A tale of two elements: effects of foliar nitrogen and phosphorus stoichiometry on plant-insect interactions

By

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To Shan, who lighted up the entire journey

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Abstract

A tale of two elements: effects of foliar nitrogen and phosphorus stoichiometry on plant-insect interactions

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The growth, survival, defense and reproduction of herbivores are influenced by plant nutrient concentrations. I integrated nutritional ecology with principles of ecological stoichiomtery and chemical ecology to explore the effects of plant quality on insect herbivores. Although nitrogen (N) is considered to be the major limiting nutrient for terrestrial insect herbivores, evidence suggests that the stoichiometric balance between N and phosphorus (P) may be equally important. In Chapter 2, I explored potential P limitation in the monarch caterpillar *Danaus plexippus* and an aphid *Aphis asclepiadis*, which are specialist insects feeding on milkweeds (*Asclepias*). I found that although the host plant, *A. syriaca* was co-limited by soil N and P, neither of the two insect species experienced induced P limitation. The body tissues of *A. asclepiadis* always exhibited higher N: P ratios than those of the plants, suggesting that the N demand of the aphid always exceeds P demand, even under high N levels. Additionally, P fertilization increased the production of latex in milkweed, which is a defense trait that negatively affected *D. plexippus* growth rate. In Chapter 3, I found that although foliar N concentration in *A. syriaca* was positively correlated with the growth rate of *D. plexippus*, this

relationship disappeared when caterpillars fed on *A. incarnata*, and became negative when fed on *A. curassavica.* The mechanism for the negative relationship was because per unit toxicity of cardenolide was higher at high foliar N levels. Because monarchs sequester cardenolides from milkweeds as defense, the increased toxicity under high N could influence sequestration efficiency. Indeed, in chapter 4, I found that the efficiency with which *D. plexippus* sequesters cardenolides from milkweed was reduced by soil fertilization. Finally, I explored feedback between herbivore activity and nutrient allocation in plants. I showed that herbivores can exert top-down effects on plant nutrient concentration by changing plant resource allocation patterns. Specifically, when under simultaneous attack by above- and belowground herbivores, *A. syriaca* was able to allocate newly absorbed N to stems and render it unavailable to future attack by both herbivores. Such nutrient reallocation may represent an important mechanism by which plants tolerate herbivore attack.

Chapter 1

Introduction

Every organism requires a set of essential elements for growth and reproduction. About 16 and 20 elements have been identified as essential for plants and animals respectively (Fitter and Hay 2002), and deficiency in any one of these elements can lead to abnormal performance compared with organisms not so deprived (Epstein and Bloom 2004). According to Liebig's law of the minimum, it is not the total amount of resources, but the scarcest resource, that limits an organism's growth rate. The fact that organisms often face nutrient deficiencies reveals that most organisms face chemical imbalances between their food supplies and their body compositions. Plants only require about 16 essential elements while there are about 80 elements in the soil; moreover, proportions of the elements in plants differ greatly from their availability in soil. For example, silicon is the second most abundant element in soil with silicon dioxide comprising 50-70% of soil mass; however, in plants silicon concentration can be as low as 0.1% (Ma and Yamaji 2006). In contrast, nitrogen comprises less than 0.5% of soil biomass, but it can exceed 3% of the dry mass of plants. For herbivores, nutrient imbalance is also severe. C: N and C: P ratios in herbivores are usually 10 times lower than those in plants (Elser et al. 2000, Cross et al. 2003), making plants very low-quality food. Recognition of this universal elemental difference has lead

to the development of stoichiometric theory, which links nutrient ratios to individual physiology, population dynamics, community structure and ecosystem processes (Sterner and Elser 2002).

Of all essential elements, nitrogen (N) and phosphorus (P) have received the most attention from ecologists. N comprises about 17% of proteins and 14.5% of nucleic acids, and P comprises 8.7% of nucleic acids; both proteins and nucleic acids are vital macromolecules for life (Sterner and Elser 2002). Traditionally, terrestrial ecosystems are considered to be N-limited while aquatic ecosystems are considered to be more P-limited, but this reflects a rather static and non-interactive view of nutrient dynamics. In the context of global environmental change, the importance of N and P balance, and interactions between these nutrients, have become increasingly apparent. Human activity has changed the global N cycle by agricultural fertilization and fossil fuel combustion, resulting in significant atmospheric N deposition to ecosystems worldwide (Vitousek et al. 1997). In North American forests, which are historically considered as N limited, N deposition can lead to nitrogen saturation (Aber et al. 1989, Lovett et al. 2000). N deposition can also induce P deficiency in temperate forests, grasslands and lakes (Mohren et al. 1986, Akselsson et al. 2008, Craine et al. 2008, Elser et al. 2009). In other words, the ecosystem consequences of N saturation can be mediated by the availability of P in ecosystems.

N: P balance has received less attention in herbivorous insects than in plants. Compared to autotrophs, heterotrophs have higher levels of stoichiometric homeostasis, showing a strict coupling of N and P (Sterner and Elser 2002). In order to maintain specific nutrient ratios, animals must selectively excrete excess elements, which itself is an energy consuming process.

This might explain why high levels of plant fertilization can sometimes lead to decreases in the performance of insects that feed on those plants (Throop and Lerdau 2004, Zehnder and Hunter 2009). Moreover, the imbalance between food and body composition can affect herbivore foraging behavior (Huberty and Denno 2006), population dynamics (Urabe et al. 2002) and energy flow efficiency (Cebrian 1999). While there is evidence to show that N and P can limit insect growth, development and reproduction (Throop and Lerdau 2004, Bertram et al. 2006), few studies have looked at how N and P simultaneously and interactively affect herbivore performances (but see Huberty and Denno 2006, Zehnder and Hunter 2009). According to Elser et al (2000), N: P ratios in terrestrial plants are almost the same as N: P ratios in insects, suggesting that P limitation should be at least as severe as N limitation for herbivores. Additionally, if atmospheric N deposition can cause increases in N concentration in plant tissues (Aber et al 1989), it should further add to relative P deficiency in insect herbivores. However, it so far remains unclear whether N deposition can lead to N saturation and P deficiency in insects.

Initially motivated by changing nutrient ratios under environmental change, I performed experiments (described in Chapter 2) to explore potential P limitation in insect herbivores induced by N deposition. Based on these results, I became interested more generally in the individual and interactive effects of plant nutrients and plant chemical defense on herbivore performance. Chapters 3 and 4 describe these interactions in detail. Finally, herbivore activity can feed back to influence the allocation of nutrients by plants. In Chapter 5, I explore changes in plant N and C allocation above and below ground that are caused by herbivore feeding.

For my study system, I chose milkweed plants (*Asclepias*) and three of their specialist herbivores. Milkweed has become a model system for studies of evolutionary biology and plant-insect interactions (Agrawal and Fishbein 2006, Agrawal and Fishbein 2008) mainly because the toxic cardenolides that characterize milkweed defense can disrupt Na^+/K^+-ATPase in animals and induce serious fitness costs (Agrawal et al. 2012). Moreover, milkweeds have physical defenses including latex and trichomes, which can also exert strong negative effects on herbivores (Levin 1973, Zalucki and Malcolm 1999, Zalucki et al. 2001). Specifically, I used three species of milkweed, *A. syriaca*, *A. curassavica* and *A. incarnata*, which all occur naturally in the U.S. The three species differ significantly in their cardenolide contents, with the highest cardenolide concentrations found in *A. curassavica* and lowest in *A. incarnata* (Sternberg et al. 2012). In addition, the foliar N and P concentrations of *A. syriaca* are much lower than those of *A. curassavica* and *A. incarnata* leaves, creating natural gradients in N and P concentration across species.

There are 13 naturally occurring insect herbivores feeding on milkweeds in the eastern US, and I selected the monarch caterpillar *Danaus plexippus*, the aphid *Aphis asclepiadis* and the larvae of the red longhorn milkweed beetle *Tetraopes tetraophthalmus* for my experiments. *D. plexippus* is a defoliator, *A. asclepiadis* is a phloem feeder and the larvae of *T. tetraophthalmus* are root feeders of milkweeds. I used the first two species in Chapter 2 to contrast their potential to experience induced P limitation under N deposition (see below). I used only used the monarch caterpillar *D. plexippus* in Chapter 3 and 4, because it is more convenient and more accurate to measure consumption, sequestration and individual growth rates of caterpillars than of other

kinds of herbivores. In chapter 5, I used both *D. plexippus* and the larvae of *T. tetraophthalmus,* because my hypotheses involved simultaneous attack by above and belowground herbivores.

Overall, I used the milkweed system to explore the relationships among nutrient availability, milkweed defenses, and the performance of milkweed herbivores. First, because of anthropogenic N deposition, I hypothesized in Chapter 2 that herbivorous insects would experience induced P limitation, but that different herbivore species would respond differentially to nutrient enrichment based on their specific requirements. Specifically, I predicted that insect species with higher N: P ratios would be less likely to experience induced P limitation than would species with low N: P ratios. To test my hypotheses, I used two insect herbivores, the monarch caterpillar, *D. plexippus* and the milkweed aphid, *A. asclepiadis*, which are both specialist herbivores feeding on common milkweed *Asclepias syriaca*. Previous surveys had shown that Lepidoptera have lower body N concentrations, but higher P concentrations, than do Hemiptera (Fagan et al. 2002, Woods et al. 2004). Therefore, I expected a greater chance of observing N-deposition induced P limitation in the caterpillar *D. plexippus* than in the aphid, *A. asclepiadis*. In a greenhouse experiment, I fertilized 500 plants with 0, 0.8, 1.6, 2.4, 3.2, 4, 4.8, 5.6, 6.4, 7.2 g/m^2/year of N, crossed with 10 P levels (0, 0.16, 0.32, 0.48, 0.64, 0.8, 0.96, 1.12, 1.28, 1.44 g/m^2/year), with 5 replicate plants per treatment. These N levels include a nitrogen deposition mimic (4 g/ m^2/year) as well as a higher level (7.2 g/m^2/year) designed to explore herbivore tolerance to imbalance. My P levels were chosen based on the fact that the N: P ratio in milkweed is around 6 to 7 (Zehnder and Hunter 2009). When the plants were three months old, I took one leaf from each plant to estimate foliar concentrations of C, N, P, and key milkweed

defense traits including latex, trichomes and cardenolide. I recorded individual and population growth rates of *D. plexippus* and *A. asclepiadis* respectively during a 10-day period. This experiment allowed me to explore the individual and interactive effects of N and P fertilization on different herbivore species, as well as their indirect effects via changing plant defense traits.

Although I focused mostly on nutrient limitation in Chapter 2, there is growing evidence that the relationship between nutrient availability and herbivore performance follows a unimodal function such that nutrient concentrations greater than optimal can lead to decreases in growth rates (Boersma and Elser 2006, Zehnder and Hunter 2009). In chapter 3, I explored the potential for high nutrient availability to reduce the fitness of a milkweed herbivore. I discovered that, across three milkweed species, *A. syriaca*, *A. curassavica* and *A. incarnata*, high foliar N concentrations resulted in reduced rates of growth by *D. plexippus* larvae. Although several mechanisms have been proposed to explain the negative effects of excessive nutrients on herbivore performance, none of them could explain the patterns in my data. Rather, I discovered a novel and significant interaction between foliar N and foliar cardenolide concentration, such that the per unit toxicity of cardenolide was higher when N concentration was high. Chapter 3 not only describes a new mechanism for the negative effects of nutrients on animal growth, but also suggests that interactions between nutrients and defense chemicals are as important as their individual effects in plant-herbivore interactions.

During co-evolution with its host plant, *D. plexippus* has evolved the ability to sequester cardenolides as a defense against its predators (Reichstein et al. 1968). Based on results in Chapter 3, illustrating that N fertilization induces higher toxicity of cardenolides, I predicted in

Chapter 4 that *D. plexippus* would exhibit a lower sequestration efficiency of cardenolides under high N conditions, either because (1) when growth rate and overall insect vigor are low, allocation to other functions is also low (Cotter et al. 2011), or (2) in order to reduce toxicity of cardenolides, *D. plexippus* may increase food passage rate, leading to lower sequestration efficiency. To test my prediction, I examined the effects of soil nutrient availability on growth, consumption, excretion and sequestration efficiency of cardenolides by *D. plexippus* larvae feeding on *A. curassavica.* In addition, I explored the effects of nutrient fertilization on foliar cardenolide concentrations. Integrating the above analyses, I was able to examine the overall effects of soil N availability on cardenolide sequestration by *D. plexippus.*

In Chapters 2 through 4, my emphasis is on the effects of nutrient stoichiometry in plants on insect growth and defense. However, there is growing evidence that herbivore activity can feed back to change plant nutritional quality by altering patterns of resource allocation (Frost and Hunter 2008); I explore such feedback in Chapter 5. In a world where herbivory is ubiquitous, plants have evolved tolerance strategies to cope with the stresses of herbivory. Specifically, tolerance is the ability of plants to maintain their fitness after damage (Rosenthal and Kotanen 1994). An important mechanism conferring tolerance in plants is the reallocation of resources following damage (Rosenthal and Kotanen 1994, Anten and Pierik 2010). Within several hours of damage by foliar herbivory, many plants can preferentially allocate newly acquired resources [mainly carbon (C) and nitrogen (N)] away from sites of attack (Babst et al. 2005, Schwachtje et al. 2006, Orians et al. 2011, Tao and Hunter 2011). In nature, many plant species are attacked by foliar and root herbivores simultaneously. Although there is ample evidence that plants can

‘‘hide’’ resources from either type of herbivore, it is not clear (1) whether plants have the capacity to reallocate resources away from both types of herbivore simultaneously and (2) if induced changes in resource allocation patterns by one herbivore type can be affected by the other (i.e., interactions between above and belowground herbivore species). I explored the idea of reallocation under simultaneous above- and below-ground herbivory by testing the following hypotheses: (1) both foliar (*D. plexippus*) and root (*T. tetraophthalmus*) herbivory change allocation of newly acquired C and N in *A. syriaca*, such that less resource is allocated to damaged sites; (2) *A. syriaca* mitigates the effects of future attack by both herbivore species simultaneously by allocating resources to stem which is unavailable to both species; and, therefore, (3) root and leaf feeding herbivores have additive effects on resource allocation in *A. syriaca*.

In my concluding chapter, I briefly summarize the key results from my work and propose avenues for future research.

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Chapter 2

Does anthropogenic nitrogen deposition induce phosphorus limitation in herbivorous insects?

Abstract

Anthropogenic nitrogen deposition has shifted many ecosystems from nitrogen (N) limitation to phosphorus (P) limitation. Although well documented in plants, no study to date has explored whether N deposition exacerbates P limitation at higher trophic levels, nor focused on the effects of induced plant P limitation on trophic interactions. Insect herbivores exhibit strict N: P homeostasis, and should therefore be very sensitive to variations in plant N: P stoichiometry and prone to experiencing deposition-induced P limitation. In the current study, we investigated the effects of N deposition and P availability on a plant-herbivorous insect system. Using common milkweed (*Asclepias syriaca*) and two of its specialist herbivores, the monarch caterpillar (*Danaus plexippus*) and milkweed aphid (*Aphis asclepiadis*) as our study system, we found that experimental N deposition caused P limitation in milkweed plants but not in either insect species. However, the mechanisms for the lack of P limitation were different for each insect species. The body tissues of *A. asclepiadis* always exhibited higher N: P ratios than that of the host plant, suggesting that the N demand of this species exceeds P demand, even under high N deposition levels. For *D. plexippus,* P addition increased the production of latex, which is an important defense negatively affecting *D. plexippus* growth rate. As a result, we illustrate that

P limitation of herbivores is not an inevitable consequence of anthropogenic N deposition in terrestrial systems. Rather, species specific demands for nutrients and the defensive responses of plants combine to determine the responses of herbivores to P availability under N deposition.

Introduction

In the last few decades, agricultural fertilization and fossil fuel combustion have changed the global nitrogen (N) cycle, resulting in significant atmospheric N deposition to ecosystems worldwide (Vitousek *et al.*, 1997). Increased N input has initially enhanced plant photosynthesis and productivity (Aber *et al.*, 1989). However, as the principle of ecological stoichiometry states, it is the relative balance between essential elements, not their absolute amount, which should predict organismal performance (Sterner & Elser, 2002). As N levels continue to rise and N limitation is subsequently alleviated, many plants are becoming more limited by other elements, most notably phosphorus (P) (Bobbink *et al.*, 2010). This "anthropogenic P limitation" (Peñuelas *et al.*, 2012; Vitousek *et al.*, 2010) has been documented in several temperate and tropical ecosystems, and is believed to be an important mechanism underlying changes in plant community composition (Bobbink *et al*., 2010) and ecosystem processes under nitrogen deposition, including changes in net primary production and nutrient retention (Aber *et al.*, 1989).

Nitrogen is an essential building block of the tissues of insect herbivores (Mattson, 1980). On average, insect body N concentrations are ten times higher than those of host plants, therefore nitrogen deposition has resulted in higher fitness and abundance of many herbivorous insects

(Throop & Lerdau, 2004). However, because P has not been manipulated independently in these previous studies, effects of N on insects cannot be separated from effects caused by changes in N:P ratios. Indeed, P is a key constituent of nucleic acids and enzymes, the limitation of which affects many aspects of insect performance, including survival (Clancy & King, 1993), body size (Huberty & Denno, 2006), development (Perkins *et al.*, 2004), growth rate (Watts *et al.*, 2006), and sexual and oviposition behavior (Bertram *et al.*, 2006). While evidence for anthropogenic P limitation in plants is accumulating rapidly, there has been no exploration to date on whether P limitation is also increasing in insects. Compared to plants, heterotrophs have very high levels of stoichiometric homeostasis (Persson *et al.*, 2010; Sardans *et al.,* 2011), and deviations from optimal dietary elemental ratios will change insect foraging behavior (Raubenheimer & Simpson, 1997) and/or post-ingestion assimilation (Woods *et al.*, 2002), both of which are energy consuming processes. While there is no simple way of determining optimal dietary N: P ratio for herbivorous insects, using body N: P ratio as a proxy can be useful (Hillebrand *et al.*, 2009). Therefore, insect performance should exhibit a hump shaped curve in relation to the stoichiometric mismatch between insect and plant tissues, with the highest insect performance achieved when the match is perfect. A survey of more than 300 species of terrestrial plants and 100 species of herbivorous insects has shown that on average, N: P ratios of insects are lower than those of plants, indicating that P limitation should be at least as severe as N limitation for herbivores (Elser *et al.*, 2000). Because plants always exhibit higher N: P ratios under increased N inputs (Morecroft *et al.*, 1994), this stoichiometric mismatch will be exacerbated further towards P limitation under N deposition.

While increased P limitation in insects by N deposition seems theoretically inevitable, we need to consider two other factors. First, insect species differ greatly in their body N and P contents. For example, Lepidoptera have the lowest N and highest P concentrations (and therefore the highest potential for P limitation) when compared to other insect orders (Fagan *et al.*, 2002; Woods *et al.*, 2004), while Hemiptera are considered to be extremely N limited (Speight *et al.*, 2008). As a result, potential P limitation may vary with the specific requirements of different herbivore taxa. Second, nutrient stoichiometry affects plant physical and chemical defenses (Karban & Baldwin, 1997). Theoretical and empirical studies of plant defense have documented that major defense chemicals vary with N availability (Bryant *et al.*, 1983; Herms & Mattson, 1992; Koricheva *et al.*, 1998). In contrast, data on the relationship between plant P and defense expression are less extensive (Koricheva *et al.*, 1998). While early theories of plant defense have focused on carbon-nutrient balance and growth/defense differentiation, biochemical studies provide a more mechanistic understanding of links between nutrient availability and plant defense. Cellular molecules containing inorganic phosphorus (Pi) are the most vulnerable P-containing molecules under P deficiency (Hidaka & Kitayama, 2011). The concentration of Pi is actively maintained by metabolic pathways (Plaxton & Carswell, 1999), such as up-regulating chorismate synthesis, which can generate 4 molecules of Pi at the expense of 1 erythrose-4-P, 1 ATP and 2 PEP molecules (Fischer *et al.*, 1993). Since chorismate is the precursor for phenylalanine, which is the precursor of phenolics in the shikimate pathway (Jones & Hartley, 1999), P limitation can indirectly induce higher total phenolic defenses. However, the opposite trend is also found in some plant species. *Rhizophora mangle* for example, has lower

concentrations of condensed tannins and total phenolics under P limitation (Feller, 1995). These mixed results illustrate the complex interplay and context dependence of carbon, nitrogen and phosphorus metabolism. It is worth pointing out that the studies cited above mimicked P limitation under natural conditions, but no study so far has aimed at correlating anthropogenic P limitation with plant defense expression. If anthropogenic P limitation decreases defense production in plants, any direct negative consequences of P limitation on insects may be offset by positive indirect effects mediated by reductions in plant defense. In contrast, effects of P limitation on insects may be particularly severe if P-limited plants also accumulate higher concentrations of defense chemicals. As a result, effects of P availability on insects under N deposition will depend on the directions and relative importance of stoichiometric mismatch and plant defense on insect performance.

To explore direct and indirect effects of anthropogenic P limitation on insect herbivores, we performed a greenhouse experiment with the common milkweed (*Asclepias syriaca*) and two specialist insect herbivores: monarch caterpillars (*Danaus plexippus*) and aphids (*Aphis asclepiadis*). A lepidopteran and a hemipteran species were selected because they are likely to differ in their N and P requirements (see above). Milkweed plants were cross-fertilized with ammonium nitrate and calcium phosphate monobasic to simulate anthropogenic nitrogen deposition and to create a phosphorus gradient within natural ranges. We explored potential P limitation in insects by correlating stoichiometric mismatch with performance of each insect species. We also explored the role of plant defense expression as a mediator. To our knowledge, this is the first attempt to investigate potential effects of anthropogenic P limitation on trophic

interactions under the dual frameworks of ecological stoichiometry and plant defense theory.

Materials and methods

Study system

The common milkweed (*Asclepias syriaca*) is a widespread native plant in eastern North America. Milkweeds reproduce both sexually and asexually and host about 12 species of insect herbivore in the eastern United States. *A. syriaca* populations have shown inconsistent response to N deposition (Pennings *et al.*, 2005), perhaps because of P co-limitation (Zehnder & Hunter, 2009). Putative defenses in *A. syriaca* include cardenolides, latex and trichomes. Cardenolides are toxic steroids that can interfere with Na+/K+-ATPase channels in animal cells, and can be negatively correlated with insect performance and lethal for mammals (Harborne, 1991). Latex is a white sticky fluid stored in high pressure non-articulated laticifers. In the genus *Asclepias,* latex is primarily composed of cardenolides, amyrin and cis-polyisoprene (Emon & Seiber, 1985). In comparison to leaf cardenolides, latex cardenolides occur at much higher concentrations (2-200 times) and are composed of more low polarity cardenolides (Seiber *et al.*, 1982). Trichomes are glandular hairs that cover both the upper and lower lamina and can deter insect feeding (Levin, 1973). The synthesis of cardenolides and latex require many phosphorus intermediates and the storage of latex in laticifers demands large amounts of ATP (Gershenzon, 1994). Therefore we predicted that chemical defenses in milkweed would be phosphorus limited.

