{(3, 37)} = 0.34$, $P = 0.79$) and P consumed ($F_{(3, 37)} = 1.9$, $P = 0.14$).

food choice (% P)	total food (mg)	phosphorus (mg)
(A) 1.4 vs 0.15	295.5 ± 30.0	2.24 ± 0.23
(B) 1.4 vs 0.05	276.2 ± 30.0	1.70 ± 0.23
(C) 1.2 vs 0.15	311.9 ± 31.7	1.67 ± 0.24
(D) 1.2 vs 0.05	315.5 ± 31.3	1.49 ± 0.24

Table 3. Experiment 3: amount of each diet consumed by grasshoppers when offered a pair of diets. Means ± SE and *P*-values from Mann-Whitney U tests for divergence from null hypothesis that equal dry mass of both diets was consumed.

food choice (%)	higher P diet (mg)	lower P diet (mg)	Mann-Whitney U
(A) 1.4 vs 0.15	143.6 ± 15.8	151.8 ± 28.0	$P = 0.30$
(B) 1.4 vs 0.05	120.5 ± 15.1	151.4 ± 26.8	$P = 0.04$
(C) 1.2 vs 0.15	90.2 ± 17.5	219.6 ± 18.8	$P = 0.04$
(D) 1.2 vs 0.05	115.6 ± 16.6	199.8 ± 17.0	$P = 0.03$

Table 4. Composition of base diet for P-manipulation. We replaced casein and peptone with amisoy and additional essential amino acids. We altered the P content by changing the ratio of various salts.

	Ingredient	grams
indigestible carbohydrates	Cellulose	39.94437
soluble carbohydrates	Dextrin	13.31479
	Sucrose	13.31479
proteins	Amisoy*	13.31479
	Egg albumen	13.31479
	Casein†, peptone†	--
amino acids	Threonine*	0.50596
	Leucine*	0.26630
	Tryptophan*	0.21304
	Glycine*	0.13315
	Proline*	0.10652
fatty acids and sterols	Wheat germ oil	2.66296
	Cholesterol	0.53259
salts	Choline chloride	0.17370
	Magnesium sulfate	1.43649
	Iron citrate	0.23941
	Sodium, Calcium, and Potassium salts ‡	--
trace salts	Potassium iodide	0.01490
	Sodium fluoride	0.01241
	Anhydrous manganese sulfate	0.00248
	Cupric sulfate	0.00124
	Anhydrous potassium aluminum	0.00124
	Zinc sulfate	0.00124
vitamins	Beta-carotene	0.08685
	Ascorbic acid	0.34741
	Thiamine	0.00278
	Riboflavin	0.00278
	Nicotinic acid	0.01114
	Pyridoxine	0.00278
	Folic acid	0.00278
	Meso-inositol	0.02788
	Ca. pantothenate	0.00557
	p-aminobenzoic acid	0.00278
	Biotin	0.00011

TOTAL	**100**

* ingredients that differ from standard Dadd diet; † ingredients in the standard Dadd diet, but not included here; ‡ see Tables 2 and 3 and methods text for composition of these salts.

Table 5. Salt mixtures for dietary phosphorus manipulation.

	Ingredient	% of salt mix
salt mix 1: $PO_4^=$ mix	sodium phosphate (monobasic)	50.00
	calcium phosphate (dibasic)	25.00
	potassium phosphate (dibasic)	25.00
salt mix 2: Cl^- /$CO_3^=$/$SO_4^=$ mix	sodium chloride	12.28
	sodium carbonate	12.28
	sodium sulfate	12.28
	calcium chloride	10.29
	calcium carbonate	10.29
	calcium sulfate	10.29
	potassium chloride	10.76
	potassium carbonate	10.76
	potassium sulfate	10.76

Table 6. Percent of elemental Ca, K, Na, and P in each salt mix.

	Ca	K	Na	P
salt mix 1: $PO_4^{=}$	7.37	11.22	9.58	23.05
salt mix 2: $Cl^{-}/CO^{=}_3/SO_4^{=}$	10.87	16.56	14.14	0.00

Table 7. Definitions of terms used in the diet calculations.

variable	unit	description
P_{max}	proportion	upper limit of dietary P for a given group of diets
P_{base}	—	P proportional content in base diet
P_{test}	—	P proportional content for a given test diet
P_c	—	P proportional content in cellulose (equal to 0)
P_{S1}	—	P proportional content in salt mix 1
P_{S2}	—	P proportional content in salt mix 2 (equal to 0)
K_c	proportion	K proportional content in cellulose (equal to 0)
K_{S1}	—	K proportional content in salt mix 1
K_{S2}	—	K proportional content in salt mix 2
M_C	grams	mass of cellulose added to the base diet (varies across diets)
M_{S1}	—	mass of salt mix 1 added to the base diet (varies across diets)
M_{S2}	—	mass of salt mix 2 added to the base diet (varies across diets)
M_{base}	grams	mass of base mix (constant across diets; see Table 5)
M_{mix}	—	mass of all salt mixes and cellulose added to the base diet (constant across diets)
M_P	—	mass of P added to the base diet (varies across diets)
M_K	—	mass of K added to the base diet (constant across diets)