EVOLUTIONARY AND ECOLOGICAL CONSEQUENCES OF NATURAL SELECTION BY HERBIVORES ON *SOLIDAGO* SPP.

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Herbivory has been shown to be a selective agent on plant secondary chemistry, and has been hypothesized to limit plant growth in an evolutionary sense by selecting for plants that allocate resources into defense at a cost to growth. Various resistance traits may be effective against different herbivores, and may be traded off with one another within a plant or over evolutionary time. The following series of studies address micro-evolutionary aspects of herbivore resistance and the evolution of resistance to different herbivores. The first paper analyzes the effects of a long-term herbivore exclusion treatment on herbivore resistance and plant growth rate. It investigates the ecological and physiological costs of herbivore resistance through correlations between resistances to two herbivores and between resistance and growth. The major findings of this study are that the correlation between growth and resistance is context dependent, and that resistance to different herbivores may evolve independently. The second paper investigates the variation of herbivore resistance along an elevation gradient, and measures the natural selection on secondary metabolites that vary with elevation. It finds that (1) the level of herbivory decreases with increasing elevation, (2) production of most secondary metabolites detected decreases with increasing elevation, although production of a few increase, and (3) there is natural selection in a low-elevation

common garden to increase production of two metabolites that correlate negatively with elevation and to decrease production of a metabolite that correlates positively with elevation. These findings strongly suggest that herbivory can be a primary agent of selection, and that variation in herbivore pressure drives variation in secondary metabolism in this system. The third paper presents a method for analyzing two types of protease inhibitors as plant anti-herbivore resistance traits, and argues that although both are induced by herbivory, only one functions against a given herbivore. The findings of this experiment suggest that a common mechanism controls the induction of two types of protease inhibitors. The last paper addresses several issues in the evolution of plant resistance, and presents the argument that opposing selective pressures can constrain evolution of increases in a given resistance trait. It further argues that tolerance and chemical diversity may not be under opposing selective pressures like some resistance traits are, so that increased tolerance or increased chemical diversity may evolve without changing the selective pressures on herbivores.

BIOGRAPHICAL SKETCH

Robert Bode was born on February 9th, 1983 in Groton, Connecticut. He graduated from King’s West High School (Bremerton, WA) in 2001. Being well-educated in creation science and how to refute the theory of evolution, he went on to study biology at Hope College (Holland, MI), and graduated with a B.S. in 2005. He began his graduate career at Cornell University the following fall, and started work with tomatoes in André Kessler’s lab. Later, he switched to working on the *Solidago* system in 2008, and plans to continue with this plant for his future research. He is currently a teaching post-doctoral associate at Canisius College in Buffalo, NY. Robert had his first child on July 4th, 2011, and considers this recent increase in fitness to be his greatest achievement.

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I thank my father, mother, and brother for teaching me patience and to love the outdoors. I especially thank my father for taking us on trips to the Adirondacks when I was young, where I would later find an excuse to hike for research. My wife, Carrie, has been instrumental in encouraging me to never surrender. Her hard work helped keep us financially stable through the leaner times, and her constant companionship was the anchor that kept me clinging to my sanity when projects failed.

None of this work could have been done without my lab members, who have been a great help when my experiments get too large for me to do alone. Thanks to Rayko Halitschke for endless help with plant chemistry and for helping me hike dry ice up a mountain, Kim Morrell and Stuart Campbell for field assistance and much needed laughs, Akane Uesugi for challenging my assumptions, and a legion of undergraduate assistants for help with the more mundane tasks. Although not strictly members of the lab, I also thank Dick Root and Bob Johnson for making me passionate about the goldenrod system and being ready sources of wisdom.

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CHAPTER 1

Herbivore Pressure on Goldenrod (*Solidago altissima*): Its Effects on Herbivore Resistance and Plant Vigor

Authorship to be published as: Robert F. Bode and André Kessler

ABSTRACT

1. Costs of resistance to herbivory are a core concept in the study of plant-insect interactions, but are still poorly understood. They are most obvious in tradeoffs in resource allocation to different growth and resistance traits. The growth-differentiation balance principle predicts trade-offs between resistance to alternate herbivores or between growth and resistance

2. We used plant genotypes collected from long-term herbivore-exclusion plots and from plots with natural herbivory to evaluate putative trade-offs between resistances to two different herbivore species, *Trirhabda virgata* and *Spodoptera exigua*, and between resistance and growth as a potential outcome of differential natural selection.

3. Genotypes from herbivore exclusion plots were less resistant to *T. virgata* than plants from control plots, but equally resistant to *S. exigua*. Resistance to one herbivore species did not correlate with resistance to another.

4. Plant growth correlated positively with resistance to *Trirhabda*, but not to *Spodoptera*, and only in plants from herbivore exclusion plots.

Synthesis: Our results suggest that correlations between growth and resistance are context-dependent and may only be apparent in populations removed from natural pressures, such as in natural populations relieved from herbivore pressure.

INTRODUCTION

General plant defense theory assumes metabolic costs for the production of plant chemical defenses. Thereby, plants are assumed to have a finite pool of resources (resource allocation principle), available to be allocated to different functions including growth, reproduction, and defense (Karban and Baldwin, 1997). It would follow that an increased allocation of resources into defense compound production should be obvious both in physiological trade-offs (e.g. with growth) as well as reproductive outputs as an integrative consequence. Although costs should be measured in terms of fitness to be interpretable in an evolutionary sense, (Koricheva, 2002; Karban, 2010), the detections of trade-offs between different life history traits (growth and reproduction) has emerged as a valuable proxy for the existence of costs of defenses. This is largely because estimating costs of plant resistance to herbivores directly is experimentally challenging for various reasons: defense metabolites may be highly effective for a low metabolic cost (Kakes, 1989), may be degraded and re-used (Mihaliak et al., 1991), or may have roles in both primary and secondary metabolism (Seigler and Price, 1976; Arnold and Targett, 2003). Measuring trade-offs also reflects feedback loops between the evolutionary and ecological levels characteristic of the evolution of herbivore resistance. Thus, the identification of potential trade-offs as well as the conditions determining them are crucial for our understanding of evolution of plant-insect interactions.

How plants allocate their resources has been extensively studied both within and among species, but with mixed results. A number of studies have found that increased resistance to herbivory is traded off with reduced growth and plant fitness (Mooney and Chu, 1974; Bentley and Whittaker, 1979; Herms and Mattson, 1992; Baldwin and Hamilton, 2000; Zavala et al., 2004; Zavala and Baldwin, 2006). In contrast, other studies have not found trade-offs between resistance and growth or reproduction, (Bowers and Stamp, 1992; Adler et al., 1995; Strauss and Agrawal, 1999; Siemens et al., 2002; Arnold and Targett, 2003; Jones et al., 2006; Lankau and Kliebenstein, 2009) or trade-offs of resistance to one herbivore with that to another (Rudgers et al., 2004; Koricheva et al., 2004). Likely reasons for the mixed messages about the trade-offs with resistance are the relative differences in the time plant populations have been exposed to a particular combination of agents of natural selection, and the environmental conditions under which trade-offs were measured. Assuming a certain mix of plant genotypes in a newly established population, natural selection is expected to alter the frequency of genotypes and so the relative abundance of particular phenotypes. Thus, trade-offs can be best measured in systems that control for (or maximize) genetic and phenotypic variability (Strauss et al., 2002) or minimize the number of interacting species for less diffuse selection (Stinchcombe, 2002; Hull-Sanders et al., 2007). More specifically, long-term selection by dominant herbivore species could make trade-offs more difficult to detect, while trade-offs with resistance to a weak (low dominance) herbivore agent of selection may be obscured by selection by other forces (Conner, 2001; Stinchcombe, 2002). In long-term experiments, selection by forces such as competition may act to minimize the trade-offs between growth and resistance.

The long-term removal of insect herbivory as an agent of natural selection from a system could be a useful means of determining whether there are trade-offs with resistance. In a constant context of no herbivory (Stinchcombe, 2002), natural selection should favor plants that invest less in resistance to the benefit of the expression of other functions, such as growth, reproduction, competition, and resistance to alternative attackers. A seminal long-term study that examined the effects of herbivore exclusion by insecticide application on plant growth of a community-dominating plant species, *Solidago altissima* (L.) and the composition of the plant community, was conducted by

Dick Root and his associates (Cain et al., 1991; Root, 1996; Carson and Root, 1999; 2000). This work revealed how long-term (7 to 10 year) exclusion of herbivores can have strong positive effects on components of plant sexual reproduction, including larger inflorescences, and a greater proportion of blooming stems (Root, 1996), as well as on components of asexual reproduction, such as production and growth of rhizomes (Cain et al., 1991), compared to plants under normal herbivore pressure. Yet it remains unclear whether populations not under selection by herbivores would consist of plants with lower resistance than would be found in populations under selection by herbivores. Moreover, comparing growth rates in plants from herbivore exclusion plots to those from plots with natural herbivory in a common garden could reveal trade-offs with resistance, since in the absence of selection by herbivores, high competition could place slow-growing, well-defended plants at a disadvantage.

Here we measure trade-offs between resistance and growth and between resistance to two herbivores in *S. altissima* plants from populations under natural herbivory and plants from populations where herbivory was excluded for a 12-year period. More specifically we evaluate mean plant resistance to one of the major and outbreaking herbivores, the chrysomelid beetle *Trirhabda virgata* (J.L. LeConte) and the rarer larvae of the noctuid *Spodoptera exigua* (Hübner) in plants from natural herbivory and herbivore exclusion regimes. Growth and defense against multiple herbivores are of particular importance in the *S. altissima* system, where dominance of canopy space ensures maximization of fitness, and a large cohort of insect herbivores are present (Root and Cappuccino, 1992). The experiments allowed us to address four major hypotheses. (A) Herbivory is a strong selective agent on herbivore resistance, so that plants from natural herbivory plots should have on average higher resistance than plants from herbivore exclusion plots. (B) There is a trade-off between growth and resistance. (C) There is a trade-off between the resistance to one herbivore species with the resistance to another. (D) The ability to detect a relationship between resistance and growth varies with the selective environment.

MATERIALS AND METHODS

Study system

Solidago altissima is a common perennial forb found in abandoned agricultural fields in eastern North America. The clonal, rhizomatous growth of *Solidago* allows for perpetual maintenance of established lines and rapid propagation of genetically identical plants. Two species of beetles (*Trirhabda virgata* and *Microrhopala vitata* (Fabricius)*,* both Coleoptera: Chrysomelidae), among others feed on *Solidago*, and are hypothesized to act as keystone herbivores, dramatically impacting plant fitness and plant community composition during outbreak years (McBrien et al., 1983; Carson and Root, 2000). To compare the effects that long-term herbivore exclusion had on plant resistance against different herbivore species we used performance of the common *T.virgata* and a low abundance generalist moth larva (*Spodoptera exigua,* Lepidoptera: Noctuidae) as two independent measures of herbivore resistance. *Trirhabda virgata* is a specialist commonly found on *Solidago* (Root and Cappuccino 1992), while *S. exigua* is a generalist less frequently found on *Solidago* (personal observation). *Spodoptera* is capable of completing its life cycle on *Solidago* and can remove substantial amounts of leaf tissue, similar to amounts removed by *Trirhabda* (personal observation). We used these species to compare resistance of two herbivore species of the same feeding guild (Maddox and Root, 1987; Hull-Sanders et al., 2007) but different relative ecological impacts on *Solidago*.

Herbivore exclusion plots and plant material

Herbivore exclusion (herbivory -) plots were established in an old field that included *S. altissima* at Whipple Farm, in Ithaca, New York, U.S.A. (42^o25' W, 76^o31'W) (as in Cain et al., 1991; Carson and Root 2000). Twelve 5 by 5 meter plots with a 2 m gap between the plots were marked off and randomly assigned a treatment regime. Six of the plots were sprayed with Fenvalerate following the manufacturer's instructions (ORTHO® Group, Marysville, OH, 2.7 µl of the active compound (Esfenvalerate) in water per m^2) every other week during the growing season (May to September) for twelve years (1996-2008). Six plots were left unsprayed (herbivory +). Fenvalerate has been shown to have no significant direct effect on plant mass or flowering (Carson and Root 2000).

We randomly collected 16 individual plants (separate genotypes) from these plots, eight from herbivory (-) plots, and eight from herbivory (+) plots. All plots were represented by at least one genotype, with multiple genotypes from some plots (2 each from 2 plots, 3 from 1 plot). All plants were derived from rhizomes taken from at least four meters apart within a plot to minimize the probability of having genotypes represented multiple times. The plants were grown in a common garden greenhouse for three cycles (one cycle = rhizome grown until flowering, rhizomes cut and re-planted) to eliminate possible maternal effects before being used in the experiment.

Plants and resistance measurements

We transplanted 14 propagated plants from each genotype into 15cm diameter azalea pots with Metro Mix® (Sun-Gro, Bellevue, Washington, USA) soil. All plants had a single ramet at the beginning of the growing season. These were grown outside under ambient light and temperatures from late April to October, which is the natural growing season in upstate New York. There was no herbivory on these plants prior to our experiment. For each genotype, we used eight plants for growth measurements and six plants for herbivore resistance measurements. For herbivore resistance measurements plants were grown for eight weeks, and then three plants of each genotype were infested with two *S. exigua* larvae (second instar out of five instars) (purchased from BioServ, Frenchtown, NJ, USA), grown on diet from BioServ (Frenchtown, NJ, USA) for six days and acclimated on *S. altissima* leaf tissue for 24 hours), and three plants were infested with two *T. virgata* larvae (second or third instar out of three instars, collected locally from field). Both larvae were weighed and an average was taken for initial mass. All plants were enclosed in mesh bags with the larvae for seven days. After the seven days, all larvae were removed and weighed again, and an average was taken from the two larvae. Growth rate of herbivores was calculated as (final mass-initial mass)/initial mass. Growth rates for herbivores were averaged on all plants of each genotype to provide genotype means. Resistance was calculated as 1/growth rate for both insects. Resistance between regimes was compared using a Student's T-test of genotype means with JMP 8.0 (SAS Institute).

Growth measurements

To measure growth, eight plants (not used in the bioassays) were surveyed from each of the 16 genotypes for aboveground shoots (ramets) in October, when all aboveground shoots that will be made in a season have been made. Because all rhizomes do not necessarily produce plants in the next season (Cain et al., 1991), and plants are able to regrow aboveground tissue after heavy leaf loss with no measurable fitness reduction (Meyer, 1998), we took ramet number as the measure of asexual reproduction rather than rhizome mass rhizome number, aboveground biomass or leaf number. We have also found that ramet number correlates well with leaf number (Linear Fit, r^2=0.336, P=0.0184, n=16) and aboveground biomass (Linear Fit, r^2=0.409, P=0.0076, n=16), signifying more canopy space dominated and thus higher competitive ability. Ramet number at season end also correlates well with the number of rhizomes produced (Linear Fit, r^2=0.463, P=0.0036, n=16) and total length of rhizomes (Linear Fit, r^2=0.405, P=0.0080, n=16). Moreover, in an old field community, asexual reproduction may be a more relevant measure of fitness, since new *Solidago* are unlikely to come from seeds (McBrien et al., 1983). Additional ramets, however, translate into a larger life-time sum of inflorescences and thus more seeds, identifying ramet number as one of the most reliable and reasonable fitness proxies for this species. We compared ramet numbers between the different genotypes and two treatment regimes using an ANOVA (JMP 8.0, SAS Institute). Relationships between ramet number and resistance were analyzed using Linear Regression with JMP 8.0 (SAS Institute).

RESULTS

Resistances to two herbivores are not correlated

Plants from the natural herbivory regime were on average more resistant to *T. virgata* larvae than plants from the herbivore exclusion regime. The larvae had a higher mass gain on plants from herbivory (-) plots than on plants from herbivory (+) plots (Student's T-test, *t*=-2.41, *P*=0.0303, n=16, Fig 1.1 A). Several larvae molted into their pupal stage or died and were excluded from measurements, since larvae begin losing mass once they molt into their pupal form. However, survivorship did not differ between the two regimes (Student's T-test, *t*=0.388, *P*=0.7040, n=16). The growth rate of *S. exigua* larvae was equal on plants from herbivory (-) plots or herbivory (+) plots (Student's T-test, *t*=-0.11, *P*=0.9166, n=16, Fig 1.1 B). There was a wide variety of larval sizes, even within herbivores feeding on the same genotype, and survivorship was lower in larvae feeding on plants from unsprayed plots, although this difference was not significant (Student's T-test, *t*=-1.82, *P*=0.1001, n=16). The growth rate of *T. virgata* larvae did not correlate with the growth rate of *S. exigua* larvae, regardless of regime (Linear fits; herbivory (-) r^2=0.027, *P*=0.6953, n=8, herbivory (+) r^2=0.030, *P*=0.6780, n=8).

