

DISSERTATION

FRAMEWORKS FOR TESTING MECHANISMS OF INVASION AND PLANT DEFENSE

Submitted by

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Graduate Degree Program in Ecology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Spring 2018

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ABSTRACT

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Invasions are an increasingly problematic ecological and economic challenge in today's world of rapid globalization and environmental change. Economists estimate that global management and damage costs of biological invasions will exceed 300 billion dollars each year. In the United States alone, plants result in annual estimated costs of greater than 33 billion dollars in management and damages, with additional annual environmental losses valued at about 150 million. Understanding how plants evolve after introduction to a new range is therefore of broad interest, as this understanding may lead to better-informed management. In turn, better-informed management may increase services of natural and agricultural settings alike, as well as increase the efficiency of the taxpayer dollars so often used to control these invasions and their impacts.

In addition to the applied implications of understanding invader evolution, biological invasions represent large-scale evolutionary experiments, as they often experience striking environmental differences between their native and introduced ranges. For example, upon introduction to a novel range, plants often experience a new climate and reduced herbivory, especially from specialists. Invasions are therefore ideal for addressing fundamental ecological and evolutionary questions, as we can use between-range comparisons to investigate how plants evolve in response to differences in selection that have remained consistent across evolutionary significant periods of time.

In general, my research is highly motivated by this concept of using invasions as a framework for testing theories of invasion and plant defense. I am especially interested in whether adaptation to herbivory or climate (or neither) best explains increased performance of introduced populations. In particular, I am intrigued by the idea that one of the most well-cited theories of invader evolution, the Evolution of Increased Competitive Ability (EICA), receives mixed support within the literature. EICA predicts that, as a result of a growth-defense trade-off, introduced populations should evolve decreased defense against herbivory in response to a decrease of natural enemies, and instead reallocate limited resources towards growth and reproduction. That investment in costly defense should decrease in the absence of herbivory makes common sense, especially given strong evidence for enemy escape. So why then is support for EICA is mixed?

In my first chapter, I aimed to better disentangle the processes that might explain why tests of invader evolution often do not match the predictions of EICA. To this end, I used two common gardens of a widespread weedy invader (*Verbascum thapsus*), one each located in its native and introduced range. This experimental design allowed me to investigate the relative importance of climate versus herbivory in driving between-range evolutionary differences in performance and defense. My finding that introduced populations did, on average, evolve increased growth as compared to their native congeners only in part matches the predictions of EICA. In contrast to predictions of EICA, I found that climate, not herbivory, best explains this between-range difference: within both common gardens, seeds collected from the native range produced smaller rosettes as the climate of origin becomes cooler and drier, while seeds collected from the introduced range produced large rosettes regardless of climate of origin. The breakdown of a potentially adaptive cline in performance after introduction to a novel range

emphasizes the need to more closely investigate the evolutionary processes that shape geographic structuring (or its absence) in both the native and introduced ranges of invasive species. In addition, this finding emphasizes that EICA is not universally applicable to all invasion scenarios, and underscores the importance of testing the underlying assumptions alongside the predictions of this hypothesis.

In my second chapter, I became increasingly interested in how the relative amount of herbivore damage between young and old leaves differed between native and introduced field populations of *V. thapsus*. I was especially curious as to whether these observations matched predictions of the optimal defense hypothesis, which states that because defense is a costly, limited resource, plants should evolve to disproportionately allocate more defense to tissue that is either more valuable to future plant reproduction or at greater risk of being attacked if undefended. One notable prediction of this hypothesis is that young leaves should be better defended than old leaves, as they are both more valuable to future plant reproduction and more vulnerable to attack if undefended. In this chapter, I answer the call to action to better disentangle the effect of adaptation to herbivore pressure from physiological processes that also likely influence within-plant allocation of defense. Specifically, I refine the optimal defense hypothesis by incorporating predictions of how, for non-annual temperate plants, the relative value of young leaves, old leaves, and roots should shift towards the end of a plant's first growing season as it prepares to overwinter. I present a different set of predictions for both mobile and immobile defenses, as each likely operates under different physiological constraints.

In my third chapter, I used *V. thapsus* to test the predictions of optimal defense I proposed in my second chapter. To this end, I conducted a potted, common garden experiment of *V. thapsus* containing introduced populations that have historically experienced a very low risk

of attack by herbivores as well as native populations that have historically experienced a very high risk of attack by herbivores. I then measured how physical and chemical traits related to defense against herbivory were invested in young versus old leaves at the beginning, middle, and end of the first growing season. *Verbascum thapsus* does more strongly defend its young leaves than its old leaves, as optimal defense predicts, but it does not match how optimal defense would predict defense to evolve between the native and introduced range or across the growing season. Overall, we show that our framework can better disentangle whether adaptation is responsible for young leaves being better defended than old leaves, which is not the case for *V. thapsus*. Innate plant physiology does explain some, but not all, of the observed variation in defense allocation to young versus old leaves. Thus, other factors in addition to adaptation to herbivory or innate plant physiology likely play a stronger role in driving variation in within-plant allocation of defense, especially across the growing season.

In my fourth chapter, I returned to a question that, to me, is at the core of an invader's success: upon introduction to a new range, what drives population spread? Population spread is strongly driven by the dispersal of the individuals that comprise that population. In this chapter, I therefore wanted to better understand how individuals are influenced by both their phenotypes and their surrounding environments when deciding how far to disperse. To this end, I used the model system *Tribolium castaneum* (red flour beetles) to test how an individual's past environment influences its response to the environment it experiences during dispersal. The role juvenile environment plays in influencing dispersal is nuanced, as juvenile environment may influence an individual's dispersal both by changing that individual's phenotype as well as by changing the phenotype of its neighbors, who play an important role in influencing how that individual decides to disperse. We found that individuals moved especially far when exposed to

poor rather than good conditions during dispersal if their phenotype (or just one-third of their neighbors' phenotypes) were shaped by a poor environment as juveniles. Juvenile environment therefore shaped an individual's dispersal both by influencing its phenotype as well as its external social environment, which suggests that the juvenile environment of even a few individuals can influence the dispersal of an entire population.

ACKNOWLEDGEMENTS

I am extremely grateful for the opportunities, both personal and professional, that I experienced as a graduate student at Colorado State University. These opportunities were often the direct result of the tireless support and guidance of my two strongest advocates, my co-advisors Ruth Hufbauer and Andrew Norton. With them as my advisors, I learned from the best how to conduct and communicate good science with poise and integrity. I will be forever grateful to them for their mentorship and friendship, and any future success I achieve will be built upon their endless guidance and support.

