Whose phenotype is it anyway?
The complex role of species interactions and resource availability in determining plant defense phenotype and community consequences.

by

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To my parents, who encouraged me to follow my dreams
And to Dave, whose constant support helps me pursue them

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ABSTRACT

Whose phenotype is it anyway? The complex role of species interactions and resource availability in determining plant defense phenotype and community consequences.

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The expression of plant defense is influenced by resource availability and biotic interactions, with consequences for herbivores and plant fitness. While the majority of plants associate with mycorrhizal fungi, which dramatically affect plant resource status, the role of these belowground interactions in shaping the expression of plant defense is poorly understood. In addition, plant-herbivore interactions affect plant growth and defense, but their effects on mycorrhizal interactions can vary dramatically. I hypothesized that changes in plant resource status and subsequent defense expression may mediate the interactions between mycorrhizal fungi and aboveground herbivores. Drawing from current knowledge of resource mutualisms, I hypothesized that the carbon costs and nutrient benefits of hosting mycorrhizal fungi would predict a nonlinear effect of mycorrhizae on the expression of plant defense. An experimental manipulation of the abundance and identity of mycorrhizal fungi associating with *Asclepias syriaca* revealed mycorrhizal colonization nonlinearly affected the expression of plant defense, although the shape of the response to increasing fungal colonization depended on the plant trait examined. In particular, traits (eg. trichomes, plant biomass) that increased with the concentration of phosphorus responded unimodally to mycorrhizal colonization as

predicted, while those traits that were putatively carbon-limited (eg. latex and toughness) declined with fungal colonization. I also manipulated carbon available to plants and examined changes in plant defense and the effects of herbivores on mycorrhizal fungi. Growth under elevated CO_2 increased plant biomass by 15% and toughness by 40%, but decreased cardenolide concentration by 20% and had little effect on trichome density. Herbivory by either aphids or caterpillars had no effect on mycorrhizal colonization when plants were grown in ambient CO_2, but herbivory dramatically increased mycorrhizal colonization under elevated CO_2. Taken together, these results indicate that fungi and aboveground herbivores interact through changes in plant resource status and defense phenotype and exert strong influence on the expression of plant defense phenotype. In addition, these experiments revealed substantial genetic variation within a single population of *A. syriaca* in the expression of plant defense and in response to mycorrhizal colonization and carbon addition, indicating the potential for evolutionary adaptation to changing environmental conditions.

Chapter I

Introduction

An organism's phenotype determines in large part how it interacts with the world around it. It has long been recognized that the expression of phenotype is a consequence of an organism's genetic composition, environment, and the interaction between the two (Fritz and Simms 1992), where the environment consists of both abiotic and biotic components (Strauss and Irwin 2004). Although biotic interactions are ubiquitous and important in both natural and managed systems (van der Heijden et al. 2008, Garibaldi et al. 2011), their effects on phenotype and performance are often context-dependent (Chamberlain and Holland 2009, Hoeksema et al. 2010). As a result, it remains difficult to anticipate when and how biotic interactions affect organism phenotype and their subsequent ecological or evolutionary consequences.

This dissertation develops a resource-based framework to understand and predict 1) how biotic interactions affect organism phenotype, 2) under what conditions biotic interactions affect ecological outcomes, 3) the evolutionary causes and consequences of variation in the outcome of biotic interactions. I consider two kinds of interactions which are both important in terrestrial ecological communities: mycorrhizae and herbivory.

Nearly all plant taxa support symbiotic fungi within their roots in an association called mycorrhizae (Smith and Read 2008). Arbuscular mycorrhizal fungi (AMF), in the Phylum Glomeromycota (Schussler et al. 2001), are among the most common mycorrhizal associations for many forbs, agricultural plants, and tropical and some temperate tree species (Smith and Read 2008). Mycorrhizal fungi exert significant influence on plant phenotype through multiple pathways (Vannette and Hunter 2009, Sikes et al. 2010). Specifically, AMF gather phosphorus (P), nitrogen (N) and

micronutrients from the soil and transfer them to plants in exchange for simple sugars. Individual plants typically benefit from this interaction, but plant responses to colonization by AMF vary with fungal identity, soil fertility, and other factors, and can sometimes result in parasitism (Johnson et al. 1997, Klironomos 2003). Despite early research that suggested that the abundance of fungi mediates the effect of fungi on plant performance (Gange and Ayres 1999), recent work fails to consider the abundance of AMF as a key variable in determining the outcome of mycorrhizal interactions (Hoeksema et al. 2010).

Plants also interact with herbivorous insects that consume plant tissue, reduce plant growth and fitness and decrease crop yield in agricultural systems. However, plants are not passive recipients of this damage (Murdoch 1966) and exhibit physical and chemical defenses that protect their tissues from consumption. Plants also actively respond to herbivory by repairing and re-growing lost tissue, and may mount an additional defense against future herbivory by increasing expression of physical or chemical traits (Karban and Baldwin 1997). Variation in plant defense, both constitutive and induced, can significantly affect plant fitness and affect the performance of herbivores and higher trophic levels (Poelman et al. 2008, de Roode et al. 2011).

The availability of soil resources affects both plant defense against herbivores (Bryant et al. 1983, Herms and Mattson 1992) and association with mycorrhizal symbionts (Treseder and Allen 2002, Treseder 2004). As a result, the effects of mycorrhizae and herbivory on plants are fluid and depend on their environmental context (Hunter and Schultz 1995, Johnson et al. 1997). The research presented here explores how AMF and insect herbivores alter plant phenotype expression, how resource availability modulates the effect of interactions on phenotype, and offers a framework for predicting the effects of resource availability, herbivory, and mycorrhizae on the expression of defense phenotype and community patterns.

In addition, coevolutionary relationships between plants and herbivores or AMF shape plant phenotype and patterns of resource allocation (Ehrlich and Raven 1964, Hoeksema

2010). Because not all functions (eg. growth, competitive ability, defense) can be maximized simultaneously, limitations imposed by resource availability and evolutionary or developmental constraints (tradeoffs) shape species and populations that vary in allocation patterns (Herms and Mattson 1992, Bergelson and Purrington 1996). Intraspecific variation in exposure to herbivores, competitors, pathogens or mutualists in space can generate and maintain phenotypic variation in traits that mediate interspecific interactions (Rausher 1984, Thompson and Cunningham 2002). Indeed, plant genotypes vary in their response to changes in the environment, and this intraspecific variation in phenotypic plasticity is fodder for the evolutionary process, and contributes to the adaptation of plant species to changing conditions (Fordyce 2006). In addition, although the ecological consequences of genetic variation are likely important in community dynamics (Bolnick et al. 2011), a lack of experimental investigations of the degree of genetic variation in response to biotic interactions limits our ability to predict its importance in ecological systems.

In this dissertation, I examine how mycorrhizal fungi and insect herbivores influence plant phenotype and the above- and belowground ecological consequences of these phenotypic changes, respectively (Fig 1.1). I hypothesize that changes in plant resource status mediate the effect of biotic interactions on the expression of plant phenotype. For this reason, the effects of interactions on plant phenotype should vary with environmental resource availability. In addition, if genetic variation exists in plant response to biotic interactions, natural selection could result in evolution within plant populations. In this way, intraspecific variation could allow local plant populations to adapt to rapidly changing abiotic and biotic conditions that now challenge many natural systems (Jump et al. 2009). To test these hypotheses, I measure the effects of biotic interactions on the expression of plant phenotype among plant genotypes and examine how the outcomes of these interactions vary along resource gradients (Fig 1.1).

This dissertation is divided into four primary chapters. Chapter II explores how carbon fertilization (elevated CO_2) changes the effects of herbivorous insects on plant defense and growth and examines the genetic variation in plant response to herbivory and carbon

fertilization. Chapter III examines how herbivory and carbon fertilization affect plant-fungal interactions and provides a quantitative comparison among potential mechanisms that may mediate the effect of aboveground herbivores on mycorrhizal fungi. In Chapter IV, I develop a model to predict how variation in the association between plants and mycorrhizal fungi affects plant defense phenotype and herbivore performance. The second part of chapter IV presents an initial test of the model. Chapter V further tests the model developed in chapter IV using an experimental manipulation of two fungal species over a range of fungal abundance, in association with multiple plant genotypes and examines how herbivore performance varies with resulting changes in plant nutrition and defense expression.

Chapter II. Genetic variation in the expression of defense phenotype may mediate evolutionary adaptation of Asclepias syriaca to elevated CO_2. If plant phenotypic response to herbivory depends on resource availability, resource addition should modify plant phenotype and induced responses to herbivory (Bazin et al. 2002, Emmerson et al. 2005, Bidart-Bouzat and Imeh-Nathaniel 2008). In addition, if plant genotypes vary in the extent to which they respond to atmospheric conditions, plant populations may be better able to adapt to rapidly changing environmental conditions associated with climate or other anthropogenic change (Jump et al. 2009). I examined the response of five genetic families of *Asclepias syriaca* (common milkweed) to elevated CO_2 and herbivory by a specialist caterpillar *Danaus plexippus* (monarch caterpillar).

I conducted a factorial experiment in which I exposed *A. syriaca* plants to herbivory by *D. plexippus* caterpillars and elevated CO_2, then examined constitutive and induced expression of plant defensive traits (Fig. 1.2a). If the phenotypic response of *A. syriaca* to herbivory differs among conditions of ambient and elevated CO_2, this would indicate that plant induced responses are dependent on carbon availability and may be subject to change under future atmospheric conditions. Additionally, if *A. syriaca* genotypes respond differentially to CO_2 or herbivory, *A. syriaca* populations may be able to adapt rapidly to changing conditions.

Chapter III. Multiple pathways mediate the effects of resource availability and herbivore identity on mycorrhizal associations. Previous research has documented that aboveground herbivores can affect plant interactions with mycorrhizal fungi (Gehring and Whitham 1994, Gange 2007) and most studies implicate carbon as the main factor that limits mycorrhizal colonization following herbivory (Hartley and Gange 2009). However, recent studies indicate that plant defense induction, rather than carbon limitation, can alter mycorrhizal colonization of plants (Kleczewski et al. 2010, de Román et al. 2011) and raise doubts on the role of carbon limitation in mycorrhizal responses to herbivory (Barto and Rillig 2010).

In order to test these alternative but potentially complementary hypotheses, we formalized multiple structural equation models that tested alternative causal pathways leading from herbivory to changes in mycorrhizal colonization. Alternative intermediates in the models included changes in carbon allocation, induction of above and belowground defense, and additional mechanisms. We compared models against the results of an experiment that measured mycorrhizal colonization of *A. syriaca* following herbivory by caterpillars and aphids, herbivores that differentially affect plant carbon status, under ambient and elevated CO_2 (Fig. 1.2b). In addition, we examined if model coefficients differed depending on herbivore identity and resource availability. If experimental data conform to the hypothesized models that contain carbon-based pathways, this is good evidence that carbon limits plant-fungal interactions following herbivory. In addition, if herbivore identity and carbon fertilization affect the pathways by which herbivores influence mycorrhizal colonization, this may lend further insight into if and how changes in resource availability and defense induction structure mycorrhizal associations.

Chapter IV. Plant defense theory re-examined: nonlinear expectations based on the costs and benefits of resource mutualisms. Variation in resource availability, including soil fertility, affects the expression of plant defense phenotype (Bryant et al. 1983, Herms and Mattson 1992). However, theoretical expectations for how defense expression varies with soil fertility have been developed with little consideration of the effects of soil symbionts. Resource mutualisms, including mycorrhizal interactions, are ubiquitous in

natural and managed systems (van der Heijden et al. 2008), and may change plant responses to soil fertility because of the costs and benefits associated with mutualistic interactions (Johnson et al. 1997). In this chapter, We develop a benefit:cost framework to anticipate how the abundance of nutrition symbionts affect plant defense expression (Fig. 1.2c). We test this model by inoculating *A. syriaca* plants with increasing abundance of mycorrhizal fungi. If plant phenotypic response to colonization corresponds with model predictions, this indicates that the exchange of resources within the mycorrhizal mutualism structures plant defense expression.

Chapter V. Mycorrhizal abundance affects plant defense expression and herbivore performance. In addition to the role of mycorrhizal abundance in shaping plant phenotype, the species identity of mycorrhizal colonists may alter the costs and benefits transferred within the mycorrhizal interaction. For example, fungal species in the Gigasporaceae allocate proportionally more resources toward the growth of extraradical hyphae than do fungal species in the Glomeraceae (Hart et al. 2001), with consequences for plant nutrient status (Powell et al. 2009). Not only may fungal species differentially affect the benefits and costs associated with mycorrhizal interactions, but plant genotypes may respond differently to the mycorrhizal interaction. In addition, the relative role of these factors in the expression of plant defense phenotype is unclear.

To explore further how increasing fungal abundance alters plant nutrition, defense expression, and herbivore performance, and if these effects depend on the identity of fungal species and plant genotype, I manipulated these factors in a large greenhouse experiment. I germinated seedlings of five genetic families of *A. syriaca*, grew them under three different mycorrhizal treatments (*Glomus etunicatum*, *Scutellospora fulgida*, and a mix of the two) with increasing fungal inoculum to generate a range of fungal colonization intensities. I quantified plant defense expression and assessed herbivore performance on plants colonized by increasing abundance of fungi. If fungal species vary in their relative effects on plant nutrition and phenotype expression, this may lend support to the cost:benefit approach and aid predictions of how fungi may affect plant defense expression and multitrophic interactions. In addition, if genetic variation exists

in plant response to fungal colonization or species identity, this may lead to complex evolutionary dynamics among plants, fungi, and insect herbivores.

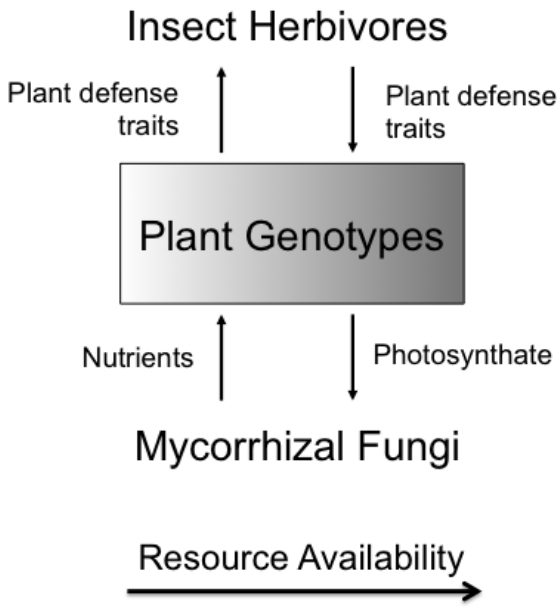

Fig. 1.1. Diagram illustrating above-belowground feedbacks within a multitrophic system. Insect herbivores are affected by plant defense traits, but can also change the expression of plant defense phenotype. Mycorrhizal fungi affect plants through the exchange of nutrients for carbon. Plant physiology and allocation patterns mediate plant responses to interactions with herbivores and mycorrhizal fungi, but plant genotypes vary in their allocation patterns, phenotype expression and response to these interactions. Both types of interactions occur across a gradient of resource availability (carbon, nutrients etc).

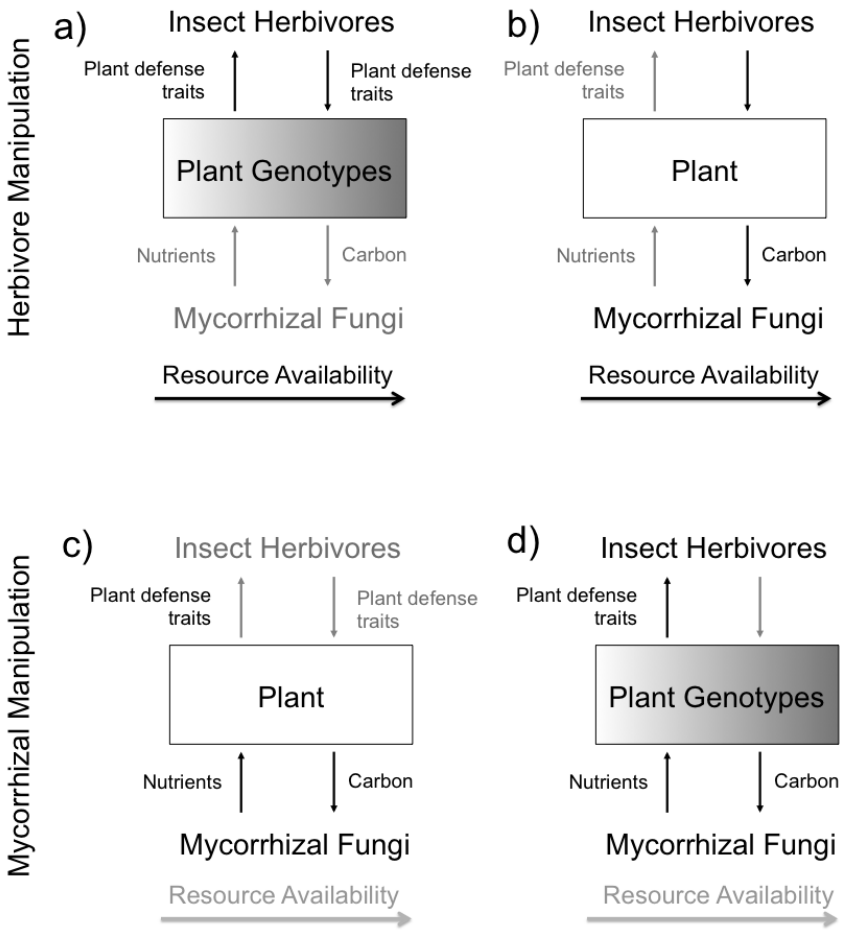

Fig. 1.2. Diagram illustrating the experiments and survey performed in Chapters II-VI, following the framework presented in Fig. 1. The arrows and words in bold indicate focal interactions or traits in each chapter. Chapter II describes the results of a manipulation of resource availability (CO_2) and herbivory on plant defense trait expression among plant families (a). Chapter III explores the effects of aboveground herbivory on mycorrhizal colonization of plants and explores the mechanisms involved in this interaction (b). Chapter IV develops expectations of the effects of mycorrhizal fungi on plant defense expression mediated by resource exchange between plants and fungi (c). Chapter V provides a test of predictions developed in Chapter IV, quantifying plant nutrients and defense trait expression among plant genotypes exposed to different fungal treatments, and exploring their effects on herbivore performance (d).

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Chapter II

Genetic variation in expression of defense phenotype may mediate evolutionary adaptation of *Asclepias syriaca* to elevated CO_2

Abstract

How species interactions may modify the effects of environmental change on evolutionary adaptation is poorly understood. Elevated CO_2 is known to alter plant-herbivore interactions, but the evolutionary consequences for plant populations have received little attention. We conducted an experiment to determine the effects of elevated CO_2 and herbivory by a specialist insect herbivore (*Danaus plexippus*) on the expression of constitutive and induced plant defense traits in five genotypes of *Asclepias syriaca*, and assessed the heritability of these traits. We also examined changes in relative fitness among plant genotypes in response to altered CO_2 and herbivory. The expression of plant defense traits varied significantly among genotypes. Elevated CO_2 increased plant growth and physical defenses (toughness and latex), but decreased investment in chemical defenses (cardenolides). We identified no effect of elevated CO_2 on plant induction of cardenolides in response to caterpillar herbivory. Elevated CO_2 affected the expression of chemical defenses (cardenolides) to a different extent depending on plant genotype. Differential effects of CO_2 on plant defense expression, rather than direct effects on relative fitness, may alter *A. syriaca* adaptation to changing climate.

Introduction

Central to ecological conservation and management remains the question: will species be able to accommodate the rapid ecological changes imposed by anthropogenic disturbance? If species can adapt to these changes, how will global change drivers interact with other selective pressures acting in species' environments to shape the evolution of species? It is clear that many global environmental changes substantially alter the environmental conditions, and thus selection regime, experienced by biota (Reusch & Wood 2007). Species can accommodate changing conditions through a variety of mechanisms, including phenotypically plastic responses, migration, and genetic change (Jackson & Overpeck 2000). While phenotypic plasticity and migration are likely responses to rapid environmental change (Parmesan 2006), habitat destruction and fragmentation have decreased the area of suitable habitat and increased dispersal distances among such habitats (Travis 2003). As a result, in-situ evolution in response to changing climate is likely to become increasingly important (Davis & Shaw 2001), but evidence for this phenomenon is scarce (Gienapp *et al.* 2007). It is unclear if natural populations host sufficient genetic variation to adapt evolutionarily to rapid environmental change (Jump *et al.* 2009, Kellermann *et al.* 2009).

In order for species to respond evolutionarily, global environmental change (GEC) drivers must differentially alter the expression of organism growth, reproductive or other phenotypic traits under selection. Additionally, the observed phenotypic traits must be both heritable and variable within populations. Recent literature documents a few examples of altered fitness responses within agricultural and native plants, insects, and bird species to global change drivers (Reusch & Wood 2007). Specifically, GEC drivers may directly alter the fitness of genotypes within a population by altering allocation to reproductive traits. For example, rice (*Oryza sativa* L.) genotypes grown under elevated CO_2 vary significantly in their grain yields as a result of intraspecific variation in photosynthetic rate (De Costa *et al.* 2007).

In reality, the performance and fitness of organisms under environmental change will reflect complex interactions among changing biotic forces (competition, predation,

disease) and changing abiotic forces (temperature, precipitation, atmospheric CO_2)(Tylianakis *et al.* 2008). For plants, the presence of competitors, herbivores, or symbionts within the environment can enhance or limit their responses to global environmental change (Brooker 2006). For example, elevated CO_2 has no effect on *Bromus erectus* growth and reproduction when plants are grown with conspecifics. In contrast, *B. erectus* plants grown with heterospecifics under elevated CO_2 show decreased growth and fitness (Steinger *et al.* 2007). In other words, competitive background and atmospheric CO_2 interact to determine *Bromus* fitness. Only by examining how species interactions modify the effects of environmental change can we begin to understand and predict the ecological and evolutionary consequences of these complex changes in natural systems.

GEC drivers are known to alter the interactions of plants with insect herbivores through changes in plant palatability and quantity (Stiling & Cornelissen 2007, Bidart-Bouzat & Imeh-Nathaniel 2008). Elevated CO_2 can alter plant-herbivore interactions by increasing plant growth, decreasing plant nutrient content, and altering the expression of plant defenses (Kinney *et al.* 1997, Agrell *et al.* 2000). However, not all plants respond to elevated CO_2 in a similar or predictable fashion (Lindroth *et al.* 1993, Hunter 2001, Bidart-Bouzat & Imeh-Nathaniel 2008) and the outcomes of plant-herbivore interactions under future atmospheric conditions remain difficult to anticipate (Petri A *et al.* 2010).

Predictions are complicated yet further if the expression of plant defense is modified by interactions with herbivores. Induced defenses, or those expressed in response to herbivore damage (Agrawal 2001) can affect plant fitness (Baldwin 1998, Agrawal 1999) and subsequent herbivore consumption (Van Zandt & Agrawal 2004a). Elevated CO_2 has been shown to increase induction of chemical defenses in *Arabidopsis thaliana* and *Brassica rapa* (Bidart-Bouzat *et al.* 2005, Himanen *et al.* 2008), but decrease induction in *Glycine max* (Zavala *et al.* 2008), and has little or no effect on herbivore induction of plant defenses in *Populus tremuloides*, *Acer saccharum*, *Lotus corniculatus*, *Gossypium hirsutum*, and *Quercus myrtifolia* (Roth *et al.* 1998, Bazin *et al.* 2002, Agrell *et al.* 2004, Rossi *et al.* 2004). From these examples, we see that the effects of elevated CO_2 on

induction are not well-understood and a general predictive theory has not yet been achieved.

Elevated CO_2 is well known to alter plant-herbivore interactions in ecological time (Lindroth *et al.* 1993, Hall *et al.* 2005, Stiling & Cornelissen 2007), whereas only a few studies have explored potential effects of elevated CO_2 on the evolutionary outcome of plant-herbivore interactions (Bidart-Bouzat 2004, Lau & Tiffin 2009). Assessing genetic variation within populations for plant defense expression, and changes in fitness under increasing CO_2, is crucial to understanding plant adaptation and plant-insect coevolution under realistic scenarios of environmental change. Since genetic variation often exists in the expression of plant defenses (Berenbaum *et al.* 1986, Simms & Rausher 1987), we expect that elevated CO_2 may differentially affect the induction of defense among genotypes (Julkunen-Tiitto *et al.* 1993; Lindroth *et al*. 2001). Additionally, elevated CO_2 may magnify or diminish differences in defense or fitness among genotypes and as a consequence, increase or decrease the strength of herbivory as a selective force on plant populations. For example, some genotypes within species may exhibit greater defense induction than do other genotypes under ambient CO_2 conditions, whereas elevated CO_2 may alleviate allocation tradeoffs such that all genotypes induce to approximately the same degree. As a consequence, elevated CO_2 may increase or decrease the fitness differences among plant genotypes. Examining the interactive effects of herbivory and CO_2 among multiple plant genotypes may allow us to anticipate both the phenotypic (ecological) and fitness (evolutionary) consequences of these forces. Only the manipulation of multiple abiotic and biotic factors will allow us to understand the complex ecological mechanisms that drive adaptation to environmental change (Tylianakis et al. 2008), and may allow us to predict how these ecological changes affect the evolution of plant populations.

To examine the potential for our focal plant population to accommodate changing atmospheric CO_2 concentrations, and the effect of elevated CO_2 on constitutive and induced plant defense, we examined the following predictions. First, we proposed that our focal plant population would contain genetic variation in the expression of

reproductive and defense traits, and that these traits would be heritable. Second, we expected that CO_2 would affect the expression of plant growth, reproduction, and constitutive and induced defense, and that plant genotypes would exhibit variation in phenotypic response to CO_2. We tested this prediction by examining variation in phenotypic responses to elevated CO_2 among plant genotypes. Third, we predicted that plant fitness (measured as plant reproductive traits) would vary among genotypes and that elevated CO_2 would alter the expression of these traits.

To address these questions, we examined intraspecific variation and heritability in the expression of growth, reproductive, and defensive traits, and the effects of elevated CO_2 on these traits, in the common milkweed *Asclepias syriaca* L. (Apocynaceae). We examined the induction of plant defenses in *Asclepias syriaca* by monarch larvae *Danaus plexippus* (Lepidoptera: Nymphalidae: Danainae), a specialist insect herbivore.

Materials and Methods

Plant and Insect Species

The common milkweed, *A. syriaca*, inhabits open fields throughout eastern North America, and reproduces asexually through rhizomatous growth belowground and sexually through the production of follicles that are fertilized by a single pollinium. As a result, *A. syriaca* pods contain full-sibling seeds. *A. syriaca* hosts at least 12 specialized insect herbivores, including chewing leaf feeders, phloem feeders, leaf miners, stem feeders, root feeders, and seed predators. Many physical and chemical traits deter herbivory or retard insect development on *A. syriaca* (Zalucki & Malcolm 1999, Zalucki *et al.* 2001, Agrawal & Fishbein 2006). High concentrations of cardenolides, toxic, bitter-tasting steroids, can decrease the survival and performance of the specialist herbivore *Danaus plexippus* (Zalucki *et al.* 2001). Latex, a sticky polyisoprene polymer that contains cardenolides and other compounds, is stored within pressurized laticifers and can engulf small herbivores and inhibit the feeding of larger ones (Zalucki & Malcolm 1999, Zalucki *et al.* 2001). Trichomes produced on the upper and lower lamina and leaf veins of *A. syriaca* may inhibit feeding by herbivores (Levin 1973). Leaf toughness, tightly correlated with specific leaf mass (SLM) (Frost & Hunter 2008), can also inhibit feeding by many insect herbivores (Coley 1983, Read & Stokes 2006). While all of the defensive traits described here consist primarily of carbon, the enzymes required to construct them require nutrients such as nitrogen and phosphorus (Gershenzon 1994). The responses of *A. syriaca* defenses to elevated CO_2 are therefore hard to predict.

Much work has been conducted on the effects of the multi-trait *Asclepias* defensive phenotype on its specialist herbivores (Zalucki & Malcolm 1999, Zalucki *et al.* 2001, Agrawal & Malcolm 2002, Agrawal 2004, Agrawal & Fishbein 2006), and it is well-established that variation in plant defensive traits affects herbivore performance and host choice in natural systems. Additionally, *Asclepias* spp. are known to respond to insect herbivory by altering their chemical phenotype (Malcolm & Zalucki 1996, Martel & Malcolm 2004, Van Zandt & Agrawal 2004a, b, Zehnder & Hunter 2007). Since we know that *A. syriaca* alters the expression of plant defenses in response to herbivory, it is

an ideal plant in which to investigate the effects of elevated CO_2 on defense expression and induction.

Asclepias syriaca is a perennial rhizomatous herb, and seedlings do not reproduce sexually for at least 2-3 years at our field site in northern Michigan (authors' unpublished data). Instead, plants reproduce asexually during this time, through rhizomatous growth and ramet production. In the fall, all aboveground plant material senesces; belowground biomass and meristem production (buds on the rhizome) constrain regrowth and ramet number the following year. Following established methods (Fagerstrom 1992), we therefore estimate *A. syriaca* fitness after one growing season using belowground biomass and the number of buds produced on the rhizome. We emphasize that this limits our conclusions about effects of treatments on plant fitness to the first few years of growth.

Experimental Design.

Asclepias syriaca pods were collected from a single population in northern Michigan at the University of Michigan Biological Station (UMBS) in Pellston, MI during Fall 2007. Five *A. syriaca* full-sibling families, hereafter referred to as genotypes, were delineated initially based on spatial clustering of their ramets and phenological, morphological, and chemical differences among genets. Subsequent excavation of rhizomes and microsatellite analyses have confirmed the existence of independent genets at our field site. During May 2008, we established 5 genotypes of *A. syriaca*, each generated from a single pod from one of the five field genotypes. Seeds were cold stratified for 4-5 weeks during spring 2008, and were germinated in May 2008 on moist filter paper at 25^{o}C. Following germination, seedlings were planted into 50 mL cells containing potting soil (SunGrow Metromix) and reared in a growth chamber for two weeks. Eighty seedlings of each genotype were planted individually into 6 inch pots containing approx 1L of a 2:1 mixture of potting soil (SunGrow Metromix) and UMBS sandy soil, respectively. Transplanted seedlings were kept in the UMBS glasshouse for 2 weeks to prevent frost damage.

