DISSERTATION

EVOLUTIONARY AND CHEMICAL ECOLOGY OF *VERBASCUM THAPSUS*

REVEAL POTENTIAL MECHANISMS OF INVASION

Submitted by

Christina Alba

Graduate Degree Program in Ecology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2011

Doctoral Committee:

    Advisor: Ruth A. Hufbauer
    Co-Advisor: James K. Detling

    M. Deane Bowers
    Alan K. Knapp

ABSRTRACT

EVOLUTIONARY AND CHEMICAL ECOLOGY OF *VERBASCUM THAPSUS*

REVEAL POTENTIAL MECHANISMS OF INVASION

Biological invasions, which occur when introduced species achieve pest status due to dramatic increases in performance, cause substantial environmental and economic damage. Invasion dynamics are extremely complex, varying in space and time, and as a function of the associations that form between introduced species and the biota present in the communities they invade. For plants, herbivores play a central role in shaping the outcome of introduction events. In particular, when plants are introduced to novel ranges, they often leave behind coevolved specialist herbivores (typically insects) that act to suppress populations in the native range. This can lead to increases in plant performance, for example when introduced plants evolving in communities devoid of enemies reallocate resources from defenses to growth and reproduction.

Because of the important biological associations that exist between plants and insect herbivores, as well as the dramatic shifts in these associations that characterize biological invasions, this research places a particular emphasis on the evolutionary and chemical ecology of plant-insect interactions. More broadly, this research quantifies several aspects of the invasion dynamics of the introduced weed

Verbascum thapsus L. (Scrophulariaceae, common mullein). I first present data from a biogeographic comparison in which a survey of more than 50 native (European) and introduced (United States) mullein populations confirms a marked increase in population- and plant-level performance in the introduced range. I also document several ecological differences between ranges, including shifts in the abundance, identity, and degree of damage caused by insect herbivores, as well as differences in the abundance and identity of plant competitors and precipitation availability.

A greenhouse experiment revealed that the increased performance observed in the field is maintained when native and introduced plants are grown from seed in a common environment; thus, a component of the performance phenotype is genetically based, or evolved. However, this increase in performance is not associated with an evolved decrease in defense investment as predicted by the *evolution of increased competitive ability* (EICA) hypothesis. Indeed, despite significant population-level variation in several defenses (trichomes, leaf toughness and iridoid glycosides), there is no evidence for the evolution of range-level differences in defense investment.

I further explored how mullein's investment in chemical defense varies in natural populations and in relationship to damage by chewing herbivores. Based on this exploration, I developed new predictions for how changes to defense allocation may result in increased performance. Natural mullein populations exposed to ambient levels of herbivory in the introduced range exhibit significant population- and plant-level variation in iridoid glycosides. In particular, young (highly valuable) leaves are more than 6× better defended than old leaves, and likely because of this incur minimal damage from generalist herbivores. The limited ability of generalists to feed

on mullein's well-defended young leaves results in negligible losses of high-quality tissue, suggesting a mechanism for mullein's increased performance in North America. Indeed, the within-plant distribution of iridoid glycosides significantly differs between native and introduced plants exposed to the different insect communities present in each range. Importantly, introduced mullein invests significantly more in the chemical defense of valuable young leaves than does native mullein, which leads to a dramatic reduction in the attack of young leaves in the introduced range relative to the native range. This optimization of within-plant investment in defense reflects the fact that introduced mullein has been released from the evolutionary dilemma posed by simultaneous attack by specialist and generalist herbivores (with specialists often being attracted to the same chemicals used to deter generalists from feeding, resulting in stabilizing selection on defense levels). In summary, this research provides evidence for a dramatic increase in the performance of introduced common mullein that is associated with several ecological differences between ranges as well as potentially adaptive shifts in mullein's chemical defense investment under natural conditions.

ACKNOWLEDGMENTS

shoulder to cry on, and intellectual stimulation along the way, including Laurel

Hartley, Amy Blair, Steve Rauth, and Dan Cariveau. Most recently, Christa Fettig

and Hannah Wilbur have been indispensable friends and colleagues.

Several members of my family have provided incredible emotional support

during this whole process. My husband Scott is himself a fantastic scientist who is

always able to share with me the burdens and joys that are a part of the scientific

process. I count a few separate occasions when I might not have made it without his

patience and encouragement. He is simply my best friend and life is wonderful

because of him. My parents have always encouraged me to pursue education and have

never once questioned the value of my following this long and winding path toward a

PhD. This is because they value knowledge and they love the world in which they

live—the more we know about its mysteries, the better. The same is true of my Aunt

and Uncle, June and Gene Lafferty. They always cared about this goal of mine (as

well as all of my endeavors from childhood) and they helped celebrate the small

victories along the way. This is a great gift, and to them, and all involved in this

process, I am grateful.

PREFACE*

Biological invasions represent one of the most important phenomena to result from the advent of globalized trade and commerce. The human-mediated transport of plant and animal species beyond their historic ranges has had several unforeseen consequences ranging from the extirpation of native species, to altered fire and hydrologic regimes, to a general homogenization of species and attendant loss of biodiversity. Because of their pronounced effect on ecosystem structure and function, as well as their value for studying the evolutionary ecology of novel interactions among species, biological invasions have drawn intense interest from the research community. However, despite many years of scientific inquiry, it remains difficult to generalize about the causes of invasion or to predict the outcome of a given introduction event. My goal with this dissertation is to continue building an understanding of the patterns and mechanisms of plant invasions by evaluating the evolutionary and chemical ecology of the introduced weed *Verbascum thapsus* L. (Scrophulariaceae, common mullein). This research places a particular emphasis on plant-insect interactions, which have important implications for plant performance, and by extension, invasion success.

There are numerous hypothesized mechanisms of invasive behavior, which is characterized by a pronounced increase in the performance of a species following its introduction to a new area. Several hypotheses posit that aggressive invaders have traits that intrinsically predispose them to succeed during introduction events, for example by producing many offspring or effectively competing for resources in

disturbed environments. It is also hypothesized that extrinsic factors mediate invasion by imposing "top down" or "bottom up" control over population dynamics. In plants, top down control is often attributable to herbivores that reduce plant performance via their feeding activities. Conversely, bottom up control results from competitive interactions between plants for water, light, space, and nutrients. Invasive populations often exhibit some sort of release from one or several of these controls following their introduction, for example by escaping from co-evolved herbivores or colonizing areas with few or poor competitors. In addition to these various biotic interactions, plant populations are regulated by abiotic factors such as precipitation and temperature, and invasions may occur when a species is fortuitously introduced to an area with a benign climate. In reality, some combination of these factors likely interacts to produce aggressive invaders. However, despite the potential for invasive species to cause pronounced economic and ecological harm, it is the case that relatively few introduction events actually produce aggressive invaders. To understand why a handful of species become invasive, it is necessary to link differences in the performance of native and introduced populations to observed shifts in a species' ecology following its introduction.

Chapter 1 of this dissertation presents a biogeographic comparison of the performance and ecology of native (European) and introduced (North American) mullein populations. In particular, I evaluated whether introduced mullein populations and individuals perform better than their native counterparts and assessed whether changes in performance are associated with escape from natural enemies (insect herbivores) and differences in resource availability (i.e., precipitation and bare

ground) and competition between ranges. The first chapter thus describes patterns associated with invasion, which is the critical first step in elucidating which mechanisms underlie the observed patterns.

The second chapter provides an experimental complement to the biogeographic comparison, which revealed that introduced mullein indeed performs better than its native counterparts, and that this increase in performance is associated with mullein's escape from several herbivores. Specifically, Chapter 2 presents the results of a greenhouse experiment designed to test whether mullein's increased performance is explained by an evolutionary (genetic) shift in the way it allocates limited resources to growth (an aspect of competitive ability) versus defense against herbivory. For invasive plants, escape from enemies may alter the selection regime such that particular defenses are no longer under positive selection, and in fact may be under negative selection if their production incurs a fitness cost. In particular, if introduced plants are predominantly attacked by generalist insects, then qualitative defenses (i.e. toxins) that deter generalists should be maintained in the new range. In contrast, quantitative defenses (i.e., structural defenses and/or digestibility-reducing chemicals), which are effective against both specialists and generalists, but are typically costly to produce, should decrease. Resulting increases in growth are then realized due to the shift in allocation from relatively costly quantitative defenses to less costly qualitative defenses. For mullein, the expectation is that qualitative defenses (iridoid glycosides) will be maintained or even increase in the introduced range, while quantitative defenses (trichomes and leaf toughness) will decrease, thereby leading to an associated increase in growth.

Chapter 3 describes population and within-plant variation in mullein's defensive chemistry against insect herbivores under natural conditions, and in relationship to attack by chewing herbivores. This is the first work to explore the chemical ecology of the iridoid glycosides aucubin and catalpol in mullein, and represents one of only a few studies to link patterns of defense investment to patterns of herbivory in wild populations of an invasive plant. In addition to describing how iridoids shape introduced mullein's ecological interactions with insect herbivores, this chapter provides a test of optimal defense theory. This theory predicts that costly defenses are optimally deployed in plants based on 1) the value of particular tissues to the plant and 2) the probability that different tissues would be attacked by herbivores in the absence of defense. Here, I evaluated whether young leaves are better defended than old leaves, which is expected based upon their greater potential lifetime contribution to fitness via photosynthesis and their high nitrogen content (Harper 1989). If young (valuable) leaves are highly defended and in turn sustain minimal attack from generalist herbivores (which cannot overcome chemical defenses to the extent that specialists present in the native range can), it suggests a mechanism for increased plant performance.

Chapter 4 further examines how optimal defense theory might be applied within the novel context of invasions by comparing within-plant variation in defenses in native and introduced mullein populations. Invasions provide an excellent system to explore how defense investment changes in response to shifts in the identity of herbivores attacking plants in their introduced ranges. Generalists, which are not tightly coevolved with the many hosts upon which they feed, are often effectively

deterred by chemical defenses. In contrast, many coevolved specialists are undeterred by these same chemicals, and in fact use them as oviposition cues and feeding stimulants. This imposes an "evolutionary dilemma" because generalists and specialists exert opposing selection pressure on plant investment in chemical defense. Thus a key combined prediction of optimal defense theory and the evolutionary dilemma model is that defense levels of young and old leaves should track the relative importance of specialist and generalist herbivores in the community. Accordingly, if specialists dominate, defenses that they use as attractants should be selected against, while if generalists dominate, those same defenses should be selected for. The predicted result is that plants growing in the introduced range will be released from stabilizing selection on defenses, allowing them to highly defend young, valuable leaves against generalist herbivores. If this is the case, it provides a mechanism by which introduced plants may exhibit fitness gains in their new range, not by increasing or decreasing overall investment in defense (as is often hypothesized), but simply by optimizing the distribution of defenses in relationship to the value of different plant tissues.

*References associated with the statements made in the preface can be found throughout the subsequent research chapters.

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CHAPTER 1

A BIOGEOGRAPHIC COMPARISON OF *VERBASCUM THAPSUS* ECOLOGY

REVEALS DIFFERENCES IN PERFORMANCE, HERBIVORY, AND

SURROUNDING PLANT COMMUNITY

OVERVIEW

It is often assumed that introduced individuals or populations perform better than

their native counterparts. However, there are relatively few biogeographic

comparisons that evaluate differences in the performance and ecology of populations

in their native and introduced ranges. It thus remains difficult to gauge whether the

assumption of increased performance generally holds, and if so, to attribute such

increases to ecological differences between ranges. We tested the assumption that

performance of introduced populations is higher than that of native populations using

Verbascum thapsus (Scrophulariaceae, common mullein), an introduced weed in

North America. We further evaluated evidence for escape from natural enemies, and

assessed whether resource availability (i.e., precipitation and bare ground) and co-

occurring vegetation differ between ranges. Introduced (western U.S.) mullein

outperforms native (European) mullein at both the population (stand density and size)

and individual (leaf number) scales. Introduced plants have escaped from several

herbivore guilds, but two guilds (a specialist thrips and grasshoppers) are more

prevalent on introduced plants. Despite this, introduced plants incur less chewing

damage than natives. There are also pronounced differences in precipitation, bare

ground, and the abundance and identity of vegetation that co-occurs with mullein in

each range. In particular, while water appears limiting to mullein in the western U.S.

portion of its introduced range, it is less limiting than the higher abundance of

vegetation with which it co-occurs in its native range. These data suggest that the

increased performance of introduced mullein is associated with both enemy escape

and a shift in the precipitation regime that leads to a reduction in potential

competitors. Such data highlight the need to design subsequent experiments that test

multiple alternative hypotheses to explain invasive behavior in introduced plants.

INTRODUCTION

A fundamental assumption of research on biological invasions is that introduced individuals or populations actually perform better than their native counterparts, i.e., that they are indeed 'invasive' (Hufbauer and Torchin 2007). While this is likely to be the case for extremely problematic species such as the red imported fire ant (*Solenopsis invicta*) or cheatgrass (*Bromus tectorum*), there are currently relatively few biogeographic data from natural populations to determine whether this assumption generally holds (Hierro et al. 2005). Indeed, recent work by Firn et al. (2011) comparing native and introduced populations of species that are not particularly problematic invaders showed that a broad range of patterns exist, including introduced populations performing better, similarly, or worse than native populations. Such variability highlights the long-acknowledged uncertainty associated with the potential outcomes of introduction events (Williamson and Fitter 1996, Mack et al. 2000). One way to minimize this uncertainty is to link comparisons of performance with measurements of how the biotic or abiotic environment differs between ranges (e.g., Ebeling et al. 2008, Cripps et al. 2010). By doing so, we can increase our understanding of which factors contribute to invasive behavior when it does exist.

Despite the acknowledged system-specific variability in mechanisms that underlie invasion (Gilpin 1990, Lodge 1993), a growing body of research reveals that shifts in interactions with higher trophic levels and availability of resources (along with its inverse, the strength of competitive interactions) often regulate patterns of invasion (Mack et al. 2000, Maron and Vilá 2001, Shea and Chesson 2002,

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Theoharides and Dukes 2007). In particular, there is evidence that introduced populations benefit from two main changes in their environment. First, they often experience release from top-down population regulation (enemy release; Elton 1958, Keane and Crawley 2002) and second, they take advantage of resource-rich sites (Hobbs and Huenneke 1992, Burke and Grime, 1996, Davis et al. 2000) or sites with few competitors (areas with low biotic resistance; Levine 2004).

Enemy release occurs when introduced populations escape from natural enemies, particularly co-evolved specialists, which in turn 'releases' them from top-down suppression (Elton 1958, Keane and Crawley 2002). Therefore, a first step in determining whether enemy release is a viable mechanism of invasion is to document natural enemy communities and levels of attack associated with populations in each range. Indeed, there is strong evidence that enemy escape is common across taxa (e.g., Memmott et al. 2000, Wolfe 2002, Torchin et al. 2003, Torchin and Mitchell 2004, Norghauer et al. 2011). However, patterns of enemy escape are not simple. In plants, the focus of our research, introduced populations often escape specialist herbivores, but may still be limited by generalists (Müller-Schärer et al. 2004; Parker et al. 2006). Because data on the composition of enemy communities *and* performance across both native and introduced populations are scant (but see Wolfe 2002, Vilá et al. 2005, Ebeling et al. 2008, Cripps et al. 2010), the degree to which escape (or a shift from specialists to generalists) is linked to invasiveness remains unclear.

A number of studies have indirectly evaluated enemy release by measuring evolutionary shifts in herbivore defense phenotypes of native and introduced

populations (e.g., tests of the *evolution of increased competitive ability* [EICA] hypothesis, Blossey and Nötzold 1995). The expectation is that introduced populations will be less well defended than natives because selection should favor the loss of costly defenses under herbivore-free (or at least depauperate) conditions. The results of such experiments show a range of evolution in defense phenotypes, including the expected decrease in defense investment (Siemann and Rogers 2001, Blair and Wolfe 2004), no change (Genton et al. 2005, Hull-Sanders et al. 2007), and actual increases in some defenses (Joshi and Vrieling 2005, Ridenour et al. 2008).

Enemy exclusion experiments also reveal that the strength of top-down regulation ranges from strong to weak depending on the system (DeWalt et al. 2004, Lewis et al. 2006, Franks et al. 2008, Williams et al. 2010). For example, DeWalt et al. (2004) confirmed that the expansion of *Clidemia hirta* L. (Melastomataceae) into forest understories in its introduced range of Hawaii is facilitated by its release from both pathogens and herbivores. In contrast, Williams et al. (2010) found that excluding enemies made only a minor contribution to increased growth rates (λ) in introduced populations of *Cynoglossum officinale* L. (Boraginaceae) relative to the much larger effect of increased resource availability.

As highlighted by the findings of Williams et al. (2010), a second main factor that can strongly shape invasion dynamics is resource availability. For example, the invasive status of some species can be explained simply by their introduction to a particularly benign environment, without needing to invoke explanations based on enemy release (e.g., Cripps et al. 2010). Additionally, there is strong evidence that disturbances such as fire and flooding promote invasion by adding resources, often in

the form of space or nutrients, to a system (e.g., Davis et al. 2000, Hierro et al. 2006, Fornwalt et al. 2011). Conversely, competition from natives, especially in diverse and functionally intact communities, can create biotic resistance to invasions (Levine 2000, Corbin and D'Antonio 2004, Levine et al. 2004, Hooper and Dukes 2010). However, while there are many studies that demonstrate the importance of resource availability in facilitating or suppressing the expansion of introduced populations, the critical data on range-level differences in resource availability are only just beginning to come to light (e.g., Bastlová-Hanzélyová 2001, Hierro et al. 2006, Cripps 2010, Williams et al. 2010).

  This research has three goals. First, we test the assumption that performance of introduced populations is higher than that of native populations using the herbaceous plant *Verbascum thapsus* L. (Scrophulariaceae, common mullein). This plant is considered troublesome in specific habitat types (Fornwalt et al. 2010), but generally is not thought of as one of the most problematic invaders (Gross and Werner 1978, Gross 1980, Reinartz 1984). Furthermore, it has a weedy habit in its native range. Thus it was not apparent from the outset whether performance would be higher, comparable, or lower in the introduced range relative to the native range. Second, we evaluate evidence for escape from natural enemies and a shift in the community of enemies (which we predicted would be more dominated by generalists in the introduced relative to the native range). Third, we assess whether resource availability (in particular precipitation and space) and vegetative cover (as a proxy for competition) differ between ranges and evaluate how they are related to population performance in each range.

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METHODS

Study system

Common mullein is a (typically) biennial forb native to Eurasia. It was
repeatedly introduced to North America, first in the 1600s by Puritan settlers who
planted it in their herb gardens because of its medicinal properties, and later by
English and German settlers in Appalachia for its effectiveness as a piscicide
(Wilhelm 1974, Gross and Werner 1978, Mitich 1989). Mullein's well-documented
ethnobotanical history and the timing of its invasion supports the contention that
Europe was the source of the introduction, as there were few trade connections
between Asia and the U.S. in the 17th century (Gumport and Smith 2006). Mullein
populations now occur in several Canadian provinces and all 50 U.S. states, with
noxious status in Colorado, Hawaii, and South Dakota.

Mullein recruits exclusively from seed following canopy-clearing
disturbances and, in the introduced range, likely depresses recruitment by co-
occurring natives in early-seral communities (Pitcairn 2000, Alba pers. obs.). It
typically grows in open sites with dry, sandy soils (Gross and Werner 1978), but it
has wide climatic tolerances, enabling it to invade high-elevation communities in
California (Parker et al. 2003) and Hawaii (Ansari and Daehler 2010). Although
mullein is widespread and locally common, it infrequently dominates sites for long
periods and thus is not often considered a management priority. Nonetheless, large
and dense infestations can persist for multiple generations in the introduced range,
especially following fire (Fornwalt et al. 2010) or in areas subject to chronic
disturbance (Alba, pers. obs.).

The few available data suggest that mullein escaped a suite of specialist herbivores upon introduction to North America. These include up to 8 species of weevil (Popov 1972, Gross and Werner 1978) that are reported from part of the native range (Poland) and the leaf-feeding larvae of *Cucullia verbasci* L. (Noctuidae) (Maw 1980). However, it is still attacked by a specialist thrips (*Haplothrips verbasci* Osborn) and seed-feeding weevil (*Rhinusa tetra* Fabricius), which were co-introduced to the new range. It is unclear whether introduced mullein has partially escaped from the co-introduced thrips and weevil in terms of their abundance (as has been reported in other systems; Memmott et al. 2000, Wolfe 2002).

In a previous test of the *evolution of increased competitive ability* (EICA) hypothesis, we found that although introduced mullein has evolved to be larger, investment in both chemical and structural defense does not significantly differ between ranges (Alba et al. 2011). The current biogeographic comparison provides the opportunity to more fully evaluate alternative hypotheses to explain these patterns (see Discussion).

Sampling design

We sampled 51 populations (21 in the native range and 30 in the introduced range) to estimate stand size; plant size (number of leaves and diameter); damage to plants by chewing herbivores; the proportion of plants hosting several insect groups; and bare ground and plant cover (a proxy for competition) adjacent to mullein plants. We also included an additional 5 populations from the native range (FR1, FR2, FR3, FR4, FR5) in the estimate of population density (Table 1). It was not feasible to

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randomly sample from the entire native and introduced ranges of this widespread

species. In the introduced range our sampling focused on several states spanning a

north-to-south gradient in the western U.S., and results thus pertain to this broad

region. Within the native Eurasian range, we focused on Europe rather than Asia to

encompass the likely provenance of introduction (Wilhelm 1974, Gross and Werner

1978, Mitich 1989). We further aimed to capture a wide range of habitats and climatic

conditions by sampling broadly across Europe (Table 1).