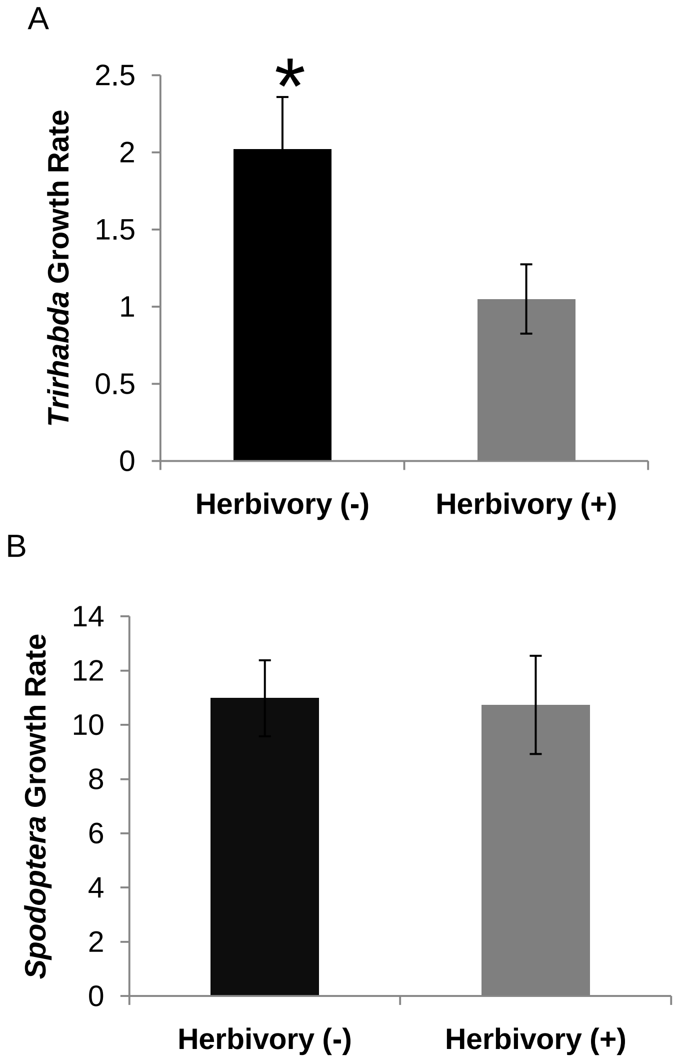

Figure 1.1 Herbivore performance on plants from long-term herbivore exclusion (Herbivory (-)) and natural herbivory (Herbivory (+)) plots. **A**) Mean mass gain (±SEM) of *T. virgata* larvae. **B**) Mean mass gain (±SEM) of *S. exigua* larvae. The asterisk (*) designates significantly different means as informed by a Student’s t- test of genotype means (*P*<0.05).

Herbivore resistance does not show a trade-off with growth

In herbivory (-) plots, resistance to *T. virgata* (1/growth of larva) was positively correlated with single-season asexual reproduction (Linear Fit, r^2=0.512, P=0.0436, n=8, Fig 1.2 A). The resistance to *T. virgata* did not correlate with ramets per plant in genotypes from herbivory (+) plots (Linear Fit, r^2=0.053, P=0.5815, n=8). Ramet production was not correlated with resistance to *S. exigua* in plants from herbivory (-) plots (Linear Fit, r^2=0.086, P=0.4808, n=8) or herbivory (+) plots (Linear Fit r^2=0.072, P=0.5195, n=8). Genotype means of growth and genotype means of resistance were used in these measurements. Variation between genotypes in the number of ramets confirmed that the genotypes were variable in growth rates (ANOVA F=4.1253, n=128, P<0.0001 Fig 1.2 B). The growth rates were not significantly different between the two treatments (Student's t-test, t=-1.78, p=0.0963).

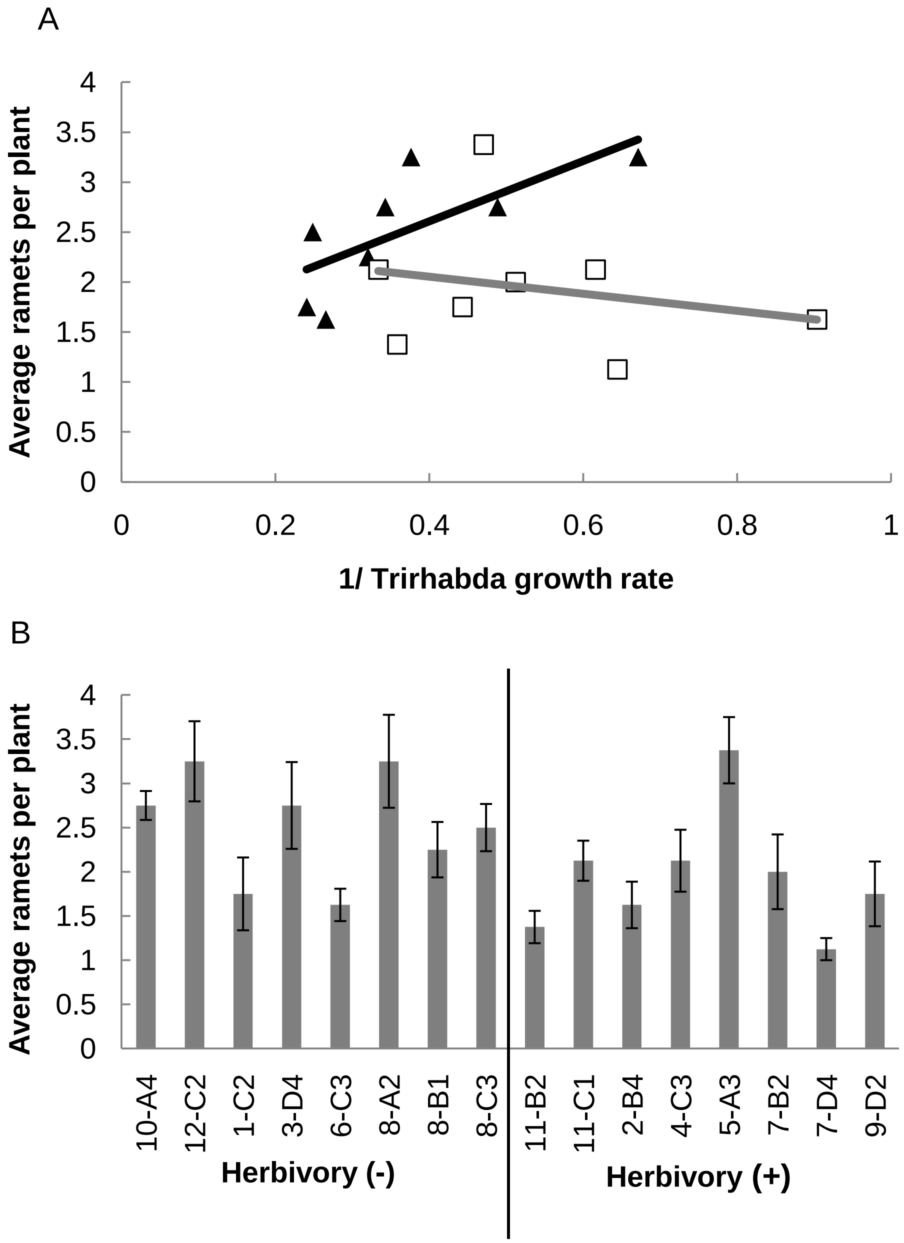

Figure 1.2 A) Relationship between plant growth (average number of ramets per plant) and resistance to *T. virgata* for genotypes from the herbivore exclusion regime (▲ , black line) and the natural herbivory plots (□ , gray line). Only the relationship in the herbivore exclusion regime is statistically significant (*P*<0.05). **B**) Variation in mean (+SEM) ramet production between genotypes propagated from herbivory (-) regime and herbivory (+) regime.

DISCUSSION

Plants from plots with natural herbivory had higher resistance to *T. virgata* than plants from herbivore exclusion plots. This suggested that herbivory is a major agent of natural selection in the *Solidago altissima* system. Although all aboveground herbivores were excluded with the insecticide treatment, it is reasonable to suggest that chrysomelid beetles like *T. virgata* are among the major selecting agents on plant resistance in the herbivore community of *S. altissima* (Carson and Root, 2000). If the removal of herbivory results in reduced mean resistance of the plants in the population it is likely a result of the missing positive selection on resistance as well as a potential negative selection on resistance traits that are costly in the absence of herbivores. Such potential costs of resistance have long been discussed and are a major concept in our understanding of plant-insect interactions (Koricheva, 2002; Koricheva et al., 2004). The resource allocation principle assumes a finite pool of resources that can be allocated into different functions, such as growth, reproduction, and resistance (Karban and Baldwin 1997). Here we indirectly tested for two potential costs of resistance; (A) that the resistance to one herbivore compromises the resistance to another herbivore and (B) that the investment into resistance compromises growth.

Our results clearly indicate that the resistance of one herbivore species does not compromise the resistance to another herbivore in this system. The performance of the generalist lepidopteran *S. exigua* was not different on plants from herbivory (+) and herbivory (-) plots. Moreover, no correlation was detected between the resistance to *T. virgata* and *S. exigua*. This result is remarkable because both herbivore species belong to the same feeding guild and do relatively similar damage. Though survivorship for *S.*

exigua was slightly lower on genotypes from unsprayed plots, suggesting that some resistance traits may be shared, this difference was not significant. Although this result does not entirely exclude the possibility that the resistance to one herbivore may be traded-off with the resistance to another herbivore or pathogen in general, it suggests that different resistance traits and mechanisms may be under selection by different herbivore species even if they are in the same feeding guild. Support for this hypothesis comes from an experiment with a close relative of *S. altissima*, *S. gigantea*. In invasive *Solidago gigantea* populations growing in Europe, plants have lower resistance to *S. exigua* (Hull-Sanders et al., 2007) but not to *T. virgata* compared to plants in America. We hypothesize that the reduced herbivore pressure in *Solidago* populations invasive to Europe resembles a natural experiment, similar to our herbivore exclusion experiment in the native habitat. The mechanisms of resistance for a coleopteran herbivore may be different from those that function against a lepidopteran herbivore (Hull-Sanders et al., 2007; Huang et al., 2010) for example, because the digestive physiology of these two orders of herbivores are not the same (Jongsma and Bolter, 1997). Thus, it is less likely that both herbivores would synergistically select for the same defenses, resulting in correlated resistance.

In addition to a trade-off between resistances between the two herbivores, we hypothesized that a trade-off between growth and defense would be a potential factor driving resistance differences in plants from herbivore exclusion plots and plants from plots with natural herbivory. The resource allocation principle predicts that decreased resistance would correlate with increased growth if plants reallocate resources from growth to herbivore resistance (Karban and Baldwin, 1997), and that, as a consequence, a trade-off of resistance with growth would be evident in plants from herbivore exclusion plots. Whereas a correlation between resistance and growth appeared to be dependent on the selection regime (herbivory (+) vs. herbivory (-)) (Fig. 1.2 A) and was only apparent among plant genotypes from herbivore exclusion plots, we did not find evidence for a trade-off between growth and resistance. Growth and resistance were instead positively correlated among plants from the long-term herbivory exclusion plots.

In our plots with natural herbivory, presumably, the strong selection by herbivores increased the overall resistance of plants to herbivory, but intraspecific competition did not decrease, and was high in all plots. Under conditions of high competition, but no herbivory (e.g. herbivory (-) plots), selection may be for genotypes that can both grow and defend, as would be expected if mechanisms of chemical resistance are linked to other functions, such as allelopathy or protection from abiotic physiological stresses (Siemens et al., 2002; Jones et al., 2006). Alternatively, plant defenses may benefit plants under competitive situations, and poorer competitors may have higher net costs of defenses, as proposed in the defense-stress benefit hypothesis (Siemens et al., 2003). Under conditions of low competition but high herbivory, one would predict a negative correlation between growth and resistance, as genotypes with extreme resistance phenotypes at the cost of growth are favored (Kato et al., 2008). The genotypes with the highest resistance also had the lowest growth rates (Figure 1.1 A), suggesting that some resistance traits may invoke costs in terms of defense. Thus, we could expect that in plots with higher herbivory, we may even see a negative correlation between growth and resistance, as in Kato et al (2008). This may explain why plants from the natural herbivory plots, which had significantly higher resistance, did not have a significantly higher growth rate.

Our study found a distinct positive correlation between growth and resistance, but only under conditions of herbivore exclusion. This finding runs contrary to expectations of the growth-differentiation balance hypothesis, which would predict that plants will either grow or defend. However, this correlation may be dependent on the context in which the plants are growing (Stinchcombe, 2002). It is possible that certain resistance traits have alternate functions in primary metabolism (Seigler and Price, 1976; Arnold and Targett, 2003) or that resistance and competitive ability are positively linked (Siemens et al., 2003; Jones et al., 2006). Probably one of the most important findings in this study is that relationships between different life functions, such as growth and resistance, may only be seen under certain environmental contexts, such as the removal of herbivore pressure (Stinchcombe, 2002). The relative strengths of the two pressures of selection and herbivory may determine the direction of the correlation between growth and resistance.

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CHAPTER 2

Effects of natural selection by variable herbivory along an elevation gradient on plant secondary chemistry and resistance in *Solidago macrophylla*

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ABSTRACT

Plants produce a diverse array of defense traits, some of which are hypothesized to protect plants from abiotic stressors, others of which are hypothesized to function in herbivore resistance. However, many plant chemicals may have multiple functions making it difficult to identify simple mechanisms underlying the evolution of plant secondary chemistry. This problem becomes particularly obvious when plant populations are studied along large spatial gradients, such as those along latitude and elevation. The difficulty in distinguishing between selection by abiotic and biotic factors on plant resistance traits represents a significant obstacle in understanding the evolution of plant-insect interactions. We studied variation in and natural selection on secondary metabolism, as well as variation in herbivore resistance in *S. macrophylla*. We measured herbivore damage and secondary metabolites along an elevation gradient of 1000 meters in the Adirondack Mountains in Northern New York. To measure resistance, we performed herbivore bioassays with plants from different elevations in common gardens. We hypothesized that biotic factors such as herbivory drive natural selection on resistance-mediating secondary metabolite traits more than abiotic factors such as UV radiation in this system, and we investigated the following components of this hypothesis.

1. Does herbivory represent a major environmental factor that varies with elevation?
2. Will plant secondary metabolite production and herbivore resistance vary with elevation?

3. Is there natural selection on plant secondary metabolite production that can be explained by environmental differences in herbivory?

We found decreasing herbivore damage with increasing elevation, a pattern reflected in decreasing secondary metabolite production and herbivore resistance in plants from higher elevations. We also found natural selection on several secondary metabolites that varied along the elevation gradient. We suggest that the variation in secondary metabolism and herbivore resistance is due to decreasing natural selection by herbivory along an elevation gradient.

INTRODUCTION

Plants produce a great diversity of secondary metabolites; many of which are hypothesized to allow plants to cope with abiotic and biotic stresses (Berenbaum, 2001; Alonso et al., 2005). However, many plant chemicals may function in coping with both types of stresses, making it difficult to suggest simple mechanisms underlying the evolution of plant chemotypes. This problem becomes particularly obvious when plant populations are studied along large spatial gradients, such as those along latitude and elevation, and selection by one agent (biotic or abiotic) may have consequences for the plants' interactions with other agents. For example, plants growing at high elevations are frequently faced with harsh abiotic conditions such as extreme temperature variation, short growing seasons, high ultraviolet radiation, and high winds (Johnson and Scriber, 1994). These factors are thought to select for plants with higher production of secondary metabolites that protect leaf tissue from UV radiation. For example, increased UV radiation at high elevations is correlated with increased levels of coumarins in foliar tissue (Stratmann, 2003, Alonso et al., 2005) and increased oxidative stress at high elevations is hypothesized to lead to higher concentrations of phenolic compounds (Close and McArthur, 2002), either by natural selection on phenolic compound production or phenotypic plasticity to growth conditions. Thus, natural selection by abiotic factors at high elevations may result in a higher frequency of phenotypes with high concentrations of secondary metabolites to protect them from abiotic stresses that are more damaging than at lower elevations (Johnson and Scriber, 1994). Additionally, the resource availability hypothesis predicts that slower growing plants are better defended because their tissues are more valuable to overall plant fitness (Coley et al., 1985; Jing and Coley, 1990). Therefore, increased resistance at higher elevations could be advantageous because the decreased plant development speed increases the relative value of each unit of tissue for overall plant fitness.

Many plant secondary metabolites are also involved in herbivore resistance, and thus, the variation in plant secondary metabolism may be due to variation in the selective pressure by herbivores. The same harsh abiotic factors that select for increases in certain secondary metabolites can also limit the ranges of herbivores, which may result in lower herbivore pressure at high elevations (McCoy, 1990; Hodkinson, 2005). Thus, if herbivory is the primary agent of selection on the production of resistance-related secondary metabolites, we would expect plants from higher elevations to be less resistant to herbivory. Generally, the greatest insect species richness and abundance occurs at the lower end of an elevation gradient, which translates into less insect herbivory with increasing elevation (McCoy, 1990; Hodkinson, 2005; (but see also (Hengxiao et al., 1999)), suggesting that their selective impact may also decrease as the level of herbivory decreases. Plants exposed to low levels of herbivory are not expected to invest resources in high levels of constitutive resistance (Karban and Baldwin, 1997). Accordingly, levels of resistance-mediating tannins decrease with increasing elevation in several tree species (Erelli et al., 1998). Other studies have also found that plant palatability increases with increasing elevation (Bruelheide and Scheidel, 1999; Hengxiao et al., 1999; Salmore and Hunter, 2001), purportedly because herbivore pressure decreases with increased elevation (Louda and Rodman, 1983; Thornber et al., 2008). Decreased natural selection by herbivory at high elevations could be driving plant resistance-mediating secondary chemistry in an opposite direction to selection by abiotic factors. However, natural selection on plant chemical resistance traits has rarely been studied (Berenbaum et al., 1986; Strauss et al., 1996; Shonle and Bergelson, 2000; Lankau, 2007; Johnson et al., 2009; Muola et al., 2010) and natural selection along an elevation gradient has not been examined for any species.

We investigated herbivory, chemistry and herbivore resistance in the montane/northern perennial herb *Solidago macrophylla* (Pursh, Asteraceae) to determine the interaction between putative selection by herbivores and resistance traits. In the Adirondack Mountains of New York, USA, *S*. *macrophylla* grows primarily as a sub-canopy plant along an elevation range of approximately 300-1500m. Prior work has shown that herbivory varies on *S. macrophylla* along an elevation gradient, with the highest herbivory occurring at low elevations (Kelly, 1998). We evaluated plants along an elevation gradient and in several common gardens to test the herbivore distribution and the plants' herbivore resistance.

We hypothesized that for this species, herbivores may drive natural selection on resistance traits and their impact would vary with altitude. Accordingly, we predicted that with increasing elevation there would be lower herbivory, lower production of secondary metabolites, and subsequently lower herbivore resistance. If herbivory is the main driver of these patterns, then we also predicted that under conditions of high herbivory, there would be selection to increase the secondary compounds related to plant resistance. If abiotic factors such as UV radiation and temperature variation select on secondary metabolites, we predicted that there would be selection to decrease these compounds at low elevations.

