Global Change Biology

Global Change Biology (2010), doi: 10.1111/j.1365-2486.2010.02316.x

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

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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 found no effect of elevated CO_2 on plant induction of cardenolides in response to caterpillar herbivory. Elevated CO_2 decreased 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.

Keywords: adaptation, *Asclepias syriaca*, climate change, herbivory, heritability, inducible defense, plant–herbivore interactions, resistance, trade-offs

Received 17 February 2010 and accepted 12 July 2010

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 (GECs) 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, 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

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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 GEC (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 *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. Because 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 *A. 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 *D. 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 multitrait *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). Because 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.

A. 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 results). 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

A. 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 five 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 °C. Following germination, seedlings were planted into 50 mL cells containing potting soil (SunGrow Horticulture, Vancouver, BC, Canada) and reared in a growth chamber for 2 weeks. Eighty seedlings of each genotype were planted individually into 6 in. pots containing approx 1 L 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 two ambient chambers using a LI-COR LI-6262 IRGA (LI-COR, Lincoln, NE, USA) 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 9 weeks of CO_2 treatment. Two weeks before the herbivory treatment was initiated (early July, 2008), when plants were approximately 2 months old, all plants were covered with a fine mesh (paint strainer bags, Mastercraft Mfg., South El Monte, CA, USA) 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 disks using a hole punch and stored them in a refrigerated incubator until use (maximum 2 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 before 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 eggs 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 h) 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 six and 22 leaves. *A. syriaca* plants have opposite leaves. All plant heights were measured, and vertical growth during 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 $-10\,^{\circ}C$ for cardenolide analysis. Five identical leaf disks 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. Latex that flowed from the first five holes punched was collected on a preweighed cellulose disk (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 belowground 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 disks 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 $\times 4$ 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 & Zalucki (1996) and Zehnder & Hunter (2007). Briefly, leaf disks were ground in methanol for 2 min using a ball mill (Retsch MM200, Haan, Germany), sonicated at 60 °C for 1 h and evaporated to dryness. Samples were resuspended in 150 μL of methanol containing 0.15 mg mL^{-1} digitoxin as an internal standard and analyzed using reverse phase high-performance liquid chromatography at high system pressures (UPLC, Waters Inc., Milford, MA, USA). 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 found 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 with regression estimates (Mousseau & Roff, 1987; Roff, 1997). We calculated heritability as two times the plant genotype variance component, divided by the total variance component ($H^2 = (2VC_{full-sib})/(VC_{full-sib} + VC_{error})$) (Lynch & Walsh 1998). 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 & 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 1). Plant genotypes varied significantly in total biomass, regrowth ability, latex exudation, trichome density, and the expression of cardenolides (Table 1). Plant traits, however, were not uniformly heritable (Table 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 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 1), that approached statistical significance ($P < 0.10$). In contrast, SLM, a proxy for leaf toughness, displayed very low heritability ($H^2 = 0.03$, Table 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 and 3), but did so to a similar extent among all plant genotypes. Atmospheric CO_2 concentration, however, 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 and 3). Despite substantial effects of CO_2 on aboveground and belowground biomass, we found no interactions between genotype and CO_2 on belowground biomass or meristem bud number produced by each genotype (Fig. 1a and b) or in response to herbivory (three-way interaction) (Table 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 $\times$ CO_2 $F_{4,90} = 2.14$, $P = 0.082$, Fig. 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 and 3, Fig. 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 ($F_{4,81} = 4.69$,

Table 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	$VC_{full-sib}$	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
Tolerance trait						
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
$^+P < 0.10$, $^*P < 0.05$, $^{***}P < 0.001$.

Table 2 Measurements of *Asclepias syriaca* growth, reproductive, and defense traits in an open-top chamber study

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

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.
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.