I am also thankful to both past and present members of the Hufbauer and Norton labs. Chrissy Alba, Ellyn Bitume, Amy Clark, Eliza Clark, Christa Fettig, Janet Hardin, Mike Koontz, Peter Leipzig-Scott, Scott McArt, Marianna Szücs, Kathryn Turner, Graham Tuttle, and Megan Vahsen were always ready to drop everything to support me in times of both struggle and celebration. In addition, I owe an especially heartfelt thank you to my extended lab family and beta readers, Esby Miller and Rachael Sitz. I am grateful to all of the above not only for their help in completing this dissertation, but for the friendships that made my graduate career such a fantastic journey.

I am also appreciative of my committee members, Deane Bowers and Cameron Ghalambor. Deane's unbridled enthusiasm for students and all things insects is always invigorating, and I am extremely appreciative for all of the time and support she dedicated towards my graduate career. Cameron's support has also been invaluable, and I am especially thankful for his continued insight into the nuances of evolution and plasticity.

Funding for this research was provided by: Colorado State University's Agricultural Research Station, Graduate Degree Program in Ecology, and the Department of Bioagricultural Sciences and Pest Management. This research was also supported by the National Science Foundation, through a Doctoral Dissertation Improvement Grant. None of this research would have been possible without the hard work and enthusiasm of more than 35 dedicated undergraduates. These students cumulatively invested more than 2,400 hours into these experiments, and their questions and insight helped me to attain a better understanding of my research than I would have achieved on my own. In addition to those mentioned above, Petr Pyšek and Franck Dayan were invaluable to the execution of the research presented in this dissertation.

Lastly, throughout this process my family, both immediate and extended, has showered me with endless support. My parents instilled a love of nature and writing in me from a very young age, and never questioned my joy at squelching through wetlands or digging up worms. Jason, my husband and occasional field assistant, has been my emotional bedrock and my biggest cheerleader. To my family: this is for you.

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CHAPTER I: BREAKDOWN OF A GEOGRAPHIC CLINE EXPLAINS HIGH
PERFORMANCE OF INTRODUCED POPULATIONS OF A WEEDY INVADER

SUMMARY

1. What drives the evolution of increased growth and fecundity in plants introduced to a novel range is not well understood.
2. We investigate between-range differences in performance for *Verbascum thapsus*, a weedy invader known to grow larger in its introduced than native range. Specifically, we question whether adaptation to herbivory or climate best explains increased performance of introduced populations.
3. We grew 14 native and 22 introduced populations of *V. thapsus* in two common garden locations: near Prague, Czech Republic (native range) and in Colorado, USA (introduced range). By removing herbivores from half of the plants within each garden we tested the prediction of the Evolution of Increased Competitive Ability (EICA) hypothesis: increased performance is driven by an evolutionary shift of resources away from defence against herbivory towards growth and reproduction. We then investigated whether genetically based clines in performance are expressed along climate gradients within both the native and introduced ranges.
4. On average, seeds produced larger rosettes when collected from the introduced versus native range. While this evolution of increased growth in introduced populations in part matches the prediction of EICA, climate, not herbivory, best explained this between-range difference. Specifically, seeds collected from the native range produced smaller rosettes as the climate of origin became cooler and drier, while there was no cline in performance amongst rosettes grown from seed collected from the introduced range, which are large regardless of climate of origin.

Thus, a climate-based cline within the native range best explains lower average performance of native compared to introduced populations.

Synthesis: The breakdown in a potentially adaptive cline emphasizes the need to more closely investigate the evolutionary processes that shape geographic structuring (or its absence) within the introduced range. In addition, EICA is not universally applicable to all invasion scenarios, and our findings underscore the importance of testing underlying assumptions alongside the predictions of this hypothesis.

INTRODUCTION

Plant populations often evolve increased growth and reproduction after introduction to a novel range (Whitney and Gabler 2008; Drenovsky *et al.* 2012). However, what drives this evolutionary pattern is not well understood (Willis *et al.* 2000, Whitney and Gabler 2008; Burton *et al.* 2010; Dunn *et al.* 2012). Many hypotheses predict that increased performance of introduced populations is driven by adaptation to novel abiotic or biotic conditions, such as fewer natural enemies or a more benign environment, in a plant's introduced as compared to native range (*e.g.*, Blossey and Nötzold 1995; Mooney and Cleland 2001; Sakai *et al.* 2001; Lee and Klasing 2004; Bossdorf *et al.* 2005; Alexander *et al.* 2012). Parsing the relative importance of these different drivers of adaptation is critical to understanding current patterns of invader evolution as well as predicting which habitats are most susceptible to invasion moving forward.

One important adaptive hypothesis posits that increased growth is the evolutionary consequence of plants escaping many of their enemies upon introduction to a new range (the Evolution of Increased Competitive Ability [EICA] hypothesis) (Blossey and Nötzold 1995). Specifically, EICA states that reduced herbivory in the introduced range should lead to an

evolutionary shift of resources away from defence against herbivory towards growth and reproduction (1995). However, despite strong evidence for enemy escape (Keane and Crawley 2002; Colautti *et al.* 2004; Lui and Stiling 2006), especially from specialists, evidence in support of EICA is mixed (Felker-Quinn *et al.* 2013; Lowry *et al.* 2013). While introduced populations do evolve decreased defence against herbivory in some plant species (Daehler and Strong 1997; Blair and Wolfe 2004), other plant species either invest similarly in defence in both ranges (Franks *et al.* 2008; Cripps *et al.* 2009; Huberty *et al.* 2014), or evolve higher defence in introduced than native populations (Stastny *et al.* 2005; Ridenour *et al.* 2008). Furthermore, increased size is often not correlated with a decrease in defence in introduced relative to native populations (Bossdorf *et al.* 2005; Felker-Quinn *et al.* 2013).

This inconsistent support for EICA must be explained if it is to remain a useful framework for understanding why many invasive populations evolve greater performance in their introduced habitat. A clear way forward is to test the two main assumptions that underlie the predictions of EICA: (1) that herbivory reduces plant fitness and (2) that defences against herbivory are costly (Cipollini *et al.* 2005; Handley *et al.* 2008; Cipollini and Lieurance 2012). Importantly, we know herbivory does not always reduce fitness; some species are quite tolerant to herbivory, particularly in benign environments (Hawkes and Sullivan 2001; Wise and Abrahamson 2007), and some even respond positively to herbivory through overcompensation (Paige 1992; Paige *et al.* 2001; Rautio *et al.* 2005); thus, herbivory should only affect plant fitness for genotypes that have not evolved high tolerance (Strauss and Agrawal 1999). In addition, evidence for costs of defence is remarkably rare (Siemens *et al.* 2010; Neilson *et al.* 2013), and is by no means ubiquitous across species (Strauss *et al.* 2002). Rigorously testing

both of the assumptions guiding the predictions of the EICA hypothesis is therefore critical for determining whether this hypothesis is even applicable to the given invasion scenario.