All populations were sampled during the 2010 growing season. Populations

were defined as discrete stands located at least 1 km away from adjacent stands, with

the exception of Sweden, where the 4 sampled populations were spaced along a trail

that was approximately 3 km in length. We aimed to sample 20 plants per population,

although due to time and weather constraints (in the introduced range) and small

population sizes (in the native range), this was not always possible (Table 1). We

sampled low-latitude populations in the introduced range (Utah and Colorado) from

22 May to 3 June; all European populations were sampled from 11 June to 2 July;

higher latitude populations in the introduced range (Wyoming, Montana, Idaho) were

sampled from 15 July to 28 July. Some populations were in the rosette stage, while

others were bolting. The two phenological stages were evenly distributed across

sampled populations (native range: n = 10 rosette stage, n = 11 bolting stage;

introduced range: n = 12 rosette stage, n = 18 bolting stage). Differences in

phenology did not qualitatively affect the model outcomes for any response variable

(see *Statistical analysis*).

Sampling protocol

Mullein performance

We estimated two metrics of population performance (population size and plant density) and two metrics of plant performance (number of leaves and plant diameter). To delineate population size, we assigned each stand to one of the following categories: < 20 plants, 21-100 plants, 101-500 plants, or > 500 plants. To estimate population density, we ran 1 or 2 transects through the longest extent of the population. The size of the patch determined the number (1 or 2) and length (5 to 20 m) of transects required to provide adequate coverage of the population. The length of each transect was walked and all plants falling within 1 m of each side were counted. In two cases (populations P1 and WY2), plants were so few and widespread as to make transects unfeasible. For these, we counted the total number of plants in the area and made a conservative visual estimate of the patch size. We selected target plants for leaf counts and measurements of diameter by throwing a pen in the air and following the direction of the pen tip until we hit the closest mullein individual (we walked a minimum of 2 m when populations were large enough to accommodate this).These same plants were used to measure herbivory and adjacent plant cover (detailed below)

Presence of insects and herbivore damage

For each plant, we documented the presence of several insect groups including caterpillars, snails, leafhoppers, aphids, weevils, grasshoppers, and thrips. We also collected specimens of each group for later identification. To estimate chewing

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damage, we used the following scoring system (after Lewis et al. 2006): 0 = no damage; 1 = minimal damage with no more than about 5% of any leaf damaged; 2 = minimal damage plus some leaves with 5-10% damage; 3 = 10-50% damage on multiple leaves, but fewer than half of all leaves affected; 4 = at least half of all leaves with 10-50% damage, and multiple leaves with more than 50% damage.

Precipitation, bare ground, and vegetation

We gathered long-term annual precipitation data for all populations to a) explore the relationship between precipitation and population performance in each range and b) to include in our assessment of the effects of co-occurring vegetation on mullein performance (see next paragraph). Data for native populations was obtained from Weatherbase (http://www.weatherbase.com/) and data for introduced populations was gathered from the Western Regional Climate Center (http://www.wrcc.dri.edu/). We searched for the closest weather stations using each population's GPS coordinates.

To estimate differences in bare ground and plant community composition between ranges, we placed a Daubenmire frame directly adjacent to each plant in the north and south directions and estimated the cover of bare ground, forbs, grasses, shrubs, mosses, rocks, and litter to the nearest five percent. We also used cover measurements to estimate the effect of bare ground and plant cover on mullein population performance in each range.

Statistical analysis

Mullein performance

We used SAS (v. 9.1, SAS Institute, Cary NC) for all statistical analyses. To test whether native and introduced populations differ in density, leaf number, and plant diameter we used analysis of variance. The original models included continent as a fixed effect and latitude and phenology as covariates. We dropped phenology from all the models because it did not contribute to a significant amount of variation in the response variables. We retained latitude only for the plant density model, as it explained a marginally significant proportion of the variation ($P = 0.09$) and lowered the model AIC value (cf. Colautti et al. 2009 for a discussion of the importance of accounting for latitudinal clines in biogeographic comparisons). Density and diameter were square root transformed to improve normality and homogeneity of variance.

To determine whether the frequency of mullein populations belonging to each of four size categories (< 20 plants, 21-100 plants, 101-500 plants, or > 500 plants) significantly differed, we performed a Chi-square test of independence using the frequency procedure with the chi square option. We included phenological stage, i.e., rosette or bolting, in the table construction to control for variation due to life stage (however, the results were qualitatively similar regardless of the inclusion of phenology).

Presence of insects and herbivore damage

We used chi-square tests of independence to determine whether 1) the proportion of native and introduced plants that harbored various insect guilds differed

and 2) the frequency distribution of damage scores differed between ranges. We used the same procedure as described above for the analyses of population size classes. We also ran a series of regressions to explore whether there was any relationship between mullein performance (population density, leaf number, and plant diameter) and level of insect herbivory, but there were no significant relationships and we did not explore this line of inquiry further.

Precipitation, bare ground, and vegetation

We tested for differences in precipitation between ranges using a mixed model ANOVA with continent as a fixed effect and population within continent as a random effect. We used the same model to test for differences in percent cover of bare ground, vegetation (composed of forbs, grasses, shrubs, and mosses), rocks, and litter adjacent to mullein. Precipitation data were square root transformed and cover variables were arcsine-square root transformed as needed.

Influence of precipitation, bare ground, and vegetation on mullein performance

We used regression analysis to explore the relationship between precipitation and mullein population density separately by range. We conducted outlier analysis by generating studentized residuals and removed one data point from the introduced range (population B1 in Utah) that had a residual of 5.3 (with outliers defined as > 2.5; Rawlings et al. 1998). The removal of this data point is biologically, as well as statistically, warranted. Sixty percent of the individuals at this site were small seedlings that had recently germinated during the spring rains. Because we sampled

this particular population right after a flush of germination, the number of individuals present was inflated relative to what would be expected following attrition due to density-dependent intraspecific competition. (Note that we also ran the plant density model with and without this data point, and the range-level difference was significant in both cases.)

We explored whether bare ground or plants that co-occur with mullein in its native and introduced ranges affect mullein performance (i.e., mullein plant density). Because precipitation differs strongly between the native and introduced ranges (see Results), and is likely to influence density, we first statistically removed the effect of precipitation on density by generating the residuals of density on precipitation using regression analysis. We then evaluated whether bare ground and total plant cover explained the residual variation in density. We analyzed these data separately for the native and introduced ranges. We also analyzed the effect of co-occurring plants on mullein density using a multiple regression with bare ground/cover and precipitation as covariates; this approach produced qualitatively similar results (data not shown; cf. Freckleton 2002).

RESULTS

Mullein performance

Native mullein populations were significantly less dense (Figure 1a; continent effect, $F_{1,48} = 6.65$; $P = 0.01$; latitude effect, $F_{1,48} = 3.03$; $P = 0.09$) and smaller (Figure 1b; Chi-square = 14.3; $P = 0.0025$) than introduced populations. Indeed, the maximum population density in the native range was 4.8 plants/m^2 while in the

introduced range it was 28 plants/m². Additionally, plants growing in the native range had significantly fewer leaves (Figure 2a; continent effect, $F_{1,48} = 51.1$; $P < 0.0001$; latitude effect, $F_{1,48} = 2.51$; $P = 0.12$) and diameters that tended to be smaller, although the difference was not significant (Figure 2b; continent effect, $F_{1,48} = 2.5$; $P = 0.12$; latitude effect, $F_{1,48} = 0.94$; $P = 0.34$).

Presence of insects and herbivore damage

The relative proportion of native and introduced plants that harbored insect enemies varied by taxonomic group. The proportion of native plants with weevils (Chi-square = 11.5; $P = 0.0007$), caterpillars (Chi-square = 23.5; $P = < 0.0001$), leafhoppers (Chi-square = 3.8; $P = 0.05$), aphids (Chi-square = 49.9; $P = < 0.0001$), and snails (Chi-square = 53.6; $P = < 0.0001$) was significantly greater than that in the introduced range. Conversely, more introduced plants had thrips (Chi-square = 176.9; $P = < 0.0001$) and grasshoppers (Chi-square = 8.4; $P = 0.004$).

The frequency distribution of damage scores significantly differed by range (Chi-square = 55.5; $P < 0.0001$). The main difference in the distributions was due to the large proportion (38%) of native plants with a high damage score of 3, a proportion twice that of introduced plants (18%; Figure 4). Additionally, the proportion of native plants with no damage was about half that (7%) of introduced plants (12%), although in both ranges there were few plants that completely escaped feeding by chewers (Figure 4).

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Precipitation, bare ground, and vegetation

Average annual precipitation was significantly greater at sites sampled in the native range (69.6 cm $\pm$ SE 4.8; range 47-111 cm) than those sampled in the introduced range (33.3 cm $\pm$ SE 1.2; range 27-45 cm; $P = 0.01$) based on records from weather stations that averaged 60.2 km $\pm$ SE 13.6 from native populations and 22.8 km $\pm$ SE 3.6 from introduced populations.

Bare ground and total plant cover (including forbs, grasses, shrubs, and mosses) were significantly higher in the native range than the introduced range (bare ground, $P = 0.04$; total plant cover, $P = 0.0001$; Table 2). In contrast, the amount of litter and rocks was significantly greater in the introduced range (illustrating why it is that bare ground and vegetation cover are not simply the inverse of each other). Breaking the vegetation into its components, the native range had a higher percent cover of forbs than the introduced range, but a lower cover of grasses.

Influence of precipitation, bare ground, and vegetation on mullein performance

In the native range, mullein density shows no relationship ($R^2 = 0.05$; $P = 0.4$) to precipitation levels ranging from 47 to 111 cm per year. Conversely, in the introduced range, where average annual precipitation ranges from 27 to 45 cm per year, mullein density significantly increases with greater precipitation ($R^2 = 0.25$; $P = 0.005$; Figure 5, a and b).

The density of native mullein populations has a significantly positive relationship with bare ground and a significantly negative relationship with plant

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cover, while the density of introduced mullein shows no relationship to bare ground or plant cover (Figure 5).

DISCUSSION

Mullein performance

Introduced mullein performs significantly better than native mullein at both the population and individual scales (Figures 1 and 2), revealing that even when introduced species infrequently dominate their new communities, they can still exhibit pronounced increases in performance. Indeed, introduced mullein populations are on average five times more dense than those in the native range, and populations are not only more dense, but larger (Figure 1b). Individual plants are also more robust in the introduced range, averaging twice as many leaves as their native counterparts (Figure 2a).

Our findings of increased performance are in accordance with others who have found that, at least on a species-specific basis, introduced populations tend to outperform their native counterparts. This pattern has been observed for several metrics including population size and density, plant size, fecundity, and seedling recruitment (e.g., Buckley et al. 2003, Paynter et al. 2003, Erfmeier and Bruelheide 2004, Jakobs et al. 2004, Ebeling et al. 2008, Beckmann et al. 2009, Herrera et al. 2011), although within a species it is not uncommon for increases in one performance metric to be accompanied by no change or even decreases in other metrics (Edwards et al. 1998, Vilá et al. 2005, Lewis et al. 2006). Additionally, because researchers often target species that are known to be problematic in the introduced range (Hawkes

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2007), the extent to which species-specific findings can be extrapolated to all introductions is unclear. Because the phenomenon of increased performance is not universal (Thébaud and Simberloff 2001, Cripps et al. 2010, Firn et al. 2011), it is important to confirm whether a shift has occurred before moving on to conduct mechanistic research on a given species.

Presence of insects and herbivore damage

Our data on natural enemies suggest that there has been a complex shift in the herbivore community on introduced mullein (Figure 3) accompanied by a reduction in the severity of attack by leaf chewers (Figure 4). Overall, introduced mullein exhibits partial or complete escape from 5 herbivore guilds (caterpillars, weevils, snails, leafhoppers, and aphids) and an increase in 2 guilds (a specialist thrips [*Haplothrips verbasci*] and grasshoppers).

Rather than showing complete escape from all of its co-evolved specialists, mullein shows variation in escape that ranges from complete (*C. verbasci*), to partial (seed-feeding weevils), to an actual increase in prevalence of *H. verbasci*. Indeed, the abundance of thrips on introduced mullein is up to 100-fold that observed in the native range (Alba and Hufbauer, unpublished data). Other studies have reported the presence of accidentally co-introduced specialists (Wolfe 2002, Memmott et al. 2000), but in these cases the specialists did not achieve the high abundances that we observed. It is possible that attack by *H. verbasci* has kept mullein from being an even more problematic invader than it is; however, its presence does not completely offset the increased performance that mullein exhibits in its introduced range.

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We also found that shifts in the generalist community are pronounced and generally indicate escape, with the exception of grasshoppers, which are more prevalent on introduced plants and cause the majority of chewing damage that we observed (Figure 3; Alba, pers. obs.). However, despite their increased prevalence, grasshoppers have only partially filled the role of the leaf-feeding specialist *C. verbasci*, as evidenced by the significant decrease in damage incurred by introduced plants. In sum, our findings of decreased damage suggest that enemy escape has occurred in this system, a pattern consistent with the findings of others who have estimated leaf damage in a biogeographic context (Vilá et al. 2005, Adams et al. 2008, Ebeling et al. 2008).

These data on enemy escape provide an interesting insight into our previous work (Alba et al. 2011; see Methods). Briefly, we found that although introduced mullein has evolved to be larger than native mullein, this increase is not accompanied by a loss of defense (as predicted by the EICA hypothesis). We hypothesized two reasons for this pattern (Alba et al. 2011). First, such findings could reflect that mullein experiences similar types and levels of herbivory in each range (i.e., there is no enemy escape), indicating that selection pressures on defense do not differ between ranges. Second, it could be that mullein did in fact escape from its natural enemies, but that this ecological shift did not elicit an evolutionary response in defense investment. Our current findings support the second hypothesis. A lack of evolutionary response could be seen for many reasons: in the case of mullein, we found little evidence of trade-offs between mullein's ability to invest in growth and defense (i.e., defenses were not measurably costly; Alba et al. 2011). Taken together,

these findings suggest that even though mullein has escaped (at least to some degree) its natural enemies, there was not a strong evolutionary response to reallocate resources from growth to defense.

Precipitation, bare ground, and vegetation

Precipitation is significantly higher in the native range sites than the introduced range sites. However, despite relatively low water availability, introduced populations and individual plants perform better than their native counterparts. This is somewhat surprising given that water limitation is considered a universal stress to plants (Chaves et al. 2002, Smith and Griffiths 1992), and indeed we found a signal of such stress within the introduced range, but not the native range (see next section).

Our comparisons of bare ground and vegetation confirm that there are range-level differences in both, which could translate into plastic or evolved responses to altered resource availability. Overall, vegetation is significantly less abundant in the introduced range, a pattern that is consistent with the findings of low precipitation. However, the relatively sparse vegetation did not directly translate into increased bare ground due to high amounts of litter and rock, and in fact bare ground is more common in the native range (Table 2). These findings suggest that although introduced mullein may experience reduced competition from neighboring plants, it does not benefit from increased availability of bare ground (e.g., as available space for recruitment). The overall reduction in vegetation is accompanied by a shift in the composition of competitors from forb- to grass dominated (Table 2). Additionally,

because mullein is more dense in the introduced range (Figure 1a), the forbs that do grow nearby are more likely to be conspecifics.

We are aware of only two other studies that have documented range-level differences in bare ground and vegetation associated with invasive plants. Similar to our findings, Bastlová-Hanzélyová (2001) reported that introduced populations of *Lythrum salicaria* occurred in sites with sparser vegetation than native populations, and often formed monocultures, which could increase the intensity of intraspecific competition. Conversely, Cripps et al. (2010) found no range-level difference in the percent cover of forbs and grasses co-occurring with *Cirsium arvense*, nor were there differences in the density of native and introduced populations, suggesting that the strength of intraspecific competition, as well as the overall identity of competitors, is similar between ranges. They also reported that bare ground was greater in the native range; however, while bare ground is known to provide important microsites for *C. arvense* recruitment (Edwards et al. 2000), it did not explain variation in population performance (in contrast to our findings; see next section).

Influence of precipitation, bare ground, and vegetation on mullein performance

We found that in the native range, where precipitation is relatively high, mullein does not appear to be limited by water availability (Figure 5a). In contrast, there was a strong positive relationship between mullein density and precipitation in the introduced range, where overall precipitation levels are quite low. These patterns suggest that although water availability limits introduced populations, it is less

limiting than the factors that regulate mullein (e.g., enemies or low light conditions) in its native range.

In addition to quantifying differences in bare ground and vegetation between ranges, we evaluated whether these factors affect mullein performance. Given mullein's life history characteristics, we expected both bare ground and vegetation to influence population density. In particular, because mullein recruits only from seed and requires disturbance and high levels of light in order to establish (Gross and Werner 1982, Reinartz 1984), we hypothesized that density would increase with more bare ground, and decrease with greater abundance of vegetation. These patterns were indeed observed for native populations (Figure 5), suggesting that bare ground represents an unused resource (e.g., safe sites for recruitment) and that co-occurring vegetation does competitively suppress mullein. In contrast, the density of introduced populations showed no relationship to bare ground or vegetation. It thus appears that in the semi-arid western U.S., beyond a threshold level of plant establishment, additional bare ground does not represent a usable resource. Moreover, the low abundance of co-occurring vegetation may be insufficient to directly limit (through interference competition) mullein's ability to establish (c.f. the maximum of 55% cover, Figure 5). We suggest that instead, water limits both mullein and co-occurring vegetation in the introduced range to the extent that the main competitive interaction is exploitation competition for water (Grace and Tilman 1990). It is widely reported that mullein performs well in dry, sandy soils, but that, being a low-statured rosette in its first year, is highly intolerant of shade (Gross and Werner 1978, Reinartz 1984). These aspects of its biology are consistent with mullein's increased performance in

the semi-arid western U.S., where primary production is relatively low and the plant canopy is sparse. Indeed, expansive and dense infestations can last for more than 10 years following fire (Alba, pers. obs.), a longevity that is not reported in the eastern U.S. (Gross 1980), where precipitation levels and competition for light likely reflect that of the native range.

In summary, we have shown that an introduced species considered to be relatively benign exhibits pronounced shifts in its performance and ecology following introduction to a new range. The data describing enemy escape, resource availability, and competition suggest putative roles for both biotic and abiotic variables in facilitating mullein's invasiveness. Such data highlight the importance of designing subsequent experiments that test multiple alternative hypotheses regarding invasion, especially with regard to enemy escape and how it may act synergistically with, or be secondary to, other important shifts in a species' ecology following an introduction event.