The monarch butterfly (*Danaus plexippus*) is a specialist herbivore on the genus *Asclepias*. Larvae feed on milkweed leaves for 10-12 days before pupating. Latex can

significantly reduce the survival rate of monarch larvae and they have evolved the behavior of trenching or severing leaf petioles before feeding (Zalucki & Malcolm, 1999). The milkweed aphid (*Aphis asclepiadis*) is a gregarious phloem feeding insect that feeds preferentially on apical leaves. Aphids generally undergo several parthenogenetic generations before sexual reproduction, sometimes resulting in more than 1000 individuals on a single milkweed ramet.

Greenhouse experiment

Milkweed seeds were collected from a natural milkweed population at the University of Michigan Biological Station in Pellston, MI in September 2009. Seeds were stored in a refrigerator at 4 °C until use. At the end of April 2010, seeds were cold stratified for six weeks and then germinated on damp filter paper in petri dishes at 25 °C. After germination, 500 seedlings were planted in 4 inch plant pots containing a 1:1:1 mixture of potting soil (SunGrow Horticulture, Vancouver, BC, Canada), sand (Kolorscape) and perlite (Miracle-Gro, Marysville, OH). They were then transported to a greenhouse at Matthaei Botanic Garden, Ann Arbor, MI. When the plants were 3 weeks old, 10 by 10 levels of nitrogen and phosphorus fertilizer were applied in a factorial design across plants (100 nutrient combinations × 5 replicates each = 500 plants). Nitrogen was added as ammonium nitrate at levels of 0, 0.8, 1.6, 2.4, 3.2, 4, 4.8, 5.6, 6.4, 7.2 g/m^2/year, and phosphorus was added as calcium phosphate monobasic at levels of 0, 0.16, 0.32, 0.48, 0.64, 0.8, 0.96, 1.12, 1.28, 1.44 g/m^2 /year. The N levels were selected to mimic predicted estimates of N deposition throughout the range of our insect species (Galloway *et al.*, 2004), and P levels were chosen based on the fact that the N: P ratio in milkweed is around 6 to 7

(Zehnder & Hunter, 2009). As a result, the experiment was designed to simulate current and future N deposition while generating plant P levels within natural ranges. Fertilizer was applied once every week for a total of 5 weeks. We expected some plant mortality, and we chose this design to ensure that we generated milkweed plants with a broad range of N: P stoichiometry for experiments with insects (below). Ultimately, we used 2 plants from each nutrient treatment for experiments with caterpillars (n = 200) and 1 plant from each nutrient treatment for experiments with aphids (n = 100). This reflects a regression design rather than a replicated factorial design.

Starting one week after the last fertilization, we measured the physical and chemical traits of the plants that we subsequently used in experiments with insects. For logistical reasons, there was a five-day gap between the start of the experiments with monarchs (one week after the last fertilization) and the experiments with aphids (12 days after the last fertilization), thus these experiments have been analyzed separately. We measured change in plant height between the first fertilization and the start of herbivore treatment as an index of plant growth. One leaf from the fourth leaf pair of each plant was harvested for chemical analysis. Specifically, six leaf discs (total 424 mm^2) were taken by a paper punch from one side of the leaf, placed immediately into 1 mL of cold methanol and stored at -10 °C for subsequent cardenolide analysis. Another six identical discs were taken from the opposite side of the same leaf and stored in glassine envelopes to estimate sample dry mass and to estimate trichome density. We used a dissecting microscope at ×4 magnification with an optical micrometer to count trichomes on both the upper and lower surfaces of each leaf disc. The numbers were then averaged to a single value for each plant. We collected latex that exuded from the first six hole punches on pre-weighed cellulose

disks (1cm diameter), that were subsequently dried and reweighed. The whole leaf was then removed from the plant, dried at 70 °C for 72 hours, and ground into fine powder for nutrient analysis.

Monarch eggs were purchased from the Butterfly Rescue International Association in Allenton, MI. They were stored in a refrigerator for 2 days to synchronize hatching. Around 200 aphids were collected from the Edwin S. George Reserve, Pinckney, MI. Insects were introduced onto plants immediately after chemical sampling. One newly hatched monarch larva was placed on each of 200 plants (above). Two aphids were introduced onto each of another 100 plants. The herbivores were confined to each plant by mesh bags. After 7 days of feeding, surviving herbivores were retrieved and kept in petri dishes at room temperature for 48 hours to void their gut contents. By 7 days, most of the monarch larvae had entered the fourth instar and aphids had completed one full generation (Mooney *et al.*, 2008). To measure insect performance, monarch larval dry mass was measured on a microbalance (Mettler Toledo, Columbus, OH), and numbers of aphids were counted to estimate aphid population growth. Perhaps due to two days of very hot weather during the experiments, the survival rates of the insects were moderate (114 out of 200 monarchs, aphids on 83 out of 100 plants). Due to logistical constraints, we could not run chemical analyses on all herbivore individuals used in our experiments. However, it is generally agreed that animals are much more strictly homeostatic than are plants (Persson *et al*. 2010; Sardans *et al*. 2011). We therefore chose only a subset of total insect samples for chemical analysis. These samples allowed us to a) verify that insects do indeed show elemental homeostasis across a broad range of nutrient availabilities and b) estimate average N and P

concentrations of our insects by which we could calculate their elemental mismatch with the treatment plants upon which they were fed. To select caterpillars, we first retrieved all of them from their treatment plants, placed them in individual petri dishes, and marked each petri dish with its treatment level. We then selected every sixth caterpillar from the total of 114 individuals, providing 19 individuals for chemical analysis covering the entire range of treatments. In contrast to caterpillars, aphids were considered as populations on plants and not collected individually. Rather, after removing aphid populations from their plants, we selected every eighth population from the total of 83 populations (plants) for a total of 8 aphid populations for chemical analysis.

Chemical analysis

Analysis of foliar cardenolide content followed Vannette and Hunter (2011a). Briefly, leaf discs were ground for 3 mins in methanol using a ball mill and sonicated at 60 °C for 1 hour. The supernatant was evaporated at 45 °C for 70 mins until dryness. Samples were resuspended in 150 μL methanol containing 0.15 mg/mL digitoxin as an internal standard and analyzed using reverse phase high-performance liquid chromatography at high system pressures (UPLC, Waters Inc., Milford, MA, USA). Running time for each sample was 9 mins. Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300nm with digitoxin as the standard. Peaks with symmetrical absorption maxima between 216 and 222nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of the

internal standard and the estimated sample mass. Carbon and N contents in plant and insect tissues were measured on a CHN analyzer (Costech, Valencia, CA) and P contents were quantified by an autoanalyzer using an acid digestion method. Plant and insect N: P ratio was calculated as molar ratio.

Statistical analysis

We used general linear models (GLM) to assess the effects of N and P fertilization and their interaction on foliar N and P concentrations and plant growth. Because the aphid and caterpillar experiments were not performed simultaneously, we ran separate analyses for each experiment. Following the methods of Sterner and Elser (2002) of regressing plant N: P ratios with those of the insects, we confirmed that both *D. plexippus* and *A. asclepiadis* exhibited strict body elemental homeostasis (slope for *D. plexippus* is 0.19, $F_{1,17}$=0.328, p=0.575; slope for *A. asclepiadis* is 0.16, $F_{1,8}$=0.512, p=0.495). Because both slopes are less than 0.25, they also fit the criteria of Persson *et al*., (2010) for homeostasis. Therefore, we used the average N: P ratio of each herbivore species in calculating the stoichiometric mismatch between plants and herbivores in all following analyses.

To test the individual and interactive effects of plant N and P on insect performance, we used GLM with plant N and P as independent variables and insect performance (mass for *D. plexippus* and numbers for *A. asclepiadis*) as dependent variables. Additionally, we hypothesized that insect performance would respond non-linearly to the N: P mismatch between plants and insect, with highest insect performance achieved when the match was perfect. We therefore

examined both linear and quadratic model fits between insect-plant N: P mismatch and insect performance. Specifically, we subtracted the log of the N: P ratio of each individual plant from the log of the average N: P ratio of aphids or monarchs to calculate the N: P mismatch between insects and individual plants. Mismatch was then the independent variable and insect performance was the dependent variable.

Measures of model fit including AIC and R^2 were extracted from each model. Model selection was performed using AIC, where differences in AIC>2 were deemed an improved fit (Burnham & Anderson, 2002). We used GLM to test the individual and interactive effects of plant N and P on each defense trait.

We then used path analysis to compare the relative strengths of direct (stoichiometric) and indirect (via plant defense) effects of N and P on insect performance. We began the modeling exercises by first constructing a saturated model for each species in which N and P were exogenous variables, and latex, cardenolides and trichomes were endogenous predictors of insect performance. We also allowed correlations between endogenous variables. We then iteratively set the least significant path to zero and examined whether removal of the path increased model fit, as judged by AIC. We continued this process until we arrived at the most parsimonious model. For *A. asclepiadis*, the most parsimonious path model excluded all three defense traits, and therefore reduced to the relationships among foliar nutrients and aphid performance under GLM (above).

Before all statistical analysis, data were evaluated by Kolmogorov-Smirnov tests for normality, and were log transformed when necessary. The path analysis was conducted in Amos

18 (SPSS Inc, Chicago, Illinois), and general linear models were conducted in the GLM package in R 2.13.2 (R Development Core team 2011).

Results

Plant nutrient content and plant growth

Our treatments effectively increased foliar N and P concentrations in *A. syriaca.* Foliar N responded linearly to N fertilization levels (in the caterpillar experiment, N fertilization effect on foliar N contents: $F_{1, 112} = 16.358$, $p < 0.001$; in the aphid experiment, $F_{1, 81} = 12.277$, $p = 0.001$). Foliar P concentrations, on the other hand, exhibited quadratic responses to P fertilization (caterpillar experiment, $F_{2, 110} = 69.90$, $p < 0.001$; aphid experiment, $F_{2, 80} = 29.31$, $p < 0.001$; Fig. 2.1). There were no interactive effects of N and P fertilization on foliar nutrient concentrations.

Plant growth increased in response to N addition but not P addition. However, there were significant interactions between N and P in their effects on plant growth (in the caterpillar experiment, N fertilization effect on plant growth, $F_{1,110} = 29.95$, $p < 0.001$; P effect, $F_{1, 110} = 0.98$, $p = 0.32$; N * P interaction $F_{1,110} = 5.87$, $p=0.017$; in the aphid experiment, N effect $F_{1, 79} = 32.77$, $p < 0.001$; P effect $F_{1, 79} = 0.90$, $p = 0.35$; N * P interaction $F_{1,79} = 19.14$, $p < 0.001$). Purely to better illustrate visually the nature of the N * P interactions, we have grouped our P levels into three categories: (1) 0-0.5 g/m^2/year; (2) 0.5-1 g/m^2/year; (3) 1-1.5 g/m^2/year. Plotting plant growth against N and P fertilization levels shows that, in both experiments, plants were more responsive to P addition at higher N fertilization levels (Fig. 2.2), suggesting that P limitation was induced by experimental N deposition in *A. syriaca.*

Effects of plant N and P concentrations on insect performance

D. plexippus had about the same tissue N concentration (11.39 ± 0.36%) as *A. asclepiadis* (10.37 ± 0.46%, independent sample t-test: $p = 0.10$), but had higher tissue P concentration (*D. plexippus,* 1.24 ± 0.08%; *A. asclepiadis,* 0.91 ± 0.02%; $p = 0.001$). *A. asclepiadis* exhibited a higher N: P ratio (25.32 ± 1.01) than the average of all experimental host plants (20.80 ± 0.83; $p < 0.001$), while *D. plexippus* had an N: P ratio (22.60 ± 2.23) similar to the average of all host plants (20.38 ± 0.64; $p = 0.24$). The performance of both *D. plexippus* and *A. asclepiadis* increased with foliar N concentration (Fig. 2.3a, c, Table 2.1). Foliar P concentration had no effect on *D. plexippus* performance and actually decreased the performance of *A. asclepiadis* (Fig. 2.3b, d, Table 2.1). Critically, there were no significant interactions between N and P concentrations and subsequent herbivore performance, suggesting that high N availability does not increase P limitation.

Effects of N: P stoichiometric mismatch on insect growth

We compared quadratic and linear model fits between the extents of N: P mismatch between insect and plant tissues and the subsequent performance of each insect species. For both species, the linear models were better fits, although the linear model was marginally non-significant in *D. plexippus* (Fig. 2.4, Table 2.2).

Effects of plant N and P concentrations on defense production

In both experiments, foliar P concentration was positively correlated with latex production, while foliar N concentration was positively correlated with cardenolide concentration (Table 2.3). There were marginally significant interactive effects of N and P on cardenolides. We also found a significant negative relationship between N and latex in the aphid experiment, but not in the caterpillar experiment. In the aphid experiment, P also correlated positively with trichome production, but the relationship was not significant in the caterpillar experiment (Table 2.3).

Decomposing direct and indirect effects of plant N and P on insect performance

Path analysis illustrated that for *D. plexippus,* the most parsimonious model included P as an exogenous factor and latex as the mediator (Fig. 2.5). Specifically, foliar P increased latex production, which influenced monarch growth rate negatively (Fig. 2.5, Table 2.4). In contrast, the most parsimonious path model for *A. asclepiadis* included only foliar N and P concentrations, reducing to the relationships illustrated previously in Fig. 2.3c, d.

Discussion

The principle of ecological stoichiometry states that the elemental balance of resources is more important to consumers than the absolute availability of single elements (Sterner & Elser, 2002). Therefore, the effects of varying concentrations of one element on organism performance depend upon the concentrations of other essential elements (Reich & Schoettle, 1988). Nitrogen and phosphorus, as biologically important elements, have been shown to be limiting for both

plants and terrestrial insect herbivores. This led us to explore potential increases in herbivore P limitation under global N deposition. However, we illustrate that P limitation of herbivores is not an inevitable consequence of anthropogenic N deposition in terrestrial systems. Rather, the stoichiometry of herbivore body tissues and the defensive responses of plants combine to determine the responses of herbivores to P availability under N deposition.

Plant-insect N: P mismatch

On average, terrestrial insect herbivores have a body tissue N: P ratio of 26.4, compared to 28.0 in plants, leading to the prediction that insect P limitation should be at least as severe as N limitation (Elser *et al.*, 2000). In our system, however, the N: P ratio of *A. asclepiadis* (26.2) was much higher than that of the average foliage of the host plant, *A. syriaca* (21.3). One concern with such a comparison for aphids is that phloem sap stoichiometry may be different from that of leaf tissues. Although we could find no study to date directly comparing leaf and phloem N: P ratios, we can combine several independent studies to explore the issue. For example, the castor bean, *Ricinus communis*, has often been used to study phloem exudates. A review summarizing 20 years of study in this species reported that the molar N: Pi ratio in leaf tissue is 50, while the N: Pi ratio in phloem exudate is 10.38 (Peuke, 2010). Assuming Pi comprises 25% of total P in terrestrial plants (Hidaka & Kitayama, 2011), and 73% of total P in phloem exudates (Hall & Baker, 1972), the N: P ratio in leaves is 12.5 compared with 7.58 in the phloem of *R. communis*. If phloem N: P ratios are also lower than foliar N: P ratios in our milkweed system, this would further exacerbate N limitation on *A. asclepiadis,* with its high

body N: P ratio. Our results suggest that N deposition does not induce P limitation in *A. asclepiadis* because N requirements surpass P demand even at N deposition levels of 8 g/m^2/year. This is illustrated in Fig. 1.4b by the paucity of data points with negative values on the x-axis; despite profound manipulation of plant N: P stoichiometry, a great majority of aphids are still N limited at high N availability.

Not only did we fail to find evidence for P limitation in *A. asclepiadis,* there was actually a negative relationship between plant P content and insect growth (Table 2.1). To meet their requirements for the most limiting resource (in this case, N), herbivorous insects may consume higher than necessary concentrations of other nutrients (Simpson *et al.*, 2004). These surplus resources are subsequently stored or excreted at the expense of energy (Boersma & Elser, 2006). Declines in aphid performance with increasing P availability may represent costs associated with eliminating excess P while meeting the demand for N. Such costs of excreting excess nutrients have been reported previously for *Aphis nerii* on milkweed (Zehnder & Hunter, 2009).

Compared with *A. asclepiadis, D. plexippus* has a higher body P concentration and therefore a lower N: P ratio, closer to that of its host plant. This is consistent with Woods *et al.* (2004) who suggest that Lepidoptera are generally richer in P than are other insect lineages. According to our original predictions, the close match between monarch and milkweed N: P ratios should increase the likelihood of observing P limitation under N deposition. Indeed, P limitation has been reported in other Lepidoptera. For example, tobacco hornworm, *Manduca sexta,* grows faster on P rich jimsonweed *Datura wrightii* (Perkins *et al.*, 2004). Likewise, in western spruce budworm *Choristoneura occidentalis,* survival rate is optimal on dietary P levels

higher than those of their natural host trees (Clancy & King, 1993). By contrast, we did not find evidence of P limitation in *D. plexippus*. Interestingly, the reason was not insensitivity to P per se, but rather through the intermediate effects of P on plant defense (Fig. 2.5). P addition appears to favor the production of latex (Table 2.3), which is known to reduce the performance of monarch caterpillars (Zalucki & Malcolm, 1999).

To date, the majority of evidence of P limitation in herbivores is found in aquatic systems. Compared to terrestrial herbivores, zooplankton and aquatic insect herbivores are more likely to be P-limited (Elser *et al.*, 2001; Frost & Elser, 2002; Sterner *et al.*, 1993). Aquatic ecosystems have lower available P, therefore N: P ratios of phytoplankton and other autotrophs can be from 1.5 to 4 times higher than those of aquatic herbivores, while average N: P ratios of plants are only 1.1 times higher than those of terrestrial insects (Cross *et al.*, 2003). Therefore, herbivores in aquatic systems should have a higher probability of P limitation when compared with terrestrial herbivores, which are more likely to be N and P co-limited.

Nitrogen, phosphorus and plant chemical defense

Plant nutrient status can affect the expression of plant chemical defense by changing relative allocation among growth, storage and defense (Bryant *et al.*, 1983; Herms & Mattson, 1992; Vannette & Hunter, 2011b). Intensive studies on the effects of N have shown that plant phenolics tend to decrease following N deposition (Koricheva *et al.*, 1998), although the effects of P on defense are less frequently reported and show inconclusive results. P availability should be vital to plant defense expression for several reasons. First, P status influences plant carbon

metabolism by affecting photosynthesis and carbon reserves (Warren & Adams, 2002). Second, many intermediates in the pathways of secondary metabolite synthesis contain P (Gershenzon, 1994), and allocation among these pathways may vary with P availability (Plaxton & Carswell, 1999). Third, plants must expend energy in the form of ATP to store defense chemicals (Gershenzon, 1994) including latex defenses such as those of *A. syriaca*. Given that there is accumulating evidence that many plants change from N limitation to P limitation under N deposition, there needs to be more exploration of the effects of P on plant defenses.

Unfortunately, there is currently a lack of integration between the fields of elemental stoichiometry and plant defense theory in the literature. In plant cells, N and P metabolism are linked because the majority of cellular P is used for translating amino acids into proteins, so P availability can regulate N dynamics in cells (Warren & Adams, 2002). In addition, N use efficiency depends on P availability (Reich *et al.*, 2009). In *Pinus strobus,* for example, N use efficiency is negatively related to the cellular N: P ratio, suggesting that P is a major regulator of N activity in plant cells (Reich & Schoettle, 1988). Therefore, the mechanisms by which the addition of N and P affect plant chemistry are complex and context dependent. Here, we report that in both aphid and caterpillar experiments, P is positively correlated with latex production, while N is positively correlated with foliar cardenolide concentration. As a result, variations in cell N and P concentrations not only can affect the absolute amounts of plant defense, but also the relative expression of different defense traits.

Nitrogen deposition and herbivorous insect communities

Long term studies on the effects of N deposition on community composition of herbivorous insects have mainly focused on how changes in plant chemistry, productivity and diversity influence insects (Haddad *et al.*, 2000; Siemann, 1998). However, even after controlling for the above variables, insect species vary in their responses to N enrichment of single host plant species (Cornelissen & Stiling, 2006; Lightfoot & Whitford, 1987; Strauss, 1987). The differential responses have been ascribed to variation in life history traits and interactions with other trophic levels (Strauss, 1987; White, 1993). Here, we suggest that constraints of stoichiometric mismatch and plant defense compounds may also play a role. Because patterns of plant resource allocation and defense are in turn influenced by herbivore feeding (Karban & Baldwin, 1997; Tao & Hunter, 2011), future work should consider feedbacks between plant and herbivore performance under N deposition and variable P availability.

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Table 2.1 Effects of foliar N and P concentrations on the performance of *Danaus plexippus* and *Aphis asclepiadis* on *Asclepias syriaca* plants

Species	Model	Df	F-ratio	P-level	R^2	Estimate
D.plexippus	Full model	3;110	3.14	0.03*	0.07	
	Nitrogen	1	6.04	0.02*		0.52 (0.21)
	Phosphorus	1	2.27	0.13		n.s.
	N × P	1	1.10	0.30		n.s.
A.asclepiadis	Full model	3;79	5.41	0.002**	0.17	
	Nitrogen	1	7.54	0.008**		0.37 (0.18)
	Phosphorus	1	8.37	0.005**		-2.75 (0.95)
	N × P	1	0.31	0.58		n.s.

For each response variable, the full model degrees of freedom, F-ratio, significance level (P) and variance explained (R^2) are given.
For each factor, the F-ratio, significance level and an estimate of the slope of the effect (± standard error) are given.
*p<0.05, **p<0.01,***p<0.001

Table 2.2 Effects of the mismatch between plant and insect N: P and the subsequent performance of *D.plexippus* and *A.asclepiadis*

Species	Model	Df	F-ratio	P-level	R^2	Estimate
D.plexippus	Linear	1;112	3.63	0.06	0.03	-0.62 (0.32)
A.asclepiadis	Linear	1;81	19.12	<0.001***	0.19	-0.04 (0.008)

For each response variable, the better fit model, degrees of freedom, F-ratio, significance level (P) and variance explained (R^2) are given.
For each model, an estimate of the slope (± standard error) is given.
*p<0.05, **p<0.01,***p<0.001

Table 2.3 Relationships between foliar N and P concentrations and the defensive traits of *A. syriaca*

Experiment	Variable	Defense trait	F	P	Estimate
Caterpillar	N	Latex	0.66	0.42	--
		Cardenolides	13.24	<0.001***	1.59 (0.60)
		Trichome	2.021	0.158	--
	P	Latex	10.35	0.002**	2.798 (0.884)
		Cardenolides	1.52	0.221	--
		Trichome	0.01	0.91	--
	N × P	Latex	2.28	0.135	--
		Cardenolides	2.88	0.093	-2.96 (0.09)
		Trichome	0.004	0.95	--
Aphid	N	Latex	5.72	0.02*	-1.41 (0.69)
		Cardenolides	3.56	0.06	2.27 (0.92)
		Trichome	0.02	0.88	--
	P	Latex	6.00	0.02*	3.12 (1.10)
		Cardenolides	2.71	0.10	--
		Trichome	7.66	0.007	7.583 (2.756)
	N × P	Latex	2.41	0.12	--
		Cardenolides	3.58	0.06	-7.10 (3.75)
		Trichome	0.33	0.57	--

For each response variable, the F-ratio, significance level (P) and estimate of the slope of the effect (± standard error) are given.
*p<0.05, **p<0.01, ***p<0.001

Table 2.4 Path analysis model selection

Model	Df	χ^2	P	AIC	R^2
Saturated model				42.00	0.17
Most parsimonious model	1	0.79	0.38	10.79	0.12

Df, degrees of freedom; AIC, Akaike information criteria. P is the significance level of the model fit, R^2 is the variance explained by the model.