Four weeks after the initial planting (June 1-2, 2008), *A. syriaca* individuals were placed in open-top controlled atmosphere chambers in the field at UMBS. The chamber array consisted of 20 chambers, with 10 maintained at ambient CO_2 concentrations, and 10 maintained at elevated CO_2 concentrations (760 ppm, dawn until dusk), dispersed uniformly within the array. Each chamber held two individual plants from each of the five plant families (10 plants per chamber), five designated for caterpillar herbivory, the others undisturbed controls. Atmospheric CO_2 concentrations were monitored daily in all elevated CO_2 chambers and 2 ambient chambers using a LI-COR LI-6262 IRGA and CO_2 was adjusted to maintain the target concentration in each elevated CO_2 chamber. Plants were watered daily and their heights measured weekly for the nine weeks of CO_2 treatment. Two weeks before the herbivory treatment was initiated (early July, 2008), when plants were approximately two months old, all plants were covered with a fine mesh (paint strainer bags, Mastercraft Mfg.) to keep any local herbivores from consuming the plants or inducing plant defenses, although nearly all plants were free of prior damage.

We captured gravid monarch butterflies from the field at UMBS, allowed them to lay eggs in the laboratory, and collected eggs on leaf discs using a hole punch and stored them in a refrigerated incubator until use (maximum two weeks). All monarch eggs came from five wild caught females of unknown provenance.

The induction treatment was initiated 5 days before harvest. A single *D. plexippus* egg that had darkened just prior to larval eclosion was 'glued' to the leaf of each treatment plant using milkweed latex. Before it dries, latex is an effective defense against herbivores, but the tiny amount added was allowed to dry and was not harmful to the larvae. Eggs were placed on a single individual of each plant family in each of 10 ambient and 10 elevated CO_2 chambers (100 plants total). The larvae hatched within hours and were allowed to eat for 5 days following eclosion, resulting in the consumption of approximately 10-20% of each plant. Both control and herbivore treatment plants remained covered in mesh during the caterpillar treatment.

Although *A. syriaca* can rapidly (24 hrs) induce cardenolide expression in response to damage (Malcolm & Zalucki 1996), extended periods of damage (up to 30 days) by aphid herbivores can also affect cardenolide expression in *Asclepias* species (Zehnder & Hunter 2007). The length of our herbivory treatment, with continual damage throughout the treatment period, should be suitable to detect changes in plant expression of defenses.

Harvest

Plants were harvested at 12 weeks of age and each had between 6 and 22 leaves. *A. syriaca* plants have opposite leaves. All plant heights were measured, and vertical growth since initiation of herbivore treatment was used to calculate net regrowth during the herbivory period as a measure of tolerance. Five hole punches (424 mm^2) of fresh leaf tissue were taken from one "side" of the two largest leaf pairs on each plant, placed immediately into 1 mL of methanol and stored at -10^oC for cardenolide analysis (below). Five identical leaf discs were taken from the opposite "side" of the leaf pairs and stored in glassine envelopes to provide estimates of sample dry weights and measures of other leaf traits (below). Latex that flowed from the first five holes punched was collected on a pre-weighed cellulose disc (1 cm. diameter), dried, and weighed.

Analysis of Plant Traits

Aboveground and belowground tissues were dried and weighed to the nearest 0.01 g as measures of above- and below-ground biomass. The number of buds on each rhizome was counted and used as a measure of clonal reproduction (Fagerstrom 1992, Wikberg *et al.* 1994). The masses of all five discs were averaged and used to calculate the specific leaf mass (SLM = mass/area) for each plant as an index of foliar toughness (Frost & Hunter 2008). The number of trichomes on five subsections of the upper and lower sides of each leaf was counted under a dissecting microscope at 4x using an optical micrometer, and averaged to a single value for each plant. The amount of leaf tissue consumed by caterpillars was estimated on scanned leaves using WinFOLIA software. Analysis of cardenolides in leaf tissue was performed using methods modified from Malcolm and Zalucki (1996) and Zehnder & Hunter (2007). Briefly, leaf discs were ground in methanol for 2 minutes using a ball mill (Rensch MM200), sonicated at 60^oC

for 1 hour and evaporated to dryness. Samples were re-suspended in 150 uL of 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). Peaks were detected by absorption at 218 nm using a diode array detector, and absorbance spectra were recorded from 200 to 300 nm with digitoxin as the standard. Peaks with symmetrical absorption maxima between 217 and 222 nm were recorded as cardenolides. Individual cardenolide compounds were separated by differences in retention time. The cardenolide peaks reported here were detected in at least 2/3 of all examined samples. 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.

Statistical Analysis

We estimated the heritability for each plant phenotypic trait using full-sibling estimates of heritability. Full-sibling heritability estimates approach narrow sense heritability measures when all genetic variance is additive, and are typically lower than broad sense heritabilities (Falconer 1981, Roff 1997). Although estimates of behavioral and physiological traits can be contaminated by dominance variation, morphological traits tend to have lower dominance variation in general. Thus, full-sib estimates of heritability are generally robust, compared to regression estimates (Mousseau & Roff 1987, Roff 1997). We calculated heritability as 2 times the plant genotype variance component, divided by the total variance component ($H^2=(2VC_{full\text{-}sib})/(VC_{full\text{-}sib}+VC_{error})$) (Lynch & Walsh 1996). We estimated the variance component explained by plant genotype using Proc Mixed (SAS v.9.1) using a one-way ANOVA, with Genotype as a random effect to extract the genotype variance component (Agrawal 2005). The significance of each heritability estimate was calculated using a z-test. Although heritability analyses conducted under controlled conditions usually yield higher estimates than do field estimates, the two are strongly correlated and heritabilities detected under lab conditions are considered meaningful under field conditions (Roff & Simons 1997). We acknowledge that a complete forecast of how trait values may change in the long-term depends on the pattern of additive genetic variances and covariances of traits (Schluter

1996). As a result, our initial analysis does not currently allow a meaningful calculation of genetic covariances, but does describe a useful prerequisite for evolutionary change.

To determine if herbivory (i.e. induction treatment), plant genotype, or CO_2 caused significant variation in plant growth or defensive traits, F-tests were performed on the trait values of all treatment (herbivory) and control plants. Residuals were examined for each model and response variables were log-transformed if necessary to improve homoscedasticity. Following Quinn and Keough (2002), we used a split-plot model, with plant genotype and herbivory treatment crossed within CO_2 treatments. The model was run using PROC MIXED in SAS v 9.1. Herbivory, plant genotype, CO_2 and their interactions were considered fixed effects, while chamber and its interactions were considered random effects. Differences among treatment means were assessed using Tukey's HSD tests. Here, we focus on plant responses to insect damage. Analysis of *D. plexippus* performance on *A. syriaca* under ambient and elevated CO_2 will be presented elsewhere.

Results

Genetic variation and heritability of growth, reproductive, and defense traits

Genotypes (full-sibling families) of *A. syriaca* collected from different genets within the same Northern Michigan population were highly variable in the expression of growth, reproductive, and defensive traits (Table 2.1). Plant genotypes varied significantly in total biomass, bud number, regrowth ability, latex exudation, trichome density, and the expression of cardenolides (Table 2.1). However, plant traits were not uniformly heritable (Table 2.1). Despite our limited ability to detect significant heritability due to the inclusion of only five full-sibling families, we have detected patterns in these data and therefore make note of moderately significant trends ($p<0.10$) in heritability estimates. For example, plant growth and reproductive traits such as biomass, root biomass, and the number of buds on the rhizome displayed very low levels of heritability (H^2=0.02-0.24, Table 2.1), while most of the defensive traits, including latex, and all cardenolide compounds examined exhibited moderate heritability estimates (H^2=0.13-0.32, Table 2.1), that approached statistical significance ($p<0.10$). In contrast, specific leaf mass, a proxy for leaf toughness, displayed very low heritability (H^2=0.03, Table 2.1).

Genotype responses to elevated CO_2: growth, reproductive, and defense traits

Elevated CO_2 increased aboveground biomass and belowground biomass by an average of 15% ($F_{1,90}$=4.82, p=0.031; $F_{1,90}$=3.98, p=0.049, Tables 2.2&2.3), but did so to a similar extent among all plant genotypes. Atmospheric CO_2 concentration had no direct effect on the number of bud meristems produced on *A. syriaca* rhizomes ($F_{1,90}$=0.89, p=0.35, Tables 2.2&2.3). Despite substantial effects of CO_2 on aboveground and belowground biomass, we identified no interactions between genotype and CO_2 on belowground biomass or meristem bud number produced by each genotype (Fig. 2.1 a&b) or in response to herbivory (three-way interaction) (Table 2.2).

In contrast to its effect on growth and reproductive traits, elevated CO_2 altered the expression of many plant defenses differently depending on plant genotype. Specifically, CO_2 tended to increase latex exudation in two of the five genotypes (Family*CO_2 $F_{4,90}$=2.14, p=0.082, Fig. 2.2). Plants grown under elevated CO_2 contained on average,

20% less cardenolide than those grown under ambient atmospheric conditions ($F_{1,90}$=4.18, p=0.04, Tables 2.2&2.3, Fig.2.3), but plant families were affected to different extents by increased CO_2. Some genotypes decreased cardenolide expression to a greater extent under elevated CO_2 than did others. Of the three cardenolide peaks detected in the majority of plants, CO_2 affected the expression of cardenolide peak 1 (the most polar compound) as well as total cardenolide concentration differently among plant genotypes. The concentration of cardenolide peak 1, comprising 5% of total cardenolide concentration, was reduced substantially in two genotypes under elevated CO_2, whereas expression levels in the other genotypes remained unchanged ($F_{4,81}$=4.69, p=0.0018, Fig. 2.3a). Similarly, total cardenolide concentration, comprised of all cardenolides in plant foliage including rare and common peaks, also declined in two of the five genotypes exposed to elevated CO_2 ($F_{4,81}$=2.58, p=0.043, Fig. 2.3d). Plants grown under elevated CO_2 decreased the expression of cardenolide peak 2 (20% of total cardenolide concentration), and this effect did not differ significantly among genotypes (CO_2 $F_{1,90}$=3.33, p=0.0714, CO_2xFamily $F_{4,90}$=1,77, p=0.14, Fig 2.3b). Elevated CO_2 increased specific leaf mass by 40% ($F_{1,90}$=60.29, p<0.0001, Table 2.2) irrespective of plant genotype (CO_2*Family $F_{4,90}$=0.20, p=0.937, Tables 2.2&2.3). Elevated CO_2 also increased the variation among genotype averages in cardenolide expression (Fig. 2.3d), but tended to decrease the variation among genotypes in the expression of latex and individual cardenolide compounds (Figs 2.2 & 2.3).

Effects of plant genotype and CO_2 on plant responses to herbivory

The amount of leaf tissue consumed by caterpillars did not differ among CO_2 treatments ($F_{1,79}$=2.05, p=0.15) or plant genotypes ($F_{4,79}$=1.42, p=0.236). Herbivory by caterpillars decreased aboveground plant biomass by 15% ($F_{1,81}$=5.6,, p=0.020, Tables 2.2&2.3). Caterpillar herbivory reduced by 20% the rate of plant net regrowth during damage (Herbivory $F_{1,81}$=9.19, p=0.0033, Tables 2.2&2.3). However, elevated atmospheric CO_2 mitigated the negative effect of insect herbivory on plant fitness, measured by the number of meristem buds produced on *A. syriaca* rhizomes (CO_2xHerbivory $F_{1,81}$=7.55, p=0.0072, Fig. 2.4).

Plant genotypes responded to caterpillar herbivory with altered physical and chemical defense expression. All plant genotypes exhibited a 55% decline in latex exudation following caterpillar herbivory ($F_{1,81}$=69.39, p<0.0001, Tables 2.2&2.3). However, elevated CO_2 ameliorated the negative effect of caterpillar herbivory on latex exudation in some genotypes (3 way interaction $F_{4,90}$=2.38, p=0.057, Table 2.3). In addition, all plant genotypes that were consumed by caterpillars displayed nearly 10% lower specific leaf mass than the control plants ($F_{2,81}$=13.71, p=0.0004, Table 2.2). Neither herbivory, CO_2, or their interaction affected substantially the density of trichomes on leaf surfaces (Table 2.2).

Herbivory by caterpillars induced increases in the concentration of some, but not all, foliar cardenolide compounds. Among all plant genotypes, caterpillar herbivory caused an average increase of 50% in the concentration of the second most polar cardenolide: peak 2 ($F_{1,81}$=4.05, p=0.047, Table 2.2). Similarly, the total concentration of cardenolides also increased by 31% in response to herbivory ($F_{1,81}$=4.81, p=0.031). The induction of chemical defenses was not significantly different among plant genotypes or modified by elevated CO_2 (Tables 2.2 & 2.3).

Discussion

Rapid environmental change has imposed novel selection regimes on most species, to which natural populations must adapt in order to persist in the face of altered conditions. Our focal plant population hosts substantial genetic variation in the expression of growth, reproductive, and defensive phenotypic traits. Despite high levels of genetic variation in nearly all traits examined, our full-sib analysis indicates that defensive traits are more heritable than growth or reproductive traits, consistent with classical theory (Mousseau & Roff 1987), and thus able to respond evolutionarily to selection. Although our analysis is limited to five genotypes, hindering our ability to detect significant heritability, our heritability estimates for defense traits in *A. syriaca* are similar to those described by Agrawal (2005), lending support to our results. Additionally, *A. syriaca* genotypes respond differently to elevated CO_2 in the expression of defense, but not growth or reproductive traits. From these results, we conclude that elevated CO_2 will not directly change genotype frequencies within this population of *A. syriaca*; rather, insect herbivory acting on altered defensive phenotype will likely shape the evolution of this plant population instead.

Field experiments demonstrate that the specialist herbivores of *A. syriaca* preferentially feed on plants depending on defense expression, previous damage or induction by previous herbivores (Van Zandt & Agrawal 2004a, Agrawal 2005), which may drive selection in *A. syriaca* populations (Agrawal 2005). For example, *A. syriaca* genotypes with high levels of latex exudation or high trichome densities typically host lower abundances of weevils, leaf miners, and leaf-feeding beetles than those with lower expression of these traits (Agrawal & Van Zandt 2003). As a result, selective herbivore damage is likely to act in combination with genotype-specific effects of elevated CO_2 to alter the evolutionary (and potentially co-evolutionary) trajectory of this plant population and its herbivore community.

Effects of CO_2 on plant defense

Elevated CO_2 substantially altered the defensive phenotype of *A. syriaca*, decreasing plant expression of chemical defense and increasing expression of physical resistance and

tolerance to herbivory. Elevated CO_2 increased plant tolerance to herbivory by mitigating the negative effect of herbivory on the number of bud meristems produced on plant rhizomes. This response has been documented in other perennial plants, but is not a universal phenomenon. *Betula pendula* (silver birch) seedlings are able to compensate to herbivory by increasing total net carbon uptake and regrowth following damage (Huttunen *et al.* 2007). In contrast, both *Arabidopsis thaliana* and *Brassica rapa* are less tolerant to insect herbivory when grown under elevated CO_2 (Marshall *et al.* 2008, Lau & Tiffin 2009). The disparate effects of CO_2 on plant tolerance identified may be due to the different metrics of tolerance assessed in these studies (seed production and regrowth), and since perennials can postpone reproductive costs until the next growing season (Huhta *et al.* 2009), the negative effects of herbivory may have not yet been manifest. Alternatively, perennials may be able to fully or overcompensate from herbivory damage with adequate access to nutrients (Huttunen *et al.* 2007), while annuals grown under elevated CO_2 actually display accelerated phenology and a decreased lifespan (Marshall *et al.* 2008). In this case, perennials may increase allocation to nutrient foraging through the growth and proliferation of fine roots or allocation to mycorrhizal symbionts, responses which Brassicaceous annuals are unlikely or incapable of performing.

With regard to plant palatability, elevated CO_2 increased leaf 'toughness' and decreased investment in cardenolide compounds among all *A. syriaca* families. We identified no effects of elevated CO_2 on the induction of cardenolides. In previous work, elevated CO_2 has been demonstrated to alter induced responses, increasing induction of N-containing glucosinolates in *A. thaliana* (Bidart-Bouzat *et al.* 2005), but also decreasing induction of proteinase inhibitors in *Glycine max* (soybean) (Zavala *et al.* 2008). However, our study, along with the bulk of work on induced responses in perennial plants, indicates no substantial effect of elevated CO_2 on induction of chemical defenses (Roth *et al.* 1998, Bazin *et al.* 2002, Agrell *et al.* 2004, Rossi *et al.* 2004). Our results indicate that plant induction under elevated CO_2 does not correspond to predictions made by simple resource availability (Bryant *et al.* 1983), but that alternative plant defense theories based on the enzymatic costs of defense and plant ontogeny must be invoked to understand these effects (Gershenzon 1994, Boege & Marquis 2005).

However, elevated CO_2 significantly reduced the constitutive expression of cardenolides in *A. syriaca*. Although the majority of studies report that elevated CO_2 increases the expression of carbon-based compounds (Bidart-Bouzat & Imeh-Nathaniel 2008), declines in carbon-based compounds have been detected as well. The reductions in cardenolide expression may be due to decreased availability of nutrients with which to synthesize enzymes (Gershenzon 1994) or resource-based tradeoffs among competing demands. In support of the trade-off mechanism, elevated CO_2 increased plant biomass and increased latex exudation in two of five plant families, but decreased cardenolide expression, which may indicate a trade-off in resource allocation among competing demands (Herms & Mattson 1992).

Alternatively, CO_2-induced changes in biomass and defensive phenotype may indicate a shift to a different ontogenetic stage where plants rely on tolerance and physical defenses rather than chemical defense (Boege & Marquis 2005). We were not able to test this hypothesis directly, since all plants were harvested at a single point in time. However, unpublished data from our field site shows that *A. syriaca* loses chemical defense in favor of structural defense with age (M.D. Hunter, unpublished data) and elevated CO_2 may accelerate this. A similar ontogenetic effect of elevated CO_2 on plant defense allocation was noted in loblolly pine (*Pinus taeda*), where elevated CO_2 directly increased *P. taeda* biomass, and indirectly increased concentrations of condensed tannins in aboveground plant material through accelerated plant growth and ontogeny (Gebauer *et al.* 1998). Elevated CO_2 often accelerates development in woody and herbaceous species (Norby *et al.* 1999, Ludewig & Sonnewald 2000), but the consequences for plant defense have rarely been considered. Further studies should investigate the effect of elevated CO_2 on ontogenetic shifts in the defensive phenotype of perennial plant species and subsequent effects on herbivores throughout the growing season (Zavala *et al.* 2009).

CO_2 x Genotype interactions

Despite intraspecific variation in phenotypic response to elevated CO_2, changing atmospheric composition did not directly affect relative fitness of genotypes within our

focal plant population. Instead, genotypes varied in the expression of defense phenotype and the effect of CO_2 on the expression of defenses was often genotype-specific.

In this study, elevated CO_2 differentially affected the expression of latex and polar cardenolide peaks among plant families. A majority of previous work documents that plant species harbor genetic variation in the degree to which defense expression responds to elevated CO_2. Clones of *Populus tremuloides* respond differentially to elevated CO_2 in the production of condensed tannins (Mansfield *et al*. 1999, Lindroth *et al.* 2001). Similarly, *Salix myrsinifolia* clones produce different amounts of salicin, salicortin, and catechin in response to elevated CO_2 (Julkunen-Tiitto *et al.* 1993). Genetic variation in the effects of CO_2 on defense induction has also been documented. Bidart-Bouzat and colleagues (2005) reported significant genetic variation in the induction of total and individual glucosinolates by *Arabidopsis thaliana* under conditions of elevated, but not ambient CO_2. In our experiment, elevated CO_2 increased variation among milkweed genotypes in total cardenolide expression, but decreased among-genotype variation in other traits, including latex exudation and some specific cardenolide compounds. Apparently, there is no simple relationship in milkweed between elevated CO_2 and the expression of genetic variance in defense traits. Some traits (e.g. total cardenolides) may exhibit more variation upon which natural selection can act whereas other traits (e.g. latex) may exhibit less.

Importantly, the genotypes in this study originated from a single population. Since anthropogenic changes to habitat matrices have, in part, limited gene flow among populations, in-situ evolution is thought to be increasingly important in determining adaptation to changing conditions (Davis & Shaw 2001). As a result, our study allows population-level prediction of evolutionary changes in this species in response to caterpillar herbivory and rising atmospheric CO_2 concentrations.

Our results emphasize the key role of interactions in evolutionary adaptation to global climate change. We present evidence that in the absence of insect herbivory, the genetic composition of plant populations should not change substantially, but that selective

herbivory dependent on plant defensive phenotype (Agrawal & Van Zandt 2003, Van Zandt & Agrawal 2004a, Agrawal 2005) could alter gene frequency within this population. Managing populations under changing global conditions will require not only an understanding of populations' genetic diversity and ability to adapt to changing conditions (Reusch & Wood 2007), but will also require that we anticipate the effects of species interactions on these adaptive responses (Hulme 2005).

Conclusions

A. syriaca families display substantial variation and heritability in the phenotypic expression of traits, especially those traits implicated in defense against herbivores. Elevated CO_2 has substantial effects on *A. syriaca* defensive phenotype, shifting it from chemical defense towards increased tolerance and expression of physical defenses. Despite significant effects of elevated CO_2 on *A. syriaca* growth and fitness components, the effects of elevated CO_2 uniformly increased growth and reproductive traits similarly among all plant families. However, elevated CO_2 affects the expression of plant defensive phenotype differently among families and increased variation in expression of cardenolides among plant families. In this way, genetic variation in defense response to elevated CO_2 and resulting changes in plant-herbivore interactions may mediate plant adaptation to changing climate.

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Table 2.1 Number of values (*N*) used to calculate trait means (*Mean*), *F*-ratio for plant genotype, variance component (VC) explained by genotype, and full-sib heritability (H^2) for growth, reproductive and defensive traits of *Asclepias syriaca* from Pellston, MI.

Plant Trait	*N*	*Mean*	*F*	$VC_{full\text{-}sib}$	VC_{error}	H^2
Growth and Reproductive Traits						
Aboveground Biomass	197	0.316	6.04***	0.00	0.02	0.11
Belowground Biomass	197	0.834	1.86	0.00	0.13	0.02
Rhizome Mass	197	0.537	1.74	0.00	0.01	0.24
Bud Number	197	27.6	1.84	3.85	197.74	0.02
Regrowth	197	0.737	2.87*	0.02	0.41	0.05
Defense Traits						
Latex	197	1.47	6.87***	17.80	117.71	0.13+
Trichome Density	197	5.89	5.15***	0.10	0.46	0.18+
SLM	197	0.0212	2.2+	0.00	0.00	0.03
Cardenolide Peak 1	188	0.06	11.49***	0.00	0.00	0.2+
Cardenolide Peak 2	188	0.27	6.03***	0.02	0.05	0.26+
Cardenolide Peak 3	188	0.5	17.49***	0.08	0.19	0.31+
Total Cardenolides	188	1.13	20.39***	0.27	0.60	0.32+

Significance of Heritability Estimates are based on z-scores, with +p<0.10, *p<0.05, **p<0.01

Table 2.2. Measurements of *Asclepias syriaca* growth, reproductive, and defense traits in an open-top chamber study. Values given are mean trait values± SE. Trait values for plant genotypes were pooled across all herbivory and CO_2 treatments, values for herbivory treatments were pooled across genotypes and CO_2 treatments, and values for CO_2 treatments were pooled across genotypes and herbivory treatments. [a]Post-hoc comparison of means using Tukey-Kramer adjustment ($p<0.05$). Means preceded by the same letter within each factor are not significantly different.

Factor	Level	Aboveground Biomass	Belowground Biomass	Rhizome Mass	Bud Number	Regrowth	ln(Latex)	Trichome Density	SLM	Cardenolide Peak 1	Cardenolide Peak 2	Cardenolide Peak 3	Total Cardenolides
Plant Genotype	A	[ab]0.340±0.023	[a]0.8696±0.059	[a]0.5716±0.048	[a]28.26±2.7	[b]0.664±0.12	[ab]2.31±0.12	[ab]5.01±0.23	[ab]0.211±0.0095	[a]0.0255±0.01	[a]0±0.046	[a]0.133±0.072	[a]0.201±0.149
	B	[b]0.353±0.023	[a]0.8697±0.057	[a]0.565±0.047	[a]24.89±2.6	[ab]0.365±0.12	[b]2.76±0.12	[a]4.46±0.23	[ab]0.222±-0.0095	[c]0.109±0.01	[a]0.0622±0.047	[ab]0.336±0.073	[b]0.698±0.15
	C	[a]0.253±0.023	[a]0.7304±0.057	[a]0.447±0.047	[a]30.04±2.6	[a]0.194±0.12	[ab]2.11±0.13	[b]5.58±0.23	[a]0.195±0.0093	[a]0.0469±0.01	[b]0.203±0.047	[b]0.552±0.074	[b]0.906±0.015
	D	[b]0.337±0.023	[a]0.7239±0.058	[a]0.0494±0.047	[a]33.05±2.6	[ab]0.341±0.12	[b]2.42±0.11	[b]5.22±0.23	[b]0.232±-.0093	[bc]0.0958±0.01	[b]0.297±0.046	[b]0.415±0.072	[b]0.910±0.15
	E	[a]0.227±0.023	[a]0.7200 ±0.058	[a]0.507±0.047	[a]27.64±2.6	[ab]0.366±0.12	[a]1.91±0.13	[b]5.20±0.23	[ab]0.211±0.0092	[b]0.0667±0.01	[b]0.33±0.047	[c]0.942±0.072	[c]1.712±0.15
Herbivory	No Herbivory	[b]0.327±0.014	[a]0.803±0.036	[a]0.513±0.037	[a]28.8±2.0	[b]0.522±0.095	[b]2.77±0.076	[a]5.15±0.18	[b]0.229±0.0059	[a]0.0629±0.006	[b]0.14±0.036	[a]0.459±0.046	[a]0.766±0.12
	Caterpillar	[a]0.277±0.014	[a]0.762±0.037	[a]0.521±0.037	[a]28.7±2.01	[a]0.250±0.095	[a]1.84±0.081	[a]5.03±0.18	[a]0.199±0.0059	[a]0.0745±0.006	[a]0.21±0.037	[a]0.492±0.046	[b]1.00±0.12
CO_2	Ambient	[a]0.279±0.014	[a]0.731±0.037	[a]0.469±0.037	[a]27.83±2.0	[a]0.391±0.097	[a]2.36±0.078	[a]4.96±0.18	[a]0.182±0.0059	[a]0.007890.006	[a]0.209±0.037	[a]0.519±0.046	[b]0.997±0.12
	Elevated	[b]0.325±0.014	[b]0.835±0.036	[b]0.564±0.036	[a]29.7±2.0	[a]0.382±0.094	[a]2.25±0.079	[a]5.22±0.17	[b]0.247±0.0059	[b]0.0584+0.006	[a]0.149±0.036	[a]0.432±0.046	[a]0.774±0.12

Table 2.3. F-ratios testing the effects of plant family, CO_2 concentration, herbivory, and their interactions on the expression of *Asclepias syriaca* growth, reproductive, and defense traits.