Further, differences in herbivore composition and abundance are not the only striking disparities between a plant's native and introduced ranges. Climate also often varies between ranges (Early and Sax 2014; Boci *et al.* 2016), and is a known driver of adaptation within invasive populations (Maron *et al.* 2004; Montague *et al.* 2008; Alexander *et al.* 2012; Zenni *et al.* 2014; Lee and Kotanen 2015). Jointly investigating EICA and climate-driven variation in performance is important for interpreting adaptive evolution for two main reasons.

First, climate may be the major driver of local adaptation within introduced populations, not reduced herbivory. For example, many plant invaders rapidly evolve predictable phenotypic variation along climatic or latitudinal clines, which often mimic pre-existing clines within the native range (Weber and Schmid 1998; Hakam and Simon 2000; Roy *et al.* 2000; Leger and Rice 2007; Dlugosch and Parker 2008b; Etterson *et al.* 2008; Montague *et al.* 2008; Keller *et al.* 2009; Hodgins and Rieseberg 2011; Konarzewski *et al.* 2012; Alexander 2013; Novy *et al.* 2013).

Second, if between-range differences in adaptation due to differences in herbivore pressure do exist, they may be obscured by unaccounted-for abiotic clines (Colautti *et al.* 2009). This is especially important as many plant populations vary predictably along climatic gradients in the two traits predicted by EICA to drive invasion success: performance and defence against herbivory. Performance tends to decrease with increasing latitude in benign environments, as plants evolve decreased biomass (associated with strong selection for earlier flowering) (Abhilasha and Joshi 2009; Colautti and Barrett 2013; Kooyers *et al.* 2015). However, herbivory too is known to vary predictably along climatic gradients; herbivory, and thus plant defense, is often hypothesized to diminish with increasing latitude (Johnson and Rasmann 2011). Although

support for this hypothesis is mixed (Moles *et al.* 2011, Anstett *et al.* 2016), defences such as trichomes (Kooyers *et al.* 2015) and secondary compounds (Pratt *et al.* 2014; Anstett *et al.* 2015; Reudler and Elzinga 2015) do often decrease with increasing latitude.

It is also the case that non-adaptive contingencies associated with introduction history may best explain between-range differences in performance. Gene flow and genetic drift also play important roles in the success of invaders relative to conspecific native populations (Simberloff 2009; Bock *et al.* 2015; Dlugosch *et al.* 2015). For example, admixture or the purging of deleterious alleles through inbreeding may increase performance of introduced populations (Verhoeven *et al.* 2011; Dlugosch *et al.* 2015). Conversely, bottleneck events or repeated introduction events may hinder adaptation, either by facilitating ongoing gene flow or by reducing genetic variation in the introduced as compared to native range (Dlugosch and Parker 2008a; Cristescu 2015). In addition, adaptation along geographic clines often occurs within 50–150 generations after introduction to a new range (Moran and Alexander 2014), and some populations simply may not have had sufficient time to adapt to their novel environment. As a consequence, genetic clines that exist within the native range may fail to reestablish within the introduced range.

Herein we evaluate the role of adaptation due to herbivory or climate, while also considering the non-adaptive role of introduction history in driving differences in performance between native and introduced populations of *Verbascum thapsus*. Specifically, we tested whether: (1) herbivory reduces plant performance, (2) defence against herbivory is costly, and (3) defence against herbivory differs between native and introduced populations. We then (4) assessed whether a population's climate of origin shapes plant performance. We hypothesized that native populations, with long evolutionary histories in their locations of origin, would

exhibit a genetically based cline in performance, while introduced populations may (via rapid adaptation) or may not (due to introduction history) exhibit a cline.

MATERIALS AND METHODS

Study system

A typically biennial forb (but see Gross and Werner 1978; Ansari and Daehler 2010), *Verbascum thapsus* L. (Scrophulariaceae) was intentionally introduced from Eurasia to North America multiple times in the early 1600s. In its native range, *V. thapsus* occurs throughout the British Isles and Europe (excluding Iceland and Crete), extending north to 64°N, east into Russia and parts of China, and south to the Western Himalayas and Caucasus Mountains (Gross and Werner 1978). Since its introduction to North America, *V. thapsus* has established within all 50 of the United States as well as all of the southern provinces of Canada (Gross and Werner 1978; Ansari and Daehler 2010), and is designated as a noxious species within Colorado and Hawaii. As reflected by its broad distribution, *V. thapsus* possesses a broad climatic tolerance, and high seed output and seed viability, though it often requires full light to germinate (Semenza *et al.* 1978; Gross and Werner 1982; Parker *et al.* 2003). *Verbascum thapsus* is often an early colonizer of disturbed habitats, is generally intolerant to shade, and prefers dry, sandy soils (Gross and Werner 1978; Reinartz 1984a). In general, first-year plants grow as low-lying rosettes, and then bolt and flower during their second year of growth (Gross and Werner 1978; Ansari and Daehler 2010).

Verbascum thapsus is ideal for investigating the role of herbivory and climate in driving invader evolution, as there is strong support for both enemy escape (Popov 1972; Gross and Werner 1978; Wolfe 2002; Alba *et al.* 2012) and evolution of greater aboveground biomass

production for introduced compared to native populations (Alba *et al.* 2011, 2012; Kumschick *et al.* 2013). In addition, *V. thapsus* populations express predictable phenotypic variation along latitudinal and climatic gradients (Ansari and Daehler 2010; Alba *et al.* 2012; Seipel *et al.* 2015), although whether these trends are driven by genes, environment, or genetically based plasticity remains less well understood (but see Reinartz 1984a,b,c).