Table 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

Bold indicates significance after Bonferroni's correction.
$^{+}P < 0.10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

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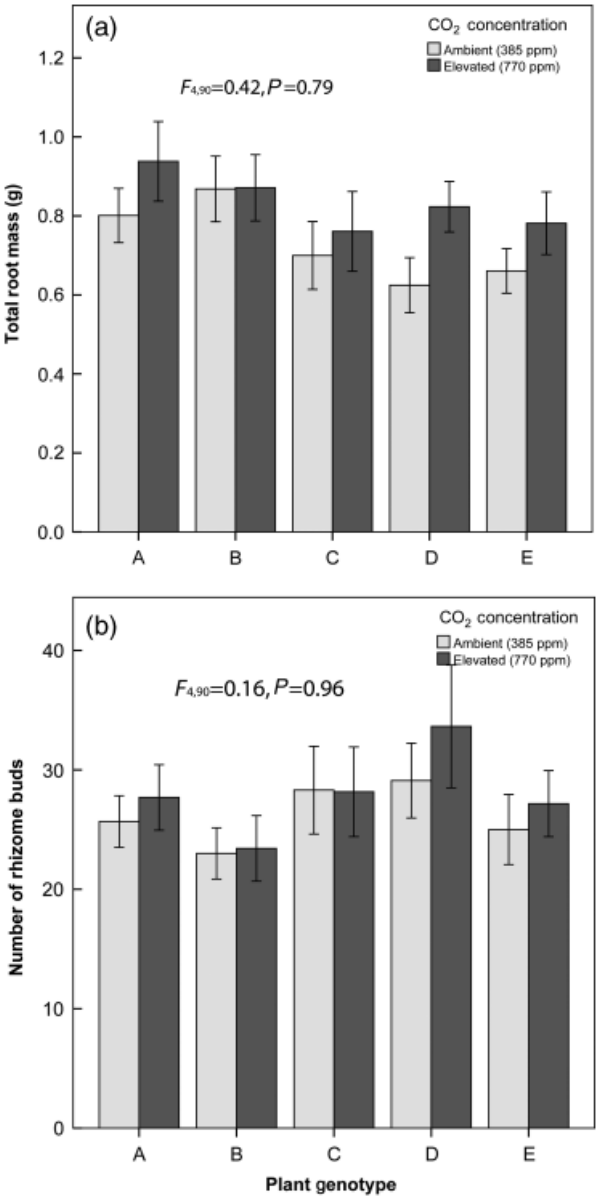

Fig. 1 The effect of elevated CO_2 on genetic families of *Asclepias syriaca* in the production of (a) belowground (rhizome plus fine root) biomass, and (b) number of meristem buds on the rhizome, both traits associated with fitness. Bars represent mean trait values ± 1 SE pooled across herbivory treatments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

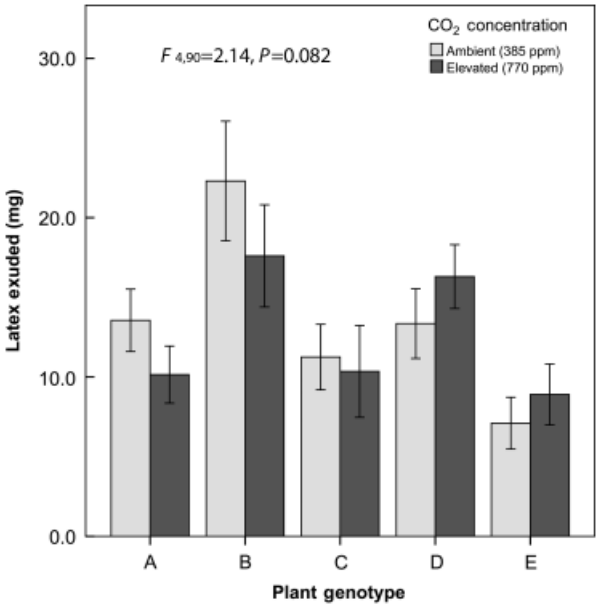

Fig. 2 The effect of elevated CO_2 on latex exudation among genetic families of *Asclepias syriaca*. Bars represent the mean dry mass of latex exuded ± 1 SE pooled across all herbivory treatments and *F*- and *P*-values are derived from the full model.