Source of Variation	df	Aboveground Biomass	Belowground Biomass	Rhizome Mass	Bud Number	Regrowth	Latex	Trichomes	SLM	Cardenolide Peak 1	Cardenolide Peak 2	Cardenolide Peak 3	Total Cardenolides
Genotype	4	**6.04*** **	1.86	1.74	1.84	2.87*	**6.87*** **	**5.15*** **	2.2+	**11.49*** **	**16.03*** **	**17.49*** **	**20.39*** **
CO_2	1	4.82*	3.98*	7.43**	0.89	0.01	0.93	2.63	**60.29*** **	5.04*	3.33+	1.84	4.18*
Herbivory	1	5.6*	0.63	0.06	0	**9.19** **	**69.39** **	0.55	**13.71*** **	1.6	4.05*	0.24	4.81*
Genotype*CO_2	4	0.44	0.42	0.79	0.16	1.04	2.14+	0.94	0.2	**4.69** **	1.77	0.85	2.58*
Genotype*Herbivory	4	1.63	0.66	0.76	1.28	0.44	0.91	0.67	1.01	0.99	0.98	0.71	0.94
CO_2*Herbivory	1	0	0.07	0.96	7.55**	0.15	3.57+	0.03	2.56	0.74	0.22	0.54	0.85
Genotype*CO_2*Herbivory	4	0.47	0.17	0.14	1.03	0.67	2.38+	0.34	0.24	0.71	0.63	0.3	1.41

+p<0.10, *p<0.05, **p<0.01, ***p<0.001, Bold indicates significance after Bonferroni correction

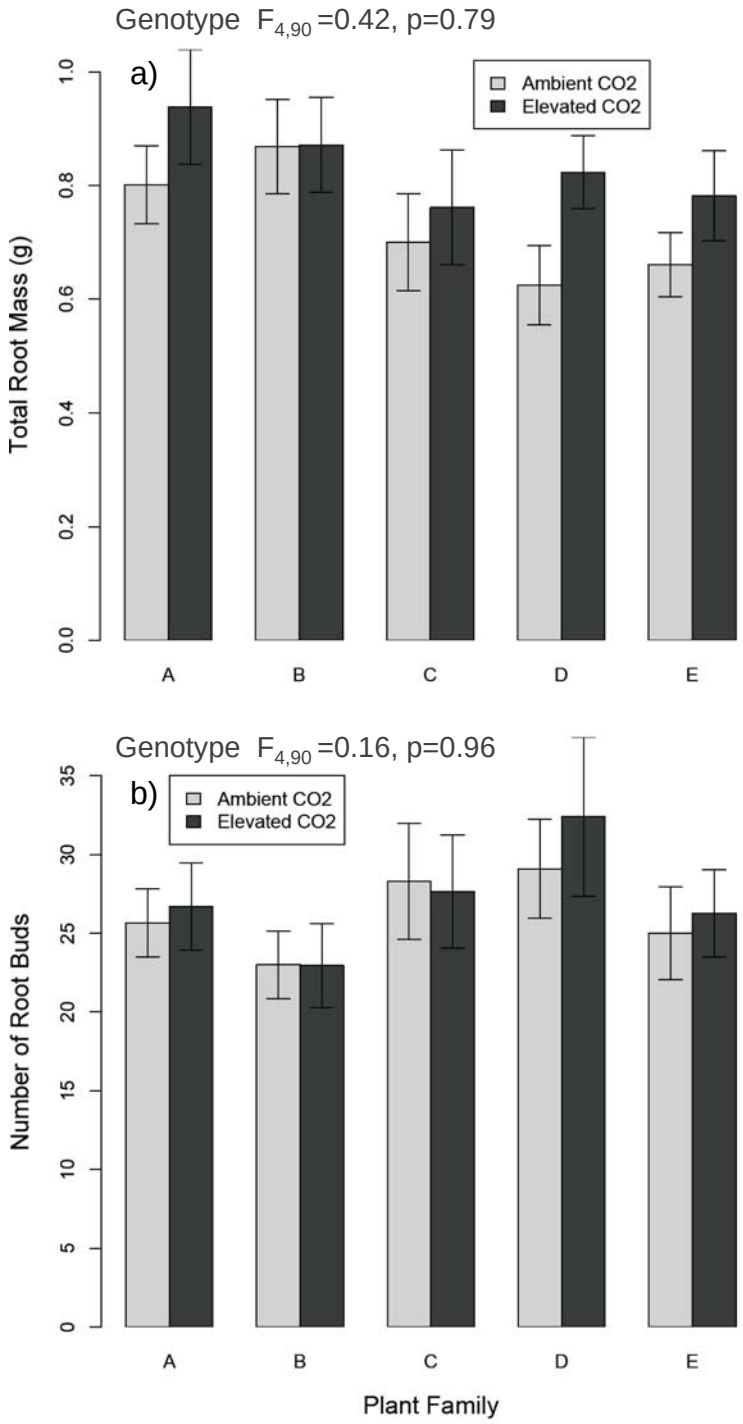

Figure 2.1. a&b The effect of elevated CO_2 on genetic families of *Asclepias syriaca* in the production of a) belowground (rhizome plus fine root) biomass, and b)number of meristem buds on the rhizome, both traits associated with fitness. Bars represent mean trait values ± 1 S.E. pooled across herbivory treatments and F and p values are derived from the full model.

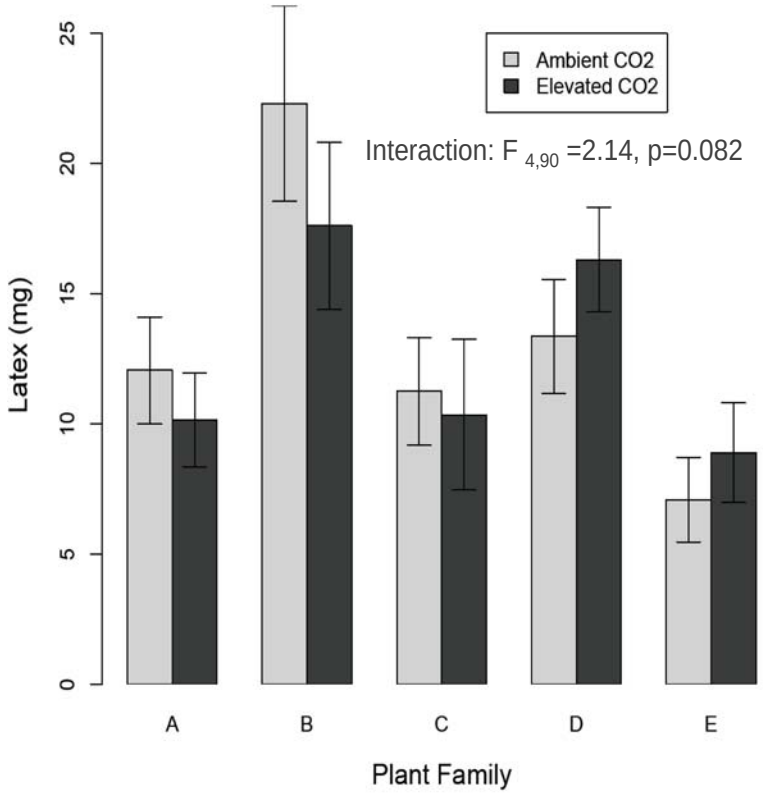

Figure 2.2. The effect of elevated CO_2 on latex exudation among genetic families of *Asclepias syriaca*. Bars represent the mean dry mass of latex exuded ± 1 S.E. pooled across all herbivory treatments and F and p values are derived from the full model.

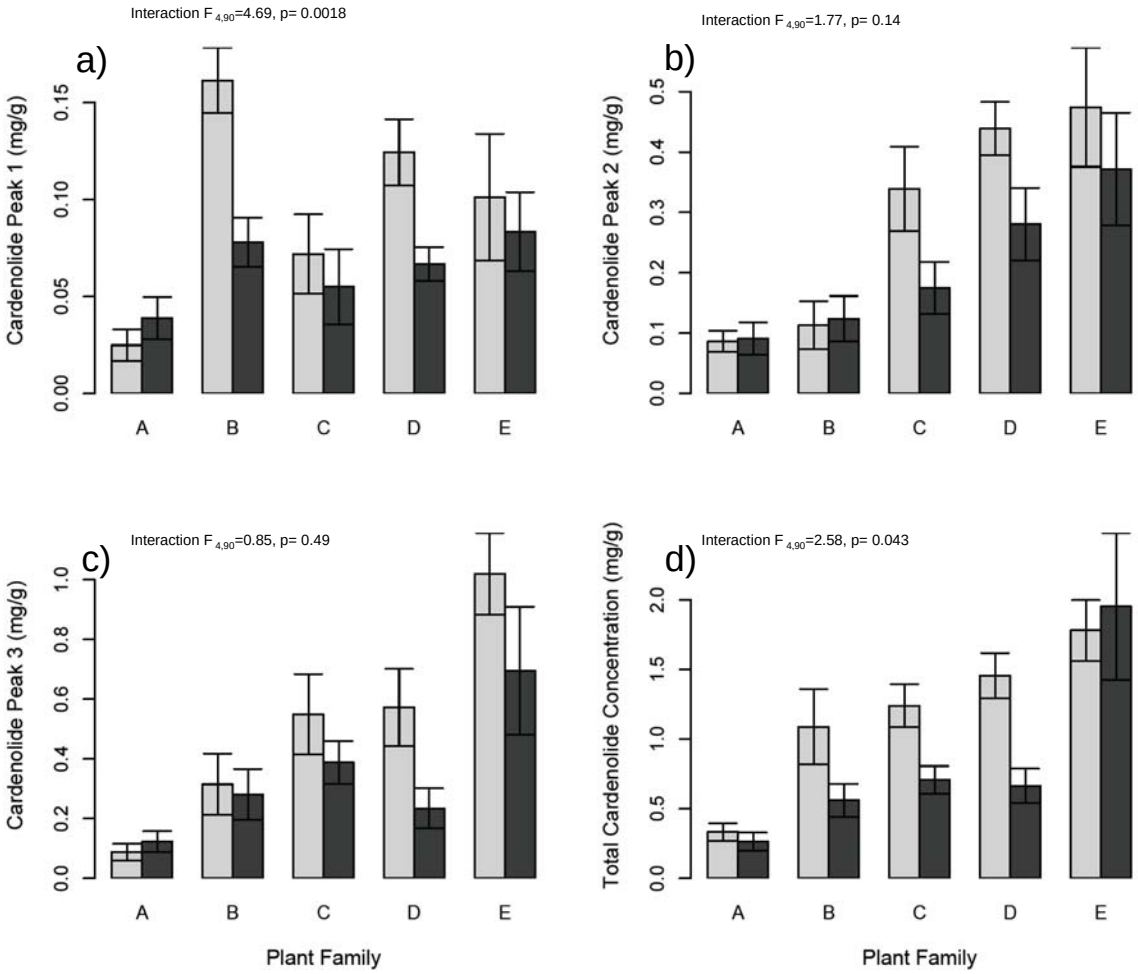

Figure 2.3. a-d The effect of elevated CO_2 on constitutive expression of cardenolide peaks 1-3 and total cardenolide concentrations (a-d) among five genetic families of *Asclepias syriaca*. Bars represent the mean concentration of cardenolides in foliar tissue ± 1 S.E. pooled across herbivory treatments and F and p values are derived from the full model. F-values listed are for genotype x CO_2 interaction. Asterisks indicate differences between ambient and elevated CO_2 treatments within families, using Tukey-Kramer adjustments for all pairwise comparisons.

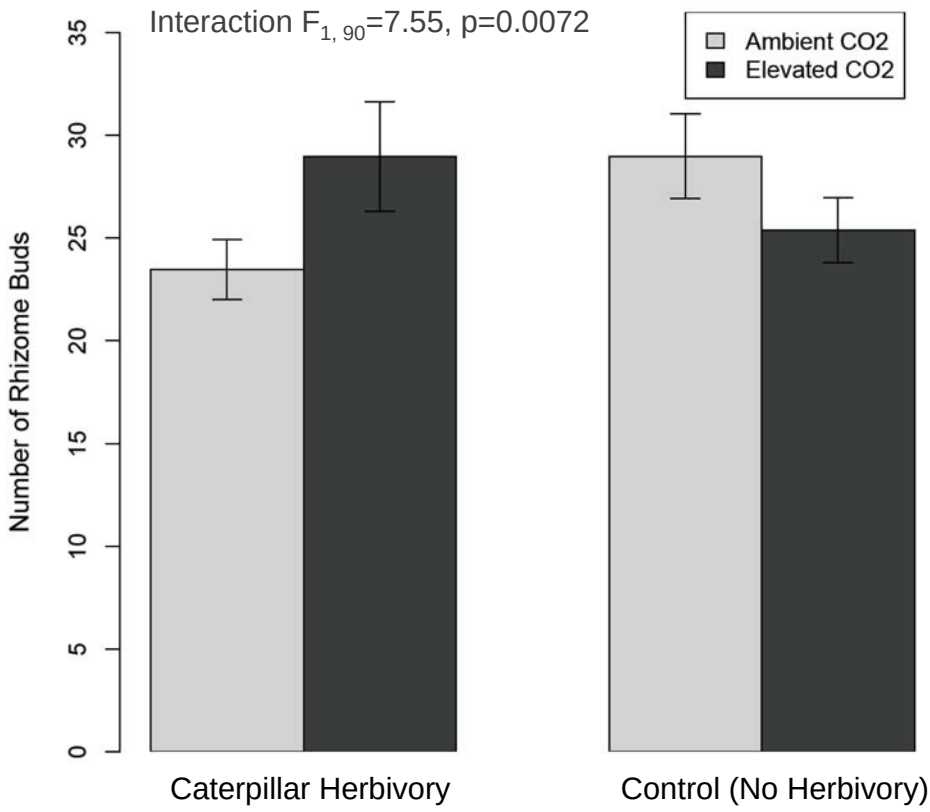

Figure 2.4. The interaction of elevated CO_2 and insect herbivory on the number of *Asclepias syriaca* rhizome buds following herbivore treatment. Bars represent mean trait values ± 1 S.E. pooled across genotypes and F and p values are derived from the full model.

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Chapter III

Multiple pathways mediate the effects of aboveground herbivory on mycorrhizae

Abstract

Plants interact with other organisms residing both above- and below ground, and can dynamically link these communities through multiple pathways. For example, aboveground herbivory can either increase or decrease colonization of plant roots by mycorrhizal fungi. Multiple mechanisms, including changes in the availability of photosynthate, may affect the strength and direction of this interaction. We formalized six structural models depicting hypothesized mechanisms by which aboveground herbivory affects plant growth, defense expression and root colonization by mycorrhizal fungi. We employed a novel application of multiple-groups structural equation modeling (MG-SEM), to explore if and how carbon fertilization (CO_2 enrichment) and herbivore identity (leaf-chewing caterpillar and phloem-feeding aphid) modify the effect of aboveground herbivory on mycorrhizal colonization. Our results show that the effect of aboveground herbivory on mycorrhizal colonization is mediated through a combination of direct and indirect pathways, including a reduction in defense expression following herbivory. Furthermore, MG-SEM revealed that aboveground herbivory by either aphids or caterpillars increases mycorrhizal colonization under elevated CO_2, but not ambient CO_2. Herbivory by caterpillars, but not aphids, strongly decreased the expression of plant defense, and as a consequence, indirectly increased mycorrhizal colonization under ambient CO_2. Our results suggest that herbivore-specific changes in the expression of defense and availability of photosynthate constrain mycorrhizal associations following herbivory.

Introduction

Feedbacks between aboveground interactions (eg. herbivory) and belowground processes (eg. decomposition) are increasingly recognized as key processes in ecological communities (Wardle et al. 2004, Lemons et al. 2005, van Dam and Heil 2011). For example, belowground herbivory can drive patterns of succession and plant community composition aboveground (Brown and Gange 1989). Conversely, insect herbivores can deposit frass, increasing nutrient mineralization belowground, which can feed back to alter plant quality (Frost and Hunter 2008). Despite the importance and prevalence of above-belowground interactions, it remains difficult to anticipate the strength or direction of these interactions and how they may respond to changing ecological conditions (van der Putten et al. 2009).

Plants link above and belowground systems through changes in physiology or resource allocation patterns (Holland et al. 1996, Henkes et al. 2008). Plants must respond to herbivore attack above ground while maintaining mutualistic associations with microbial partners below ground (Hamilton & Frank 2001). The majority of plant taxa rely on mycorrhizal fungi for growth, survival (Smith and Read 2008), reproduction (Stanley et al. 1993), tolerance to damage (Kula et al. 2005) and resistance to herbivores (Vannette and Hunter 2011b) and plant pathogens (AzconAguilar and Barea 1996). The abundance and diversity of mycorrhizal fungi with which plants associate can strongly affect plant community composition (van der Heijden et al. 1998), primary productivity (van der Heijden et al. 2008) and soil carbon (C) storage (Wilson et al. 2009). The degree of mycorrhizal colonization also can strongly influence both plant and fungal performance (Gange and Ayres 1999, Johnson et al. 2010). For example, low colonization can limit nutrient uptake (Sanders et al. 1977, Lekberg and Koide 2005) and protection against root-feeding nematodes (de la Pena et al. 2006). Conversely, high levels of fungal colonization may decrease plant growth (Douds et al. 1988), tolerance to damage (Garrido et al. 2010), and resistance to herbivores (Vannette and Hunter 2011b). As a result, the degree of association between plants and mutualistic fungi may drive population, community, and ecosystem-level processes. Mycorrhizal interactions are maintained in large part through allocation by plants of labile C to mycorrhizal fungi

(Nehls et al. 2007, Baier et al. 2010). Because mycorrhizal fungi are considered to be obligate symbionts of their plant hosts (Smith and Read 2008), C availability is generally thought to limit plant-fungal associations and structure mycorrhizal interactions (Smith and Read 2008).

Herbivores that consume above and belowground plant tissue (Strong et al. 1984) and by removing nutrient-rich tissue, photosynthetic apparatus, or consuming directly the carbon that plants use to support mycorrhizal symbionts, herbivory can reduce mycorrhizal colonization (Gehring and Whitham 1994, Markkola et al. 2004, Gange 2007). Carbon availability can strongly affect plant response to defoliation in some systems; for example, extreme defoliation strongly decreases ectomycorrhizal colonization in *Betula pubescens* (white birch) (Markkola et al. 2004) and extensive herbivory by rabbits decreases mycorrhizal colonization of grasses (Wearn and Gange 2007). However, the effect of herbivory on mycorrhizal colonization varies among and within studies. In contrast to the studies mentioned above, heavy grazing by cattle (Eom et al. 2001) and herbivory by grasshoppers (Kula et al. 2005) both increased mycorrhizal colonization of prairie grasses. While the divergent outcomes among studies may simply be due to variation in carbon limitation imposed by herbivores, a recent meta-analysis identified no relationship between the proportion of tissue damaged and the effect on mycorrhizal fungi, and called into question the generality of the carbon limitation hypothesis (Barto and Rillig 2010). In addition, other mechanisms by which herbivores may affect mycorrhizae have recently been proposed. For example, herbivory can induce changes in plant secondary metabolism belowground (Bezemer and van Dam 2005) and induction of general or specific defenses may negatively affect mycorrhizal associations (Kleczewski et al. 2010, de Román et al. 2011). Additionally, hormonal signaling can mediate plant response to herbivory and may directly or indirectly alter mycorrhizal colonization (Tejeda-Sartorius et al. 2008, Kiers et al. 2010). We may begin to reconcile these seemingly contradictory studies and alternate approaches by examining and comparing among multiple pathways that may mediate the effects of herbivores on mycorrhizal fungi.

In order to understand and better predict how aboveground herbivory affects mycorrhizal associations and what mechanisms cause these changes, we formulated testable models from recently proposed hypotheses and used structural equation modeling (Cronin et al. 2009, Schumacker and Lomax 2010) to compare among them using data from an experiment. We then used a novel application of multiple group SEM (MG-SEM) (Schumacker and Lomax 2010) to examine whether resource availability and herbivore identity alter the strength and direction of pathways between aboveground herbivory and mycorrhizal colonization.

Model Specification

We formalized six alternative causal models that integrate potential mechanisms by which aboveground herbivory affects belowground defense and mycorrhizal associations. **Model 1 (Damage Model, Fig 3.1a)** summarizes potential effects of herbivores on mycorrhizal colonization mediated by damage to aboveground tissue, and implicit regrowth costs. Plant tissue damaged by, or lost to, herbivores must be repaired or replaced, which may decrease primary metabolites available for overall plant growth, allocation to mycorrhizal fungi and belowground plant defense (Gehring and Whitham 1994, Gange 2007).

Model 2 (Root Allocation, Fig 3.1b). Plants under attack may instead allocate primary metabolites belowground (Dyer et al. 1991) to protect these resources from herbivores (Tao and Hunter 2011). The flux of carbon belowground may increase defense synthesis (Kaplan et al. 2008) and allocation to mycorrhizal fungi.

Model 3 (Induction Costs, Fig 3.1c). Upon herbivore attack, plants exhibit a wide variety of physiological and genetic defense responses including the up-regulation of defense genes and synthesis of secondary metabolites above or belowground (Karban and Baldwin 1997, Dicke and Hilker 2003). Induction responses may be costly and are predicted to decrease plant growth (Heil 2001, Walters & Heil 2007) and resources available to support mycorrhizal fungi (Gehring and Whitham 1994).

Model 4 (Toxicity, Fig 3.1d). In contrast to Model 3, the main effect of herbivores on mycorrhizal colonization depicted in Model 4 is mediated by higher concentrations of plant defense toxins within roots. Chemical defenses in roots and shoots can co-vary (Rasmann and Agrawal 2011) and defense induction above ground can subsequently increase defense expression below ground (Soler et al. 2005). Because the activation of generalized or specialized defense-related genes and protein products in roots may directly affect mycorrhizal colonization through toxicity or other mechanisms (Kleczewski et al. 2010, de Román et al. 2011), the concentration of defense is predicted to negatively affect mycorrhizal fungi (Strauss et al. 2002).

Model 5 (Water Stress, Fig 3.1e). Herbivores may increase water loss or decrease turgor pressure in plants by compromising waterproof barriers during damage or by direct consumption of phloem (Schmidt et al. 2009). As a consequence, plants may close stomata, decreasing photosynthetic rates (Quick et al. 1992), and reduce C flow to mycorrhizal fungi or other demands.

Model 6 (Full Model, Fig 3.1f). Because all of the mechanisms described above may act simultaneously, we compared Models 1 through 5 to a full model that included all of the above processes.

In all of the models described so far, we consider the interactions between herbivores and mycorrhizal fungi to be indirect, mediated by resource allocation for other functions. However, plants are known to target resources directly to soil symbionts in response to defoliation (Holland et al. 1996, Hamilton and Frank 2001). In mycorrhizae, such direct interactions may be fine tuned by expression of invertases (Wright et al. 1998). We therefore tested each of the above models with and without the presence of a direct pathway from herbivores to mycorrhizal colonization (paths not shown in Fig 3.1 for ease of interpretation).

Context-Dependence: Herbivore Identity and Resource Availability

The relative strength of the mechanisms proposed above may vary with environmental context. For example, the identity of herbivore may affect plant defense and resource allocation patterns based on herbivore feeding mode or the plant organ consumed (Stout et al. 1994). Aphids consume photosynthate-rich phloem, whereas caterpillars consume nutrient-rich foliar tissue. Plants respond differently to carbon or nutrient limitation (Bloom et al. 1985), so while nutrient limitation can increase allocation to mycorrhizal fungi (Treseder and Allen 2002), carbon limitation imposed by phloem-feeders may instead decrease mycorrhizal colonization (Delvecchio et al. 1993). We therefore hypothesized that herbivory by caterpillars and aphids would differentially affect mycorrhizal colonization. In addition, if C availability limits allocation to mycorrhizal fungi following herbivory, we hypothesized that C fertilization (plant growth under elevated CO_2) would alleviate C-based tradeoffs and increase plant allocation to mycorrhizal fungi among all treatments.

Analysis Overview

To investigate how aboveground herbivory affects mycorrhizal associations, we compared 6 causal models (above) in which aboveground herbivory affects measurable plant traits and ultimately mycorrhizal colonization. We tested these alternative models against the results of an experiment *(n* = 400 plants), in which plants were grown in ambient and elevated CO_2 and herbivory was imposed by either caterpillars or aphids to determine which causal model was best supported. Then we separated these data into four treatment groups and examined the effects on mycorrhizal colonization of aphid herbivory under ambient CO_2, aphid herbivory under elevated CO_2, caterpillar herbivory under ambient CO_2, and caterpillar herbivory under elevated CO_2 (n = 100 for each group). We used MG-SEM to test the hypothesis that resource availability and herbivore identity alter the strength and direction of pathways linking above and belowground interactions.

Materials and Methods

Study System

The common milkweed, *Asclepias syriaca,* inhabits open fields throughout eastern North America, and associates with mycorrhizal fungi throughout its range (Medve 1984, Chapter IV). *Asclepias syriaca* is attacked by specialized insect herbivores, including leaf chewers, phloem feeders, leaf miners, stem feeders, root feeders, and seed predators. Many physical and chemical traits deter milkweed herbivores and the expression of defense traits can be affected by herbivory in *A. syriaca,* in both above (Malcolm and Zalucki 1996, Martel and Malcolm 2004, Van Zandt and Agrawal 2004, Zehnder and Hunter 2007) and belowground plant tissue (Rasmann et al. 2011). In milkweed, the expression of toxic cardenolides in both above and belowground tissues covary (Rasmann and Agrawal 2011). Plant defenses may be costly to produce, either in terms of carbon substrate or nutrients required for enzymatic synthesis (Gershenzon 1994), and defense expression may trade off with growth or other plant functions (Herms and Mattson 1992) and may negatively affect plant mutualists (Strauss et al. 2002), including mycorrhizal fungi. In our system, *A. syriaca* is known to increase growth at the expense of some defenses under CO_2 enrichment (Chapter II).

Experimental Design

Asclepias syriaca pods were collected from a single population in northern Michigan at the University of Michigan Biological Station (UMBS) in Pellston, MI during Fall 2007. During May 2008, we established 5 genetic families of full siblings, each generated from a single pod from one of five field clones (Kabat et al. 2010). Seeds were cold stratified for 4-5 weeks during spring 2008, and were germinated in May 2008 on moist filter paper at 25^{o}C. Following germination, seedlings were planted into 50 mL cells containing potting soil (SunGrow Metromix) and reared in a growth chamber for two weeks. Eighty seedlings of each family were planted individually into 6 inch pots containing approx 1L of a 2:1 mixture of potting soil (SunGrow Metromix) and unsterilized UMBS Rubicon, respectively; plants were not fertilized. Soil was homogenized in a cement mixer to ensure equal inoculation with local mycorrhizal fungi across treatments. Transplanted seedlings were kept in the UMBS glasshouse for 2 weeks to prevent frost damage.

Four weeks after the initial planting (June 1-2, 2008), *A. syriaca* individuals were placed in controlled atmosphere chambers in the field at UMBS. The chamber array consisted of 40 chambers, with 20 maintained at ambient CO_2 concentrations, and 20 maintained at elevated CO_2 concentrations (760 ppm, dawn until dusk). Each chamber held two individual plants from each of the five plant families (10 plants per chamber). Twenty chambers (10 ambient, 10 elevated) were assigned to caterpillar treatments while 20 chambers (10 ambient, 10 elevated) were assigned to aphid treatments. Atmospheric CO_2 concentrations were monitored daily in all elevated CO_2 chambers and 2 ambient chambers using a LI-COR LI-6262 IRGA and CO_2 was adjusted to maintain the target concentration in each elevated CO_2 chamber. Two weeks before the herbivory treatments were initiated (early July, 2008), when plants were approximately two months old, all plants were covered with a fine mesh (paint strainer bags, Mastercraft Mfg.) to keep any local herbivores from consuming the plants or inducing plant defenses.

Aphids were reared asexually from a single *Aphis asclepiadis* female taken from a natural population at UMBS and maintained on *Asclepias syriaca* plants in the greenhouse. We captured five gravid monarch butterflies from the field at UMBS, allowed them to lay eggs in the laboratory, and collected eggs on leaf discs using a hole punch and stored them in a refrigerated incubator until use (maximum two weeks). All monarch eggs came from five wild caught females of unknown provenance.

The aphid and caterpillar treatments were initiated on July 15, 2008, 5 days before plants were harvested. Aphid treatments were initiated with two to five 4^{th} or 5^{th} instar aphids per treatment plant, and final aphid densities reached an average of 65.5 ±5.05 aphids/g of aboveground plant biomass, well within field densities at our site, and significantly higher than the aphid densities found on bagged control plants (1.5 ± 1.05 aphids/g.). For caterpillar introduction, a single *D. plexippus* egg that had darkened just prior to larval eclosion was 'glued' to the leaf of each treatment plant using milkweed latex. Eggs were placed on a single individual of each plant family in each of 10 ambient and 10 elevated CO_2 chambers (100 plants total). The larvae hatched within hours and were allowed to eat for 5 days following eclosion, resulting in the consumption of approximately 10-20%

of each plant. Both control and herbivore treatment plants remained covered in mesh during the aphid and caterpillar treatments.

Harvest

Plants were harvested at 12 weeks of age and each had between 3 and 11 pairs of opposite leaves. All plant heights were measured, and vertical growth since initiation of herbivore treatment was used to calculate plant growth rate during herbivory. Five hole punches (424 mm^2) of fresh leaf tissue were taken from one "side" of the two largest leaf pairs on each plant, placed immediately into 1 mL of methanol and stored at -10^oC for cardenolide analysis (below). Five identical leaf discs were taken from the opposite "side" of the leaf pairs and stored in glassine envelopes to provide estimates of sample dry weights and measures of other leaf traits (below). Latex that flowed from the first five holes punched was collected on a pre-weighed cellulose disc (1 cm. diameter), dried, and weighed. All plants were separated into above and belowground tissues, dried, and weighed.

Analysis of Plant Traits

Plant traits were assessed as described in Chapter II. Above and belowground plant tissue was dried and weighed to the nearest 0.01 g as measures of above- and below-ground biomass. Analysis of cardenolides in leaf and root tissue was performed using methods modified from Malcolm and Zalucki (1996) and Zehnder & Hunter (2007). Plant material was ground in methanol for 2 minutes using a ball mill (Rensch MM200), sonicated at 60^oC for 1 hour and evaporated to dryness. Samples were re-suspended in 150 uL of 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) using an Acquity column (1.7 μm, 2.1x50 mm, Waters, Milford, MA, USA). Peaks were detected by diode array at 218 nm, and absorbance spectra were recorded from 200 to 300 nm with digitoxin as the standard. Peaks with symmetrical absorption maxima between 217 and 222 nm were recorded as cardenolides. Total cardenolide concentration was calculated as the sum of all individual cardenolide peaks, corrected by the concentration of the internal standard and the sample mass.

A subset (c. 0.5 g) of fresh fine root tissue was sampled from each plant, cleared with 10% KOH for 10 minutes, acidified using 2% HCl and stained in 0.05% trypan blue in 1:1:1 water: glycerine:lactic acid. Roots were mounted on slides and scored using the magnified gridline intersect method (McGonigle et al., 1990). A site was considered colonized if AM hyphae, arbuscules, or vesicles were present.

Statistical Analysis

Prior to analysis, we transformed response variables as necessary to meet the assumptions of normality and homogeneity of variance, and examined bivariate correlations for linearity, as required for linear SEM models (Schumacker and Lomax 2010). We estimated each plant's total investment in aboveground defense by summing the z-scores (centered and standardized values) for all measured aboveground defensive traits including foliar cardenolide concentration, specific leaf mass, trichome density, and latex exudation (Agrawal and Fishbein 2006). Total root cardenolides were calculated by multiplying root cardenolide concentration by total root mass. Data were imported into AMOS (Arbuckle 2006), where all SEM analyses were performed. To avoid listwise deletion, we used AMOS ML imputation to estimate missing data points (<3%) (Allison 2002). Analysis of data after listwise deletion gave qualitatively similar results.