Common gardens

In summer 2012 we planted *V. thapsus* populations in two gardens: one located in the Czech Republic, Průhonice (native range) at the Institute of Botany of The Czech Academy of Sciences (49.99423°N, 14.56734°E) and the other in Fort Collins, CO (introduced range) at Colorado State University's Agricultural Research, Development and Education Center (40.65261°N, 104.99699°W). Each garden included populations representative of the plant's broader distribution, containing individuals grown from seed collected from two maternal lines from each of 14 European and 22 North American populations located across a large geographical range (Table 1.1). The herbivore communities in each garden reflected broader continental trends, with more, mostly specialist, insects in the Czech Republic garden (*i.e.*, clay groundling [*Nothris verbascella*] and mullein moth caterpillars [*Cucullia verbasci*]) and fewer, mostly generalist, leaf chewers in the United States garden (*i.e.*, grasshoppers [*Melanoplus* spp.] and the palestriped flea beetle [*Systema blanda*]).

Both gardens followed a modified split-plot design blocked by maternal line, in which population was randomly assigned at the whole-plot level (Fig. 1.1). Within each whole-plot were six plants: three randomly assigned to a reduced herbivory treatment, and three randomly assigned to an ambient herbivory treatment (6 plants × 2 maternal lines × [14_{European} and 22_{North}

American populations] \times 2 gardens = 336 European and 528 North American plants). Since maternal lines coincided with our two spatial blocks, within our analyses “block” encompasses both biologically important variation and incidental variation associated with the position within each common garden. Our design therefore does not allow us to compare differences *between* maternal lines within a population. However, it does allow for a much stronger comparison of performance *within* maternal lines, as plants from the same maternal line are tightly grouped together within the garden. This is essential for estimating tolerance and trade-offs between defense and performance, which is a main focus of our study. In addition, blocks were immediately adjacent and encompassed only a small section of long-standing agricultural plots (*i.e.*, all blocks within each garden have been treated similarly for the past several decades). Between-block differences are therefore expected to be small, as the maximum distance between plants of different blocks was less than 60 meters.

In our analyses, we treat garden location as a blocking factor (see below) instead of using a between-garden comparison. With this approach, garden allows us to account for random variation due to differences in abiotic (*e.g.*, temperature, rainfall, seasonality) and biotic (*e.g.*, soil biota) conditions at each garden location. Because there are only two gardens we cannot test for differences in plant performance due to the range in which the experiment was performed, as this is an un-replicated comparison. Importantly, we recognize that variation in herbivore type likely differs predictably between our two gardens, with plants experiencing specialist-dominated herbivory in the native range and generalist-dominated herbivory in the introduced range. However, our approach is conservative, as it adds environmental heterogeneity and investigates broad patterns that emerge despite random variation as well as predictable differences in herbivore communities.

Plant propagation

The following methods were simultaneously implemented within both gardens unless stated otherwise.

The first week of April 2012 we seeded 10 replicates of moistened Jiffy peat pellets (25mm in diameter) with 5–10 seeds for each of two maternal lines from each population. We then placed the peat pellets in germination chambers for two weeks on a 12-hour light/dark cycle, respectively set to 25/15°C, and watered as needed. We re-randomized pellets within the germination chamber every several days. After two weeks, we thinned each pellet down to two individuals and as soon as the buds of the first true leaves became visible to the naked eye we calculated the area of each cotyledon pair to estimate maternal provisioning ($\pi \times \text{cotyledon length} \times \text{cotyledon width}$). A t-test revealed no significant differences (Colorado garden: $t_{285.76} = 1.28$, $P = 0.2$; Czech Republic garden: $t_{335.91} = -0.57$, $p = 0.6$) in size between cotyledons grown from seed collected from the native (Colorado garden: $M = 18.5$, $SE = 0.000035$; Czech Republic garden: $M = 27.2$, $SE = 0.00058$) and introduced range (Colorado garden: $M = 19.1$, $SE = 0.000028$; Czech Republic garden: $M = 30.4$, $SE = 0.00042$). This suggests that observed performance differences between the native and introduced range are unlikely to be due to differences in maternal provisioning.

Four weeks after seeding, we transplanted peat pellets into 10-cm-diameter pots, randomly thinned the number of individuals down to one, and grew the remaining seedlings under similar greenhouse conditions for another two weeks. We then moved plants to an outside shelter and allowed them to acclimate for one week. The third week of May, we planted individuals into rows of white plastic weed barrier to minimize interspecific competition, with at least 1m between each plant to minimize intraspecific competition. We allowed at least 0.75-m-

wide strips of vegetation to grow between each row of weed barrier in order to encourage herbivore establishment, but regularly trimmed this vegetation to prevent light competition with experimental plants. Since Colorado experiences a much drier and hotter growing season than Průhonice, plants in the United States garden were regularly irrigated throughout the growing season, while plants in the Czech Republic garden were only irrigated until June 11, 2012 to encourage establishment.

Herbivory treatment

We assigned plants to either: (1) a reduced herbivory treatment (sprayed with systemic insecticides to minimize insect feeding damage), or (2) an ambient herbivory treatment (sprayed with water only). We diluted all insecticides according to the manufacturer's recommended concentrations, but we were restricted in our choice of insecticides due to conflicting regulations concerning pyrethroids between the United States and the Czech Republic. For plants within the reduced herbivory treatment, we applied a soil drench one week after individuals were transplanted into the gardens (Hi-Yield Grub Free Zone [applied 1 tsp of 0.5% imidacloprid granules at the base of each plant] in the United States, and Confidor 200 OD [0.00772% imidacloprid in a water solution] in the Czech Republic). We also applied a foliar insecticide, using Bayer Advanced Complete Insect Killer For Soil & Turf (0.001125% imidacloprid and 0.005625% β -Cyfluthrin in a water solution) in the United States, and a combined treatment of Confidor 200 OD (0.00772% imidacloprid in a water solution) and Cyperkill 25 EC (0.0125% cypermethrin in a water solution) in the Czech Republic. For both treatments, we sprayed each plant until run-off every 2 weeks until mid-October. Although insecticides may alter plant physiology, a previous greenhouse experiment found no difference in biomass between *V.*

thapsus sprayed weekly until runoff with Bayer Advanced Dual Action Rose & Flower Insect Killer or sprayed with a water control (Wilbur *et al.* 2013). In addition, evidence suggests the used insecticides do not enhance, and may even reduce, growth in other plant species (Palumbo and Sanchez 1995; Xia *et al.* 2006; Ahemad and Khan 2011).

Finally, we applied a one-time foliar application of spinosad in both gardens to control for thrips (Captain Jack's Deadbug Brew [0.007813% spinosad in a water solution] on May 1 in the United States, and SpinTor [0.001710% spinosad in a water solution] on July 11 in the Czech Republic). In the Czech Republic garden we also applied a one-time application of Vanish Molluscicide on June 5 to control for slugs, which were not active within the United States garden.