METHODS AND MATERIALS

Study System

Solidago macrophylla is an herbaceous, perennial forb native to northeast North America, from Labrador to the Catskill Mountains of New York (Fernald, 1950). *Solidago macrophylla* forms rosettes, and does not produce a bolting stalk every year. Asexual reproduction occurs via rhizome and re-growth from corms, and plants are typically found in clusters of clones, generally connected belowground via rhizomes, some of which are bolting, others of which are in the rosette stage (personal observation). These aspects of *S. macrophylla* biology facilitated propagation and enabled us to use genetically identical clones in separate common gardens. Previous studies have found aphids (Hemiptera) and seed predators (Diptera) feeding on *S. macrophylla* (Kelly, 1998). We also observed leaf miners (Diptera), stem gallers (Lepidoptera), slugs (Gastropoda), leaf chewing lepidopteran larvae, and mammalian herbivores such as white tailed deer (*Odocoileus virginianus*) and groundhogs (*Marmota monax)*. We studied herbivory and foliar chemistry of plants growing at different elevations along the Dix Mountain trail in the Adirondack Mountains of New York, USA (Table A1.1). *Solidago macrophylla* is native to this environment, and could be readily found growing at elevations from 357 to 1469 m.

Herbivory Measurements

To confirm previously observed patterns of reduced herbivore diversity at higher elevations (Hodkinson; 2005); we surveyed the plants at Dix Mountain for herbivore damage repeatedly. Sites for measurements were chosen by walking the trail and finding plants that could be accessed without straying from the trail. Elevations for each site are listed in Table A1.1. Once every two weeks in 2009 (June 24th to August 17th) and 2010 (June 16th to August 9th) we surveyed herbivore damage for approximately three plants at each of the sites, revisiting the same plants each time (21 sites, 63 plants in 2009; 27 sites, 79 plants in 2010). We added two sites for the final survey and plant collection in 2009 to increase sample sizes for common gardens. In 2009, both rosette and bolting plants were included in surveys, in 2010, only rosette plants were included.

Herbivore damage was categorized by type (chewing, piercing/sucking, leaf mines, or leaf galls). Because one herbivore can damage between 1 to 100% of a leaf, we used a standardized unit of “herbivory” in our analyses to quantify herbivore activity. Our measurement is a summation of all herbivore attacks on a per-leaf basis and includes all types of leaf herbivory (chewing, piercing/sucking (visible as small white dots where cell content had been removed), or mining) under a single measurement. We calculated "herbivory" by adding up each incident of herbivory on a plant and dividing by the total number of leaves on that plant. We considered that larger plants may have attracted more herbivores (Feeny, 1976), and attempted to control for this by dividing by number of leaves. Leaves were scored as having an herbivory level of 1 if they had damage by one type of herbivore. A leaf with damage by two or more types of herbivores was counted as two or more incidents of "herbivory". For all data shown, no leaves had more than 10% of their tissue removed by chewing. Damage by other sources (such as falling debris or human impact) was excluded from analyses. We measured herbivory along the elevation gradient three times in 2009 and five times in 2010 (Table A1.2, A and B). We used a repeated-measure ANOVA in R (R development core team, 2009) for each year's survey to determine the relationship between herbivory and elevation over the season. Time and elevation were independent variables and herbivory was the dependent variable.

Secondary metabolite analysis

To quantify secondary metabolites of plants growing along the elevation gradient, we collected a half leaf (27 to 144 mg fresh weight) from three plants at each Adirondack survey site and froze them on dry ice on July 17th, 2009. To ensure that compound production was not altered due to potentially damage- induced metabolite production in response to herbivory, only undamaged tissue was collected from undamaged or when not possible, very lightly damaged plants (one herbivore attack). We have not seen inducibility in phenolic compounds in *Solidago macrophylla*, and the level of damage on plants did not correlate with increased levels of phenolics (unpublished data). Phenolic compounds were extracted with 80% methanol and were analyzed using an Agilent 1100 HPLC (Santa Clara, CA, USA) on a 4.6 mm by 150 mm Gemini (Phenomenex, Torrance, CA, USA) column as described by Keinanen et al (2001). Each detected compound was assigned to a compound class according to the observed UV absorption spectrum that matched known compounds, and quantified as peak area per mg of leaf tissue. Selected compounds were identified by additional analyses using identical chromatographic conditions on a Quantum Access triple-quadrupole system (ThermoScientific, West Palm Beach, FL, USA) at the Chemical Ecology Core Facility (Cornell University, Ithaca, NY, USA). Retention times, UV spectra, characteristic molecular ions and MS/MS breakdown fragments were compared with published data and commercially available standards. Unknown compounds were also quantified as peak area per mg leaf tissue. Correlations between compound production and elevation were calculated using Linear Regression (JMP 8.0, SAS Institute) and Pearson's coefficient of correlation (STATVIEW, SAS Institute).

Plant collection

To examine the relationship between resistance and elevation in common gardens, corms were collected from one plant at each of the 21 sites surveyed and two additional sites (September 16th, 2009) for 23 plants total. A corm was removed from the soil, wrapped in a moist paper towel, placed in a plastic bag, and transferred back to Ithaca (NY, USA). Corms were transplanted into Metro Mix® soil (Sun-Gro, Bellevue, Washington, USA) and grown in the greenhouse at 21-27° C, 16 hours daylight. Plants were cloned (corm tissue was split off, transplanted into Metro Mix® soil in a new pot and allowed to grow into a new plant) twice after three and six months, so that each genotype was represented once in each of three common garden experiments.

Common Gardens

To examine the resistance of plants to herbivores in natural environments, we used the greenhouse-cloned plants in a series of three common gardens, two at the extremes of the elevation gradient in the Adirondacks and one at a low elevation, high herbivory site in Ithaca, NY. Each plant (80 total) was weighed before transplanting. The low elevation garden was at King Philip Spring (44^{o}4’460” N, 73^{o}39’ W, elevation 357 m) in the Adirondack Mountains. The high elevation garden was on the summit of Dix Mountain (44^{o}4’983” N, 73^{o}47’ W, elevation 1469 m). These two Adirondack gardens had clones from 23 and 22 (due to plant death) of the sites surveyed in 2009, respectively, with one replicate per genotype. Plants were placed in six rough approximations of rows, with rows about 1.5 meters apart, and plants within rows at least 10 cm apart, allowing for intervening rocks and logs.

A third common garden was at Fall Creek Natural Area (42^{o}45’329” N, 76^{o}44’586” W, elevation 304 m) in Ithaca, NY, USA where the plant also naturally grows (Permission received through Cornell Plantations management). Plants in the Fall Creek Natural Area had more herbivore attacks than at either Adirondack garden, and this common garden allowed us to analyze plants under conditions of high herbivory. This common garden had plants in six rows approximately 10 cm apart. Two plants from

19 of the sites surveyed in 2009 were planted with the exception of three sites, for a total of 35 plants.

In each garden, plants were transplanted when naturally growing *S. macrophylla* were of equal size; mid-May for the low elevation and Fall Creek gardens and early June for the high garden. Biweekly surveys of damage were done from planting until late August, with a final survey and plant collection on September 16th 2010 for the Adirondack sites, and on October 2nd, 2010 for the Fall Creek site. Because herbivores can prefer undamaged plants (Wise, 2009), we used the first survey as a metric for resistance for all common gardens. Later surveys were used to confirm that plants were still alive, and to measure herbivory. All plants were weighed after collection, and relative growth was calculated as (starting mass-final mass)/starting mass. All plants in the Fall Creek garden lost mass during the growing season, all mass lost was to herbivory.

Greenhouse Bioassay

To assess how resistance varied in plants from along the elevation gradient, we grew plants in a greenhouse and measured resistance as performance of the generalist herbivore *Spodoptera exigua.* This herbivore has been used before for bioassays on *Solidago* (Hull-Sanders et al., 2007; Tooker et al., 2008), and it is not found on plants along the Adirondack elevation gradient, meaning that it likely lacks a co-evolutionary history with our population. *Spodoptera exigua* eggs and diet were purchased from Benzon Research Inc. (Carlisle, PA, USA) the eggs were hatched in the greenhouse. Larvae fed on diet (Benzon Research Inc., Carlisle, PA, USA) in the greenhouse, and

14 days after egg arrival, when larvae were on average 3.9 mg, two larvae were weighed and transferred to leaves of each of 21 plants (1/site) grown from corms collected along the elevation gradient as described above. Two larvae per plant were restrained to a single leaf (the most recently fully expanded leaf) by a clip cage. They fed for one week after which they were removed and weighed again. Relative growth was calculated as (final mass-initial mass)/initial mass, and the larval masses were averaged for each plant. Relationships between larval growth and plant source elevation were analyzed using Linear Regression with JMP 8.0 (SAS Institute).

We collected leaf tissue (between 42 and 200 mg per plant) from undamaged clones of the plants used for the bioassay. Phenolic compounds were extracted, analyzed, and quantified as for the field-collected tissue. Relationships between larval growth and plant secondary metabolites were analyzed using Linear Regression with JMP 8.0 (SAS Institute).

Natural selection on plant defensive chemistry

To measure natural selection on defensive chemistry in *S. macrophylla*, we collected 37 plants from 11 populations in Tompkins County, NY along with ten greenhouse-grown clones from plants harvested along the elevation gradient in the Adirondacks to set up a common garden at Whipple Farm, Tompkins County, NY, USA (42^{o}25’ W, 76^{o}31’W, elevation 350 m). The greenhouse-grown plants were the same as for other common gardens and were included to increase diversity. The source elevations were not taken into account during analysis. This approach had two advantages; first by collecting plants from multiple populations we potentially increased the phenotypic variation with which to detect selection and second using a common garden should have reduced biases due to environmental correlates with the traits and fitness (Rausher, 1992). Prior to transplanting, one leaf was removed from each plant for secondary metabolite analysis as above. Additionally, the roots were washed, leaves and ramets counted, and the whole plant weighed before planting. If any leaves were damaged, they were scored as above. To facilitate later collection of belowground biomass, plants were placed in 10 cm pots with Metro Mix® soil (Sun-Gro, Bellevue, Washington, USA) and sunk into the ground at Whipple Farm on May 5th, 2010 (42^{o}25’ W, 76^{o}31’W, elevation 350 m). Plants were organized into rows of 4 plants each, 10 cm apart, in a forest. On October 19th, all plants were harvested, washed, and reweighed. Because no plants flowered during the course of our experiment, we used mass gain (starting mass-final mass) as a fitness proxy (plants which were dead at collection were assigned a final mass of zero), since higher mass gain is hypothesized to lead to more growth, and potentially higher sexual reproduction in the next season (Cain, 1990). Plant loss and missing data led to a final sample of 42 plants.

To measure natural selection on chemical traits, we first ran univariate selection differential models for each of 18 compounds identified from leaf tissue collected along the elevation gradient. Two compounds found in the Adirondacks were not detectable in this common garden and therefore could not be included in the analyses. Selection differentials measure total selection on a trait and were regressions of relative fitness (relative mass gain/population mean) on each standardized trait (mean of zero, standard deviation of one). Unfortunately, we did not have the power to also use a multivariate regression approach to determine which chemical traits were the targets of selection (Lande and Arnold, 1983). However, we did measure selection gradients (β) by including both the final number of leaves and rhizomes in multivariate regression models with each compound to account for plant vigor. By accounting for plant vigor, we were able to determine whether selection on the chemical traits was being driven by correlations with vigor, which could improve our power to detect selection (Parachnowitsch and Kessler, 2010). We also measured quadratic selection (stabilizing or disruptive selection) by running univariate regression models that included a term for each the compound and a term for the compound*compound regressed on relative fitness. We found significant disruptive selection on one compound (retention time 14.699; 0.585 ± 0.247, $P = 0.02$). However, given the number of tests of quadratic selection (18), we question the significance of this finding and do not present the rest of the values. All selection analyses were done with SAS (Cary Institute, version 9.2).

RESULTS

Herbivory and secondary metabolites along the elevation gradient

We found that herbivory decreased with increased elevation in *S. macrophylla*. A repeated measures ANOVA showed variation in the level of herbivory with elevations in both 2009 (F=2.5002, p=0.0061) and 2010 (F=2.6071, p=0.0005). We found a negative correlation between herbivory and elevation over both growing seasons (Figure 2.1). We present a single survey for each year when bolting plants began to flower, and herbivory was at its peak as representatives of the general pattern. During both of these surveys, there was a significant correlation between herbivory and elevation (2009, Figure 2.1 A; r^2=0.09, p=0.0067 and 2010 Figure 2.1 B; r^2=0.05, p=0.0351). Data for other surveys is presented in Supplementary Table 2.2. Damage by chewing herbivores such as microlepidopteran larvae, geometrid moth larvae, and mollusks were the most frequent damage type observed along the elevation gradient. We saw no differences in growth rates for plants growing at different elevations.

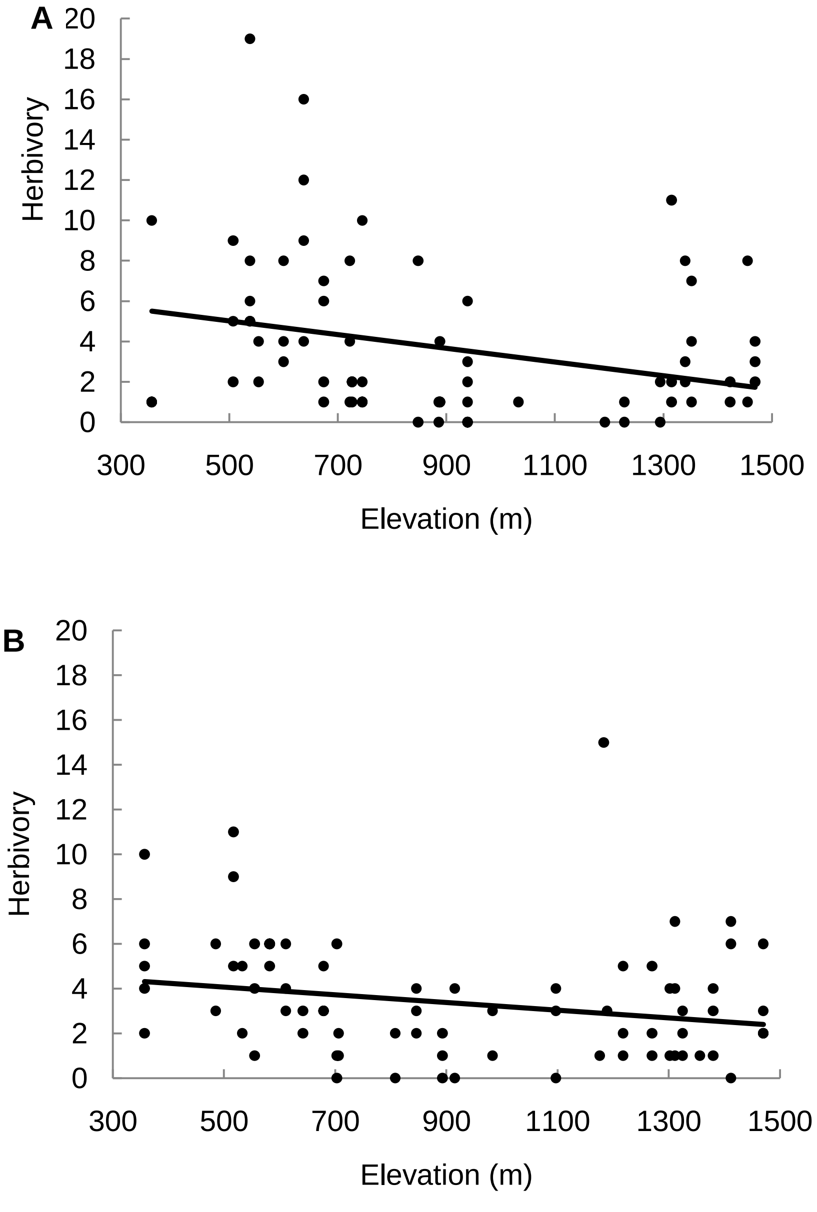
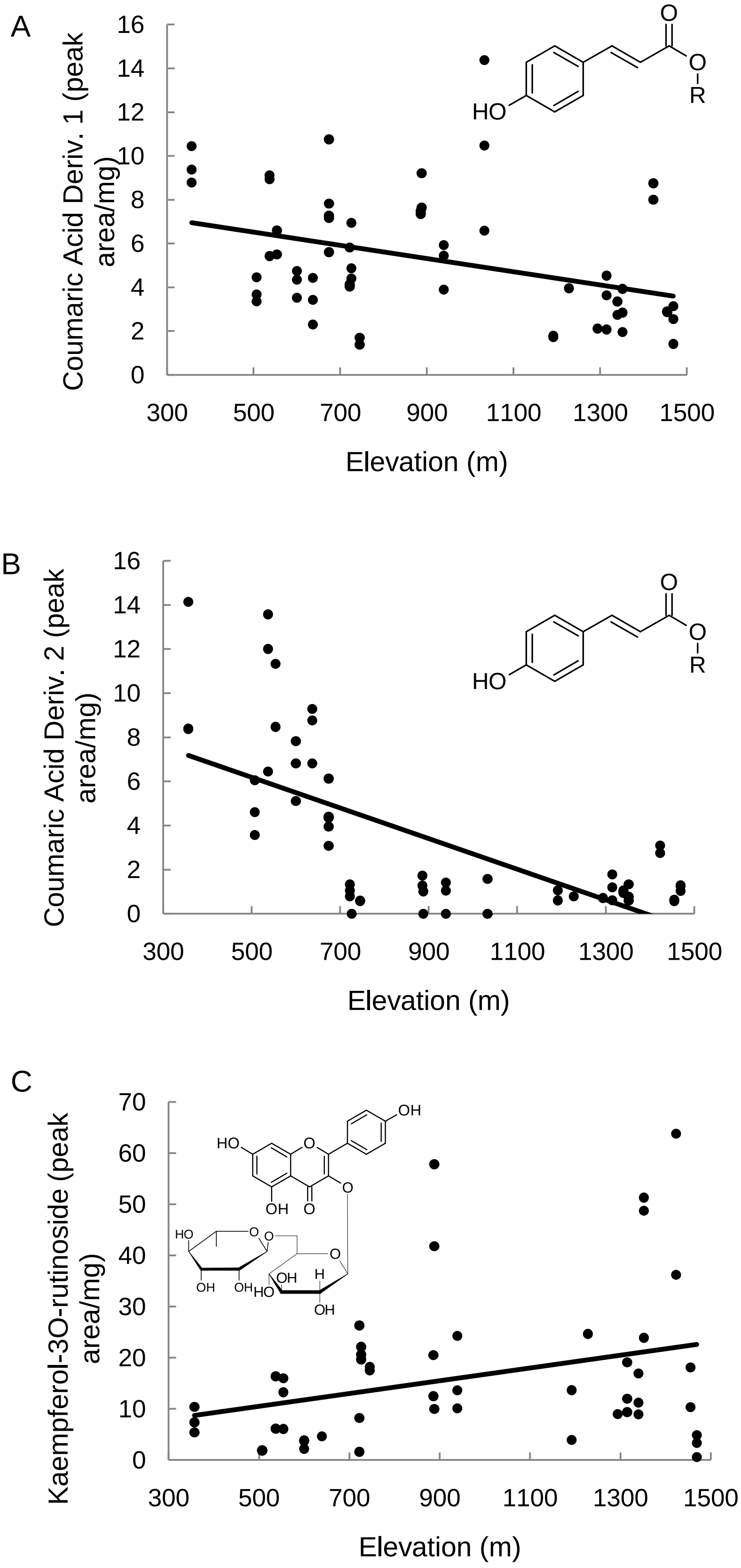


Figure 2.1: Herbivory on *Solidago macrophylla* decreased with increasing elevation in both (**A**) 2009 and (**B**) 2010. Each point represents one plant measured at the peak of herbivory (mid-July).