To choose among causal models from our six proposed alternatives, we first required the SEM to be a good fit to the observed data. There is no single best measure of fit for SEM, but models are typically considered to be a good fit when χ^2 p-value > 0.10, CFI is maximized and RMSEA and AIC are minimized (Schumacker and Lomax 2010). We report multiple fit indices for all candidate models (Table 3.1) and confidence intervals for RMSEA as reported by AMOS (Arbuckle 2006). We then used the single best-supported model chosen among the multiple causal models as a baseline for multiple group analysis (MG-SEM) in AMOS. We tested for multigroup invariance, that the treatment groups differ in their regression coefficients or factor covariances (Jöreskog 1971, Byrne 2004). Testing for invariance is a multistep process whereby increasingly invariant models (those with more constraints) are tested against 'free' models (where parameters, intercepts etc. may vary among treatment groups), and the difference in fit is

assessed (Byrne 2004). We tested the fit of the unconstrained model against increasingly constrained models (Table 3.2). We then chose the best supported of these models and examined the regression coefficients to assess if herbivore identity or resource availability altered the effects of herbivory on mycorrhizal colonization (Fig. 3.2).

Results

Best-fit model

Using SEM to compare among alternative models using the entire dataset, we found that adding a direct pathway from herbivory to mycorrhizal colonization significantly improved the fit of all proposed models, so this pathway was included in the final structural model. Multiple fit statistics indicated that the covariance structure of the data best fit that proposed by the Induction Costs model (Table 3.1, Fig 3.1c). In this model, aboveground herbivory affects mycorrhizal colonization through changes in aboveground plant defense expression and total root defense (cardenolides). The Induction Costs model was well-supported using the full dataset and provided a good fit to the data using established criteria (Schumacker and Lomax 2010). Root Allocation, Toxicity, Water Stress, and the Full model were poor fits to the data (Table 3.1). The Damage Model (Fig. 3.1a), which implicitly includes carbon costs, also provided an adequate, though weaker, fit to the data (Table 3.1).

Multiple-Groups Comparison

The MG-SEM performed on the Induction Costs model revealed that the least constrained model, containing four groups in which pathways were allowed to vary among each treatment group, best fit the data (Table 3.2). This indicates that herbivory and resource availability affect the direction and/or strength of pathways (mechanisms) that mediate interactions between aboveground herbivores and mycorrhizal colonization.

Effects of Herbivore Identity and Resource Addition on Model Coefficients

Because the unconstrained four-group model was a better fit to our data than the single-group model, we compared the path coefficients among the four treatment groups to assess the direct (unmeasured plant mediated effects) and indirect (measured changes in plant growth and defense traits) pathways that mediate the effect of aboveground herbivory on mycorrhizal associations. Carbon fertilization strongly increased mycorrhizal colonization following herbivory by both *D. plexippus* and *A. asclepiadis* (Fig. 3.2 c & d) through direct routes (Fig. 3.3). However, the strength of the indirect pathways varied most depending on herbivore species identity (Fig. 3.3), and the

direction of the indirect effect was determined by carbon fertilization (Fig. 3.3). Specifically, herbivory by *D. plexippus* decreased total plant defense expression above and belowground under both ambient and elevated CO_2 (Fig. 3.2 b & d), driven mostly by a reduction in latex exudation and specific leaf mass. However, the indirect effect of caterpillar herbivory on mycorrhizal fungi, mediated by changes in defense, depended on CO_2 fertilization (Fig. 3.2 b & d). Under ambient CO_2, decreased defense expression was positively associated with mycorrhizal colonization, while under conditions of elevated CO_2, decreased defense expression did not lead to increased mycorrhizal colonization. Herbivory by *A. asclepiadis* aphids did not significantly change aboveground defense expression, and as a result, aphid herbivory affected mycorrhizal fungi mainly through direct pathways (Fig. 3.3).

Total aboveground defense and root cardenolide expression were positively correlated in all but one of the groups. Conversely, the total expression of root cardenolides tended to be negatively correlated to mycorrhizal colonization in ambient aphid and elevated caterpillar treatments, but positively correlated in elevated aphid and ambient caterpillar treatments (Fig. 3.2). Aboveground defense was negatively correlated with plant growth rate in all treatment groups (Fig. 3.2).

Discussion

We compared among alternative causal models to determine the pathways by which aboveground herbivory affects mycorrhizal colonization, and if herbivore identity or carbon fertilization affected the strength and direction of those pathways. A comparison among SEM models identified strong support for the Induction Costs model, in which changes in plant defense phenotype (indirect pathway) and a direct (unmeasured) pathway combine to mediate the effects of aboveground herbivores on mycorrhizal colonization. This model was also a good fit statistically to each individual treatment group and the best model overall for 3 of the 4 treatment groups. We then explored if the strength of each pathway varied among treatment groups using MG-SEM and examined the path coefficients within each group. This analysis revealed that herbivory by caterpillars, but not aphids, strongly reduced the expression of plant defense above- and below-ground and was linked to changes in mycorrhizal colonization. However, the direction of this indirect effect depended on the carbon fertilization treatment: under ambient CO_2, caterpillar-induced reduction in aboveground defense increased mycorrhizal colonization, whereas this effect was negligible under elevated CO_2. In addition, elevated CO_2 enhanced the positive direct effect of both caterpillar and aphid herbivory on mycorrhizal colonization. We suggest that variation in the strength of the multiple possible direct and indirect pathways is mediated by herbivore identity and the availability of photosynthate and may reconcile reports of positive and negative effects of plant damage on mycorrhizal colonization (Delvecchio et al. 1993, Eom et al. 2001, Gange et al. 2002, Wearn and Gange 2007).

Carbon limitation

Resource availability can limit plant growth, the expression of defense phenotype, and other demands, such as allocation to mycorrhizal fungi through allocation tradeoffs (Herms and Mattson 1992, Gehring and Whitham 1994). However, the extent to which carbon limitation structures mycorrhizal colonization following herbivory has been called into question (Barto and Rillig 2010). In our study, growth under elevated CO_2 allowed plants to increase mycorrhizal colonization substantially following aboveground herbivory, regardless of herbivore identity. Although we did not directly quantify plant

carbon availability, two lines of evidence offer support for the carbon limitation hypothesis proposed by Gehring and Whitham (1994)--that carbon availability structures plant associations with mycorrhizal fungi. First, our best-supported model includes tradeoffs between the expression of defense and mycorrhizal colonization, and plant growth rate. Secondly, specific path coefficients within the MG-SEM point towards carbon-based tradeoffs between the expression of plant defense and growth or mycorrhizal colonization. In particular, the expression of aboveground defense was negatively correlated with plant growth rate in all four treatment groups, although only significantly so in plants grown under ambient CO_2 and exposed to aphids (Fig. 3.2a). Furthermore, all models included a negative correlation between aboveground or belowground defense and mycorrhizal colonization, indicating a tradeoff or cost associated with the expression of defense.

The negative correlation between defense and colonization identified through structural equation modeling was also evident as a tradeoff among plant genotypes (Fig. 3.4). This is a pattern similar to that reported by De Deyn and colleagues in the expression of iridoid glycosides and mycorrhizal colonization among lines of *Plantago lanceolata* (2009). In order to distinguish the cost of defense expression from direct effects of defense on mycorrhizal fungi (ie. toxicity), a thorough test should experimentally examine the effects of cardenolides on mycorrhizal fungi. However, the results of the SEM may inform this relationship as an initial step. First, the data provided little support for the Toxicity model (Fig. 3.1d), which explicitly examined the effects of root defense on mycorrhizal colonization. Second, root cardenolide expression did not consistently affect negatively root colonization by mycorrhizal fungi, which would be expected if cardenolides were toxic to these fungi. Rather, when allocation to cardenolides overall was high (above and below ground), mycorrhizal colonization was correspondingly low.

Taken together, our results strongly suggest that the availability of photosynthate limits mycorrhizal colonization following herbivory. In this way, our results are consistent with other studies that have documented tradeoffs among the expression of defense, growth and mycorrhizal colonization (Laird and Addicott 2007, Kempel et al 2010). Our findings

contrast with those documented by de Roman and colleagues (2011), who demonstrated that the application of acibenzolar-S-methyl, a compound that induces plant systemic acquired response to pathogens, induces a transient decrease in mycorrhizal colonization of soybean (*Glycine max* L.). These authors reported that the decrease in mycorrhizal colonization was associated with an upregulation of pathogenesis-related genes and increased glucanase activity rather than a decrease in root sugar content. We suggest that mycorrhizal responses to herbivory and pathogen infection may differ because of the specific signaling pathways triggered (Dicke and Hilker 2003), defenses upregulated, and the cost associated with mounting defenses against each challenger. However, we do not rule out the potential direct effects of root defense on mycorrhizal fungi that may occur in other systems. For example, increased expression of structural defense in tree roots may physically prevent colonization by ectomycorrhizal fungi (Kleczewski et al. 2010). Moreover, fungal species may be differentially susceptible to plant toxins, and fungi that have coevolved with host plants may be better adapted to plant secondary metabolites than are novel fungi (Callaway et al. 2008).

Herbivore identity mediates plant response belowground

It is well established that herbivore species can differentially affect plant growth, fitness, and defense expression (Stout et al. 1994, Kessler and Halitschke 2007) driven by variation in the amount and type of damage inflicted, plant hormonal signaling, and the eventual phenotypic response to herbivory (Bingham and Agrawal 2010). Our results extend the species-specific effects of herbivores belowground, and demonstrate that herbivores can differentially affect mycorrhizal colonization through species-specific changes in plant defense expression. Differences in the extent of damage inflicted can also vary among herbivore species. For example, Wearn and Gange documented that herbivory by rabbits, but not insects, increased mycorrhizal colonization of grasses, and attributed this difference to the extent of damage inflicted and subsequent root carbon dynamics (Gange 2007, Wearn and Gange 2007). However, we add that differential effects of herbivores on defense expression may contribute to the availability of C belowground and subsequent effects on mycorrhizal colonization. In support of this view, a recent meta-analysis reported that the type of defoliation significantly affected

mycorrhizal response to damage--real herbivory tended to negatively affect mycorrhizal colonization, whereas simulated defoliation did not cause this reduction, but the percent foliar damage did not explain this variation (Barto and Rillig 2010). Combined, these results suggest that the induction (or reduction) of plant defenses in response to herbivory likely contributes to changes in mycorrhizal colonization following herbivory. A few of the mechanisms that may underly these differences have been identified. For example, herbivore species induce different signaling pathways (Heidel and Baldwin 2004), and these hormonal signal molecules may directly modulate mycorrhizal colonization (Kiers et al. 2010) or may affect subsequent defense expression and mycorrhizal colonization. Our experiment and analysis cannot disentangle the role of each mechanism, but we suggest that a better understanding of the effects of herbivory on plant hormonal signaling and its effects belowground may explain some of the variation in the effect of herbivores on mycorrhizal fungi.

Implications and caveats

We documented large (~20%) increases mycorrhizal colonization following herbivory in just a few days. Longer term, increased allocation to belowground symbionts may increase plant nutrient gain, the expression of defense (Chapter V), and the ability to tolerate herbivory or regrow following damage by herbivores (Holland et al. 1996, Hamilton and Frank 2001). The increase in mycorrhizal associations that we observed, particularly pronounced under elevated CO_2, may fuel this regrowth response (Gavito et al. 2000, Compant et al. 2010). Indeed, in previous work, milkweeds grown under elevated CO_2 were better able to sustain regrowth during herbivory (Chapter II). Our results suggest that plants growing under future atmospheric conditions may be better able to gain nutrients and respond to herbivory through increased associations with belowground symbionts (Phillips et al. 2011).

In this study, we only report the effects of herbivores on mycorrhizal colonization, but fungal extraradical hyphal growth or sporulation may also respond to defoliation (Allsopp 1998). In addition, the identity of fungi within plant roots may also change with herbivory or resource availability (Saravesi et al. 2008, Pestana and Santolamazza-

Carbone 2011). Different species of mycorrhizal fungi may vary in their responses to C allocation or other changes in plant physiology and may also provide different benefits to plants under attack (Bennett and Bever 2007). Additionally, our experiment only examined mycorrhizal colonization five days after the onset of herbivory and the responses we observed may vary over time (Nishida et al. 2009). Although these temporary responses belowground are likely to improve short-term nutrient acquisition and regrowth, future studies should examine the duration of such effects and examine the effects of temporal changes in the abundance and community composition of mycobionts.

Conclusions

Unravelling the complex interactions among herbivores, plants, and soil mycobionts is a daunting challenge, but quantifying the strength of multiple causal pathways under varied experimental conditions can offer insights into the multiple pathways that mediate these ubiquitous interactions. Our results suggest that both herbivore-specific changes in the expression of defense and the availability of photosynthate constrain mycorrhizal associations following herbivory. Understanding the pathways by which aboveground herbivores affect mycorrhizal fungi will not only improve our understanding of multitrophic interactions, but may also increase our ability to predict changes in plant and fungal communities and the ecosystem functions which these taxa mediate.

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Table 3.1. Results of comparison between hypothesized structural models that include the direct allocation pathway. Df indicates model degrees of freedom, a nonsignificant p-value indicates that observed and modeled variance-covariance structures do not differ. The root-mean-square error of approximation (RMSEA) is a measure of global fit and should be less than 0.05. 90% CI indicates the confidence interval around the RMSEA. The comparative fit index (CFI) is a parsimony-adjusted measure of fit based on the correlation among variables; larger values indicate a better fit to the model. Chg. AIC indicates the difference between the hypothesized model AIC and the AIC for the saturated model.

Model Description	**df**	χ^2	**p-value**	**RMSEA**	**90% CI**	**CFI**	**Chg. AIC**
a) Damage Model	3	2.32	0.51	0.00	0-0.077	1.00	-3.68
b) Root Allocation	1	1.34	0.25	0.03	0-0.14	0.98	-0.66
c) Induction Costs	4	3.01	0.56	0.00	0-0.066	1.00	-5.00
d) Toxicity	1	1.97	0.16	0.05	0-0.15	0.94	-0.03
e) Water	4	13.12	0.01	0.08	0.03-0.12	0.23	5.12
f) Full Model	13	218.09	0.00	0.20	0.17-0.22	0.09	192.10

Table 3.2. Results of comparison among increasingly constrained structural models. Df indicates model degrees of freedom, a nonsignificant p-value indicates that observed and modeled variance-covariance structures do not differ. The root-mean-square error of approximation (RMSEA) is a measure of global fit and should be less than 0.05, and 90% CI indicate the confidence interval around the RMSEA. 90% CI indicates the confidence interval around the RMSEA. The comparative fit index (CFI) is a parsimony-adjusted measure of fit based on the correlation among variables; larger values indicate a better fit to the model. The AIC is a parsimony-adjusted measure of model fit used to compare among nested models.

Model	df	χ^2	p-value	RMSEA	90% CI	CFI	AIC
Unconstrained	16	20.1	0.21	0.026	0-0.056	0.75	148.1
Structural weights	31	50.1	0.02	0.039	0.017-0.059	0.00	148.1
Structural intercepts	43	125.1	0.00	0.069	0.055-0.084	0.00	199.1
Structural means	46	125.1	0.00	0.066	0.052-0.080	0.00	193.1
Structural covariances	49	125.1	0.00	0.063	0.049-0.076	0.00	187.1
Structural residuals	61	221.9	0.00	0.082	0.070-0.093	0.00	259.9
Saturated model	0	0.0	-	-	-	1.00	160.0
Independence model	60	76.6	0.07	0.026	0-0.043	0.00	116.6

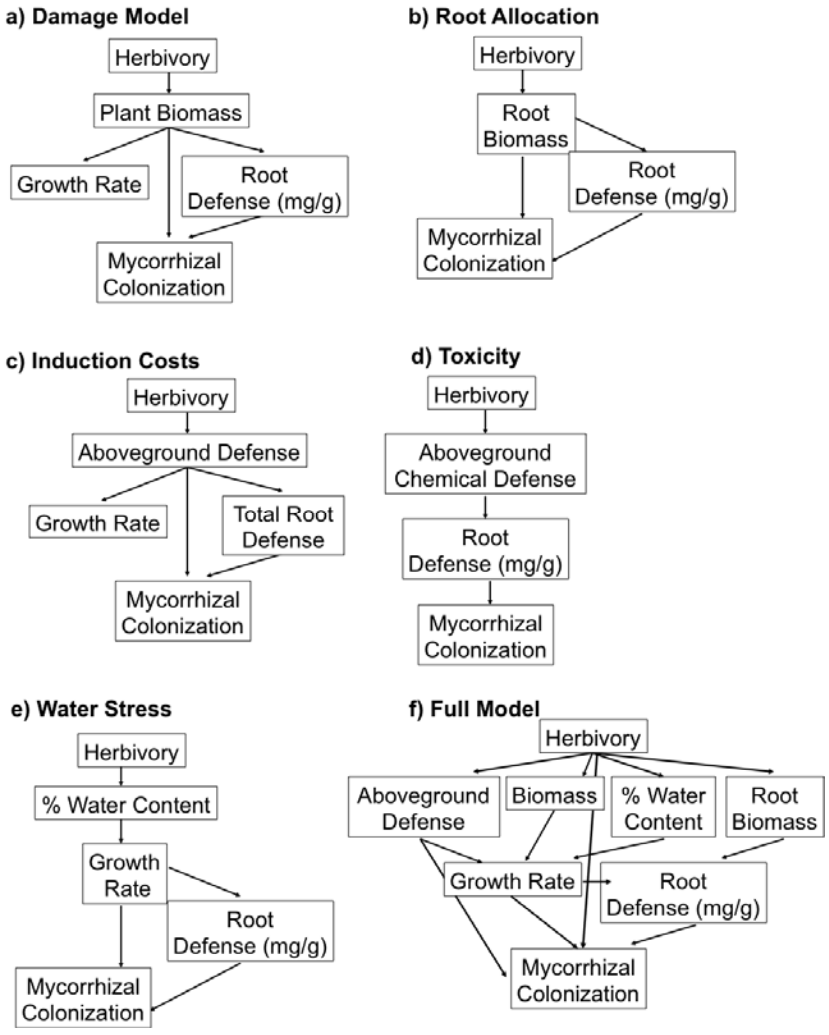

Figure 3.1. Structural models that illustrate the relationships implied by above-belowground models.

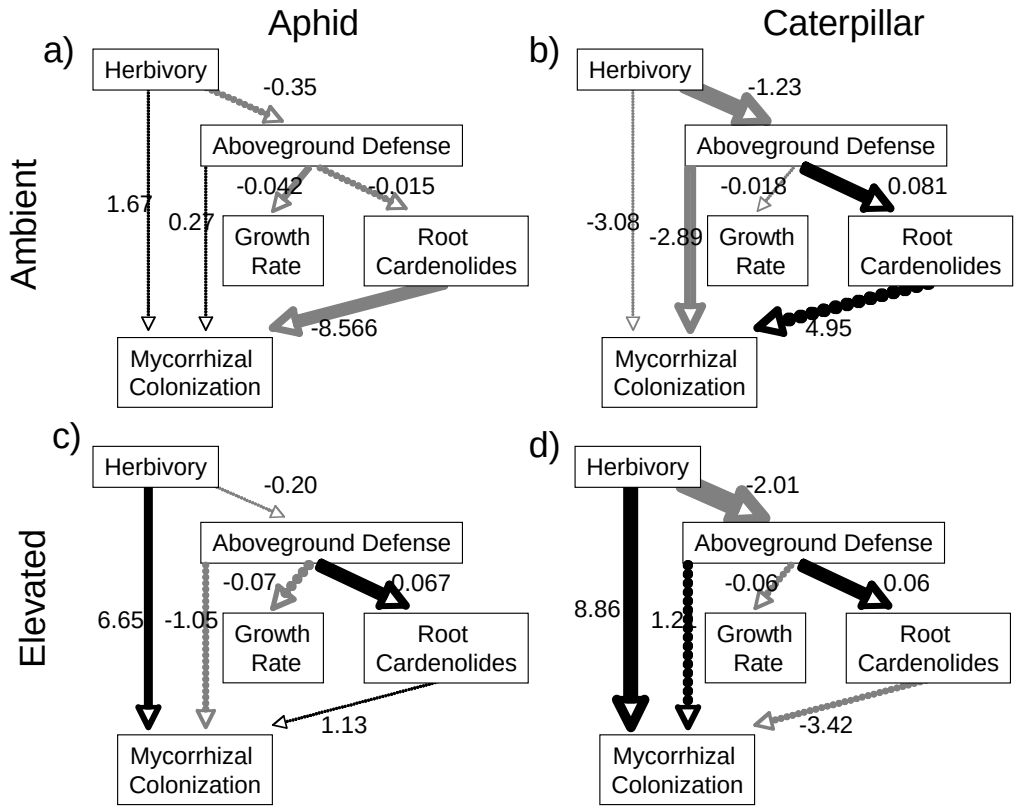

Figure 3.2. Path coefficients from Induction Costs model describe the effects of herbivory on mycorrhizal colonization mediated by changes in defense phenotype among treatment groups. The width of lines represents effect size (standardized coefficients), solid lines represent significant effects ($p<0.05$) dotted lines are nonsignificant. Numbers represent unstandardized coefficients. Gray lines depict negative effects and black lines depict positive effects. All analyses were performed in AMOS.

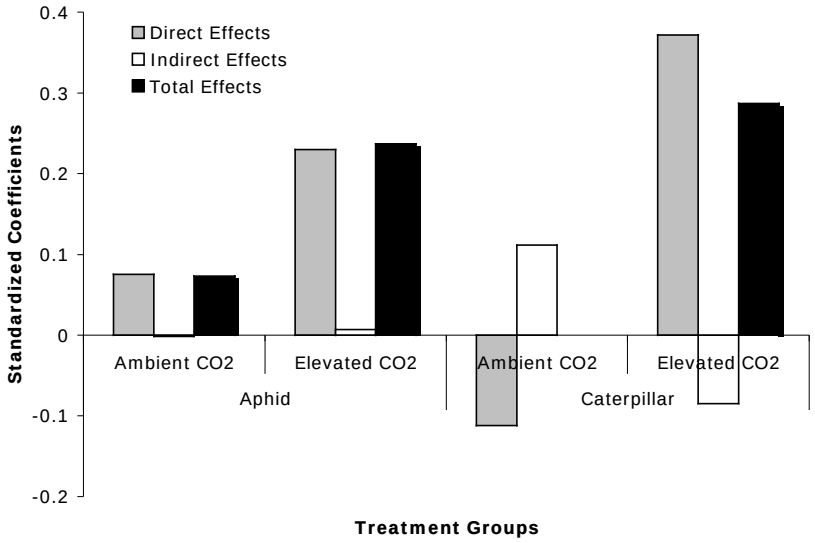

Figure 3.3. Standardized direct and total effects of aboveground herbivores (*Aphis asclepiadis* and *Danaus plexippus*) on mycorrhizal colonization of *Asclepias syriaca* plants under ambient and elevated atmospheric CO_2 concentrations. Direct effects (gray) comprise plant-mediated unmeasured pathways, while the total effect (black) is the sum of the direct and indirect (measured changes in plant growth and defense traits) pathways on mycorrhizal colonization. Effect coefficients are taken from the four group model run in AMOS.

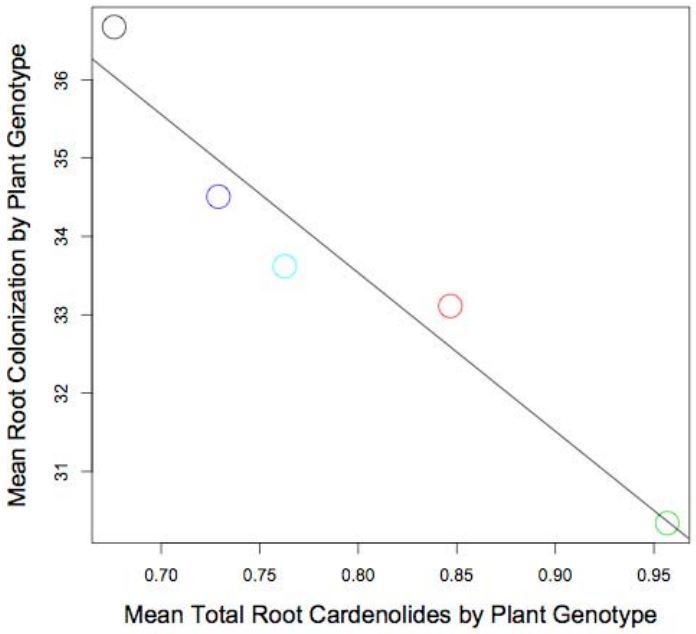

Figure 3.4. Correlation between cardenolide concentration (mg/g) in the roots of *Asclepias syriaca* and proportion root colonized by mycorrhizal fungi (%). Circles represent mean trait values calculated for each genotype of *A. syriaca.*

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Chapter IV

Plant defense theory re-examined: non-linear expectations based on the costs and benefits of resource mutualisms

Abstract

General theories of plant defense often fail to account for complex interactions between the resources required for defense expression. For example, the carbon that is used for carbon-based defense is acquired using nutrient-rich photosynthetic pigments, while nutrient gain itself requires substantial carbon allocation belowground. We should therefore expect the expression of plant defense to reflect the tight linkage between carbon and nutrient gain, yet mechanistic studies linking resource gain with plant defense theory have been slow to emerge. The overwhelming majority of plants participate in nutrition mutualisms with fungal or bacterial symbionts. We propose the resource exchange model of plant defense (REMPD) in which the costs and benefits associated with nutrition mutualisms affect plant resource status and allocation to growth and defense. The model predicts quadratic relationships between mutualist abundance and expression of defense. Within plant genotypes, both plant biomass and defense expression are maximized at optimal nutrient exchange among mutualistic partners, and as a consequence, the two are positively associated.

We tested the model by growing *Asclepias syriaca*, the common milkweed, with two mycorrhizal fungal species in nine fungal abundance treatments. Plant growth and defense traits and mycorrhizal colonization were quantified after 14 weeks of plant growth. Linear, quadratic, saturating and exponential decay models were fit to curves relating the proportion of root colonized by mycorrhizal fungi to plant traits, and compared using AICc.

As predicted by our model, increasing colonization by *Scutellospora pellucida* produced quadratic responses in plant growth, latex exudation and cardenolide production. In contrast, *Glomus etunicatum* appeared to act as a parasite of *A. syriaca*, causing exponential decline in both plant growth and latex exudation. As predicted by our model, plant growth was positively correlated with all defenses quantified.

The REMPD combines cost—benefit analysis of mutualisms with plant resource acquisition strategies to predict the expression of plant defense. The effects of *S. pellucida* and *G. etunicatum* on defense expression differ, but both provide support for the model and suggest that resource mutualisms will affect the expression of defense in a predictable nonlinear fashion.

Introduction

Predicting the expression of plant defense against herbivores in natural and managed ecosystems is essential to modeling and managing these systems. However, current general theories of plant defense are incomplete (Hamilton et al., 2001; Stamp, 2003). Recent advances have improved our understanding of the evolution of defensive strategies among plant species and genotypes (Fine et al., 2006; Agrawal & Fishbein, 2008), but the prediction of individual phenotypic expression of plant defense remains challenging. While early hypotheses relied on plant nutrient availability in ecological time to predict plant allocation to defense or growth (Bryant et al., 1983), many of the underlying assumptions have since been challenged (Gershenzon, 1994; Hamilton et al., 2001). Plant nutrient status alters not only the availability of precursor compounds for the synthesis of defense, but also changes plant physiology and allocation patterns (Bloom et al., 1985; Herms & Mattson, 1992; Shipley & Meziane, 2002) and influences the ability of plants to acquire other resource types (Hamilton et al., 2001). For example, *Populus tremuloides* plants grown under elevated CO_2 are limited by nitrogen (N) availability, but plants that are able to acquire more N through increased carbon (C) allocation belowground improve subsequent C acquisition through increased photosynthesis (Zak et al., 2000). Carbon and nutrient acquisition are coupled through alternate allocation to roots and shoots (Ingestad & Agren, 1991). When resource acquisition is uncoupled and resources become limiting, trade-offs become evident (Herms & Mattson, 1992; Mole, 1994; Donaldson et al., 2006). Here, we develop a general model that integrates the coupled acquisition and expenditure of resources in an ecological context and generates novel predictions regarding the expression of defense. By incorporating into defense theory the complex interactions among nutrients during resource acquisition and allocation, we may gain a better understanding of phenotypic variation in defense expression (Glynn et al., 2007).

Current models of plant defense (Stamp, 2003), as well as models of optimal resource allocation within plants (Shipley & Meziane, 2002), fail to incorporate the biotic interactions that mediate resource acquisition and alter plant allocation patterns between growth and defense. Soil microbes are intimately coupled with root function, but can

induce changes in plant physiology not predicted by models of nutrient uptake by roots alone (Wright et al., 1998a). For example, mycorrhizal fungi and rhizobia can act to stimulate plant photosynthesis (Kaschuk et al., 2009) and net assimilation rate (Wright et al., 1998a) independently of plant nutrition. Conversely, symbionts also require resources for their own growth, which can result in parasitism and growth depressions in host plants (Peng et al., 1993; Johnson et al., 1997). As a result, incorporating microbial associations into models of plant defense expression may result in novel predictions.

Plant Resource Acquisition through Nutrition Mutualisms

Over 80% of land plants acquire mineral nutrients from soil microbes at the expense of C (Wang & Qiu, 2006; Smith & Read, 2008). As a consequence, general theories of plant defense should include the feedback among resources mediated by plant-microbe interactions. Additionally, resource mutualisms represent a convenient framework in which to examine plant allocation patterns, the interactions among nutrients, and resulting effects on plant defense expression. Incorporating resource exchanges among organisms into plant defense theory will increase our understanding and prediction of plant defense expression in an ecological context.

Plant interactions with mycorrhizal fungi are among the most common nutrition mutualisms and provide an excellent opportunity to explore the interactions among primary currencies and the expression of plant defenses. More than 80% of all plant species examined host symbiotic fungi within their roots (Wang and Qiu 2006) and transfer hexose sugars to fungal partners in exchange for mineral nutrients and water (Smith and Read 2008). We focus on the interaction between arbuscular mycorrhizal fungi (AMF) and their plant partners because this symbiosis is the most common nutrition mutualism among plant species (Wang & Qiu, 2006). Although the exact currencies of transfer may vary, other types of nutrition mutualisms, including symbiotic N-fixing bacteria and additional types of mycorrhizal fungi, are likely to function comparably in their effects on defense (Kempel et al., 2009), and our model seeks to generalize to plants participating in these mutualisms as well. Because the vast majority

of land plants exchange vital resources with soil symbionts, these interactions may be a key (and underappreciated) variable in the expression of plant defense.