Fig. **2.1** *Asclepias syriaca* foliar N and P concentrations in response to fertilization levels in experiments with caterpillars (a, b) and aphids (c, d). Regression lines signify significant relationships.

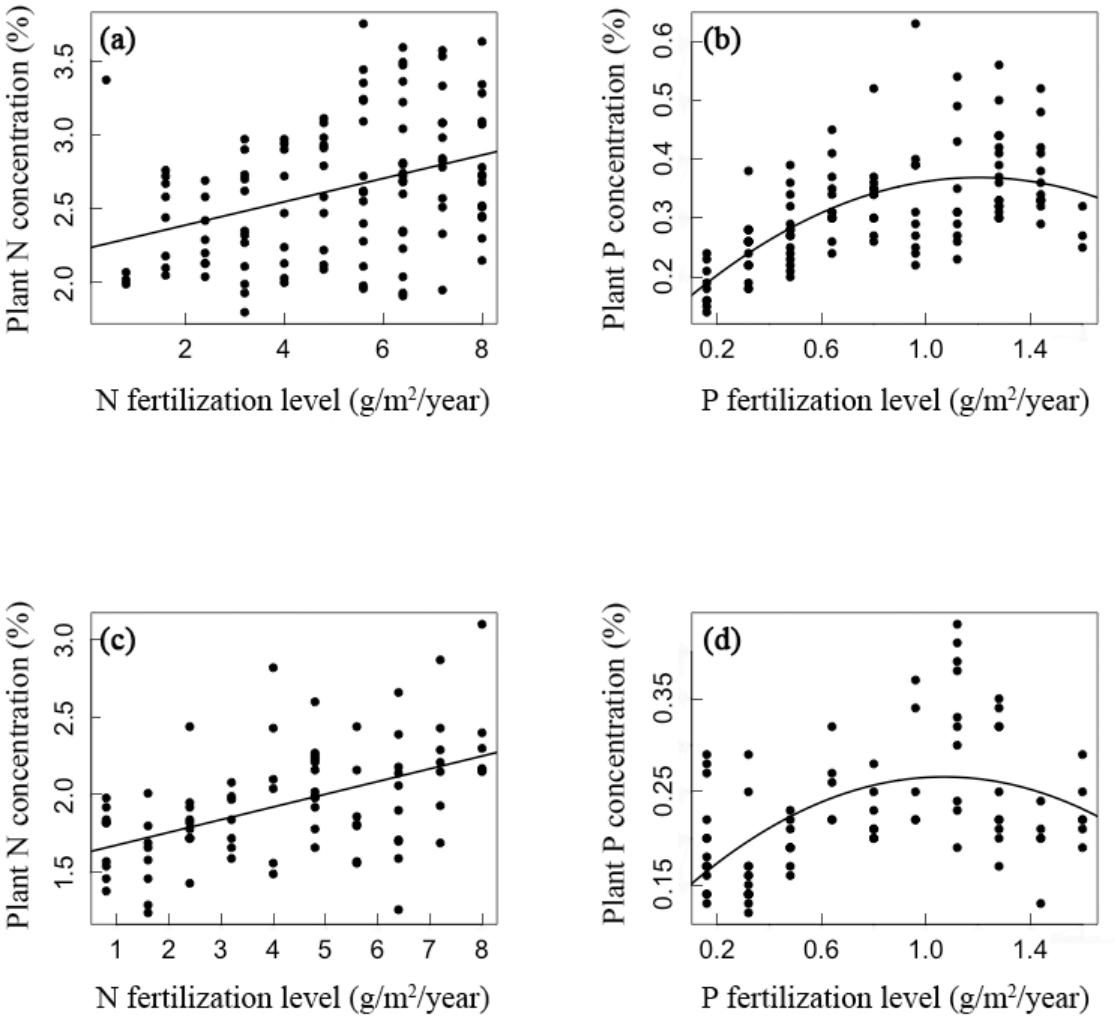


Fig. **2.2** Growth of *A. syriaca* plants in response to N and P fertilization levels in experiments with caterpillars (a) and aphids (b). Statistical analyses were conducted on 10 levels of P fertilization, but P levels are shown here in 3 groups for ease of illustration.

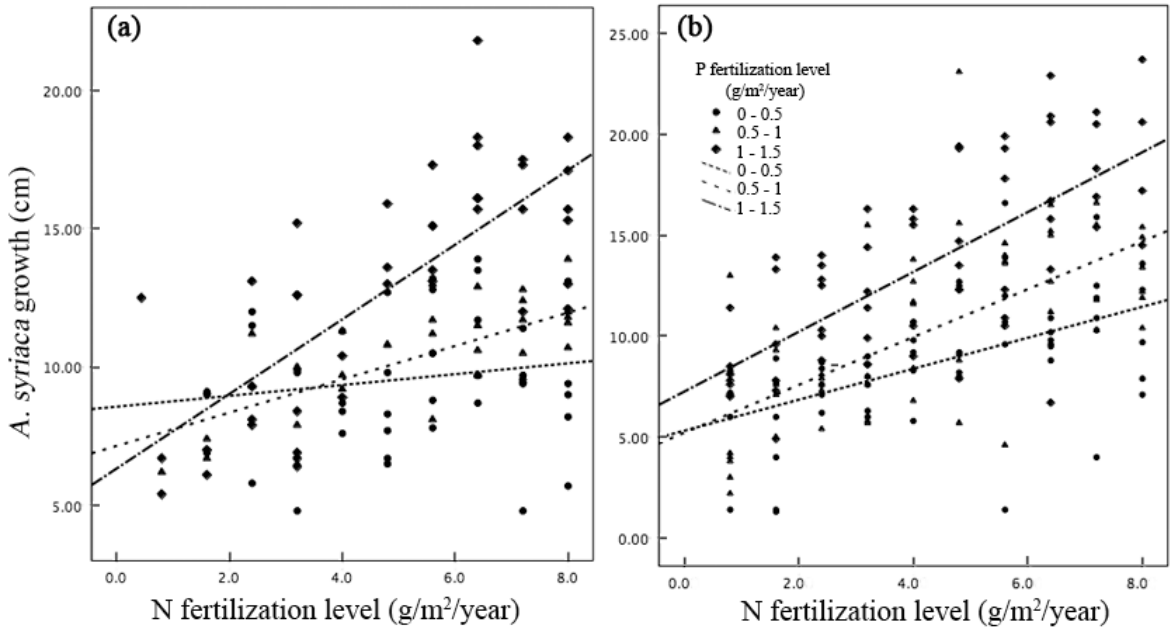


Fig. **2.3** Performance of *D. plexippus* (a,b) and *A. asclepiadis* (c,d) in response to foliar nitrogen and phosphorus concentrations (%) of their host plants. Regression lines signify significant relationships.

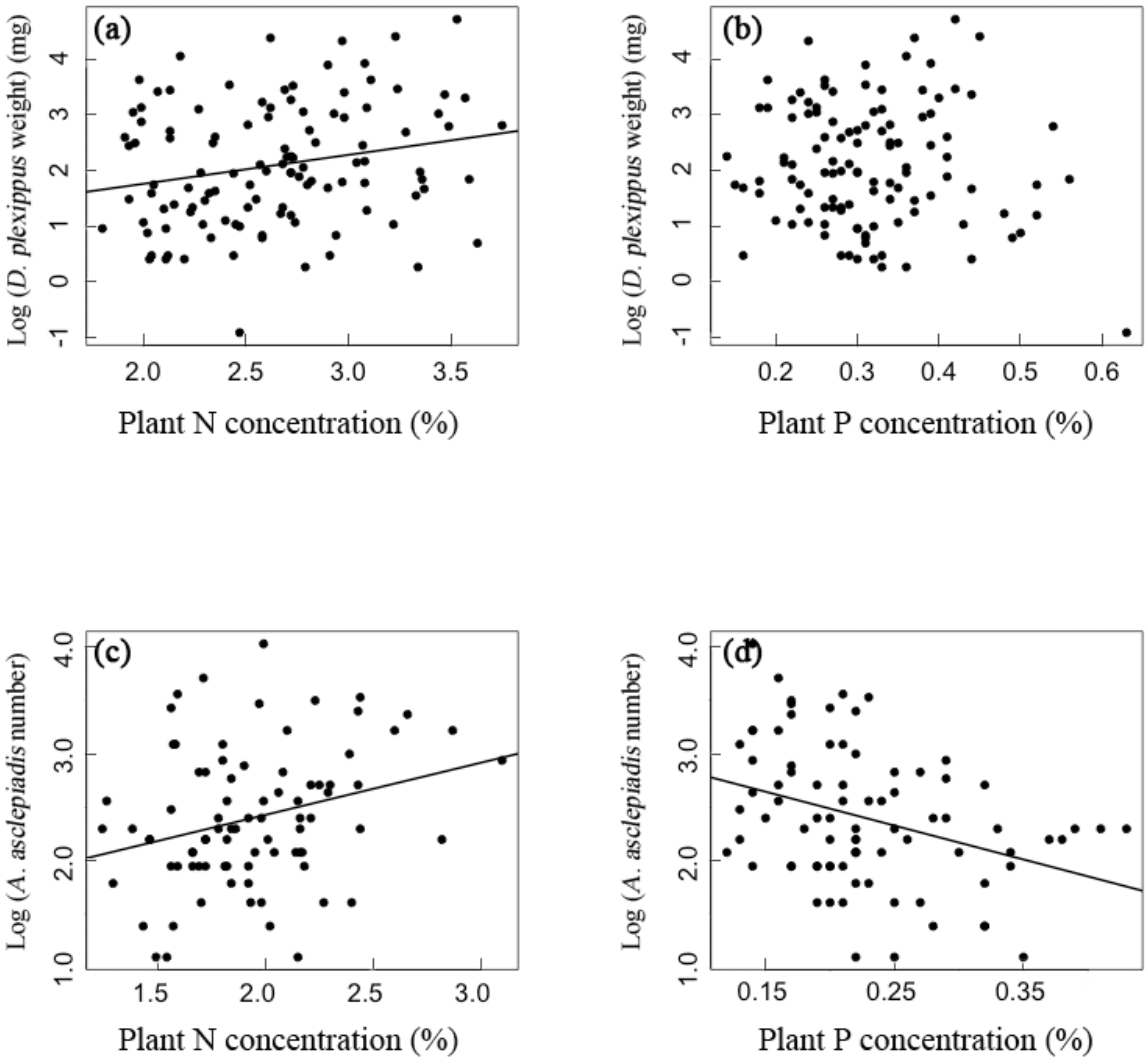
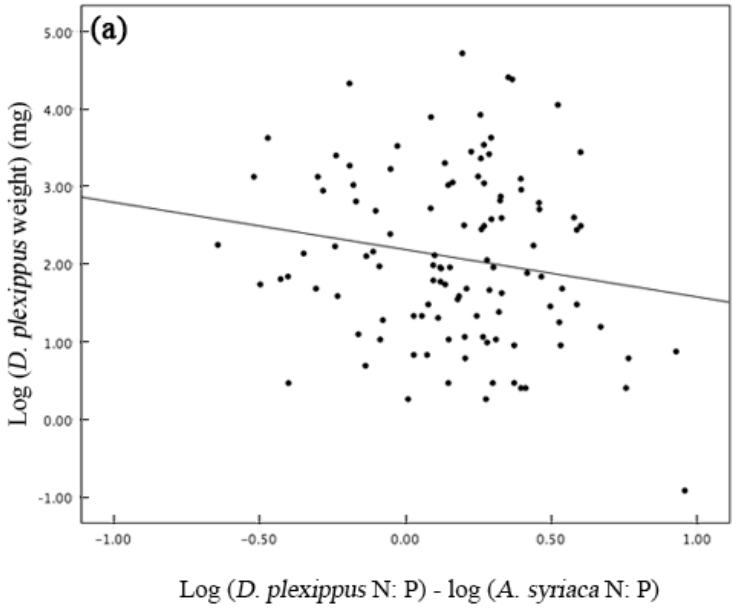


Fig. **2.4.** Performance of *D. plexippus* (a) and *A. asclepiadis* (b) in relation to the N: P stoichiometric mismatch between their tissues and those of their host plants. Regression lines signify significant relationships (in the case of *D. plexippus,* the relationship was marginally significant).



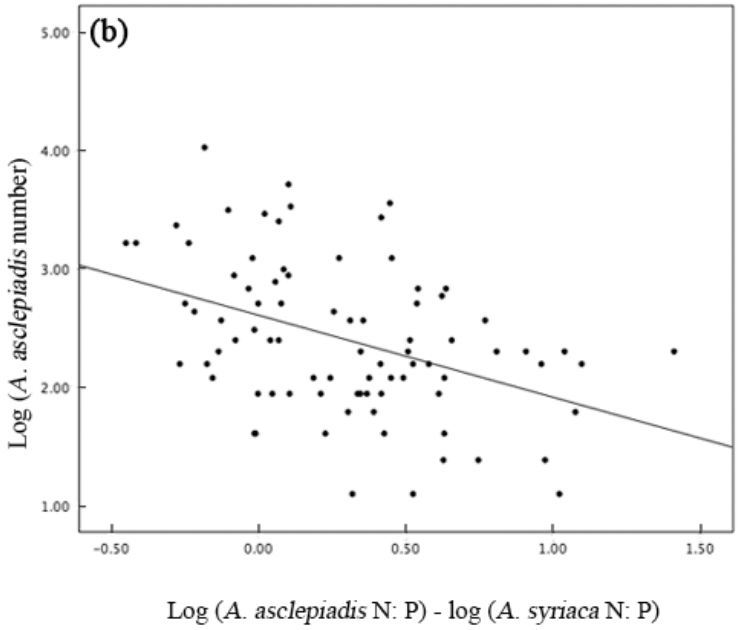
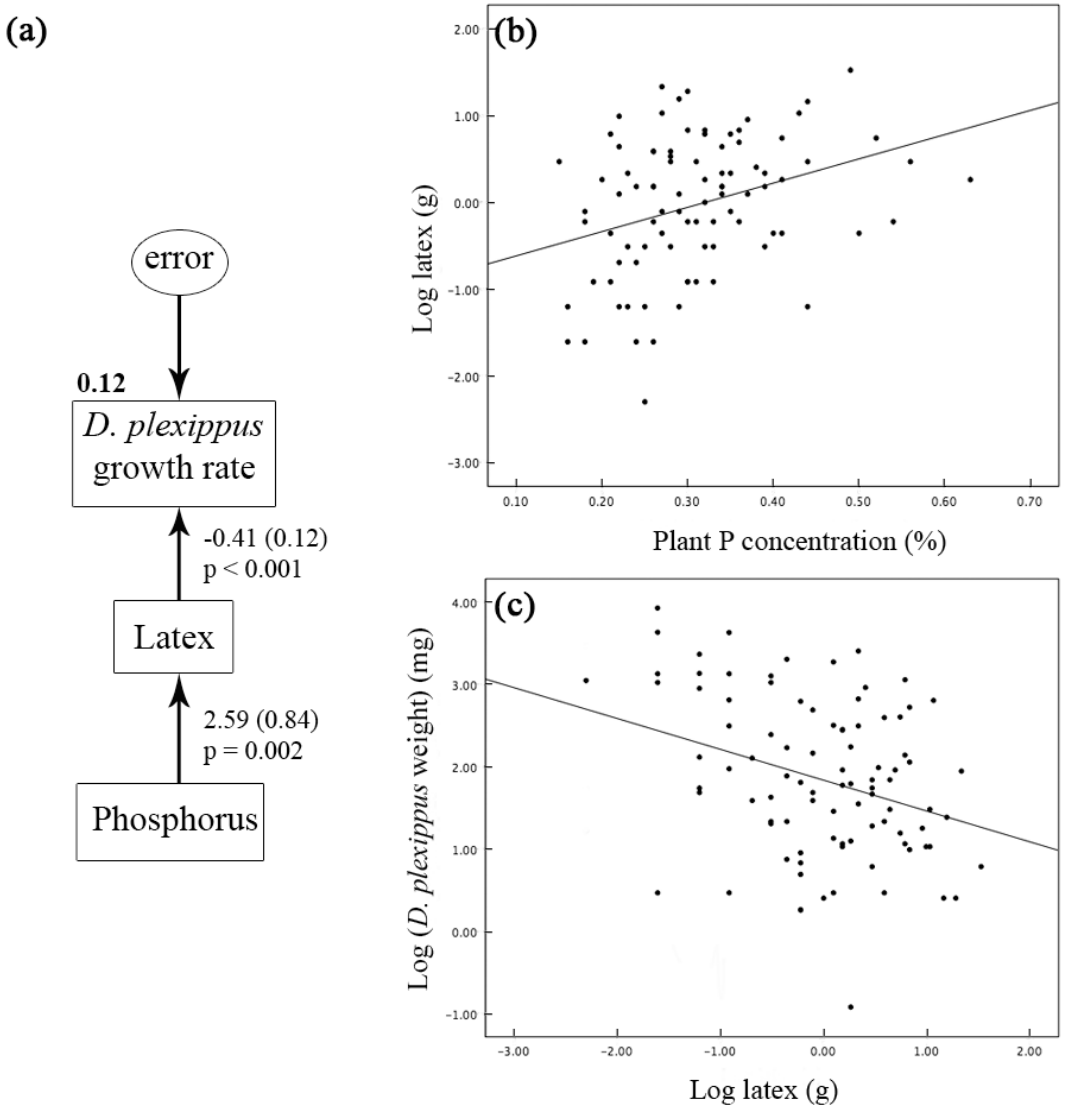


Fig. **2.5** Path analysis of the effects of foliar N and P concentrations on the growth rate of *D. plexippus*. (a) Path diagram for the most parsimonious model. Unstandardized path coefficients (standard deviation in parenthesis) are given beside each arrow. The bold number indicates the variance explained by the model. (b) Relationship between foliar P concentration and latex production in *Asclepias syriaca*. (c) Negative association between latex production in *Asclepias syriaca* and the growth rate of *D. plexippus* larva.



Chapter 3

Why does a good thing become too much? Interactions between foliar nutrients and toxins determine performance of an insect herbivore

Abstract

In terrestrial ecosystems, nitrogen (N) and phosphorus (P) are generally believed to be the most limiting nutrients for organisms across different trophic levels. However, accumulating evidence suggests that dietary nutrient concentrations higher than optimal can lead to decreases in consumer growth rate**.** In the current study, we explored mechanisms underlying the negative effects of high nutrient availability on the performance of a specialist herbivore. Specifically, we investigated the responses of the monarch caterpillar *Danaus plexippus* to natural and experimental variation in N and P concentrations of three species of milkweed plants (*Asclepias syriaca, A. curassavica* and *A. incarnata*) that also varied in their foliar toxin concentrations. We found that high foliar N concentrations in milkweed were associated with decreases in the growth rate of *D. plexippus* larvae. However, such negative effects of N were only found when larvae were feeding on *A. curassavica,* which also had high foliar concentrations of cardenolide, a widespread chemical defense in the genus *Asclepias*. Foliar N concentration was not correlated with cardenolide concentration. Rather, the per unit toxicity of cardenolide was higher as N increased in excess of demand, resulting in deleterious effects of N. Our results suggest that

interactions between nutrient concentrations in excess of demand and high dietary toxin concentrations provide an additional mechanism by which high nutrient availability can reduce the performance of consumers.

Introduction

Since von Liebig's time (von Liebig 1840), ecologists have been studying the nature and consequences of nutrient limitation in organisms. From the classic Law of the Minimum to the Multiple Limitation Hypothesis (Gleeson & Tilman 1992) and the Principle of Ecological Stoichiometry (Sterner & Elser 2002), our increasing understanding of nutritional ecology has shed much light on species interactions and ecosystem processes. In terrestrial ecosystems, nitrogen (N) and phosphorus (P) are generally believed to be the most limiting nutrients for organisms across different trophic levels (Denno & Fagan 2004; Sterner & Elser 2002; Elser et al. 2007). In the context of plant-insect herbivore interactions, foliar N and P concentrations are on average 10 times lower than those in herbivore tissues (Elser et al. 2007), therefore increases in foliar N and P concentration can result in higher performance by herbivores (Mattson 1980; Hunter, Watt & Docherty 1991; Speight et al. 2008).

However, the principle of ecological stoichiometry predicts there to be an optimal concentration of each nutrient for each organism at which maximal growth rate is attained (Boersma & Elser 2006). As a result, nutrient concentrations higher than optimal can lead to decreases in growth rate. Negative effects of nutrient enrichment on consumer growth rates have been recorded in a number of organisms. For example, high N concentrations can lead to

decreases in the fitness of grasshoppers (Joern & Behmer 1998), butterflies (Fischer & Fiedler 2002) and aphids (Zehnder & Hunter 2009), all of which are often considered limited by N (Mattson 1980). Similarly, high P concentrations can result in negative effects on the growth rates of aquatic organisms, which are traditionally thought to be P limited (Boersma & Elser 2006).

Several mechanisms have been proposed to explain such negative relationships between nutrient availability and organismal performance at high nutrients. First, organisms have an intake target in multivariate resource space (Simpson & Raubenheimer 1993). Therefore, when consuming food whose nutrient composition deviates from the target, they will regulate their total intake to strike a balance between excess and limitation of each nutrient (Raubenheimer & Simpson 1999; Simpson et al. 2004). In other words, by preventing the excess uptake of one nutrient, other essential elements may be in deficit. This is especially true in heterotrophs, which obtain their nutrients in mixtures rather than as separate elements. Second, excretion and storage of excess nutrients may impose metabolic costs (Boersma & Elser 2006). Third, concomitant changes in other physical/chemical properties of the diet that may potentially decrease growth rate (Plath & Boersma 2001). Specifically, the levels of nutrients and secondary metabolites in food sources are often tightly correlated (Bryant, Chapin & Klein 1983; Mattson & Herms 1992; Vannette & Hunter 2011). As a result, the deleterious effects of high nutrients on consumers may indirectly result primarily from higher levels of plant resistance rather than high nutrients themselves (Tao & Hunter 2012).

