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RESEARCH ARTICLE

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

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ABSTRACT

Comparisons of the carbon, nitrogen and phosphorus (P) content of plants and insect herbivores suggests that P limitation and herbivore foraging to balance P intake could be common. However, the 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 content 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.

KEY WORDS: Ecological stoichiometry, Geometric framework, Grasshopper, Phosphate, 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 rate and abundance 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 content (%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). 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 abundance (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% (Elser et al., 2000a). Joern et al. (2012) compared elemental compositions of grasses and forbs collected from central Nebraskan grasslands and found a P range from about 1500 to 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 threefold 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.

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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 a higher phosphate content (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 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 a high body P content 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 non-linear. 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 reflect 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 dietary N:P ratios ranged from 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 content 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 was conducted within the third 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: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 these pairs in such a way that it obtains the same amount of the nutrient, the animal can be considered to be behaviorally 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 behavioral tests allow the animal itself to '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 20 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 'Development of the P-flexible synthetic diet', below). P 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 1 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 96 l aluminium cages (N=20 individuals per treatment). Air temperature was 31±1°C. Light was provided by individual fluorescent tubes beside each cage and set to 14 h:10 h light:dark schedule. Water was supplied by gravity feed into a dish with a 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 3 days (once daily for lettuce-fed animals). Phosphate levels were increased by adding greater amounts of an equimolar mix of calcium phosphate and potassium phosphate and included 0.02%P, 0.05%P, 0.10%P, 0.25%P, 0.50%P 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 salts 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 grasshopper body mass at the start and end of the experiment, respectively, and dt is the time between weighing in days. Because grasshoppers were not tracked individually, we estimated initial body mass by taking the average mass of all grasshoppers at the beginning of the experiment.

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Table 1. Composition of base diet for phosphorus (P) manipulation

	Ingredient	Mass (g)
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 aluminium	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
	Calcium pantothenate	0.00557
	p-Aminobenzoic acid	0.00278
	Biotin	0.00011
Total		100

We replaced casein and peptone with amisoy and additional essential amino acids. We altered the P content by changing the ratio of various salts.
*Ingredients that differ from standard Dadd diet.
‡Ingredients in the standard Dadd diet, but not included here.
§See Table 2–4 and Materials and methods for composition of these salts.

Table 2. Salt mixtures for dietary P manipulation

	Ingredient	% of salt mix
Salt mix 1: PO_4^{2-} mix	Sodium phosphate (monobasic)	50.00
	Calcium phosphate (dibasic)	25.00
	Potassium phosphate (dibasic)	25.00
Salt mix 2: $Cl^-/CO_3^{2-}/SO_4^{2-}$ 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

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 1 week after hatching on the standard lettuce/bran diet. Then, animals were switched to the 0.50%P artificial diet until the test instar (third or final juvenile instar). On day 1 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%P to 1.50%P. In these experiments, P was increased by addition of sodium phosphate salts 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 chloride, carbonate and sulfate and potassium chloride, carbonate and sulfate. For the 1.00%P diet, we also tested a diet in which %P was increased using calcium phosphate and potassium phosphate (as in experiment 1), to test whether these specific cations affected the results. After 3 days (instar duration was about 5 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 weighing. P ingestion rates were calculated by multiplying food consumption by the P content of the diet. Third instar treatment groups started with 10 animals; final instar groups started with 8. Animals that died or molted during the experiment were excluded. Eight grasshoppers molted and 14 died out of 144. Mortality and molting were distributed across treatment groups and no treatment group had more than two mortalities or molts.

All fecal pellets were collected, dried and weighed, after which the P contained in the pooled sample of all fecal 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^{-1}) by multiplying the total fecal mass by the average fecal P proportion and dividing by the duration of the experiment. Phosphate assimilation rates (μg day^{-1}) were calculated by subtracting the rate of P excretion from the rate of P ingestion.