Although other models of defense incorporate resource uptake from roots (Herms and Mattson 1992), resource dynamics resulting from exchange with mycorrhizal fungi differ from those of nutrient uptake via roots (Wright et al., 1998b), in part because there can be fitness conflicts between partners (Kiers & Denison, 2008). We propose that the fundamental exchange of resources within the mycorrhiza mediates the expression of plant defense. How might variation in mycorrhizal associations alter the expression of plant defense?

Mycorrhizal associations are typically classified as mutualistic interactions, but intrinsically involve both costs and benefits (Koide & Elliott, 1989; Fitter, 1991). Plant responses to colonization are largely a function of these exchanges. The costs and benefits of the currencies transferred, and therefore the outcome of mycorrhizal associations, vary within natural and agricultural systems (Johnson et al., 1997). Plant and fungal identity, ontogeny and abiotic resource availability alter the costs and benefits of association among partners, and therefore mediate the outcome of mycorrhizal interactions (Johnson et al., 1997; Hoeksema et al., 2010). However, one aspect of the mutualism notably missing from this discussion is the importance of partner abundance (but see Gange & Ayres, 1999). While the abundance of mutualist partners can affect mutualist performance and population dynamics (Holland et al., 2002), and is tied to partner performance in non-mycorrhizal systems (Morris et al., 2010), recent work on mycorrhizae has not emphasized the importance of fungal abundance. We argue that the factors that alter resource exchange (costs and benefits of association) between soil mutualists and plants in large part determine the effect of soil mutualists on the expression of plant defense (Jones & Last, 1991).

Experimental evidence demonstrates that mycorrhizal fungi can substantially alter insect performance (Goverde et al., 2000; Gange, 2001), often increasing aphid performance and that of specialist insects, while decreasing the performance of generalist chewing

insects (Hartley & Gange, 2009; Koricheva et al., 2009). However, the effect of mycorrhizal fungi on insect herbivores and secondary metabolites varies substantially among studies (Hartley & Gange, 2009). Our model offers a framework for interpreting and reconciling these results in terms of resource stoichiometry and effects on plant defense.

Model description – the resource exchange model of plant defense

Plants are predicted to allocate optimally to obtain limiting resources (Bloom et al., 1985), and have associated for 465 million years with mycorrhizal fungi (Brundrett, 2002), which aid in acquisition and uptake of macro and micronutrients (Smith & Read, 2008). Although mycorrhizal fungi confer multiple benefits to plants including pathogen protection and improved water relations (Auge, 2001; Borowicz, 2001), we focus on nutrient benefits, a key factor in predicting the outcome of AMF symbioses (Johnson, 2010) and the expression of plant defense (Herms & Mattson, 1992; Gershenzon, 1994).

When mineral nutrients limit plant growth, plants increase C allocation belowground, increasing the root:shoot ratio (Bloom et al., 1985; Shipley & Meziane, 2002) or allocation to mycorrhizal fungi (Treseder & Allen, 2002). We refer to allocation to fungi as the carbon 'cost' associated with nutrient acquisition through mycorrhizal symbionts. In return, mycorrhizal fungi transfer phosphorus (P), nitrogen (N) and micronutrient 'benefits' to plants (Smith & Read, 2008). The cost associated with hosting mycorrhizal fungi can be substantial, from 4 to 20% of net photosynthetic intake (Jakobsen & Rosendahl, 1990). Nutrient returns are also considerable: some plants receive in excess of 50% of total P inflow from AMF (Li et al., 2006), and substantial N influx via AMF has been documented as well (Govindarajulu et al., 2005). A stronger C sink in roots and an increase in nutrients with which to construct photosynthetic apparatus allow plants to increase the rate of photosynthesis so that under some circumstances, fungi essentially 'pay' for themselves (Kaschuk et al., 2009).

Previous models have described the costs and benefits of mycorrhizal fungi (Koide & Elliott, 1989; Fitter, 1991; Gange & Ayres, 1999), and postulated that they may alter the

expression of plant defense against herbivores (Jones & Last, 1991; Bennett et al., 2006), and we build upon these previous efforts. Specifically, we propose that the costs and benefits of symbiosis are dynamic, depend intrinsically upon the abundance of soil mutualists, and affect the expression of plant defenses. After describing the basic model, we illustrate how environmental or biotic variation may shift the shape of the cost or benefit curves and alter plant defense expression. We conclude by incorporating our predictions with those of the growth-differentiation balance hypothesis (GDBH) (Herms and Mattson 1992) to generate the novel predictions of the REMPD.

Mycorrhizal fungal abundance varies substantially within and among ecosystems (Treseder & Cross, 2006). As a result, plant associations with fungi also vary among habitats and ecosystems. Limited fungal abundance in the environment can constrain the formation of the mycorrhiza and associated resource exchange in greenhouse, agricultural and natural systems (Lekberg & Koide, 2005). Greater fungal abundance can increase colonization of plant roots and resource exchange (Sanders et al., 1977; Fitter, 1991) (Fig 1 a), due to a greater extraradical biomass and nutrient flux to the root. Indeed, the proportion of root colonized is significantly correlated with AMF biomass, quantified using phospholipid fatty acids (van Diepen et al., 2007) and hyphal length outside the root (Miller et al., 1995). We use the proportion of root length colonized as a proxy for the abundance of a single fungal species with which a plant associates (Hart & Reader, 2002a) because it is easily quantified and reported in most studies. Although we acknowledge that the proportion of root colonized does not perfectly represent nutrient flux between partners (Li et al., 2008), we use it to represent the maximum nutrient transfer rate within the symbiosis. Using this assumption, we hypothesize benefit and cost curves based on fungal colonization of root tissue.

As mycorrhizal fungal abundance in the environment increases and plants increasingly associate with these fungi, mycorrhizal interactions and nutrient exchange between plants and fungi increases, but the carbon cost associated with hosting fungi also increases (Fig 4.1a), owing mainly to the construction and maintenance costs of fungal tissue (Douds et al., 1988; Peng et al., 1993). Some plants have developed adaptations to limit the extent

of root colonization to prevent parasitism (Koide & Schreiner, 1992), while others are unable to limit fungal colonization and exhibit growth depressions (Klironomos, 2003).

We use the ratio of the gross carbon cost to nutrient benefit afforded by the mycorrhiza to represent the net effect of the mycorrhizal symbiosis (Fig. 4.1b). At low fungal densities, nutrient return for C investment is high, and increased photosynthetic capability can allow plants to keep up with or overcompensate for the C cost of the fungi (Kaschuk et al., 2009). However, at high colonization density and fungal abundance, carbon costs of fungal tissue construction and respiration can exceed P benefits (Douds et al., 1988) and result in net parasitism (Johnson et al., 1997). As a result, the cost:benefit ratio curve (Fig. 4.1b) suggests that benefits obtained from mycorrhizal fungi are maximized at intermediate colonization densities, where carbon costs are balanced by nutrient gains associated with the mycorrhiza. Optimal colonization density will depend on plant and fungal identity, as well as abiotic context. What then are the consequences for defense?

Specifically, the resource exchange model predicts three zones of fungal abundance, nutrient transfer and associated zones of plant defense expression (Fig 4.1c). First, when plants are colonized by no or few fungal propagules, both growth and defense are limited by nutrient and carbon availability. Carbon costs associated with the symbiosis are low and balanced by any increase in nutrients transferred within the mycorrhiza. Within this zone, increasing nutrient acquisition should increase the expression of both growth and defense (Glynn et al., 2007).

The second zone of fungal abundance represents maximal C:nutrient exchange efficiency and an optimal association with soil mutualists (Zone II, Fig 4.1b &c). Within this range, photosynthetic rates are maximal, plants are co-limited by C and nutrients, and we predict that defense expression is also maximized (Fig 4.1c). High nutrient availability facilitates enzymatic synthesis of both carbon and nutrient-based defenses (Gershenzon, 1994), and precursor molecules are also predicted to be available. Within this zone, plant genotypes may vary in their relative allocation to growth and defense (Fig. 4.1d), but both should be expressed maximally within any individual plant. In other words, we expect genetic

tradeoffs between growth and defense, but that individual-based tradeoffs will not be manifest in this zone. Coevolved plant—fungal symbioses at equilibrium are predicted to function primarily in zone II (Johnson et al., 2010).

The third zone represents fungal parasitism. Arbuscular mycorrhizal fungi, as obligate symbionts, must acquire carbon from plants in order to grow and reproduce, and although some plants can decrease allocation to AMF, others are unable to limit the extent of infection (Koide & Schreiner, 1992; Johnson et al., 1997). As a result, plants can exhibit growth depressions associated with supporting the construction and maintenance costs of a large amount of mycorrhizal fungi (Peng et al., 1993). We predict that at high levels of fungal colonization, the expression of defenses, and potentially plant growth, will decline (Fig 4.1c) due to a reduction in C available for the construction of primary and secondary metabolites.

Predictions

From the conceptual model presented above, the following predictions can be made regarding the expression of defense. First, the relationship between defense expression and fungal colonization will be nonlinear, increasing to a local maximum, and decreasing at high fungal abundance. The shape of this relationship should hold both for plant growth and defense, as plants that are exchanging nutrients at an optimal rate will grow and defend maximally. However, we expect the expression of defense to decline earlier than any decline in growth at high levels of AMF colonization (Herms & Mattson, 1992; Glynn et al., 2007).

Secondly, since nutrient benefits conveyed by mycorrhizal symbioses are contingent upon abiotic nutrient availability (Johnson et al., 1997), the shape of the cost and benefit curves will depend on soil fertility. Specifically, plants that can access sufficient N and P without AMF will experience only a C cost to hosting mycorrhizal fungi, and therefore experience parasitism at most levels of colonization by mycorrhizal symbionts. We predict that increasing environmental P availability will diminish the benefits gained through mycorrhizal fungi, and as a result, decrease the ideal AMF abundance. In

addition, the trade balance model of AMF functioning (Johnson, 2010) predicts that the costs and P benefits of association with AMF are dependent on N availability. With sufficient N, rates of photosynthesis compensate for the C cost of AMF, and plants are more likely to exhibit positive growth responses to elevated P. The cost:benefit curves in Figure 1 may be extended to a plane with two or more nutrients to represent the interactions among these resources (see Discussion for integration with the GDBH). What are the consequences for defense? In high-nutrient environments, plants are not likely limited by nutrient availability, but fungal parasitism may limit the C available for defense expression in those plants unable to control C flow to fungi. In contrast, plants growing in nutrient-poor environments may rely heavily on mycorrhizal fungi, and may not experience fungal parasitism. Plant defense expression in these plants would be positively correlated with fungal colonization and nutrient benefits.

The REMPD was developed for plants hosting a single species of mycorrhizal fungus, but fungal species vary in nutrient gathering ability and carbon demand (Hart & Reader, 2002b). The balance of nutrients conveyed and the carbon required to support the construction of a hyphal network determine the net benefit of the interaction. In reality, plants are associated with multiple species of fungi (Opik et al., 2006), which may access a greater range of nutrients than a single fungal species (Koide, 2000; Jansa et al., 2008). As a result, the slope and maximum of the nutrient benefit curve may increase, but the costs to hosting multiple fungi may also be greater. Plant defense expression will still be determined by the net benefit:cost ratio curve.

An initial test of the resource exchange model of plant defense

Study system

As an initial test of the REMPD, we inoculated *Asclepias syriaca* L. (common milkweed) plants with a series of mycorrhizal fungal soil treatments. *Asclepias syriaca* is a perennial herb that grows throughout eastern North America and is associated with mycorrhizal fungi throughout its range (Landis et al., 2004). *Asclepias syriaca* is attacked by a variety of insect herbivores and expresses traits that deter damage by herbivores or reduce herbivore growth and reproduction (Dussourd & Hoyle, 2000;

Zalucki et al., 2001; Agrawal, 2005). Cardenolides, toxic, bitter-tasting steroids, can decrease the survival and performance of the specialist herbivore *Danaus plexippus* (Zalucki *et al.*, 2001). Latex, a sticky polyisoprene polymer that contains cardenolides and other compounds, is stored within pressurized laticifers and can engulf small herbivores and inhibit the feeding of larger ones (Zalucki & Malcolm, 1999; Zalucki et al., 2001). Trichomes, produced on the upper and lower lamina and leaf veins of *A. syriaca*, may inhibit feeding by herbivores (Levin, 1973). These defensive traits are primarily composed of carbon, but synthesis of such compounds and structures requires nutrient-rich enzymes (Gershenzon, 1994). While *A. syriaca* does not require mycorrhizal fungi for growth, plants at our field site are associated with AMF in colonization levels ranging from 10-80% root length colonized (authors' unpublished data). Mycorrhizal fungal species *Glomus etunicatum* and *Scutellospora pellucida* associate with *A. syriaca* at our field site.

Materials and Methods

To investigate the effect of mutualist abundance on the expression of plant defenses, we manipulated the density of mycorrhizal fungi available to milkweed clones. We delineated five genets of *A. syriaca* growing in a natural population in northern Michigan (Pellston, MI, USA) based on morphological, phenological, and chemical similarity. Clonal structure at this site has since been verified using microsatellite markers (Kabat et al., 2010). Rhizomes of *A. syriaca* were unearthed, bleached in 5% bleach solution, and freed from all fine roots. This process removes mycorrhizal fungi from *A. syriaca* roots. Rhizomes were then overwintered at 3 ˚C in a refrigerator. Cultures of *Glomus etunicatum* (MI210B) and *Scutellospora pellucida* (NC118), were obtained from INVAM and cultured on *Sorghum* roots to obtain sufficient inoculum. In spring, rhizomes were cut into 5 cm pieces containing meristem buds and were planted into fungal density treatments. Rhizome biomass was recorded and did not differ among fungal treatments (ANOVA for *S. pellucida*: $F_{1,87}$=0.08, p=0.77, and *G. etunicatum*: $F_{1,145}$=0.78, p=0.37). Conical Deepots ™(Steuwe and Sons Inc., Tangent, Oregon, USA), with a diameter of 6.4 cm and depth of 25cm, were filled with 600 mL 1:1 autoclaved Sunshine Metromix:sand including mycorrhizal fungal inoculum which contained spores, hyphae,

and colonized sorghum root pieces, in 9 dilutions ranging from 150 mL to 4 mL mixed inoculum/pot. These inoculation densities were determined from an initial trial with *A. syriaca* in order to generate a wide range of colonization densities. Arbuscular mycorrhizal fungi (AMF) density treatments were established separately with *Glomus etunicatum* and *Scutellospora pellucida* species. Due to some plant mortality, sample sizes varied among treatments (*G. etunicatum* N=9-22, *S. pellucida* N=4-17 per fungal density) and clones were pooled to provide replicates of AMF treatments. Rhizome pieces were planted in inoculated soil, maintained in a greenhouse and watered daily.

Harvest and Analysis of Plant Traits

At the end of four months, plants were destructively harvested, foliar defense levels were assessed and above- and below-ground biomass measured. Five hole punches (424 mm^2) of fresh leaf tissue were taken from one half of the two largest leaf pairs on each plant, placed immediately into 1 mL of methanol and stored at -10 ˚C for cardenolide analysis (below). Five identical leaf discs were taken from the opposite half of the leaf pairs and stored in glassine envelopes to provide estimates of sample dry mass and measures of other leaf traits (below). Latex that flowed from the first five holes punched was collected on a pre-weighed cellulose disc (1 cm. diameter), dried and weighed. Trichomes on the lower surface of the leaf were counted under a dissecting microscope. Plant chemical defenses were assessed following established protocols (Zehnder & Hunter, 2007). Briefly, cardenolides were separated and quantified by extracting plant material in methanol. Samples were run on a HPLC (Waters Inc, Milford, MA, USA) with digitoxin as an internal standard, and peaks with symmetrical absorbance between 218 and 222 nm were quantified as cardenolides. Total cardenolides were calculated as the sum of individual peaks.

A subset (c. 0.5 g) of fresh fine root tissue was sampled from each plant, cleared with 10% KOH for 10 minutes, acidified using 2% HCl and stained in 0.05% trypan blue in 1:1:1 water: glycerine:lactic acid. Roots were mounted on slides and scored using the magnified gridline intersect method (McGonigle et al., 1990). A site was considered colonized if AM hyphae, arbuscules, or vesicles were present. Non-AMF hyphae were

also detected at low levels (<0.05%), and occurrence did not differ among treatments. Above- and below-ground plant tissues were collected, dried and weighed; total biomass was calculated from dry mass plus estimates of tissue removed for cardenolide and root analysis.

Statistical Analysis

The resource exchange model of plant defense (REMPD) predicts that plant defenses will respond non-linearly to changes in AMF colonization (Fig. 4.1c). We therefore examined a series of linear and non-linear model fits to the plant traits measured during our experiments (Motulsky & Ransnas, 1987). We fit linear, quadratic, Michaelis-Menton, and negative exponential functions to relationships between defense traits and AMF density using the stats package in R (v. 2.11.0) (Team, 2010). The first three models were fit to increasing or null relationships, but only linear and exponential decay functions were fit to decreasing relationships in order to limit regressions to hypothesized and biologically realistic relationships. Mean trait values at each colonization density were weighted by variance^{-1} in the trait value and fit to either a linear (y=a+bx), quadratic (y=a+bx+cx^2), Michaelis-Menten (y=ax/(k+x)), or negative exponential (y=ae^bx) model. Data were plotted and log-transformed if necessary to reduce heteroscedasticity. We used weighted regression (Sokal & Rohlf, 1995), because fungal colonization followed neatly the treatments imposed (Fig. 2). Measures of model fit including AICc (McQuarrie & Tsai, 1998), and adjusted R^2 were extracted from each model using package qpcR (Spiess & Ritz, 2010). Adjusted R^2 was calculated as 1-(1-R^2) n-1/(n-p-1), where n=sample size and p is the total number of regressors. R^2, defined broadly, was calculated for all models as 1-Residual Sums of Squares/Total Sums of Squares. Model selection was performed using AICc; models with the lowest AICc are presented in the results.

Additionally, we assessed correlations among plant biomass and defense traits among all plants from all treatments using Pearson product moment correlations using the stats package in R (v. 2.11.0).

Results

As is required to test REMPD, we succeeded in generating a wide range of AMF colonization densities on *A. syriaca* plants for both fungal species (Fig 4.2a & b). *Glomus etunicatum* colonized *A. syriaca* root length to a greater extent than did *S. pellucida*, with maxima of 45% and 28% root length colonized, respectively. Proportion root length colonized by arbuscules was correlated with total mycorrhizal colonization ($F_{1,127}$=360.8, p<0.0001, R^2=0.73). Plant growth and defense traits varied in the shapes of their responses to AMF abundance, from linear through saturating to quadratic. The statistics underlying model fits are provided in detail in Tables 4.1 and 4.2. Below, we report general trends and refer back to the Tables for statistical support.

As predicted by REMPD, *A. syriaca* biomass responded nonlinearly to colonization by *S. pellucida* (Fig. 4.3a, Table 4.1), increasing at low to mid fungal abundance, and decreasing at high fungal abundance. In contrast, an exponential decay model best represented the relationship between *A. syriaca* biomass and colonization by *G. etunicatum*, as if *G. etunicatum* was acting only as a parasite (Fig. 4.3b, Table 4.2). As predicted by REMPD, the relationship between latex exudation by *A. syriaca* and colonization by *S. pellucida* was best represented by a quadratic function (Fig. 4.3c, Table 4.1), maximized at intermediate levels of fungal colonization. The expression of foliar cardenolides was also best represented by a quadratic function (Fig. 4.3e), maximized at intermediate *S. pellucida* density (Table 4.1). In contrast, colonization by *G. etunicatum* tended to decreased latex exudation, best represented by an exponential decay model (Fig. 4.3d, Table 4.2). *Glomus etunicatum* did not affect cardenolide expression in *A. syriaca* (Fig. 4.3f, Table 4.2). Trichome density was not statistically related to the abundance of either fungal species (Tables 4.1 and 4.2), but tended to increase in response to colonization by *S. pellucida* (Table 4.1).

Analysis of Pearson correlations revealed that all defense traits measured, including latex exudation, foliar cardenolide expression and trichome density, were positively correlated with plant biomass (Table 4.3), as predicted by REMPD. Cardenolide concentration was

negatively correlated with trichome density, while other relationships among defense traits were not statistically significant (Table 4.3).

Discussion

Our model incorporates ecologically realistic nutrient exchange dynamics between plants and soil mutualists to generate novel predictions regarding the expression of defense. An initial test of the model provides good support for REMPD with the fungal species *S. pellucida,* but results contrary to expectations with the fungal species *G. etunicatum*. We detected quadratic responses in *A. syriaca* biomass, latex exudation and cardenolide expression in response to colonization by *S. pellucida* (Figs 4.3a,c,e), as predicted by REMPD (Fig. 4.1c). In contrast, increasing colonization by *G. etunicatum* led to exponential declines in both plant biomass and latex exudation. The quadratic relationships predicted by our model are based on the assumption that soil symbionts act as mutualists over some range of colonization densities; in this case, *G. etunicatum* appears to be acting only as a parasite. As a consequence, we should expect to see only the 'right-hand side' of Figure 4.3c expressed. Both growth (Fig. 4.3b) and latex defense (Fig. 4.3d) declined with *G. etunicatum* colonization, suggesting that the increasing carbon cost associated with hosting *G. etunicatum* seems to have outweighed any nutrient benefits received from the interaction. The different plant responses to the two fungal species were likely due to intrinsic differences in the biology of the fungi. *Glomus* species tend to invest heavily in intraradical structures and relatively little outside the root and as a result tend to confer fewer nutrient benefits (Powell et al., 2009). In contrast, *Scutellospora* species often display lower rates of root colonization but more extensive extraradical hyphal growth (Hart & Reader, 2002c), and tend to increase plant growth. These differences in fungal biology were reflected in the plant phenotypic response. Fungal life-history and allocation patterns may aid predictions of the effects of other fungal species on plant growth and defense expression.

The positive correlations between plant biomass and defense traits also support REMPD. Although cardenolides, latex and trichomes are all composed primarily of carbon, the benefits associated with *S. pellucida* colonization at intermediate densities allowed for increased allocation to both growth and defense. In consistent fashion, parasitism by *G. etunicatum* decreased resource availability for allocation to both growth and latex defense. Overall, these results suggest that allocation to growth and defense are coupled,

as the model predicts. However, defense traits were not uniformly correlated with one another and some defense traits may receive preferential allocation over others. We recognize that 'defense' is not a univariate trait and suites of traits may co-occur or trade-off (Rasmann & Agrawal, 2009). Specifically, resistance and tolerance (Vandermeijden et al., 1988), as well as constitutive and induced resistance (Karban & Baldwin, 1997) should be included in the broad definition of defense. They should be quantified in future work to construct a complete description of *A. syriaca* defense across a range of AMF densities. Overall, we predict that defense viewed and quantified broadly will respond nonlinearly to fungal colonization and resource exchange.

Additional variation in our results may be due to multiple plant genotypes used in our experiment. Previous work has demonstrated that *A. syriaca* genotypes vary in the expression of growth and defense traits (Agrawal, 2005; Vannette & Hunter, accepted). Genotypic differences in allocation patterns and nutrient requirements may interact with fungal nutrient exchange dynamics to shift the shape of plant response to fungal colonization (Fig. 4.1d) (Garrido et al., 2010). Future experiments will allow us to partition variation in defense among effects of plant genotype, fungal colonization, and their interaction.

Additional support for our model can be found in previous research that documents the effects of fungal density on plant phenotype. Gange & Ayres (1999) proposed that the increasing costs and diminishing benefits conveyed by mycorrhizal fungi would result in a nonlinear response of plant biomass to fungal abundance. They describe numerous examples where plant 'benefit' was nonlinearly related to arbuscular colonization intensity. More recently, Garrido and colleagues (2010) manipulated the density of mycorrhizal fungi within the roots of *Datura stramonium* (jimson weed), and documented a curvilinear response—increasing, then decreasing—of root mass, seed production and leaf area to increasing fungal colonization. However, the tolerance of jimson weed to herbivory decreased with increasing mycorrhizal colonization. Although plant tolerance of simulated herbivory did not seem to follow our predicted pattern, we suggest that unmeasured plant resistance traits may respond in kind with root biomass

and reproduction. It is a combination of tolerance and resistance traits that define the defensive strategy of plants; this combination should follow the predictions of REMPD.

Synthesis

While previous plant defense theory has ignored the role of soil mutualists, these symbionts play a crucial role in mediating nutrient acquisition for the majority of plants (Smith & Read, 2008). The identity and abundance of soil symbionts vary, and accordingly, alter nutrient exchange with plants. Although AMF are an integral part of roots, the cost:benefit ratio of the association can change dramatically depending on plant and fungal genotypes and environmental resource availability (Johnson et al., 1997; Hoeksema et al., 2010). As a consequence, AMF can act parasitically (Johnson et al., 1997), a condition not accounted for in models of plant defense based only on optimal allocation models.

The model we develop here (REMPD) offers both complementary and novel predictions when compared with previous theories of plant defense. In order to facilitate a comparison of REMPD to GDBH (Herms & Mattson, 1992), we present both models independently in Figure 4 (note the difference in the x-axes) and their integration in Figure 5. Both models predict a nonlinear response in plant defense to environmental variation that is ultimately linked to internal nutrient availability (Fig. 4.4). However, the models differ in two specific ways. First, two different mechanisms may account for decreasing defense expression: a resource-based tradeoff between growth and defense, as posited by GDBH (Fig. 4.4a), or the increasing resource demands of soil microbial symbionts (REMPD). Second, REMPD predicts that plant growth rate may also decline at fungal abundance, as a result of the increasing cost of root symbionts. Our results (Figs 4.3a &b), as well as those from other studies (Gange & Ayres 1999), confirm that plant biomass can decline with increasing fungal colonization.

Additionally, the costs and benefits of fungal colonization may be altered by soil fertility (Hoeksema et al., 2010), especially in plants unable to limit fungal colonization at high nutrient availability. To facilitate predictions of the integrative effects of symbiotic

exchange and varying environmental nutrient availability on defense expression (Kleczewski et al., 2010), we constructed a response surface to illustrate plant defense as a function of environmental nutrient availability and fungal abundance (Fig. 4.5), using the following assumptions: 1) When resource availability is very low, the effect of AMF on defense should be quadratic (this is our basic model). 2) Likewise, when AMF density is very low, the effect of resources on defense should be quadratic (this is the prediction from GDBH). 3) When resource availability is high, and nutrient gain is therefore already saturated, the only effect of AMF on plants is carbon parasitism and defenses should decline with increasing AMF (Fig. 4.5). 4) When AMF density is high, nutrient gain has already saturated, and increasing resources will have no effect on defense (Fig. 4.5). Therefore, one important difference between the predictions of our combined model and that of GDBH is that we predict that defense expression will be insensitive to soil fertility at the highest levels of fungal colonization. In Fig. 4.5, we extend our model predictions to a single dimension of soil fertility but acknowledge that extending the model to consider multiple soil nutrients would also be valuable (Johnson, 2010).

In summary, REMPD proposes that positive feedbacks mediated by ecological interactions between nutrient and C availability can increase the availability of precursor compounds and enzymes available for growth and the synthesis of defense (Gershenzon, 1994), and increase allocation to both demands (Bennett et al., 2006). Both our initial experiment and data from previous work in mycorrhizal systems support the potential for the resource exchange mechanism as a useful framework for understanding plant defense expression and tritrophic interactions. In addition, the model makes novel predictions about the ecological costs that may limit defense expression and it offers insight into the interactions among resources that control defense expression. Further experimental tests of REMPD will determine the generality of the cost:benefit approach and its effects on plant defense expression.

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Table 4.1. Best-fit regression models and their adjusted R^2 values of the effects of *Scutellospora pellucida* colonization on *Asclepias syriaca* growth and defense traits. Best-fit models were selected using AICc from weighted linear, quadratic, negative exponential and Michaelis—Menten regression analyses. All analyses were performed in R (v. 2.11)

Trait	Best-fit Model	Adj R^2
Plant Biomass	Quadratic*	0.58
Foliar Cardenolides	Quadratic*	0.54
Latex	Quadratic*	0.64
Trichomes	Linear	0.21

+ p<0.10, *p<0.05, **p<0.01, ***p<0.001

Table 4.2. Best-fit regression models and their adjusted R^2 values of the effects of *Glomus etunicatum* colonization on *Asclepias syriaca* growth and defense traits. Best-fit models were selected using AICc from weighted linear, quadratic, negative exponential and Michaelis—Menten regression analyses. All analyses were performed in R (v. 2.11)

Trait	Best-fit Model	Adj R^2
Plant Biomass	Negative Exponential**	0.76
Foliar Cardenolides	Linear	0
Latex	Negative Exponential	0.15
Trichomes	Linear	0

+ p<0.10, *p<0.05, **p<0.01, ***p<0.001

Table 4.3. Pearson product-moment correlations between plant biomass and the expression of various defense traits in *Asclepias syriaca*. N=234

Plant Trait	Latex	Cardenolides	Trichomes
Plant Biomass	0.328***	0.134+	0.1423*
Latex	1	0.0017	0.0503
Cardenolides		1	-0.174*
Trichomes			1

+ p<0.10, *p<0.05, **p<0.01, ***p<0.001

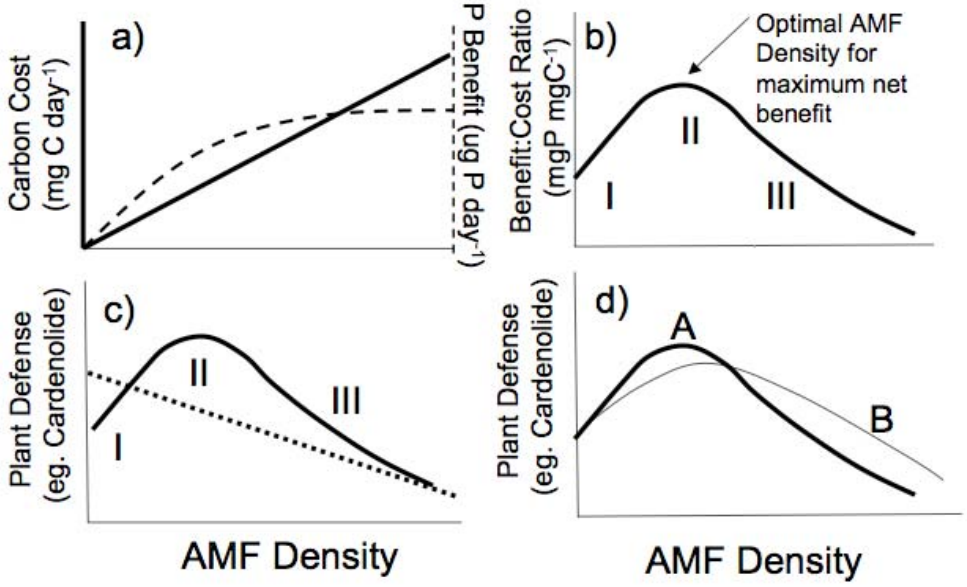

Figure 4.1. Hypothesized relationships between increasing arbuscular mycorrhizal fungi (AMF) mutualist density and a) carbon costs and nutrient benefits. Costs increase with increasing mutualist density, while benefits saturate. As a result, b) the benefit:cost ratio is nonlinearly related to mutualist density. Zone I represents limited fungal abundance and nutrient transfer, zone II represents optimal exchange with mutualistic fungi and maximal nutrient benefits, and zone III represents fungal parasitism, where carbon costs exceed nutrient benefits. The benefit:cost ratio translates directly to the c) expression of plant defenses predicted by our model (solid), in comparison to CNB (dotted). d) The shapes of the phenotypic response curves to fungal abundance vary among plant genotypes (A and B).