In the current study, we explored mechanisms underlying the negative effects of high nutrient availability on the performance of a specialist herbivore. Specifically, we investigated the responses of the monarch caterpillar *Danaus plexippus* to natural and experimental variation in N and P concentrations of three milkweed species that also differed in their foliar toxin concentrations. In contrast to predictions based on the mechanisms described above, we found that the deleterious effects of high N concentration on monarch performance were only expressed under high foliar toxin concentrations. Our results suggest that interactions between nutrient concentrations in excess of demand and high dietary toxin concentrations provide an additional mechanism by which high nutrient availability can reduce the performance of consumers.

Materials and methods

We carried out experiments with three species of milkweed: *Asclepias syriaca, A. incarnata* and *A. curassavica*. Under control conditions, these milkweed species exhibit a broad range of foliar N (1.1-5.2%) and P concentrations (0.1-0.8%), with one species expressing low N and P concentrations (*A. syriaca*, N: 1.62 ± 0.28%; P: 0.18 ± 0.02%), and two species expressing high N and P concentrations (*A. curassavica,* N: 3.32 ± 0.26%, P: 0.35 ± 0.08%; *A. incarnata,* N: 3.68 ± 0.26%; P: 0.27 ± 0.02%; Fig. S1). In milkweeds, the major defensive chemicals are cardenolides, which can disrupt animal Na^+/K^+ ATPase (Agrawal et al. 2012). Our chosen milkweeds vary in foliar cardenolide concentrations from low (*A. incarnata*) through intermediate (*A. syriaca*) to high (*A. curassavica*) (Agrawal & Malcolm 2002; Sternberg et al.

2012).

A. syriaca seeds were collected in September 2010 from a natural population at the University of Michigan Biological Station in Pellston, MI and stored in a refrigerator at 4 °C until use. *A. incarnata* and *A. curassavica* seeds were purchased from Butterfly Inc, San Ramon, California. At the end of March 2012, *A. syriaca* and *A. incarnata* seeds were cold stratified for 6 weeks and then germinated on damp filter paper in petri dishes at 25 °C. *A. curassavica* seeds, which do not need stratification, were germinated simultaneously. After germination, seedlings were planted in 4 inch plant pots containing a 1:1 mixture of potting soil (SunGrow Horticulture, Canada) and sand (Kolorscape). Plants were kept in a controlled growth chamber at 25 °C and an L16:D8 light cycle. When plants were 6-weeks old, 3 by 3 levels of nitrogen and phosphorus fertilizer were applied in a fully factorial design. Nitrogen was added as ammonium nitrate at total levels of 0, 4, 8 g m^{-2}, and phosphorus was added as calcium phosphate monobasic at total levels of 0, 0.4, 0.8 g m^{-2} Fertilizer was applied once every week for a total of 5 weeks; each plant received 1/5 of its total fertilizer allocation each week. We have shown previously that these levels of fertilization generate a broad but realistic range of C:N:P stoichiometry in milkweed plants (Tao & Hunter 2012). For *A. incarnata* and *A. curassavica*, we used 10 replicates per treatment for a total of 90 plants for each species (9 nutrient treatments * 10 plants each = 90 plants). For *A. syriaca,* due to a low germination rate, we used 6 replicates per treatment for a total of 54 plants (9 nutrient treatments * 6 plants each = 54 plants). In this experiment, N fertilization resulted in significant increases in foliar N concentrations ($F_{2, 187}$=35.98, $p<0.001$), while the effects of P fertilization on foliar P concentration were not

significant ($F_{2, 187}$=0.80, p=0.45; Fig S3.1a, d). As a result, we have used foliar N and P concentrations, rather than treatment levels as independent variables in regression analyses. This provides us with considerable variation in foliar nutrient concentrations (Fig. S3.1) with which to examine effects on herbivore performance.

D. plexippus eggs were obtained from a colony maintained in our lab. 250 eggs were collected for the experiment and stored in a refrigerator for 2 days prior to the experiment to synchronize hatching. Each neonate caterpillar was assigned randomly to receive foliage from a single plant. Caterpillars were maintained individually in 163 ml plastic containers. Each day, we retrieved fresh leaves from each plant and fed them *ad libitum* to their associated caterpillars. The experiment lasted for 7 days in total. This period represents 50% of the average larval period of monarchs under our rearing conditions: plants were not large enough to rear all caterpillars through to pupation, so we kept the number of rearing days constant to better compare growth rates among treatments. Effects of foliage quality on monarch growth are known to be most important during early instars (Zalucki et al. 2001). After 7 days of feeding, all caterpillars were starved for 24h to void their gut contents. They were then oven dried and their dry mass measured on a microbalance. The average daily growth rate of each caterpillar was calculated by dividing its log transformed mass by 7.

To measure foliar cardenolide concentrations, the major defensive chemicals in the genus *Asclepias,* we followed Zehnder & Hunter (2009). Briefly, 6 leaf disks from the fourth pair of leaves of each plant were taken and ground in methanol using a ball mill and sonicated at 60 °C for 1 h. Another 6 leaf disks were taken and oven dried to provide estimates of sample dry

weights. The supernatant from samples in methanol was evaporated at 45 °C for 70 min until dryness. Samples were then resuspended in 150 uL methanol containing 0.15 mg/mL digitoxin as an internal standard and analyzed using reverse phase ultra high performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA). Running time for each sample was 9 min. Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300 nm. Peaks with symmetric absorption maxima between 216 and 222 nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of the internal standard and the estimated sample mass. For the same leaves from which disks were taken, the remaining leaf material was oven dried and ground for subsequent carbon (C), N and P measurements. Foliar C and N concentrations were measured on a CHN analyzer (Costech, Valencia, CA, USA) and foliar P concentrations were quantified by an autoanalyzer using an acid digestion method (Tao & Hunter 2012). Only N and P concentrations were used in subsequent analyses because insect herbivores are generally considered to be N and/or P limited (Denno & Fagan 2004; Sterner & Elser 2002).

Statistical Analysis

To examine the effects of plant traits on *D. plexippus* growth rates, we used general linear models with N, P, cardenolide concentration, plant species identity and all possible interactions as fixed factors, and growth rate of *D. plexippus* as the dependent variable. Because of expectations that caterpillar responses to nutrient availability would be non-linear (see

introduction), we also included quadratic terms for both N and P and their interactions with other terms. A minimal model was derived using a backward selection procedure (Kleinbaum et al. 2003) in which terms were retained in the model if their removal significantly ($p<0.05$) reduced the explanatory power of the model (Crawley 2007). To test potential effects of N, P and species identity on the concentrations of cardenolide in leaves, we used the three factors and their interactions as independent variables and cardenolide concentration as the dependent variable.

Because of strong interactive effects of plant species and foliar traits on insect performance, we developed species-specific models to explore the effects of foliar N, P, cardenolide concentration and their interactions on the growth rate of *D. plexippus*. Because we found strong interactive effects of foliar N and cardenolide concentrations on monarch growth rates feeding on some plant species (below), we illustrated the nature of these interactions with contour plots using weighted least squares to fit a local polynomial trend surface to approximate the changes in caterpillar growth rate across N and cardenolide gradients in each milkweed species. We provide additional scatterplots (Fig. S3.3) illustrating the interactions between foliar N and cardenolide concentration on caterpillar growth rate.

All statistical analyses were performed in R 2.13.0 (R Development Core Team 2011), and contour plot were generated by the Lattice package.

Results

The growth rate was greatest at intermediate foliar N concentrations, initially increasing and then decreasing as foliar N concentration increased (Table 1; linear term, $F_{1,185} = 55.53$, $p <$

0.001, slope = 0.242; quadratic term, $F_{1,185} = 4.83$ p = 0.03, slope = -0.031). This combination of slopes indicates that, across the three milkweed species, the highest caterpillar growth rates were achieved when foliar nitrogen concentration was around 3.9%. Caterpillars that fed on *A. incarnata* and *A. curassavica* had higher growth rates (3.14 ± 0.02 and 3.13 ± 0.01, respectively) than did caterpillars feeding on *A. syriaca* (2.84 ± 0.02, units are log μg/day for all species) (Species effect $F_{2,185} = 34.43$, $p < 0.001$). Across all three milkweed species, caterpillar growth rates declined with increasing foliar cardenolide concentration (cardenolide effect: $F_{1,185} = 4.42$, p = 0.04; slope = -0.02 ± 0.008). In addition, the effects of foliar N concentration on the growth rate of *D. plexippus* varied among milkweed species ($F_{2,185} = 4.63$, p = 0.01, see full details below). Together, these five variables explained 43.58% of variations in the growth rate of *D. plexippus*. There were neither independent nor interactive effects of foliar P concentration on the growth rate of *D. plexippus* larvae and its effects on larval performance are not considered further.

A. curassavica and *A. incarnata* had similar foliar N concentrations (3.32 ± 0.26% and 3.68 ± 0.26%, respectively), while *A. syriaca* had significantly lower foliar N concentrations than the other species (1.62 ± 0.28%) ($F_{2, 193} = 100.05$, $p<0.001$). On average, foliar N concentration in *A. syriaca* was 105% and 127% lower than that in *A. curassavica* and *A. incarnata*, respectively. Cardenolide concentrations were highest in *A. curassavica* at 2.79 ± 0.16 mg/g, followed by *A. syriaca* (0.57 ± 0.05mg/g) and *A. incarnata* (0.05 ± 0.01mg/g) ($F_{2, 190} = 290.67$, $p < 0.001$). In other words, the foliar cardenolide concentration of *A. curassavica* was 4.9 and 56 times higher than the foliar cardenolide concentrations of *A. syriaca* and *A. incarnata*,

respectively. There was no relationships between foliar N, nor its interaction with milkweed identity, and the concentration of cardenolides in milkweeds (Table S3.1; N: $F_{1,181} = 0.14$, $p = 0.71$; N by species interaction: $F_{2,181} = 0.01$, $p = 0.99$), illustrating that N levels did not affect cardenolide production in any of the milkweed species that we studied. However, there was a significant positive relationship between foliar P concentration and cardenolide concentration ($F_{1,181} = 14.22$, $p<0.001$, slope = 5.28 ± 0.99; Table S3.1, Fig. S3.2).

We also developed species-specific general linear models using foliar N, P, cardenolide concentration and all possible interactions as independent variables (Table 3.2). The results from species-specific models corroborate those from the full model that the effects of foliar N concentration on caterpillar growth rate differed among species. In *A. curassavica,* increases in foliar N concentration were associated with decreases in the growth rate of *D. plexippus* ($F_{1,59} = 9.42$, $p = 0.003$, slope = -0.16 ± 0.1, Fig. 3.1a). In *A. incarnata*, there was no significant relationship between the growth rate of *D. plexippus* and variation in foliar N concentrations ($F_{1,69} = 0.35$, $p = 0.55$, Fig 3.1b). In contrast, increasing N concentrations were associated with an increase in larval growth rate in *A. syriaca* ($F_{1,41} = 5.45$, $p = 0.02$, slope= 0.34 ± 0.17, Fig. 3.1c).

We have illustrated the interactive effects of foliar N concentration and cardenolide on larval growth rates by contour plots (Fig. 3.2, S3.3). In *A. curassavica,* at low cardenolide levels, changes in *D. plexippus* growth rates were relatively minor across gradients in foliar N concentration. However, at high cardenolide concentrations, *D. plexippus* growth rates declined steeply with increasing foliar N concentration (Fig. 3.2a, S3.3a) (N × cardenolide interaction $F_{1,63} = 4.93$, $p = 0.03$, Table 3.2). In other words, the deleterious effects of excess N on larval growth

were expressed increasingly at high cardenolide levels. The complimentary view is that reductions in larval growth rate caused by increasing cardenolide concentrations are greatest at high N levels, suggesting that the per unit toxicity of cardenolide increases as N increases in excess of the optimum. In contrast, the cardenolide concentrations are too low in *A. incarnata* (0 - 0.32 mg/g) to facilitate any negative effects of high N concentration on larval growth (N × cardenolide interaction $F_{1,73} = 0.18$, $p = 0.66$, Fig. 3.2b). Finally, foliar N concentrations in *A. syriaca* are always below those that interact significantly with foliar cardenolide (N × cardenolide interaction $F_{1,45} = 0.003$, $p = 0.96$, Fig. 3.2c).

Discussion

High foliar N concentrations in milkweed were associated with decreases in the growth rate of *D. plexippus* larvae. However, such negative effects of N were found only when larvae were feeding on *A. curassavica,* which also had high foliar concentrations of cardenolide, a widespread chemical defense in the genus *Asclepias*. Thus, for this species, the per unit toxicity of cardenolide was higher when N occurred in excess of optimum concentrations for larval growth, providing an additional mechanism by which high nutrient availability can cause declines in consumer fitness (Boersma & Elser 2006). When feeding on *A. incarnata,* which had similar high foliar N concentrations, excess N did not have significant negative effects on larval growth, presumably because cardenolide concentrations are on average 40 times lower than those of *A. curassavica*. In addition to illustrating a new mechanism for the negative effects of excess nutrients on consumers, our work emphasizes that future studies of species interactions

should focus increasingly on interactions between nutrients and defense chemicals (Raubenheimer & Simpson 2009).

During pre-ingestion periods, nutrients in excess of demand can inhibit food consumption, resulting in lower organismal growth rates, as has been shown for some grasshoppers (Raubenheimer & Simpson 1999). In *D. plexippus,* high nitrogen concentration has been shown to reduce consumption of *A. syriaca* (Lavoie and Oberhauser 2004). However, we do not think reduced consumption is the mechanism underlying the negative effects of N on caterpillar growth in our study. First, the N fertilization levels in Lavoie and Oberhauser (2004) were about 10 times higher than those in our study. Second, if the high foliar N levels in our study acted to reduce consumption, we should have seen similar declines in caterpillar growth rates on *A. incarnata* as we saw on *A. curassavica* as they share similar foliar N concentrations. However, caterpillar growth rates were unaffected by high foliar N concentrations in *A. incarnata.* A second mechanism to explain reductions in consumer growth under high nutrient concentrations proposes that post-ingestive costs of excreting excess nutrient may translate into reduced fitness (Boersma & Elser 2006). Although there is indirect evidence for this mechanism operating in aphids (Zehnder & Hunter 2009), it is difficult to measure costs of excretion directly. As before, our current experiment suggests that this is an unlikely explanation for our data. If operating, we would expect similar excretion costs in caterpillars fed on *A. incarnata* and *A. curassavica*, which had similarly high foliar N concentrations. A third proposed mechanism, which perhaps is relatively common, is that nutrient enrichment results in correlated changes in foliar defenses that themselves influence consumer performance (Plath & Boersma 2001; Tao & Hunter 2012).

Although fertilization by N:P:K (potassium) fertilizers has been shown to reduce total concentrations of cardenolide across several milkweed species (Agrawal et al. 2012), associations between foliar nutrient concentrations and foliar cardenolide concentrations are less well established. In our experiment, N concentrations were not correlated with cardenolide production in any of the three species that we studied ($p=0.73$, 0.89, 0.93 for *A. curassavica, A. incarnata* and *A. syriaca* respectively). Rather, we observed increases in foliar cardenolides associated with high foliar P concentrations. Because foliar N and foliar cardenolide concentrations are unrelated in our study, we can discount the possibility that negative effects of high foliar N on monarchs result from concomitant increases in foliar cardenolides. In addition, by removing individual leaves before feeding them to the caterpillars, we removed any potential effects of latex on the growth rate of *D. plexippus.*

In general, relationships between foliar nutrients and chemical defenses are not as simple as once thought (Bryant, Chapin & Klein 1983). Rather, the effects of nutrient availability on chemical defense are now recognized to vary with plant functional group and life history, and cannot easily be predicted from current theories of plant defense (Koricheva et al. 1998; Hamilton et al. 2001; Vannette & Hunter 2011). Studies that associate variation in foliar defense with foliar nutrient availability should include a broad mixture of intra- and interspecific studies, to measure the importance of phylogenetic signal and phenotypic plasticity in such responses (Agrawal & Fishbein 2006). The fact that cardenolide production is correlated with foliar P concentration in milkweeds suggests that synthetic pathways of cardenolides may be tightly controlled by P containing intermediates and/or cell energetic needs. As anthropogenic N

deposition increases, understanding the mechanisms by which nutrient availability influences foliar chemical defense will become increasingly important (Throop & Lerdau 2004; Zehnder & Hunter 2008).

In our study, the strong interaction between foliar N and cardenolide concentrations suggest that the negative effects of high N concentration on monarch growth arise from increasing toxicity of cardenolides in *A. curassavica.* Although there is accumulating evidence that dietary nutrients can affect the impact of secondary metabolites on herbivore performance (Raubenheimer & Simpson 2009), the majority of such evidence to date has involved low nutrient levels. For example, during pre-ingestion periods, inhibitory effects of chemical defenses on feeding may be greater at low nutrient levels, because the stimulatory effects of nutrients are not enough to counteract inhibitory effects of defenses (Glendinning & Slansky 1994). The result is a higher expression of deterrence per unit concentration of chemical defense when the nutrient is limiting (Simpson & Raubenheimer 2001, Cruz-Rivera & Hay 2003). Alternatively, during post-ingestion periods, there can be direct chemical interactions between nutrients and secondary metabolites, such that effective concentrations of nutrients vary with levels of defense chemicals. For example, responses of brushtail possums to N variability in *Eucalyptus* leaves depend on the foliar concentration of condensed tannins (DeGabriel et al. 2009). When foliar N levels are high, possums suffer less per unit increase in condensed tannin concentrations, because after precipitation by condensed tannins there remains enough protein for the herbivore. In our study, foliar N concentrations were lowest in *A. syriaca.* We found no interactive effect of foliar N and cardenolide on caterpillar growth on *A. syriaca,* presumably

because cardenolide concentrations are also relatively low. In contrast, monarch growth rate appeared more strongly affected by foliar cardenolide concentration on *A. incarnata* under low P concentrations (Table 3.2). While this might suggest that cardenolide toxicity is higher under low P concentrations, we oberved no such effect on either *A. syriaca* or *A. curassavica*, suggesting some alternative mechanism. Future studies should include adding cardenolides experimentally to low N and P milkweed species to explore in more detail this end of the N/P/cardenolide spectrum.

We are only aware of one other published example where foliar chemical defenses appear more toxic under high dietary nutrients. In their work with African grasshoppers, Simpson & Raubenheimer (2001) found that excess protein led to higher toxicity of condensed tannins during post ingestion periods. In our system, we also speculate that the interaction between cardenolide and excess N occurs during post ingestion periods because cardenolides in milkweed act as feeding stimulants rather than as deterrents (Zalucki, Bower & Malcolm 1990), making pre-ingestion effects of high cardenolide on monarchs unlikely. To test this hypothesis, future studies should include direct measures of consumption, excretion and growth efficiency of herbivores.

Summary & Conclusions

Although many classic studies have explored the independent effects of foliar nutrients and secondary chemicals on consumers, studies of their interactions remain scarce. Here, we show that high foliar N concentrations increase the per unit toxicity of a potent plant defense, reducing the growth rate of a specialist insect herbivore. With increased nutrient loading in both

aquatic and terrestrial ecosystems, such interactions between chemical defense and plant nutrients may become increasingly prevalent. Exploring the nature and mechanisms underying such interactions will be important in future studies of plant defense and co-evolution between plants and herbivores.

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Table 3.1 Results of a general linear model examining the effects of foliar nitrogen (N) concentration, milkweed species, foliar cardenolide concentration and their interactions on the growth rate of *D. plexippus* larvae feeding on *A. syraica, A. incarnata* and *A. curassavica.*

	F	p
N	$F_{1,185}$=55.53	<0.001
Species	$F_{2,185}$=34.43	<0.001
Cardenolide	$F_{1,185}$=4.42	0.04
N	$F_{1,185}$=4.83	0.03
N × Species	$F_{2,185}$=4.63	0.01

Table 3.2 Results of general linear models examining the effects of foliar nitrogen (N) concentration, phosphorus (P) and cardenolide concentration and their interactions on the growth rate of *D. plexippus* larvae on individual species of *Asclepias*.

		A. syriaca	*A. curassavica*	*A. incarnata*
N	F	$F_{1,41}$=5.45	$F_{1,59}$=9.42	$F_{1,69}$=0.35
	p	0.02	0.003	0.55
	slope	0.34 (0.17)	-0.16 (0.1)	0.08 (0.07)
P	F	$F_{1,41}$=3.56	$F_{1,59}$=0.12	$F_{1,69}$=0.17
	p	0.07	0.73	0.69
	slope	4.01 (1.96)	-2.99 (1.66)	0.58 (0.91)
Cardenolide	F	$F_{1,41}$=2.78	$F_{1,59}$=2.67	$F_{1,69}$=0.21
	p	0.10	0.11	0.65
	slope	-0.13 (0.48)	-0.16 (0.13)	-1.87 (3.81)
N × P	F	$F_{1,41}$=1.16	$F_{1,59}$=0.03	$F_{1,69}$=0.13
	p	0.27	0.85	0.72
	slope	-1.62 (0.80)	0.76 (0.39)	-0.24 (0.23)
N × Cardenolide	F	$F_{1,41}$=0.22	$F_{1,59}$=5.83	$F_{1,69}$=0.29
	p	0.65	0.02	0.59
	slope	-0.25 (0.24)	-0.007 (0.002)	-0.12 (0.88)
P × Cardenolide	F	$F_{1,41}$=1.37	$F_{1,59}$=0.21	$F_{1,69}$=6.18
	p	0.24	0.65	0.02
	slope	-5.14 (2.52)	0.03 (0.03)	11.76 (15.49)
N × P × Cardenolide	F	$F_{1,41}$=3.01	$F_{1,59}$=3.49	$F_{1,69}$=0.03
	p	0.09	0.07	0.87
	slope	1.78 (1.03)	-0.02 (0.12)	-0.54 (3.37)

Table S3.1 Results from a general linear model examining the effects of milkweed species, foliar nitrogen (N) concentration, foliar phosphorus (P) concentration, and their interactions on foliar cardenolide concentrations in *A. syriaca, A. incarnata* and *A. curassavica.*

Species	N	P	Species*N	Species*P	N*P
$F_{2,181}$=241.43 p<0.001	$F_{1,181}$=0.14 p=0.71	$F_{1,181}$=14.22 p<0.001	$F_{2,181}$=0.01 p=0.98	$F_{2,181}$=1.79 p=0.18	$F_{2,181}$=0.33 p=0.56

Figure 3.1 Relationship between foliar nitrogen (N) concentration and the growth rate of *D. plexippus* larvae feeding on *A. curassavica* (a), *A. incarnata* (b) and *A. syriaca* (c). Regression lines illustrate significant relationships.