Plants growing at high elevations have lower levels of secondary metabolites when compared to those growing at a lower elevation. We indentified 21 plant phenolics in the leaf tissue collected in the Adirondacks. Most of these metabolites were caffeic acid derivatives (9 compounds), coumaric acid derivatives (4 compounds), or flavonoids (3 compounds), with 4 unidentified compounds. Fourteen of the peaks varied significantly with elevation (including one marginally significant), and 12 decreased with increasing elevation. The pattern for coumaric acid derivative 1 (r^2=0.14, p=0.0032, Figure 2.2 A) and 2 (r^2=0.42, p<0.0001, Figure 2.2 B) which decreased in production with increasing elevation and kaempferol-3O-rutinoside (r^2=0.09, p=0.0253, Figure 2.2 C) which increased with increasing elevation are shown because we also found them to be under natural selection in a common garden (see below). The two largest peaks, a caffeic acid derivative and a coumaric acid derivative, contributing together, on average, 44% of total peak areas, also both decreased significantly with increasing elevation (r^2=0.12, p=0.0077, and r^2=0.26, p<0.0001, respectively).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Elevation (m)
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B
Coumaric Acid Deriv. 2 (peak area/mg)
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Figure 2.2: *Solidago macrophylla* secondary metabolite concentrations vary with elevation. (**A**) Correlations between elevation and Coumaric Acid Derivative 1 and (**B**) Coumaric Acid Derivative 2. (**C**) Kampferol-3O-rutinoside shows increasing concentration with increasing elevation. Each point represents a single plant along the gradient. Proposed chemical structures are superimposed on graphs, R has tentatively been identified as quinic acid.

Resistance in common gardens

In the Adirondacks, herbivory differed between the low and high common gardens with higher damage levels in the lower garden than in the higher garden for each genotype (Wilcoxon Signed Rank T=-125.5, $p<0.0001$). Tissue loss was not significantly different between plants in the low or high elevation Adirondack common gardens; however, our ability to detect significant differences in damage may have been hampered by the relatively low variability in mass loss. In the low elevation garden, plants were subjected to heavy herbivory, with 0.62±0.05 herbivore attacks per leaf in the first survey. Several unidentified slugs and one unidentified lepidopteran larva were directly observed feeding at the low common garden. Furthermore, genotypes from lower elevations received less herbivory than those from high elevations in the early season ($r^2=0.37$, $p=0.0023$, Figure 2.3 A) and this trend generally persisted through the season (data not shown). In the high garden, there was low damage (mean incidents per leaf) to all plants. Slugs were observed feeding on plants at the high garden; however, damage by slugs and lepidopteran larvae is indistinguishable, thus we cannot rule out the presence of lepidopteran larvae at the high garden. Unlike the low elevation garden, damage occurred roughly evenly across all plants, regardless of source elevation (r^2=0.057, p=0.2835, Figure 2.3 B).

In the Fall Creek common garden, there was high herbivory, with 0.66±0.08 herbivore attacks per leaf on average, and all plants receiving damage by the first survey. As in the low elevation Adirondack garden, the level of herbivory correlated with source elevation (r^2=0.55, p<0.0001, Figure 2.3 C). Herbivory at this garden was primarily by slugs, although there were 15 incidents of groundhogs digging up plants to feed on roots and rhizomes, resulting in six plant deaths. Early herbivore resistance correlated positively with final mass lost for the Fall Creek common garden (r^2=0.398, p=0.0050, Figure A1.1). In the Fall Creek common garden, plants from lower elevations lost significantly less mass to herbivory than plants from high elevations (r^2=0.247, p=0.0302, Figure 2.3 D), with 24 plants experiencing complete loss of above and belowground biomass (mortality). Growth rates of all plants were similar, plants from high elevation sites did not grow any faster or slower than plants from low elevation sites. This pattern was true for all common gardens.

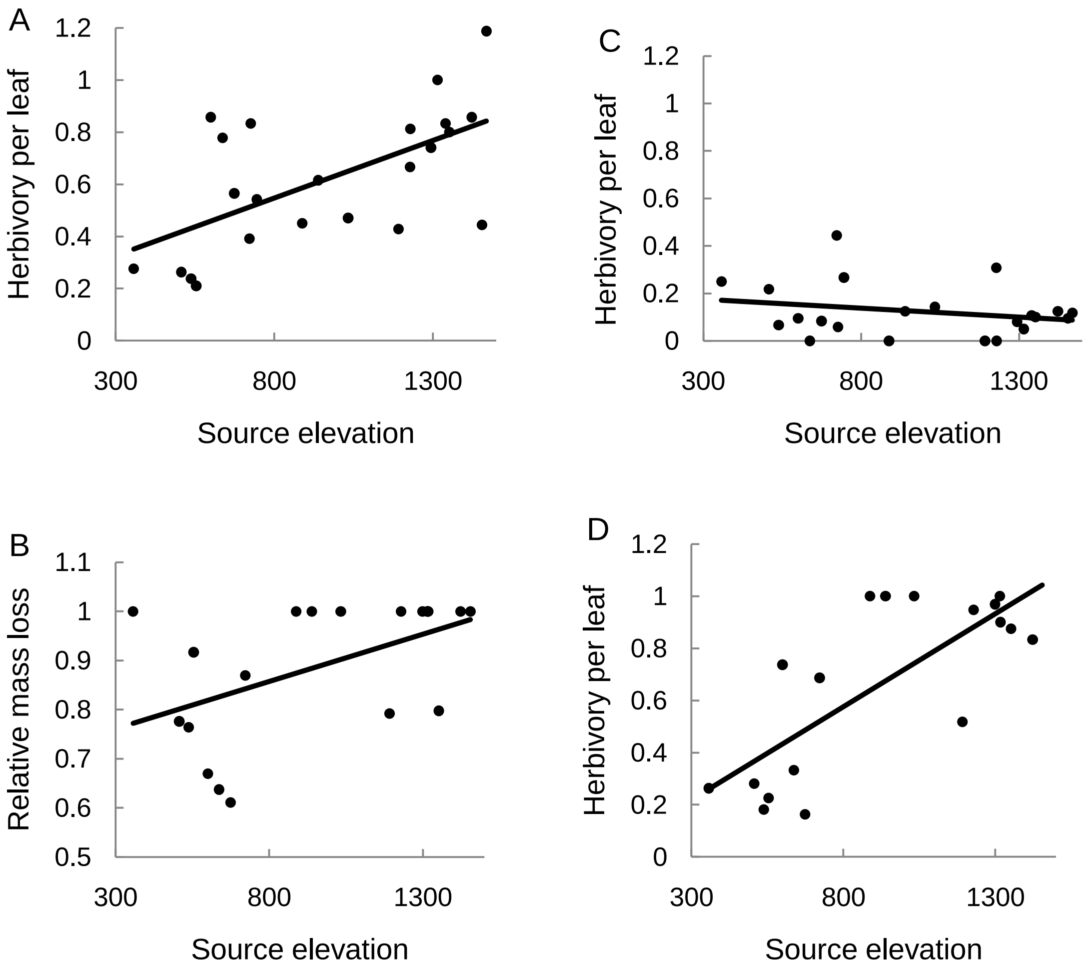

Figure 2.3: Herbivore attacks on *S. macrophylla* in common gardens were more frequent on plants from high elevations. Correlation between source elevation and herbivore attacks per leaf in the (**A**) low, (**B**) high and (**C**) Fall Creek common gardens. (**D**) Correlation between mass lost to herbivory and source elevation.

Plant resistance to a generalist herbivore

In *S. macrophylla*, resistance to herbivory (*S. exigua* larval mass gain) varied with the source elevation from which the genotypes were collected. Larval mass gain correlated positively with elevation (r^2=0.46, p=0.0014, Figure 2.4). Plants from the highest elevations had the lowest resistance to this generalist herbivore and plants from the lower elevations had the highest resistance. We compared production of several secondary metabolites in plants growing in the greenhouse with *Spodoptera* growth, and found negative correlations between larval growth and production of one of the two coumaric acid derivatives (Coumaric Acid Derivative 1, r^2=0.42, p=0.0089, Figure A1.2 A), but no correlation with kaempferol-3O-rutinoside (r^2=0.03, p=0.5495, Figure A1.2 B). We did not see production of Coumaric Acid Derivative 2 in plants growing in the greenhouse.

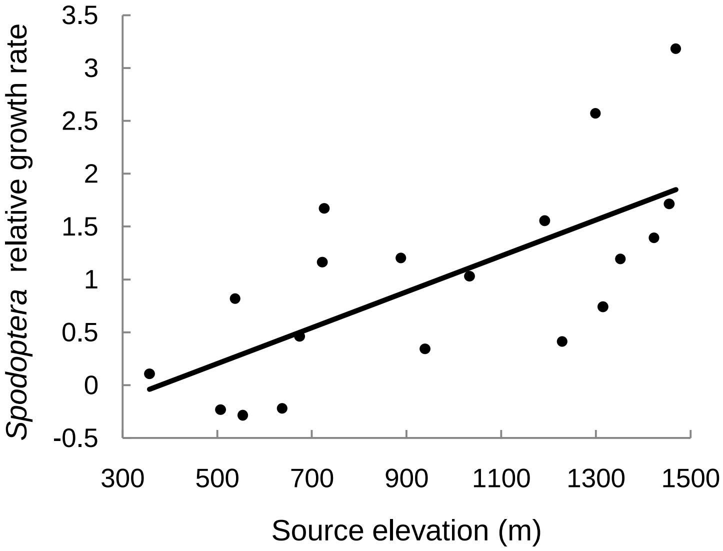

Figure 2.4: Relationship between plant genotype source elevation and *Spodoptera exigua* relative larval growth rate.

Natural selection on secondary metabolites

We found significant phenotypic selection on 8 of the 18 compounds produced by *S. macrophylla* in a common garden, meaning that plants with higher (or lower) levels of these compounds had higher levels of growth. There was natural selection to increase four of the compounds and to decrease four others (see Table 2.1). However, when we controlled for plant vigor by including the final number of leaves and rhizomes in the model, only selection on three compounds (retention times 16.688 min. (coumaric acid derivative 1), 17.229 min. (coumaric acid derivative 2) and 17.624 min. (kaempferol-3O-rutinoside)) remained significant. We found selection to increase the two coumaric acid derivatives (1 and 2) and to decrease the flavonoid. Interestingly, these coumaric acid derivatives decreased with elevation in the Adirondack survey while the flavonoid increased with elevation (Table 2.1).

Retention Time	Compound class	S	β	*R*
11.253	Caffeic Acid Deriv.	**-0.697±0.297**	-0.189±0.293	**-0.226**
13.389	Caffeic Acid Deriv	**-0.616±0.302**	-0.301±0.296	**-0.341**
14.966	Caffeic Acid Deriv.	**0.607±0.303**	0.398±0.341	**-0.508**
15.325	Caffeic Acid Deriv.	**0.542±0.306**	0.511±0.261	**-0.486**
16.688	Coumaric Acid Deriv.	**0.710±0.297**	**0.612±0.294**	**-0.374**
17.229	Coumaric Acid Deriv.	**0.872±0.286**	**0.707±0.291**	**-0.656**
17.624	Flavonoid	**-0.893±0.294**	**-0.644±0.303**	**0.118**
18.699	Flavonoid	**-0.947±0.280**	-0.517±0.293	0.077

Table 2.1: Retention times, compound classes, natural selection differentials (S), gradients (β) and Pearson correlations with elevation (R^2) for the 8 compounds of *Solidago macrophylla* with significant selection differentials in a low elevation/high herbivory common garden. Significant differentials, gradients, and correlations ($P < 0.05$) are in **bold**. The correlation between compounds and elevation is from surveys in the Adirondacks, NY, USA in 2009.

DISCUSSION

We found support for the hypothesis that natural selection by herbivory on plant secondary chemistry leads to a negative correlation between resistance and elevation in the montane/northern herb *S. macrophylla*. Herbivory and herbivore resistance decrease with increasing elevation. The production of most secondary metabolites also decreased, although we saw production of a flavonoid increase. In common gardens with high herbivory, plants from high elevations were preferred by herbivores. We also saw natural selection on plant secondary metabolites in a common garden experiment. The similarities between the pattern of natural selection in a common garden and the variation in secondary metabolism with elevation strongly support the hypothesis that natural selection by herbivores drives the pattern of production for most secondary metabolites. We hypothesize that UV radiation may drive the pattern of production for others that increased with increasing elevation.

Consistent with patterns of decreasing resistance with increasing elevation, we found decreasing levels of 12 out of 21 phenolic compounds detected in plants growing along the elevation gradient. However, we saw production of a flavonoid increasing with increasing elevation, and hypothesized that there may be different functions for the metabolites that decrease and those that increase. For compounds that decreased with increasing elevation, the patterns of resistance and phenolic production were more likely to be due to natural selection by herbivores than by natural selection by abiotic factors in this system. Phenolics have been previously demonstrated to have anti-herbivore functions (Bennett and Wallsgrove, 1994). Further support for this was found in a correlation between production of one of these compounds and herbivore resistance (Figure A1.2). If the pattern seen for these traits was due to changes in abiotic environmental factors, and herbivores were not limited to low elevations, we would have expected a similar pattern of herbivory as was seen in Hengxiao et al (1999), where less-resistant plants at high elevations received more herbivore attack in the field. The pattern seen here is similar to other studies that have shown variation in defense chemistry with elevation (Erelli et al., 1998; Salmore and Hunter, 2001). Kaempferol-3O-rutinoside, for which production increased with increasing elevation, has been indicated as protecting against UV-B irradiation (Chappell and Hahlbrock, 1984; Buchanan et al., 2000). Ultraviolet radiation is known to increase with increasing elevation, and such a function would explain why this compound had increased production with increased elevation.

In the common gardens, plant origin had no influence on final plant size, suggesting that plants were not more stressed if they were further removed from their source elevation. This suggests that the resource allocation in plants from different elevations did not differ significantly (Herms and Mattson, 1992). The slow-growth/high defense hypothesis (Coley et al., 1985) would not serve as the best framework for our results, since plants from high elevations did not grow slower in common gardens. Herbivory levels differed with elevation between the common gardens in the Adirondack Mountains (McCoy, 1990; Hodkinson, 2005). In the lower garden, plants from high elevations were less resistant to herbivory than plants from low elevations. However, in the high garden damage was distributed evenly, suggesting herbivores did not choose between plants based on their resistance at this site. If herbivores selected on plants in the high garden in the same manner as in the low garden, we would expect similar secondary metabolite production, even if the level of selection was lower. The lack of a similar pattern in damage between the gardens suggests that pressures of natural selection are not the same at both sites.

We found natural selection to increase two compounds and to decrease one compound. We hypothesized that the natural selection to increase defense-related metabolites in this system was due to herbivory, as has been seen in other experiments (Mauricio and Rausher, 1997; Latta and Linhart, 1997; Shonle and Bergelson, 2000; Johnson et al., 2009). It is possible that the production of kaempferol-3O-rutinoside comes at a cost in terms of production of other secondary metabolites when UV-B radiation is low and herbivory is high, as in our common garden, and this may be why we saw selection to decrease production of this compound.

However, herbivore levels may vary from year to year and measurements of natural selection by herbivores may vary in strength or direction, depending on the herbivore community (Strauss and Irwin, 2004). By taking a measurement of how secondary metabolite production varied along a gradient of herbivory, we were able to suggest that even with variation in herbivore pressure from year to year; the overall pattern of natural selection was for reduced production of most secondary metabolites with increasing elevation.