Table 1.1 Sampling locations for comparisons of performance, herbivory, and competition

Population Code	Country/State	Plants Sampled	Latitude (N)	Longitude (E)	Elevation (m)	Mean Annual Precipitation (cm)
Native (EU)						
BE1	Belgium	22	51.039483	5.757936	31	82.0
BE2	Belgium	15	50.927214	4.424692	16	81.0
P1	Czech Rep.	13	50.181333	13.415575	346	110.6
P2	Czech Rep.	6	49.999106	14.559139	300	47.0
P3	Czech Rep.	8	49.874744	14.437797	254	66.7
P4	Czech Rep.	11	50.785525	14.458558	423	47.0
P5	Czech Rep.	12	50.064025	14.485831	228	47.0
LR	France	5	43.888381	3.56825	551	73.0
MO	France	6	43.672003	3.864839	51	70.0
NA	France	6	42.430811	1.977811	1210	67.0
SJ	France	4	43.587011	3.828942	50	70.0
*FR1	France	N/A	43.698197	3.853719	67	70.0
*FR2	France	N/A	43.677228	3.85595	67	70.0
*FR3	France	N/A	43.93055	3.702453	67	70.0
*FR4	France	N/A	43.725167	3.794783	67	70.0
*FR5	France	N/A	43.701794	3.854169	67	70.0
MA1	Macedonia	10	41.297903	21.097853	674	49.0
MA2	Macedonia	10	41.062711	21.174872	946	49.0
SE	Serbia	10	44.572944	20.560178	180	65.8
SW1	Sweden	4	60.258389	17.636114	41	54.0
SW2	Sweden	12	60.259086	17.656744	41	54.0
SW3	Sweden	2	60.251239	17.669442	41	54.0
SW4	Sweden	3	60.2422	17.682306	41	54.0
CH1	Switzerland	20	47.359325	7.364636	416	107.0
CH2	Switzerland	5	47.335256	7.115103	695	107.0
CH3	Switzerland	7	47.341156	7.317797	442	107.0
Introduced (US)			Latitude (N)	Longitude (W)		
BC	CO	20	40.800472	105.379528	2252	38.4
HG	CO	20	40.701733	105.314656	2092	41.8
HT	CO	20	40.539767	105.178628	1784	38.4
LO	CO	20	40.650061	102.741167	1269	45.8
LOR	CO	20	40.583006	105.174506	1671	38.4
SL	CO	15	40.986139	107.381667	2041	25.6
ID	ID	10	44.137006	112.222542	1828	30.2
MT1	MT	15	45.686333	110.512472	1444	26.0
MT2	MT	15	45.424972	110.651167	1849	36.8
MT3	MT	15	45.499556	110.547694	1649	36.8
MT4	MT	10	45.673972	110.540056	1427	36.8
MT5	MT	15	46.225694	111.377917	1394	27.3
MT6	MT	10	46.253611	111.297333	1493	27.3
MT7	MT	15	46.286917	111.532	1377	27.3
MT8	MT	15	46.318722	111.629111	1506	27.3
MT9	MT	15	47.404667	111.327917	1135	40.0
MT10	MT	15	47.432056	111.319944	1402	37.1
MTA	MT	15	45.097417	112.776972	1689	33.3
B1	UT	20	38.304111	112.837642	2043	28.3
B2	UT	20	38.419186	112.713906	2043	28.3
B3	UT	20	38.429372	112.643167	2039	30.9
D1	UT	20	40.403056	110.343056	2252	23.2
D2	UT	20	40.211389	110.41	2593	24.0
ST	UT	20	40.182913	111.050444	1765	38.3
WY1	WY	3	41.130667	109.213472	2358	22.8
WY2	WY	16	41.055056	109.352417	2495	22.8
WY3	WY	20	43.429583	110.781583	2129	40.2
WY4	WY	15	43.404	110.751556	1898	40.2
WY5	WY	15	43.386222	110.725833	1928	40.2
WY6	WY	13	43.428861	110.777028	1885	40.2

*These locations used for plant density analyses only

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Table 1.2. Percent cover of bare ground, vegetation, litter, and rocks in plots adjacent to native and introduced mullein plants

	Native Mean (±SE)	Introduced Mean (±SE)	F-statistic	DF	P-value
Bare ground	42.4	24.6	4.3	1,47	0.04
Forbs	32.8	9.6	88.2	1,47	<0.0001
Grasses	9.7	15.0	8.8	1,47	0.005
Shrubs	1.08	1.27	0.7	1,47	0.68
Mosses	1.10	0.23	5	1,47	0.03
Total Vegetation	44.7	26.1	17.1	1,47	0.0001
Litter	11.3	33.5	14	1,47	0.0005
Rocks	1.5	15.4	8.6	1,47	0.005
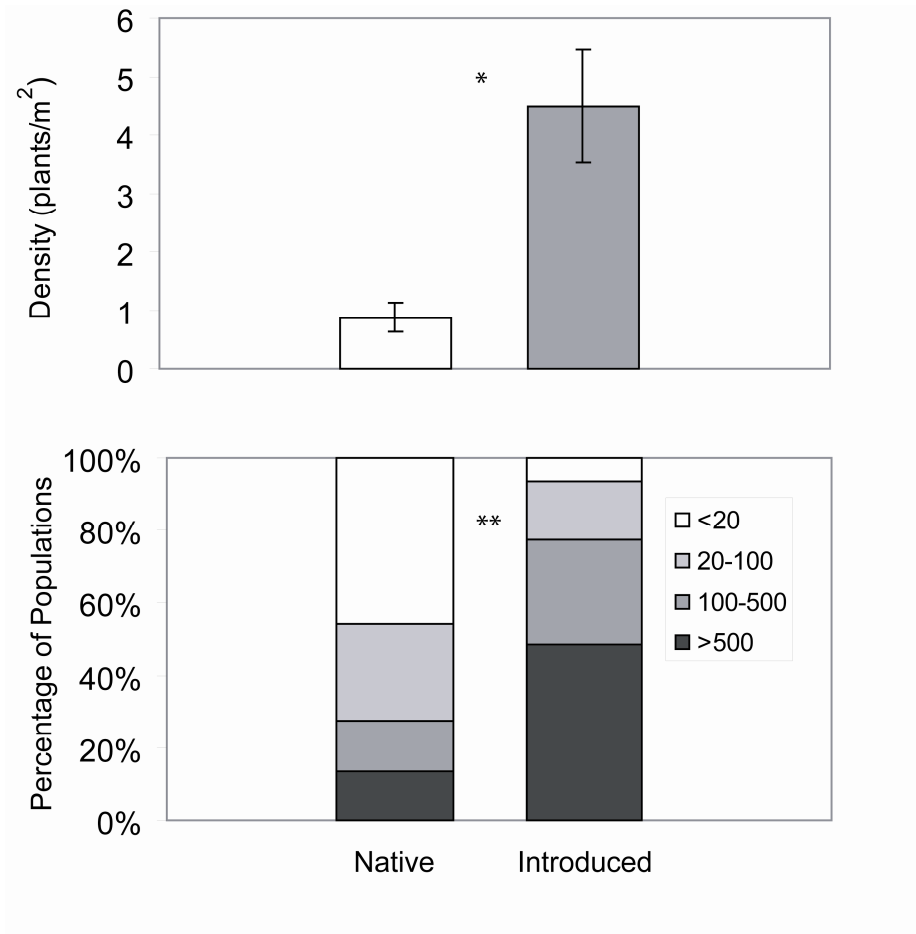
Figure 1.1. (a) Mullein density (mean ± SE) and (b) size distributions of populations present in the native (Europe) and introduced (U.S.) ranges. Single asterisk denotes a significance of $P < 0.05$. Double asterisk denotes a significance of $P < 0.001$.

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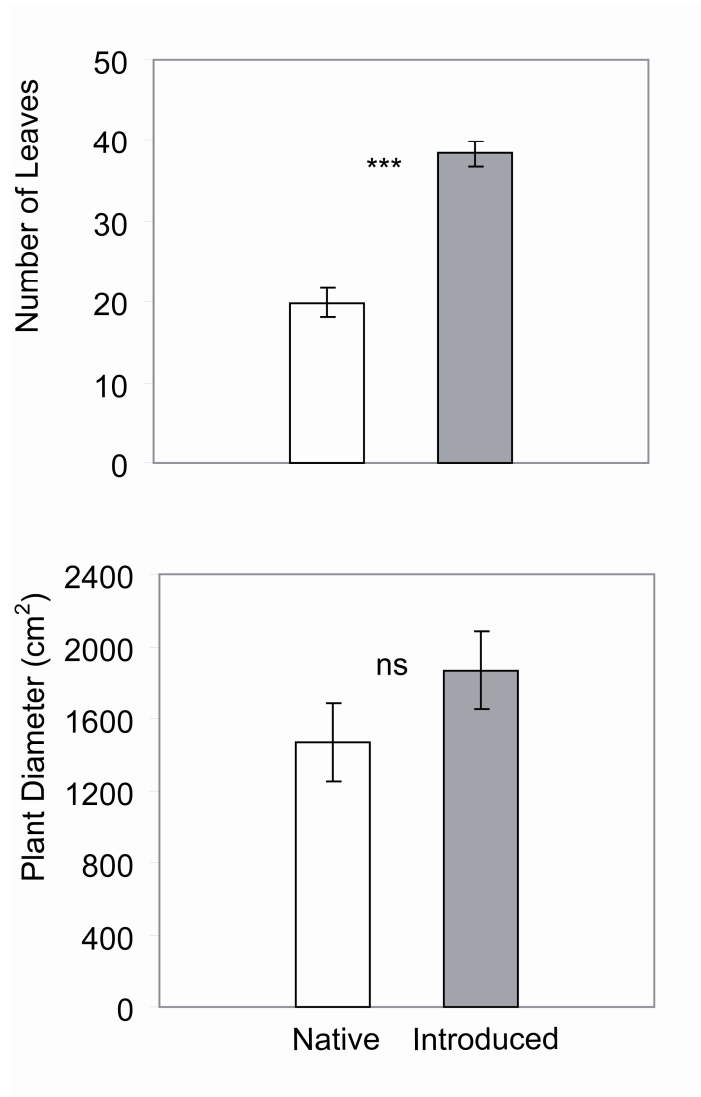
Figure 1.2. Number of leaves (mean ± SE) and diameter (mean ± SE) of mullein plants growing in the native (Europe) and introduced (U.S.) ranges. Triple asterisks indicate a significance of $P < 0.0001$; ns = not significant.
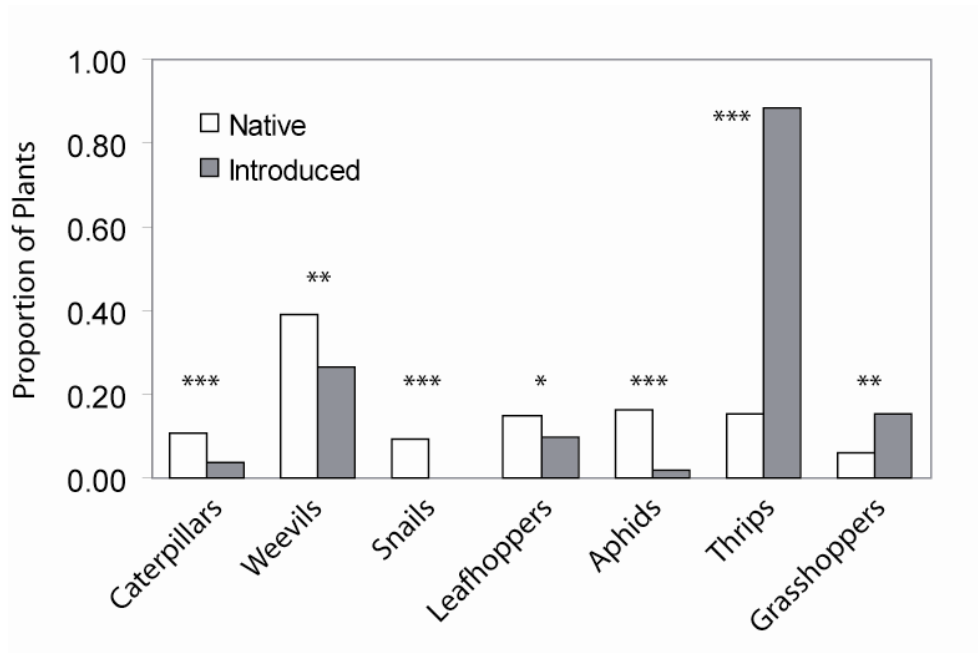
Figure 1.3. Proportion of plants sampled in the native (Europe) and introduced (U.S.) ranges that hosted various insect groups. Triple asterisks denote a significance of $P < 0.0001$; double asterisks denote $P < 0.001$; a single asterisk denotes $P < 0.05$.
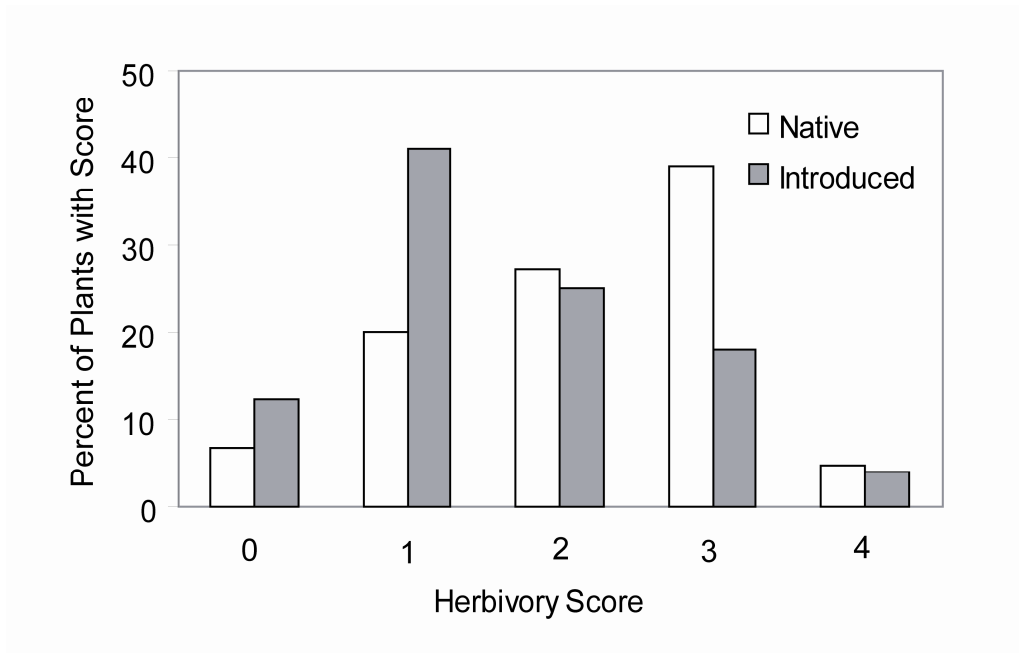
Figure 1.4. Proportion of native (Europe) and introduced (U.S.) plants that exhibit a chewing damage score of 1-4 (see text for details; after Lewis et al. 2006). The frequency distributions of the native and introduced ranges significantly differ (chi-square 55.47; $P < 0.0001$), with native plants experiencing heavy damage (score of 3) about twice as often as introduced plants.
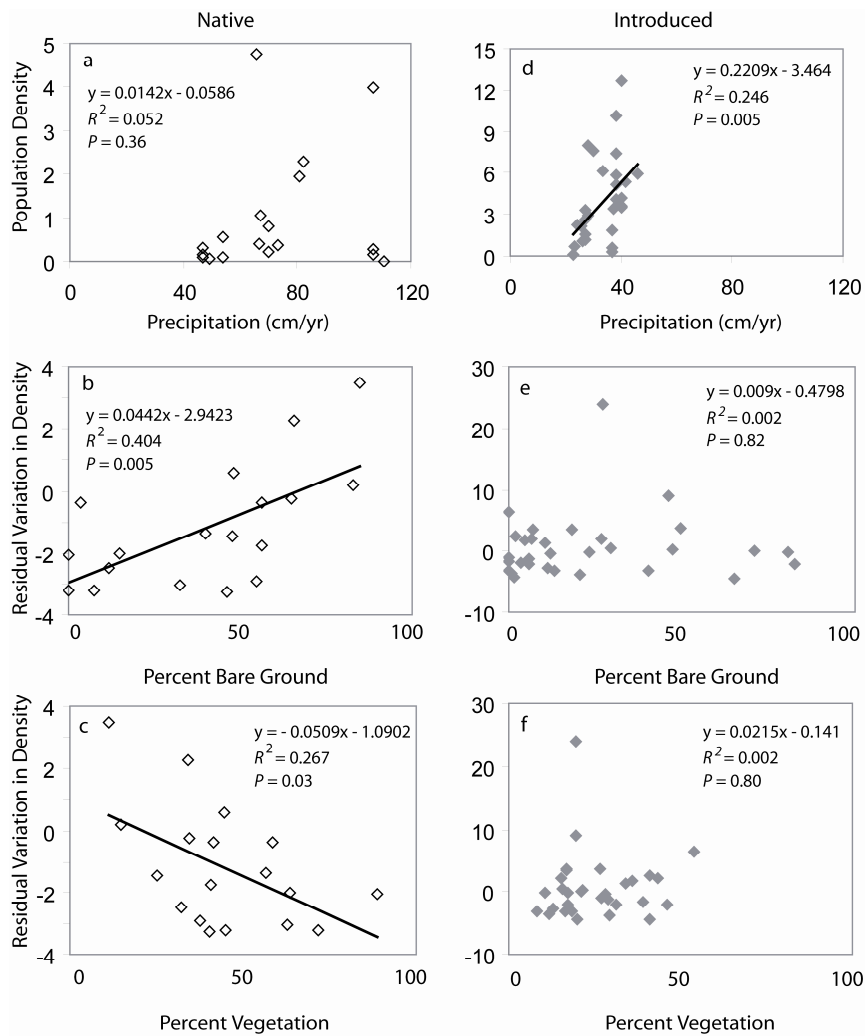
Figure 1.5. Plots of the regression of precipitation on mullein density (top panels) and of the residuals generated by the regression of mullein density on precipitation against percent bare ground (middle panels) and percent total plant cover (bottom panels). In the middle and bottom panels, the Y-axis shows the residual variation in mullein density after removing the confounding effect of precipitation. Regressions represented by open diamonds (panels a-c) are populations from the native (Europe) range. Regressions represented by closed diamonds (panels d-f) are populations from

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the introduced (U.S.) range. Note that the percent total cover in the introduced range

has a maximum of 55%.

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CHAPTER TWO

EVOLUTION OF GROWTH BUT NOT STRUCTURAL OR CHEMICAL

DEFENSE IN *VERBASCUM THAPSUS* (COMMON MULLEIN) FOLLOWING

INTRODUCTION TO NORTH AMERICA

OVERVIEW

Post-introduction evolution of increased growth or reproduction has been observed in many species of invasive plants; however, it is not consistently associated with a loss of defense, as predicted by the influential *evolution of increased competitive ability* (EICA) hypothesis. Inconsistent support for the EICA hypothesis likely reflects the fact that, although invasive plants are released from attack by some enemies, typically specialists, they often do not escape attack from generalists. Thus, different types of defense (e.g., structural versus chemical) may evolve in different directions following introduction. We used a common garden experiment to test whether a shift in allocation among defenses (as opposed to a simple increase or decrease in a single defense) is associated with increased growth in introduced *Verbascum thapsus* populations. Introduced populations had significantly greater shoot biomass than natives. However, root biomass was similar between ranges, and highly variable, resulting in only marginal differences in total biomass. Mean investment in all three defenses was remarkably similar between the native and introduced populations, providing no evidence for range-wide, post-introduction evolution of defense. This finding was consistent with the fact that, despite significant population-level variability for all defenses, there was little evidence of trade-offs between growth and defense or among different types of defense. These results suggest that evolution of increased growth in *V. thapsus* is not fueled by decreased allocation to defense, and that selection on defense may vary more at the population scale than the continental scale.

INTRODUCTION

Invasive species commonly escape many of their natural enemies, leading to a release from top-down population regulation (e.g., Elton 1958, Memmott et al. 2000, Keane and Crawley 2002, DeWalt et al. 2004; but see Colautti et al. 2004, Chun et al. 2010). For invasive plants, release from enemies may alter the selection regime such that particular defenses are no longer under positive selection, and in fact may be under negative selection if their production incurs a fitness cost (Strauss et al. 2002, Müller-Schärer et al. 2004). This can then result in an evolutionary loss of defenses, which is predicted to favor growth and reproduction (*evolution of increased competitive ability* or EICA hypothesis; Blossey and Nötzold 1995).

Post-introduction evolution of increased growth or reproduction has been observed in many species, as predicted by the EICA hypothesis (e.g., Leger and Rice 2003, Wolf et al. 2004, Blumenthal and Hufbauer 2007). However, it is not consistently associated with a loss of defense (reviewed in Hinz and Schwarzlaender 2004, Bossdorf et al. 2005), perhaps because introduced plants are often attacked by generalist herbivores in the new range, and thus do not completely escape herbivory (Memmott et al. 2000, Müller-Schärer et al. 2004, Parker et al. 2006). If introduced plants are predominantly attacked by generalists (here we focus on insects), then qualitative defenses (i.e. toxins), which deter generalists and are relatively inexpensive for a plant to produce, should be maintained in the new range. In contrast, quantitative defenses (i.e., structural defenses and/or digestibility-reducing chemicals), which are effective against specialists and generalists, but are typically costly to produce, should decrease (Müller-Schärer et al. 2004, Joshi and Vrieling

2005). Resulting increases in growth are then thought to stem from a shift in allocation from relatively costly quantitative defenses to less costly qualitative defenses (Müller-Schärer et al. 2004).

There are few studies that have tested the expanded EICA hypothesis (sensu Müller-Schärer et al. 2004) by measuring chemical and structural defense in addition to some aspect of growth or reproduction (but see Joshi and Vrieling 2005, Franks et al. 2008, Ridenour et al. 2008). Most tests of the EICA hypothesis directly quantify only one type of defense (e.g., Joshi and Vrieling 2005, Lewis et al. 2006, Cano et al. 2009; but see Franks et al. 2008, Ridenour et al. 2008) or use more general feeding assays that cannot pinpoint which specific defensive traits might differ between ranges (e.g., Blossey and Nötzold 1995, Siemann and Rogers 2003, Leger and Forister 2005). Studies that do provide a detailed test of the expanded EICA hypothesis (sensu Müller-Schärer et al. 2004), are, taken together, inconclusive (Joshi and Vrieling 2005, Franks et al. 2008, Ridenour et al. 2008). For example, in clear support of the hypothesis, Joshi and Vrieling (2005) found that introduced populations of *Senecio jacobaea* (Asteraceae) grew larger than their native counterparts, and in addition were better protected against generalist herbivores (*Mamestra brassicae* and *Spodoptera exigua*) while less protected against a specialist (*Tyria jacobaeae*). In contrast, Ridenour et al. (2008) reported that introduced populations of *Centaurea maculosa* (Asteraceae) are not only larger, but also better defended, against both specialists and generalists than their native counterparts. They interpreted these findings to be evidence for a lack of trade-offs between growth and defense.

The Ridenour et al. (2008) findings contribute to a long-standing debate regarding the existence of costs associated with trade-offs between plant growth and defense (e.g., Mole 1994, Bergelson and Purrington 1996, Herms and Mattson 1992, Koricheva 2002, Strauss et al. 2002). This debate is directly relevant to the predictions of the EICA hypothesis: if trade-offs are weak or imperceptible in a system, there is little reason to expect an increase in growth or reproduction to come at the expense of investing in defense. The first study to provide a detailed analysis of fitness costs associated with defense (Bergelson and Purrington 1996) reported that plants exhibit a trade-off in only 33% of cases. However, a follow-up review that included more recent work reached a quite different conclusion, showing that costs are detectable in 76% of cases (Strauss et al. 2002). A recent meta-analysis (Koricheva 2002) highlighted that several factors determine the shape of the function describing costs, including environmental factors and the type of defense compounds explored. Because investigations of the EICA hypothesis lend themselves to correlation analysis, they provide a tool to directly test for inverse relationships between growth and defense or between different types of defense.