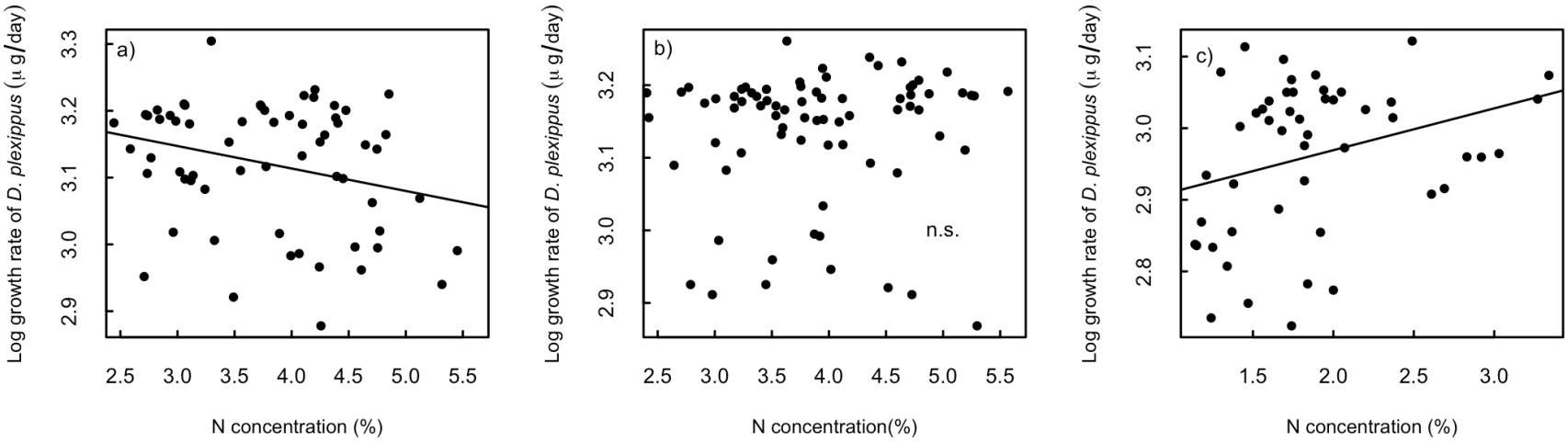


Figure 3.2 The growth rate of *D. plexippus* larvae across variation in foliar nitrogen (N) and cardenolide concentrations. In the contour plots, the x-axis represents N concentration (%), the y-axis represents cardenolide concentration (mg/g), and color represents the larval growth rate of *D. plexippus* (log μg/day). Responses are illustrated for larvae on *A. curassavica* (a), *A. incarnata* (b) and *A. syriaca* (c).

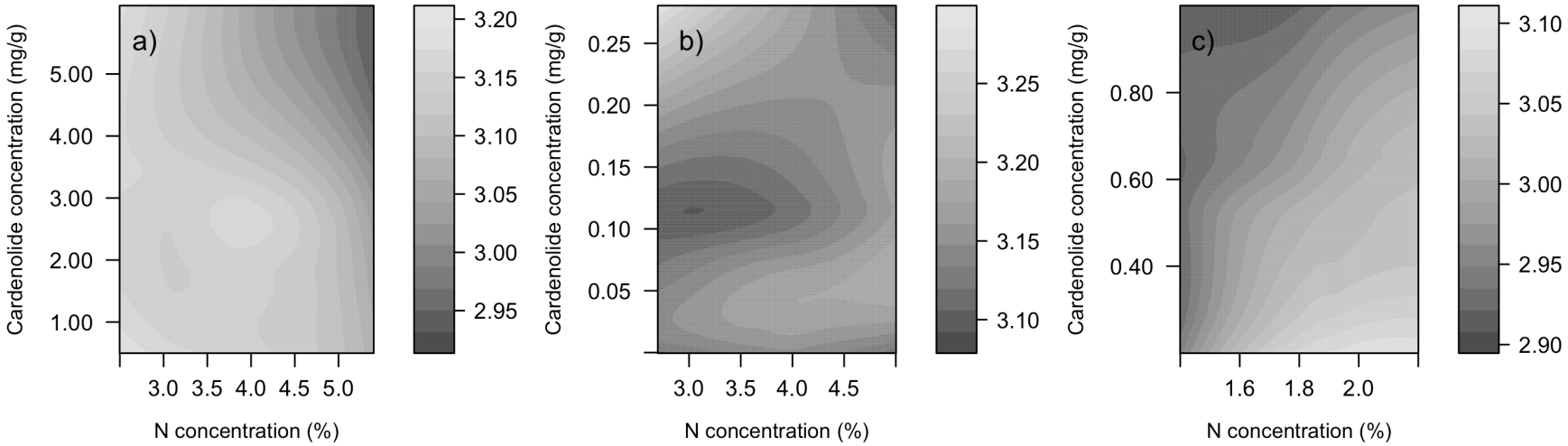


Figure S3.1 Effects of N (a, b) and P (c, d) fertilization on foliar N (a, c) and P (b, d) concentrations of *A. syriaca, A. curassavica* and *A. incarnata.*

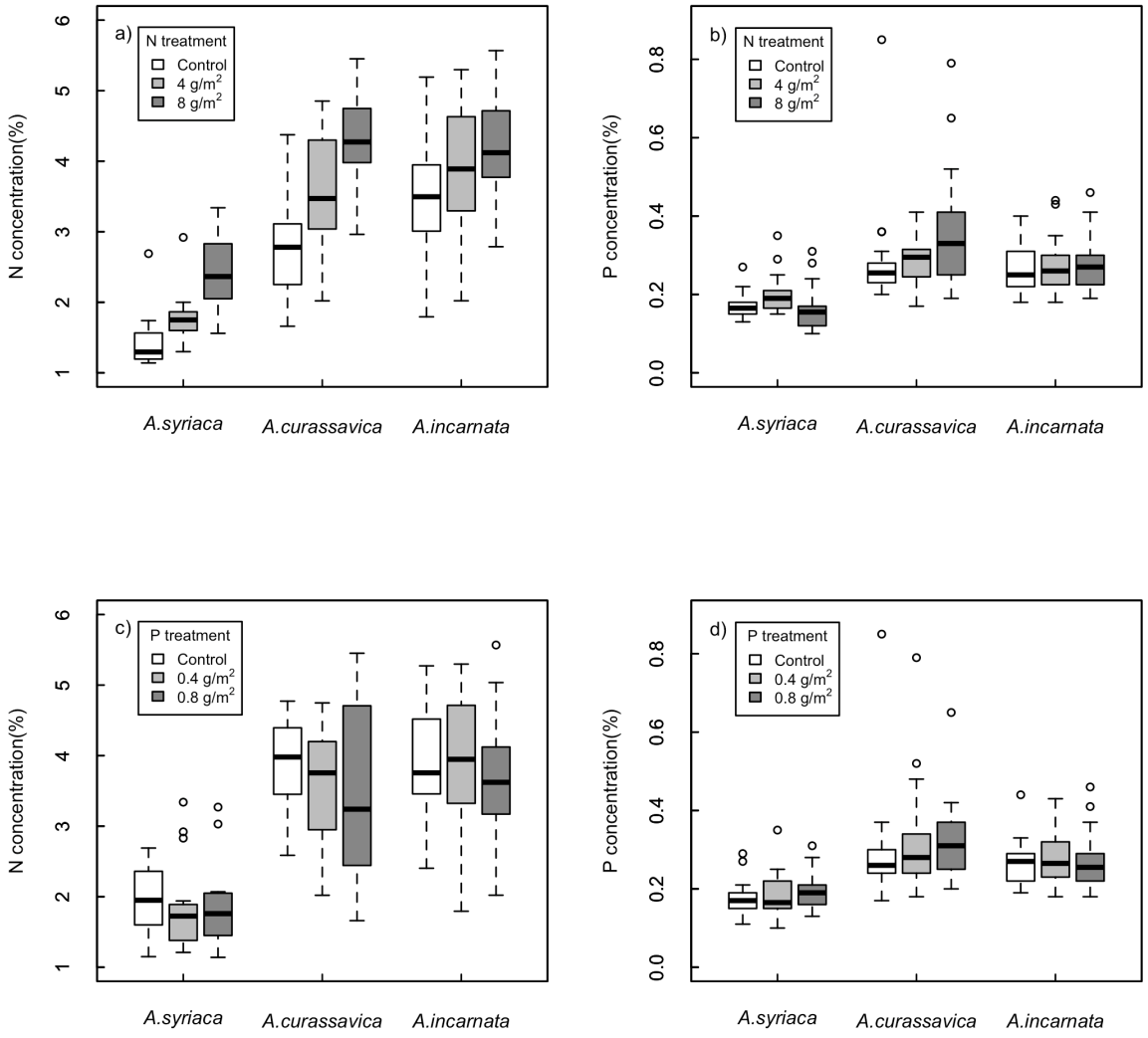


Figure S3.2 Relationship between foliar P concentration (%) on foliar cardenolide concentration (mg/g) in *A. curassavica* (a), *A. incarnata* (b) and *A. syriaca* (c).

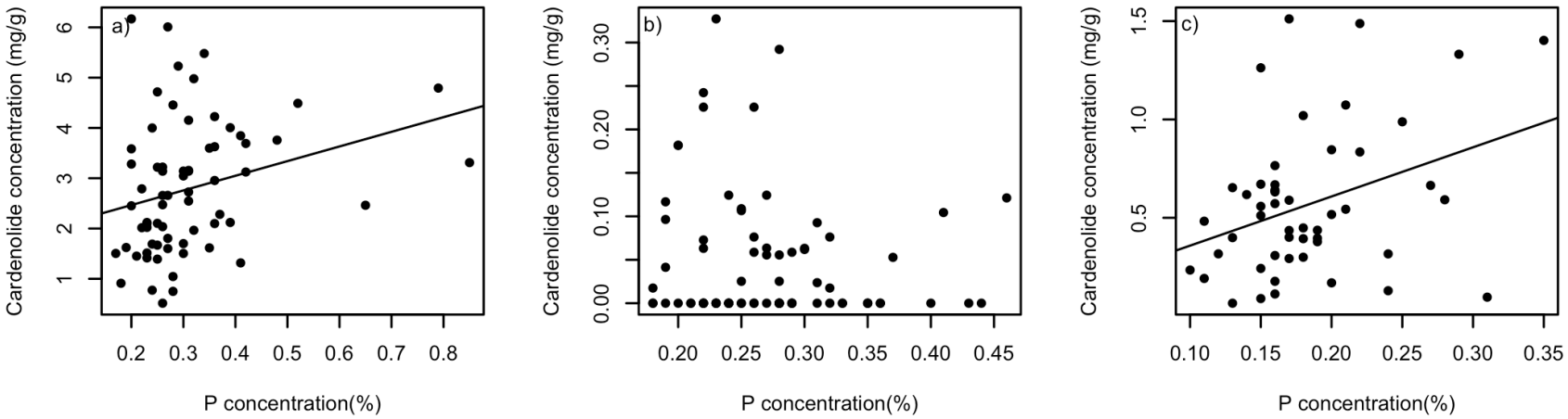
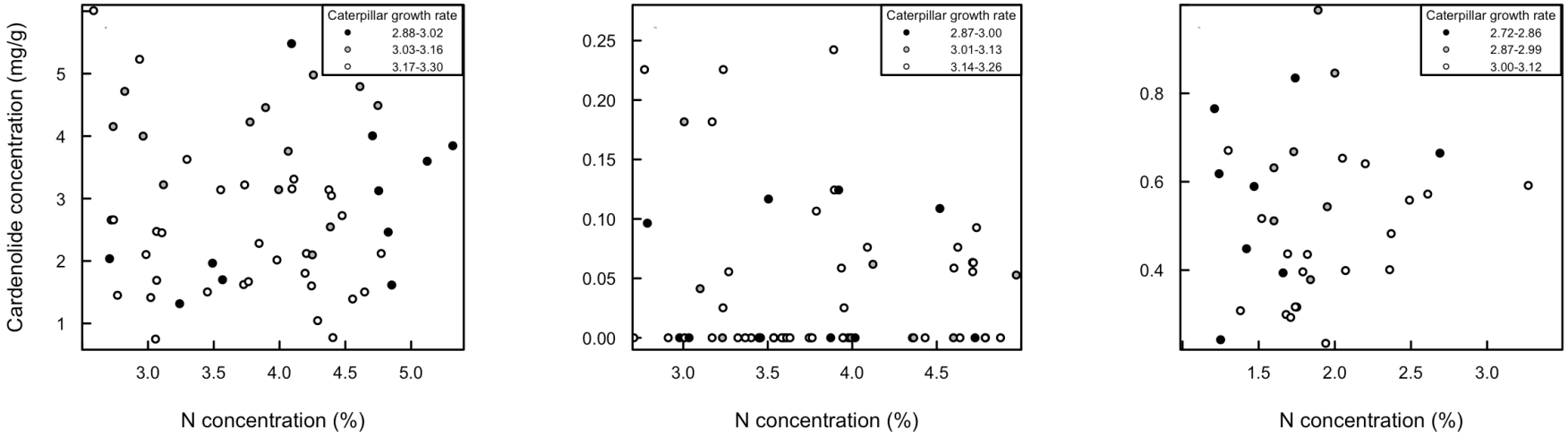


Figure S3.3 Scatterplot showing the growth rate of *D. plexippus* larvae across variation in foliar nitrogen (N) and cardenolide concentrations in (a) *A. curassavica*, (b) *A. incarnata* and (c) *A. syriaca*.



Chapter 4

Effects of soil nutrients on the sequestration of plant defense chemicals by the specialist insect herbivore, *Danaus plexippus*

Abstract

Although anthropogenic nitrogen enrichment has significantly changed the growth, survival and reproduction of herbivorous insects, its effects on the defensive sequestration of secondary chemicals by insect herbivores are less well understood. Previous studies have shown that soil nutrient availability can affect sequestration directly through changing plant defense concentrations or indirectly through altering growth rates of herbivores, but there has been less exploration of its effects on consumption of secondary chemicals and sequestration efficiency. In the current study, we examined the overall effect of soil N availability on cardenolide sequestration by *D. plexippus*. Specifically, we measured the effects of soil nutrient availability on growth, consumption, excretion and sequestration efficiency of cardenolides by the monarch caterpillar *Danaus plexippus* fed on the tropical milkweed *Asclepias curassavica.* We also measured the effects of soil nutrient availability on foliar cardenolide concentration. We found that soil N and P fertilization significantly reduced the growth rate and the

sequestration efficiency of cardenolides by monarch caterpillars feeding on *A. curassavica*. The lowered sequestration efficiency was accompanied by higher concentrations of cardenolides in frass, suggesting that higher cardenolide excretion rates under high N and P fertilization levels may help to reduce toxicity of cardenolides to the caterpillars; cardenolide toxicity is higher under high foliar N concentrations (Chapter 3). Although the total cardenolide contents of caterpillars were lower under high N or P fertilization levels, caterpillar cardenolide concentrations were constant across fertilization treatments because of lower growth rates (and therefore lower body mass) under high fertilization. We conclude that anthropogenic N deposition may have multiple effects on insect herbivores, including their ability to defend themselves from predators with sequestered plant defenses.

Introduction

Plants produce secondary chemicals as defenses against herbivory (Rhoades 1979, Karban and Baldwin 1997). However, many specialist insect herbivores have evolved the ability to detoxify (Dowd et al. 1983), and even sequester plant-derived secondary compounds for their own defense (Nishida 2002, Opitz and Müller 2009). Sequestration plays an important role in ecological and evolutionary interactions between insects and other species (Bowers 1992, Nishida 2002), and it is important therefore to explore the ecological factors that affect the efficiency of sequestration.

Soil nutrient availability has significant potential to influence the sequestration of foliar defense chemicals by insects. The effects of soil N availability on sequestration has become increasingly relevant because anthropogenic N deposition has significantly changed the input of N to terrestrial ecosystems (Vitousek et al. 1997), Although there have been many studies of N deposition on the individual growth of insect (Throop and Lerdau 2004), its effects on the interaction between herbivores and their predators are less well understood. Existing studies suggest that soil N availability can affect sequestration through two mechanisms. First, soil nutrient availability can affect the concentrations of secondary chemicals in plant tissues, which in turn can result in changes in levels of sequestration. For example, nitrogen (N) fertilization reduces the production of aucubin (an iridoid glycoside) in ribwort plantain (*Plantago lanceolata*), which subsequently leads to lower levels of aucubin sequestration by common buckeye caterpillars, *Junonia coenia* (Prudic et al. 2005). Second, soil nutrient availability can indirectly affect sequestration by changing insect growth rate. In *Linaria dalmatica* for example, foliar concentration of iridoid glycosides are unaffected by N fertilization. However, because the body weight of the toadflax caterpillar (*Calophasia lunula*) is significantly higher under N fertilization, the concentration of sequestered iridoid glycosides is significantly lower (Jamieson and Bowers 2012).

Although previous studies have shown that soil nutrient availability can affect sequestration directly through changing plant defense concentrations or indirectly through altering growth rates of herbivores, there has been less exploration of its effects

on sequestration efficiency. Sequestration efficiency describes the proportion of ingested defense chemical that is retained by the herbivore (Bowers and Collinge 1992, Camara 1997), which is related to insect ontogeny (Bowers and Collinge 1992), host plant chemistry (Burghardt et al. 2001, Bowers 2003), insect genotype (Camara 1997) and insect gender (Burghardt et al. 2001). As a result, variation in sequestration efficiency can affect sexual selection and life history evolution of insect herbivores. In addition, sequestration efficiency reflects the ability of insect herbivores to avoid auto-toxicity and eliminate excess secondary metabolites, which should be actively selected for during the evolution of sequestration (Camara 1997). Therefore, to fully understand the mechanisms by which soil nutrient availability can influence sequestration, it is important to examine how it influences sequestration efficiency.

Previously, we found that when a cardenolide sequestering insect, the monarch caterpillar *Danaus plexippus* fed on the tropical milkweed *Asclepias curassavica*, the toxicity of cardenolide increased with foliar N concentration (Tao and Hunter in revision). Consequently, we predicted that under high N conditions, *D. plexippus* would exhibit a lower sequestration efficiency of cardenolides. This prediction could be realized through at least two mechanisms First, when insect growth rate and overall vigor are low, allocation to other functions is also generally low (Cotter et al. 2011). Second, in order to reduce the toxicity of cardenolides, *D. plexippus* may increase food passage time, leading to lower sequestration efficiency. Although we could not differentiate between these alternatives here, we were able to test our overall predication that sequestration efficiency

would decline with increasing N availability. We examined the effects of soil nutrient availability on growth, consumption, excretion and sequestration efficiency of cardenolides by *D. plexippus* fed on *A. curassavica.* In addition, we explored the effects of N fertilization on foliar cardenolide concentrations. Integrating the above analyses, we were able to examine the overall effect of soil N availability on cardenolide sequestration by *D. plexippus.*

Materials and methods

The tropical milkweed, *Asclepias curassavica* is a natural host plant for the monarch caterpillar, *Danaus plexippus.* The specialist monarch has evolved resistance to cardenolides (Holzinger and Wink 1996) and the ability to sequester them as a defense against predators (Reichstein et al. 1968). Previous studies have shown that the sequestration of cardenolide by *D. plexippus* is highly selective, such that sequestration efficiency is subject to change with variation in foliar cardenolide concentration and composition (Malcolm et al. 1989).

We purchased *A. curassavica* seeds from Butterfly Encounters, Inc. The seeds were germinated on damp filter paper in petri dishes at 25 °C. After germination, seedlings were planted in 4 inch plant pots containing a 1:1 mixture of potting soil (SunGrow Horticulture, Canada) and sand (Kolorscape). Plants were kept in a controlled growth chamber at 25 °C and an L16:D8 light cycle. When plants were 3-weeks old, 3 by 3 levels of N and phosphorus (P) fertilizer were applied in a factorial design. N was added

as ammonium nitrate at levels of 0, 4, 8 g m^{-2} yr^{-1}, and P was added as calcium phosphate monobasic at levels of 0, 0.4, 0.8 g m^{-2} yr^{-1}. Fertilizer was applied once every week for a total of 5 weeks; each plant received 1/5 of its total fertilizer allocation each week. We have shown previously that these levels of fertilization generate a broad but realistic range of C:N:P stoichiometry in milkweed plants (Tao and Hunter 2012). We used 10 plants per treatment, resulting in a total of 90 plants.

We started our experiment two days after the last fertilization, when plants were just over 8 weeks old. Before feeding the leaves to individual caterpillars, we measured foliar cardenolide concentrations from each plant using methods described in Zehnder & Hunter (2009). Briefly, 6 leaf disks from the fourth pair of leaves of each plant were ground in methanol using a ball mill and sonicated at 60 °C for 1 h. Another 6 leaf disks were taken, weighed for fresh weight and then oven dried to provide estimates of foliar fresh-dry weight ratios for each plant. The supernatant from samples in methanol was evaporated at 45 °C for 70 min until dryness. Samples were then resuspended in 150 uL of methanol containing 0.15 mg/mL digitoxin as an internal standard and analyzed using reverse phase ultrahigh-performance liquid chromatography (UPLC, Waters Inc., Milford, MA, USA). Running time for each sample was 9 min. Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300 nm. Peaks with symmetric absorption maxima between 216 and 222 nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all separated cardenolide peaks, corrected by the concentration of the internal standard

(digitoxin) and the estimated sample mass. For the same leaves from which disks were taken, the remaining leaf material was oven dried and ground for subsequent carbon (C), N and P measurements. Foliar C and N concentrations were measured on a CHN analyzer (Costech, Valencia, CA, USA) and foliar P concentrations were quantified on an autoanalyzer using an acid digestion method (Tao and Hunter 2012).

D. plexippus eggs were obtained from a colony maintained in our lab. Around 100 eggs were collected for the experiment and stored in a refrigerator for 2 days prior to the experiment to synchronize hatching. Each neonate caterpillar was assigned randomly to receive foliage from a single plant. Caterpillars were maintained individually in 163 ml plastic containers. Each day, we retrieved fresh leaves from each plant and fed them *ad libitum* to their associated caterpillars. To quantify consumption, we measured the fresh weight of leaves fed to each caterpillar, and converted it to dry weight using the fresh-dry conversion described above. Before the next feeding, we retrieved the leftover leaves from the previous day, oven-dried and weighed them. Consumption was then calculated as the difference in dry mass between amounts fed to each caterpillar and leftover leaves. The experiment lasted for 7 days in total, which represents 50% of the average larval period of monarchs under our rearing conditions; plants were not large enough to rear all caterpillars through to pupation, so we kept the number of rearing days constant to better compare growth rates among treatments. Effects of foliage quality on monarch growth are known to be most important during early instars (Zalucki et al. 2001). After 7 days of feeding, all caterpillars were starved for 24h to void their gut contents. They were then

oven dried and their dry mass measured on a microbalance. Frass from each caterpillar was also collected, dried and weighed. Caterpillar and frass cardenolide concentrations were measured by UPLC as described above.