Even though our natural selection experiment was small, the sample size was sufficient to detect significant natural selection on putative defensive compounds in *S. macrophylla*. The pattern of selection was consistent with the hypothesis that herbivores are agents of selection. Under this hypothesis, we expected to see natural selection for compounds that decreased with elevation in our surveys, since selection by herbivores would also decrease with elevation. Compounds for which we saw positive selection in the common garden showed negative correlations with increasing elevation in 2009 field measurements along the elevation gradient. Kaempferol-3O-rutinoside showed a positive correlation with increasing elevation in plants collected from the field. Since the patterns of natural selection in our common garden experiment matched patterns of production in the field, we believe that natural selection by herbivory acts on the defense-related metabolites that decrease in production along an elevation gradient. We hypothesize that the variability in herbivory along the elevation gradient resulted in variability in herbivore resistance and the production of defense-related metabolites.

SUMMARY

In this study, we found that herbivory decreases along an elevation gradient of 1000 m, confirming in our system prior findings that herbivore damage decreases with increasing elevation (Louda and Rodman, 1983; McCoy, 1990; Kelly, 1998; Bruelheide and Scheidel, 1999; Hodkinson, 2005; Thornber et al., 2008). We hypothesized that the natural selection by herbivory on herbivore resistance and defense metabolism in plants would be weaker at the high elevations. Herbivore resistance and defense-related metabolite production decreased with increasing elevation at our site, as has been seen in previous studies (Louda and Rodman, 1983; Erelli et al., 1998; Hengxiao et al., 1999). In a common garden, we found that there was selection on defense-related metabolites, and this selection followed the pattern of production in the field, with selection to increase metabolites that decreased with elevation, and selection to decrease a metabolite that increased with elevation. We conclude that natural selection by herbivory, and variations in the herbivore distribution results in the variability that we saw in resistance and defense-related metabolite production along our elevation gradient.

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CHAPTER 3

The induction of two types of protease inhibitors by a Lepidopteran and a Coleopteran Herbivore in *Solidago altissima* (L)

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ABSTRACT

Plant protease inhibitors are some of the best known resistance traits plants use against their herbivores. There are different types of plant protease inhibitors, with putatively specific functions against the different types of proteases of insect guts. Serine protease inhibitors (SPIs) and cysteine protease inhibitors (CPIs) are hypothesized to function against lepidopteran and coleopteran herbivores, respectively. Here we investigate whether *Solidago altissima* can specifically induce these protease inhibitors in response to damage by a lepidopteran or a coleopteran herbivore. We also investigate whether the pattern of protease inhibitor induction correlates with plant resistance to *Spodoptera exigua*, a generalist lepidopteran herbivore. We found that both SPIs and CPIs are induced in response to damage, regardless of the herbivore doing the damage. However, we hypothesized that only SPIs were effective against *S. exigua*. We found that SPI concentration negatively correlated with *S. exigua* performance, and lower *S. exigua* gut protease activity. These patterns were not seen with CPIs, leading us to conclude that although both PIs were induced by *S. exigua* herbivory, only one functions against *S. exigua*. We present a rapid and effective method for analyzing both SPIs and CPIs from a single leaf sample, and use this method with insect gut proteases to demonstrate that plant protein extract can inhibit insect gut proteases.

INTRODUCTION

Plants have evolved a diverse array of defense-related metabolites, including a multitude of toxic secondary metabolites and anti-digestive proteins that inhibit an herbivore’s ability to digest plant material after ingestion. These defenses are expressed constitutively in plant tissue, and many can be induced to a higher level when plant tissue is damaged (Karban and Baldwin, 1997). A well characterized group of defensive proteins are protease inhibitors (PIs). Protease inhibitors act as competitive inhibitors of herbivore gut proteases (Green and Ryan, 1972), and can represent up to 12% of total leaf protein, the high concentration hypothetically due to their role as defenses, rather than as regulators of intracellular processes (Gustafson and Ryan, 1976; Jongsma and Bolter 1997). Insect herbivores feeding on plants with high PI levels have reduced growth and survival (Broadway and Duffey, 1986; Bolter and LatoszekGreen, 1997; Hilder et al., 1987; Zavala et al., 2004a) when compared to those feeding on plants with low PI levels. Reduced herbivore growth was also observed when PI-containing diet was fed to *Helicoverpa armigera* larvae (Bhavani *et al.*, 2007). Moreover, (Glawe *et al.*, 2003) showed that herbivores prefer plant genotypes with lower PI concentrations over genotypes with higher PI concentrations. Genetic manipulation of plant protease inhibitor production decreases herbivore performance (Goulet et al., 2008) and the damage done to the plant (Hilder et al., 1987; Zavala et al., 2004a), implicating PIs as effective in plant resistance to insect herbivores (Broadway, 1997).

Protease inhibitors are effective resistance traits, but a single type of PI cannot serve as a defense against all insect herbivores, since insect gut proteases are not universally conserved, and some types of gut proteases are unaffected by some types of PIs. Different taxonomic clades of arthropods have different protease types that function optimally in guts with different pH (Wolfson and Murdock, 1987; Jongsma and Bolter, 1997). While gut pH and protease types differ between arthropod orders, they seem to be relatively well-conserved among species within each order; at least in the two orders with the most herbivores, Lepidoptera and Coleoptera (Clark, 1999). Most coleopteran herbivores tested thus far have an acidic midgut and primarily cysteine or aspartate proteases, while lepidopteran herbivores have a basic midgut and primarily serine proteases (Wolfson and Murdock, 1987; Gruden et al., 1998). The taxon specificity of insect gut proteases is contrasted by the diversity of different PIs produced in plant tissue (Garcia-Olmedo et al., 1987; Strukelj et al., 1992). The presence of both cysteine and serine PIs in the same plant could increase plant resistance to different types of herbivores. Because the production of PIs is costly (Zavala et al., 2004b) plants that can regulate the expression of PIs in a manner specific to the amount of damage and to the attacking herbivore should be able to regulate their use of resources more effectively.

Plants are known to induce some defenses specifically to the herbivore attacking them (Stout et al., 1998; Agrawal and Sherriffs, 2001; Van Zandt and Agrawal, 2004; Voelckel and Baldwin, 2004; Tooker et al., 2008). Specifically responding plants may become less attractive to later herbivores of the same species that first damaged the plant, or other herbivores in the community (Viswanathan et al., 2005; Poelman, 2008). However, it is also possible that plants, once induced by one herbivore species, become less able to induce resistance to another, later arriving herbivore species. Thus, a specific induction to an early herbivore may result in an inability to induce defenses to a later herbivore (Viswanathan et al., 2005). From a more mechanistic point of view, it is also possible that plants may not be physiologically able to specifically induce their PIs, because the induction of different types of PIs may be regulated through the same endogenous plant wound signaling pathways (Farmer and Ryan, 1990; Bolter, 1993;

Koiwa et al., 1997). Both SPI and CPI have been hypothesized to be induced by jasmonic acid (JA), but not all compounds that are inducible by JA are co-induced (Dammann et al., 1997). Thus, PIs may be regulated independently, depending on the type of damage (Sanchez-Hernandez et al., 2004; Turra et al., 2009), or may be co-induced with damage. This is because a damage signal is more than just induction of JA, but is modulated by a host of other compounds (Bowles, 1998; Leon et al., 2001), including herbivore-specific chemical elicitors (Kessler and Baldwin 2002), allowing a plant to temper its response to an attacker.

Here we test the hypothesis that plants can specifically induce PI production with the ecological model system *Solidago altissima* (tall goldenrod) and the herbivores *Spodoptera exigua* (Lepidoptera, Noctuidae) and *Trirhabda virgata* (Coleoptera, Chrysomelidae). We predict that if plants induced their PIs specifically, we would see serine protease inhibitors (SPIs) induced in response to attack by *S. exigua* but not to *T. virgata* and that we would see the opposite pattern of induction for cysteine protease inhibitors (CPIs). However, if there was no specificity of induction, which may happen if both SPIs and CPIs are regulated by the same mechanism, we expected to see induction of both CPIs and SPIs in a damage-dependent manner, regardless of what herbivore was doing the damage. Last, we expected SPIs to reduce *S. exigua* gut protease activity and therefore to see resistance to *S. exigua* correlate with the pattern of SPI induction, but not with CPI induction, and SPIs to reduce *S. exigua* gut protease activity.

MATERIALS AND METHODS

Plant and insect material

Solidago altissima is a common perennial forb found in abandoned agricultural fields in eastern North America. The clonal, rhizomatous growth of *Solidago* allows for perpetual maintenance of established lines and rapid propagation of genetically identical plants. For this experiment, 384 individual *S. altissima* plants, consisting of 24 clones each from 16 genotypes collected from a local natural population (Whipple Farm, in Ithaca, New York, U.S.A. (42^{o}25’ W, 76^{o}31’W)), were grown outside under ambient light and temperature from late April to October, which is the natural growing season in upstate New York. All plants were grown in 15cm azalea pots with Metro Mix® (Sun-Gro, Bellevue, Washington, USA) soil. Plants were watered as needed and fertilized weekly with Jacks 21-5-20 N-P-K fertilizer (Comfort House, Newark, NJ, USA) at 150 ppm N. We controlled for genotype by collecting a single rhizome in 2008 from 16 distinct plants and cloning it repeatedly through three growth cycles (one cycle means grown until seed set, rhizomes harvested and made into new plants). *Spodoptera exigua* larvae were purchased from BioServ (Product #E9219, Frenchtown, NJ, USA), and grown on BioServ Lepidoptera medium (Product #F9772) for six days and acclimated on *S. altissima* leaf tissue for 24 hours. *Trirhabda virgata* larvae were collected from local populations found feeding on *S. altissima*.

Treatments

Eight weeks after planting, plants were divided into four treatments, with 6 plants from each genotype in each treatment. Undamaged plants received no herbivores. Plants in the *Spodoptera* treatment received two *S. exigua* larvae (second out of five instars), Plants in the *Trirhabda* treatment received two instar *T. virgata* larvae (second instar out of three instars). And plants in the *Spodoptera* + *Trirhabda* treatment received one larva of each species. All plants were enclosed in mesh bags with the larvae. After seven days, all larvae were removed, total leaves and total damaged leaves were counted, and one damaged leaf (or ontologically similar leaf from Undamaged plants) was collected, weighed, flash-frozen in liquid N_2 and kept at -80^o C until extraction. For two genotypes, less than 24 plants survived, these genotypes did not receive the *Spodoptera* + *Trirhabda* treatment. For analyses using genotype means of treatments, there was an N of 62 (14 genotypes X 4 treatments + 2 genotypes X 3 treatments).

Herbivore resistance bioassay

We used *S.exigua* relative growth rate as a standardized measurement of herbivore resistance. Two *S. exigua* (second instar, grown on diet and acclimated on *S. altissima* leaf tissue for 24 hours) were weighed and added to each of 3 plants from each treatment (for a total of 12 plants for each of the 16 genotypes; 192 plants total). The mesh bags were replaced, and the larvae were allowed to feed for seven days before being removed and weighed again. Genotype means of relative mass gain ((final mass-start mass)/start mass) was used as a measure of herbivore resistance. Herbivore resistance was compared between treatments using a Tukey/Kramer post-hoc test (STATVIEW, SAS Institute). Relationships between larval growth and PI concentration/activity were analyzed using Pearson's coefficients (STATVIEW, SAS Institute) and linear regression (JMP 7.0, SAS Institute).

Protein extraction

We added 900 mg zirconium beads (2.3 mm, BioSpec) and 1 mL of extraction buffer to each leaf tissue sample and used a Fastprep® machine to homogenize sample and buffer. The extraction buffer for all samples was a 25 mM sodium phosphate buffer (pH 7.2) containing 150 mM sodium chloride, 2.0 mM EDTA, with 2 mg/mL phenylthiourea, 5 mg/mL diethyldithiocarbamate and 50 mg/mL polyvinylpolypyrrolidone (PVPP). After centrifugation (30 min, 14000 rpm), 500-700 *μL* of supernatant was transferred to a 1.5 ml Eppendorff® sample vial. Samples were kept at -80^{o} C until further analysis.

Protein quantification

For protein quantification we used the Bradford assay (Bradford, 1976) modified for microplate as follows: sample extracts were diluted 1:10 in 0.1 M TRIS, which gave a protein concentration between 0.5 and 0.033 mg/mL. Ten µL of each diluted sample were combined with 200 µL of Bradford reagent (Sigma, St. Louis, MO, USA). The samples were incubated at 25^{o}C for 10 minutes, and absorbance at 595 nm was measured using a Synergy HT multi-detection microplate reader (Bio-Tek). Standards of immunoglobulin (Sigma, St. Louis, MO, USA) were prepared at 0.5, 0.25, 0.125, 0.068 and 0.033 mg/mL in 0.1 M TRIS buffer for quantification.

Serine protease inhibitor quantification

The method for measuring Serine Protease Inhibitor (SPI) concentration was modified from the cysteine protease inhibitor activity measurements in (Zhao *et al.*, 1996) for use in a microplate assay. SPI activity was measured by combining 20µL of reaction buffer (0.1 M TRIS) 10 µL of 0.25 mg/mL trypsin in 0.1 M TRIS, and 20 µL sample, shaking gently to mix, and incubating at 37.5^{o}C for 5 minutes. Then 20 µL of 3.1 mg/mL *N*-benzoyl-DL-arginine-b-naphtylamide in DMSO was added to each sample on the microplate, and incubated for another 20 minutes at 37.5^{o}C. The reaction was terminated by adding 100µL of 2% HCl in ethanol (1 mL 12M HCl in 49 mL 200 proof EtOH) and the absorbance at 540 nm was measured, to control for the background of each sample, using the Synergy HT microplate reader. Last, 100 µL of 0.06% *p*-dimethylaminocinnamaldehyde in ethanol was added to develop the sample. The dye reaction was allowed to proceed for 30 minutes at room temperature before the total absorbance was measured at 540 nm. A positive reaction control (with trypsin, no sample), standards (Soybean trypsin inhibitors (Sigma, St. Louis, MO, USA) of six concentrations (0.24, 0.12, 0.06, 0.03, 0.015 and 0.0075 mg/mL in 0.1 M TRIS), and a negative reaction control (including no trypsin and no sample) were run on the same microplate, and SPI concentration was determined using a standard curve, which was calculated from the standard solutions and the reaction controls. The SPI concentration was expressed as mg SPI per mg protein. We analyzed the relationship between SPI concentration and larval performance with linear regression (JMP 7.0, SAS Institute). We analyzed differences between the treatments using a Tukey/Kramer post-hoc test (STATVIEW, SAS Institute).

Cysteine protease inhibitor quantification

CPI activity was determined using a microplate method similar to the method for SPI quantification. For this assay, we used 0.25 M sodium phosphate buffer (pH 6.0) with 2.5 mM EDTA instead of TRIS as the reaction buffer and papain (1 mg/mL papain in a 1:1 mixture of sodium phosphate buffer and distilled water, diluted to 250 µg/mL papain in sodium phosphate buffer) instead of trypsin in 0.1 M TRIS as the enzyme. Cysteine protease inhibitor activity was measured as a percentage of the positive reaction control inhibited (100-(absorbance in sample/absorbance in positive reaction control)*100) per mg protein after subtracting absorbance of the negative reaction control and of the background per sample from total absorbance of each sample rather than as a total concentration as for SPI. We analyzed the relationship between CPI concentration and larval performance with linear regression (JMP 7.0, SAS Institute), using each plant with surviving *S. exigua* larvae as a sample (N=129 for SPI, 138 for CPI). We analyzed differences between the treatments using a Tukey/Kramer post-hoc test (STATVIEW, SAS Institute).

Insect gut protease assay

We grew separate *S. exigua* larvae on BioServ Lepidoptera diet for gut protease collection, when these *S. exigua* larvae reached the 4th instar, they were chilled on ice. The midguts were removed, homogenized and centrifuged at 14000 rpm for 4 minutes. The supernatant was removed and used in a 1:10 dilution with 0.1 M TRIS in place of trypsin for SPI microplate assays as described above. We used the midgut content in place of trypsin to analyze the inhibition of gut content directly instead of measuring trypsin inhibition as a proxy for *S. exigua* gut protease inhibition.

RESULTS

Different types of protease inhibitors are not specifically induced

All treatments induced SPI (F=30.47, *p*<0.0001, N=62, Figure 3.1 A) and *Trirhabda* damage alone induced SPIs significantly less than the *Spodoptera* and *Spodoptera* + *Trirhabda* treatments, but SPI concentration was still significantly higher than in undamaged plants (Student's T-test, *p*=0.0012). *Spodoptera* and *Spodoptera* + *Trirhabda* damage induced CPI (F=4.45, *p*=0.0044, N=62, Figure 3.1 B), however, *Trirhabda* damage alone did not induce CPI's (*p*=0.1733).