Here we quantify variation among populations and between ranges in three types of defense (two structural and one chemical) in the introduced weed *Verbascum thapsus* L. (Scrophulariaceae; common mullein). We used a common garden approach to test whether a shift in allocation among defenses (as opposed to a simple increase or decrease in a single defense) is associated with increased growth in introduced populations. We predicted that introduced mullein would invest more in biomass, more or similarly in chemical defense (iridoid glycosides) against

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generalists, and less in structural defense (trichomes and leaf toughness) against specialists and generalists, than do native populations. By simultaneously measuring growth and several defense traits, we were also able to explore whether there is a negative relationship between growth and defense or between different types of defense. A negative relationship provides evidence for an underlying assumption of the EICA hypothesis: that defenses are costly and impose a trade-off between the ability to grow and defend (sensu Herms and Mattson 1992).

METHODS

Study system

Common mullein is a monocarpic perennial (typically biennial) forb that was repeatedly introduced to the eastern United States, first by Puritan settlers in the 1600s for its medicinal properties and later by English and German settlers for use as a piscicide (Wilhelm 1974, Gross and Werner 1978, Mitich 1989). It was also directly imported to the U.S. from Germany in the early 1900s (Henkel 1917). It now occurs in all 50 of the United States, having spread rapidly from its points of introduction in the east to Michigan by 1839 and the Pacific Coast by 1876 (Brewer et al. 1879; Gross and Werner 1978). It is designated as noxious in Colorado, Hawaii, and South Dakota. Mullein has a large native range, occurring throughout Europe and Asia. Although there are currently no molecular reconstructions of its introduction history, the timing of its introduction and its well-documented ethnobotanical history support the contention that Europe was the source of the introduction, especially since there were few trade or travel connections between Asia and the U.S. in the 17th century.

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Mullein has several characteristics typical of weedy invaders. It produces up to 175,000 seeds per plant and forms long-lived seed banks (Gross and Werner 1978). Mullein flourishes in response to disturbance, and therefore may depress recruitment by co-occurring natives in early-seral communities (Pitcairn 2000). Although this species tends to be fugitive, infestations can persist for many years in the introduced range, especially following fire (Fornwalt et al. 2010) or in areas subject to chronic disturbance (e.g., black-tailed prairie dog [*Cynomys ludovicianus*] colonies; Alba, pers. obs.). A recent biogeographic comparison of native (n = 21) and introduced (n = 32) populations showed that introduced populations are significantly larger and more dense than native populations, with larger individual plants (Alba and Hufbauer, cf. Chapter 1.). Additionally, introduced plants are less severely damaged by insect herbivores than their native counterparts (Alba and Hufbauer, cf. Chapter 1.), and they have been released from attack by several specialist insects, including *Cucullia verbasci* L. (Noctuidae) (Maw 1980) and several species of weevil (Gross and Werner 1978; Alba and Hufbauer, cf. Chapter 1.).

Mullein invests heavily in both structural and chemical defense against herbivores. Mullein leaves are covered with dense trichomes, structures that reduce feeding by many insects including caterpillars (e.g., Khan et al. 1986, Agren and Schemske 1993), leafhoppers (reviewed in Levin 1973), beetles (e.g., Dimock and Tingey, 1988) and grasshoppers (Woodman and Fernandes 1991). Another potentially important structural defense is leaf toughness, which has been shown to deter insect feeding (Coley 1983, Choong 1996) and to reduce insect performance (Feeny 1970, Clissold et al. 2009) on multiple plant species. Mullein also produces

toxic secondary metabolites including the iridoid glycosides aucubin and catalpol (Khuroo et al. 1988, Pardo et al. 1998). These chemicals deter generalists (e.g., Bowers and Puttick, 1988) and can attract specialists that use them as oviposition and feeding cues and are able to detoxify or sequester them (e.g., Bowers 1984, Bowers and Puttick 1988, Pereyra and Bowers 1988, Nieminen et al. 2003). Catalpol is the final product of the biosynthetic pathway (Damtoft et al. 1983), suggesting that higher proportions of catalpol reflect greater energetic investment by the plant. Additionally, catalpol is more strongly deterrent to generalists than aucubin (Bowers and Puttick 1988). As such, the ratio of aucubin to catalpol may be an important driver of herbivore feeding preferences in addition to their total amount.

Experimental design

　　We used a common garden approach to explore whether mullein populations exhibit variation in biomass, trichome length, leaf toughness, and iridoid glycoside content, with the specific aim to test whether introduced and native mullein populations differ for these traits. Plants from 10 introduced and 4 native sites were grown in a greenhouse from field-collected seed (see Table 1 for locations of sample sites). Although limited samples were available from the native range, the sites are within the geographic range reported to be the source of mullein introductions into North America (see *Study System*). Despite this, the relatively low replication requires some caution in interpreting the experimental results. We grew three replicates of each of 10 maternal lines per site (with the exception of the Romania and Ithaca, NY sites, which had 5 and 6 maternal lines, respectively) for a total of 393 plants. We

measured above-ground biomass, trichome length, and leaf toughness on all three replicates of each maternal plant, while root biomass and iridoid glycosides were measured on one replicate of each maternal plant.

In June 2008, seeds were sown into germination trays containing Sunshine #3 germination mix (DWF Grower Supply, Denver CO) and placed on a mist bench (average daytime temp., 24.8 °C; average daytime relative humidity, 59.5%; average nighttime temp., 19.9 °C; average nighttime relative humidity, 77.4%). Excess seed was sown and seedlings were thinned as necessary to avoid competition. The length of one cotyledon per seedling was measured with calipers to provide an estimate of maternal provisioning. We took this measurement to help us determine whether observed differences in biomass between native and introduced plants might be a result of maternal effects. Germination trays were re-randomized at regular intervals to avoid micro-climatic effects. At four weeks, seedlings were transplanted into 1-gallon pots containing a mixture of 75% Sunshine #2 potting soil (DWF Grower Supply, Denver, CO), 15% turface (L.L. Johnson Distributing Co., Fort Collins, CO), and 10% sand (Bath Garden Center, Fort Collins, CO) and moved to greenhouse benches (average daytime temp., 21.9 °C; average daytime relative humidity, 64.5%; average nighttime temp., 18.4 °C; average nighttime relative humidity, 72.6%) for the remainder of the experiment, where they were re-randomized once every two weeks. Plants were watered as needed and fertilized once with Osmocote (a slow-release NPK fertilizer) per the manufacturer's directions. To control an outbreak of thrips and fungus gnats, all plants were treated a single time with a Permethrin-based (2.5%)

multipurpose insecticide and Gnatrol (a biocontrol insecticide using *Bacillus thuringiensis*), respectively.

Plants were harvested for growth and defense measurements at 8 weeks of age. Mullein rosettes must reach a threshold size in order to successfully overwinter (Gross 1980); thus, the rate at which biomass is accumulated early in life has a critical influence on final fitness. Indeed, individuals that germinate early in the season (and thus can achieve greater rosette size before overwintering) produce larger inflorescences and more seed than those germinating later in the season (Gross 1980). We also note that, although we conducted our common garden experiment in the introduced range, it is unlikely that the greenhouse conditions or potting soil favored introduced populations.

Biomass Measurements

All rosettes were oven dried at 50 °C to a constant mass and then weighed. We measured root biomass on a subset of individuals (1 individual of each maternal line in each population, n = 131 individuals). Roots were gently washed free of their potting soil prior to drying and weighing.

Defense Measurements

Measurements of trichome length and leaf toughness were made on freshly harvested leaves. We controlled for differences in defense due to leaf age and size by harvesting leaves of similar rank, randomly choosing from the two leaves within a rank, and measuring the length of each leaf to include as a covariate in statistical

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analyses. Leaves were cut in half and each half randomly assigned to trichome or leaf toughness measures. Trichome length was measured under an ocular micrometer at 40× magnification (Woodman and Fernandes 1991). We removed a 0.6-cm-diameter circle of tissue from between the second and third secondary veins (moving away from the leaf tip), near the midrib. The circle was gently held on end with tweezers, and the length of trichomes was measured from the epidermal layer out. The length of the trichomes did not include the occasional longer hairs, but was taken to be the dominant layer or mat of hairs (sensu Woodman and Fernandes 1991).

Leaf toughness measurements were made at the same location on the other half of each leaf using a Lloyd LF-Plus universal testing machine customized to work as a leaf penetrometer. The penetrometer forces a blunt circular probe (7.0686 mm^2) through the leaf at a constant speed, and measures force applied to the probe continuously with a 20 Newton load cell, accurate to within 1% of the force measurement. We recorded both the total work required to puncture a leaf and the maximum force required to puncture a leaf, but report only the latter (load at maximum load in kN), as it was less sensitive to measurement error. For simplicity, we use the term "leaf toughness" throughout.

Iridoid glycosides were quantified in a subset of individuals (1 individual from each maternal line in each population, n = 131) using gas chromatography (detailed in Gardner and Stermitz 1988, Bowers and Stamp 1993). Briefly, we ground dried rosettes to a fine powder and extracted 50-mg subsamples in methanol. The extract was then partitioned between water and ether to remove chlorophyll and hydrophobic compounds. We added an internal standard (phenyl-β-D-glucose) to the

remaining sample, which was then derivatized with Tri-Sil-Z (Pierce Chemical,

Rockford Illinois, USA) prior to injection on a gas chromatograph (Hewlett Packard

5890 equipped with an autoinjector).

Statistical analyses

All statistical analyses were conducted in SAS, v. 9.1 (SAS, Cary Institute,

NC 2002). We first tested for differences in cotyledon size due to invasive status (i.e.,

continent of origin) using mixed model ANOVA with continent of origin as a fixed

effect and site with continent as a random effect. After ruling out continent-level

differences in maternal provisioning based on cotyledon size, we continued with the

remaining analyses.

We tested for differences in biomass and levels of defense due to continent of

origin using mixed model ANOVA. We evaluated the use of latitude as a covariate in

the model for shoot biomass and altitude as a covariate in the models for trichome

length and leaf toughness. As we found no effect of latitude and altitude on the

response variables (latitude effect on shoot biomass, $P = 0.39$; altitude effect on

trichome length, $P = 0.68$; altitude effect on leaf toughness, $P = 0.27$), we analyzed

shoot biomass, trichome length, and leaf toughness with continent of origin as a fixed

effect and population within continent and maternal line within population as random

effects. The models for root biomass, total biomass, shoot:root ratio, and iridoid

glycoside content did not include the random effect of maternal line within site

because we did not have replication at that level. Models testing for differences in

trichome length and leaf toughness included leaf length as a covariate to control for

differences in leaf age. We used the least square means statement to test for differences based on continent of origin. To test the significance of the random effects of site and maternal line, we generated likelihood-ratio statistics and compared them against a chi-square distribution with one degree of freedom (Littell et al. 1996). When necessary, data were transformed (square root: shoot biomass, root biomass, and leaf toughness; arcsine square root: aucubin and catalpol proportions) to improve normality and homogeneity of variance.

To test for trade-offs between biomass and the three defenses, and between the three defenses themselves, we generated correlation coefficients using the PROC CORR procedure (Table 2). We used family means when possible (for shoot biomass, trichome length, and leaf toughness). We did not have replication within families for total biomass, iridoid glycoside content, and the proportion of iridoids made up of catalpol. "Global" trade-offs" were evaluated by generating correlations that included data points from all populations in the two ranges (Table 2). We additionally evaluated trade-offs separately for each population to ensure that the global correlation coefficients did not obscure any trade-offs present at the population scale. To test whether native and introduced populations had significantly different global correlation coefficients, we used a mixed model ANOVA (fixed effect = continent; random effect = population with continent).

RESULTS

Maternal effects

      Cotyledon size did not differ between introduced and native populations (introduced, 3.25 mm ± SE 0.03; native, 3.45 mm ± SE 0.04; $P = 0.27$), providing no evidence that maternal provisioning differed between continents.

Biomass

      Introduced plants had significantly greater shoot biomass than native plants ($F_{1,12} = 10.43$; $P = 0.007$; Fig. 1a), but root biomass was similar between populations from the two ranges ($F_{1,12} = 0.21$; $P = 0.66$; Fig. 1b). As a result of this, the difference in total biomass was only marginally significant ($F_{1,12} = 2.02$; $P = 0.09$; Fig. 1c). The shoot:root ratios did not significantly differ ($F_{1,12} = 0.63$; $P = 0.43$; Fig. 1d). There was no significant population-level variation in biomass (shoot, $P = 0.32$; root, $P = 0.5$) or shoot:root ratios ($P = 0.22$), nor was there significant within-population (maternal plant) variation in shoot biomass ($P = 0.38$). As such, we present only the continental means for the biomass data.

Defense

      Defenses were remarkably similar between the native and introduced ranges. There were no significant differences between introduced and native populations for trichome length *($F_{1,12} = 0.12$; $P = 0.74$;* Fig. 2a), leaf toughness ($F_{1,12} = 0.05$; $P = 0.83$; Fig. 2b), percent dry weight of iridoid glycosides ($F_{1,12} = 0$; $P = 0.99$; Fig. 2c), or the proportion of total iridoids made up of catalpol *($F_{1,12} = 0.77$; $P = 0.40$;* Fig. 2d).

In contrast to the striking similarity of defense investment at the continent scale, there was highly significant among-population variation for all defenses (trichome length, $P = 0.005$; leaf toughness, $P < 0.0001$; percent dry weight of iridoids, $P < 0.0001$; proportion catalpol, $P = 0.005$; Fig. 2, a-d). There was no significant within-population (maternal plant) variation in the defenses with replication at that level (trichome length: $P = 0.5$; leaf toughness: $P = 0.5$).

Cost of defense

Correlation coefficients expressing the relationship between all pairwise comparisons of total biomass, shoot biomass, trichome length, leaf toughness, and iridoid glycoside content revealed no compelling evidence of trade-offs.. The only significant global correlation coefficients (generated using all data points from all populations in both ranges) were positive (Table 2). When evaluating populations separately, (14 populations $\times$ 13 pairs of traits = 182 comparisons), we detected only 8 significantly negative correlations (cf. Table 2 for populations exhibiting negative trade-offs). A mixed model ANOVA showed that correlation coefficients did not significantly differ between native and introduced populations for any pair of traits (data not shown).

DISCUSSION

Our goal was to provide a detailed test of the expanded EICA hypothesis (Müller-Schärer et al. 2004) by quantifying growth plus several types of defense that are predicted to deter mainly specialist (trichomes, leaf toughness) or generalist

(iridoid glycosides) insects. We found partial support for the EICA hypothesis in that shoot biomass of introduced plants was significantly greater than that of natives (Fig. 1a). However, root biomass was similar between ranges, and highly variable, resulting in only marginal differences in total biomass (Fig. 1c). The different conclusions reached based on the results for shoot biomass (clear support for EICA) versus total biomass (weak support for EICA) highlights the importance of estimating whole-plant growth rather than only aboveground growth, which is sometimes done, likely because of logistical constraints (e.g., Blumenthal and Hufbauer 2007, Cano et al. 2009). Our results also suggest that aboveground biomass was more strongly selected to increase in the introduced range than was belowground biomass; this indicates that in some invasive populations, potentially adaptive changes in plant architecture (e.g., a shift in the relative investment in above- versus belowground parts) may be present even if total investment in growth is similar between ranges.

We detected no difference in trichome length, leaf toughness, or iridoid glycoside content when comparing plants from mullein's native and introduced ranges. That none of the traits showed evidence of post-introduction evolution provides a compellingly consistent pattern—one that stands in contrast to the equally clear pattern of significant population-level variation present for each defense. It is possible that our low population replication for the native range failed to capture existing differences in defense phenotypes at the continent scale, and this interpretation cannot be ruled out given the variation that exists among populations. However, two lines of evidence suggest that our findings are accurate. First, there were no non-significant trends toward differences in defense. In fact, the means for

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all three types of defense were virtually identical between ranges (Figure 2). Second, correlation analysis suggests that our findings of no effect reflect a biological reality of the system: there is only very weak evidence of trade-offs between either biomass and defense, or between the three types of defense (Table 2). Overall, populations with large plants also tended to have plants with relatively tough leaves, and high concentrations of chemical defenses. Adler et al. (1995) found results similar to ours, in that they detected no trade-off between allocation to biomass and iridoid glycoside content in *Plantago lanceolata*. This finding may well reflect a true lack of a physiological trade-off; alternatively, it could reflect a greater degree of variation among genotypes in the ability to assimilate carbon than variation in the allocation of carbon to growth versus defense (Adler et al. 1995). Although the conditions under which trade-offs manifest are complicated (Koricheva 2002), and their existence can be difficult to detect (Bergelson and Purrington 1996, Strauss et al. 2002), it is nonetheless striking that we found little evidence for trade-offs between any of the several traits measured (Table 2).

Although the EICA hypothesis explicitly predicts differences at the continent scale, considering all levels of genetic structuring (including among- and within-population variation) can help researchers interpret either the presence or absence of differences between ranges. For example, here we found that none of the biomass traits (shoot, root, and total biomass) exhibit significant population variation, with a mean difference in shoot biomass instead manifesting at the continent scale (Figure 1a). Conversely, all of the defenses showed significant population-level variation, with no indication of mean differences between ranges. If our sample populations

accurately capture mean investment in defense, it suggests that selection operating at local or regional scales may be stronger than the directional selection predicted to operate at the continent scale.

There are many examples of geographic variability in selection ("selection mosaics") on plant-insect interactions (e.g., Berenbaum and Zangerl 1998, Gomulkiewicz et al. 2000, Thompson and Cunningham 2002). Such geographic structuring leads to population differentiation for traits associated with the interactions, thereby precluding a "globally favored" phenotype spanning all populations of a species (or in the context of invasions, all populations in a species' native or introduced ranges; Thompson 1997). We also found no maternal variation for any of the traits that had replication of maternal lines (shoot biomass, trichome length, leaf toughness). A lack of within-population variation suggests that, even if selection were acting on these traits, populations may not possess the requisite genetic variability to respond rapidly. In the case of shoot biomass, the combination of continent-scale genetic differentiation and minimal within or between population variation may reflect the introduction of pre-adapted genotypes rather than a rapid response to selection following introduction.

Although several studies do provide support for the EICA hypothesis (e.g., Siemann and Rogers 2003, Blair and Wolfe 2004; Wolfe et al. 2004), the balance of studies, including ours, provide partial or no support (reviewed in Hinz and Schwarzlaender 2004, Bossdorf et al. 2005). The next step is to determine *why* the hypothesis appears to explain invasion dynamics in some systems but not others. While common garden experiments effectively measure the *results* of evolutionary

processes, they cannot directly quantify the source, direction, or strength of selection on traits that are relevant to invasion (Endler 1986, Kalisz 1986), nor discriminate between rapid adaptation and other modes of genetic divergence such as the differential introduction of pre-adapted genotypes. Here we have suggested that it can be useful to directly test the conditions required for EICA, such as variation in and tradeoffs between growth and defense, both within and among populations. We would also suggest that the next generation of studies in this area should incorporate direct measurements of selection gradients on traits associated with competitive ability and defense (cf. Franks et al. 2008, Murren et al. 2009) so that the identity and role of putative selection pressures (e.g., specialist and generalist enemies) acting in each range can be confirmed.

Table 2.1 Collection locations for seeds used in the common garden experiment

Site Name	State/Country	Coordinates		Elevation (m)
Introduced				
Gold Creek Rd.	Montana	46° 54' 02" N	115° 00' 17" W	1609
Worley	Idaho	47° 33' 14" N	116° 54' 58" W	777
Edgewater	Maryland	38° 54' 05" N	76° 33' 20" W	18
Ithaca	New York	42° 27' 44" N	76° 26' 39" W	314
Poudre Canyon	Colorado	40° 39' 55" N	105° 13' 09" W	1603
Narrows	Colorado	40° 41' 23" N	105° 25' 54" W	1966
Dadd Gulch	Colorado	40° 41' 58" N	105° 32' 38" W	2138
Lake John	Colorado	40° 46' 54" N	106° 28' 42" W	2238
Steamboat Springs	Colorado	40° 30' 07" N	106° 55' 21" W	2009
Hewlett Gulch	Colorado	40° 41' 21" N	105° 18' 36" W	1775
Native				
Muntschemier	Switzerland	46° 59' 13" N	7° 08' 24" E	814
Grissheim	Germany	47° 53' 15" N	7° 34' 52" E	210
Oradour-sur-Glane	France	45° 57' 12" N	01° 00' 82" E	254
Iasi	Romania	47° 08' 51" N	27° 38' 25" E	37

Table 2.2. Global correlation coefficients (including data points from all populations in mullein's native and introduced ranges) describing relationships between biomass and defense traits. Positive coefficients indicate no evidence for trade-offs between investment in two traits while negative coefficients indicate a trade-off. The EICA hypothesis assumes a trade-off between growth and defense (see text for details), but there is little evidence of that for the traits measured in this study.