Measuring plant performance and herbivory

From mid-May to mid-September, we measured rosette area and damage by herbivores every six weeks. We calculated rosette area using the formula for a circle, $A = \pi \cdot r^2$, where r is one half the average rosette diameter.

To estimate herbivory, we used the second non-senesced pair of leaves up from the base of the plant. First, we estimated percent chewing damage for each leaf in the pair using the following categories: 0%, 1–5%, 5–10%, and increments of 10% thereafter. Then, we estimated herbivory on each plant as the average mid-point of each the two damage categories. Finally, we averaged our estimate of leaf herbivory for each leaf pair across our four sampling periods.

Quantifying climate of source populations

Climate-driven variation is often documented in introduced populations using latitude or elevation as proxies for climate (Colautti *et al.* 2009; Monty and Mahy 2009; Alexander 2010; Moroney *et al.* 2013). However, the current accessibility of geographic information system (GIS) maps linked to environmental variables allows us to directly evaluate the influence of a broad suite of climatic variables (Kozak *et al.* 2008) on performance. Here we use 19 bioclimatic variables associated with temperature, precipitation, and their seasonality (WorldClim database; www.worldclim.org) to elucidate potential clines in *V. thapsus* performance. We accounted for correlations among these 19 variables by creating linear combinations of the original variables using Principle Component Analysis. Specifically, we applied the loadings of three Principle Components (PCs) previously developed by Dupin *et al.* (2011) to the 19 bioclimatic values extracted from each of our collection sites (see Table 3 of Dupin *et al.* (2011)). This process created 3 PC values for each of our populations, one for each linear component. The first linear component (PC1) is attributed mainly to temperature, the second (PC2) to precipitation during wet or warm periods, and the third (PC3) to precipitation during dry and warm periods. These first 3 linear combinations of the variables explained nearly 84% of the variation present in the original variables (Dupin *et al.* 2011).

By using the loading values from Dupin *et al.* (2011) our PCs were constructed using a much broader dataset (>2,000 locations across Europe and North America) than the 36 collection sites represented within our manuscript. These PCs are therefore more likely to accurately capture biologically meaningful variation across the distribution of *V. thapsus* than PCs constructed from our collection sites alone. In fact, Dupin *et al.* (2011) show that these PCs

significantly improve species distribution models of a similarly-distributed invader, western corn rootworm.

Statistical analyses

We performed analyses using SAS 9.4 (SAS Institute, Cary, NC). In all models, garden location was included as a random effect. We accounted for unequal variation within the United States versus Czech Republic garden by assigning garden location to the group option within the random statement, which allowed the values of the covariance parameters to vary by garden. We also excluded plants in the United States garden that were overwatered as a result of rodent damage to the irrigation system.

To evaluate whether insecticide effectively reduced herbivory, we used a two-step process. Herbivory data were zero-inflated, with many plants exhibiting no measurable damage. We thus first investigated whether plants differed in whether they were attacked by analyzing presence of herbivory as a binary response. This model included origin (native or introduced), treatment (reduced or ambient herbivory), climate of the source population (the continuous climatic variables of PC1, PC2, and PC3), as well as two-way interactions between origin and each of these fixed effects. For plants that were attacked, we then used a generalized linear mixed model to examine how these same variables affected the amount of leaf tissue herbivores consumed. For both models, random effects included block, garden, and interactions between: (1) block and population nested within origin of the source population (hereafter referred to as origin), and (2) block, population nested within origin, and treatment (*i.e.*, reflects a split-plot design with two spatial blocks, with population assigned to the whole-plot level, and treatment to the sub-plot level).

i. Does herbivory reduce plant performance?

We focused on two response variables: rosette area at the end of the first growing season, and survival to the beginning of the second growing season. We used a linear mixed model to test whether rosette area, log transformed to improve assumptions of normality, was influenced by origin, treatment, or climate (the continuous climatic variables of PC1, PC2, and PC3), as well as two-way interactions between origin and treatment, and origin and each of the three climate PCs. We also used a generalized linear mixed model with a binomial response to evaluate the role of the same predictor variables in survival to the beginning of the second growing season. To investigate the degree to which climate improved model fit, we ran a likelihood ratio test between the full model and the model of log-transformed rosette size excluding the climate PCs. For both models, we used the same random effects described above for models of herbivore damage.

ii. Is defence against herbivory costly?

To evaluate the cost of defence (herein constrained to constitutive resistance) we determined whether, in the absence of herbivory (when defences have no benefit), more-resistant plants have reduced performance (Simms and Rausher 1987; Strauss and Agrawal 1999). This could occur if, in the absence of herbivory, well-defended plants are smaller than plants that are less well defended, as they have invested more in unnecessary, costly defence. For each maternal line within each population we measured performance as average rosette area of plants protected from herbivores, and resistance of unprotected plants within each population. Each population was therefore represented by four data points: one for each maternal line within each garden. We defined ‘resistance’ as the cumulative effect of traits that allow a plant to avoid or reduce

herbivory (Agrawal *et al.* 2004), which we defined as $\left(\frac{1}{\text{percent herbivore damage}}\right)$. We then examined whether there were range-level patterns in the correlations between resistance and performance that were measured for each maternal line. We used a generalized linear model to evaluate whether a population's average plant size in the absence of herbivory was positively correlated with average resistance when exposed to herbivory. We ran this model using a lognormal distribution within proc glimmix. For both models, random effects included population nested within origin and garden location, which was assigned to the group option within the random statement.

iii. Does defence against herbivory differ between native and introduced populations?

In comparing defence against herbivory between ranges, we investigated both resistance and tolerance to herbivory. We defined resistance as above. We used a generalized linear mixed model to test whether resistance was affected by origin, treatment, or climate (the continuous climatic variables of PC1, PC2, and PC3), as well as two-way interactions between origin and treatment, and origin and each of the three climate PCs. We used a lognormal distribution within proc glimmix, as values of resistance are continuously distributed values with non-negative values (Limpert *et al.* 2001).

We defined tolerance as the slope of the line of log-transformed rosette area (Wise and Carr 2008) plotted against percent herbivore damage experienced by each plant (*sensu* Strauss and Agrawal 1999). For each garden, we calculated a slope for each maternal family within each population. A negative slope indicates low tolerance, a slope not different from zero indicates high tolerance, and a positive slope indicates overcompensation. To evaluate whether tolerance to herbivory differed between populations of native and introduced origin we used a generalized

linear mixed model to assess whether the coefficient of the size-damage slope (calculated for each maternal line within each garden) was influenced by origin, climate of the source population, or the interaction between origin and each of the climate PCs. The observed tolerance coefficients were weighted by the $1/\sigma^2$ of each linear regression (Williams *et al.* 2014). For both models, population is the unit of replication, and random effects included block and garden location.

iv. Does climate of origin shape plant performance?