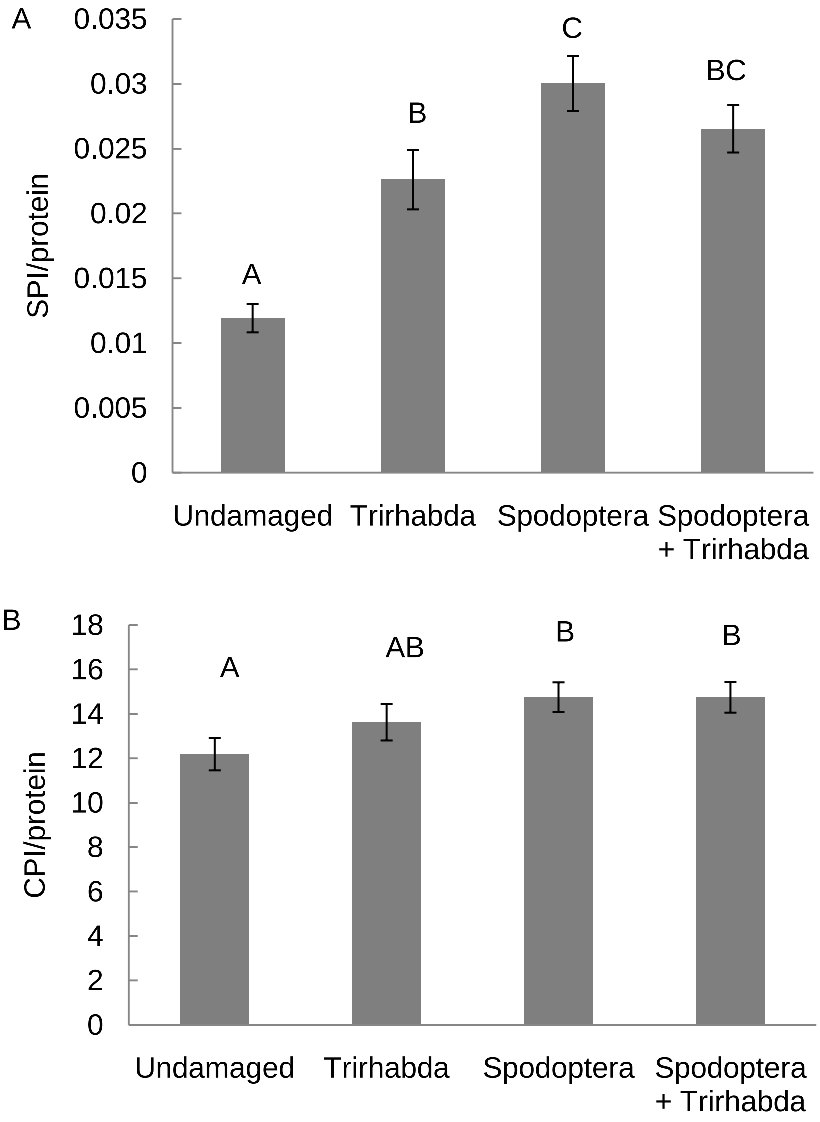

Figure 3.1: Variation in PI induction between the four treatments (Undamaged, *Spodoptera*, *Trirhabda*, and *Spodoptera* + *Trirhabda*) (A) levels of SPIs and (B) levels of CPIs. Significant differences are indicated by different capital letters and were inspected with a Tukey/Kramer post hoc test of an ANOVA.

Spodoptera ***larvae gain less mass on plants previously damaged by*** **Spodoptera**

S. exigua larvae feeding on plants that had previously received *S. exigua* herbivory, whether in the *S. exigua* or Spodoptera + Trirhabda treatments, grew significantly less than larvae feeding on plants from the Undamaged and *Trirhabda* treatments (F=15.03, *p*<0.0001, N=62, Figure 3.2).

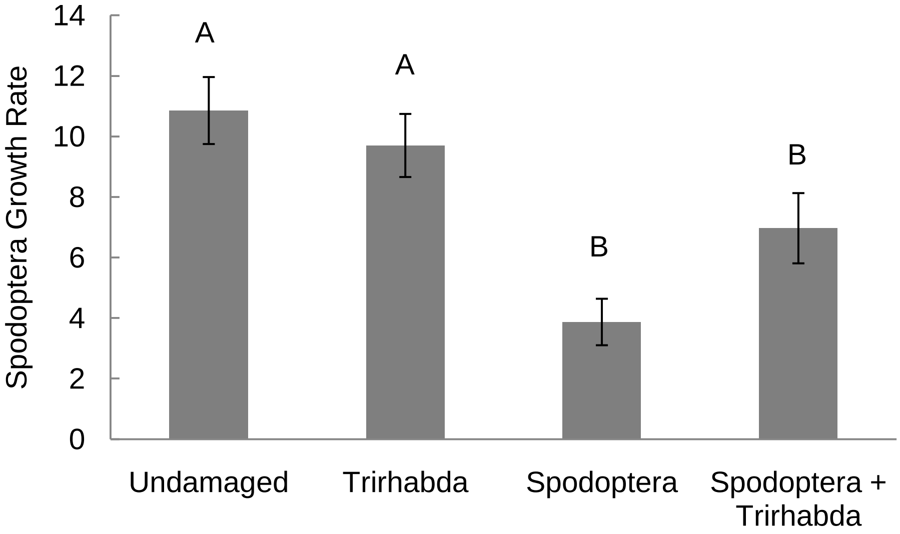

Figure 3.2: Variation in relative *S. exigua* growth in the four treatments (Undamaged, *Spodoptera*, *Trirhabda*, and *Spodoptera* + *Trirhabda*). Significant differences are indicated by different capital letters and were inspected with a Tukey/Kramer post hoc test of an ANOVA.

Protease inhibitor induction correlates with amount of damage

Both SPIs (*r*=0.304, *p*<0.0001, N=62, Figure 3.3 A) and CPIs (*r*=0.245, *p*=0.0033, N=62, Figure 3.3 B) correlated with the percent of leaves damaged. The production of both types of PIs correlate with each other (*r*=0.515, *p*<0.0001, N=62, Figure 3.3 C). Genotype means for each of the four treatments were used for this statistical test.

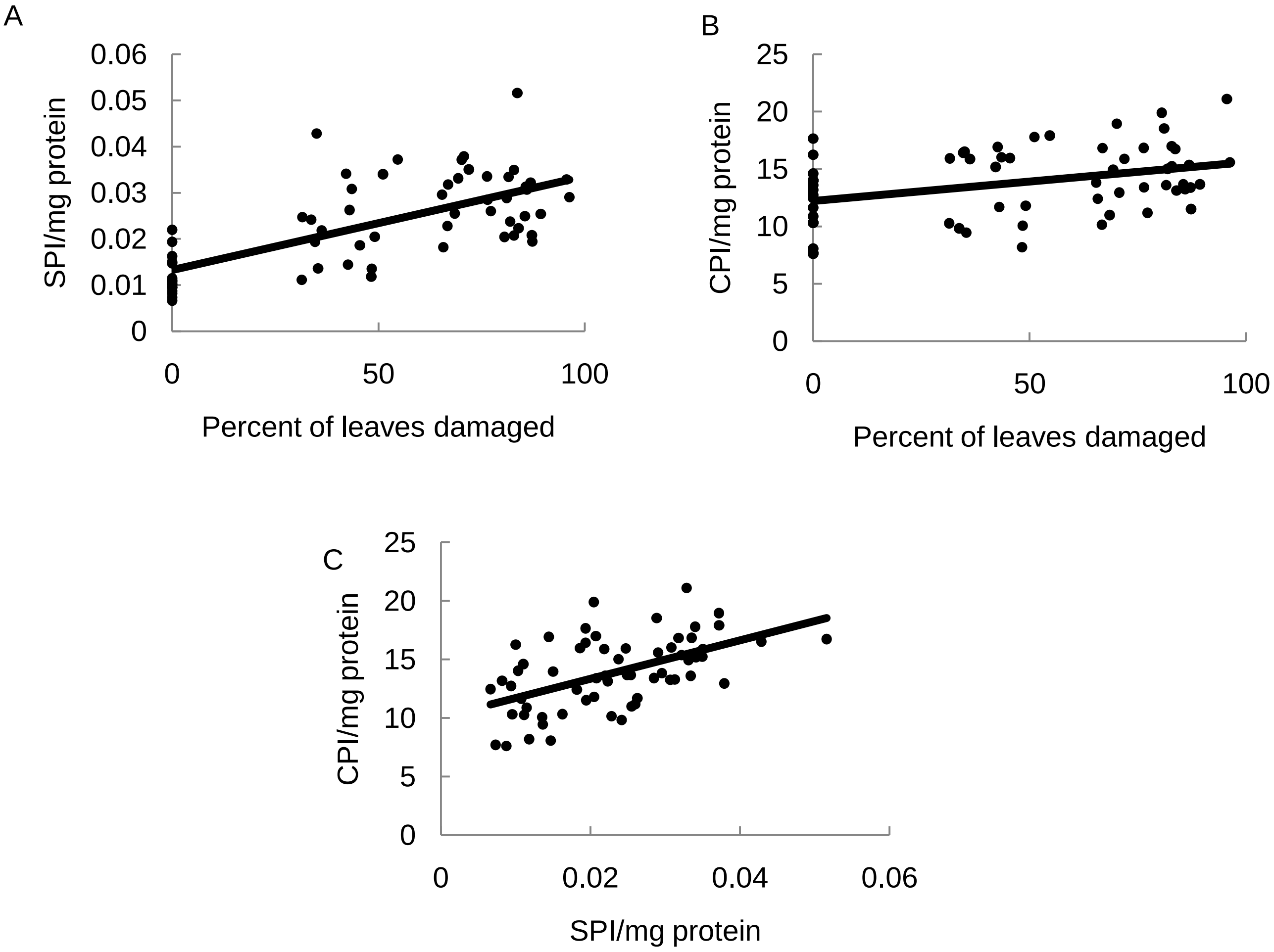

Figure 3.3: Relationships between (A) the percentage leaves damaged and SPI level, (B) percentage leaves damaged and CPI level, and (C) between levels of SPIs and CPIs

Spodoptera *growth rate correlates with SPI but not CPI*

To confirm that SPIs and not CPIs were effective resistance traits against *S. exigua*, we plotted levels of SPIs (Figure 3.4 A) and CPIs (Figure 3.4 B) against *S. exigua* growth rate for all plants. The growth rate of *S. exigua* larvae correlated negatively with SPI concentration (r^2=0.095, *p*=0.0004, N=129, Figure 3.4 A) but did not correlate with CPI activity (r^2= 0.013, *p*=0.1773, N=138, Figure 3.4 B).

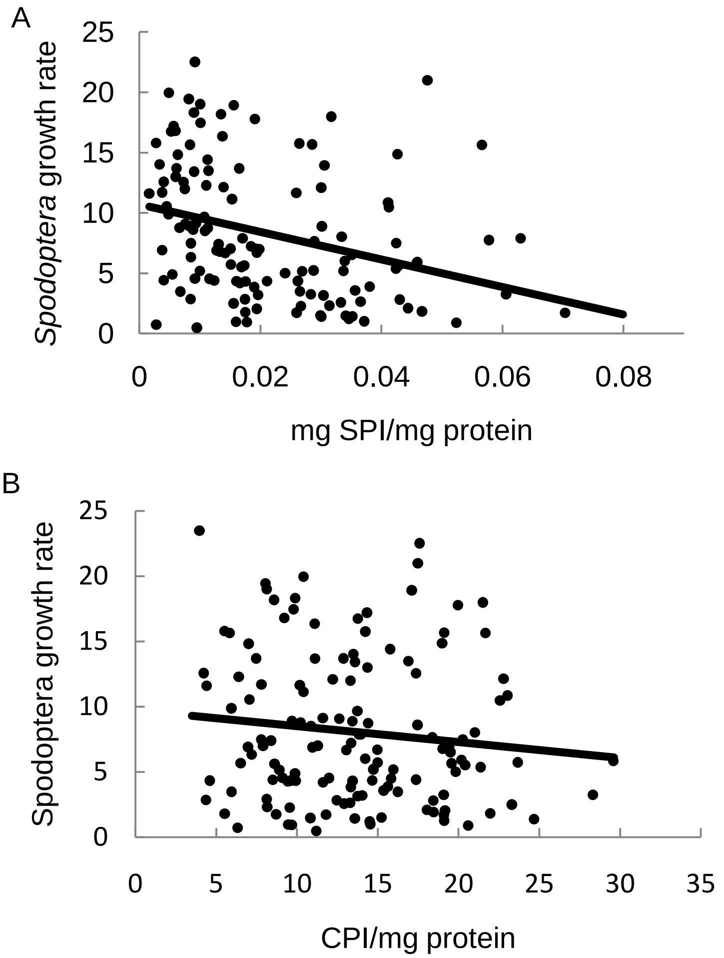

Figure 3.4: Relationship between *Spodoptera* growth rate and (A) the concentration of SPIs or (B) CPI activity)

Spodoptera *gut proteases and larval growth are inhibited by SPI*

There was a significant, positive correlation between the inhibition of trypsin and the inhibition of *S. exigua* gut activity (*r*=0.303, *p*<0.0001, N=28, Figure 3.5). The growth rate of *S. exigua* larvae correlates negatively with the percent of gut activity inhibited by samples extracted from leaves of the same plants (*r*= -0737., *p*=0.0103, N=26, Figure 3.6).

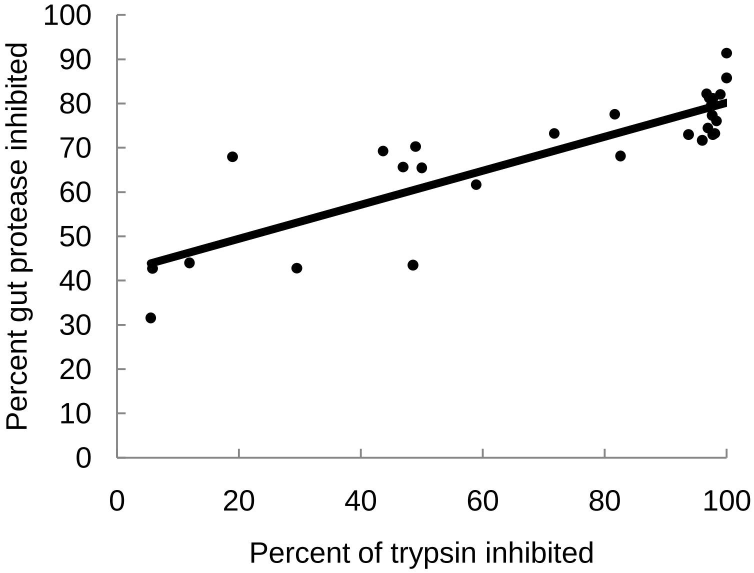

Figure 3.5: The relationship between gut content inhibition and the inhibition of trypsin.

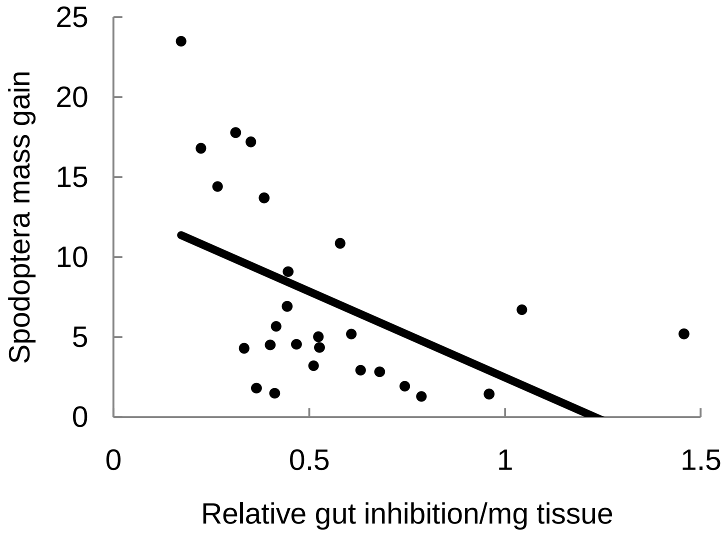

Figure 3.6: The relationship between *Spodoptera* growth and the relative gut inhibition per mg leaf tissue

DISCUSSION

We found that SPIs were induced by all treatments, albeit to a lower level in the *Trirhabda* treatment (Figure 3.1 A). These results supported the hypothesis that PIs are induced generally in response to damage. In further support of this, CPIs were induced significantly only in the *Spodoptera* and *Spodoptera* + *Trirhabda* treatments, but not significantly in the *Trirhabda* treatment (Figure 3.1 B). The *Trirhabda* treatment had lower damage than the other two (Appendix 2), which may mean that induction of SPI and CPI is damage-induced in this system (Farmer and Ryan, 1990; Bolter, 1993; Koiwa et al., 1997), and may not be differentially regulated in response to an herbivore (Sanchez-Hernandez et al., 2004; Turra et al., 2009). Since both SPIs and CPIs were induced by damage, they may be under similar regulation. The correlation between both types of PIs (Figure 3.3 C) strongly suggested that these defensive proteins were induced by damage, and their production may be similarly regulated by endogenous signaling. Previous studies have shown that both SPI and CPI induction are regulated by jasmonic acid (Farmer and Ryan, 1990; Bolter, 1993; Koiwa et al., 1997).

We found that not all treatments resulted in induced resistance to *S. exigua* larvae. As was expected, only plants that had been exposed to *S.exigua* larvae, whether in the *Spodoptera* or the *Spodoptera* + *Trirhabda* treatments, had significantly higher resistance to *S.exigua* larvae compared to undamaged plants (Figure 3.2). Not only was induced resistance limited to those two treatments, but there were no significant differences in larval growth on plants from the *Spodoptera* or *Spodoptera* + *Trirhabda* treatments. The pattern of resistance suggests that damage by *T. virgata* does not result in increased resistance to *S. exigua*. We confirmed that the induction of SPIs correlated with increased resistance to *S. exigua* (Broadway and Duffey, 1986), and that the low level of induction in the *Trirhabda* treatment (Figure 3.1 A) may not have been enough to induce significant resistance to *S. exigua*. The same could have been hypothesized for levels of CPIs, but since *S. exigua* is a Lepidopteran herbivore, it is believed to have an alkaline midgut and serine proteases (Ahmad et al., 1976; Jongsma and Bolter, 1997), which would be unaffected by CPI. Thus, we did not expect CPI induction to correlate with resistance to *S. exigua.*

To confirm this, we tested for specificity of effect; i.e. if SPI would function in induced resistance to *S. exigua* while CPI would not. The levels of SPI correlated with *S. exigua* growth, but levels of CPI did not (Figure 3.4 A and B), suggesting that SPIs function against *S. exigua* gut proteases, while CPIs do not. The functionality of SPI in herbivore resistance is expected to be through inhibition of gut proteases (Broadway and Duffey, 1986). Further tests of gut inhibition confirmed that SPI levels correlated with inhibition of *S. exigua* gut content (Figure 3.5), and that this inhibition of gut content correlated with larval performance (Figure 3.6). Thus, although we did not see specific induction of PIs in response to feeding by *S. exigua*, we did find that only SPIs were effective putative resistance traits against *S. exigua* (Broadway and Duffey, 1986). Cysteine protease inhibitors are induced by *S. exigua*, but are not hypothesized to function as resistance traits against Lepidopteran larvae (Jongsma and Bolter, 1997). This was an interesting result, because, through analyzing the levels of SPI and CPI in the same leaf tissue, we were able to tease apart the effect (or lack thereof) of the two types of PI induced by *Spodoptera*. Although herbivores can alter their gut proteases in response to challenge by plant PIs (Broadway, 1997), they are not expected to change their gut pH and change from serine to cysteine PIs, thus, we expect that CPI induction would not result in increased resistance to *S. exigua*.