	Total biomass (g)	Shoot biomass (g)	Trichome length (mm)	% total iridoid glycosides	Proportion catalpol	Leaf toughness (kN)
# Total biomass (g)	1	0.58922*	-0.07871 (SS)	0.24208*	0.20112*	0.19835* (PC)
♦ Shoot biomass (g)		1	-0.05945 (Iasi)	0.20919* (Wor)	0.08505	0.20653*
♦ Trichome length (mm)			1	-0.01429 (PC, Griss)	0.02935 (Griss)	-0.1174 (Nar)
# % total IGs				1	0.16179	0.28488*
# Proportion catalpol					1	-0.02427
♦ Leaf toughness (kN)						1

*$P < 0.05$
♦ Indicates traits with family means
# Indicates traits without family means

Single populations with significantly negative correlation coefficients are in parentheses following the global correlation coefficient;
Griss = Grissheim; Iasi = Iasi; Nar = Narrows; PC = Poudre Canyon; SS = Steamboat Springs; Wor = Worley; See Table 1 for location information.
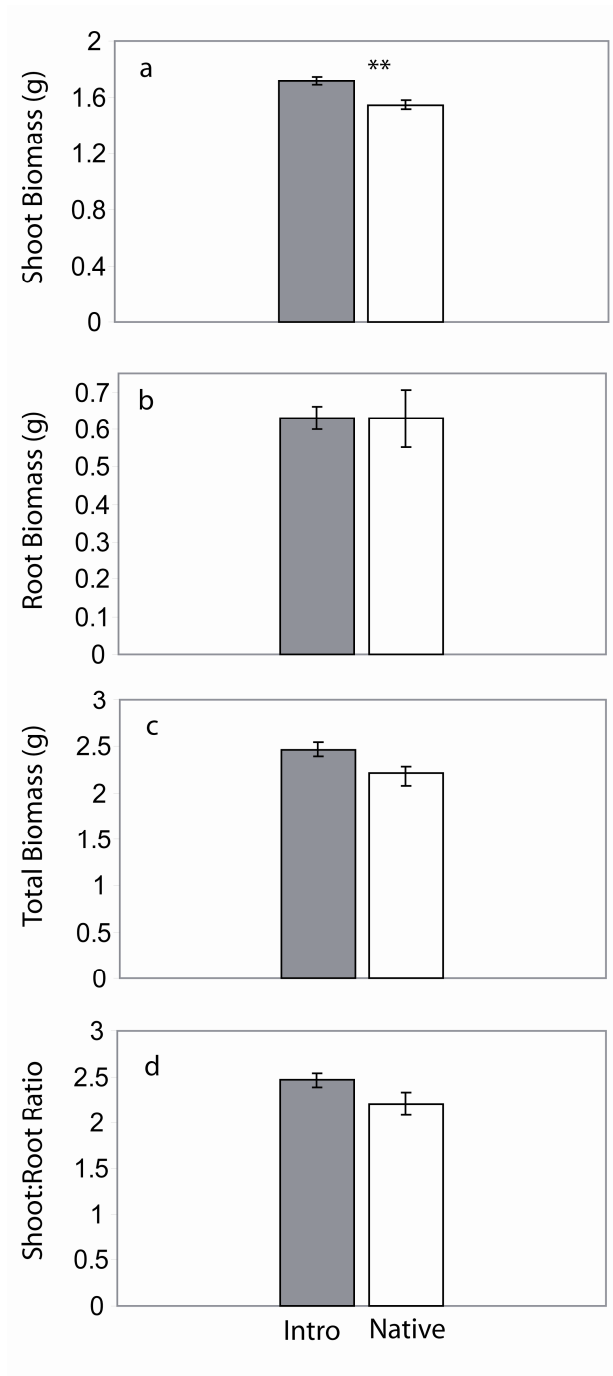
Figure 2.1. Continent means (± SE) of (a) shoot biomass, (b) root biomass, (c) total biomass, and (d) shoot:root ratio. Asterisks denote *P* < 0.01.
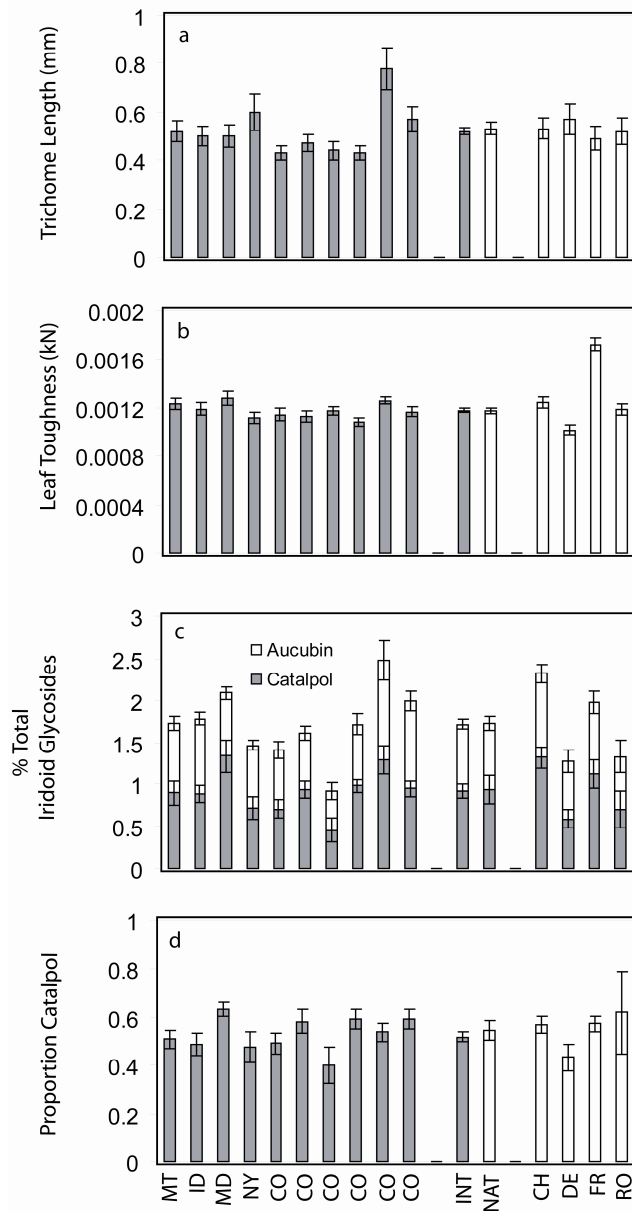
Figure 2.2. Continent and population means (± SE) of (a) trichome length, (b) leaf toughness, (c) iridoid glycoside content (with separate standard error bars presented for aucubin and catalpol), and (d) the proportion of iridoids composed of catalpol. Continent means are not significantly different ($P < 0.05$) in any case. In contrast, there is significant population-level variability for all defenses (see Results). Populations ordered as in Table 1.

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CHAPTER 3

POPULATION AND LEAF-LEVEL VARIATION OF IRIDOID GLYCOSIDES IN

THE INVASIVE WEED *VERBASCUM THAPSUS* L. (COMMON MULLEIN):

IMPLICATIONS FOR HERBIVORY BY GENERALIST INSECTS

OVERVIEW

Plant-insect interactions, which are strongly mediated by chemical defenses, have the potential to shape invasion dynamics. Despite this, few studies have quantified natural variation in key defensive compounds of invasive plant populations, or how those defenses relate to levels of herbivory. Here we evaluated variation in the iridoid glycosides aucubin and catalpol in naturally occurring, introduced populations of the North American invader, *Verbascum thapsus* L. (common mullein; Scrophulariaceae). We examined two scales that are likely to structure interactions with insect herbivores—among populations and within plant tissues (i.e., between young and old leaves). We additionally estimated the severity of damage incurred at these scales due to generalist chewing herbivores (predominantly grasshoppers and caterpillars), and evaluated the correlation between iridoid glycoside content and leaf damage. We found significant variation in iridoid glycoside concentrations among populations and between young and old leaves, with levels of herbivory strongly tracking investment in iridoids in old leaves (i.e., herbivory was negatively correlated with iridoid concentrations), but less so in young leaves. This pattern reflects the fact that young leaves were highly defended by iridoids (averaging 6.5× the concentration present in old leaves, and containing higher proportions of the more toxic iridoid, catalpol) and suffered only minimal damage from (naïve) generalists. In contrast, old leaves were significantly less defended and therefore more substantially utilized. These findings reveal that novel interactions have developed between introduced mullein and native generalist herbivores in North America. However, the limited ability of generalists to feed on mullein's well-defended young leaves results in

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minimal losses of high-quality tissue, suggesting a mechanism for mullein's increased

performance in North America.

INTRODUCTION

Plant-insect interactions have long been of interest to scientists because they are diverse in mode (including herbivory, pollination, and seed dispersal), have strong implications for the structuring of communities, and provide an excellent framework for studying taxonomic diversification and coevolution (Ehrlich and Raven, 1964; Strong et al., 1984; Fine et al., 2004; Becerra, 2007). Given these attributes, it is not surprising that research on biological invasions, which aims to understand how novel species interactions shape community assembly and contemporary evolution, often focuses on plant-insect interactions. In particular, the long-standing interest in how plant defenses influence herbivory and thus plant performance has become an important aspect of research on invasions. For example, the *novel chemistry*, *evolution of increased competitive ability*, and *resource-enemy release* hypotheses propose that the unique or optimal deployment of plant defenses facilitates invasion (Müller-Schärer et al., 2004; Blumenthal, 2006; Cappuccino and Arnason, 2006). However, while much research on invasions assigns a central role to the chemical ecology of plant-insect interactions, few studies have quantified natural variation in defense compounds in introduced plant populations (but see Darrow and Bowers 1997; Zangerl et al., 2008; Barto et al., 2010; Jamieson and Bowers 2010).

In some cases the phytochemical uniqueness of introduced plants facilitates their invasion, for example because naïve herbivores do not recognize invaders as potential hosts (Strong et al., 1984; Cappuccino and Arnason, 2006; but see Lind and Parker, 2010). However, herbivores often successfully feed on invasive plants, for example if they are taxonomically or chemically similar to co-occurring natives (e.g.,

Feeny, 1976; Thomas et al., 1987; Bowers et al., 1992; Courant et al., 1994), or if herbivores can rapidly adapt to overcome their unique defenses (Karowe, 1990). In such cases, novel ecological interactions that have the potential to affect plant performance and ultimately evolution may develop. Indeed, several herbivorous insects in North America have successfully incorporated introduced plants into their diets (e.g., Strong and Lawton, 1984; Singer et al., 1993; Graves and Shapiro, 2003). Given that novel interactions such as these have the potential to shape invasion dynamics, it is critical to quantify the amount, distribution, and ecological relevance of chemical defenses in introduced plants.

Defense phenotypes are determined by several intrinsic and extrinsic factors including plant genotype and ontogenetic development, resource availability, seasonality, and herbivore attack (e.g., Coley, 1983; Denno and McClure, 1983; Coley et al., 1985; Fritz and Simms, 1992; Karban and Baldwin, 1997; Boege and Marquis, 2005). These myriad sources of variation ultimately give rise to the defenses deployed against herbivorous insects in the wild, which in turn regulates the severity and distribution of damage incurred. Several major groups of defensive compounds, including iridoid glycosides, glucosinolates, and pyrrolizidine alkaloids, are known to vary across multiple scales (e.g., among populations, individuals, branches, and leaves), with such variation having demonstrated effects on herbivore feeding decisions and plant performance (e.g., Bowers and Puttick, 1989; Mauricio et al., 1993; van Dam et al., 1995; Donaldson and Lindroth, 2007; Bidart-Bouzat and Kliebenstein, 2008).

In this study, we 1) evaluated phenotypic variation in the iridoid glycosides aucubin and catalpol in introduced populations of the North American invader, *Verbascum thapsus* L. (common mullein; Scrophulariaceae) and 2) related variation in defense investment to damage by generalist herbivores. Here we focus on aucubin and catalpol for several reasons. First, iridoid glycosides, a group of cyclopentanoid monoterpene-derived compounds, are extremely common, occurring in more than 50 plant families (including Caprifoliaceae, Dipsacaceae, Gentianaceae, Plantaginaceae, Scrophulariaceae, Valerianaceae, and Verbenaceae; Bowers 1991) with widespread geographic distributions, and which contain several species that are successful invaders in North America (e.g., *Linaria* spp., *Plantago* spp., and *Verbascum* spp.). Second, iridoid glycosides mediate plant-insect interactions in several systems (e.g., L'Empereur and Stermitz, 1990; Biere et al., 2004; De Deyn et al., 2004; Beninger et al., 2008), with leaf-tissue concentrations reaching high levels in terms of percent dry weight (e.g., up to 17.4% in *Linaria dalamatica*, Jamieson and Bowers, 2010). In particular, aucubin and catalpol have been shown to act as deterrents to generalist herbivores (e.g., *Spodoptera eridania* and *Limantria dispar*; Bowers and Puttick, 1988; Puttick and Bowers, 1988) as well as attractants to specialist herbivores (e.g., *Euphydryas chalcedona* and *Junonia coenia*, Bowers 1983, 1984; Bowers and Puttick, 1988). Catalpol is more toxic to generalists than aucubin (Bowers and Puttick, 1988); as such, the ratio of aucubin to catalpol, as well as the total amount of aucubin and catalpol present, is likely to influence herbivore feeding preferences.

We measured variation in iridoid glycoside content at two distinct scales that are likely to structure ecological interactions with insect herbivores, among

populations and within individual plants. At the within-plant level, we compared young and old leaves. Young leaves are critical to the future growth of a plant, and tend to be well-defended, while older leaves are typically less-well defended (e.g., McKey, 1974, 1979; Coley, 1983; Krischik and Denno, 1983). For example, in *Plantago lanceolata*, another introduced North American weed that contains aucubin and catalpol, young leaves are consistently better defended by iridoids than old leaves (e.g., Bowers and Stamp, 1992; Stamp and Bowers, 1994; Adler et al., 1995). In addition to defenses, we estimated the severity of damage incurred by young and old leaves across populations due to generalist, chewing herbivores (predominantly grasshoppers [Acrididae] and to a lesser extent caterpillars [Noctuidae]) present in mullein's introduced range. We hypothesized that there would be significant variation in both iridoid glycoside investment and herbivory among populations. We further hypothesized that young leaves would contain more iridoid glycosides than old leaves, and that if aucubin and catalpol effectively deter generalist herbivores, then 1) patterns of chewing damage would track differential investment in iridoid glycosides between young and old leaves, and 2) increasing levels of iridoid glycosides would be correlated with decreasing levels of attack.

METHODS

Study System

Mullein is a (typically) biennial forb that was repeatedly introduced to the United States and Canada by European settlers who used it for its medicinal properties and as a piscicide (Wilhelm, 1974; Gross and Werner, 1978; Turker, 2005).

Introductions date back to the early 1600s (Mitich, 1989), and populations are now

established in all 50 states, with noxious status in Colorado, Hawaii, and South

Dakota. Mullein has a large native range, with populations present throughout the

British Isles and Europe (including Scandinavia), to the east in Russia and China, and

to the south in the Caucasus Mountains and Western Himalayas (Clapham et al.,

1952; Werner and Gross, 1978). Plants in both the native and introduced ranges

typically grow in dry, sandy soils and often require canopy-opening disturbance to

facilitate recruitment (Gross, 1980). Individuals form a basal rosette in the first year

and overwinter before developing a large flowering stalk that can produce up to

175,000 seeds (Gross and Werner, 1978).

Ecological differences exist between native and introduced mullein, with

introduced mullein exhibiting increased population- and plant-level performance

(Alba and Hufbauer, cf. Chapter 1). This increased performance is associated with a

shift in the prevalence and identity of herbivore enemies on introduced relative to

native mullein populations (Alba and Hufbauer, cf. Chapter 1). In particular,

introduced populations have partially escaped from several insect guilds including

caterpillars, weevils, leafhoppers, and aphids, and they have completely escaped

attack by snails, across a widespread and arid portion of their introduced range (sensu

the *enemy release* hypothesis, Elton, 1958; Kean and Crawley, 2002). Additionally,

introduced mullein incurs significantly less damage by chewing herbivores than its

native counterparts (Alba and Hufbauer, cf. Chapter 1). This reduced herbivory in

part reflects the fact that introduced mullein has escaped from the specialist caterpillar

Cucullia verbasci L. (Noctuidae), which causes substantial damage in the native

range (Maw 1980, Alba, pers. obs.). The main leaf chewers in the introduced range are generalist grasshoppers (Woodman and Fernandes 1991, Alba, pers. obs.) and generalist noctuids (e.g., *Autographa californica* [alfalfa looper] and unidentified cutworms, Alba, pers. obs.). While grasshoppers do not completely fill the role of the specialist *C. verbasci* in terms of causing consistent and substantial damage across mullein populations, they can, under some conditions, cause significant damage (e.g., up to an average of 25% leaf area missing in some populations [n = 20 plants]; Alba, unpublished data). Introduced mullein is also attacked by two co-introduced specialists, the seed-feeding weevil *Rhinusa tetra* (syn. *Gymnetron tetrum*) and the cell-content feeder, *Haplothrips verbasci*.

In addition to its chemical defenses, mullein is densely covered by trichomes that deter feeding by generalist grasshoppers (Woodman and Fernandes 1991) and caterpillars (Alba et al., unpublished data). In particular, trichomes can affect patterns of feeding within a plant when old leaves with few trichomes are preferred over young leaves that are more completely covered (Woodman and Fernandes 1991, Alba et al. unpublished data).

Collection of Leaf Tissue for Iridoid Glycoside Analysis

We harvested young and old leaves from 10 randomly chosen, overwintered rosettes from each of five mullein populations in Utah and Colorado (see Table 1 for sampling dates and population characteristics) for analysis of iridoid glycoside concentrations. Young leaves were positioned at the 2nd or 3rd rank out from the center of the rosette and old leaves were positioned at the 2nd or 3rd rank in from the

outermost whorl of the rosette. Leaves were harvested after herbivory scores were assigned (see below). In many cases, we had to harvest more young leaves than were used to estimate herbivory in order to ensure that we had enough tissue for chemical analysis. In those cases, we harvested leaves from adjacent leaf ranks, which typically had similar levels of herbivory. All plant tissues were oven-dried at 50 °C to a constant mass and weighed to the nearest 0.01 g. To assess variation in concentrations of iridoid glycosides, young and old leaves from each plant were separately ground into a fine powder from which we removed leaf trichomes by passing samples over a mesh screen. We then prepared 50-mg subsamples for chemical extraction and analysis by gas chromatography following previously described methods (e.g., Bowers and Stamp 1993). Briefly, the subsamples were extracted overnight in methanol and the extract was filtered off of the remaining tissue under a vacuum. We added an internal standard (phenyl-β-D-glucose) to the remaining sample and partitioned the extract between water and ether to remove chlorophyll and hydrophobic compounds. An aliquot of the remaining solution was removed, evaporated, and derivatized with Tri-Sil-Z^{TM} (Pierce Chemical Company) and injected into a HP 7890A gas chromatograph (Agilent Technology) using an Agilent DB-1 column (30 m, 0.320 mm, 0.25 µm particle size). Concentrations of these compounds were quantified using ChemStation B-03-01 software and they are presented as percent dry weight for comparative purposes (e.g., Fuchs and Bowers, 2004; Barton, 2007; Jamieson and Bowers 2010).

Herbivory Estimates

We estimated chewing damage by insect herbivores on the same leaves that were collected for iridoid glycoside analysis (although, as noted above, in some cases we harvested additional young leaves to ensure that we had enough tissue for analysis). Damage estimates were made on three young and three old leaves per plant using the following scoring system: 0 = no leaf tissue missing; 1 = 1-10% of tissue missing; 2 = 11-50% of tissue missing; 3 = 51-75% of tissue missing; and 4 = greater than 75% of tissue missing.

Statistical Analysis

All statistical analyses were conducted in SAS (v. 9.1, Cary, NC). Because aucubin and catalpol were correlated (Pearson's correlation coefficient = 0.86; $P <$ 0.0001), we initially assessed population and leaf-age variation in the percent dry weight of both iridoids using multivariate ANOVA (proc glm), with a repeated measures statement to account for young and old leaves collected from the same plant. We included population, leaf age, and a population × leaf age interaction as fixed effects. All effects were significant using MANOVA (between-subject effects: population, age, and population × age, $P <$ 0.0001; within-subject effects: plant, plant × population, plant × age, and plant × population × age, $P <$ 0.0001). As such, we followed up with univariate ANOVA for each iridoid, as well as for the total percent dry weight of iridoids, and the proportion of iridoids composed of catalpol (cf. Jamieson and Bowers, 2010). All univariate ANOVAs included population, leaf age, and a population × leaf age interaction as fixed effects, and treated young and old

leaves from the same plant as repeated measures (see Littell et al. [1996] for a description of how to construct repeated measures using the glm procedure). We did not include plant diameter or number of leaves as a covariate in the models because there was no relationship between plant size and investment in iridoid glycosides (Pearson's Correlation Coefficient for diameter = -0.09, $P = 0.51$; leaves = 0.22, $P = 0.12$). We used least mean squares with a Tukey adjustment to test all pairwise comparisons of the fixed effects. All data were arcsine-square root transformed to meet assumptions of normality.

The herbivory data contained only zeros for young leaves at site B2 and for young and old leaves at site ST, and did not meet assumptions of normality following data transformation. We therefore used the raw herbivory scores to create two categories: undamaged leaves (herbivory score of zero) and damaged leaves (herbivory scores of 1-4). Treating the data this way allowed us to use a generalized linear mixed model (GLMM) with a binomial distribution and logit link function (proc glimmix) to test for differences in herbivory among populations and between young and old leaves (Schabenberger, 2011). The GLMM procedure uses an "events per trial" syntax, where an event is damage to a leaf (scores 1-4) and trials are the number of leaves sampled. Therefore, each of the 10 plants sampled per population had 3 trials for young leaves and 3 trials for old leaves, resulting in 30 trials for each leaf age in each population. We included population, leaf age, and a population × leaf age interaction as fixed effects in the model, and treated young and old leaves from the same plant as repeated measures. Correlation analysis showed no relationship between damage and plant diameter (Pearson's correlation coefficient = 0.22; $P =$

0.12), so we did not include plant size as a covariate in the model. In order to allow the model to converge on a solution, we included one event in the B2 and ST datasets for young leaves (i.e., 1 herbivory event out of 30 trials, rather than 0 events out of 30 trials), and one event in the ST dataset for old leaves. This change is conservative with respect to our analyses, as it reduces differences in herbivory between young and old leaves. We used least square means with a Tukey adjustment to test all pairwise comparisons of the fixed effects.