$P = 0.0018$, Fig. 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. 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 $F_{1,90} = 3.33$, $P = 0.0714$, $CO_2 \times$ Family $F_{4,90} = 1.77$, $P = 0.14$, Fig. 3b). Elevated CO_2 increased SLM by 40% ($F_{1,90} = 60.29$, $P < 0.0001$, Table 2) irrespective of plant genotype ($CO_2 \times$ Family $F_{4,90} = 0.20$, $P = 0.937$, Tables 2 and 3). Elevated CO_2 also increased the variation among genotype averages in cardenolide expression (Fig. 3d), but tended to decrease the variation among genotypes in the expression of latex and individual cardenolide compounds (Figs 2 and 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 and 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 and 3). Elevated atmospheric CO_2, however, mitigated the negative effect of insect herbivory on plant fitness, measured by the number of meristem buds produced on *A. syriaca* rhizomes ($CO_2 \times$ Herbivory $F_{1,81} = 7.55$, $P = 0.0072$, Fig. 4).

Plant genotypes responded to caterpillar herbivory with altered physical and chemical defense expression.

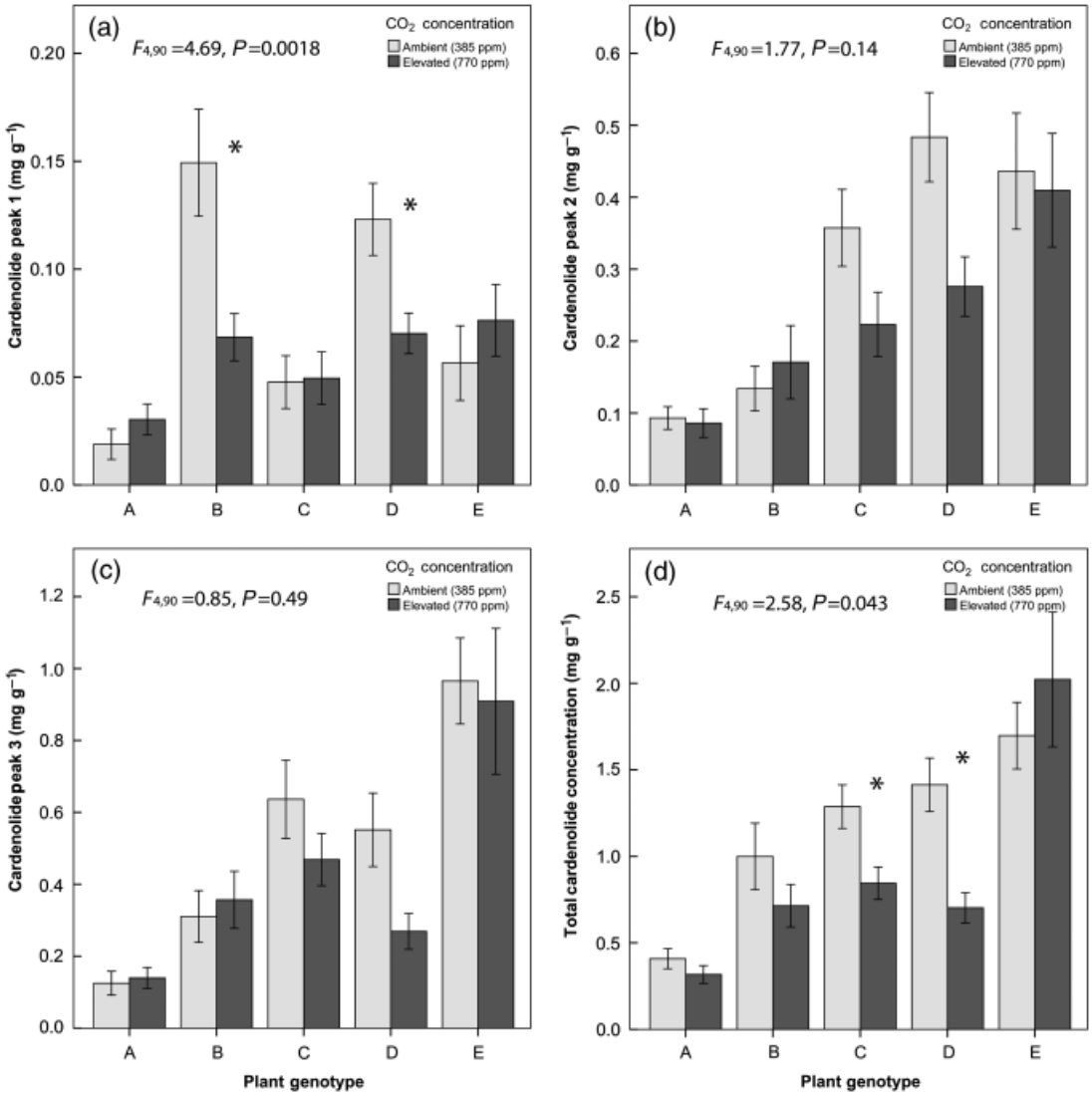

Fig. 3 The effect of elevated CO_2 on constitutive expression of cardenolide peaks 1–3 and total cardenolide concentrations (a–d) among five genetic families of *Asclepias syriaca*. Bars represent the mean concentration of cardenolides in foliar tissue ± 1 SE pooled across herbivory treatments and *F*- and *P*-values are derived from the full model. *Differences between ambient and elevated CO_2 treatments within families, using Tukey–Kramer adjustments for all pairwise comparisons.

All plant genotypes exhibited a 55% decline in latex exudation following caterpillar herbivory ($F_{1,81} = 69.39$, $P < 0.0001$, Tables 2 and 3). Elevated CO_2, however, ameliorated the negative effect of caterpillar herbivory on latex exudation in some genotypes (three-way interaction $F_{4,90} = 2.38$, $P = 0.057$, Table 3). In addition, all plant genotypes that were consumed by caterpillars displayed nearly 10% lower SLM than the control plants ($F_{2,81} = 13.71$, $P = 0.0004$, Table 2). Neither herbivory, CO_2, or their interaction affected substantially the density of trichomes on leaf surfaces (Table 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). 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 and 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

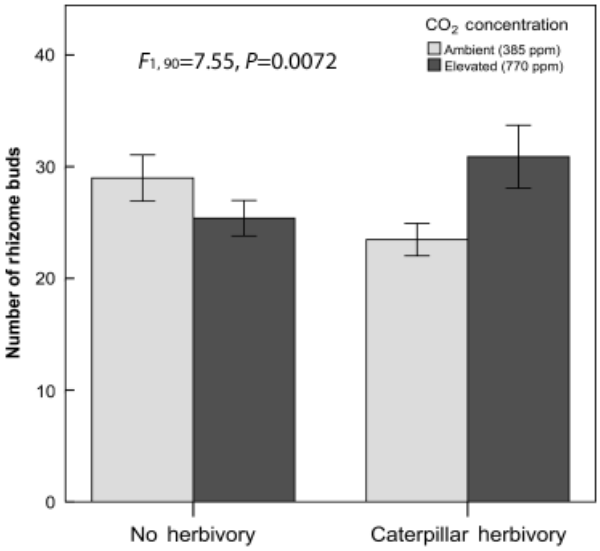