Experiment 3: testing for a P intake target

We collected grasshoppers from the colony population within 6 h of molt to the final juvenile instar. All individuals collected had molted after 24:00 h on a given day and food and water were withheld for that evening. Trials were started the following morning at 08:00 h. Individuals were given a choice of two artificial diets for a total of 6 days, with food dishes being replaced on day 2. 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 phosphate, potassium phosphate and calcium phosphate salts, with cations kept constant across diets by reciprocal addition of sodium salts, potassium salts and calcium salts of chloride, carbonate and sulfate. We made the following diets, expressed as %P dry mass: 1.40%P, 1.20%P, 0.15%P 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: (A) 1.40+0.15, (B) 1.40+0.05, (C) 1.20+0.15 and (D) 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 of N=42. The four treatment groups were blocked within the environmental chamber to avoid any bias caused by light, temperature or disturbance.

Development of the P-flexible synthetic diet

To determine whether our P-flexible diet would yield similar growth and performance to 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 1). 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 2. 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 content 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

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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 3 for definitions of terms used in the diet calculations). At P_{max}:

$$M_{S1} = (M_{base} \times (P_{max} - P_{base}))/(P_{S1} - P_{max}). \quad (1)$$

We then calculated the value for M_K (the mass of K added to the base diet), which we kept constant across diets. At P_{max} (where only salt mix 1 is added to the base diet):

$$M_K = (K_{S1} \times M_{S1}). \quad (2)$$

Keeping M_K 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 4). 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}:

$$M_{S1} = M_{mix}. \quad (3)$$

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, **AB**=**C**:

$$\mathbf{A} = \begin{vmatrix} 1 & 1 & 1 \\ P_C & P_{S1} & P_{S2} \\ K_C & K_{S1} & K_{S2} \end{vmatrix},$$

$$\mathbf{B} = \begin{vmatrix} M_C \\ M_{S1} \\ M_{S2} \end{vmatrix},$$

$$\mathbf{C} = \begin{vmatrix} M_{mix} \\ M_P \\ M_K \end{vmatrix}.$$

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** show 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** shows the grams of cellulose (M_C), salt mix 1 (M_{S1}) and salt mix 2 (M_{S2}). **C** shows 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.

Table 3. 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	g	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}	g	Mass of base mix (constant across diets; see Table 1)
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)

Table 4. %Elemental Ca, K, Na and P in each salt mix

	Ca	K	Na	P
Salt mix 1: PO_4^{2-}	7.37	11.22	9.58	23.05
Salt mix 2: $Cl^-/CO_3^{2-}/SO_4^{2-}$	10.87	16.56	14.14	0.00

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:

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

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 content 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 non-linearly affected grasshopper body mass, survival and performance, measured on the final collection point on day 57 (Figs 1, 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%P to 0.50%P, and then decreased for the 1.00%P diet (ANOVA, $F_{4,48}$=30.29, P<0.001; Fig. 1A). There was a significant difference in survival rate among the treatment groups (χ^2_5survival test=47.64, P<0.001, followed by pairwise Cox F-tests corrected for multiple comparisons; Fig. 1B). Again, the effect was non-linear, 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 molted to adults during the experiment (0.02%P, 0.05%P and 0.10%P; Fig. 1C). There were no differences in the rates at which grasshoppers molted to adults among the remaining three treatment groups (0.25%P, 0.50%P, 1.00%P) (χ^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%P to 0.25%P, where it plateaued through 0.50%P and then decreased at 1.00%P (ANOVA, $F_{4,48}$=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 analyzed separately for the effect of dietary P on food consumption, P consumption, P assimilation and P excretion, the statistical significance of the results was the same as when instars were pooled. Therefore, we concentrated our interpretations on pooled results for the two instars, using ANOVAs on mass-specific data (Table 5). Grasshoppers maintained similar total food consumption rates on all dietary P treatments, with third instars eating more on a mass-

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specific basis than final instar juvenile nymphs (Table 5). 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 5).

We analyzed mass-specific P excreted and assimilated using ANOVAs (Table 5). 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 5). 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 content by plotting P excreted and P assimilated versus P consumed (Fig. 3). We transformed mass-specific data (μg 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 as a consequence of eating diets with higher P concentrations.