We explored whether and how the concentration of iridoid glycosides present in young and old leaves and the severity of damage to those same leaves were correlated with one another. We used correlation analysis because field data preclude the *a priori* establishment of cause and effect between levels of defense investment and herbivory (i.e., high levels of iridoids could deter herbivory, or high levels of herbivory could induce iridoids; see Discussion). We conducted two correlation analyses. First, we determined the correlation between defense and damage using all data points from the five populations (n = 50 data points for young leaves and 50 for old leaves). Second, we used population averages of iridoid levels and damage to generate relationships at the population scale (n = 5 data points for young leaves and 5 for old leaves). To estimate damage levels, we averaged the herbivory scores (0-4) for the group of leaves for which iridoid content was measured. A square-root transformation of the data improved normality and homogeneity of variance.

Iridoid Glycoside Content

Plant chemical defenses, measured as the concentration of total iridoid glycosides, as well as separately for aucubin, catalpol, and the proportion of iridoids made up of catalpol, significantly varied among populations and as a function of leaf age (Figures 1 and 2, Table 2). For example, when averaging over leaf age, population-level variability in the mean ($\pm$ SE) concentration of iridoid glycosides ranged from $1.63 \pm 0.43\%$ dry weight in population LG to $5.03 \pm 1.12\%$ dry weight in population B2. Additionally, leaf age was an important source of variation, with young leaves containing on average $6.5 \times$ more iridoid glycosides than old leaves (Table 2). Indeed, young leaves contained a significantly higher percent dry weight of iridoids than old leaves in every population except ST (Figure 1). There was, however, significant variability in iridoid concentrations within age classes (with young leaves averaging from 3 to almost 10% dry weight, and old leaves ranging from 0.2 to 2% dry weight; Figure 1) depending on the population of origin (cf. the significant population $\times$ age interaction, Table 2). Finally, the proportion of the more toxic iridoid catalpol was significantly greater in young leaves (0.75-0.85) than old leaves (0.48-0.58) across all populations (Figure 2; note the lack of a population $\times$ age interaction, Table 2).

Herbivory

The proportion of mullein leaves damaged by herbivores varied significantly as a result of population and leaf age (Table 2). The main source of variation among

populations was the complete lack of damage to any plant in population ST. Across

populations, the proportion of young leaves with chewing damage was typically zero

and never exceeded 17% (population HG) of the leaves sampled in a given

population. Conversely, the proportion of old leaves damaged was substantial,

ranging from 57% (population LG) to 87% (population HG). As such, the proportion

of young leaves damaged was significantly less than that of old leaves in all

populations except ST, where none of the leaves were attacked, regardless of age

(Figure 3; Table 2). There was not a significant interaction between population and

leaf age (Table 2), suggesting that overall, the distribution of damage between young

and old leaves did not differ across populations (with the only significant pairwise

differences being that old leaves of population ST were significantly less damaged

than old leaves of all other populations).

We note here that although it is possible that the observed differences in

chewing damage between young and old leaves reflect the fact that old leaves were

simply exposed to herbivory for a longer period of time, there is compelling evidence

that suggests otherwise. First, as part of a related project, 30 introduced (U.S.) and 21

native (European) mullein populations were surveyed for damage using the same

basic protocol as detailed above, beginning only one week after the completion of the

surveys discussed here. We found that more than half of young leaves on European

plants were damaged, in large part by the specialist herbivore *Cucullia verbasci*

(Noctuidae), relative to only 11% of young leaves on introduced plants (Alba and

Hufbauer, unpublished data). This provides strong evidence that low herbivory levels

on young leaves in the introduced range reflect true avoidance behaviors by generalist

herbivores. Second, in laboratory feeding trials, the generalist *Trichoplusia ni* (Noctuidae) significantly preferred old mullein leaves to young when given a choice (Alba and Hufbauer, unpublished data; n = 20; *P* = 0.02).

Relationship between Iridoid Glycoside Content and Herbivory

Across all populations, there was a weak negative relationship between the concentration of iridoid glycosides and feeding damage to young leaves (Figure 4a; Pearson's Correlation Coefficient = -0.24, n = 50, *P* = 0.09) and a much stronger negative relationship between iridoid glycosides and herbivory in old leaves (Figure 4b; Pearson's Correlation Coefficient = -0.60, n = 50, *P* = < 0.0001). A similar trend was seen for the population averages (Pearson's Correlation Coefficient for young leaves = -0.25, n = 5, *P* = 0.69; Pearson's Correlation Coefficient for old leaves = -0.89, n = 5, *P* = 0.04; see square symbols in Figures 4a and b for population averages).

DISCUSSION

We found significant variation in iridoid glycoside concentrations, as well as in the proportion of iridoids composed of the more toxic catalpol, at both large (population) and small (within-plant) scales of phenotypic structuring (Figures 1 and 2; Table 2). There was also significant variation in herbivory at both of these scales (Figure 3; Table 2), with the main source of variation at the population scale being the absence of damage to plants in population ST. Correlation analysis revealed that at the population scale, higher levels of iridoids are associated with lower levels of

herbivory in old leaves, but not young (see square symbols in Figure 4 for population averages). However, these results are based on five sites only, one of which is particularly influential (population ST). As such, they must be interpreted with caution, especially given that many factors other than defenses likely contribute to variation in herbivory across sites (e.g., top-down controls over herbivore population dynamics; Rosenheim, 1998; Mooney et al., 2010). Further research surveying a larger number of introduced populations across mullein's introduced range would shed light on the ubiquity of the patterns observed here.

At the within-plant scale, the distribution of herbivore damage clearly tracked investment in defense, with damage being significantly skewed toward old leaves that had low concentrations of iridoid glycosides and lower proportions of catalpol (Figures 1-3). Correlation analysis also revealed that increasing investment in iridoid glycosides is associated with decreasing amounts of damage in old leaves (Figure 4). This relationship was less apparent for young leaves, in part because damage levels were often zero. In fact, it appears that above a threshold of ~6% dry weight of total iridoids, herbivores completely avoided feeding on mullein (Figure 4). Overall these findings reveal that generalist insects present in mullein's introduced range encounter both among-population and within-plant variation in host plant quality, and selectively feed on tissue lower in iridoid glycoside content.

Defensive compounds often vary among populations for a variety of reasons ranging from underlying genetics and phenology to any number of environmental factors that elicit a plastic response in plants (e.g., Coley, 1983; Coley et al., 1985; Fritz and Simms, 1992; Karban and Baldwin, 1997; Boege and Marquis, 2005). It is

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likely that many or most of these sources contributed to the variable iridoid glycoside levels that we observed across our study locations. For example, it has been previously shown that mullein populations exhibit genetically based variation in iridoid glycosides, with average concentrations across 14 populations ranging from <1% to ~2.5% (in plants grown from seed under greenhouse conditions; Alba et al., 2011). Additionally, aucubin and catalpol are known to be inducible in *Plantago lanceolata* (Darrow and Bowers, 1999; Wurst et al., 2008; Fuchs and Bowers, 2004), which suggests that the population variation we observed was potentially mediated by differential levels of insect attack across sites. Furthermore, differences in certain environmental factors such as soil nutrient availability, UV light, and temperature can alter iridoid content (Darrow and Bowers 1999, Jarzomski et al. 2000, Tamura 2001). Regardless of the source of variation, these results indicate that introduced mullein represents a heterogeneous food source for native (naïve) generalist herbivores. Such population-level variation is ubiquitous across several plant species and defensive compounds (e.g., Krischik and Denno, 1983; Zangerl and Berenbaum, 1990) and has been demonstrated in other invasive weeds that contain iridoid glycosides (e.g., *Plantago lanceolata*, Adler et al., 1995; Darrow and Bowers, 1997; Barton, 2007; and *Linaria dalmatica*, Jamieson and Bowers, 2010). Given that native herbivores often adopt introduced species as host plants (e.g., Strong and Lawton, 1984; Bowers et al., 1992; Singer et al., 1993; Graves and Shapiro, 2003), quantifying such variation is of interest within the context of herbivore population dynamics as well as plant invasion dynamics.

In addition to pronounced population-level variation, there exists a marked difference in iridoid glycoside investment between young and old leaves. Highly defended young leaves are typical of several other systems (e.g., Rhoades and Cates, 1976; Coley, 1983), which is consistent with the fact that young leaves are often more valuable to a plant than old leaves (sensu Optimal Defense Theory; McKey 1974, 1979; Feeny, 1976). For example, young leaves typically contain more nitrogen or have higher photosynthetic rates than old leaves, and thus make a potentially greater contribution to future fitness than do old leaves (reviewed in Denno and McClure, 1983). Additionally, damage to expanding (meristematic) tissue can be particularly problematic if it depresses subsequent growth (Ehrlen, 1995). Indeed, in mullein's native range, where plants can sustain severe chewing damage to young leaves at the center of the rosette (by the specialist noctuid *Cucullia verbasci*), its ability to bolt appears compromised (Alba, pers. obs.). More generally, mullein has low survival and slow re-growth capacity if plants are mechanically defoliated while in the rosette stage (van der Meijden et al., 1998). This lack of tolerance to leaf damage suggests that resistance to attack via chemical defenses is a crucial strategy in mullein.

The observed patterns of damage to young and old leaves (Figure 3) reflect previous work showing that generalist and specialist insects have different feeding preferences (e.g., Cates 1980). Typically, generalist insects, which are not tightly co-evolved with their host plants and therefore may be more susceptible to the plant's defenses, prefer old leaves with low concentrations of toxins. In contrast, specialist insects that have evolved to tolerate chemical defenses prefer young leaves that are very nutritious (Cates 1980). For example, similar to our findings, van Dam et al.

(1995) reported a significant decrease in pyrrolizidine alkaloids with leaf age in *Cynoglossum officinale*; this decrease was associated with the clear preference of several generalist herbivores with different feeding modes (*Helix aspera, Lyriomyza trifolii, Frankliniellia occidentalis,* and *Spodoptera exigua)* for poorly defended older leaves. However, this pattern is not always seen. In a study with *Plantago lanceolata*, Bowers and Stamp (1993) reported that the generalist herbivore *Spilosoma congrua* preferred young leaves with high iridoid glycoside content to old leaves with low content. However, these leaves are also higher in water and nitrogen, which may also be important for generalist herbivore feeding preferences (Bowers and Stamp 1993). Such findings reveal that even for the same chemical compounds (aucubin and catalpol), feeding preferences can greatly differ depending on both the plant and herbivore species involved in the interaction. However, given that the damage estimates presented herein capture the feeding behaviors of a suite of native herbivores on introduced mullein, the avoidance of young leaves appears to be a robust pattern in this case.

The correlation analysis reveals that increasing amounts of iridoid glycosides are associated with decreasing amounts of herbivore damage within leaf age classes (Figure 4). These results enable us to more clearly assign a deterrent effect to iridoid glycosides, which we aimed to do because the distribution of trichomes on mullein plants covaries with iridoid glycoside content (i.e., young leaves are better defended by both trichomes and iridoids than old leaves; Woodman and Fernandes 1991; Alba et al., unpublished data). In particular, there appears to be a significant deterrent effect of iridoids to herbivores feeding on old leaves (Figure 4) that are not well

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protected by trichomes. Structural defenses such as trichomes can be costly to produce and maintain (Hare et al., 2003). In this case, the high investment in both trichomes and iridoids in young mullein leaves provides further evidence that these tissues are highly valuable to the plant. By extension, the limited ability of generalist chewing herbivores to feed on young leaves may represent an important contribution to mullein's increased performance in North America (Alba and Hufbauer, Chapter 1).

Here we demonstrated that chemical defenses in the invasive weed *Verbascum thapsus* vary significantly across populations and plant tissues (i.e., leaf age), and that within-plant variation in iridoids is a key feature explaining patterns of herbivory. These findings reveal that ecological interactions between introduced plants and generalist herbivores have the potential to affect plant performance, and subsequently, invasion dynamics.

Table 3.1. Summary of sampling locations for herbivory and iridoid glycoside content of mullein plants

Location	State	Site ID	Sample Date	Latitude (N), Longitude (W)	Elevation (m)	Site Characteristics
Beaver 1	UT	B1	6/1/2010	38.304111, 112.837642	2043	Burned (2007) area on BLM land; sagebrush-dominated uplands; sedge and forb-rich lowlands
Beaver 2	UT	B2	6/1/2010	38.419186, 112.713906	2043	Burned (2007) area on BLM land; steep slope with weedy plants including *Bromus inermis* and *Bromus tectorum*.
Strawberry Reservoir	UT	ST	6/2/2010	40.182913, 111.050444	1765	Roadside population; highly disturbed with minimal vegetation
Logan	CO	LG	5/22/2010	40.650061, 102.741167	2092	Conservation Reserve Program land; seeded with erosion-controlling bunchgrasses; weedy plants present (e.g., *Bromus tectorum, Medicago sativa*)
Hewlett Gulch	CO	HG	5/27/2010	40.701733, 105.314656	1269	Trailside population on burned (2005) National Forest Service land; mixture of weedy plants (e.g., *Bromus tectorum*) and native species

Table 3.2. ANOVA results of the effect of population, leaf age, and the interaction of population and leaf age on the percent dry weight of the iridoid glycosides (IG) aucubin and catalpol, the total percent dry weight of IGs, the proportion of IGs composed of catalpol, and the proportion of leaves damaged by herbivores.

Source	Aucubin		Catalpol		Total IG		Proportion Catalpol		Herbivory	
	$F(df)$	P	$F(df)$	P	$F(df)$	P	$F(df)$	P	$F(df)$	P
Fixed Effects										
Population	14.5(4)	<0.0001	24.5(4)	<0.0001	23.2(4)	<0.0001	8.5(4)	<0.0001	2.5(4)	0.05
Leaf Age	198.9(1)	<0.0001	767.1(1)	<0.0001	625.1(1)	<0.0001	434(1)	<0.0001	17.1(1)	<0.0001
Population x Leaf Age	38.7(4)	<0.0001	31.3(4)	<0.0001	35.9(4)	<0.0001	3.2(4)	0.02	0.82(4)	0.52
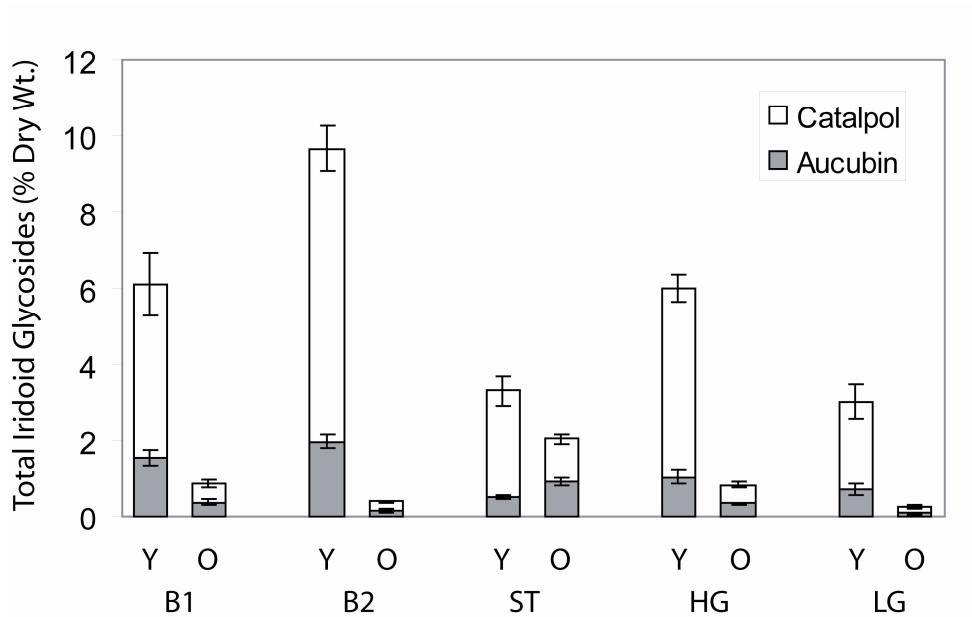
Figure 3.1. Mean percent dry weight (± SE) of the iridoid glycosides aucubin and catalpol in the young (Y) and old (O) leaves of plants (n = 10) from five mullein populations in Utah and Colorado, USA. Population abbreviations as in Table 1. See Table 2 for ANOVA results.

Figure 3.2. Mean proportion of catalpol (± SE) present in young and old leaves of plants (n = 10) from five mullein populations in Utah and Colorado, USA. Population abbreviations as in Table 1. See Table 2 for ANOVA results.
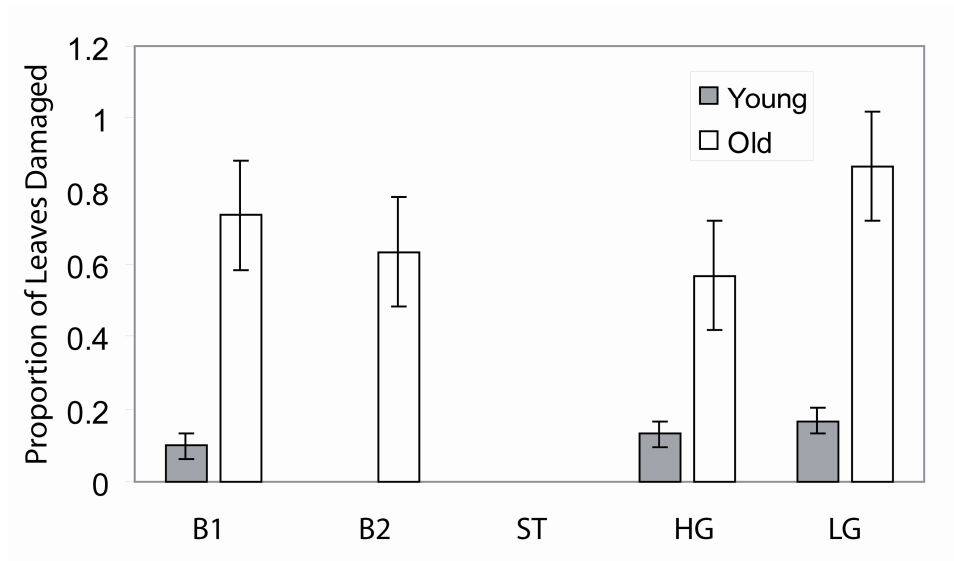
Figure 3.3. Mean proportion (± SE) of young and old leaves damaged by chewing

herbivores in five mullein populations in Utah and Colorado, USA. Population

abbreviations as in Table 1. See Methods for details of how damage was scored and

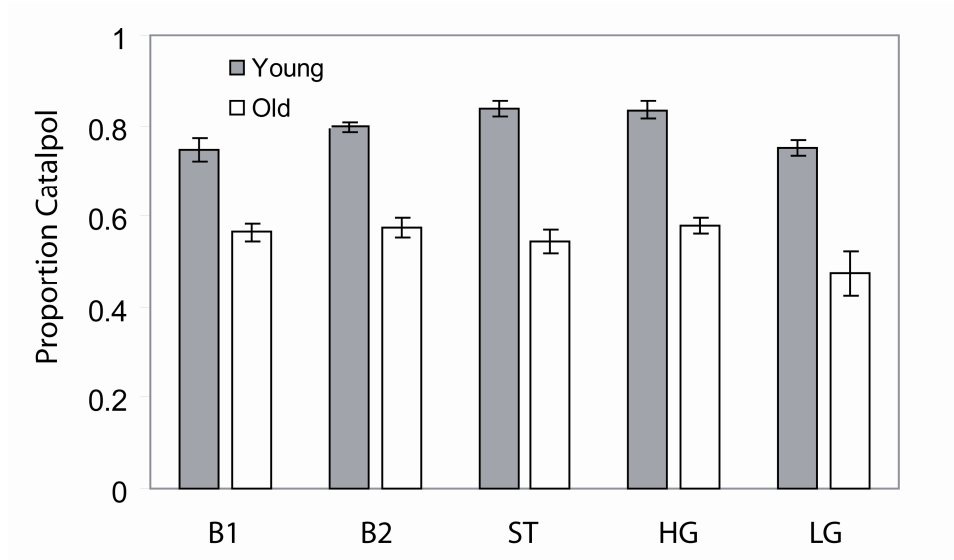analyzed.
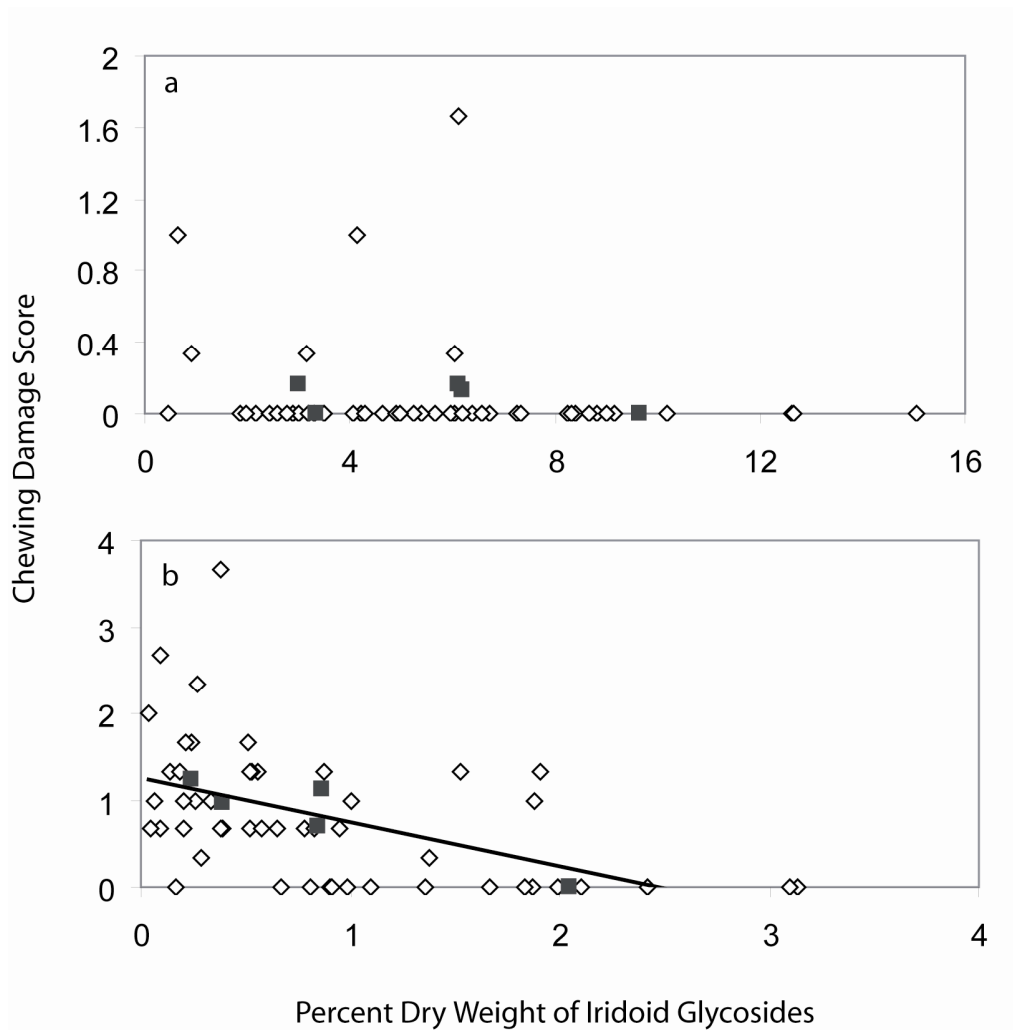
Figure 3.4. Correlations between the percent dry weight of total iridoid glycosides present in (a) young and (b) old leaves and the average damage score assigned to those leaves. Open diamonds represent data points from each plant (n = 10) sampled in each of five mullein populations (line is shown to depict the trend in the data for the open diamonds). Filled squares represent population averages. See Results for correlation coefficients and associated *P* values. Note the different scales on the axes in panels a and b.