To evaluate whether herbivory or climate best explain plant performance, we then calculated the relative likelihood, defined as $e^{-\frac{1}{2}\Delta i}$, of the model of plant performance described above, where Δi is the difference between Akaike's Information Criterion for the model without climatic variables as compared to the full model including climate-related variables (Burnham and Anderson 2002).

RESULTS

Insecticide applications effectively reduced leaf herbivory by half; about 58% of sampled leaves experienced herbivory in the ambient herbivory treatment, as compared to only 29% of sampled leaves within the reduced herbivory treatment ($F = 47.65_{1,127}$, $P < 0.0001$; Fig. 1.2a). If leaves were attacked, the insecticide treatment also significantly reduced the amount of leaf tissue consumed ($F = 37.95_{1,127}$, $P < 0.0001$; Fig. 1.2b).

Herbivory reduced plant performance

Plants within the reduced herbivory treatment were about 12% larger than plants within the ambient herbivory treatment ($F = 3.95_{1,115}$, $P = 0.05$; Fig. 1.2c). The probability of plants surviving to the beginning of the second growing season was also 1.3 times higher for plants experiencing reduced as compared to ambient herbivory ($F = 3.69_{1,136}$, $P = 0.06$; Fig. 1.2d). Herbivory affected performance of plants from the native and introduced origin similarly (see non-significant interactions, Table 1.2).

We found no support for a cost of resistance for plants of native or introduced origin

In fact, counter to our prediction, more-resistant populations produced larger rosettes under reduced herbivory, irrespective of range of origin ($F = 7.46_{1,37}$, $p = 0.01$; Fig. 1.3; Table 1.3).

Native and introduced populations were similarly defended against herbivory

Resistance did not differ between native and introduced populations (Fig. 1.4a). In addition, neither attack rate nor the amount of tissue consumed once leaves were attacked differed by origin (Fig. 1.2a,b; Table 1.2). Tolerance to herbivory also did not differ between native and introduced populations (Fig. 1.4b; Table 1.4).

Climate of origin played a more important role than herbivory in mediating between-range differences in size

Plants of introduced origin were about 27% larger than plants of native origin. Although rosettes of introduced origin were large irrespective of their climate of origin, a clear cline in size

is seen across native European populations, such that rosettes became smaller as their climate of origin became cooler and drier (significant interactions between: (1) origin and PC1, which encompasses variation in temperature [$F = 4.49_{1,355}$, $P = 0.03$; Fig. 1.5], as well as (2) origin and PC2, which is mostly attributed to precipitation during wet or warm periods [$F = 4.86_{1,355}$, $P = 0.03$; Table 1.2]). The likelihood of the reduced model excluding all variables related to climate (*i.e.*, PC1, PC2, PC3, and their respective interactions with origin) was 0.03. Including climate thus made the model 33 times more likely to be a better fit for predicting rosette area than the model without any climate-related terms. Although we cannot make an inference about differences between gardens, we did find that patterns (or lack thereof) of clinal variation in herbivory and performance were similar between the two gardens (Table 1.5, Fig. 1.6).

DISCUSSION

Our findings, based on a robust experimental design and thorough test of the EICA hypothesis, suggest that herbivory does not drive an evolved increase in performance of introduced versus native populations of *V. thapsus*. In addition, we found no evidence for rapid adaptation to local climates within introduced populations, which instead consistently produced large rosettes regardless of their climate of origin. In contrast, among native populations rosettes from cooler and drier locations were smaller than those from warmer and wetter locations. Thus, perhaps counterintuitively, climate-based clines within the native range best explain lower average performance of native compared to introduced populations.

We initially investigated whether the evolution of increased size within introduced populations is the result of decreased defence against herbivory as predicted by EICA (Blossey and Nötzold 1995). Our results support EICA's first assumption that herbivory reduces plant

performance (shown also for this species by Wilbur *et al.* 2013). This finding is consistent with results from other study systems, *e.g.*, *Alliaria petiolata* (Bossdorf *et al.* 2004), *Centaurea diffusa* (Turner *et al.* 2014), *Melaleuca quinquenervia* (Franks *et al.* 2008), *Senecio inaequidens* (Bossdorf *et al.* 2008), *Solidago gigantea* (Meyer and Hull-Sanders 2008), *Triadica sebifera* (Huang *et al.* 2010; 2012), *Persicaria perfoliata* (Guo *et al.* 2011), *Peuraria montana* var. *lobata* (Yang *et al.* 2014), and *Phytolacca americana* L. (Huang and Ding 2016). However, many species show high tolerance to herbivory, *e.g.*, *Solidago canadensis* (van Kleunen and Schmid 2003) and *Chromolaena odorata* (Zheng *et al.* 2015), emphasizing that it is critical to test whether herbivory reduces plant fitness, as herbivory cannot drive selection if it does not reduce plant fitness.

We found no support for EICA's second assumption that defence against herbivory is metabolically costly to produce and maintain. If defences were costly, in the absence of herbivory well-defended plants should be smaller than plants that are less well defended (Strauss and Agrawal 1999). We instead found that larger rosettes were also more resistant to herbivory, which suggests that, instead of a trade-off, well-defended genotypes are more fit overall. A positive correlation between performance and defence can arise if traits that confer resistance to herbivory also provide fitness benefits independent of defence, and is especially likely if defence traits incur little or no metabolic cost, such as with secondary metabolites (Müller-Schärer *et al.* 2004; Joshi and Vrieling 2005). Alternatively, high plant performance can drive high defence if plants' 'general vigour' (Agrawal 2011) enables them to produce defences despite their potential physiological cost. Given this positive correlation between performance and resistance, we perhaps unsurprisingly do not see the expected decrease in defence within introduced populations. Instead, both native and introduced populations of *V. thapsus* experienced similar

levels of insect damage (*i.e.*, similar levels of resistance), and exhibited similar levels of tolerance to herbivore damage.