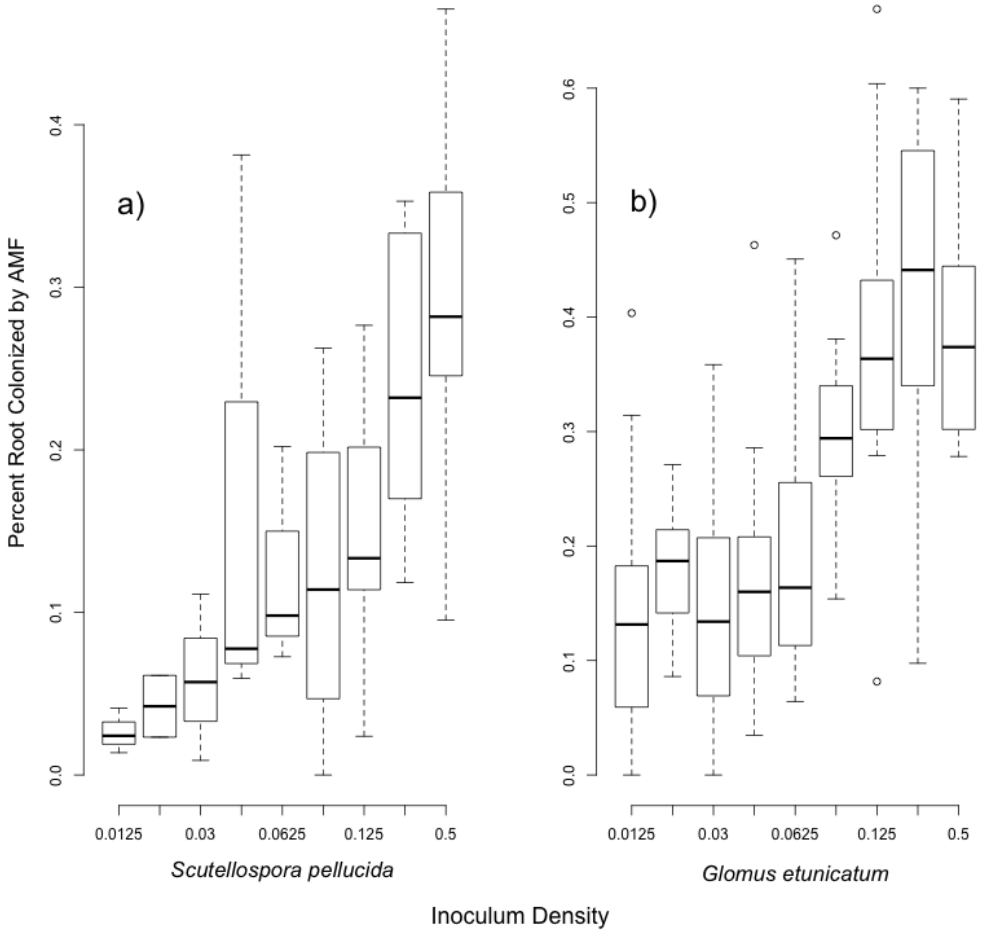

Figure 4.2. Box and whiskers plot of *Asclepias syriaca* root tissue colonized by a) *Scutellospora pellucida* and b) *Glomus etunicatum* in response to experimental inoculum manipulation.

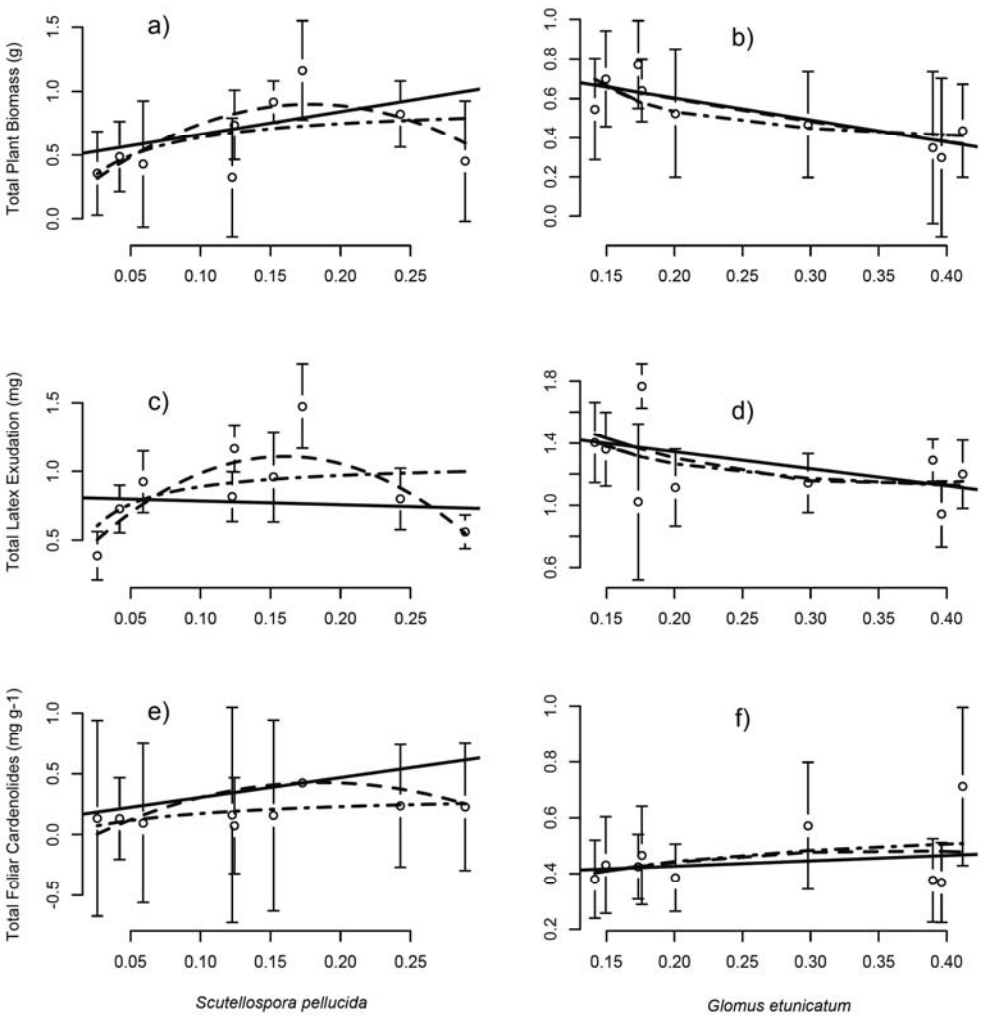

Figure 4.3. Expression of *Asclepias syriaca* defensive traits when grown under experimental manipulation of fungal inoculum density. The left column illustrates responses to colonization by *Scutellospora pellucida*, while the right column illustrates responses to colonization by *Glomus etunicatum* inoculum. Solid lines represent the best-fit linear regression model, dashed lines represent the best-fit quadratic regression model, while dotted and dashed lines represent the nonlinear best fit Michaelis-Menten or negative exponential regression model. Trait means ± 1 SD represented are a &b) plant biomass, c&d) latex exudation, and e&f) total foliar cardenolide concentration.

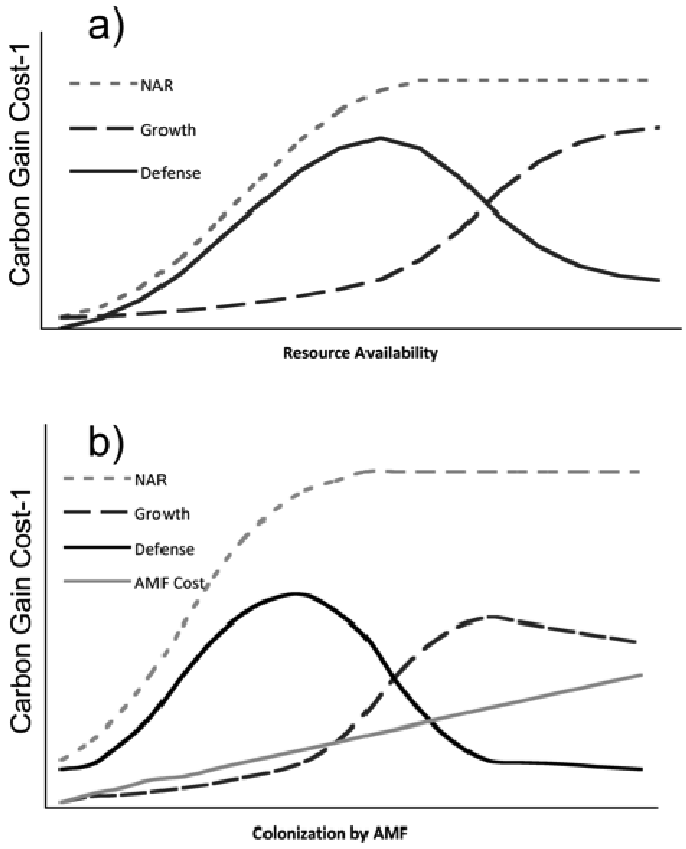

Figure 4.4. Comparison of (a) the Growth Differentiation Balance Hypothesis (GDBH, after Herms & Mattson 1992) and (b) Resource Exchange Model of Plant Defense (REMPD). Note the different x-axes in the figures. In b) mycorrhizal colonization is assumed to increase plant internal nutrient availability and increase net assimilation rate (NAR). REMPD predicts that increasing arbuscular mycorrhizal (AM) costs will decrease defense expression, and decrease plant growth at high colonization levels.

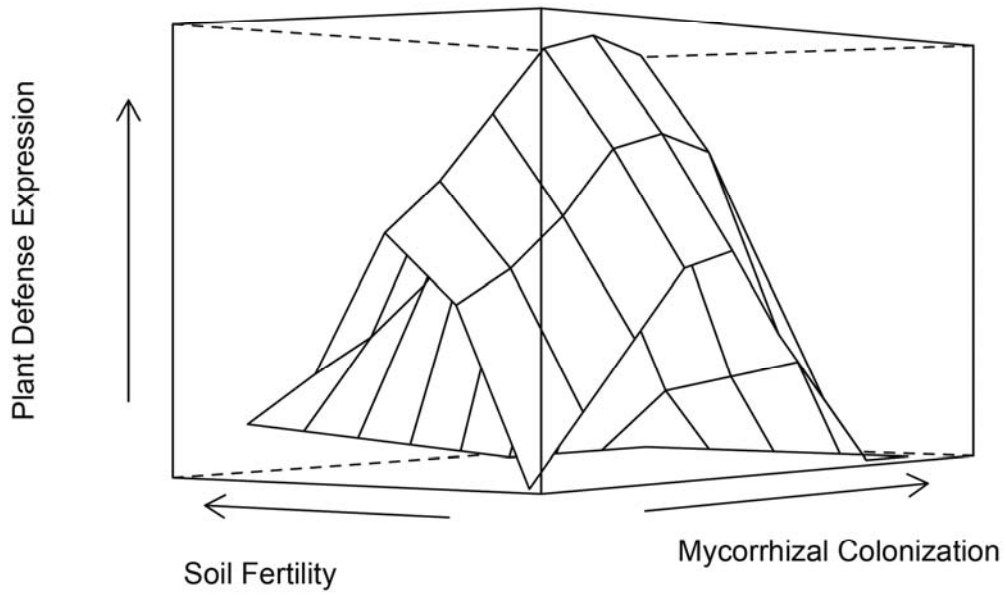

Figure 4.5. An integration of the Growth Differentiation Balance Hypothesis with the Resource Exchange Model of Plant Defense. Soil fertility alters the benefits associated with mycorrhizal fungal colonization and the subsequent effects on defense expression. When soil fertility is very high, mycorrhizal fungi act only as parasites, and increasing mycorrhizal costs result in declines in defense expression. When mycorrhizal colonization is very high, defense expression is insensitive to variation in soil fertility.

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Chapter V

Mycorrhizal abundance affects the expression of plant defense and herbivore performance

Abstract

Mutualisms intrinsically involve costs and benefits, with the potential for dynamic outcomes for partnering organsims. Increasing abundance of belowground symbionts, eg. arbuscular mycorrhizal fungi (AMF), should increase both carbon costs and nutrient benefits to plants, but the effects of AMF abundance on multitrophic interactions are not well understood. Although our understanding of mycorrhizal interactions is based on studies that manipulate the presence or absence of fungi, plants in natural systems are nearly always colonized by AMF to some extent. Increasing AMF colonization should improve plant phosphorus nutrition, affect nonlinearly the expression of plant defenses, and influence the performance of a specialist insect herbivore. To examine how AMF abundance affects plant defense and herbivore performance, we grew *Asclepias syriaca* seedlings with *Glomus etunicatum*, *Scutellospora fulgida*, and a mix of the two species in one of ten abundance treatments. We quantified plant phosphorus (P), defense expression and the performance of specialist herbivore *Danaus plexippus*. Colonization by *S. pellucida* increased foliar P and chemical defense (cardenolides), decreased latex exudation and specific leaf mass and unimodally affected trichome density. *Glomus etunicatum* affected unimodally foliar P and trichome density and tended to decrease specific leaf mass and increase cardenolide expression. Colonization by the mix of AMF decreased specific leaf mass and increased trichome density. Mycorrhizal colonization explained more variation in the expression of most plant traits than did fungal species identity or plant genotype. Mycorrhizal colonization strongly increased caterpillar growth rate, driven by a decline in specific leaf mass. We conclude that variation in mycorrhizal

colonization can profoundly influence the expression of plant defense and herbivore performance.

Introduction

Interactions between plants and other organisms are ubiquitous and mediate essential functions in natural and managed systems (Bronstein 1994b, van der Heijden et al. 2008, Garibaldi et al. 2011). Nearly all plant species associate with belowground microbes, including bacteria and mycorrhizal fungi, in nutrition symbioses that are often characterized as mutually beneficial. Nevertheless, many of these interactions intrinsically involve both costs and benefits, and as a result, can vary from mutualism to antagonism (Bronstein 1994a, Johnson et al. 1997). Belowground symbionts influence plant nutrition and growth, ultimately affecting plant-plant interactions, and thus community composition (van der Heijden et al. 1998, Smith and Read 2008) and plant-consumer interactions (Gange 2007). However, the mechanisms by which nutrition symbionts affect trophic interactions are not well understood (Hartley and Gange 2009). Developing this understanding is important, because most plants are consumed by insect herbivores and greater than 90% of plants associate with belowground symbionts.

The presence of nutrition symbionts can substantially increase plant nutrient content and thereby increase the performance of nutrient-limited herbivores (Borowicz 1997, Goverde et al. 2000). However, nutrition symbionts can also alter the expression of plant defenses. For example, inoculation with mycorrhizal fungi increases alkaloid concentration in *Castanospermum australe* (Abu-Zeyad et al. 1999) and nodulation with rhizobia increases cyanogenic potential in *Phaseolus lunatus* (lima bean) (Thamer et al. 2011). Nutrition symbionts may also affect the expression of carbon-based defenses. For example, colonization by arbuscular mycorrhizal fungi (AMF) increases the concentration of the iridoid glycosides acubain and catalpol in *Plantago lanceolata* (Gange and West 1994). Although most studies that investigate the effects of nutrition symbionts on multitrophic interactions examine chemical defenses, changes in physical traits may also affect herbivores. Indeed, herbivores may respond to microbially-induced increases in nutritional quality or changes in chemical or physical defense, depending on their feeding mode and degree of specialization (Hartley and Gange 2009), but the relative roles of changing nutrition and defense in plant protection and herbivore

performance remain elusive, since changes in plant resistance and nutrition are often confounded or not concurrently quantified.

In addition, most studies simply manipulate the presence or absence of these microbes. However, plants in natural systems are rarely, if ever, free of microbial colonists, instead varying in the degree of association with and the identity of belowground symbionts (Gange and Ayres 1999). To develop a quantitative understanding of plant-symbiont interactions and their multitrophic effects, we generate a realistic gradient of symbiont abundance and examine its effects on plant defense, nutrition and herbivore performance.

Arbuscular mycorrhizal fungi (AMF, Phylum Glomeromycota) are common belowground symbionts and associate with the majority of plant species examined to date (Wang and Qiu 2006). Plant hosts supply photosynthate to fungi (Schussler et al. 2001) and in return, are provided with resources gathered from the soil, most often phosphorus (P), micronutrients, and water (Smith and Read 2008). It is clear that the identity of AMF species colonizing plants affects plant resistance to herbivory (Gange 2001), tolerance to herbivory (Bennett and Bever 2007), and the performance of herbivores (Goverde et al. 2000). In contrast, the effect of mycorrhizal abundance on plant-herbivore interactions is not well understood. Earlier work suggests that the abundance of mycorrhizal fungi alters plant performance (Gange and Ayres 1999), and indeed, greenhouse and some field studies document strong effects of the abundance of mycorrhizal fungi on plant growth and nutrition (Wright et al. 1998, Lekberg and Koide 2005). However, most recent studies of the effects of mycorrhizal fungi on multitrophic interactions continue to rely on presence/absence manipulations of AMF.

Previously, we used a cost-benefit approach (Morris et al. 2010) to develop expectations for how AMF abundance may affect plant defense against herbivores in the Resource Exchange Model of Plant Defense (REMPD; Vannette and Hunter 2011b). Increasing colonization by AMF or other beneficial microbes is predicted to increase plant carbon costs and nutrient benefits—both components of plant defense traits (Fig. 5.1a). When plants associate with a greater abundance of mycorrhizal fungi, they often receive more

nutrients from their symbionts (Fitter 1991), but are required to expend more carbon to support the growth and reproduction of these fungi (Douds et al. 1988). The benefits gained by the plant can saturate with increasing mycorrhizal abundance because competition for nutrients (Violi et al. 2007) and constraints on fungal foraging distance (Drew et al. 2003) may limit the amount of nutrients gathered by fungi. However, the carbon costs associated with maintaining mycorrhizal symbiosis are unlikely to saturate and therefore can result in net parasitism and growth depressions in plant hosts (Peng et al. 1993, Klironomos 2003). As a result, the benefit:cost ratio shifts with symbiont abundance (Fig. 5.1b) and REMPD predicts that plants will respond unimodally to increasing association with AMF in growth and defense expression (Fig. 5.1c) (Vannette and Hunter 2011b). Moreover, colonization by different symbionts may shift the benefit:cost ratio because of interspecific variation in nutrient-gathering ability or maintenance and construction costs (Hart and Reader 2002a) (Fig. 5.1d). As a result, colonization by different fungal species, particularly those that differ in life-history or allocation strategies may produce different phenotypic responses in plants (Fig 5.1d) (Powell et al. 2009).

Here, we provide an experimental test of the relationships illustrated in Figure 5.1. We hypothesize that increasing levels of colonization by AMF will improve plant phosphorus nutrition and nonlinearly affect the expression of plant defenses. Additionally, we hypothesize that AMF identity will differentially affect plant responses to varying AMF abundance in a unimodal relationship. Finally, we hypothesize that changes in plant phosphorus and defense mediated by increasing AMF abundance will influence the performance of a specialist insect herbivore.

Study System

To test these hypotheses, we inoculated *Asclepias syriaca* L. (common milkweed) seedlings with a series of AMF soil treatments. *Asclepias syriaca* is a perennial herb that grows throughout eastern North America and associates with AMF throughout its range (Landis et al. 2004). *Asclepias syriaca* is attacked by a variety of specialist insect herbivores and expresses several traits that can deter damage by herbivores or reduce the

growth and reproduction of even specialists (Dussourd and Hoyle 2000, Zalucki et al. 2001, Agrawal 2005). Cardenolides, toxic, bitter-tasting steroids, can affect the survival and performance of monarch butterflies *Danaus plexippus (*Zalucki et al. 2001, De Roode et al. 2008) and aphids (de Roode et al. 2011). Latex, a sticky polyisoprene polymer that contains cardenolides and other compounds, is stored within pressurized laticifers and can engulf small herbivores and inhibit the feeding of larger ones (Zalucki and Malcolm 1999, Zalucki et al. 2001). Trichomes, produced on the upper and lower lamina and leaf veins of *A. syriaca*, may inhibit feeding by herbivores (Levin 1973). While all these defensive traits are primarily composed of carbon, their synthesis requires nutrient-rich enzymes (Gershenzon 1994).

Although *A. syriaca* does not require AMF for growth, plants at our field site in northern Michigan, USA, associate with AMF at colonization levels ranging from 10-80% of root length colonized (authors' unpublished data). Most arbuscular mycorrhizal fungi provide phosphorus to the plant, and fungal species may vary in their nutrient-gathering ability and carbon cost to the plant (Hart and Reader 2002a). In addition, families of *A. syriaca* vary substantially in defense expression (Chapter II) and may respond differentially to mycorrhizal colonization or AMF identity.

Materials and Methods

To investigate the effect of symbiont abundance on the expression of plant defenses among plant families, we manipulated the density of AMF available to milkweed plants. We delineated five genets of *A. syriaca* growing in a natural population in northern Michigan (University of Michigan Biological Station, Pellston, MI) based on morphological, phenological, and chemical similarity. Clonal structure at this site has since been verified using microsatellite markers (Kabat et al. 2010). Follicles containing full-sibling seeds were collected from five different genets at our field site, cold moist stratified for at least three months, and germinated. Pure cultures of *Glomus etunicatum* and *Scutellospora fulgida*, AMF species that associate with *A. syriaca* at our field site (R.L. Vannette personal obs.), were obtained from INVAM (http://invam.caf.wvu.edu/) and cultured on *Sorghum* roots to obtain sufficient inoculum for experiments.

We generated a range of mycorrhizal colonization by limiting the availability of fungal inoculum to plants. Seedlings were planted in conical Deepots ™(Steuwe and Sons Inc.), with a diameter of 6.4 cm and depth of 25cm, filled with 600 mL 1:1 autoclaved Sunshine Metromix:sand containing mycorrhizal fungal inoculum. To each pot was added 150 mL (1/4 pot volume) of fungal inoculum consisting of spores, hyphae, and colonized sorghum root pieces in densities ranging across 11 AMF inoculation densities from 100% autoclaved inoculum to 100% live inoculum (Table 5.1). The top and bottom of each pot contained 225 mL of autoclaved soil mixture to prevent contamination. These inoculation densities were determined from an initial trial with *A. syriaca* to generate a wide range of colonization intensities. AMF density treatments were established separately for *Glomus etunicatum*, *Scutellospora fulgida*, and a mix of the two species. (N=10 replicates/ treatment =1550 plants - see Table 1 for replication level by family and AMF treatment).

Herbivore Assay

Eggs of monarch butterflies, *D. plexippus*, were obtained from Michigan Monarchs (**http://www.mi-monarchs.com**) and attached to the leaves of a subset of plants to assess fungal-induced changes in plant nutrition and defense. Eggs were applied to 4 replicates

of each plant family x AMF treatment to expose caterpillars to a range of fungal abundance treatments (Table 1, N=4 replicates/treatment=320 caterpillars). A single egg was applied to one leaf of the fourth expanded leaf pair from the apical meristem and held to the leaf using a single drop of water. The entire leaf was enclosed with a small mesh cage, as were those on paired plants from the same mycorrhizal abundance treatments (n=4/treatment, 320 total), but with no monarchs, to control for the effect of the cage on plant performance and trait expression. Eggs hatched within 2 days and the date of eclosion was recorded. Caterpillars were allowed to feed for five days, after which they were collected, allowed to void their guts and frozen. Caterpillars were subsequently freeze-dried and weighed using a microbalance (Mettler Toledo, Columbus, Ohio, USA). Caterpillar growth rate was calculated by dividing total caterpillar biomass by the number of days the caterpillar had fed following eclosion.

Harvest and Analysis of Plant Traits

At the end of three months of growth, plants were destructively harvested, and plant growth, phosphorus content, and defense responses to mycorrhizal treatments were quantified. For all analyses presented here, defense traits were quantified on control (herbivore-free) plants. Five hole punches (424 mm^2 total) of fresh leaf tissue were taken from one half of the two largest leaf pairs on each plant, placed immediately into 1 mL of methanol and stored at -10^oC for cardenolide analysis (below). Five identical leaf discs were taken from the opposite half of the leaf pairs and stored in glassine envelopes to provide estimates of sample dry mass and measures of other leaf traits (below). Latex that flowed from the first six holes punched was collected on a pre-weighed cellulose disc (1 cm. diameter), dried, and weighed. Trichomes on the lower surface of the leaf were counted under a dissecting microscope. Plant chemical defenses were assessed following established protocols (Zehnder and Hunter 2007). Briefly, cardenolides were separated and quantified by extracting plant material in methanol. Samples were analyzed by HPLC (Waters Inc) with digitoxin as an internal standard, and peaks with symmetrical absorbance between 218-222 nm were quantified as cardenolides. Total cardenolides were calculated as the sum of individual peaks.

To quantify levels of AMF colonization that resulted from inoculation treatments, a subset (approx 0.1 g) of dried fine root tissue was sampled from two control plants from each plant family from all AMF species x abundance treatments (n = 310). Roots were rehydrated for 24 hours in water, cleared with 10% KOH for 10 minutes, acidified using 2% HCl and stained in 0.05% trypan blue in 1:1:1 water: glycerine:lactic acid. Stained roots were mounted on slides and scored at 200x using the magnified gridline intersect method (McGonigle et al. 1990) using a Nikon compound microscope (Melville, NY, USA). A site was considered colonized if AM hyphae, arbuscules, or vesicles were present. Non-AMF hyphae were also detected at low levels (< 0.05%).

Quantifying Benefits and Costs

In order to quantify one currency of the benefits conferred by AMF to plants, we examined foliar phosphorus. We ground foliar tissue from two control plants of each plant family from all AMF x abundance treatments (n =310). Foliar phosphorus in ground samples was converted to soluble P using acid reflux and quantified using the molybdenum method with ascorbic acid reduction by Mike Grant at the laboratory in Pellston, MI. All analyses of constitutive defenses and phosphorus were performed on control plants that were not exposed to herbivores. Previous work has demonstrated that root colonization by mycorrhizal fungi is correlated with fungal biomass in roots (van Diepen et al. 2007) and carbon costs associated with hosting mycorrhizal fungi (Peng et al. 1993). The proportion of *A. syriaca* root colonized by AMF is also predictive of fungal biomass in roots, measured using fatty acid 16:1ω5c (R^2 = 0.48 for colonization by *G. etunicatum*) (author's unpublished data). We use mycorrhizal colonization as an estimate of fungal abundance within the root and carbon costs associated with hosting AMF (Gange and Ayres 1999).

Statistical Analysis

We hypothesized that plant defense expression would respond unimodally to variation in AMF colonization (Fig. 5.1c) and that this response would depend on AMF identity. To explore the shape of plant response to variation in AMF colonization, we examined a series of linear and non-linear model fits between plant traits and AMF colonization

(Motulsky and Ransnas 1987). We used weighted regression to analyze the effects of mycorrhizal colonization on plant defense expression (Sokal and Rohlf 1995) using average mycorrhizal colonization for each AMF x abundance treatment because only a subset of the plants were examined for mycorrhizal colonization and foliar phosphorus. To examine how AMF colonization affected plant defense expression, we compared the following full weighted regression equations, fit separately for each plant trait and AMF treatment using the stats package in R (v. 2.11.0) (R Development Core Team 2010). Only linear and quadratic models were fit to increasing relationships between AMF colonization and defense.

1) $D=F_i + C + F_i*C$

2) $D=F_i + C + C^2 + C*F_i + C^2*F_i$

3) $D=e\text{^}(C+F_i)$

where D=plant defense trait, F_i= each plant family, and C= AMF colonization.

Mean trait values at each colonization density, for each plant family were weighted by variance^{-1} in the trait value. Measures of model fit, including AIC and adjusted R^2, were extracted from each model. Adjusted R^2 was calculated as $1-(1-R^2)\ n-1/(n-p-1)$, where n=sample size and p is the total number of regressors. AIC was used to select the best-fit model.

To examine the relative contribution of AMF identity, colonization, and plant family identity to the expression of plant traits, we used ANCOVA to partition the variance in defense traits explained by each predictor (Hunter et al. 1997) by calculating the Explained Sums of Squares/Total Sums of Squares by AMF species. Mean trait values were used as above, and weights were applied as described previously. Since all traits exhibited different best-fit curves, we modified Eqn. 2 above to include AMF identity and interactions with plant genotype and AMF colonization.