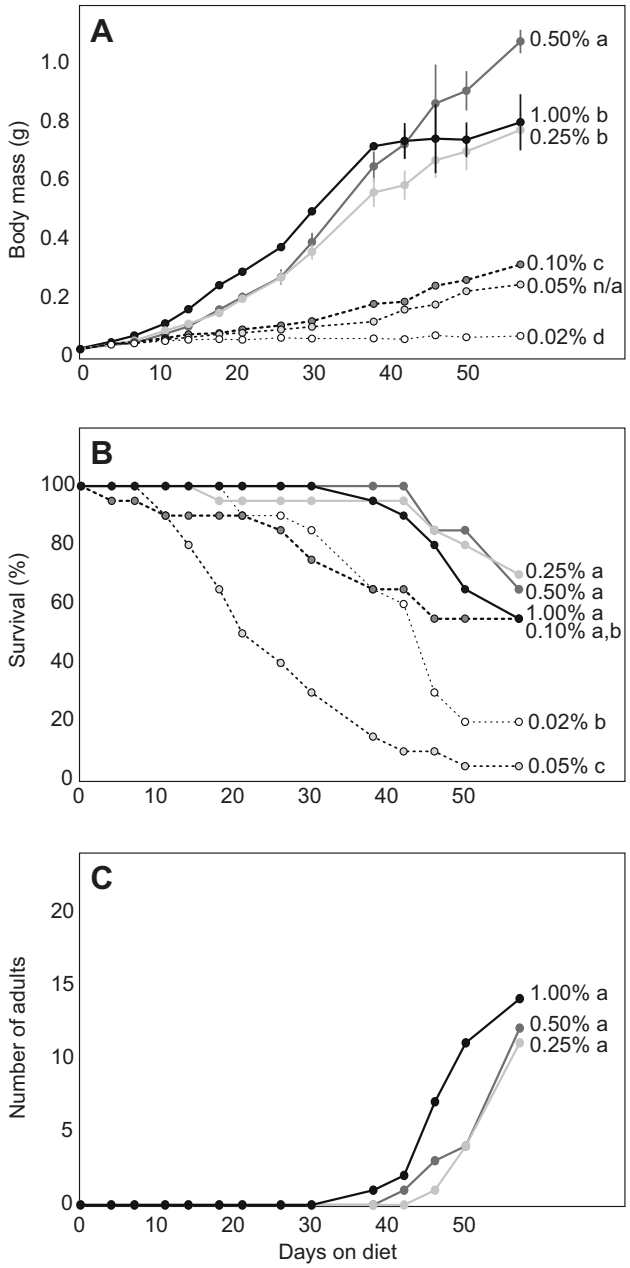

Fig. 1. Experiment 1: effect of phosphorus content (%P) of diets on grasshopper growth, development and survival. (A) Change in body mass, (B) survival rate and (C) number developed into the adult stage for grasshoppers reared for 60 days on the different %P diets. N=20 individuals per treatment. Here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

Experiment 3: testing for a P intake target

In the intake target experiments, all treatment groups consumed similar amounts of P and total food, despite their differing diet combinations (Table 6). 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 7). We also compared the amount of phosphate (PO_4^{2-}) versus the amount of replacement anions (Cl^-, CO_3^{2-} and SO_4^{2-}) 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 Materials and methods, 'Development of the P-flexible synthetic diet', for details). There was no effect of diet pairing on the amount of PO_4^{2-} or Cl^-, CO_3^{2-} and SO_4^{2-} consumed (MANCOVA, $F_{6,72}$=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 s.e.) by balancing feeding among the low and high P diets.

Development of the P-flexible synthetic diet

There were no significant differences in survival rates among the diet treatment groups (Fig. 5A). However, grasshoppers fed the amisoy diet without the essential amino acids added attained a lower

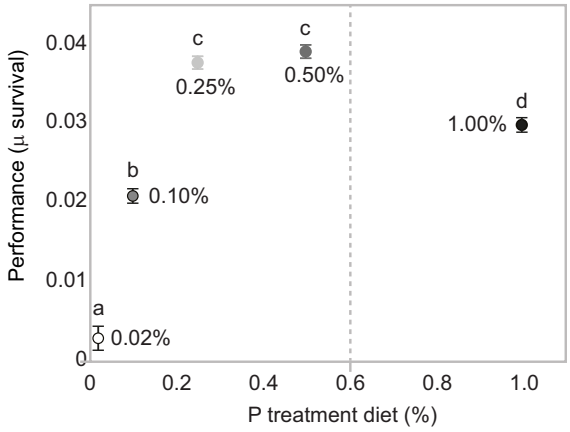

Fig. 2. Experiment 1: long-term grasshopper performance on different %P diets. Performance responses of grasshoppers reared for 60 days on different %P diets. The dashed line indicates their self-selected %P intake target. To calculate performance, we multiplied the specific growth rate (μ) of an individual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