However, our measure of defence has limitations. Costs of defence are notoriously hard to detect (Cipollini *et al.* 2003; Zangerl 2003; Agrawal 2011), and may be expressed via trade-offs with traits either not measured or not expressed within our two gardens. For example, one interpretation of enemy escape is that the evolution of decreased defence predicted by EICA should be specific to defence against specialist, but not necessarily generalist, herbivores (van der Meijden 1996; Müller-Schärer *et al.* 2004; Joshi and Vrieling 2005). Our experimental design did not allow us to investigate ecological costs of herbivory driven by differences in the relative abundance of specialists and generalists. In addition, optimal defence theory predicts that between-range differences in defence should be most pronounced in young leaves (McKey 1974; Rhoades and Cates 1976), a pattern shown to be true for iridoid glycosides within field populations of *V. thapsus* (Alba *et al.* 2012). We focused on resistance (differences in amount of herbivory) on older leaves, as they experienced more herbivory overall. However, the patterns for young leaves were similar, with no difference in resistance between native and introduced populations (Fig. 1.7). Thus, despite limitations, our data imply that costs of herbivore defence, specifically resistance, are not the most important factor driving variation in rosette size.

An alternative explanation for increased performance of introduced populations of *V. thapsus* is climate. If climate drives local adaptation we would expect a genetically based cline within the introduced range that parallels that observed within the native range. However, we instead found that climate strongly explains native, but not introduced, plant performance; introduced rosettes are instead consistently large regardless of their climate of origin.

This evident breakdown in a potentially adaptive cline is contrary to many observations of invader evolution (Weber and Schmid 1998; Hakam and Simon 2000; Roy *et al.* 2000; Leger and Rice 2007; Dlugosch and Parker 2008b; Etterson *et al.* 2008; Montague *et al.* 2008; Keller *et al.* 2009; Hodgins and Rieseberg 2011; Konarzewski *et al.* 2012; Alexander 2013; Novy *et al.* 2013). However, clines are not always present in the introduced range (Ebeling *et al.* 2011) and indeed were not found by Alba *et al.* (2016), who studied germination of the *V. thapsus* seeds produced by our Czech Republic garden. The absence of genetically based clines among introduced populations may result from at least five different processes. First, this pattern is consistent with non-adaptive processes being stronger than adaptation. For example, bottlenecks or repeated introductions from a single source population could impede local adaptation by reducing genetic diversity or by swamping out locally adapted phenotypes (Simberloff 2009; Bock *et al.* 2015; Dlugosch *et al.* 2015).

Second, introduced populations may not have had enough time or genetic variation to adapt. However, adaptation along geographic clines often occurs within 50–150 generations after introduction to a new range (Moran and Alexander 2014) and *V. thapsus* was introduced to North America almost 400 years ago, suggesting that enough time has passed for a mostly biennial plant. Additionally, introduced populations of *V. thapsus* have evolved clinal differences in phenology, with southern populations having more annual genotypes, and northern populations more triennial genotypes (Reinartz 1984a), suggesting that neither time nor genetic variation are limiting.

Third, introduced populations may have higher fitness overall, and if that is true across the introduced range, selection for adaptive clines along climate gradients could be weak (Richards *et al.* 2006). Higher fitness could be achieved through a reduction in genetic load,

which can occur if bottlenecks and inbreeding in conjunction with natural selection purge deleterious mutations (Crnokrak and Barrett 2002; Facon *et al.* 2011; Marchini *et al.* 2016). Alternatively, if only the most robust genotypes survive the introduction and establishment phases (te Beest *et al.* 2012, Blackburn *et al.* 2015, Iles *et al.* 2016), a particularly robust genotype may invade. Introduced populations of *V. thapsus* allocate more resources to belowground biomass (Kumschick *et al.* 2013), and invest in fewer, but larger, leaves than native populations of *V. thapsus* (Fig. 1.8), traits that may be linked to higher overall vigour than native populations.

Fourth, the absence of a cline in performance among introduced populations may be due to the potential non-independence of herbivory and climate-driven selection. Latitudinal clines in herbivory are common, but often differ in direction both within and among plant species (Moles *et al.* 2011, Anstett *et al.* 2016). Discordant clines may exist due to differences in herbivore type and specificity (Anstett *et al.* 2014, Moreira *et al.* 2015) as well as geographical variation in the cost of defense (Kooyers *et al.* 2017). This has clear implications for between-range differences in clinal variation of an invader, as populations typically experience selection from specialist-dominated herbivore communities in the native range as opposed to generalist-dominated herbivore communities in the introduced range (van der Meijden 1996; Müller-Schärer *et al.* 2004; Joshi and Vrieling 2005). In fact, some co-occurring native and introduced plant populations differ in clinal variation, with introduction populations often expressing no cline in herbivory (Nunes *et al.* 2016; Allen *et al.* 2017). However, these studies test for differences in clines of herbivory among introduced and native populations that coexist within North America; how genetically based clines in herbivory differ between the North American and European

range remains less well understood, and would require large-scale experiments consisting of multiple common gardens within each range.

Fifth, the cline detected within the native range may not be adaptive, and we should therefore not expect this cline to be replicated within the introduced range. Although geographic clines in performance are often thought of as evidence of local adaptation (Turrison 1930; Clausen et al. 1940), rigorous estimates of the strength of selection, and its consequences, are often lacking (Savolainen et al. 2007; Moran and Alexander 2014). Populations may still perform suboptimally in their ‘home’ as compared to ‘away’ environments if low fitness occurs at range margins or if intraspecific and interspecific competition limits dispersal (Savolainen et al. 2007). Geographic structuring of genetic variation can also be an artifact of past range expansion, not local adaptation, as repeated founder effects often lead to gene surfing at expanding population edges (Slatkin and Excoffier 2012; Peter and Slatkin 2015).

Future studies should use molecular approaches to clarify introduction history, and thus the relative contribution of local adaptation versus non-adaptive processes in driving genetically based variation along abiotic clines. This is especially important within the introduced range (van Kleunen and Fischer 2008; Moran and Alexander 2014; Colautti and Lau 2015) where introduction history may either impede adaptation through bottleneck events or facilitate adaptation if admixture leads to increased genetic diversity (Genton *et al.* 2005; Lavergne and Molofsky 2007; Marrs *et al.* 2008; Verhoeven *et al.* 2011). Further exploration of whether, and if so how, climate mediates adaptation of introduced plant populations would provide valuable insight into which climate-related variables play the most important role in invader evolution. A strength of our study is that we incorporated climate into our models using three Principle Component axes (PCs) developed from 19 bioclimatic variables (Dupin *et al.* 2011), and

sampled broadly across both the native and introduced ranges (Table 1.1). This approach allowed us to move forward from using latitude or elevation as a proxy, to instead directly accounting for a wide breadth of climatic variables; this may explain why previous studies find an increase in plant size, independent of latitude, in the introduced range (Blumenthal and Hufbauer 2007; Alba *et al.* 2011; Kumschick *et al.* 2013), while we find no evidence of this difference after accounting for a much broader suite of climatic variables.