4) $D=F_i + C + C^2 + C^2*F_i + S + S*F_i + S*C^2 + S*C^2*F_i$

where D=plant defense trait, F_i= each plant family, and C= AMF colonization, and S=AMF species (eg. *Glomus etunicatum*, *Scutellospora fulgida*, or the mix).

To assess the correlation among plant traits, we examined Pearson product-moment correlations among all measured plant traits on mean trait values. Mean trait values for each AMF species x colonization x plant genotype combination (N=155) were used for the correlation analysis. In addition, plant defense can be represented as a suite of traits that may act in concert to reduce herbivore consumption and performance (Rasmann and Agrawal 2009). To examine changes in the plant multi-trait defensive phenotype induced by mycorrhizal fungi, we used permutational MANOVA in the package vegan (Oksanen et al. 2010) in R to assess if mycorrhizal colonization, AMF identity, or their interaction alters the overall expression of milkweed defensive phenotype. Mean trait values were used as for the correlation analysis.

To examine how caterpillar growth rate varies with increasing mycorrhizal colonization among AMF species, we used ANCOVA on caterpillar growth rates averaged for each plant family x AMF species x colonization treatment (Table 5.1, N=75). To further assess how changes in plant nutrition and defense traits affect caterpillar growth, we used multiple regression to assess the effects of individual plant traits on log-transformed caterpillar growth rate. Correlations among variables were examined for multicollinearity using Pearson correlations (above), and since the expression of traits was not highly collinear ($r>0.80$), we used all traits in the multiple regression. For this analysis, we calculated average caterpillar growth rates for each plant family x AMF species x colonization and regressed these mean values on average plant traits, since traits were not measured on the same plants that caterpillars consumed. We first examined the fit of the full model, which contained all measured plant traits as predictors, then used the step function, which uses AIC to choose among models using a combination of forward and reverse selection and AIC values.

Results

Inoculum treatments with either *Glomus etunicatum* or *Scutellospora fulgida* generated a wide range of AMF colonization intensities (0-35% and 0-28% respectively), whereas the range of colonization intensities generated by the mixed species inoculum was narrower (0-15%) (Fig. 5.2). We identified characteristic fungal structures (http://invam.caf.wvu.edu) for each fungal species in their respective treatment, but not in the other's single-species treatment, and structures characteristic of both fungal species were clear in the mix treatment. Arbuscular colonization was consistent within AMF inoculum treatments (ANOVA $F_{20,236}$=4.39, $p < 0.0001$) and mycorrhizal colonization levels did not vary significantly among plant families. Total colonization and arbuscular colonization were highly correlated ($p<0.0001$, R^2=0.79), and since arbuscules represent the functional structure of the mycorrhiza, we used mean arbuscular colonization values for each AMF x abundance treatment (y-values in Fig. 5.2) were used as measures of AMF abundance in all subsequent analyses (Gange and Ayres 1999). Plant phosphorus increased linearly in response to colonization by *S. fulgida* ($F_{1,159}$=55.23, $p<0.001$), responded unimodally to colonization by *G. etunicatum* ($F_{1,159}$=3.74, p=0.025), but did not respond strongly to colonization by a mix of fungal species ($F_{1,160}$=0.28, p=0.59) (Fig. 5.3).

Effects of mycorrhizal colonization on individual plant traits

With a few exceptions (noted below) plant families responded in similar ways to AMF treatments (Fig. 5.4). However, we observed substantial variation among plant defense traits in their responses to AMF colonization. Moreover, the identity of AMF species determined the shape of defense responses to increasing levels of AMF colonization (Table 5.2, Fig. 5.4). Among most plant families, increasing colonization by *S. fulgida* decreased latex exudation and specific leaf mass (Fig. 4 a & d), but increased cardenolide concentration (Fig. 5.4j). Trichome density responded unimodally to colonization by *S. fulgida*, first increasing then decreasing (Fig. 5.4g). Similarly, colonization by *G. etunicatum* increased foliar cardenolide concentration, affected unimodally trichome density (among most genotypes), and decreased nonlinearly plant expression of specific leaf mass (Fig. 5.4 e, h, k). Plant families varied in their response to increasing

colonization by *G. etunicatum* in latex--some families increased, but others decreased latex exudation with increasing mycorrhizal colonization (Fig. 5.4 b). Colonization by a mix of fungal species decreased latex exudation and strongly decreased specific leaf mass (Fig. 5.4 c, f), but tended to increase trichome density and did not predict cardenolide concentration (Fig. 5.4 j, l).

Plant family identity and mycorrhizal colonization influence plant defense expression

The full model that included AMF colonization, identity and plant family explained a large proportion of the variation in plant traits, from 10-92% of variation explained, depending on the plant trait examined (Adjusted R^2 presented in Table 5.2). In general, mycorrhizal colonization and plant genotype explained more variation in plant trait expression than did AMF identity, revealed by the ANCOVA used to partition the variance explained by these predictors (Table 5.3). AMF colonization explained the most variation of all predictors in latex exudation, trichome density, specific leaf mass, and foliar phosphorus concentration (Table 5.3). While the identity of AMF colonist significantly affected the expression of all measured traits, this effect was outweighed by both fungal colonization and plant family identity in latex exudation, trichome density, and foliar phosphorus concentration. Plant family identity explained the greatest amount of variation in the expression of cardenolides, followed by AMF colonization and AMF identity.

Correlations among traits and effects of mycorrhizal colonization on multivariate plant defense expression

Pearson correlations revealed that foliar phosphorus was positively associated with trichome density, but negatively associated with specific leaf mass and latex. In addition, the expression of latex and specific leaf mass were positively correlated (Table 5.4). Cardenolide expression was not significantly associated with phosphorus concentration or other defense traits. Plant defense modeled as a multivariate trait also responded strongly to fungal colonization (perMANOVA AMF $F_{2, 144}=11.6$, $p<0.001$, Colonization $F_{1,154}=19.8$, $p<0.001$, Interaction $F_{2,154}=4.26$, $p<0.001$). In general mycorrhizal colonization shifted plant defense phenotype away from physical defenses, such as latex

and specific leaf mass, and increased trichome density, foliar P content and cardenolide concentration.

Effects of mycorrhizal colonization and plant traits on herbivore performance

The growth rate of the specialist herbivore *D. plexippus* varied among fungal treatments (ANCOVA: Interaction $F_{2,72}=7.41$, $p<0.001$) (Fig. 5.5), but was in general greater on mycorrhizal plants than nonmycorrhizal plants. Caterpillar growth rates varied unimodally with colonization by *G. etunicatum* and *S. fulgida*. The response of caterpillars to fungal colonization by the mix of AMF species was not continuous, but rather increased sharply with the presence of fungi. There was not enough variation in colonization by the mix treatment to determine the effect of AMF colonization on herbivore performance in this treatment.

Caterpillar growth rate was strongly negatively associated with specific leaf mass (Table 5.5), as revealed by multiple regression. In addition, caterpillar growth rate tended to be positively affected by phosphorus concentration and trichome density (Table 5.5) and negatively associated with latex exudation and cardenolide concentration, although these effects were not significant. Only specific leaf mass, a measure of leaf toughness (Frost and Hunter 2008), was retained as a significant predictor of caterpillar growth rate in the stepwise regression (Table 5.5).

Discussion

We hypothesized that increasing mycorrhizal colonization would nonlinearly affect the expression of plant defenses, mediated by increased foliar phosphorus (P), but we found that the direction and strength of mycorrhizal effects on plant defense varied among fungal treatments and with the particular defense trait examined. First, colonization by either species of fungi alone generally increased plant 'benefit' (P); colonization by *G. etunicatum* increased foliar P while *S. fulgida* weakly affected affected foliar P in a unimodal relationship. However, trichome density was the only trait to respond unimodally to colonization by AMF as predicted by the benefit:cost ratio. In contrast, specific leaf mass (toughness) and latex declined with mycorrhizal colonization, and cardenolide expression increased exponentially in response to the two single-species fungal treatments. These changes in plant defense expression were correlated with changes in foliar P--trichome density was positively correlated with leaf P, whereas leaf toughness and latex exudation were negatively correlated with leaf P. Mycorrhizal colonization also increased the performance of a specialist herbivore, *D. plexippus* apparently by reducing leaf toughness. Interestingly, fungal abundance explained a greater proportion of the variation in most defense traits than did fungal identity or plant family.

Effects of mycorrhizal colonization on the expression of defense

Most studies that examine the ecological role of mycorrhizal fungi in an experimental setting manipulate the presence or absence of fungi. However, our results suggest that fungal abundance may be an overlooked, but key aspect of the mycorrhizal mutualism (Gange and Ayres 1999, Violi et al. 2007, Garrido et al. 2010) and explain more variation in plant phenotype than other factors more commonly manipulated (Karst et al. 2008, Hoeksema et al. 2010). In addition, differences among fungal species in their effects on plant phenotype and subsequent ecological dynamics that have been attributed to species identity may be confounded by differences in the proportion of root colonized rather than fungal identity per se.

Despite the effect of fungal abundance on plant P and the expression of plant defense, the reponse of most traits differed from expectations generated by the benefit:cost analysis in the resource exchange model (REMPD) (Fig. 5.1). Although both our estimates of the costs (mycorrhizal colonization) and benefits (foliar P) were associated with changes in defense expression, only trichome density responded unimodally to increasing abundance of either single species of fungi as predicted. We suggest a few complementary reasons for these deviations from predictions. First, similar to the results reported by Garrido and colleagues (2010), the effects of AMF colonization on plant trait expression in our study depended on the specific trait examined. Variation in the shape of phenotypic response among plant traits may result from the nutrient requirements for construction of specific physical structures or chemical compounds (Gershenzon 1994, Donaldson et al. 2006). For example, while phosphorus was negatively associated with latex and specific leaf mass, foliar phosphorus was positively associated with trichome density (Table. 5.4). In constrast, the expression of physical defense traits, such as toughness and latex have been shown to increase with CO_2 fertilization (Chapter II), suggesting that the expression of these may be limited by the availability of photosynthate. As a result, we may improve trait-specific predictions by tailoring them to the specific nutrient requirements of each trait and assessing trait-specific benefit:cost ratios. Second, mycorrhizal colonization of *A. syriaca* in natural populations ranges from 10-80% of the root length colonized, with a mean of approximately 30% (author's unpublished data). The proportion of root colonized that we generated in our experiment only spanned a portion of these values, and as a result, may not capture the entire range of effects and shape of the curve hypothesized by REMPD. In addition, the proportion of root colonized may not adequately represent the carbon costs imposed by mycorrhizal fungi (Gavito and Olsson 2003). Finally, the relative benefits and costs associated with hosting mycorrhizal colonization may depend on soil fertility (Johnson et al. 1997, Violi et al. 2007). In our experiment, plants were minimally fertilized with phosphorus-free fertilizer, but soil fertility may still have been greater than field conditions. We suggest that variation in soil nutrient availability likely mediates the effects of mycorrhizal fungi on defense, may explain the variation in results between this chapter and Chapter IV, and is the subject of current investigation.

Taken together, these explainations suggest that our current experiment may not have explored the entire benefit:cost response curve and that the 'optimal' colonization density for maximum trait expression may vary among plant traits due to differences in resource requirements. Future studies should attempt to generate a greater range of colonization densities under a range of soil fertility to more fully assess the assumptions of the model.

Species-specific effects on expression of plant defense

In our experiment, AMF species varied in their effects on plant phosphorus and defense, and this may be attributed to species-specific carbon requirements and efficacy of nutrient foraging (Hart and Reader 2005), which is phylogenetically conserved (Hart and Reader 2002b, Powell et al. 2009). Specifically, fungal species in the Gigasporaceae (eg. *Scutellospora fulgida*) are more effective at increasing host nutrition when compared with those in the Glomeraceae (eg. *Glomus etunicatum*) (Powell et al. 2009). Consistent with this finding, colonization by *S. fulgida* in our experiment linearly increased leaf P, whereas colonization by *G. etunicatum* affected unimodally leaf P (Fig. 5.3). Moreover, the two AMF species induced slightly different responses in plant defense traits (Fig. 5.4), which were tied to changes in phosphorus concentration. These results lend support to the finding that association with different symbionts can generate divergent responses in plant partners, in part mediated by costs and benefits associated with different species (Stanton and Palmer 2011).

We were unable to generate a wide range of root colonization intensities with the mix of fungi and as a result, cannot fully evaluate the effect of fungal abundance in this treatment. In addition, the proportion of root colonized by a mix of fungal species was lower than colonization by either species separately. Other studies have been able to generate a range of colonization intensities with a mix of fungal species (Garrido et al. 2010), and some document greater arbuscular colonization by a mix of fungi than single-species inoculum (Bennett and Bever 2009). We suggest that interspecific interactions among fungi within or outside the root (Cano and Bago 2005, Bennett and Bever 2009, Kennedy et al 2009) may in part explain the smaller proportion of root colonized by the

mix of fungal species. Furthermore, we identified no evidence of complementarity, displayed by increased P or enhanced defense expression in the mix treatment. This was contrary to our expectations, since previous work suggests that increased fungal diversity, specifically phylogenetic diversity (Maherali and Klironomos 2007), may promote plant nutrition and growth due to complementarity in resource use (Jansa et al. 2008). However, our results are consistent with those described by Violi and colleagues, who reported that colonization by a mix of *G. intraradices* and *Scutellospora heterogama* reduced the P benefits conveyed by either fungal species alone (Violi et al. 2007).

Effects of AMF colonization on plant resistance and the maintenance of mutualism

In our study, mycorrhizal colonization increased the growth rate of *D. plexippus*, consistent with predictions that mycorrhizal colonization should increase the performance of specialist herbivores (Gehring and Bennett 2009). However, in our study, *D. plexippus* growth was more closely associated with a decrease in physical defense rather than simple changes in nutrition (Koricheva et al. 2009). In natural systems, , *D. plexippus* may oviposit preferentially on plants that contain high concentrations of cardenolides (Lefevre et al. 2010), which increased with mycorrhizal colonization. Indeed, the sharp decrease in the expression of physical defense and increased chemical defense in response to mycorrhizal colonization seems ecologically and evolutionarily unfavorable in light of herbivory by specialist herbivores and unlikely to contribute to the maintenance of this mutualism (Kiers and van der Heijden 2006). How then is this mutualism maintained?

Mycorrhizal colonization may enhance plant fitness through other mechanisms or when generalist herbivores are considered. For example, increased expression of chemical defenses in *A. syriaca* may deter generalist herbivores such as the deer that are common at our field site and can severely reduce plant fitness. In addition, high concentrations of cardenolides can also reduce the growth and survival of specialist herbivores in some situations (Malcolm and Zalucki 1996). While mycorrhizal colonization did not improve *A. syriaca* resistance to specialist herbivory in this experiment, increased mycorrhizal colonization may improve plant tolerance to and regrowth following herbivory (Bennett

et al. 2006, Vannette and Hunter 2009). No clear pattern has emerged from previous studies that examine the effects of mycorrhizal fungi on plant tolerance (Kula et al. 2005, Bennett and Bever 2007, Garrido et al. 2010). In a previous study with *A. syriaca*, we describe increased plant regrowth following herbivory in plants grown under elevated atmospheric CO_2 (Chapter II), associated with increased levels of mycorrhizal colonization (Chapter III). We suggest that mycorrhizal fungi may favor tolerance and regrowth following herbivory in *A. syriaca*, but future experiments must test experimentally this hypothesis. Alternatively, mycorrhizal colonization may provide other benefits to maintain the mutualism including increasing plant reproduction or resistance to pathogens (Sikes et al. 2009) and root herbivores (Rasmann and Vannette, in prep).

Plant families vary in response to mycorrhizal colonization

Plant families varied significantly in phenotypic response to AMF species in the expression of trichomes and cardenolides, and in response to mycorrhizal colonization in the expression of latex. Although plant family by AMF interactions explained substantially less variation in defense traits than did main effects, they may still contribute to eco-evolutionary dynamics. In contrast to previous work that has documented variation among genotypes in the association or dependence on AMF in weeds (Ramos-Zapata et al. 2010) agricultural cultivars (Graham et al. 1997) and other plants (reviewed in Hoeksema 2010), we identified little variation among genotypes in the proportion of root colonized. However, our results demonstrate that plant genotypes can vary in their phenotypic responses to variation in AMF. This finding is relevant because insect herbivores can exert significant selection pressure on *Asclepias syriaca* (Agrawal 2005). As a result, the soil biotic environment may interact with plant genotype to determine the expression of defense and may lead to complex evolutionary dynamics within this system.

Conclusions

Our results demonstrate that increasing mycorrhizal abundance exhibits strong trait-specific effects on the expression of plant defense, mediated in part by an increase in

plant phosphorus status, and in turn, increases the performance of a specialist herbivore. Although further research should examine plant responses to a greater range of colonization densities and soil fertility to thoroughly test the assumptions of REMPD, the results presented here suggest that trait expression responds to changes in the costs (C) and benefits (P) associated with mycorrhizal colonization, although not in a unimodal fashion as predicted. In addition, genetic variation in phenotypic response to mycorrhizal colonization may also allow for selection by herbivores on plant-fungal associations. Overall, our results emphasize that mycorrhizal abundance can profoundly influence plant defense and the performance of herbivores, with implications for the outcome of the mycorrhizal mutualism (Holland et al. 2002).

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Table 5.1. Fungal treatments consisted of live and autoclaved whole fungal inoculum (homogenized root pieces, spores, hyphae) from *Glomus etunicatum*, *Scutellospora fulgida*, or a 1:1 mix of the two species. Total Plant Replicates describe the total number of seedlings planted, while Plant Trait Replicates describe the number of plants used for analysis of constitutive defense traits. Total Caterpillar Replicates is the total number of caterpillars (1/plant) used to assess plant defenses.

Treatment	Live Inoculum (mL/pot)	Autoclaved Inoculum (mL/pot)	Total Plant Replicates	Plant Trait Replicates	Total Caterpillar Replicates
0	0	150	50 (N=10/family)	30 (N=6/family)	20 (N=4/family)
1	0.5	149.5	150 (N=10/family/AMF)	150 (N=10/family/AMF)	0
2	1	149	150 (N=10/family/AMF)	90 (N=6/family/AMF)	60 (N=4/family/AMF)
3	2	148	150 (N=10/family/AMF)	150 (N=10/family/AMF)	0
4	5	145	150 (N=10/family/AMF)	90 (N=6/family/AMF)	60 (N=4/family/AMF)
5	10	140	150 (N=10/family/AMF)	150 (N=10/family/AMF)	0
6	25	125	150 (N=10/family/AMF)	90 (N=6/family/AMF)	60 (N=4/family/AMF)
7	40	110	150 (N=10/family/AMF)	150 (N=10/family/AMF)	0
8	60	90	150 (N=10/family/AMF)	90 (N=6/family/AMF)	60 (N=4/family/AMF)
9	100	50	150 (N=10/family/AMF)	150 (N=10/family/AMF)	0
10	150	0	150 (N=10/family/AMF)	90 (N=6/family/AMF)	60 (N=4/family/AMF)

Table 5.2. Results of weighted linear, quadratic, and negative exponential regressions examining the effect of average arbuscule colonization on defense trait expression in *Asclepias syriaca* seedlings (Eqns 1-3). Negative exponential models were fitted only to decreasing relationships. Models include effect of plant family. Average arbuscule colonization (shown in Fig. 2) was calculated for each inoculum treatment level for each AMF species x abundance treatment separately. The best-fit model for each trait x fungal species combination was chosen using AIC. The symbols indicate the significance of the mycorrhizal colonization term. Adjusted R^2 for the entire model is presented. All analyses were performed in R v. 2.11.

	Model					
Glomus etunicatum	**Linear**		**Quadratic**		**Negative Exponential**	
Defense Traits	AIC	Adj. R^2	AIC	Adj. R^2	AIC	Adj. R^2
Latex	44.1	0.22	45.5	0.21	92.9	0.11
Trichome Density	201.1	0.12	194.2	0.27	---	---
SLM	-530.6	0.18	-557.9	0.51	-55.7	0.17
Cardenolides	17.8	0.82	15.2	0.84	---	---
Scutellospora fulgida	**Linear**		**Quadratic**		**Negative Exponential**	
Defense Traits	AIC	Adj. R^2	AIC	Adj. R^2	AIC	Adj. R^2
Latex	32.4	0.32	31.8	0.32	86.9	0.42
Trichome Density	172.8	0.82	168.7	0.83	---	---
SLM	-517.4	0.67	-528.9	0.74	-45.0	0.71
Cardenolides	52.0	0.38	45.5	0.47	---	---
Mix	**Linear**		**Quadratic**		**Negative Exponential**	
Defense Traits	AIC	Adj. R^2	AIC	Adj. R^2	AIC	Adj. R^2
Latex	26.5	0.40	24.6	0.41	60.0	0.35
Trichome Density	161.2	0.30	163.5	0.28	---	---
SLM	-557.3	0.57	-559.8	0.59	-68.9	0.48
Cardenolides	37.6	0.26	39.3	0.23	---	---

Table 5.3. Variance in plant trait values explained by AMF identity, colonization and plant family identity determined by F-tests and sums of squares derived from weighted ANCOVA. Variance explained was calculated by dividing the sums of squares explained by each predictor by the total sums of squares for each trait. Df column indicates error df for each analysis, and df row indicates the df for each predictor. All analyses were performed on the family average trait value for AMF x abundance treatment level and weighted by variance $^{-1}$. AMF treatments indicate fungal species treatments including *Scutellospora fulgida*, *Glomus etunicatum*, and an equal mix of the two species. Plant family denotes different genetic families of *Asclepias syriaca*. Colonization is average arbuscule colonization (y-values from Fig. 2) calculated separately for each inoculation treatment for each AMF x colonization treatment. AMF Colonization^2 refers to the quadratic term in the model. All analyses were performed in R v. 2.11.

Plant Trait	df	AMF Species	AMF Colonization	AMF Colonization^2	Plant Family	AMF Species x Colonization^2	AMF x Plant Family	Colonization^2 x Plant Family	AMF x Colonization^2 x Plant Family
		2	1	1	4	2	4	4	8
Latex	134	**0.083*****	**0.089*****	---	**0.16*****	0.043*	---	0.046**	---
Trichome Density	133	**0.030*****	**0.38*****	**0.063*****	**0.25*****	---	0.025*	---	---
SLM	134	**0.089*****	**0.29*****	**0.24*****	**0.075*****	**0.027*****	---	---	---
Cardenolides	90	**0.11*****	**0.026*****	**0.17*****	**0.56*****	0.011**	0.019+	---	---
Phosphorus (not weighted)	134	0.046**	**0.14*****	0.038**	---	**0.073*****	---	---	---

*p<0.05, **p<0.01, ***p<0.001, Bold indicates that F-values are significant after Bonferroni adjustment.

Table 5.4. Pearson product moment correlations coefficient calculated pairwise for traits expressed by *Asclepias syriaca*. Correlations were calculated from average trait values for each plant family x AMF species x AMF colonization combination (N=155). SLM stands for Specific Leaf Mass, and cardenolides and phosphorus refer to total foliar cardenolide or phosphorus concentration, respectively. All tests were performed in R v. 2.11.

	Trichome Density	SLM	Cardenolides	Phosphorus
Latex	0.29***	0.29***	-0.07	-0.13+
Trichome Density	1.00	-0.17*	-0.19	0.19*
SLM		1.00	-0.088	-0.39***
Cardenolides			1.00	-0.039

+ p<0.10, *p<0.05, **p<0.001, ***p<0.0001

Table 5.5. Partial unstandardized regression coefficients from multiple regression examining the effect of measured *Asclepias syriaca* traits on the log-transformed growth rate of *Danaus plexippus* caterpillars. Regression was conducted on the family means for each AMF x inoculum treatment combination (N=75). Coefficients and their significance are reported from both the full model and stepwise best-fit model. All regression analyses were performed in R v. 2.11.

Predictors	Full Model	Stepwise
Latex	-0.23	---
Trichome Density	0.12	---
SLM	-284.40***	-324.15***
Cardenolide Concentration	-0.25	---
Phosphorus	1.75	---

+p<0.10, *p<0.05, **p<0.001, ***p<0.0001

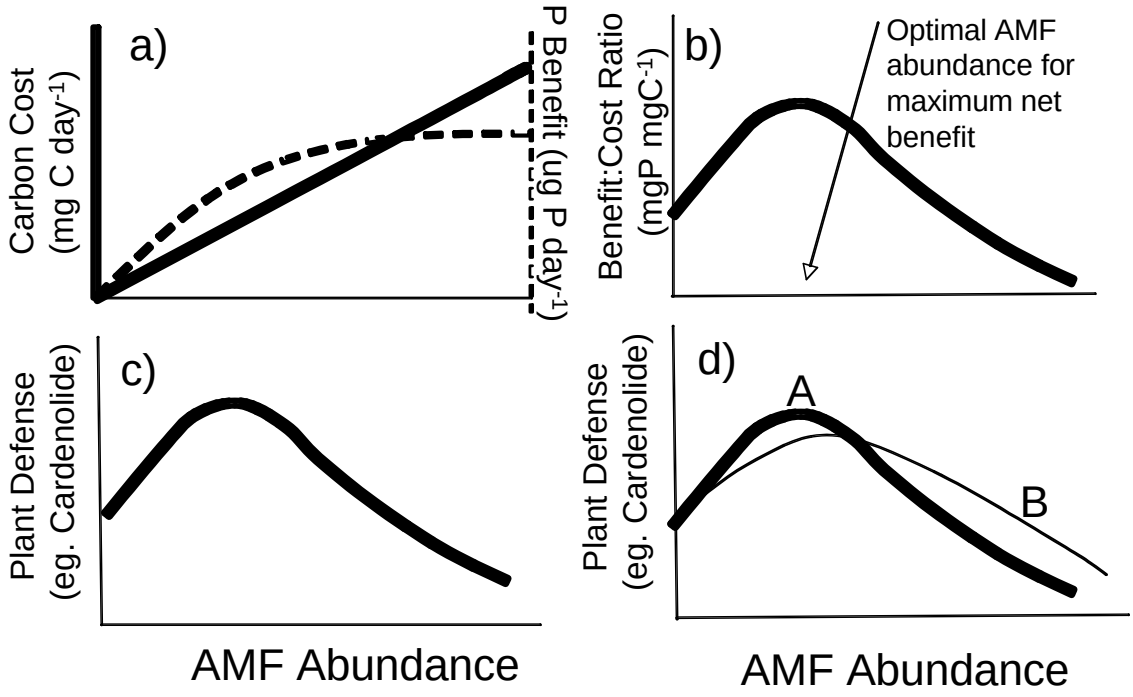

Figure 5.1. Hypothesized relationships between increasing mycorrhizal symbiont density and a) carbon costs and nutrient benefits. Costs increase with increasing mycorrhizal density, while benefits saturate. As a result, b) the benefit:cost ratio is nonlinearly related to mycorrhizal density. The benefit:cost ratio translates directly to the c) expression of plant defenses predicted by Vannette and Hunter (2011b) d) The shapes of the phenotypic response curves to mycorrhizal abundance vary in response to fungal species (A and B).

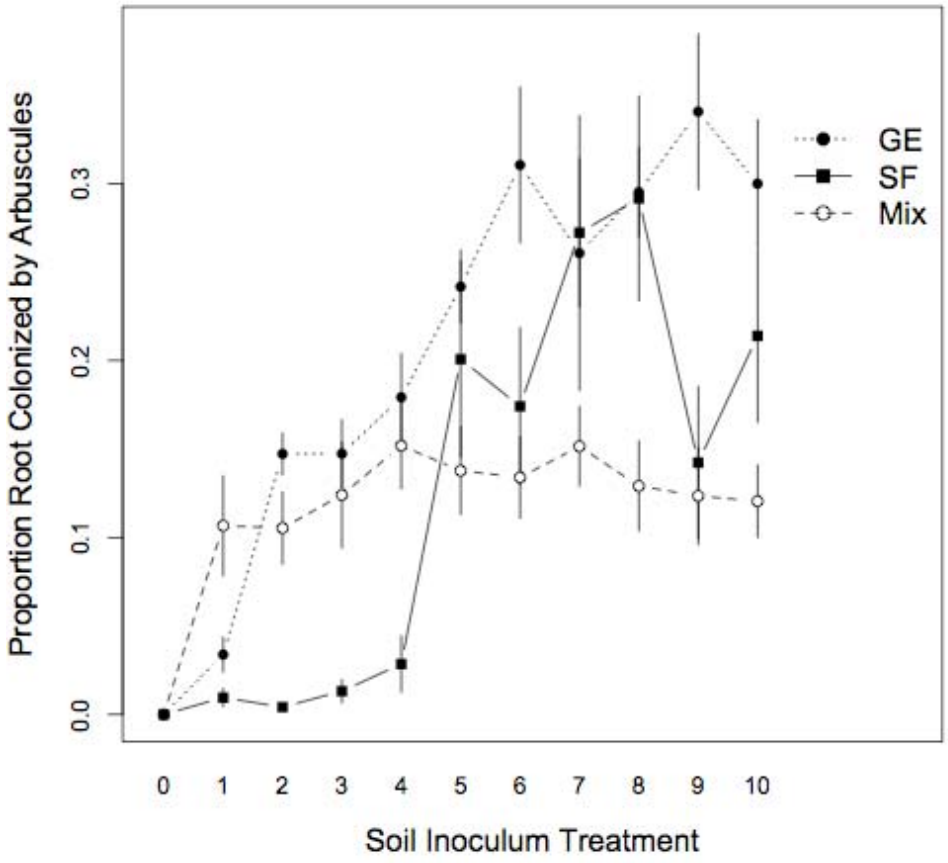

Figure 5.2. Proportion of *Asclepias syriaca* root colonized by mycorrhizal fungi +/- 1SE. Fungal treatments imposed included *Glomus etunicatum* (GE), *Scutellospora fulgida* (SF) and a mix of the two species (Mix). Inoculum treatments ranged from 150 mL autoclaved inoculum to 150 mL live inoculum (1/4 total pot volume).