Statistical analysis

Total consumption was calculated as the sum of consumption on each day, and total consumption of cardenolides was calculated as the product of foliar cardenolide concentration and total consumption. The average daily growth rate of each caterpillar was calculated by dividing its log transformed mass by 7. Total cardenolide content of each *D. plexippus* individual was calculated as the product of caterpillar weight and cardenolide concentration. Sequestration efficiency was calculated by dividing total cardenolide content of caterpillars by their total consumption of foliar cardenolide. During the experiment, 20 caterpillars died; in addition, we lost 3 samples during cardenolide processing, leaving 67 caterpillars for analysis.

To explore the effects of our N and P fertilization treatments on foliar chemical traits, we used N and P treatment levels and their interactions as independent variables, and foliar N, P and cardenolide concentrations as dependent variables in separate general linear models. To examine how fertilization and foliar cardenolide concentration affected (a) growth rate, (b) total consumption of plant material, (c) consumption, excretion and sequestration efficiency of cardenolides, (d) cardenolide concentration and (e) total cardenolide contents of *D. plexippus*, we used the above measurements as dependent

variables in separate general linear models, and N, P fertilization level, foliar cardenolide concentration and their interactions as independent variables. All analyses were conducted in R 2.15.2 (2012).

Results

N fertilization significantly increased foliar N and P concentrations in *A. curassavica* (Fig. 4.1a, b, Table 4.1; $F_{2,81}$=51.46, p<0.001; $F_{2,81}$=7.08, p=0.001). Foliar cardenolide concentration was also affected by N fertilization ($F_{2,78}$=15.54, p<0.001). Specifically, the highest cardenolide concentration occurred in plants under low N treatment, and the lowest concentration occurred in plants under intermediate N fertilization (Fig. 4.1c). Although P fertilization did not have any significant independent effects on foliar N, P or cardenolide concentrations, there were significant interactive effects of N and P fertilization on all three foliar traits (Table 4.1). Specifically, under low and intermediate N fertilization levels, P fertilization decreased foliar N concentration; under high N levels, P fertilization increased foliar N concentration (Fig. 4.1a). Similarly, P fertilization increased foliar P concentration only under intermediate and high N fertilization levels (Fig. 4.1b). Finally, under high N fertilization, P fertilization increased foliar cardenolide concentration, although it decreased cardenolide concentration under low and intermediate N fertilization levels (Fig. 4.1c).

Total consumption of plant material declined with foliar cardenolide concentration (Table 4.2, Fig. 4.2a; $F_{1,49}$=7.67, p=0.008). In addition, consumption was affected by N

fertilization (Table 4.2; $F_{2,49}$=6.10, p=0.004) such that caterpillar consumption was greatest under medium N fertilization (Fig. 4.2b), presumably because cardenolide levels were lowest (Fig. 4.1c). However, total consumption of cardenolide was unaffected by N fertilization (Table 4.2; $F_{2,49}$=1.89, p=0.16).

Caterpillar growth rate was reduced by N fertilization (Table 4.2, Fig 4.2c; $F_{2,49}$=12.34, p<0.001). P fertilization also tended to reduce caterpillar growth rate, although the effects were marginally non-significant (Table 4.2, Fig 4.2d; $F_{2,49}$=2.89, p=0.07). There was a strong positive correlation between caterpillar growth rate and sequestration efficiency of cardenolide (Fig. 4.2e; $F_{1,60}$=32.53,p<0.001).

Sequestration efficiency was negatively correlated with foliar cardenolide concentration (Table 4.2, Fig. 4.2f; $F_{1,49}$=17.12, p<0.001). Moreover, N fertilization reduced sequestration efficiency of caterpillars (Table 4.2, Fig. 4.3a; $F_{2,44}$=4.38, p=0.02). High P, but not intermediate P fertilization level, also reduced sequestration efficiency (Fig. 4.3b; $F_{2,49}$=7.07, p=0.002). Because the total amount of cardenolide consumed by caterpillars was unaffected by fertilization (see above), the effects of N and P fertilization on total cardenolide contents of *D. plexippus* were similar to their effects on sequestration efficiency (Table 4.2, Fig. 4.3c, d). N fertilization increased cardenolide concentration in frass (Table 4.2, Fig. 4.3e; $F_{2,49}$=9.75, p<0.001). Similarly, under high P fertilization levels, frass cardenolide concentrations were greater than those under low or medium P levels, although the effect was marginally non-significant (Table 4.2, Fig. 4.3f; $F_{2,}$

$_{49}$=2.50, p=0.09). There were no effects of N, P, cardenolide or any of their interactions on cardenolide concentrations in *D. plexippus* (Table 4.2, Fig. 4.3g, h).

Discussion

In the current study, soil N and P fertilization significantly reduced the growth rate and the sequestration efficiency of cardenolides by monarch caterpillars feeding on *A. curassavica*. Generally, growth rate was positively related with sequestration efficiency, suggesting that allocation to sequestration was limited when growth rate was low. In addition, the lowered sequestration efficiency was accompanied by higher concentrations of cardenolides in frass. Combined with our previous finding that cardenolides were more toxic when foliar N concentration was high (Tao & Hunter, in revision), higher cardenolide excretion rates under high N and P fertilization levels may help to reduce toxicity of cardenolides to *D. plexippus* caterpillars. Although the total cardenolide contents of caterpillars were lower under high N or P fertilization levels, caterpillar cardenolide concentrations were constant across fertilization treatments because of lower growth rates (and therefore lower body mass) under high fertilization.

Previous studies on the effects of soil nutrient availability on sequestration have focused primarily on changes in the concentration and composition of foliar secondary chemicals (e.g. Prudic et al. 2005, Jamieson and Bowers 2012). However, the concentrations of secondary chemicals that are ultimately sequestered by herbivores depend not only on plant chemical concentration, but also on consumption and

sequestration efficiency (Camara 1997). These are traits that can vary with environmental and genetic factors, as well as with other properties of the diet. For example, previous studies have shown that when *D. plexippus* is feeding on plants with low cardenolide concentrations, sequestration efficiency is relatively constant across the lower ranges of cardenolide concentrations. However, when feeding on plants with high cardenolide concentrations, sequestration efficiency declines as foliar cardenolide concentrations increase (Lynch and Martin 1993). The net result is that cardenolide concentrations in caterpillars are positively correlated with those in the foliage of low cardenolide plants, but unrelated to foliar concentrations in the foliage of high cardenolide plants (Lynch and Martin 1993). Although in our study we did not observe any direct effects of N and P fertilization on cardenolide concentrations in *D. plexippus* caterpillats, it did not mean that soil nutrient availability did not affect the sequestration process. Rather, soil nutrient availability changed multiple factors simultaneously, including foliar cardenolide concentration, consumption by caterpillars, sequestration efficiency and caterpillar body mass. The offsetting effects of these variables yielded no overall effect on cardenolide concentration in caterpillars.

Sequestration is an active process that is accomplished by transport proteins in the hemolymph (Frick and Wink 1995, Hartmann 2004). Therefore, insects have the ability to control the uptake of different defense compounds as well as their total amounts. For example, flea beetles in the genus *Longitarsus* selectively store simple pyrrolizidine alkaloid (PA) monoesters as opposed to more complex compounds (Dobler et al. 2000).

Similarly, the catalpa sphinx caterpillar *Ceratomia catalpae* selectively stores catalpol instead of catalposide from its host plant *Catalpa bignonioides* (Bowers 2003). Such selective sequestration may have evolved to avoid auto-toxicity of the sequestered chemicals. For example, the chrysomelid beetle, *Oreina cacaliae*, absorbs and stores non-toxic N-oxide pyrrolizidine alkaloids, while pro-toxic free base pyrrolizidine alkaloids are detoxified by glucosylation (Hartmann et al. 1999). Similarly, in our system, *D. plexippus* can selectively sequester cardenolides with intermediate polarity as opposed to low polarity cardenolides (Malcolm and Brower 1989), presumably because non-polar cardenolides are of greater toxicity to the insect. Previously we have found that under high foliar N concentrations, toxicity of cardenolides to *D. plexippus* increases (Tao and Hunter in revision). Hence, the reduction in sequestration efficiency when cardenolide concentration increases may reflect another example of regulation of sequestration to avoid auto-toxicity.

Alternatively, the fact that N fertilization reduced sequestration efficiency could also be explained by lower caterpillar growth rates under high N availability. In some insect species, sequestration is a costly process (Bowers 1992) while in other species, sequestration does not incur significant costs (Kearsley and Whitham 1992, Camara 1997). In monarch caterpillars, the findings are mixed. While physiological studies have suggested that processing cardenolides incurs few costs (Erickson 1973, Vaughan and Jungreis 1977), ecological studies have found that cardenolide sequestration is negatively correlated with body mass (Cohen 1985) and migration distance (Brower et al. 1972,

Brower and Glazier 1975). In the current study, we also found a negative relationship between caterpillar growth rate and cardenolide concentration in *D. plexippus* (linear regression P=0.02, r^2=0.08, data not shown). Based on the above evidence, we suggest that cardenolide absorption and transportation may be of negligible cost to *D. plexippus*, but that sequestering large amounts of cardenolide incur auto-toxicity and high storage costs. Therefore, allocation to sequestration, as measured by sequestration efficiency, may be limited when growth rate is low, but less restricted when growth rate is high (Fig. 4.2e).

During sequestration, secondary chemicals can be absorbed directly, metabolized, or excreted (Nishida 2002, Hartmann 2004, Optiz and Muller 2009). The reduction in sequestration efficiency that we observed can be explained at least in part by increases in excretion of cardenolides (Fig. 4.3e, f), which could result from faster gut passage time and/or lower absorption efficiency of the food. Faster food passage time significantly reduces the mortality rate in gypsy moth *Lymatria dispar* caused by nuclear polyhedrosis virus (Keating et al. 1988). Similarly, infection rate by *Ophryocystis elektroscirrha*, a protozoan parasite of *D. plexippus* may become lower as gut passage rate increases because caterpillars are infected by parasites via the midgut. Because parasite infection is also related to dietary cardenolides (Lefevre et al. 2010, de Roode et al. 2011), this suggests an interesting direction for future research that incorporates cardenolide toxicity, nutrient availability and parasite infection in tritrophic studies of plant-insect-parasite interactions.

In the current study, we only measured the total concentration of cardenolides in caterpillars. However, cardenolide composition, especially polarity, is also important in cardenolide sequestration (Malcolm and Bower 1989) and interactions between *D. plexippus* and its protozoan parasite (de Roode et al. 2011). Therefore, future studies should place more emphasis on the effects of soil nutrient availability on chemical composition of foliar cardenolides to explore whether higher toxicity (Tao and Hunter in revision) and lower sequestration efficiency result from changes in cardenolide polarity.

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Table 4.1 Effects of N and P fertilization, and their interaction, on foliar N, P and cardenolide concentration of *Asclepias curassavica*

	Soil N		Soil P		Soil N × P	
	F	p	F	p	F	p
Foliar N	$F_{2,81}$=51.46	<0.001	$F_{2,81}$=3.00	0.06	$F_{4,81}$=6.90	<0.001
Foliar P	$F_{2,81}$=7.08	0.001	$F_{2,81}$=2.65	0.08	$F_{4,81}$=5.13	<0.001
Cardenolide	$F_{2,78}$=15.54	0.001	$F_{2,78}$=1.39	0.38	$F_{4,78}$=7.67	<0.001

Table 4.2 Effects of foliar cardenolide, N and P fertilization and their interactions on growth, consumption, excretion and sequestration of cardenolide by *Danaus plexippus.* Numbers represent F-values (and P-values) of general linear models.

	Plant Cardenolide	Soil N	Soil P	N × P	Plant Cardenolide × N	Plant Cardenolide × P	Plant Cardenolide × N × P
Total consumption	7.67 (0.008)	6.10 (0.004)	1.69 (0.19)	0.24 (0.79)	0.58 (0.57)	0.32 (0.86)	0.57 (0.68)
Consumption of cardenolide	68.74 (<0.001)	1.89 (0.16)	2.89 (0.07)	0.16 (0.96)	0.77 (0.47)	0.50 (0.61)	0.39 (0.81)
Growth rate	3.86 (0.06)	12.34 (<0.001)	2.89 (0.07)	1.06 (0.39)	1.13 (0.33)	0.30 (0.74)	0.77 (0.55)
Sequestration efficiency	17.12 (<0.001)	4.38 (0.02)	7.07 (0.002)	1.79 (0.18)	1.32 (0.28)	0.37 (0.83)	2.12 (0.09)
Cardenolide concentration in frass	3.57 (0.06)	9.75 (<0.001)	2.50 (0.09)	1.49 (0.23)	0.72 (0.49)	0.80 (0.45)	0.20 (0.89)
Total cardenolide content	2.61 (0.11)	7.44 (<0.001)	7.64 (<0.001)	0.42 (0.80)	2.85 (0.07)	0.61 (0.55)	3.19 (0.02)
Cardenolide concentration	0.61 (0.44)	0.38 (0.69)	0.20 (0.82)	1.33 (0.27)	0.93 (0.40)	0.99 (0.42)	1.24 (0.31)

Fig. 4.1 Effects of N and P fertilization on foliar N, P and cardenolide concentration of *A. curassavica*. Columns represent the means of 10 samples and bars represent standard errors.

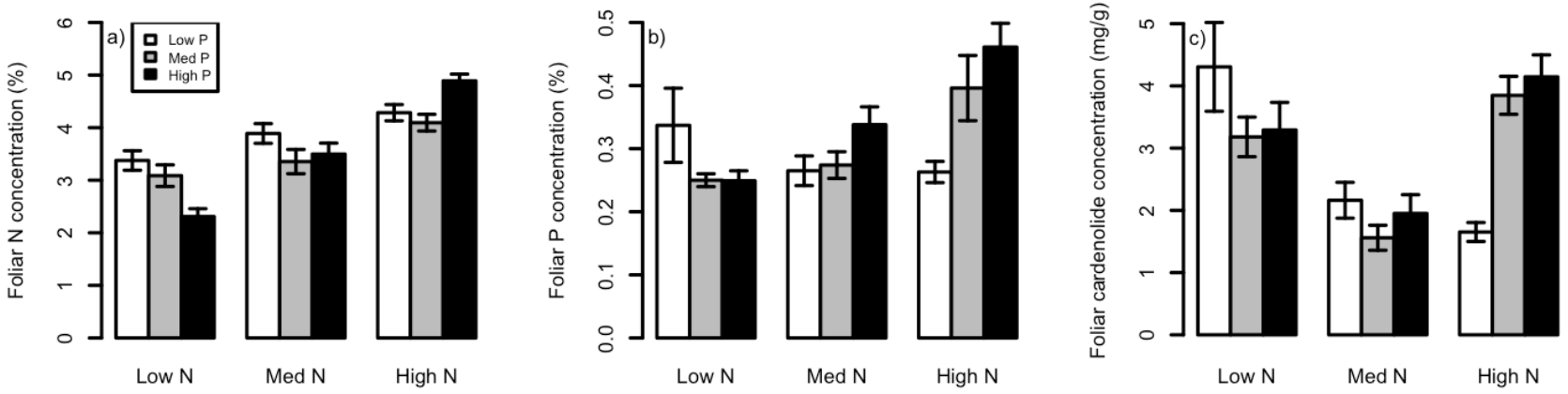


Fig. 4.2 Relationship between foliar cardenolide concentration and total consumption (a); effects of N fertilization on consumption (b), growth rate (c), and effects of P fertilization on growth rate (d) of *D. plexippus*. Columns are the means of 30 samples and bars represent standard errors. (e) illustrates the positive relationship between caterpillar growth rate and sequestration efficiency and (f) illustrates the negative relationship between foliar cardenolide concentration and sequestration efficiency.

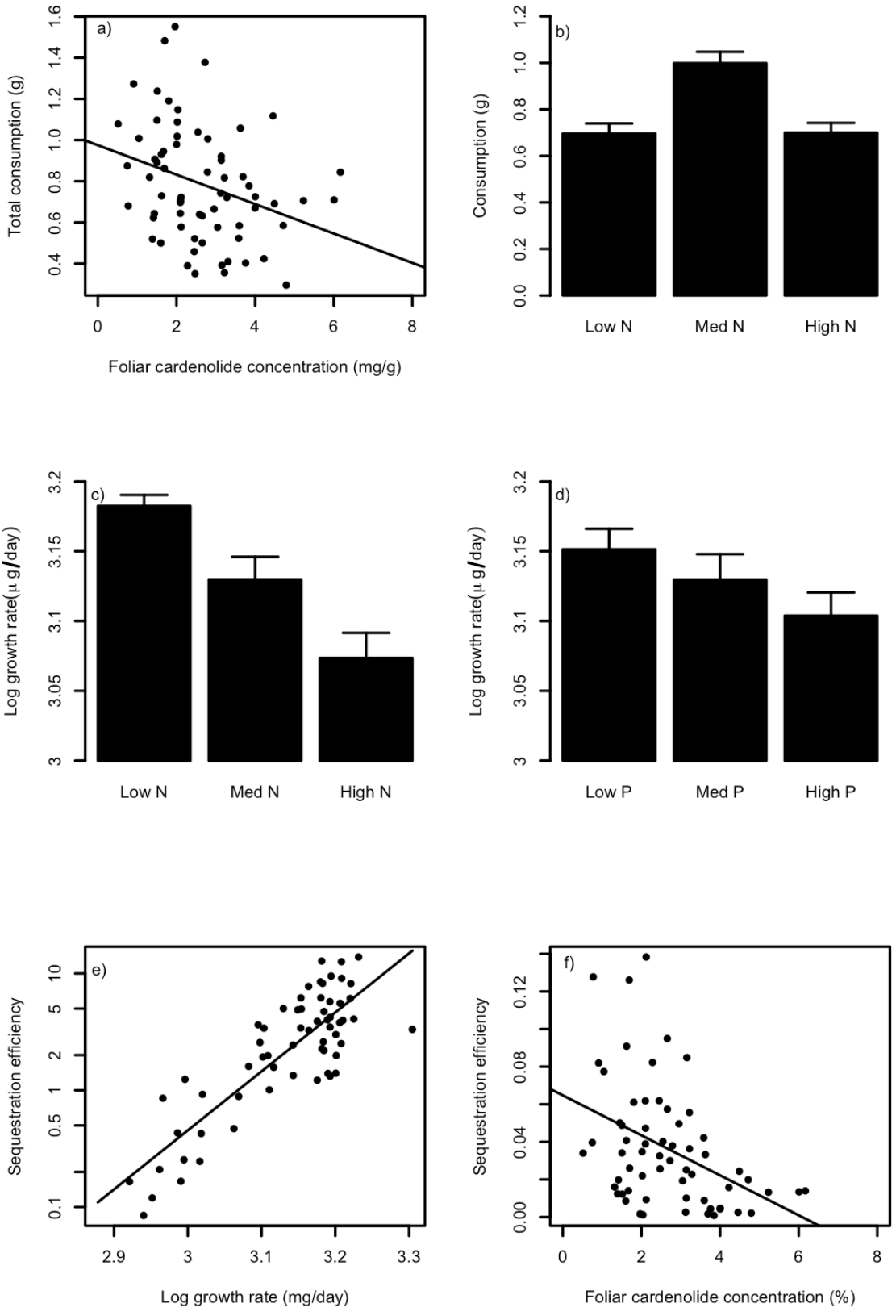

a)
Total consumption (g)
0.4
0.6
0.8
1.0
1.2
1.4
1.6
0
2
4
6
8
Foliar cardenolide concentration (mg/g)
b)
Consumption (g)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
Low N
Med N
High N
c)
Log growth rate(μ g/day)
3
3.05
3.1
3.15
3.2
Low N
Med N
High N
d)
Log growth rate(μ g/day)
3
3.05
3.1
3.15
3.2
Low P
Med P
High P
e)
Sequestration efficiency
0.1
0.5
1
2
5
10
2.9
3
3.1
3.2
3.3
Log growth rate (mg/day)
f)
Sequestration efficiency
0.00
0.04
0.08
0.12
0
2
4
6
8
Foliar cardenolide concentration (%)

Figure 4.3 Effects of N (a, c, e, g) and P fertilization (b, d, f, h) on sequestration efficiency (a, b), total contents of sequestered cardenolide (c, d), cardenolide concentration in frass (e, f) and cardenolide concentration (g, h) in *D. plexippus*. Columns are the means of 30 samples and bars represent standard errors.