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Table 5. Experiment 2: 3 day feeding experiment in the third and final instars

Treatment	Specific growth rate (μ)	Food consumption (mg day^{-1} g^{-1})	P consumption (μg day^{-1} g^{-1})	P excreted (μg day^{-1} g^{-1})	P assimilated (μg day^{-1} g^{-1})
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×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**

Summary of 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.

body mass than those on the other three diet treatments (Fig. 5B). Adding essential amino acids to the amisoy diet increased body mass 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%P 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 1 week. Thus, this synthetic diet allows testing of the behavioral and growth responses of grasshoppers from 0.02%P to 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 phosphate and potassium phosphate and the other with calcium phosphate and potassium phosphate. We found no differences between the two diets for growth rate or for the amount 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 non-linearly 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 they are 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, 4). However, diets with a P content 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' behavior 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 levels 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 behavior and performance of this grasshopper.

Effects of dietary P on growth, development and survival

When *S. americana* were confined to single artificial diets over their lifetime, their growth 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. P is needed to produce RNA, DNA, membranes and proteins; our data suggest that long-term consumption of diets with less than 0.1%P does 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.

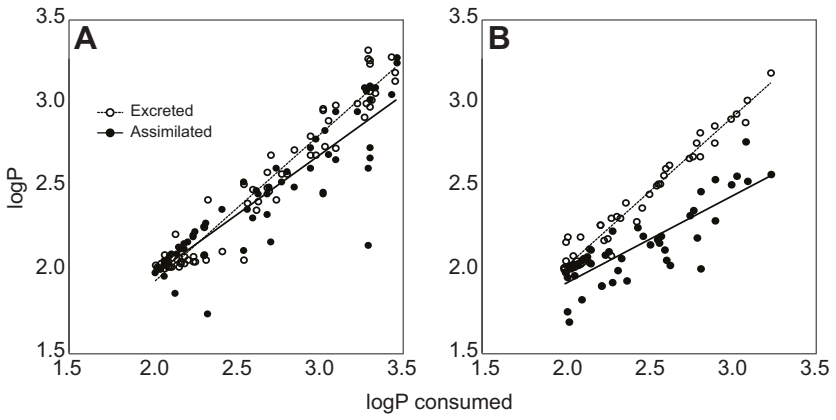

Fig. 3. Experiment 2: P assimilation and excretion versus P consumption for grasshoppers fed single diets for 3 days as either third instar or final instar nymphs. (A) Third instar nymphs; (B) final instar nymphs. We transformed mass-specific data (μg day^{-1} g^{-1}) by adding 100 to remove negative values and then taking the log. The slopes of the assimilated and excreted lines for third instars 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 Materials and methods for treatment groups and sample sizes.

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Table 6. Experiment 3: P and total food consumption by grasshoppers in the target experiment

Food choice (%P)	Total food (mg)	P (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

Means±s.e. from ANCOVA for total food consumed ($F_{3,37}$=0.34, P=0.79) and P consumed ($F_{3,37}$=1.9, P=0.14).

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 *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 fifth). 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 3 days within a single instar after feeding for multiple instars on diets with 0.50%P. Possibly, body P stores compensated for short-term

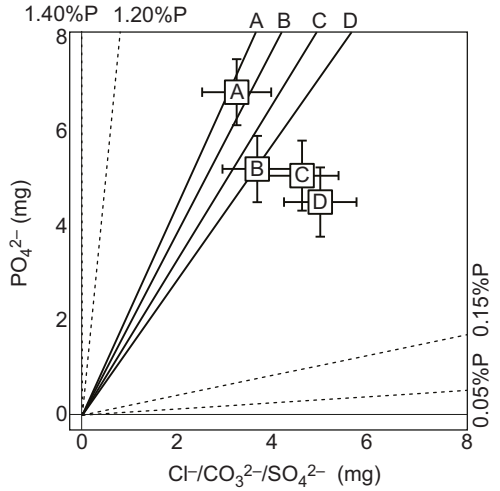

Fig. 4. Experiment 3: mean P versus alternative anion intake by grasshoppers given one of four pairings of synthetic diets. Dashed lines show the composition of the four diets and solid lines show expected intake if equal quantities of each diet in a given pair were eaten. Letters indicate the diet pairing (see Table 6 and 7 for statistics). Note that phosphate concentrations are higher than P concentrations because of differences in molecular weight. For diet pairings A and B, N=11; for diet pairings C and D, N=10.