Fig. 4 The interaction of elevated CO_2 and insect herbivory on the number of *Asclepias syriaca* rhizome buds following herbivore treatment. Bars represent mean trait values ± 1 S.E. pooled across genotypes and *F*- and *P*-values are derived from the full model.

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 found by Agrawal (2005), lending support to our results. Additionally, we found that *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 coevolutionary) 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 *A. thaliana* and *B. 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 because 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 found 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 *G. 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).

Elevated CO_2 did, however, significantly reduce 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, we found that 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, because 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 results) 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 × 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, we identified heritable intraspecific variation in the expression of defense phenotype and found that the effect of CO_2 on the expression of defenses was often genotype-specific.

Specifically, we found that elevated CO_2 differentially affected the expression of latex and polar cardenolide peaks among plant families. A majority of previous work has found that plant species harbor genetic variation in the degree to which defense expression responds to elevated CO_2. Clones of *P. 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 *et al*. (2005) reported significant genetic variation in the induction of total and individual glucosinolates by *A. 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. Because 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 toward 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.

Acknowledgements

We gratefully acknowledge Dr David Karowe and the UMBS for the use of their CO_2 chamber array and other facilities. We also thank S. Kabat, S. Breed, E. Wason, M. Tyner, and R. Bienek for their help with experimental setup, rearing caterpillars, harvesting, and sample processing. We thank the reviewers for their constructive comments and suggestions. This work was supported by funding from NSF-IGERT BART to RLV and NSF DEB 0814340 to MDH and RLV.

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