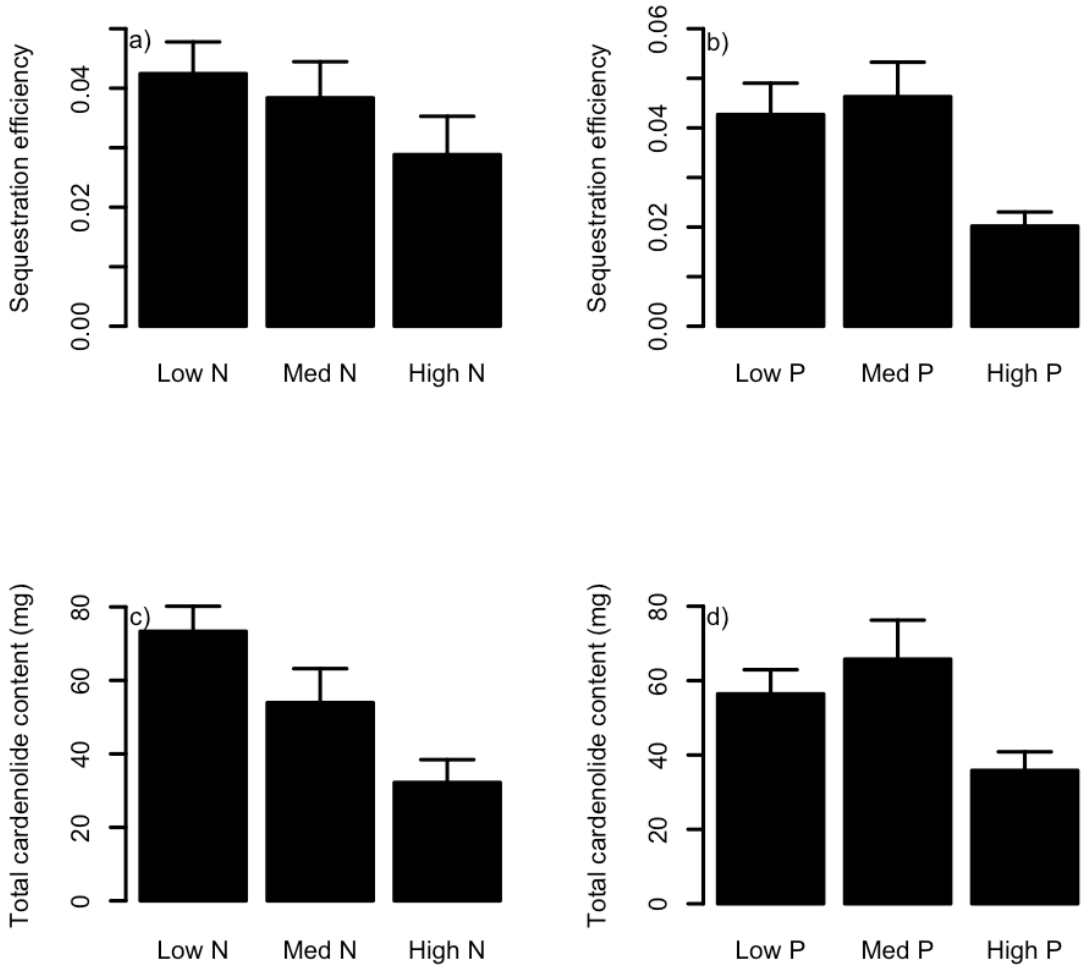
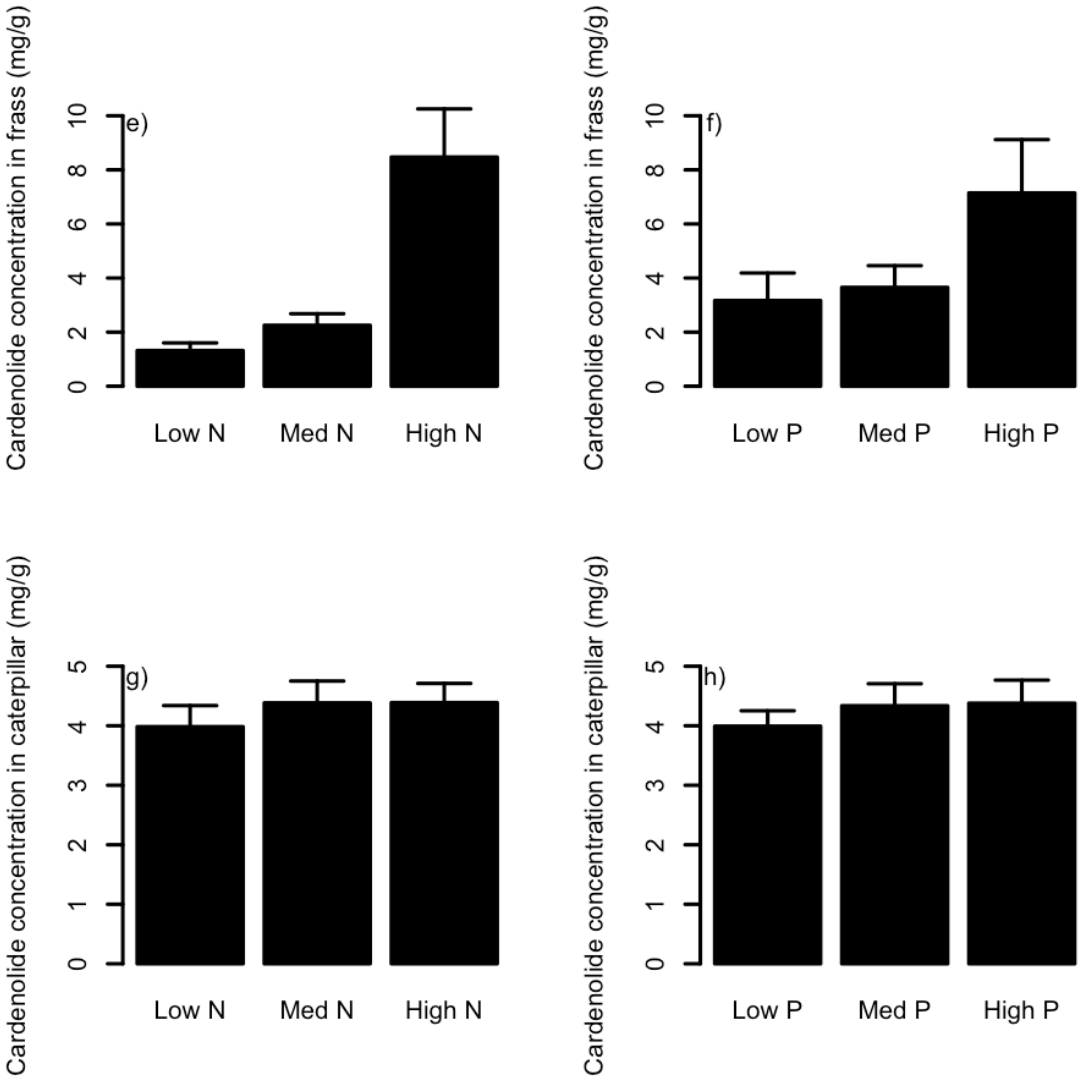


Fig. 4.3 continued.



Chapter 5

Allocation of resources away from sites of herbivory under simultaneous attack by aboveground and belowground herbivores in the common milkweed, *Asclepias syriaca*

Abstract

Following herbivory, plants can preferentially allocate newly-acquired resources away from attacked sites as an important mechanism conferring tolerance. Although reported previously for both aboveground and belowground herbivores, it remains unclear whether plants can simultaneously allocate resources away from both kinds of herbivore attack, and whether they have interactive effects on plant resource allocation. In the current study, we used dual-isotopic techniques to compare the allocation of newly acquired carbon (C) and nitrogen (N) by the common milkweed *Asclepias syriaca* following attack by an aboveground herbivore, the monarch caterpillar *Danaus plexippus* and a belowground herbivore, larvae of the red milkweed beetle *Tetraopes tetraophthalmus*. Both species induced significant changes in the allocation of C and N in *A. syriaca*. Specifically, *A. syriaca* increased allocation of new N to stems at the expense of allocation to damaged tissues (i.e. leaf or root). When under simultaneous attack, the allocation of resources to stems was greater than that induced by either herbivore alone, suggesting that (1) the herbivores have additive effects on allocation patterns by *A. syriaca*, and

(2) *A. syriaca* was able to mitigate the effects of future attack by both herbivore species simultaneously.

Introduction

In a world where herbivory is ubiquitous, plants have evolved defensive strategies to cope with the stresses of herbivory. For example, a wide range of responses in plants, including increases in the expression of physical, chemical, and indirect defenses, can be induced by herbivores, which may decrease their fitness (Baldwin and Schultz 1983; Karban and Baldwin 1997). However, herbivores have also evolved traits to minimize the impact of plant resistance (Brattsten 1988) and most plants are still subjected to tissue loss. Because they are unable to escape herbivore damage completely, many plants have evolved mechanisms of tolerance.

Tolerance is the ability of plants to maintain their fitness after damage (Rosenthal and Kotanen 1994). An important mechanism conferring plant tolerance is the reallocation of resources (Rosenthal and Kotanen 1994; Anten and Pierik 2010). Within several hours of damage by foliar herbivory, many plants can preferentially allocate newly-acquired resources (mainly carbon (C) and nitrogen (N)) away from sites of attack (Babst et al. 2005; Schwachtje et al. 2006; Orians et al. 2011; Tao and Hunter 2011). These resources may be allocated to sites of storage (Orians et al. 2011), to the growth of other tissues (Moreira et al. 2012), to the synthesis of defense compounds (Arnold and Schultz 2002)or to the stimulation of soil microbes (Holland et al. 1996). In addition to reallocation of plant resources induced by foliar feeders, root feeders have also been reported to affect patterns of plant resource allocation. For example, in the

spotted knapweed *Centaurea maculosa*, short-term allocation of newly acquired N to roots is almost halved after attack by the root boring sulphur knapweed moth (*Agapeta zoegana*) (Newingham et al. 2007). Changes in patterns of plant resource allocation induced by above- and belowground herbivores can not only reduce the immediate risks of further resource loss to herbivores, but also increase the potential for regrowth in the long term.

In nature, many plant species are attacked by foliar and root feeders simultaneously. Although there is ample evidence that plants can "hide" resources from either type of herbivore, it is not clear (1) whether plants have the capacity to reallocate resources away from both types of herbivore simultaneously, and (2) if induced changes in resource allocation patterns by one herbivore type can be affected by the other (i.e. interactions between above- and belowground herbivore species). Resource reallocation induced by herbivory can be systemic, such that foliar damage can increase resource allocation to roots and root damage can increase allocation to leaves (Newingham et al. 2007; Gómez et al. 2010; Gómez et al. 2012). Under simultaneous attack however, such reciprocal changes are unlikely to be adaptive. Simultaneous evasions would instead require the deposition of resources in sites unavailable to both herbivore types. In vascular plants, stems may be a good option for reallocation because they are a major site for storage and are generally isolated from direct leaf- and root feeding. Under such circumstances, foliar and root feeders are unlikely to have interactive effects as they only change the allocation of resources between the damaged site and storage site.

In the current study, we used dual-isotopic labeling to explore the allocation of newly acquired C and N in a fully factorial manipulation of damage by root and foliage feeding

herbivores on the same plant species. We also measured overall changes in C and N concentrations of plant tissues to assess whether allocation of existing resources are qualitatively in concordance with those of new resources. Specifically, we exposed the common milkweed *Asclepias syriaca,* to an aboveground herbivore, the monarch caterpillar *Danaus plexippus* and a belowground herbivore, larvae of the red milkweed beetle *Tetraopes tetraophthalmus*. Because specialist insects such as these have largely overcome the chemical defenses of milkweed, tolerance is an important strategy to cope with herbivory in *Asclepias* plants (Agrawal and Fishbein 2008). Moreover, because both insect species have chewing mouthparts, we can avoid confounding results that may arise from using herbivores that feed in different ways. We explored the idea of simultaneous reallocation under herbivory testing the following hypotheses: (1) both foliar and root herbivory change allocation of newly acquired C and N in *A. syriaca*, such that less resource is allocated to damaged sites; (2) *A. syriaca* mitigates the effects of future attack by both herbivore species simultaneously by allocating resources to stem which is unavailable to both species, and therefore (3) root and leaf feeding herbivores have additive effects on resource allocation in *A. syriaca*.

Materials and methods

Study system

The common milkweed *A. syriaca* is a widespread native perennial in eastern North America. *A. syriaca* can reproduce both asexually via rhizome or sexually when plants are older than two years. The monarch caterpillar *Danaus plexippus* is a specialist herbivore feeding on

the foliage of *Asclepias*. In southeastern Michigan where our experiment was conducted, *D. plexippus* larvae emerge from eggs from early June to late August. At our local field sites, larval density rarely exceeds one or two individuals per plant (Tao, personal observation). Larvae of the red milkweed beetle, *Tetraopes tetraophthalmus* are specialist herbivores of the fine roots and rhizomes of *Asclepias syriaca* (Gardiner 1961). During mid June to early August, *T. tetraophthalmus* females lay eggs into hollow stems of neighboring grasses. After hatching, larvae dig into the ground and feed exclusively on milkweed roots and rhizomes. They overwinter in the soil as pre-pupae (Chemsak 1963). In southeastern Michigan, the density of *T. tetraophthalmus* larvae can reach as high as 30 individuals per ramet (Tao, personal observation).

Milkweed seeds used in this experiment were collected at the University of Michigan Biological Station at Pellston, Michigan in September of 2008. After germination in May 2009, 60 seedlings were planted in 4 inch pots containing potting soil (SunGrow Horticulture, Vancouver, Canada). The plants were then kept in a controlled growth chamber at 24 ºC and a L16:D8 light cycle, with regular watering and biweekly 1:1:1 NPK fertilization. *D. plexippus* eggs were collected from the E. S. George Reserve in Pinckney, MI. 10 mating pairs of *T. tetraophthalmus* were collected from the University of Michigan campus to provide larvae for the experiment.

Greenhouse experiment

In late July 2009, when they were three months old, plants were moved to a greenhouse at Matthaei Botanic Gardens of the University of Michigan in Ann Arbor, MI. After approximately one week, plants were assigned at random to one of four treatments representing all combinations of the presence or absence of both herbivore species. From our initial 60 seedlings, 52 survived, allowing us to establish 13 replicates of each of the four treatments. For the aboveground herbivory treatment, one second instar *D. plexippus* larva was introduced to the fourth pair of leaves of the plant. By the time of the experiment, most plants had 6-7 pairs of leaves, and the fourth pair had finished expanding. The larvae were confined to a single leaf of the fourth pair by small mesh bags; all plants in the experiment received such bags (whether or not they contained a caterpillar) to control for any effects of the bags themselves on resource allocation. For the belowground herbivory treatment, five second instar *T. tetraophthalmus* larvae were introduced to the soil of each pot. Herbivores were allowed to feed on the plants for 48 hours, after which the caterpillars were removed by hand and *T. tetraophthalmus* larvae were killed by applying 2 mL of 0.05 mL/L chlorpyrifos solutions (Maron 1998). Chlorpyrifos is the active ingredient in the commercial soil insecticide, Dursban. Chlorpyrifos was applied to all 52 pots to control for any effects of insecticide on plant nutrient allocation. The numbers of herbivores and duration of damage were selected to generate comparable putative signals of herbivory within plants without radically altering plant biomass. We were more interested in the responses of plants to the signals of attack than potential changes in allocation caused by generating extensive damage and their accompanying large nutrient sinks. Analyses presented below show that the herbivore species caused comparable amounts of damage.

Immediately after herbivores were removed, each pot was injected with a total of 4 ml of 0.5 M solution of 99atom% $^{15}NH_4Cl$ (Isotec Inc, Illinois) by injecting 1 ml into each quarter of the pot at a depth of 3 cm. After N injection, all plants were transferred into a sealed chamber of 1.0m × 1.0m × 0.6m in size. The chamber was constructed of transparent polyethylene over PVC frames. 1 liter of 99atom%$^{13}CO_2$ (Cambridge Isotope Laboratories, Massachusetts) was then pumped into the chamber. A second identical pulse-labeling event occurred 6 hours later to ensure adequate labeling (Frost and Hunter 2008). The chamber was kept sealed for 2 days to maximize uptake of $^{13}CO_2$ and plants were watered through the injection ports as necessary. After 2 days, all plants were destructively sampled and separated into four parts: leaves, stem, rhizomes and fine roots. We chose two days because changes in allocation can be induced within several hours of damage (Gómez et al. 2010) and last for at least several days (Frost and Hunter 2008). Such short-term reallocation responses are important to study because they illustrate how plants may sequester or "hide" newly acquired resources in response to the immediate danger of herbivore attack; the presence of either of these herbivore species on milkweed is likely to be followed immediately by more tissue loss, and rapid allocation of resources away from sites of attack may significantly reduce the costs of herbivory. In the current experiment, we defined fine roots as those with a diameter less than 2mm. Each plant tissue was gently rinsed in distilled water and then dried at 70 °C for 72 hours. We then measured the dry mass of each tissue, and ground the samples to a fine powder in a ball mill for subsequent chemical analysis.

All plant samples were analyzed on a Finnigan Delta Plus isotope ratio mass spectrometer (IRMS) connected with a Finnigan Conflo II interface to a CE Elantech NC 2500

Elemental Analyzer in the soil ecology lab at University of Michigan. For each sample, we measured C and N contents and $\delta^{15}N$ and $\delta^{13}C$.

Statistical analyses

Analyses of allocation of newly gained C and N to different tissues followed Frost & Hunter (2008) and Newingham et al. (2007). Namely, for each tissue, mass of ^{13}C and ^{15}N was calculated by the following equation: atom % ^{13}C (or ^{15}N) × tissue C (or N) content × biomass. The allocation was then determined by dividing mass of ^{13}C (or ^{15}N) of each tissue by the total ^{13}C (or ^{15}N) gained. To explore the effects of herbivory on the biomass of each tissue, we used two-way ANOVA with above- and belowground herbivory as class variables, and biomass of each tissue as dependent variables.

To explore patterns of resource allocation among plant tissues, we used multivariate analysis of variance (MANOVA) with above- and belowground herbivory as class variables to examine their individual and interactive effects on ^{13}C and ^{15}N partitioning among tissues. We chose Pillai's trace as the reported statistic (Zar 1999). The total allocation of any single resource among tissues must sum to 100%, which restricts the using of MANOVA for all four tissues simultaneously. We therefore used allocation to three of four tissues as dependent variables and then inferred changes in allocation to the fourth tissue. To confirm the validity of our analyses, we performed MANOVA with all possible combinations of three tissues and obtained identical quantitative results with each set of three.

We also used MANOVA with above- and belowground herbivory as class variables to explore their individual and interactive effects on total (i.e. old and new) C and N concentrations among the four plant tissues. Before all statistical analyses, data were evaluated by Kolmogorov-Smirnov tests for their fit to assumptions of normality, and were log transformed when necessary. All analyses were performed in R 2.13.2 (R Development Core team 2011).

Results

Overall, neither *D. plexippus* nor *T. tetraophthalmus* caused any major declines in plant biomass (Table 5.1). When averaged across treatments with and without root damage, *D. plexippus* reduced plant leaf biomass by 9.5%. Likewise, when averaged across treatments with and without foliar damage, *T. tetraophthalmus* reduced root biomass by 8.3%. However, leaf and root biomass were not significantly different between control and herbivore treatments, and there was no interaction between the herbivore types in influencing tissue biomass. These results conformed to our goal of (1) creating similar and low damage level by both species; and (2) exploring herbivore-induced nutrient allocation without radically changing basic source/sink sizes.

D. plexippus did not change the allocation of newly acquired C (i.e.^{13}C) (Fig. 5.1a, Table 5.2, Pillai's trace=0.13, $F_{1,48}$=2.38, p=0.08). However, *T. tetraophthalmus* significantly altered ^{13}C allocation (Pillai's trace=0.21, $F_{1,48}$=4.01, p=0.01). Specifically, *T. tetraophthalmus* reduced ^{13}C allocation to roots and rhizomes by18.6% and 14.4% respectively, while increasing allocation to stems by 14.5%, when compared with control plants. There was no interaction

between *D. plexippus* and *T. tetraophthalmus* in the allocation of ^{13}C (Pillai's trace=0.07, $F_{1,48}$=1.09, p=0.36). Both species significantly altered the allocation of ^{15}N among tissues (Fig. 5.1, Table 5.2, Pillai's trace=0.48, $F_{1,48}$=13.96, p<0.001; Pillai's trace=0.46, $F_{1,48}$=13.26, p<0.001 for *D. plexippus* and *T. tetraophthalmus*, respectively). Specifically, *D. plexippus* decreased ^{15}N allocation to leaves by 36.6%, and increased ^{15}N allocation to stems by 16.8%. The relative allocation of N to rhizome and root was unchanged. Similarly, *T. tetraophthalmus* decreased allocation of ^{15}N to roots by 37.2% and increased allocation to stems by 64%. There was no interaction between *D. plexippus* and *T. tetraophthalmus* in the allocation of ^{15}N (Pillai's trace=0.04 , $F_{1,48}$=0.64, p=0.59). Rather, *D. plexippus* and *T. tetraophthalmus* had statistically additive effects. Under simultaneous attack, the allocation of ^{15}N to stems (39.7%) was higher than that induced by *D. plexippus* damange (23.9%) or by *T. tetraophthalmus* damage (28.9%) alone, compared to 15.1% of new N allocated to stems under control conditions (Fig. 5.1b).

We also examined the effects of herbivory on total (old and new) C and N concentration among tissues of *A. syriaca*. In no case did above- and belowground herbivory interact to influence tissue C and N concentrations (Table 5.3). Consequently, we graphically present the results as separate main effects without further reference to interaction terms (Fig 5.2). Damage by *D. plexippus* had no significant effects on C and N concentrations among tissues of *A. syriaca* (Table 5.3, Fig. 5.2a, b, Pillai's trace=0.05, $F_{1,48}$=0.63, p=0.64; Pillai's trace=0.09, $F_{1,48}$=1.17, p=0.34 for C and N, respectively). Similarly, *T. tetraophthalmus* did not affect C concentrations (Fig. 5.2c, Pillai's trace=0.18, $F_{1,48}$=1.63, p=0.19). However, damage by *T. tetraophthalmus,*

reduced concentrations of total N in both leaves and roots, and increased N concentrations in stems and rhizomes (Fig. 5.2d, Pillai's trace=0.39, $F_{1,48}$=7.19, p<0.001).

Discussion

By using stable isotopic techniques to track the allocation of newly acquired C and N in plants, we established that both above- and belowground herbivores reduced the allocation of newly acquired N to sites of attack in *A. syriaca*. Instead, stems represented major sinks for new N when plants were under attack. In addition, the effects of the herbivore species on resource allocation were additive, as simultaneous attack resulted in a greater proportion of newly acquired N deposited in stems. Such additive responses should serve to mitigate future losses of N to herbivores under simultaneous attack from above and below ground. Our results add to the growing body of evidence that belowground herbivory is an important factor affecting plant physiology, and provide a preliminary comparison between flexible allocation responses to above- and belowground herbivory in plants. Although our experiment addressed only short-term "escape" of nutrients from sites of ongoing herbivore attack, such changes in C and N partitioning after damage may be important traits conferring plant tolerance to herbivores, and may have significant implications for subsequent interactions between spatially separated herbivores.

The majority of studies focusing on short-term changes in plant resource allocation following herbivory have used labeled C, with increasing attention to labeled N only in recent years (Newingham et al. 2007; Frost and Hunter 2008; Gómez et al. 2010). Although connected

in many biochemical reactions, N and C metabolism, transport and partitioning in plants show considerable independence. For example, there is no correlation between the allocation of newly acquired C and N in red oak (*Quercus rubra*) after defoliation (Frost and Hunter 2008). Similarly, in the current study, we found that the effects of herbivores on the allocation of N are greater than their effects on C, as both species allocated significantly less ^{15}N to sites of attack, and *T. tetraophthalmus* reduced total N concentration in roots. In addition, although marginally non-significant ($p=0.077$), *D. plexippus* damage resulted in slightly higher total ^{15}N concentrations in plants, suggesting an increase in N uptake after attack. This may reflect difference in the relative importance of C and N as limiting resources for both plants and herbivores. N is often a limiting nutrient for plants and in turn has strong influences on insect performance and population dynamics (Mattson 1980). Therefore, by preferentially transporting N away from sites of attack, plants can not only protect their N from consumption, but also reduce their nutritional value and appeal to insect herbivores (Tao and Hunter 2011).

Despite the technical difficulties of working with belowground herbivores, and the relative scarcity of data on their ecological effects, there is accumulating evidence that herbivory belowground can impose strong negative influences on plant performance in many ecosystems (Hunter 2001; Blossey and Hunt-Joshi 2003; Hunter 2008). In a recent meta-analysis quantifying plant responses to belowground insect herbivory, the average negative effect of root feeders on plant performance was slightly larger than that of aboveground herbivores (Zvereva and Kozlov 2012). In our system, *T. tetraophthalmus* larvae can induce great fitness costs to *A. syriaca.* For example, experimental additions of the herbivore at natural densities can generate strong

negative effects on greenhouse *A. syriaca*, where rhizome biomass, as a measurement of clonal reproduction (Fagerström 1992), was reduced by more than 30% (Matter 2001). At our field sites in Michigan, clonal reproduction is the only mechanism of reproduction in *A. syriaca* until a genet is more than 2 - 3 years old, and remains a major form of reproduction thereafter. Moreover, stored nutrients in rhizomes can be used to replenish lost tissues aboveground over the long term (Hochwender et al. 2000). Attack by *T. tetraophthalmus* can not only exhaust local storage of a ramet, but also sever physical connections to other potential sources (other ramets within the genet). As a result, *T. tetraophthalmus* may represent a significant threat to *A. syriaca* fitness, leading to the physiological responses by plants that we observed.