Table 7. Experiment 3: amount of each diet consumed by grasshoppers when offered a pair of diets

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

Means±s.e. and P-values from Mann-Whitney U-tests for divergence from null hypothesis that an equal dry mass of the two diets was consumed.

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 content 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.

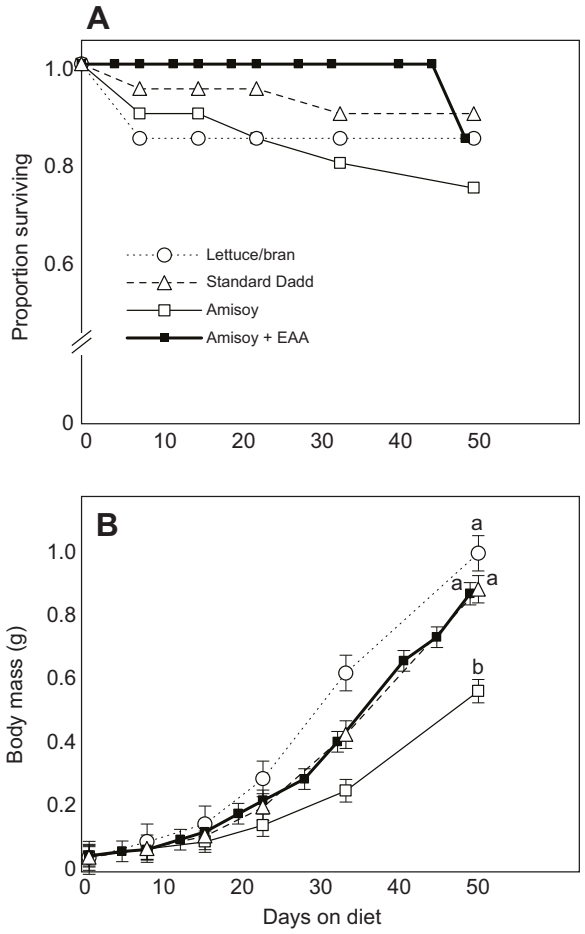

Fig. 5. Development of the P-flexible synthetic diet. Survival rate (A) and grasshopper body mass (B) over the duration of the feeding experiment. There were no differences in survival rate among the treatment groups (χ^2_3 survival test=1.03 P=0.79). There were significant differences in body mass (mean± s.e.) on the final day of the experiment (ANOVA, $F_{3,63}$=7.01, P=0.0004). Tukey *post hoc* analyses revealed that grasshoppers fed the amisoy diet without essential amino acids (EAAs) added had a lower body mass than those in the other three groups, as indicated by lower case letters in the figure. N=20 individuals per treatment.

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Effects of dietary P on food consumption

Many animals forced to consume a diet deficient in only 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 content (Visanuvimol and Bertram, 2011). There was no evidence for such compensatory feeding for P in this study when measured over 3 days (Table 5). 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, *G. 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, 4). However, as one of the diet pairings did not result in consumption significantly different from random, the target behavior 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 a 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%P, 0.66%P and 1.1%P of dry diet and that crickets prioritized balancing protein and carbohydrate over P intake in the range 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 hemolymph 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 behavior in the field. Clearly, new studies of the sensory and behavioral mechanisms associated with the 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 hemolymph, suggesting a possible site for 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 *M. 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 5). 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 behavior and possibly abundance 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 behavior 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). However, Joern et al. (2012) found that plant P content was the most important element predicting overall grasshopper abundance in the central Nebraskan grasslands. Moreover, fertilization of natural plants with P directly stimulates 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.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

A.J.C., J.J.E., M.F. and J.F.H. designed the study; A.J.C. and M.F. carried out the experiments; A.J.C. and J.F.H. performed data analyses; and A.J.C., J.J.E. and J.F.H. interpreted the findings and drafted the article.

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