Assuming that PI production is costly to a plant (Zavala et al., 2004b), plants should be under selection to only induce PIs that function in resistance against the herbivore doing the damage. Plants are capable of specific induction of some resistance traits (Viswanathan et al., 2005; Poelman, 2008). However, there may be constraints on whether certain defenses can be specifically induced, especially if they are under the control of the same regulatory pathways. It is possible, that in the *S. altissima* system, the induction of PIs evolved under selection by many herbivores, and a broad-spectrum induction is more beneficial to the plant than induction of discrete defense-related metabolites. Alternately, it is possible that *S. altissima* induces some defenses specifically (Tooker et al., 2008), but others are induced in a general manner by the same regulatory mechanisms, such as jasmonic acid (Farmer and Ryan, 1990; Bolter, 1993; Koiwa et al., 1997). If different types of PI are induced simultaneously, this may make damaged plants less attractive (Glawe et al., 2003; Wise, 2009), which may be a benefit greater than the cost of inducing multiple PIs in response to a single herbivore. The specificity of induction may vary depending on what induced defenses are studied, what regulates the induction of defenses, and the ecological consequences of induction.

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CHAPTER 4

Constraints of coevolution: ecological and physiological costs in the evolution of plant resistance

Authorship to be published as: Robert F. Bode and André Kessler

ABSTRACT

Many tactics are used by plants to reduce damage by herbivores. Herbivores must still eat plants to survive, and to do so, have evolved counter-resistance measures, in an evolutionary arms race that has spanned the history of angiosperms. However, this arms race often pits plants against multiple herbivores, which may exert opposing selective pressures on plant defenses. Plants must also balance their allocation of resources between growth and defense. These opposing pressures on plant defenses may constrain the evolution of increases in an herbivore resistance trait. However, although evolutionary increases in the production of defense-related metabolites may be constrained by opposing selective pressures, a diverse array of similar defense metabolites may itself constrain the evolution of counter-resistance in defenses. There may still be selection for a diversification of defenses, even if the selection on a single defense trait is constrained by opposing pressures. Plants can also become more tolerant of herbivory, which does not necessarily come at a cost to resistance. Both higher chemical diversity and increased tolerance may serve to constrain the evolution of herbivore counter-resistance. Thus, although the evolution of increases in a resistance trait may be constrained by opposing selective pressures, strategies such as chemical diversity and tolerance may be under positive selection by herbivory.

INTRODUCTION

Plants use a diverse array of traits to resist herbivory. While these traits have been assumed to yield fitness benefits for the plants, studies that show direct positive fitness consequences of expressing chemical defenses are rare (Berenbaum et al., 1986; Strauss et al., 1996; Agrawal, 1999; Shonle and Bergelson, 2000; Bailey et al., 2007; Lankau, 2007; Johnson et al., 2009; Muola et al., 2010). Plants and insects are hypothesized to co-evolve with one another, with increased resistance, often in levels of plant secondary metabolites, yielding fitness benefits for the plant, and counter-resistance yielding fitness benefits for the herbivore (Ehrlich and Raven, 1964). This explanation is attractive, but works best where interactions are limited to two interactors (Futuyma and Slatkin, 1983). However, the majority of plant species have a complex community of insect herbivores (Futuyma and Gould, 1979), meaning that there will be limits to how much natural selection results from a pairwise arms race between two interactors. This is not to say that chemical defenses do not yield fitness benefits, or that insect herbivores are not significant selective agents on plant resistance traits (Kulman, 1971), but rather that the evolution of plant defenses is frequently subject to selection by multiple independent agents from a diverse herbivore community. This diverse herbivore community is likely to include both specialist and generalist herbivores, which may exert opposing selective pressures on the production of a given resistance trait (Strauss and Irwin, 2004). In cases where a diverse herbivore community represents opposing and equally strong selective pressures (although these pressures may vary over time) we expect that the ecological costs of increased or decreased production of a defense-related metabolite will constrain evolution of that trait. Additionally, allocation of resources to growth or to defense may limit the production of costly defense-related metabolites (Berenbaum et al., 1986; Herms and Mattson, 1992). Thus, in systems with a suite of herbivores exerting opposing selective pressures, or in systems where physiological limitations force tradeoffs between resistance and fitness, we hypothesize that there will be stabilizing selection on defense traits rather than directional selection for increased production of defense-related metabolites. Tolerance and high chemical diversity may limit the evolution of counter-resistance (Jones et al., 1983; Stinchcombe and Rausher, 2002; Espinosa and Fornoni, 2006), and may not come with their own physiological and ecological costs (Van Dam et al., 1995; Leimu and Koricheva, 2006 Nunez-Farfan et al., 2007; Stevens et al., 2007). Thus, we hypothesize that directional selection by herbivores is more likely to be for chemical diversity and tolerance than for increased production of a resistance trait, especially in plants with a diverse herbivore community, or under conditions where high concentrations of a compound are costly to a plant.

Defense- Traits that decrease the negative fitness consequences of attacks – from the plant's point of view (Karban and Baldwin 1997)

Resistance- Traits that reduce herbivore survival, reproductive output or preference for a plant – from the herbivore's point of view (Karban and Baldwin 1997)

Counter-resistance- Herbivore traits that reduce the effectiveness of resistance – from the herbivore's point of view (Karban and Agrawal 2002)

1. Opposing selection pressures from ecological and physiological costs of resistance may constrain directional selection on a single resistance trait.

Most plant species have a community of insect herbivores composed of generalists and specialists (Lawton and Schroder, 1978). The community composition is affected by many factors including plant resistance, and may vary within a season and between growing seasons. A diverse herbivore community represents a potential challenge for a plant: if defenses are too low, the plant will be less resistant to generalist herbivores (Van Dam *et al.*, 1995), but if defenses are too high, the plant could be more attractive to specialist herbivores (Gupta and Thorsteinson, 1960; Hare and Futuyma, 1978;Futuyma and Slatkin, 1983; Reed et al., 1989). Some generalist herbivores may feed on a plant with high defenses because it represents an enemy free space (Singer *et al.*, 2004). A prime example of the challenge of opposing selective pressures comes from the mustard family (Brassicaceae), where glucosinolates are effective in resistance to generalist herbivores, but stimulate feeding in some specialist Lepidoptera (Gupta and Thorsteinson, 1960; Reed et al., 1989). As a consequence there should be positive selection on glucosinolates in the presence of generalists (who are deterred), and negative selection in the presence of specialists (who are attracted). Indeed, the selection pattern for sinigrin was seen to vary depending on the presence of specialist (aphid) or generalist (mollusk) herbivores as well as depending on competition in *Brassica nigra* (Lankau, 2007). Since both specialists and generalists may be present at the same time in the same plant population, there may be both positive and negative selection on a resistance trait, leading to a diffuse pattern of selection on this resistance trait (Mauricio and Rausher, 1997).

Another evolutionary restriction on plant resistance comes with the “To Grow or Defend” hypothesis (Herms and Mattson, 1992). In this hypothesis, plants are assumed to proportionately allocate resources into growth or into defense, while investment into either comes at a cost to the other. In evolutionary time, this could mean conflicting directions of selection; selection by an herbivore to increase a compound and selection to decrease that same compound and allocate resources to seed production. An example of this dichotomy can be seen in *Pastinaca sativa* (wild parsnip) and *Depressaria pastinacella* (parsnip webworm), where there is selection by the herbivore to increase the concentration of coumarins, but any increase in the concentration of these compounds results in decreased seed set (Berenbaum *et al.*, 1986). In this case, the diffuse selection by an herbivore and resource allocation is called a “stalemate” in the evolutionary arms race (Berenbaum et al., 1986), since further increases in defense metabolism result in decreased seed set, thus negating the fitness benefits of defense (Shonle and Bergelson, 2000).

In systems where there is alternating selection on a resistance trait by multiple types of herbivores, or where the trait is costly in terms of fitness, there are two distinct and opposite selective pressures on the resistance trait (Figure 4.1). So long as both pressures are roughly equal, selection may vary in direction from year to year, and will not result in a measurable direction of evolution over time. Although in each year, it would be possible to see positive or negative selection on a trait; this may be reversed in the following year. Overall, the opposing selective pressures by two herbivores or by allocation costs may constrain the evolution of increased production of a resistance trait. Opposing selective pressures may also select for diversification of trait expression, such as multiple compounds with similar functions, or induction of compounds when and if necessary (Karban and Baldwin, 1997).

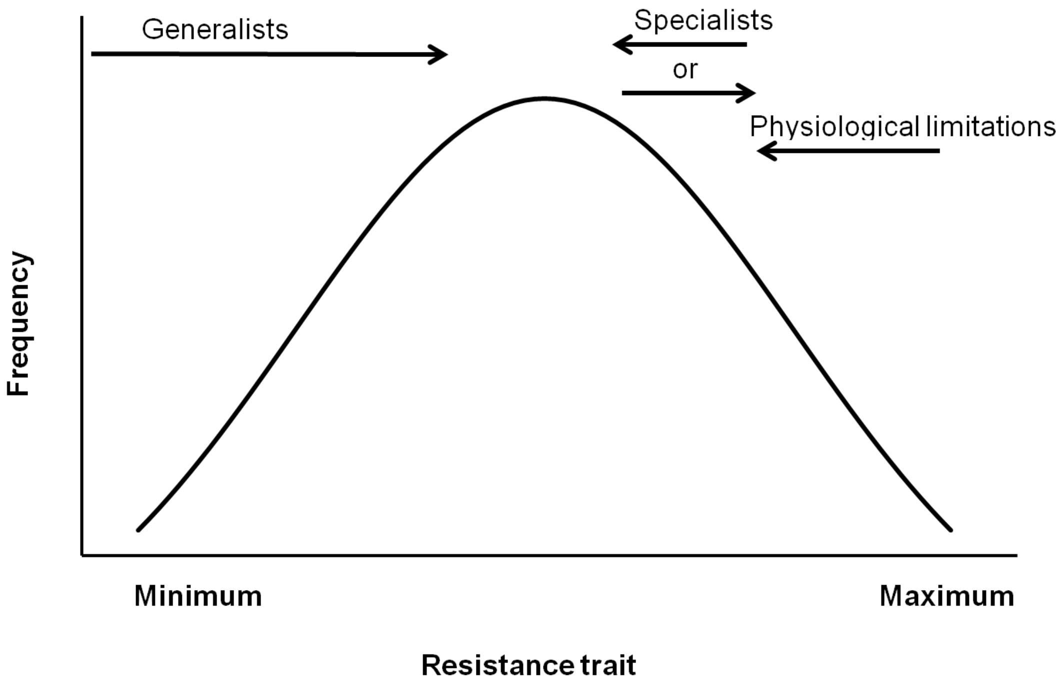

Figure 4.1: Different selective pressures are hypothesized to act in opposing directions (here symbolized by arrows) on a trait. For a resistance trait with a normal distribution, natural selection by different agents constrains the evolution of the trait in any single direction.

2. Plants with opposing selective pressures will experience variable and diffuse natural selection on individual resistance traits, leading to overall trait stability

The simple question of what resistance traits are under natural selection by herbivory defies a simple explanation. Selection on resistance traits is context dependent (Lankau and Strauss, 2008), and the context of herbivory changes with a changing herbivore community (Muola et al., 2010). The context for natural selection can change as much from year to year as from community to community (Parachnowitsch, 2011). If natural selection on a trait changes from year to year, it is possible that selection affects plants with different growth styles differently. For annual plants, the traits selected for in one generation may be selected against in the following generation. For biannual plants, the fitness in the second year may be determined by selective pressures in the first year as much (or more, depending on resource acquisition) as in the second year. For perennial plants, measuring long-term selection may be impossible, since multiple generations of herbivores may feed on a single plant, doing sub-lethal damage each year. Variable levels of selection on a resistance trait here could mean variable levels of fitness for the same plant from year to year. Thus, traits under diffuse selection may remain roughly the same in evolutionary time, although single-year measurements are likely to show directional selection on the trait.

3. Herbivore-mediated selection on resistance traits is dependent on the makeup of the herbivore community

Examples abound of insect herbivores that have large effects on the plant community, especially during periods when these herbivores exist in high population densities (Morrow, 1977; Louda *et al.*, 1990; Marquis, 1992; Carson and Root, 2000). These periods of high population density may represent a temporary increase in the pressure of natural selection by the herbivore on certain resistance traits, although during periods of extreme outbreaks, when plants do not induce resistance, all plants may be eaten, regardless of resistance (Mattson, 1991). Thus, not all herbivores have equal impacts during a growing season. If the traits under selection during the period of high population density limit the reproduction of the herbivore, then the increased plant resistance in the year after may negatively affect the density of that herbivore. If resistance traits exist at a tradeoff to one another (Strauss *et al.*, 2002) or if they evolve independently (Rudgers *et al.*, 2004), the resistance to other herbivores may be lower or no higher than before the period of high population density. Other herbivores may become more frequent during following seasons, and this would represent another increase in selection on another suite of resistance traits, either counter to or independent of the previous selection pressure. As a consequence rapid evolution can function back on ecological processes. While composition of the herbivore community affects the evolution of plant resistance traits, the evolution of plant resistance traits may affect the composition of the herbivore community.

Not all herbivores have equal timetables; during a growing season, a plant will be exposed to a series of herbivore attacks. The interaction between the evolution of resistance and sequential herbivore attacks is mediated by the herbivore preferences and by the inducibility of resistance. Herbivores, whether of the same or a different species, may (A) prefer undamaged plants (Agrawal, 1999; Gonzalez-Megias and Gomez, 2003; Van Zandt and Agrawal, 2004; Wise, 2009), or (B) prefer previously damaged plants (Damman, 1989; Pilson, 1992; Cappuccino and Martin, 1994; Agrawal and Sherriffs, 2001). Plants may (1) be able to induce resistance (Green and Ryan, 1972); (Karban and Baldwin, 1997), or (2) not induce resistance in response to herbivory. If (A) and (1), than the herbivore community will feed on undamaged plants, and induced defenses may be under positive selection. In the case where (A) and (2), the herbivore population may constrain the evolution of higher resistance, as later herbivores compete for undamaged tissue, regardless of resistance to the first herbivore (Wise, 2009). If (B) and (1), however, then plants may be under selection to have higher constitutive resistance, and thus avoid early attack. An exception to this may occur if the resistance trait is a feeding stimulus (Reed et al., 1989), and does not become induced to the point of toxicity, in which case higher initial levels may increase the likelihood of later damage. Last, if (B) and (2), plants will again be under selection to have higher constitutive defenses, and to avoid the early damage. When herbivores prefer damaged plant tissue, plants may also be under selection to become more tolerant of herbivore attack (Strauss and Agrawal, 1999). Evolution of resistance is thus dependent on two contexts: that of herbivore preferences, and that of the inducibility of resistance.

4. A diverse suite of herbivores limits selection on plant defenses; a diverse suite of defenses limits selection by herbivores

Just as the diverse herbivore community may exert opposing selection on a defense trait, multiple defense traits may exert opposing selection on herbivores. Feeny (1976a) predicted that specialists may vary in sensitivity to different types of defense compounds in a single compound class. This means that an adaptation that allows an herbivore to overcome one of the plant's defenses would not necessarily confer an advantage in terms of a second defense. However, the multiple types of compounds within a single class may be similar enough to confer resistance to non-adapted herbivores, regardless of relative quantities of the compounds (Van Dam *et al.*, 1995). This would mean that plants with only a single compound in one class may have the same defense against non-adapted herbivores as plants with two variations of the compound that add up in terms of defense benefits to the single compound. However, if herbivores evolve counter-resistance to a single compound, plants with the two variations of the compound will be better defended, because the herbivore only has counter-resistance to one compound. Through this somewhat-convoluted pathway, plants with two variations of a defense-related compound will suffer no loss of resistance to non-adapted herbivores, and will have a benefit in the case of the evolution of counter-resistance to one variation. Having a broad suite of distinct secondary compounds can also mean that an herbivore that is able to deal with one will necessarily be unable to deal with another by virtue of the compound properties (Jones *et al.*, 1983).

A potential cost to chemical redundancy comes when plants must make tradeoffs between levels of two compounds with a similar function. In *Pastinacea sativa*, two angular furanocoumarins, bergapten and sphondin, are negatively genetically correlated in concentration (Berenbaum et al 1986). Selection by herbivores for an increase in either compound is selection for decreasing the other. However, the frequency of plants with high levels of each compound varies over time, which may limit the ability of herbivores to overcome both resistance traits, much as variable and opposite selective pressures by multiple herbivores may limit the evolution of plant defense traits (Strauss and Irwin 2004).

Most plants have a diverse suite of defensive compounds. There are many types of glucosinolates in Brassicaceae, sesquiterpene lactones in Asteraceae, and cardiac glycosides in Asclepiadaceae. There is also broad diversity in compounds found in multiple families, such as phenylpropanoids. This variation in defense chemistry may be a means of constraining the evolution of herbivore counter –resistance. The evolution of counter –resistance to one trait may increase selection on plants that have low levels of that trait, but high levels of another similar trait. If there is no net loss of resistance by trading one trait for another, the evolution of counter-resistance is foiled. Plants that have high levels of one defense-related metabolite may be less vulnerable to certain herbivores. An increase in the frequency of these plants could result in reduced numbers of those herbivores, which could result in an increase in other herbivores (Gonzalez-Megias and Gomez 2003). This could then increase selection on other, similar defense-related metabolites, increasing the frequency of plants with high levels of those metabolites, and reducing the numbers of susceptible herbivores. Diversification in plant defense chemistry may be under positive selection, even when there are herbivores that can detoxify certain variants of a defense-related metabolite (Benderoth et al., 2006). Higher chemical diversity can also increase the effectiveness of each individual defense metabolite (Feeny, 1976b). Alternatively, the variation in defense chemistry may be the result of selection by a diverse herbivore community, where each herbivore selects on different metabolites (Iason et al 2011).