Consistent with several previous studies (Hougen-Eitzman and Rausher 1994; Maron 1998; Ayres et al. 2007), we showed here that above- and belowground herbivory affect plant physiology additively. Specifically, allocation of newly acquired N to stems was greater under simultaneous attack than under damage by either herbivore species alone. Why are the results additive? When feeding alone, the leaf feeder had insignificant effects on allocation of new resources in roots. Likewise, when feeding alone, the root feeder had negligible effects on the allocation of new resources in leaves (Fig. 5.1, Table 5.2). The additive effects of the herbivores on nutrient allocation may simply emerge because both species drive allocation of new resources away from the sites of attack to stems.

In our study, the labeling process lasted for 48 hrs. As a result, labeled N was less than 5% of the total N within all plant tissues. Nevertheless, we still observed significant effects of belowground herbivory on the concentrations of total N (old and new) among tissues, which are

unlikely to have been caused by changes in allocation of new N alone. Rather, our observations may reflect the remobilization of stored N. Compared with new resources, pre-damage assimilates represent a larger pool, therefore reallocation of the "old" resources should be at least as important as the fate of new resources in conferring plant tolerance. In *Festuca rubra*, for example, increased C exudation in the rhizosphere induced by defoliation mainly comes from pre-damage resources stored in the plants, rather than from newly gained C (Paterson et al. 2005). Therefore, we suggest that future studies of plant tolerance to herbivory should not only focus on the partitioning of new resources, but also on remobilization and redistribution of "old" resources.

The large changes in total N concentration induced by *T. tetraophthalmus* could have important implications for its interaction with *D. plexippus.* We observed that although N concentration was significantly increased in stems following root damage, total N concentration was reduced in leaves. In previous work, we have shown that high foliar N concentrations favor *D. plexippus* fitness (Tao and Hunter 2012); therefore, *T. tetraophthalmus* could negatively influence the performance *D. plexippus.* By contrast, *D. plexippus* is unlikely to affect the performance of *T. tetraophthalmus* because we observed no effects of *D. plexippus* damage on nutrient allocation in roots. However, we did not measure induced changes in chemical defense*s* in *A. syriaca,* which can influence interspecific interactions. For example, in a previous study, *T. tetraophthalmus* attenuated the induction of latex by *D. plexippus* (Rasmann et al. 2009); latex is an important chemical defense negatively affecting *D. plexippus* growth rate (Zalucki et al. 2001; Tao and Hunter 2012). As a result, the net effects of *T. tetraophthalmus* on *D. plexippus*

performance will depend on the relative magnitudes of changes in nutrient allocation and defense expression and their subsequent consequences for insect growth.

Generalizing outcomes of species interactions between above- and belowground herbivores from either nutrient partitioning or defense allocation models alone is difficult (Masters et al. 1993; Bezemer and van Dam 2005). The few examples that exist of studies investigating both in combination have shown that their importance varies among systems (Soler et al. 2005; Kaplan et al. 2008). This is probably due to the context dependency of plant physiology, herbivore identity, life history and susceptibility to chemical defense (Kaplan et al. 2008; Soler et al. 2005; Wurst and van der Putten 2007). Therefore, increasing efforts to assess nutrient partitioning and concomitant changes in secondary metabolites simultaneously at broader scales are required to increase our understanding of plant-mediated interactions between above- and belowground herbivores.

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Table 5.1 Biomass in grams (±SE) of *Asclepias syriaca* tissues in a fully factorial design including damage by above- (*Danaus plexippus*) and below-ground (*Tetraopes tetraophthalmus*) herbivores.

	Control	+*D. plexippus*	+*T. tetraophthalmus*	*D. plexippus* +*T. tetraophthalmus*
Leaf	0.33±0.06	0.32±0.05	0.42±0.04	0.34±0.07
Stem	0.39±0.04	0.45±0.04	0.44±0.05	0.59±0.06
Rhizome	0.36±0.06	0.41±0.04	0.31±0.03	0.35±0.04
Root	0.21±0.03	0.27±0.02	0.19±0.01	0.23±0.02
Total	1.29±0.12	1.45±0.11	1.36±0.09	1.51±0.14

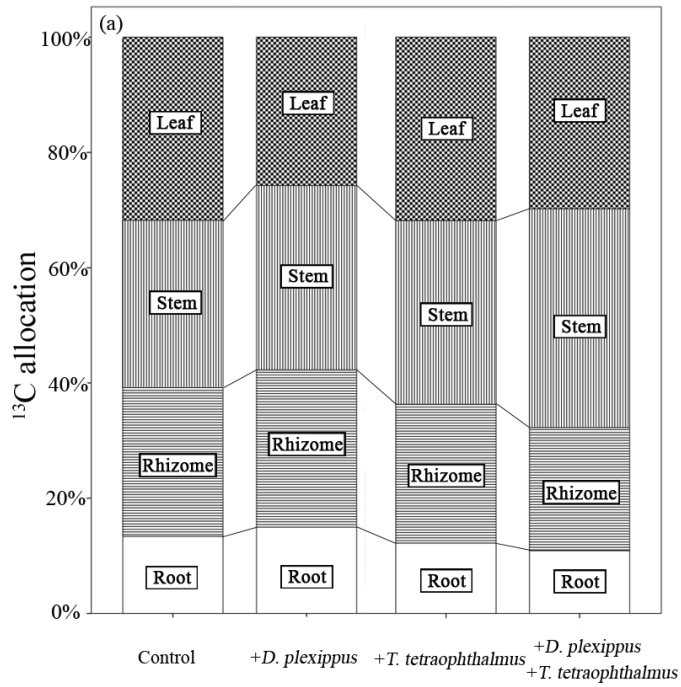
Table 5.2 Results (Pillai's trace, assocaited F values, degrees of freedom and associated p -values) of MANOVA of the individual and interactive effects of herbivory by *Danaus. plexippus* and *Tetraopes tetraophthalmus* on ^{13}C and ^{15}N allocation among *Asclepias syriaca* tissues.

	Factor	Pillai's trace	$F_{1,48}$	p
^{13}C	*D. plexippus*	0.13	2.38	0.08
	T. tetraophthalmus	0.21	4.01	0.01
	D × *T*	0.07	1.09	0.36
^{15}N	*D. plexippus*	0.48	13.96	<0.001
	T. tetraophthalmus	0.46	13.26	<0.001
	D× *T*	0.04	0.59	0.64

Table 5.3 Results (Pillai's trace, associated F values, degrees of freedom and associated p -values) of MANOVA of the individual and interactive effects of herbivory by *Danaus. plexippus* and *Tetraopes tetraophthalmus* on total C and N concentrations among *Asclepias syriaca* tissues.

	Factor	Pillai's trace	$F_{1,48}$	p
C	*D. plexippus*	0.05	0.63	0.64
	T. tetraophthalmus	0.18	1.63	0.19
	D × *T*	0.12	1.53	0.21
N	*D. plexippus*	0.09	1.17	0.34
	T. tetraophthalmus	0.39	7.19	<0.001
	D× *T*	0.11	1.45	0.23

Figure 5.1. The effects of damage by *Danaus plexippus* and *Tetraopes tetraophthalmus* on the allocation of (a) ^{13}C and (b) ^{15}N among tissues of *Asclepias syriaca.*



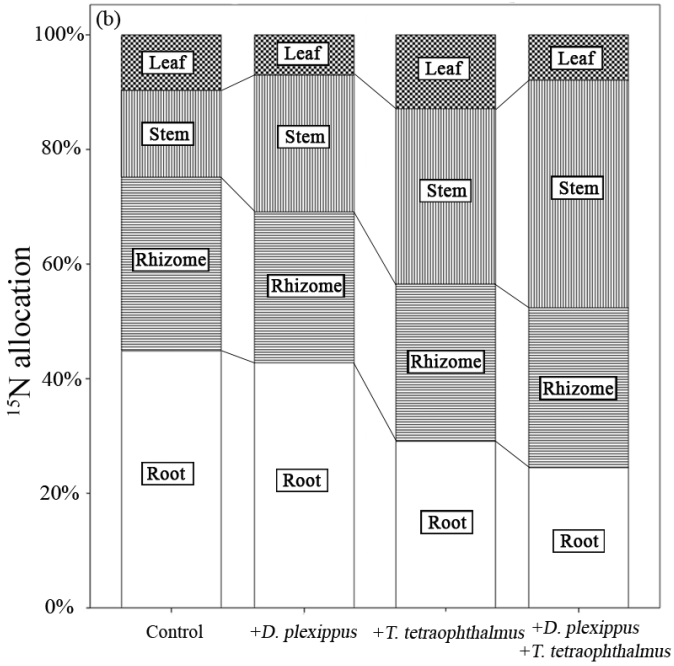
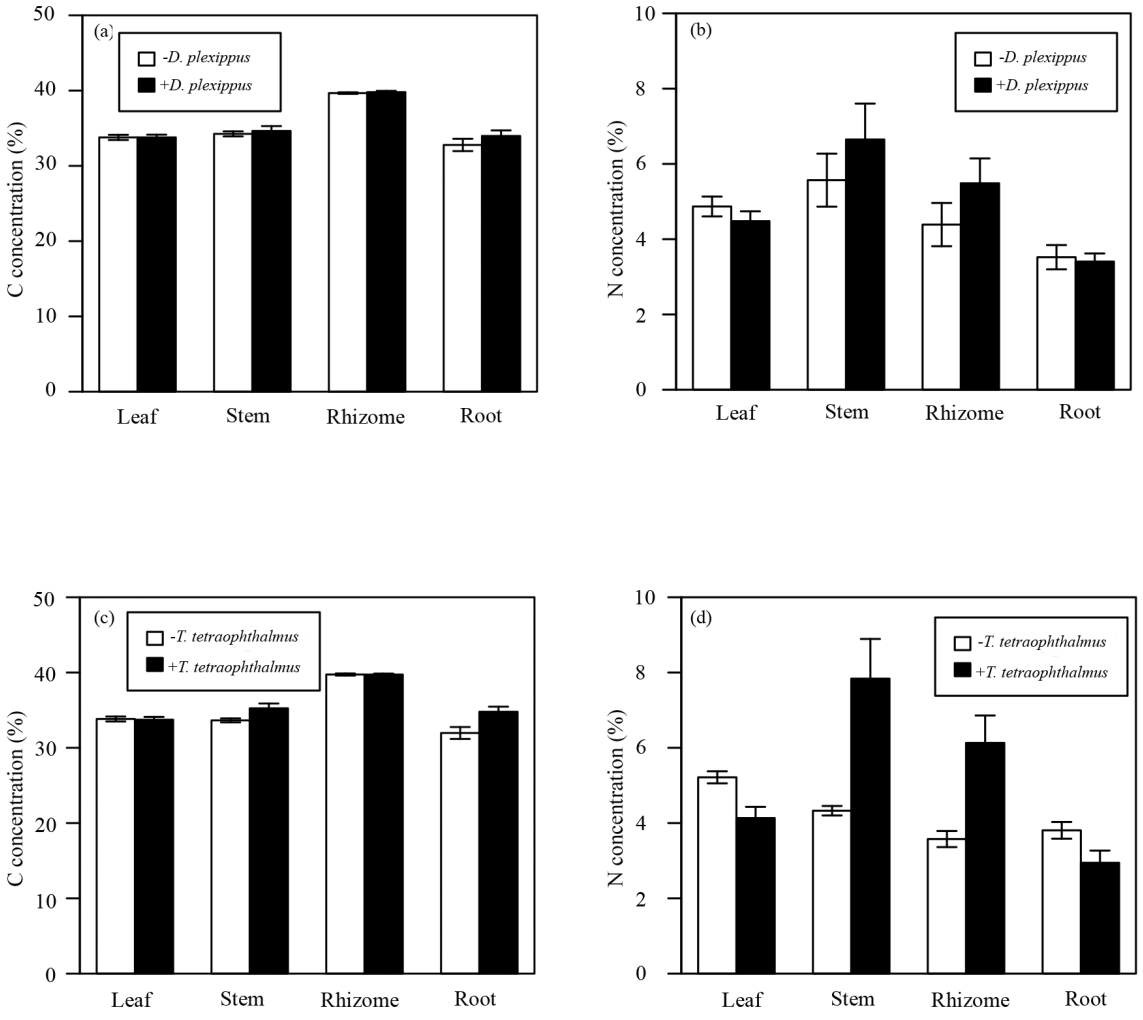


Figure 5.2. The effects of damage by *Danaus plexippus* (a, b) and *Tetraopes tetraophthalmus* (c, d) on total C (a, c) and N (b, d) concentrations in the tissues of *Asclepias syriaca.*



Chapter 6

Conclusions

Resource acquisition is essential for the survival of organisms, and resource quality has important consequences for many ecological and evolutionary processes, including species interactions and coevolution (Lundberg and Astrom 1990, Kay et al. 2005), sexual selection (Morehouse et al. 2010), invasion (González et al. 2010), community assembly (Moe et al. 2005) and ecosystem functioning (Sardans et al. 2012). Especially in the field of plant-herbivore interactions, the effects of plant nutrients on insect performance have been and remain topical due to the profound differences between the tissue compositions of animals and plants (Mattson 1980, Elser et al. 2000). In recent years, such resource-oriented studies have become increasingly relevant, because anthropogenic activities have significantly altered nutrient cycles in natural ecosystem (Vitousek et al. 1997, Throop and Lerdau 2004, Urabe et al. 2010).

In addition to foliar nutrients, other important plant traits that influence herbivore performance include foliar secondary chemicals, many of which are used as defense against herbivory (Rhoades 1979). Therefore, plant-herbivore interactions can only be fully understood if the individual and interactive effects of nutrients and toxins, as well as their co-variation, are all studied together (Raubenheimer and Simpson 2009).

My dissertation has focused on plant nutrients, plant defenses, and their interactions as they influence herbivores, with particular reference to global change biology. Specifically, I used milkweed (*Asclepias syriaca, A. curassavica* and *A. incarnata*) and three of their specialist herbivores (the monarch caterpillar *Danaus plexippus,* the aphid *Aphis asclepiadis* and the red longhorn milkweed beetle *Tetraopes tetraophthalmus*) as my study system. By using echniques from insect nutritional ecology, chemical ecology and stable isotope ecology, I was able to explore the complex effects of foliar nutrients and defense chemicals on growth and defense of the herbivores, as well as top-down effects of herbivory on plant quality.

In Chapter 2, although theories predict that P limitation in insect herbivores would be induced by anthropogenic N deposition, I found that neither of two insect species (*A. asclepiadis* and *D. plexippus*) that feed on the common milkweed *A. syriaca* experienced induced P limitation. The mechanisms underlying the lack of induced P limitation differed between species. The body tissues of *A. asclepiadis* always exhibited higher N : P ratios than did those of their host plants, suggesting that the N demand of aphids exceeds P demand, even under high N deposition levels. P addition increased the production of latex by milkweed plants, which is an important foliar defense negatively affecting *D. plexippus* growth rate. Therefore, my results illustrate that P limitation of herbivores is not an inevitable consequence of anthropogenic N deposition in terrestrial systems. Rather, species-specific demands for nutrients and the defensive responses of plants combine to determine the responses of herbivores to P availability under N deposition.

Although in Chapter 2, I found that foliar N concentration in *A. syriaca* was positively

correlated with growth rate of *D. plexippus*, the relationship disappeared when caterpillars were fed on *A. incarnata*, and became negative when caterpillars fed on *A. curassavica* (Chapter 3). The major difference among the three milkweed species is that N and P concentrations are much lower in *A. syriaca* compared to those of the other two species. Although there is accumulating evidence that too much nutrient can lead to decreases in organism growth by either reducing total food consumption or changing other properties of the diet (Joern and Behmer 1998, Boersma and Elser 2006), neither of the above mechanisms could explain my data. There were no effects of foliar N on consumption of plant material by *D. plexippus*, and foliar cardenolide was not correlated with foliar N concentration. Rather, the per unit toxicity of cardenolide was higher as foliar N concentration increased in excess of demand, resulting in deleterious effects of N. When feeding on *A. incarnata,* which had similar high foliar N concentrations, excess N did not have significant negative effects on larval growth because cardenolide concentrations are on average 40 times lower than those of *A. curassavica*. Although previous studies have found that in some systems, toxicity of plant defense chemicals are higher when nutrients are limiting for the herbivore, my results are among the first to show that toxicity can also be greater at the other end of nutrient spectrum. In addition to illustrating a new mechanism for the negative effects of excess nutrients on consumers, my work also emphasizes that future studies of species interactions should focus increasingly on interactions between nutrients and defense chemicals (Raubenheimer & Simpson 2009).

Given that high N levels resulted in higher toxicity of cardenolides in *A. curassavica*, high N levels could potentially affect the efficiency of cardenolide sequestration by *D. plexippus*.

Consistent with this expectation, I illustrate in Chapter 4 that N and P fertilization reduced cardenolide sequestration efficiency. There was a positive relationship between caterpillar growth rate and sequestration efficiency, which may be because when growth rate is low, allocation to defense is limited. Furthermore, cardenolide concentrations in caterpillar feces (frass) were much higher under high N and P fertilization levels, suggesting that *D. plexippus* was able to reduce the negative effects of cardenolides by a higher excretion rate/lower absorption rate. Although total amounts of sequestered cardenolides were lower under high N levels, the concentration of cardenolides in *D. plexippus* tissue were unaffected because their body weights were also lower. Given that final body concentrations of sequestered cardenolides were unaffected by soil nutrient levels, the protective effects of cardenolides against vertebrate predators (Reichstein et al. 1968) may also be unaffected. However, interactions between *D. plexippus* and other enemies may be changed. Specifically, if food passage time is high under high nutrient conditions, infection rate by *Ophryocystis elektroscirrha*, a protozoan parasite of *D. plexippus*, may become lower. Because parasite infection is also related to dietary cardenolides (Lefevre et al. 2010, de Roode et al. 2011), this illustrates an interesting future direction of incorporating cardenolide toxicity, nutrient availability and parasite infection in tritrophic studies of plant-insect-parasite interactions.

In Chapter 5, I used dual-isotopic techniques to compare the allocation of newly acquired C and N by *A. syriaca* following attack by the aboveground herbivore *D. plexippus* and the belowground herbivore, larvae of *T. tetraophthalmus*. Both species induced significant changes in the allocation of C and N. Specifically, *A. syriaca* increased allocation of new N to stems at

the expense of allocation to damaged tissues (i.e., leaf or root). When under simultaneous attack, the allocation of resources to stems was greater than that induced by either herbivore alone, suggesting that (1) the herbivores have additive effects on resource allocation patterns by *A. syriaca* and (2) *A. syriaca* was able to mitigate the effects of future attack by both herbivore species simultaneously. My results add to the growing body of evidence that belowground herbivory is an important factor affecting plant physiology, and provide a preliminary comparison between flexible allocation responses to above and belowground herbivory in plants. Although my experiment addressed only short-term ‘‘escape’’ of nutrients from sites of ongoing herbivore attack, such changes in C and N partitioning after damage may be important traits conferring plant tolerance to herbivores, and may have significant implications for subsequent interactions between spatially separated herbivores.

Some Final Thoughts

While I have tried to explore relationships among nutrient availability, plant chemical defense and herbivore performance, I suspect that I have found more new questions than I have answered. For example, an important assumption in many empirical studies of nutrient stoichiometry, including my Chapter 2, is that stoichiometric requirements are equivalent to elemental body composition. In reality, organisms have different utilization efficiencies of different elements, so that the optimal ratio is likely to differ from body elemental ratios (Sterner and Elser 2002). In other words, to fully understand whether N and P are co-limiting for insect herbivores, and to predict potential P limitation in different systems, it is also important to study

N and P utilization efficiency. If differences indeed exist in these efficiencies, it could shed light on the evolutionary forces underlying differential nutrient use efficiencies in nature.

Additionally, generalist insects that can actively choose their host plants during development can also mix their diets to maintain their intake of N and P (Jonas and Joern 2008). For specialist insects like *D. plexippus,* which can complete the larval stage on a single plant, diet mixing is much less likely. Moreover, intraspecific variation in N: P stoichiometry may be much less than interspecific variation, further restricting the evolution of mixing of plant diets with different nutrient levels. Therefore, it would interesting to explore if specialist herbivores are more prone to nutrient limitation, and if they have a greater potential to experience induced P limitation than do generalist herbivores that mix their diets.

In Chapter 3, I showed that cardenolide toxicity is a function of foliar N concentration, but the mechanism underlying this relationship is unclear. The only other existing study that found higher toxicity of secondary chemicals when dietary N was high (Simpson and Raubenheimer 2001), showed that N growth efficiency----a measure of retaining N from the food and incorporating it into herbivore biomass----was more affected by condensed tannins when dietary N was high. Due to logistic constraints in my study, I did not measure N concentrations in the frass and body of each caterpillar, preventing me from testing this potential mechanism. In addition, foliar N is marginally correlated with cardenolide non-polarity in milkweed ($p=0.10$), suggesting that the positive correlation between foliar N and cardenolide toxicity might result from changes in cardenolide composition. Future studies should therefore separate the co-variation between both foliar nutrient and toxin concentration (Chapter 3), and between

nutrient concentration and toxin composition. Using artificial diets may help with untangling these complex relationships.

To increase the relevance of lab-based research on the nutritional ecology of insect herbivores to population and community ecology, it is important to incorporate other trophic levels. Specifically, I would like to conduct additional work on the parasites of insects and their influence on nutrient-toxin interactions. For example, parasites have been shown to affect nutrient foraging by insects (Thompson et al. 2001). Moreover, immunological responses in insects depend strongly on dietary nutrient composition (Siva-Jothy and Thompson 2002, Cotter et al. 2011), suggesting that the optimal dietary nutrient composition can change with the presence of parasites. In addition, in mesocosm studies, natural enemies have been shown to affect N cycling (Hawlena et al. 2012), which may modify the effects of soil N on insect herbivores. Such interactions provide much opportunity for future research.

In summary, nutrient mediated interactions between plants and animals are more complex than simple nutrient limitation. The responses of herbivores to nutrient enrichment depend upon specific requirements of each herbivore, concomitant changes in other properties of the diet, and interactions between nutrients and plant toxins. These effects influence not only the growth rate of herbivores, but may also affect their defenses against enemies. To add another layer to this already complex picture, the effects of nutrient enrichment on herbivores are likely to be dynamic, because herbivory can change plant quality by affecting plant resource allocation patterns. Understanding the above relationships will not only help us to untangle the complex

relationships between herbivores and their host plants, but also enable us to predict responses of herbivores and plants to global environment change.

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