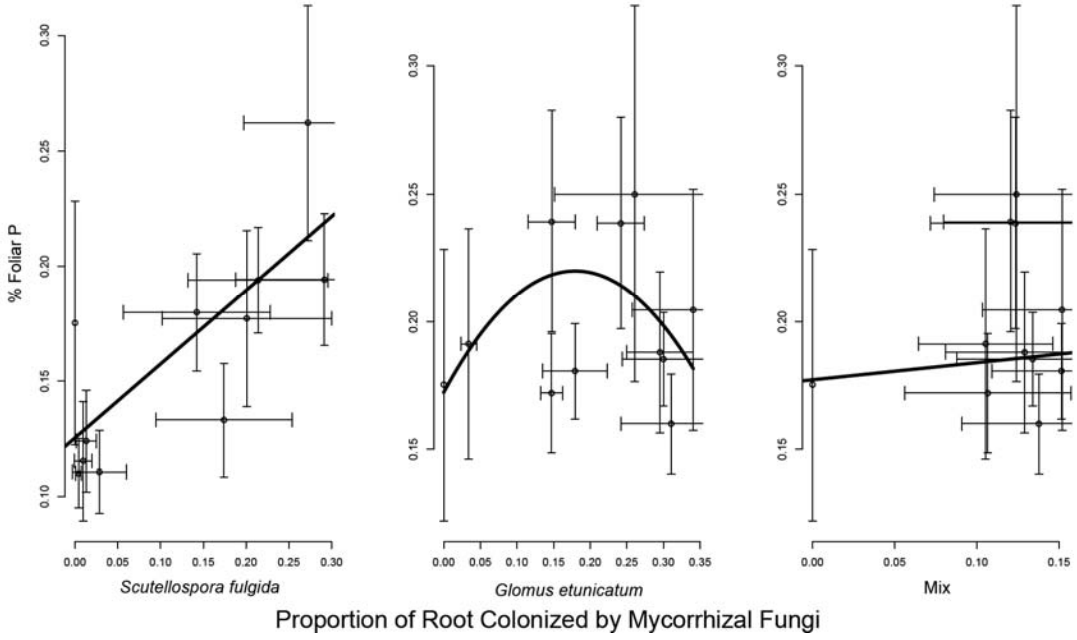

Figure 5.3. The effect of mycorrhizal colonization on *Asclepias syriaca* foliar phosphorus (P) varies among fungal treatments. Increasing arbuscular colonization by a)*Scutellospora fulgida* increases foliar P content linearly ($F_{1,159}$=55.23, p<0.0001, R^2=0.25). Colonization by b) *Glomus etunicatum* affects P content nonlinearly ($F_{2,160}$=3.74, p=0.026, R^2=0.045), while c) the mix of two species has no significant effect on plant P ($F_{1,162}$=0.29, p=0.59, R^2=0.001).

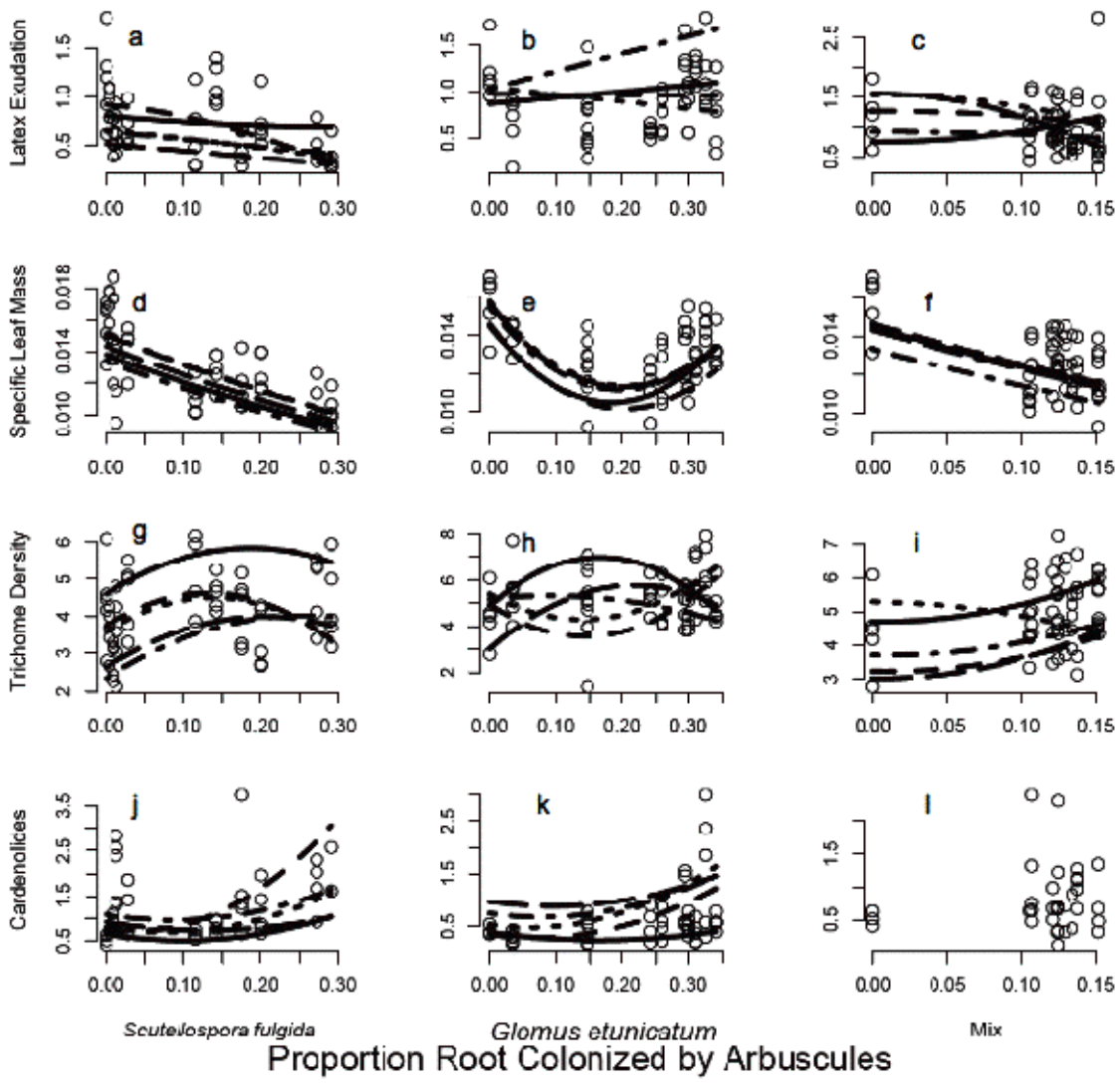

Figure 5.4. Effects of increasing colonization by *Scutellospora fulgida*, *Glomus etunicatum*, and a mix of the two species on the expression of defense traits among genetic families of *Asclepias syriaca*. Points represent the mean trait value for each of five half-sibling genetic families at a given level of colonization. Lines represent the best-fit model for each plant genotype determined using weighted regression. Best-fit lines were chosen from Table 5.2 based on AIC values, and line is absent if the best-fit full model was not significant (p-value>0.05).

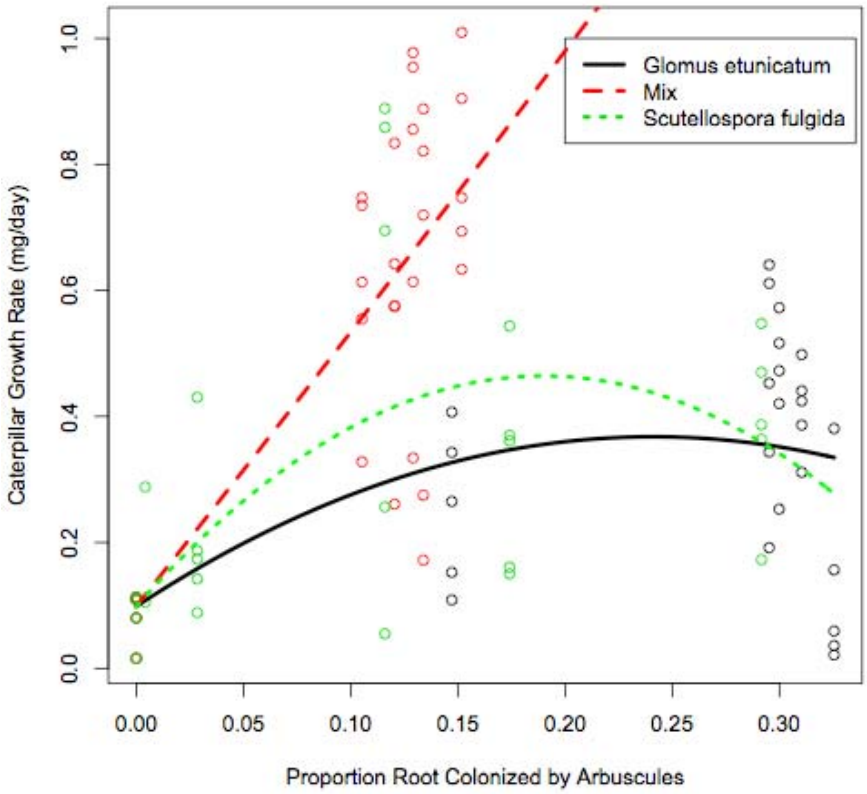

Figure 5.5. Caterpillar *Danaus plexippus* growth rate increases with mycorrhizal colonization of host plant *Asclepias syriaca*. Increasing abundance of AMF *Scutellospora fulgida* and *Glomus etunicatum* affects caterpillar growth in a unimodal fashion, while colonization by a mix of fungal species linearly increases herbivore performance. Points represent mean caterpillar growth rate for live caterpillars recovered from individual plant families on each AMF abundance treatment.

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Chapter VI

Conclusion

Biotic interactions can influence the phenotype of partner organisms, with important ecological and evolutionary consequences (Fritz and Simms 1992). However, despite a large body of work both at experimental and theoretical levels (Strauss and Irwin 2004), it remains difficult to anticipate when and how species interactions drive ecological outcomes (Hoeksema et al. 2010), especially when these interactions encompass both above and belowground communities (van der Putten et al. 2009) and involve multiple trophic levels (Hartley and Gange 2009).

In this dissertation, I integrate a series of manipulative experiments to examine the importance of biotic interactions, for the expression of plant defense and begin to examine the community consequences of these multitrophic interactions. To understand the mechanisms by which biotic interactions affect plant defense phenotype, I quantify changes in plant defensive traits and manipulate the availability of resources that may underlie plant responses to these interactions. I also examine whether plant responses vary among plant genotypes, in order to examine the potential for evolutionary change in response to variation in biotic interactions. First, I use this framework to assess how aboveground herbivory affects the expression of plant defensive phenotype, if plant responses are limited by resource availability, and how phenotypic changes may affect above-belowground interactions and the evolution of plant populations. I then develop and test predictions for how belowground nutrition symbionts affect plant defense expression and the performance of aboveground herbivores. Below I summarize the results from my four primary chapters.

Chapter II. Genetic variation in the expression of defense phenotype may mediate evolutionary adaptation of* Asclepias syriaca *to elevated CO_2. Plant responses to herbivory may be limited by resource availability (Walls et al. 2005) with the consequence that resource addition may alter plant response to herbivory (Bidart-Bouzat and Imeh-Nathaniel 2008). In addition, genetic variation in plant responses to herbivory or resource availability could indicate the potential to respond evolutionarily to selection imposed by changing conditions. In this chapter, I investigated how herbivory by the specialist herbivore *D. plexippus* interacts with carbon fertilization imposed by elevated CO_2 to affect defense expression among *A. syriaca* plant families.

Growth under elevated CO_2 increased plant biomass and changed plant defense phenotype: increasing expression of the physical defenses of toughness and latex, and decreasing expression of toxic cardenolide compounds. Elevated CO_2 did not modify the expression of plant resistance traits in response to caterpillar herbivory, but did increase plant growth during herbivory, thereby increasing plant tolerance to herbivory. Importantly, genetic families of *A. syriaca* varied in their defense responses to elevated CO_2. For example, some plant families reduced expression of chemical defenses by 50% while others actually increased chemical defense expression with carbon fertilization.

The results of this experiment demonstrate that carbon availability changes constitutive plant defense expression, but not the expression of *A. syriaca* resistance traits following herbivory. In addition, this population hosts genetic variation in phenotypic response to rising CO_2 concentration, which will likely allow *A. syriaca* to adapt evolutionarily to elevated CO_2, mediated by herbivory rather than differences in growth responses to changing atmospheric conditions.

Chapter III. Multiple pathways mediate the effects of resource availability and herbivore identity on mycorrhizal associations. Nearly all plants interact simultaneously with aboveground herbivores and belowground mycobionts (Fritz and Simms 1992, Gange 2007, van der Heijden et al. 2008). Interactions among these organisms span above and belowground ecological communities (Gehring and Bennett 2009) and are

linked by plant resource allocation and changes in phenotype (Hartley and Gange 2009). Although carbon availability has been assumed to structure plant associations with mycorrhizal fungi following herbivory, recent work suggests that other mechanisms may mediate this above-belowground interaction instead (Barto and Rillig 2010, de Román et al. 2011). We formalized alternative causal pathways by which aboveground herbivory affects mycorrhizal colonization and tested these models against the results of an experiment. In addition, we explored whether herbivore identity or carbon fertilization altered the strength of the pathways by which herbivory affects mycorrhizal fungi.

Structural equation modeling revealed that aboveground herbivory by aphids and caterpillars affects mycorrhizal colonization through multiple pathways, including changes in aboveground and belowground defense expression. Herbivore identity mediated the strength of defense-related mechanisms—caterpillar herbivory decreased aboveground defense expression, indirectly increasing mycorrhizal colonization. In contrast, carbon availability mediated the total effect of herbivory on mycorrhizal fungi—when grown under elevated carbon, plants strongly increased mycorrhizal colonization following herbivory. The results presented here suggest that the amount of photosynthate available can limit plant associations with mycorrhizal fungi following herbivory, and that specific responses to herbivore species can differentially affect root-associated fungi.

Chapter IV. Plant defense theory re-examined: nonlinear expectations based on the costs and benefits of resource mutualisms. Resource availability is predicted to affect plant defense against herbivores, but despite the ubiquity of resource mutualists and their profound influence on plant resource status, current theory does not account for these interactions and their potential effects on plant defense expression (Stamp 2003). We combine the documented effects of mycorrhizal fungi, common nutrition symbionts, on plant resource status using a benefit:cost framework to predict how resource mutualisms affect plant defense expression. Specifically, the model predicts that the nutrition (phosphorus) benefits associated with hosting mycorrhizal fungi will saturate with increasing fungal abundance, whereas the carbon costs to the plant will continue to rise with increasing fungal colonization. The ratio of the two predicts that plant benefit, in

terms of defense expression, is maximized at intermediate levels of fungal colonization and decreases at high levels of fungal colonization. We tested this model using *A. syriaca* propagated from rhizomes, inoculated with increasing densities of mycorrhizal fungi *Scutellospora pellucida* and *Glomus etunicatum.*

In general agreement with our model, colonization by *S. pellucida* caused plant defense to vary unimodally with increasing fungal abundance, whereas increasing colonization by *G. etunicatum* in general, decreased plant defense expression in a negative exponential relationship. We suggest that variation in the effects of fungal species on plant defense may be mediated by differences in the carbon costs or nutrient benefits conferred to plants. Finally, combining realistic levels of variation in mycorrhizal colonization and a mechanistic understanding of resource exchange between plant and fungal partners may improve our predictions of the expression of plant defense.

Chapter V. Mycorrhizal abundance affects plant defense expression and herbivore performance. To more fully test the assumptions and generality of the model developed in Chapter IV, and to compare the relative importance of AMF abundance to AMF identity and plant genotype, we conducted a large-scale experiment. We quantified the benefits [phosphorus] conveyed by mycorrhizal fungi, subsequent effects on plant defense expression, and assayed herbivore performance on plants colonized by a range of mycorrhizal fungi. We planted *A. syriaca* seedlings from five genetic families into soil containing three fungal communities of increasing fungal density. These fungal treatments included increasing densities of single species inoculum of *Glomus etunicatum, Scutellospora fulgida,* or a mix of the two species.

Colonization by either single-species of fungi increased plant phosphorus concentration, but at high colonization intensity, the benefits (foliar P) conveyed by *Glomus etunicatum* saturated, and declined to a small extent. In contrast, colonization by a mix of fungal species did not significantly affect plant P. Plant defense expression varied in response to increasing colonization according to fungal treatment and the individual trait examined. In general, colonization by *S. fulgida* decreased latex exudation and leaf toughness, but

increased cardenolide expression. Similarly, increasing colonization by *G. etunicatum* decreased latex, toughness, and increased cardenolide expression. Colonization by either species affected unimodally the density of trichomes. Plant families responded similarly to colonization by mycorrhizal fungi in the expression of most traits, but varied in their response to mycorrhizal fungi in the expression of trichomes. The growth rate of the specialist herbivore *D. plexippus* increased with decreasing plant toughness and was higher on plants colonized by mycorrhizal fungi compared to uninoculated control plants. We conclude that nutrient benefits in part explain the effects of mycorrhizal fungi on plant defense expression and herbivore performance. Our results suggest that AMF abundance is a key variable in determining the role of mycorrhizal fungi in multitrophic systems. This experiment provided limited support for REMPD, and we suggest that specific nutrient requirements for defensive traits must be combined with the cost:benefit framework to aid predictions of the effects of mycorrhizae on plant defense.

Synthesis This dissertation illustrates how aboveground herbivores and mycorrhizal fungi interact through changes in plant photosynthate, phosphorus, and subsequent effects on the expression of plant defense. The results presented here document how a realistic range of mycorrhizal colonization influences the availability of plant photosynthate and phosphorus status, plant defense phenotype and herbivore performance. These results suggest that biotic interactions, including mycorrhizal colonization and herbivory, exert strong influence on the expression of plant phenotype and extended phenotype (Dawkins 1982), including herbivore performance. In *Asclepias syriaca*, the effects of these biotic interactions on defense can exceed effects of plant genotype, despite substantial genotypic variation within this system. In addition, resources, including external availability and plant resource status, mediated the effects of biotic interactions on the expression of plant defense. Specifically, aboveground herbivores exert strong influence on mycorrhizal colonization of plants under elevated, but not ambient CO_2 concentrations, indicating that photosynthate limits the belowground response of plants to herbivory. In addition, the effects of mycorrhizal colonization on the expression of plant defense were in part mediated by changes in plant phosphorus status and the benefit:cost ratio associated with hosting these mycobionts.

A few overall trends in the phenotypic response of *A. syriaca* to treatments were noteworthy. Specifically, elevated CO_2 and mycorrhizal colonization exert opposing forces on defense expression. For example, growth under carbon fertilization increased plant physical and structural defenses (toughness and latex exudation) but decreased the concentration of chemical defenses in plant tissue. In contrast, mycorrhizal colonization generally reversed this trend, decreasing physical defenses and increasing chemical defense and plant P concentration. Moreover, increases in mycorrhizal colonization triggered by carbon allocation following herbivory may maintain plant growth and nutrient gain in the face of damage by insect herbivores. As a result, changes in *A. syriaca* defense expression and association with mycorrhizal fungi can be predicted in terms of changes in plant resource status.

Another conclusive finding from this dissertation is that substantial genotypic variation exists in the response of *A. syriaca* to carbon fertilization and mycorrhizal interactions in the expression of some defense traits. For example, *A. syriaca* genotypes vary widely in cardenolide expression and in their response to mycorrhizal colonization and carbon fertilization in the expression of cardenolides. The genetic variation in both the magnitude and shape of plant response to biotic (mycorrhizae) and abiotic (CO_2) context, may allow for evolutionary adaptation within milkweed populations in response to selection by herbivores on plant defense expression. In addition, results described in chapter V suggest that plant genotypes vary in phenotypic response to the identity and abundance of soil fungi, which may allow for complex evolutionary dynamics in this multitrophic system. Future research should evaluate experimentally the consequences of multitrophic interactions and resource availability for the evolution of plant defense expression.

Caveats and future directions The results presented in this dissertation strongly support a central role of plant resource status in mediating plant phenotype expression. The experiments described here demonstrate clear effects of carbon fertilization on plant phenotype and the role of phosphorus in predicting the effects of mycorrhizal fungi on defense expression. However, plant defense expression did not exactly follow the precise

benefit:cost curve outlined in Chapters IV and V. To reconcile the variation in the response of traits to mycorrhizal colonization and carbon fertilization, I suggest that 1) costs and benefits associated with fungi be more precisely measured, 2) soil fertility must be taken into consideration and 3) the range of costs and benefits must be fully explored. First, the results in this dissertation provided a rough estimate of the costs associated with mycorrhizal colonization, but may either over or underestimate the costs involved. A complete quantification of carbon costs should include measurements of extraradical hyphae, also constructed from carbon derived from plants. On the other hand, plants in natural systems often have the benefit of tapping into established hyphal networks (van der Heijden and Horton 2009) and may not bear the primary carbon cost associated with constructing a large network. As a long-lived perennial, *Asclepias syriaca* may construct or tap into an existing network and contribute over multiple years, and as a result, the carbon cost is likely to vary through time. Second, soil fertility can influence the costs and benefits associated with hosting AMF (Graham et al. 1997, Graham and Eissenstat 1998), Fig. 4.5. Our experiments may have been conducted at a relatively high level of nutrient availability and thus our results would only capture the parasitic effects of mycorrhizal fungi and little of the benefits (Region III in Fig. 4.1b). We are currently investigating how carbon costs, nutrient benefits, and the effects of AMF on plant fitness change over time and are influenced by nutrient availability in soil. Third, the shape of the response curve for individual traits may vary with nutrient requirements. As a result, not all traits will likely respond in kind to mycorrhizal colonization, but these traits all should be limited by nutrients or carbon at some point. To fully explore the shape of the response of plant defense to mycorrhizal colonization, a greater range of fungal colonization densities should be generated, under a larger range of nutrient availability.

For the maintenance of mutualisms in evolutionary time, mycorrhizae must offer fitness benefits or come at low cost to both partners involved (Hoeksema and Bruna 2000, Kiers and van der Heijden 2006, Hoeksema 2010). However, under some experimental conditions, we failed to identify fitness benefits from colonization by AMF. Under current investigation is how the effects of AMF on *A. syriaca* may be dependent on other factors, including soil fertility, the duration of the association and the presence of root

herbivores. Initial evidence suggests that mycorrhizal colonization decreases the incidence of root herbivory by a generalist herbivore and increases the survival of *Asclepias* spp. following root herbivory (Rasmann and Vannette in prep). In addition, the results of chapters II and III suggest that mycorrhizal fungi can increase plant tolerance and regrowth following herbivory, and that growth in future atmospheric conditions may augment this effect. Future studies could also examine the role of AMF in drought stress (Auge 2001) and protection against pathogens (Borowicz 2001), which are both likely important to this long-lived clonal plant (Burdon et al. 2006) that often grows in dry conditions. Accounting for these additional benefits conveyed by AMF may tip the benefit:cost ratio in favor of mutualistic stability over a wider range of conditions.

Not only can the degree of plant association with mycorrhizal fungi change with resource availability or herbivory (Barto and Rillig 2010), but fungi community composition may also change (Saravesi et al. 2008, Gehring and Bennett 2009). Initial evidence from this system indicates that herbivory by *D. plexippus* or *A. asclepiadis* affects the community of mycorrhizal fungi within the roots of *A. syriaca* (Fig. 6.1.). Ongoing work will shed light on the changes in fungal community composition caused by aboveground herbivory in field and greenhouse studies. Future studies should synthesize this knowledge of how herbivory shifts AMF community composition with the consequences for plant phenotype and fitness to improve our understanding of above-belowground feedbacks within this multitrophic system and potential consequences for community dynamics and ecosystem function (eg. Bever 2002, Rillig 2004).

The experiments described here were conducted in pots, and as a result, plants were not exposed to competition from conspecifics or heterospecifics or other soil organisms, which could mediate plant responses to fertilization or biotic interactions (Amthor 2001). However, field data provide evidence that at least some of these findings are relevant in natural systems. Clones of *A. syriaca* vary widely in mycorrhizal colonization of roots in the field (~20-80%), and in defense phenotype (R. Vannette, Hunter Lab, unpublished data). In addition, experimental manipulation of herbivory by larvae of *D. plexippus* increased mycorrhizal colonization of *A. syriaca* roots one month after herbivory under

growth in the field (Fig. 6.2.). Additional studies are ongoing and will more fully explore patterns of herbivory, mycorrhizal colonization and interactions between them in *A. syriaca* in the field. In addition, our experiments did not generate the same range of colonization densities as found in the field (0-35% in experiments, compared to 10-80% in the field). Future experiments should include a greater experimental duration or number of fungal species to capture the variation present in field conditions.

Despite these caveats, the work described in this dissertation adds to our ability to predict the importance and outcome of interspecific interactions by quantifying their effects on plant resource status and expression of defense phenotype. A better understanding of multitrophic interactions that span above and belowground systems can guide practices for agricultural, horticultural, or restoration applications and inform the ecological and evolutionary dynamics within these systems.

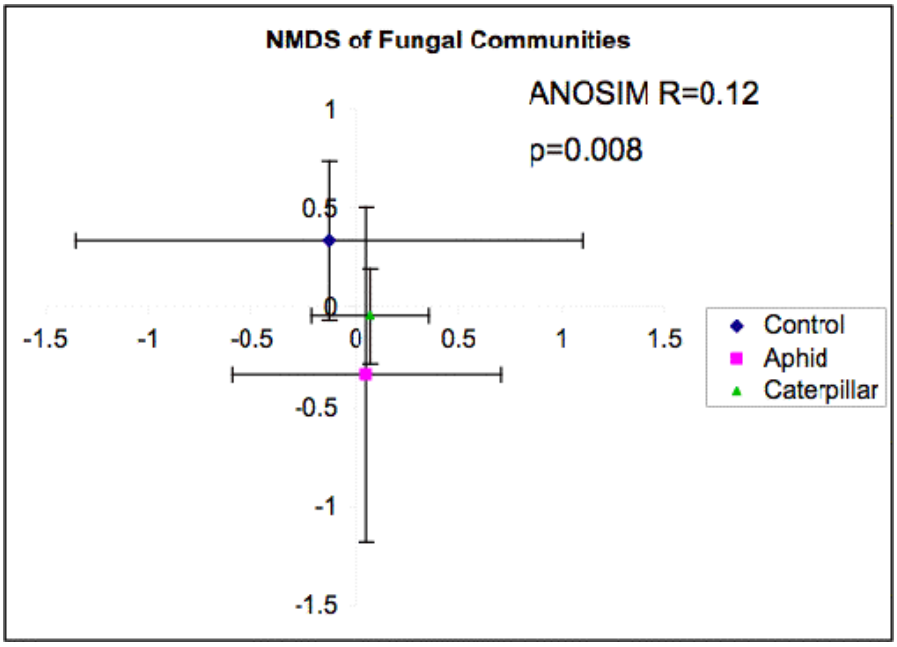

Figure 6.1. The effect of herbivory by *Danaus plexippus* and *Aphis asclepiadis* on fungal community composition in the roots of *Asclepias syriaca* seedlings measured using Terminal-Restriction Fragment Length Polymorphism (T-RFLP). Plants were grown as described in Chapter III and harvested five days following herbivory by *D. plexippus* and *A. asclepiadis*. DNA was extracted from roots of *A. syriaca* and methods for T-RFLP followed those by Aldrich-Wolfe (2007). Points represent mean NMDS coordinates for fungal communities of plants receiving no herbivory or herbivory by aphids or caterpillars ±1 SD. Analysis of Similarity was performed in the package vegan using R v. 2.11.0.

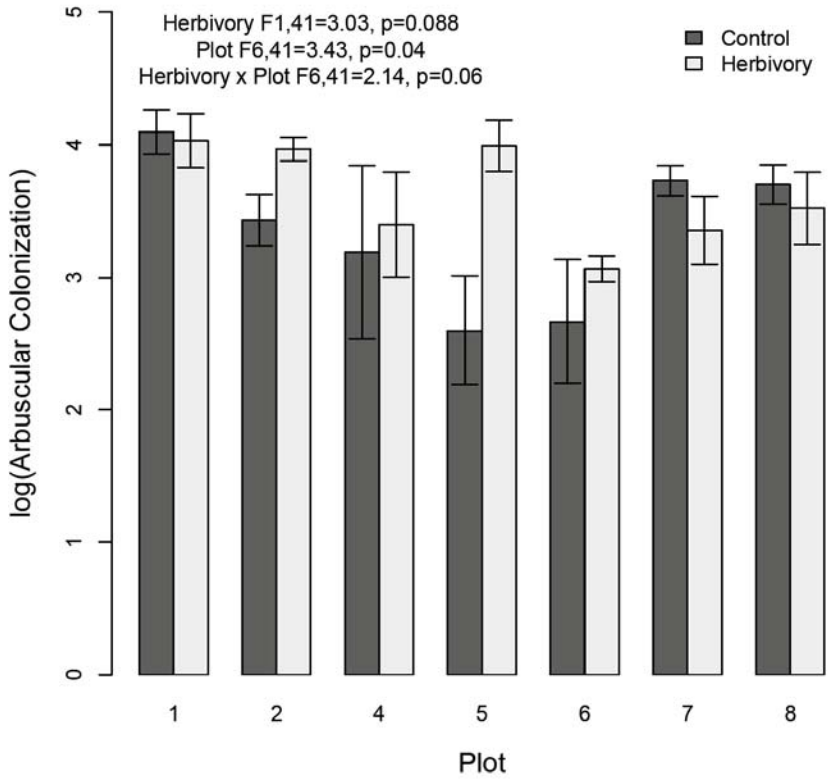

Figure 6.2. Herbivory by *Danaus plexippus* larvae increases the proportion of arbuscules within the roots of two-year-old *Asclepias syriaca* plants. Four genotypes of *A. syriaca* seedlings were planted in plots within the field at the University of Michigan Biological station in Pellston, MI. Caterpillars were allowed to eat ~20% of leaf tissue during the first and second year of plant growth, and plants were harvested one month following the second herbivory treatment.

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