5. Herbivore evolution reduces the effectiveness of resistance traits but not of tolerance

The co-evolutionary hypothesis posited that the evolution of a novel chemical defense that successfully repelled herbivores allowed a plant species to enter an herbivore-free zone. However, Ehrlich and Raven (1964) also noted that herbivores that evolved counter-resistance would enter a competitor-free zone. In such a zone, they would no longer be limited by interactions with other herbivores. Sequestration of toxic plant compounds can also reduce predation on the herbivores. In short, escape from herbivory opened up a niche for new herbivores to exploit. An “herbivore-free zone” to a plant is a “competitor-free zone” to an herbivore, and in evolutionary time, these two factors were hypothesized to drive co-evolution between a phylogeny of plants and a phylogeny of herbivores, in at least some systems (Ehrlich and Raven, 1964); (Berenbaum, 1983; Becerra, 1997; but see also (Braby and Trueman, 2006)).

Central to the co-evolutionary hypothesis is the concept that resistance traits can lose their effectiveness over evolutionary time. But plants do not rely on resistance alone to mitigate the effects of herbivory. Tolerant plants are able to receive damage and re-grow in such a way as to overcome negative fitness effects of damage. Tolerance is not hypothesized to come at a cost in terms of resistance (Leimu and Koricheva, 2006; Nunez-Farfan et al., 2007; Stevens et al., 2008), and plants can exhibit mixed strategies of tolerance and resistance. Tolerance is a trait that has been shown to have no negative effects on herbivore growth and fitness (Tiffin, 2000; Stinchcombe, 2002; Espinosa and Fornoni, 2006), thus, there should be no selection for herbivores to overcome plant tolerance traits. If herbivores do not evolve traits to reduce the effectiveness of plant tolerance, then unlike resistance traits, tolerance will not lose its’ effectiveness over evolutionary time.

6. Plants will evolve to be more tolerant, but without losing resistance

Plants are not limited to a single defense strategy, but rather may use strategies of constitutive resistance, induced resistance, and tolerance to deal with herbivory. These strategies can be evolutionary flexible, and there may be tradeoffs between different types of defense strategies (Lebreton, 1982), or not (Mauricio et al., 1997; Nunez-Farfan et al., 2007). The evolution of tolerance may occur at the same time and be independent from the evolution of resistance, since there is little evidence for a tradeoff between the two. Thus, even if selective pressures on a resistance trait are variable and diffuse there may still be directional selection on tolerance. In cases where herbivores do not select on resistance traits but still do ample damage, there may still be selection to increase tolerance (Mattson et al., 1991).

Tolerance can complement existing resistance strategies by reducing the selective pressure on the herbivore community. As mentioned above, herbivores do no worse on re-growth tissue of tolerant plants than on original tissue (Tiffin, 2000; Stinchcombe and Rausher, 2002; Espinosa and Fornoni, 2006), and thus it is unlikely that herbivores have any need to cope with tolerance as they need to cope with resistance. The evolution of counter-resistance is hypothesized to be slower on tolerant plants (Stinchcombe and Rausher, 2002; Espinosa and Fornoni, 2006). Thus, tolerance could function to slow the evolution of counter-resistance, as well as being selected for as an adaptive trait in itself (Mattson et al., 1991**,** Stowe et al., 2000; Lennartsson and

Oostermeijer, 2001; Weinig et al., 2003)

SUMMARY

A diverse herbivore community and tradeoffs between growth and defense constrain the evolution of plant resistance traits effective against each single attacker species through diffuse selection. The evolution of plant resistance may be constrained when herbivores prefer undamaged plants and plants do not induce resistance. The co-evolutionary hypothesis posits that resistance traits open up competitor-free (and sometimes predator-free) space for herbivores to exploit if they can evolve counter-resistance. With these constraints and counters to the evolution of resistance traits, it would seem that natural selection on resistance traits is unlikely to lead to increases in a resistance trait in evolutionary time.

However, the evolution of increased chemical diversity and tolerance to herbivory are both likely to reduce the evolution of counter-resistance by herbivores. These traits may both be under selection in themselves, and can complement existing resistance traits. Although resistance traits are likely to be under diffuse and variable selection, plants may still evolve to reduce the impact of herbivory.

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Appendix 1

Additional Data and Correlations for Chapter 2

Figure A 1.1: Relationship between early herbivory and final relative plant biomass loss to herbivory.

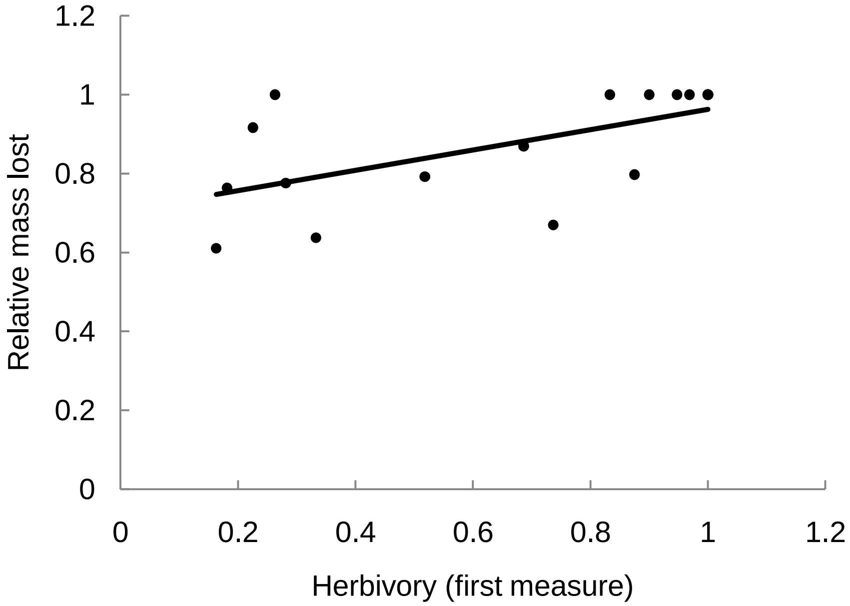
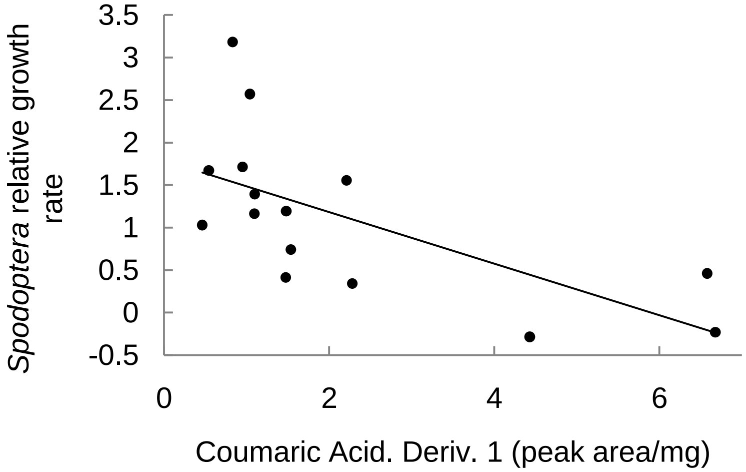


Figure A 1.2: Relationship between production of (**A**) Coumaric Acid Derivative 1, (**B**) Kaempferol-3O-rutinoside and *Spodoptera* growth from the greenhouse assay.

A



B

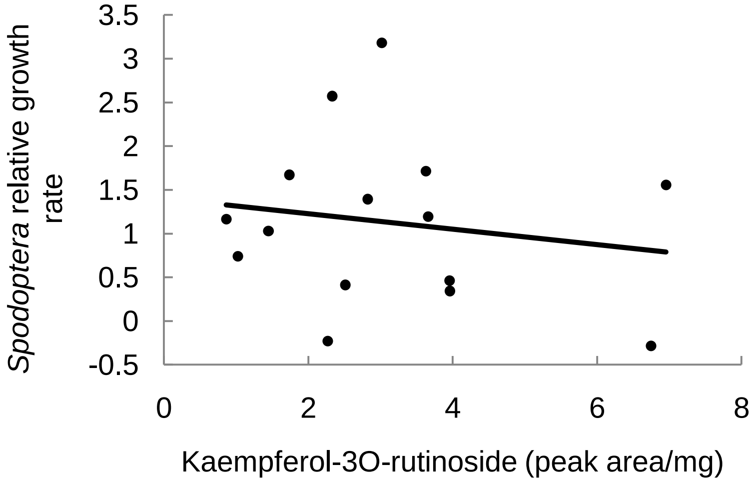


Table A 1.1- Mean (±SE) measurements from each site from (A) 2009 and (B) 2010 surveys, including number of leaves, leaves damaged by chewing herbivores, mining herbivores, and all herbivores combined per leaf.

A

Elevation	Total leaves	Chewed Leaves	Mined Leaves	Herbivory
1469	13.11±0.44	2.89±0.29	0.00±0.00	0.27±0.04
1455	12.33±0.33	0.50±0.50	0.50±0.29	0.53±0.17
1423	5.72±0.71	0.83±0.17	0.00±0.00	0.31±0.03
1352	13.56±0.44	1.89±0.78	0.00±0.00	0.25±0.04
1340	25.00±0.33	4.33±0.88	0.11±0.11	0.34±0.04
1315	14.00±0.51	3.22±0.29	0.00±0.00	0.33±0.07
1294	6.00±0.58	0.33±0.00	0.00±0.00	0.17±0.07
1228	6.56±0.29	0.56±0.29	0.00±0.00	0.29±0.06
1192	5.89±0.40	0.56±0.40	0.00±0.00	0.20±0.08
1033	14.67±1.26	0.44±0.22	0.22±0.22	0.12±0.08
939	15.89±0.70	1.36±0.47	0.14±0.07	0.14±0.08
888	14.22±0.62	4.33±1.64	0.44±0.29	0.31±0.09
886	16.17±0.60	1.83±0.73	0.17±0.17	0.29±0.07
848	13.00±0.29	0.17±0.17	0.00±0.00	0.26±0.14
745	11.08±0.94	2.00±0.43	0.25±0.14	0.10±0.02
726	17.00±1.02	0.22±0.11	0.89±0.11	0.16±0.04
722	14.83±0.30	4.42±0.74	0.58±0.22	0.35±0.06
674	13.27±0.35	2.47±0.52	1.47±0.29	0.28±0.12
637	24.67±2.09	4.17±0.22	2.08±0.82	0.28±0.14
600	19.67±1.58	1.67±0.33	2.22±0.11	0.28±0.10
554	17.00±1.76	0.22±0.11	1.44±0.48	0.16±0.06
538	22.87±0.24	1.33±0.55	0.67±0.13	0.20±0.09
507	7.25±2.01	2.17±0.51	1.50±0.80	0.39±0.15
357	20.00±0.88	1.11±0.48	0.67±0.19	0.23±0.09

B

Elevation	Total leaves	Chewed Leaves	Mined Leaves	Herbivory
1473	4.87±0.25	1.00±0.21	0±0.00	0.60±0.15
1460	5.17±0.54	1.10±0.23	0.33±0.21	0.54±0.11
1424	6.27±0.32	1.20±0.20	0.20±0.08	0.59±0.12
1393	5.20±0.43	0.40±0.27	0.27±0.19	0.25±0.11
1349	6.00±1.01	1.47±0.57	0.30±0.08	0.52±0.10
1333	6.87±0.64	1.93±0.67	0.33±0.18	0.60±0.17
1323	6.07±0.44	1.53±0.25	0.10±0.10	0.54±0.10
1259	5.20±0.34	1.00±0.28	0.20±0.13	0.48±0.14
1231	5.27±0.23	1.50±0.19	0.25±0.22	0.43±0.05
1020	6.60±0.46	1.67±0.28	0±0.00	0.46±0.08
955	6.17±0.19	1.33±0.44	0±0.00	0.30±0.10
932	6.27±0.45	1.00±0.43	0±0.00	0.21±0.06
887	7.27±0.39	1.87±0.48	0.13±0.13	0.43±0.09
850	6.00±0.28	1.40±0.37	0.13±0.13	0.39±0.11
737	6.33±0.49	0.93±0.34	0.20±0.08	0.27±0.07
732	5.17±0.32	0.67±0.00	0.17±0.11	0.30±0.11
714	6.47±0.34	1.80±0.27	0.13±0.08	0.38±0.05
676	5.65±0.58	1.07±0.22	0.35±0.12	0.53±0.04
642	5.80±0.92	1.53±0.23	0.13±0.08	0.46±0.09
614	6.93±0.57	2.27±0.12	0.40±0.19	0.58±0.07
589	6.93±0.51	2.97±0.29	0.33±0.21	0.72±0.08
559	8.00±1.06	3.40±0.52	0.40±0.32	0.64±0.09
542	6.47±0.65	3.20±0.23	0.33±0.15	0.74±0.07
523	5.00±0.00	3.00±0.00	0.25±0.16	0.71±0.06
514	5.96±0.34	3.67±0.50	0.21±0.11	1.19±0.21
485	5.63±0.19	2.37±0.53	0±0.00	0.69±0.13
328	5.56±0.31	3.23±0.35	0±0.00	1.05±0.15

Table A 1.2: Mean (±SE) damaged leaves from each site during each survey for (A) 2009 and (B) 2010.

A

Elevation	July 17th	August 3rd	August 17th
1469	0.26±0.04	0.33±0.02	0.29±0.06
1455	0.23±0.23	0.19±0.19	0.75±0.25
1423	0.14±0.07	0.31±0.19	0.26±0.08
1352	0.14±0.04	0.31±0.16	0.25±0.13
1340	0.22±0.12	0.36±0.04	0.28±0.08
1315	0.23±0.16	0.24±0.14	0.31±0.05
1294	0.07±0.07	0.06±0.06	0.17±0.17
1228	0.00±0.00	0.20±0.20	0.41±0.10
1192	0.00±0.00	0.10±0.05	0.36±0.15
1033	0.09±0.04	0.00±0.00	0.10±0.06
939	0.05±0.04	0.18±0.03	0.26±0.04
888	0.10±0.08	0.35±0.20	0.45±0.18
886	0.03±0.03	0.43±0.12	0.19±0.11
848	0.00±0.00	0.53±0.48	0.03±0.03
745	0.26±0.10	0.11±0.06	0.07±0.05
726	0.15±0.09	0.19±0.08	0.09±0.05
722	0.25±0.07	0.40±0.08	0.43±0.06
674	0.30±0.09	0.47±0.04	0.31±0.11
637	0.46±0.11	0.45±0.15	0.37±0.17
600	0.40±0.18	0.33±0.15	0.42±0.17
554	0.18±0.08	0.17±0.03	0.26±0.12
538	0.38±0.11	0.35±0.09	0.22±0.09
507	0.64±0.17	0.58±0.11	0.50±0.06
357	0.21±0.09	0.31±0.12	0.19±0.07

B

Elevation	June 17th	June 28th	July 14th	July 27th	August 9th
1473	0.17±0.17	0.52±0.02	0.48±0.02	1.08±0.08	0.75±0.25
1460	0.25±0.00	0.40±0.23	0.58±0.18	0.89±0.11	0.58±0.08
1424	0.24±0.17	0.39±0.06	0.67±0.35	0.83±0.20	0.81±0.11
1393	0.00±0.00	0.00±0.00	0.41±0.12	0.29±0.04	0.55±0.16
1349	0.40±0.05	0.66±0.09	0.20±0.00	0.58±0.09	0.75±0.05
1333	0.20±0.11	0.53±0.03	0.29±0.04	0.83±0.16	1.14±0.00
1323	0.46±0.20	0.28±0.07	0.75±0.25	0.41±0.01	0.79±0.20
1259	0.06±0.06	0.27±0.13	0.72±0.20	0.80±0.20	0.56±0.22
1231	0.39±0.04	0.27±0.13	0.53±0.24	0.51±0.25	
1020	0.24±0.13	0.45±0.25	0.33±0.17	0.65±0.04	0.60±0.06
955	0.00±0.00		0.30±0.15	0.35±0.03	0.54±0.27
932	0.07±0.07	0.32±0.22	0.22±0.22	0.37±0.19	0.10±0.05
887	0.20±0.11	0.53±0.13	0.21±0.15	0.63±0.04	0.57±0.14
850	0.30±0.07	0.00±0.00	0.56±0.06	0.48±0.17	0.61±0.22
737	0.00±0.00	0.43±0.03	0.26±0.14	0.33±0.06	0.32±0.09
732	0.12±0.06		0.48±0.26		
714	0.52±0.13	0.29±0.16	0.40±0.31	0.28±0.15	0.43±0.04
676	0.55±0.24	0.40±0.10	0.52±0.02	0.67±0.08	0.54±0.24
642	0.28±0.21	0.25±0.07	0.53±0.03	0.75±0.14	0.51±0.13
614	0.43±0.14	0.62±0.12	0.54±0.11	0.83±0.10	0.47±0.12
589	0.52±0.12	0.62±0.13	0.94±0.06	0.66±0.06	0.86±0.32
559	0.59±0.05	0.72±0.15	0.34±0.20	0.88±0.29	0.68±0.02
542	0.92±0.17	0.87±0.14	0.55±0.05	0.79±0.21	0.59±0.12
523	0.80±0.20		0.61±0.20		
514	0.55±0.05		1.22±0.33	1.66±0.38	1.32±0.19
485	0.47±0.13	0.40±0.31	0.82±0.12	0.68±0.07	1.10±0.33
328	0.53±0.08	1.33±0.18	0.95±0.20	1.32±0.22	1.10±0.35

APPENDIX 2

Variation in damage among treatments

Figure A2.1: The relative levels of damage (percentage of leaves with damage) in the four treatments (Undamaged, *Spodoptera*, *Trirhabda*, and *Spodoptera* + *Trirhabda*). Data are genotype means, different letters indicate significant differences.