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CHAPTER FOUR

CHEMICAL DEFENSES IN *VERBASCUM THAPSUS* (COMMON MULLEIN) ARE

OPTIMALLY DISTRIBUTED IN RELATIONSHIP TO SPECIALIST AND

GENERALIST HERBIVORES IN ITS NATIVE AND INTRODUCED RANGES

OVERVIEW

Optimal defense theory posits that insect herbivores act as a major selective force on their plant hosts, and that plants with limited resources deploy defenses based on the value of different plant tissues (e.g., young versus old leaves) and their probability of attack. However, what constitutes optimal defense depends in large part on the identity of the herbivores involved in the interaction. In particular, generalists, which are not tightly coevolved with the many hosts upon which they feed, are often effectively deterred by chemical defenses, while many coevolved specialists use these same chemicals as oviposition and feeding cues. This imposes an "evolutionary dilemma" because generalists and specialists exert opposing selection pressure on plant investment in chemical defense, and therefore act to stabilize defenses at intermediate levels. Here we take advantage of the natural shift in herbivore community composition that typifies many plants invasions to test a key combined prediction of optimal defense theory and the evolutionary dilemma model: that defense levels of young and old leaves track the relative importance of specialist and generalist herbivores in the community. We use natural populations of *Verbascum thapsus* (common mullein) exposed to ambient herbivory in its native range (where specialists and generalists are prevalent) and introduced range (where generalists are prevalent) to illustrate significant differences in the way iridoid glycosides are distributed among young and old leaves. Importantly, high-quality young leaves are 6.5× more highly defended than old leaves in the introduced range, but only 2× more highly defended in the native range. This differential investment in defense of young

and old leaves is tracked by patterns of chewing damage to those same tissues, with damage restricted mostly to low-quality old leaves in the introduced range, but not the native range. Given that overall investment (averaging over leaf age) in defense investment does not differ between ranges, there exists the potential for introduced mullein to benefit from a fitness gain simply by optimizing the within-plant distribution of defenses in the absence of important specialist herbivores.

INTRODUCTION

The role that plant-insect interactions serve in shaping the abundance and distribution of plants has received much attention by ecologists (Crawley 1989), both historically and in light of the fact that herbivores have the potential to affect invasion dynamics (e.g., Maron and Vilá 2001, Keane and Crawley 2002). A major cornerstone of research on the evolutionary ecology of plant-insect interactions is optimal defense theory (McKey 1974, Rhoades and Cates 1976). This theory states that herbivores represent a key selection pressure on plant defenses, and that, because resources are limited, plants will optimally deploy defenses in relationship to 1) the value of different tissues and 2) the probability that such tissues would be attacked if not chemically defended. The ability of optimal defense theory to predict the within-plant distribution of chemical defenses has proven robust across plant taxa and different classes of chemical compounds (Van Dam et al. 1996, Zangerl and Rutledge 1996, Ohnmeiss and Baldwin 2000, cf. the recent meta-analysis by McCall and Fordyce 2010). In particular, numerous studies have demonstrated that nutritious, high-quality young leaves are better defended than old leaves (e.g., Coley 1983, Krischick and Denno 1983, McCall and Fordyce 2010), and yet may be more heavily attacked by herbivores that can overcome chemical defenses (Coley 1980, Coley 1983). Given that young leaves represent a greater potential contribution to a plant's lifetime fitness than do old leaves (Denno and McClure 1983, Harper 1989), their preferential attack by herbivores should impose strong selection for high levels of defense. This scenario leads to the observed pattern that high investment in defenses

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is nonetheless associated with high levels of attack. Though robust, this pattern is not always seen, for example when herbivores prefer to feed on less defended (albeit less nutritious) mature leaves (Louda 1984, van Dam et al. 1995, Andrew and Hughes 2005). As such, the relationships between leaf age and herbivore damage depends in large part on the herbivores involved in the interaction (Cates 1980).

Indeed, not all herbivores are created equal, and a major challenge to plants is that they are attacked by both generalist and specialist enemies. Generalists, which are not tightly coevolved with the many hosts upon which they feed, are often effectively deterred by chemical defenses (Cates 1980). In contrast, many coevolved specialists are undeterred by these same chemicals, and in fact use them as oviposition cues and feeding stimulants (Cates 1980). This imposes an "evolutionary dilemma" (van der Meijden 1996) because generalists and specialists exert opposing selection pressure on plant investment in chemical defense (van der Meijden 1996, Lankau 2007). Plants that have developed a rich insect community comprising both generalists and specialists (e.g., those that are relatively abundant and widespread, with long evolutionary histories in their native communities; Strong et al. 1984) are therefore predicted to undergo stabilizing selection on chemical defense (van der Meijden 1996, Lankau 2007). By extension, it can be predicted that a major shift in the insect community on a plant should elicit changes (either fixed or environmentally plastic) in chemical defense in order to maximize fitness in the new environment. Thus a key combined prediction of optimal defense theory and the evolutionary dilemma model is that defense levels of young and old leaves should track the relative importance of specialist and generalist herbivores in the community. Accordingly, if

specialists are prevalent, defenses that they use as attractants should be selected against, while if generalists dominate, those same defenses should be selected for. Evidence for such shifts would validate both optimal defense theory and the evolutionary dilemma model, but unfortunately, most research on these ideas focuses on patterns of defenses found in greenhouse experiments or among natural populations that do not differ systematically in herbivore composition (McCall and Fordyce 2010).

Empirical validation of the optimal defense and evolutionary dilemma models has direct relevance in the context of invasions, because both theories are foundational to the branch of invasion research that focuses on plant-insect interactions. Most notably, the *evolution of increased competitive ability* (EICA; Blossey and Nötzold 1995) hypothesis draws on the tenets of optimal defense theory by invoking herbivores as the main selective agent on costly plant defenses. EICA predicts that invasive species evolving in communities devoid of enemies will reallocate resources from defenses to growth and reproduction. More than a decade of research in this area has revealed that while post-introduction evolution of plant competitive ability is common, attendant changes in defense allocation are variable, ranging from the proposed decrease in defense investment, to no change, to actual increases (reviewed in Hinz and Schwarzlaender 2004, Bossdorf et al. 2005, Orians and Ward 2010). Müller-Schärer et al. (2004) provided a needed refinement to the EICA hypothesis by stressing that invasive plants are often colonized by generalists in their new range, and must therefore continue to invest in chemical defenses against them. Indeed, introduced plants that are released from the evolutionary dilemma of

simultaneous attack by specialists and generalists are poised to invest more highly in chemical defense than their native counterparts (van der Meijden 1996). Under these conditions, increased competitive ability is thought to stem from decreased investment in quantitative defenses effective against specialists (Müller-Schärer et al. 2004). The refined EICA hypothesis has been supported in several systems (e.g., Joshi and Vrieling 2005, Stastny et al. 2005). However, it is still the case that evolved increases in growth and reproduction are not necessarily associated with *any* significant shifts in plant chemical defense (as measured directly or via feeding damage to native and introduced genotypes; Willis et al. 1999, Buschmann et al. 2005, Genton et al. 2005, Hull-Sanders et al. 2007, Alba et al. 2011). In sum, the lack of a consistent pattern makes it difficult to generalize about the role that escape from enemies serves in shaping defense phenotypes and ultimately fitness in introduced plants.

Here we propose that a key gap in evidence for optimal allocation of defenses can be filled using the natural experiments represented by plant invasions in which the native and introduced ranges differ in the relative importance of specialist and generalist herbivores. Simultaneously, the predictive framework established by the EICA hypothesis and its later refinement would be strengthened if they more fully incorporated the predictions of optimal defense theory by taking into account within-plant variation in chemical defense.

To our knowledge, variation of defenses in plant tissues of different value has not been assessed in native and introduced individuals of an invasive species, despite the fact that adaptive shifts in defense are likely to manifest at this scale. We used

natural populations of *Verbascum thapsus* (common mullein) exposed to ambient

herbivory in its native and introduced ranges to illustrate significant differences in the

way chemical defenses (iridoid glycosides) are distributed among leaves of different

value, although average investment in defense does not differ between ranges. We

also show that differential investment in defense among leaves is tracked by patterns

of chewing damage to those same leaves, and that damage is restricted mostly to

lower-quality leaves in the introduced range, but not the native range. We additionally

illustrate that specialist and generalist herbivores respond to chemical defenses in a

predictable manner, with generalists typically showing avoidance behavior and

specialists showing attraction.

METHODS

Study System

Mullein is a monocarpic perennial (typically biennial) forb that was

introduced to the United States from Western Europe (Gross and Werner 1978). It is

widely distributed in its native range, present throughout the British Isles and Europe

(including Scandinavia), to the east in Russia and China, and to the south in the

Caucasus Mountains and Western Himalayas (Clapham et al. 1952, Gross and

Werner 1978). Since its introduction it has established populations in all 50 states in

the US and is designated as noxious in Colorado, South Dakota, and Hawaii.

Mullein's ethnobotanical history is well documented because it contains several

useful compounds, including saponins, which are used as an expectorant to treat

coughs; rotenone, which is toxic to fish and can be used as a piscicide; and iridoid

glycosides, the focal compounds of this study, which are used medicinally in various contexts (Wilhelm 1974, Turker 2005). Mullein was introduced to the northeastern U.S. in the early 1600s by English settlers who brought it with them to plant in their herb gardens (Mitich 1989). A second introduction event occurred in the 1700s, when English and German settlers of Appalachia, who had long used mullein as a piscicide, included it in their New-World botanical arsenal (Wilhelm 1974, Gross and Werner 1978). Mullein quickly spread from its points of introduction, reaching Michigan by 1839 and the Pacific Coast by 1876 (Brewer et al. 1879, Gross and Werner 1978). Mullein has several characteristics of weedy invaders including wide climatic tolerances, prodigious seed output, and heavy recruitment in response to disturbance (Gross 1980, Parker et al. 2003). It typically grows in sandy, well-drained soils, requires full light to germinate, and is relatively intolerant to shade (Gross and Werner 1978, Reinartz 1984a).

Marked ecological differences exist between native and introduced mullein populations. In a previous study including 21 native and 30 introduced populations (of which the populations evaluated herein form a subset), we showed that introduced populations are larger and more dense than native populations, and introduced plants have significantly more leaves and tend to have larger diameters (cf. Chapter 1). This increase in performance is associated with mullein's partial or full escape from several herbivore guilds (including caterpillars, weevils, leafhoppers, aphids, and snails) that are important in the native range. In that earlier study, we estimated herbivore damage to whole plants (in contrast to the more refined estimates in the current study focused on young and old leaves – see below) and found a significant

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reduction in chewing damage to leaves (cf. Lewis et al. 2006 for an explanation of the scoring system used). Importantly, the main leaf chewer in the native range is a specialist caterpillar (*Cucullia verbasci* [mullein moth, Noctuidae]), which feeds almost exclusively on *Verbascum* spp., and rarely on other iridoid-containing plants in the Scrophulariaceae (Maw 1980). *Cucullia* was once considered as a biological control agent on mullein because of its specificity and ability to cause substantial damage (Maw 1980). In contrast, the main leaf chewers in the introduced range are generalist grasshoppers (Acrididae) and to a lesser extent generalist caterpillars (e.g., *Autographa californica* [alfalfa looper, Noctuidae] and unidentified cutworms, Alba, pers. obs.). Indeed, grasshoppers are actually more prevalent on introduced than native mullein (cf. Chapter 1) and under certain conditions cause substantial damage (e.g., up to an average of 25% leaf area missing in some Colorado populations; Alba, unpublished data). Introduced mullein is also attacked by two co-introduced specialists, the seed-feeding weevil *Rhinusa tetra* (syn. *Gymnetron tetrum*) and the cell-content feeder, *Haplothrips verbasci*. The majority of weevils and the thrips migrate from the vegetative portions of the plant to the inflorescence once bolting initiates.

Mullein produces the iridoid glycosides aucubin and catalpol. Iridoid glycosides are a group of cyclopentanoid monoterpene-derived compounds that are present in many plant families with wide geographic distributions (e.g., Caprifoliaceae, Dipsacaceae, Gentianaceae, Plantaginaceae, Scrophulariaceae, Valerianaceae, and Verbenaceae; Bowers 1991). Iridoids deter generalist herbivores (e.g., *Spodoptera eridania*, Puttick and Bowers, 1988) while at the same time

attracting specialists that use them as feeding and oviposition cues (e.g., *Euphydryas chalcedona* and *Junonia coenia*, Bowers and Puttick, 1988). Because catalpol is more toxic to generalists than aucubin (Bowers and Puttick, 1988), the ratio of these two compounds, as well as their total amount, is likely to influence herbivore feeding preferences. In a previous study with mullein, we found that native and introduced plants grown from seed under common greenhouse conditions exhibit significant population-level variation in whole-plant iridoid glycoside content. However, in contrast to what is predicted by the EICA hypothesis and its later refinement, we did not find any difference in whole-plant iridoid investment, or in investment in two structural defenses (trichomes and leaf toughness), between ranges. Despite the lack of a range-level shift in defense investment, we found that introduced populations had significantly greater shoot biomass (Alba et al. 2011).

Collection of Leaf Tissue for Iridoid Glycoside Analysis

To estimate within-plant variation in chemical defense, we harvested young and old leaves from 8 to 10 bolting plants in each of 6 native and 6 introduced populations (native range, n = 57 plants; introduced range, n = 58 plants; see Table 1 for location information). Young leaves were collected from the 2nd or 3rd rank from the top of the bolt and old leaves were collected from the 2nd or 3rd rank in from the base of the stalk, taking care to avoid senesced leaves. Leaves were harvested after herbivory scores were assigned (see below). In many cases (in both ranges) we had to harvest more young leaves than were used to estimate herbivory to ensure that we had enough tissue for chemical analyses. In those cases, we harvested leaves from

adjacent ranks. All plant tissues were oven-dried at 50 °C to a constant mass and weighed to the nearest 0.01 g. The dried material was ground to a fine powder and leaf trichomes were removed by passing samples over a mesh screen. We then prepared 50-mg subsamples for chemical extraction and analysis by gas chromatography following previously described methods (e.g., Bowers and Stamp 1993). Subsamples were extracted overnight in methanol and the remaining material was separated from the extract by filtering the samples under a vacuum. We added an internal standard (phenyl-β-D-glucose) to the extract and partitioned it between water and ether to remove chlorophyll and hydrophobic compounds. An aliquot of the remaining solution was evaporated and derivatized with Tri-Sil-Z^{TM} (Pierce Chemical Company) prior to injection into a HP 7890A gas chromatograph (Agilent Technology) using an Agilent DB-1 column (30 m, 0.320 mm, 0.25 µm particle size). Iridoid compounds were quantified using ChemStation B-03-01 software.

Herbivory Estimates

Herbivore chewing damage was estimated on the same leaves that were collected for iridoid glycoside analysis (although, as noted above, in some cases we harvested additional young leaves to ensure that we had sufficient tissue for analysis). Damage estimates were made on three young and three old leaves per plant using the following scoring system: 0 = no leaf tissue missing; 1 = 1-10% of tissue missing; 2 = 11-50% of tissue missing; 3 = 51-75% of tissue missing; and 4 = greater than 75% of tissue missing. Damage intensity was then calculated by averaging the 3 damage scores assigned to each set of young and old leaves per plant.

Statistical Analyses

Iridoid Glycosides

All statistical analyses were conducted in SAS (v. 9.1, Cary, NC). Because aucubin and catalpol were correlated (Pearson's Correlation Coefficient = 0.58; $P <$ 0.0001), we initially used multivariate ANOVA to assess the effects of range (native or introduced), leaf age, and a range $\times$ leaf age interaction on the percent dry weight of aucubin and catalpol. We constructed the error term [age*population(range)] such that populations were treated as the equivalent of a split plot from which both young and old leaves were drawn. When significant effects were found using MANOVA, we proceeded with univariate ANOVA for aucubin and catalpol separately, as well as for the total percent dry weight of iridoid glycosides and the proportion of iridoids composed of catalpol. We constructed mixed models with the same fixed effects used in the MANOVA, but with a modified random (error) term [age*plant(population range)] that treated plants as the equivalent of a split plot from which both young and old leaves were drawn (insufficient degrees of freedom constrained fitting this term in the MANOVA above). All dependent variables were arcsine-square root transformed.

Herbivory

We first evaluated the proportion of leaves damaged by herbivores and the intensity of herbivore damage using the full complement of populations for which we sampled herbivory (native range, n = 14; introduced range, n = 21; Table 1). We then ran the same models again using only the subset of populations for which we conducted iridoid glycoside analyses (n = 6 in each range). We used the same fixed

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and random effects as in the univariate mixed models for iridoid glycosides. We used a generalized linear mixed model (GLMM) with a binomial distribution and logit link function (proc glimmix) to test for differences in the proportion of leaves damaged (Schabenberger 2011), and a mixed model ANOVA to test for differences in damage intensity. Damage intensity scores were square-root transformed prior to analysis.

Correlation between iridoid glycoside content and herbivory

We generated Pearson's Correlation Coefficients to evaluate the relationship between 1) percent total iridoid glycosides and 2) the proportion of iridoids composed of catalpol and damage intensity to young and old leaves in each range. The iridoid variables were arcsine-square root transformed and the herbivory scores were square-root transformed. We used correlation analysis because it is difficult to establish cause and effect between levels of defense investment and herbivory in the field (i.e., high levels of iridoids could deter feeding, or feeding could induce high levels of iridoids).

RESULTS

Iridoid Glycoside Content

The percent total iridoid glycoside content of young leaves ranged from a minimum of 1.2% in native population CH1 to a maximum of 9.5% in introduced population WY3. Old leaves in both ranges had lower average iridoid content than young leaves, ranging from 0.50% in introduced population MT9 to 3.8% in native population MA1. For both ranges and leaf ages, catalpol was the more prevalent of

the two iridoids, ranging from a minimum of 0.12% in old leaves in the native range to a maximum of 7.6% in young leaves in the introduced range (in comparison to a minimum of 0.07% aucubin in old leaves in the introduced range and a maximum of 0.94% in young leaves in the introduced range). The MANOVA results showed that range did not explain a significant proportion of the variability in aucubin and catalpol (Wilk's $\lambda = 0.94$; $F = 0.26_{(2,9)}$; $P = 0.77$), while leaf age (Wilk's $\lambda = 0.21$; $F = 0.17.2_{(2,9)}$; $P = 0.0008$) and the range × leaf age interaction (Wilk's $\lambda = 0.33$; $F = 9.3_{(2,9)}$; $P = 0.006$) were highly significant.

Univariate ANOVA of percent aucubin and catalpol showed that neither compound significantly differed by range (aucubin: native mean = 0.26, 95% CI =0.14-0.41; introduced mean =0.19, 95% CI = 0.09-0.33; catalpol: native mean = 1.13, 95% CI = 0.41-2.22; introduced mean = 1.07, 95% CI = 0.37-2.13; Table 2). For aucubin, there was a significant effect of age (young leaves, mean = 0.27, 95% CI = 0.18-0.37; old leaves, mean = 0.18, 95% CI = 0.11-0.27), and a pronounced range × age interaction (native range: young leaves, mean = 0.24, 95% CI = 0.13-0.38; old leaves, mean = 0.28, 95% CI = 0.16-0.43; introduced range: young leaves, mean = 0.30, 95% CI = 0.18-0.46; old leaves, mean = 0.11, 95% CI = 0.04-0.21). This significant interaction illustrates that while in the native range aucubin is higher in old leaves than in young, the opposite is true in the introduced range. Percent catalpol also varied significantly by leaf age (young leaves, mean = 2.34, 95% CI = 1.57-3.26; old leaves, mean = 0.32, 95% CI = 0.08-0.72), but there was not a significant range × age interaction (Table 2). The percentage of leaf dry weight made up of total iridoid glycosides did not differ by range, but as with aucubin and catalpol, there was a

significant effect of age (Table 2, Figure 1a). There was also a significant range × age interaction (Figure 1a) showing that young leaves are better defended in the introduced range than in the native range, despite the fact that average investment in defense does not differ by range. The proportion of iridoids composed of catalpol differed only as a function of leaf age (Table 2; Figure 1b). Finally, the random effect of population was highly significant for all dependent variables (Table 2).

Herbivory

Averaging over leaf age, the proportion of leaves damaged in the native range (mean ± SE = 0.58 ± 0.12) was significantly greater than the proportion damaged in the introduced range (mean ± SE = 0.36 ± 0.09; $F = 9.6_{(1,33)}$; $P = 0.004$). In both ranges the proportion of young leaves damaged was less than the proportion of old leaves ($F_{(1,763)} = 219.3$; $P < 0.0001$ for both ranges), but a significant range × leaf age interaction ($F_{(1,763)} = 27.9$; $P < 0.0001$; Figure 2) illustrates the much larger discrepancy in attack between young and old leaves in the introduced relative to the native range (Figure 2). For the model that included only the populations used in the iridoid glycoside analyses, the range effect became non-significant ($F_{(1,11)} = 3.5$; $P = 0.09$), but the age ($F_{(1,311)} = 102$; $P < 0.0001$) and range × age interaction ($F_{(1,311)} = 8.1$; $P = 0.005$) remained highly significant. The severity of damage to leaves did not significantly differ between ranges when averaging over leaf age ($F_{(1,33)} = 3.6$; $P = 0.07$) but did differ as a function leaf age ($F_{(1,763)} = 268$; $P < 0.0001$ for both ranges), and there was again a significant range × age interaction ($F_{(1,763)} = 19.3$; $P < 0.0001$) that followed the same pattern as found for the proportion of leaves damaged. The

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results for the model that included only the populations used in the iridoid glycoside analyses were qualitatively similar (range: $F_{(1,10)} = 3.1$; $P = 0.11$; age: $F_{(1,210)} = 77.1$; $P < 0.0001$; range $\times$ age: $F_{(1,210)} = 8.1$; $P = 0.005$).

Correlation between iridoid glycoside content and herbivory

For young leaves in the native range, there was a significant positive correlation between percent catalpol, percent total iridoid glycosides, and the proportion of iridoids composed of catalpol and the severity of damage (Table 3). In contrast, none of the iridoid measurements were related to the severity of herbivory to young leaves in the introduced range, in large part because young leaves were so rarely attacked (Table 3; Figure 2b).

Percent aucubin was not related to the severity of damage to old leaves in the native range, but was highly negatively correlated with damage in the introduced range (Table 3). Percent catalpol was negatively correlated to damage to old leaves in both ranges. There was only a marginally significant negative relationship between total iridoid glycosides and herbivory in the native range (Table 3; $P = 0.07$), and a much stronger negative relationship in the introduced range (Table 3). Finally, the proportion of iridoids composed of catalpol was negatively related to damage in the native range, but not in the introduced range (Table 3).

DISCUSSION

Here we have shown that iridoid glycosides are distributed in native and introduced mullein plants as predicted by optimal defense theory (Mckey 1974, Rhoades and Cates 1976). In both ranges, young leaves are significantly better defended than old leaves, both in terms of total iridoid content and the proportion of iridoids composed of the more toxic catalpol (Figure 1, a and b). However, while young leaves of native plants have an average iridoid glycoside content that is ~2× greater than old leaves, young leaves of introduced plants have an average 6.5× greater content (Figure 1a). This dramatic shift in allocation is consistent with introduced mullein having been released from the evolutionary dilemma that arises when the same compounds that are used to defend against generalists simultaneously attract specialists (van der Meijden 1996). Specifically, we hypothesize that in the absence of the specialist leaf chewer, *Cucullia verbasci*, introduced mullein is free to deploy higher maximum iridoid concentrations than is viable in the native range. Given that overall investment (averaging over leaf age) in the percent total iridoid glycosides does not differ between ranges, there exists the potential for introduced mullein to enjoy a fitness gain simply by optimizing the within-plant distribution of defenses in its new (*Cucullia*-free) environment. The difference in defense allocation apparent in natural field populations could be either fixed or an environmentally plastic response to attack. However, levels of investment in many chemical defenses, including iridoid glycosides, are heritable (e.g., Berenbaum et al. 1986, Marak et al.

2000, Wright et al. 2004); as such, the variation is likely to be at least partially genetically based.

The proportion of iridoids made up of the more toxic compound catalpol is also higher in young than old leaves in both ranges (Figure 2b). However, unlike the percent total iridoids, the proportional investment in catalpol is nearly identical between ranges. This could represent a constraint on the biosynthetic pathway that produces catalpol from its precursor aucubin (Damtoft 1994). Alternatively, it appears that herbivores in the introduced range are deterred simply by the presence of either iridoid, avoiding both aucubin and catalpol that is present in old leaves (Table 3). Given that converting aucubin to catalpol represents an extra step in the biosynthetic pathway and likely an increased cost of defense (Gershenzon 1994), there may be little selection for increased proportions of catalpol in the introduced range.

Chewing damage by herbivores is less prevalent and less severe on young than old leaves in both ranges. However, the discrepancy between attack to young and old leaves is much smaller in the native range, illustrating that native plants lose significantly more high-quality tissue than their introduced counterparts (Figure 2, a and b). One prediction of optimal defense theory is that the best-defended tissues should also be those most vulnerable to attack in the absence of chemical defense (McKey 1974). This part of the theory is not often considered in the context of invasions, where the probability of attack will differ depending on whether generalists or specialists are important herbivores. In mullein's native range, where *C. verbasci*, as a native specialist herbivore, likely uses iridoids as feeding and oviposition cues (as has been found for other specialists on iridoid glycoside-containing plants,

reviewed in Bowers 1991), an absence of iridoids would impair the moth's ability to find its host plant, leading to less feeding damage. In the introduced range, where mullein is free from specialist chewers, a reduction in iridoids would lead only to increased attack by generalists. As such, young leaves in the introduced range can be effectively protected from attack with increasing levels of iridoids, while young leaves in the native range risk attack whether they are defended or not. The significant range × leaf age interaction for percent total iridoid glycosides (Figure 1a) thus provides support for optimal defense theory in a unique context.

The correlation analysis between iridoid glycoside content and the severity of herbivore damage reveals several interesting patterns. In the native range, the relationship between iridoid investment and herbivory clearly differs between young and old leaves. For young leaves, percent catalpol, percent total iridoids, and the proportion of iridoids composed of catalpol are all positively correlated with feeding damage. This pattern is consistent with the specialist *C. verbasci* being attracted to leaves with high iridoid content. Conversely, herbivory to old leaves of native plants is negatively correlated with percent catalpol, percent total iridoids, and the proportion of iridoids composed of catalpol. This indicates a deterrent effect and suggests that generalist feeders are involved in the interaction. It further suggests that native generalists partition themselves onto older leaves with lower iridoid content. In the introduced range, there was no relationship between any measure of iridoid content and herbivory to young leaves, which is due to the fact that young leaves were rarely attacked at all (Figure 2a). In contrast, generalist herbivores were able to feed on older leaves with lower iridoid content (Figure 2, a and b), although

increasing amounts of aucubin, catalpol, and total iridoids all had a significant

deterrent effect on feeding (Table 3). Interestingly, higher concentrations of percent

aucubin were associated with decreased feeding damage in the introduced range, but

not the native range; conversely, higher proportions of iridoids composed of catalpol

were associated with diminished damage in the native range, but not the introduced

range (Table 3). This suggests that while iridoids have an overall deterrent effect on

generalist feeders in both ranges, different aspects of the chemical profiles inform

feeding decisions in each range.

By taking advantage of the natural shift in the enemy communities associated

with mullein in each range, we were able to test optimal defense theory in a novel

way. Our findings provide support for the theory, both in terms of the within-plant

distributions of defense and herbivory with respect to plant tissue value, and more

broadly by illustrating that herbivores indeed appear to represent a major selective

force on plant chemical defenses. Our findings also provide an additional refinement

to the EICA hypothesis, and have the potential to explain the variable trajectories

associated with post-introduction shifts in defense investment. For example, several

previous studies have reported increased performance in introduced plants that do not

exhibit associated changes in defense investment (Willis et al. 1999, Buschmann et al.

2005, Genton et al. 2005, Hull-Sanders et al. 2007, Alba et al. 2011). In these studies,

defenses were measured at the whole-plant scale, without considering the distribution

of defenses among plant tissues of different value (nor differences in herbivory

among tissues of different value). Here we have illustrated that changes at the within-

plant scale may be particularly common in the contexts of invasions, and represents a

potentially important adaptive shift that deserves further study.

Table 4.1. Mullein populations sampled for herbivory and iridoid glycoside content*

Population Code	Country/State	Plants Sampled for Herbivory	Latitude (N)	Longitude (E)	Elevation (m)	Mean Annual Precipitation (cm)
Native (EU)						
MA1	**Macedonia**	**10**	41.297903	21.097853	**674**	**49.0**
CH2	Switzerland	5	47.335256	7.115103	695	107.0
CH3	Switzerland	7	47.341156	7.317797	442	107.0
CH1	**Switzerland**	**20**	47.359325	7.364636	**416**	**107.0**
P3	**Czech Rep.**	**8**	49.874744	14.437797	**254**	**66.7**
P2	Czech Rep.	6	49.999106	14.559139	300	47.0
P5	**Czech Rep.**	**12**	50.064025	14.485831	**228**	**47.0**
P4	Czech Rep.	11	50.785525	14.458558	423	47.0
BE2	Belgium	15	50.927214	4.424692	16	81.0
BE1	**Belgium**	**22**	51.039483	5.757936	**31**	**82.0**
SW4	Sweden	3	60.2422	17.682306	41	54.0
SW3	Sweden	2	60.251239	17.669442	41	54.0
SW1	Sweden	4	60.258389	17.636114	41	54.0
SW2	**Sweden**	**12**	60.259086	17.656744	**41**	**54.0**
Introduced (US)			Latitude (N)	Longitude (W)		
LOR	CO	10	40.583006	105.174506	1671	38.4
BC	CO	20	40.800472	105.379528	2252	38.4
SL	**CO**	**15**	**40.986139**	**107.381667**	**2041**	**25.6**
WY2	WY	16	41.055056	109.352417	2495	22.8
WY1	WY	3	41.130667	109.213472	2358	22.8
WY5	**WY**	**15**	**43.386222**	**110.725833**	**1928**	**40.2**
WY4	WY	15	43.404	110.751556	1898	40.2
WY6	WY	13	43.428861	110.777028	1885	40.2
WY3	**WY**	**20**	**43.429583**	**110.781583**	**2129**	**40.2**
ID	**ID**	**10**	**44.137006**	**112.222542**	**1828**	**30.2**
MTA	MT	15	45.097417	112.776972	1689	33.3
MT2	MT	15	45.424972	110.651167	1849	36.8
MT3	MT	15	45.499556	110.547694	1649	36.8
MT4	MT	10	45.673972	110.540056	1427	36.8
MT1	**MT**	**15**	**45.686333**	**110.512472**	**1444**	**26.0**
MT5	MT	15	46.225694	111.377917	1394	27.3
MT6	MT	10	46.253611	111.297333	1493	27.3
MT7	MT	15	46.286917	111.532	1377	27.3
MT8	MT	15	46.318722	111.629111	1506	27.3
MT9	**MT**	**15**	**47.404667**	**111.327917**	**1135**	**40.0**
MT10	MT	15	47.432056	111.319944	1402	37.1

*Populations in bold analyzed for iridoid glycoside content

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Table 4.2. Results of ANOVA testing the effects of range (native or introduced), leaf age, and the range x leaf age interaction on variation of the iridoid glycosides aucubin and catalpol in *Verbascum thapsus* (common mullein)

Source	Aucubin		Catalpol		Total Iridoid Glycosides		Proportion Catalpol	
	F value	P value	F value	P value	F value	P value	F value	P value
Range	0.62(1,10)	0.45	0.01(1,10)	0.92	0.01(1,10)	0.92	0.02(1,10)	0.9
Leaf Age	6.5(1,180)	0.01	130(1,179)	<0.0001	177.1(1,203)	<0.0001	200.5(1,179)	<0.0001
Range x Leaf Age	14.3(1,180)	0.0002	2.9(1,179)	0.09	33(1,203)	<0.0001	1.33(1,179)	0.25
Random*	χ2	P value	χ2	P value	χ2	P value	χ2	P value
Population	24.9	<0.0001	51.7	<0.0001	77.1	<0.0001	67	<0.0001

*The random effect χ-square is a one-sided test with a single degree of freedom which tests the hypothesis that the variation due to the effect is greater than zero.

Table 4.3. Pearson's Correlation coefficients describing the relationship between iridoid glycoside content of young and old leaves of native and introduced mullein and the severity of chewing damage to those same leaves

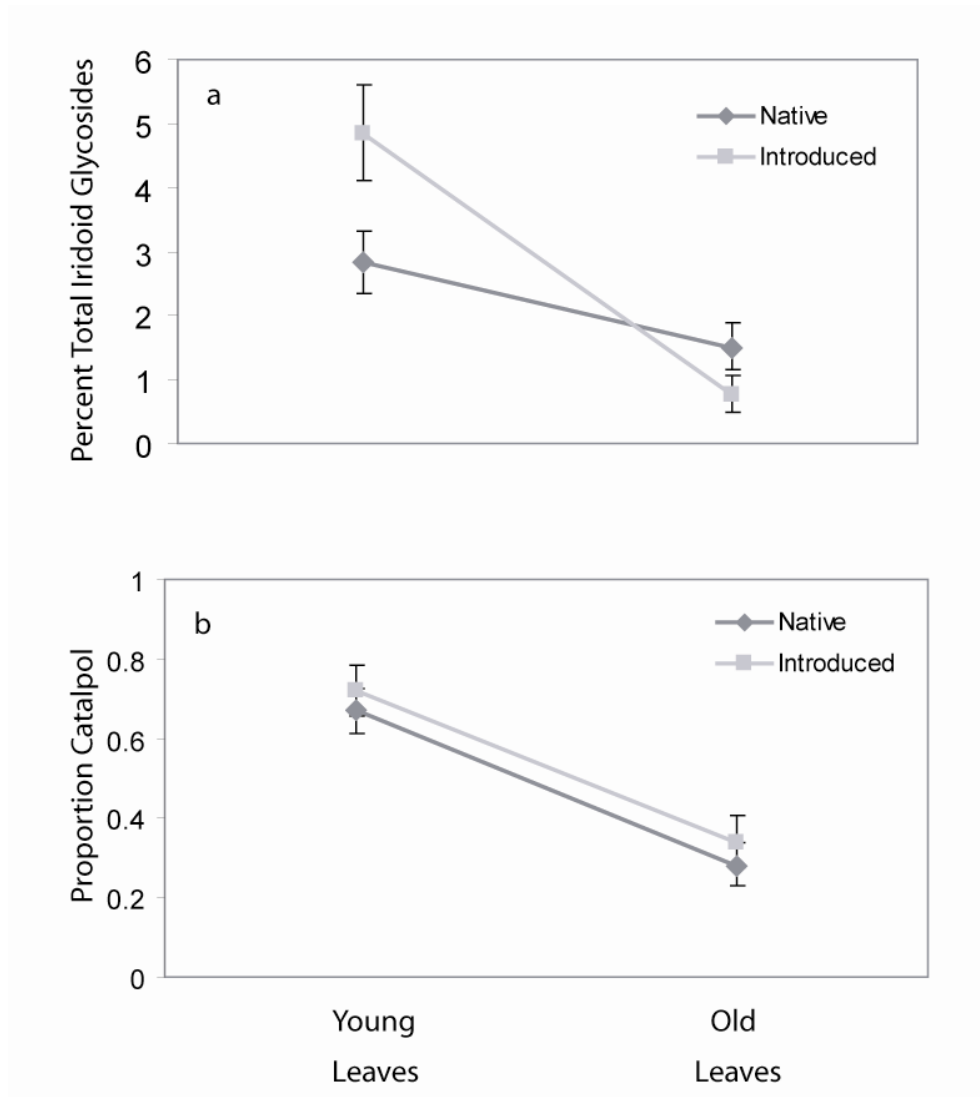|	Native		Introduced	
	Young	Old	Young	Old
Percent Aucubin	-0.07	-0.037	-0.08	-0.51 ***
Percent Catalpol	0.39 **	-0.27 *	0.14	-0.36 **
Percent Total IGs	0.36 **	-0.25	-0.002	-0.46 **
Proportion catalpol	0.4 **	-0.31 *	0.15	-0.13

$*P < 0.05$; $**P < 0.01$; $***P < 0.0001$

Figure 4.1. Mean (± SE) (a) percent total iridoid glycosides and (b) proportion of iridoids composed of catalpol in young and old leaves of native and introduced *Verbascum thapsus* (common mullein) populations. See Table 2 for ANOVA results of the effect of range, leaf age, and the range × leaf age interaction on iridoid glycoside content.
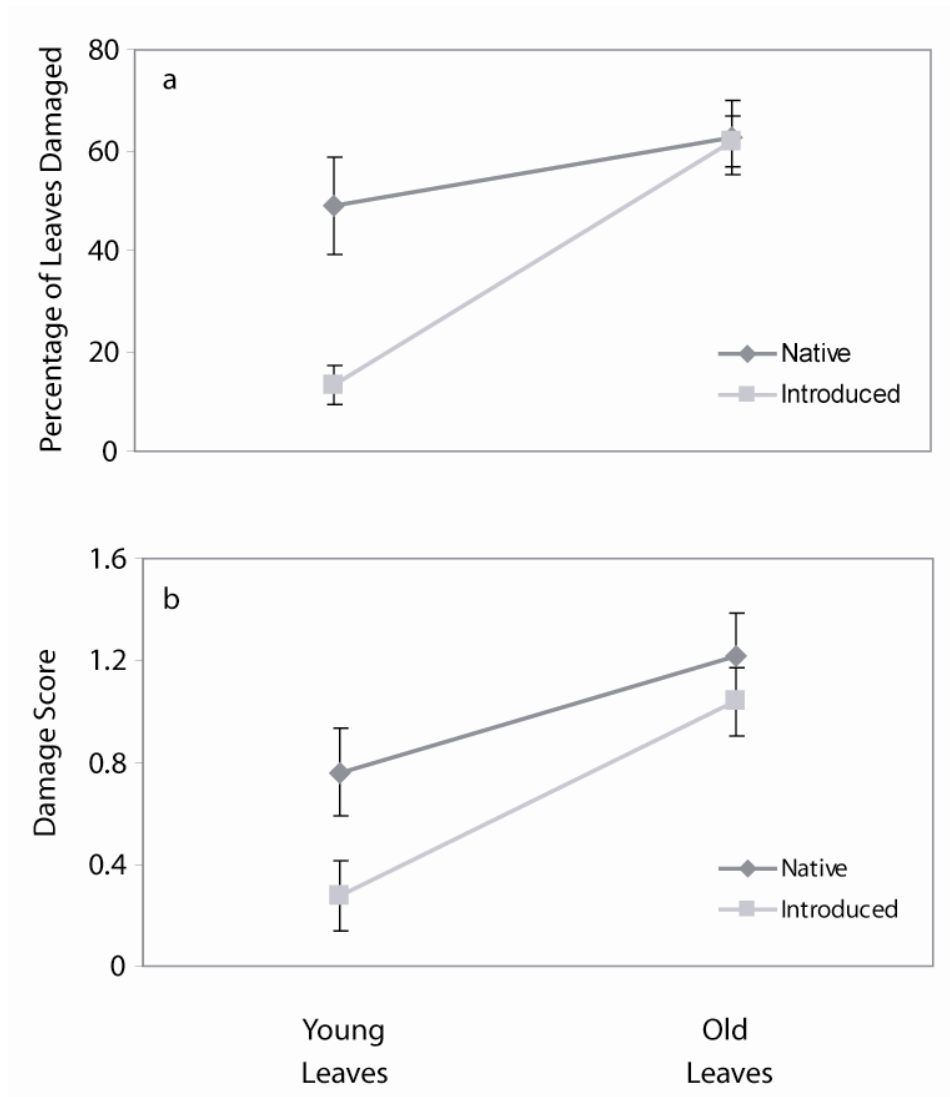
Figure 4.2. Mean (± SE) (a) percentage of leaves damaged and (b) damage scores
(i.e., damage intensity) for young and old leaves of *Verbascum thapsus* (common
mullein) experiencing ambient herbivory by chewing herbivores in its native (Europe)
and introduced (U.S.) ranges. See text for details of scoring system and statistical
significance of the effects of range, leaf age, and the range × leaf age interaction on
levels of herbivory.

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