Overall, our study shows that evolutionary differentiation of introduced populations of *V. thapsus* from their native conspecifics is not driven by the reallocation of defence investment to increased competitive ability. Although between-range differences in performance are strongly explained by climate, this pattern results from a breakdown of genetically based clines along climate gradients. Thus, while climate and herbivory are known to be important drivers of invader evolution, investigating other adaptive and non-adaptive processes would strengthen understanding of invader evolution moving forward.

TABLES AND FIGURES:

Table 1.1 Collection locations for the 14 native (European) and 22 introduced (North American) populations of *V. thapsus*. To account for climate of the source populations, we used three linear Principle Components (PCs) that were developed from 18 bioclimatic variables taken from the WorldClim database (www.worldclim.org). The first linear component (PC1) was attributed mainly to temperature, the second (PC2) to precipitation during the wet or warm periods, and the third (PC3) to precipitation during periods of drought. PCs and elevation for populations 11 and 16 are based on GPS approximations.

Population ID	Origin	Location	Latitude	Longitude	Elevation (m)	PC1	PC2	PC3
1	Introduced	Hawaii, USA	19.68785	-155.46444	2000	0.69083917	0.81520826	2.51158953
2	Introduced	Tennessee, USA	35.55754	-84.01096	273	0.33217898	-0.22985937	1.60471988
3	Introduced	North Carolina, USA	35.76076	-78.67743	347	0.45071676	-0.16318308	1.16186213
4	Introduced	North Carolina, USA	35.86973	-82.04298	833	0.12609689	-0.09818333	1.79809868
5	Introduced	Maryland, USA	38.90028	-76.55556	18	0.26821721	-0.17950794	1.21854198
6	Introduced	Kansas, USA	39.20829	-96.5847	336	0.04894232	0.36152032	-0.46547973
7	Introduced	Illinois, USA	40.0165	-88.25696	236	-0.03121997	0.07596859	0.45731336
8	Introduced	Colorado, USA	40.38054	-106.80385	2463	-0.05449945	-1.24874568	1.09693837
9	Introduced	Pennsylvania, USA	40.43699	-79.94445	310	0.06672297	-0.06627461	1.02537143
10	Introduced	Colorado, USA	40.66529	-105.21944	1603	0.11539105	-0.67091089	-0.12658414
11	Introduced	Wyoming, USA	41.08	-109.18	2286	0.04613079	-1.14066756	0.38706273
12	Introduced	Ohio, USA	41.41744	-81.36583	346	-0.00059035	-0.19873257	0.93243963
13	Introduced	Massachusetts, USA	42.20102	-71.77729	134	-0.11379627	-0.28258729	1.73770452
14	Introduced	Oregon, USA	42.6638	-122.8263	467	0.35570750	-0.32210922	0.41347238
15	Introduced	New Hampshire, USA	42.84138	-71.67622	78	-0.15351161	-0.32902113	1.53949368
16	Introduced	Wyoming, USA	43.46667	-110.75	2073	0.01059600	-0.98862100	0.84529200
17	Introduced	Toronto, Canada	44.0292	-79.53742	295	-0.22833000	-0.22378641	0.76478267
18	Introduced	Wisconsin, USA	44.53314	-92.05947	217	-0.32944143	0.27930400	-0.41213733
19	Introduced	Oregon, USA	45.531	-121.0919	289	0.17250167	-0.18506013	0.46223226
20	Introduced	Montana, USA	47.06978	-111.97194	1078	0.02702288	-0.57699466	-0.20737687

Population						Elevation		
ID	Origin	Location	Latitude	Longitude	(m)	PC1	PC2	PC3
21	Introduced	Montana, USA	47.43206	-111.31994	1058	-0.00898109	-0.59286284	-0.08020727
22	Introduced	Idaho, USA	47.55414	-116.91631	777	0.13967800	-0.66917014	0.85385013
23	Native	France	43.67723	3.85595	67	0.49328300	-0.69727051	0.92036241
24	Native	France	45.94939	1.01531	268	0.37359545	-0.85359466	1.59350574
25	Native	Switzerland	46.84713	7.17373	584	-0.19494238	0.30356354	1.88201439
26	Native	Switzerland	46.98717	7.14019	453	-0.19494238	0.30356354	1.88201439
27	Native	Romania	47.14608	27.63928	54	0.02820559	-0.25488710	0.27429718
28	Native	Germany	47.88756	7.58136	217	-0.05630821	0.11035503	1.36094439
29	Native	Czech Republic	50.1797	13.37999	343	0.01495890	-0.55586785	0.78348118
30	Native	Finland	60.2061	25.13333	17	-0.23681052	-0.45496073	0.75757152
31	Native	Sweden	60.2296	17.70548	33	-0.14395426	-0.51036072	0.69397432
32	Native	Sweden	60.25826	17.71917	53	-0.14395426	-0.51036072	0.69397432
33	Native	Sweden	60.25868	17.63611	40	-0.14395426	-0.51036072	0.69397432
34	Native	Sweden	60.25931	17.66047	40	-0.14395426	-0.51036072	0.69397432
35	Native	Finland	61.00024	24.4161	121	-0.31137472	-0.32167828	0.39155805
36	Native	Finland	61.35197	24.83544	100	-0.32953167	-0.29102200	0.42948890

Table 1.2 Generalized linear mixed models results for the effect of range of origin, treatment (*i.e.*, reduced versus ambient herbivory), PC1 (temperature), PC2 (precipitation during wet or warm periods), and PC3 (precipitation during periods of drought) on herbivory and plant performance. Additional fixed effects also included the interaction between range of origin and treatment, as well as between range of origin and each linear bioclimatic component (*i.e.*, PC1, PC2, PC3). The first two response variables represent whether or not the plant experienced visible leaf herbivory, and if so, how much leaf tissue was consumed. The second two response variables represent rosette size at the end of the first growing season, and survival to the beginning of the second growing season. Random effects are not shown here, but include maternal line, as well as interactions between: (1) maternal line and population nested within range, and (2) maternal line, population nested within range, and treatment.

Fixed Effects	Binomial Attack Rate		Percent Damage		Rosette Size		Survival to 2nd Growing Season	
	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>
Origin	0.54 (1,126)	0.4621	0.14 (1,126)	0.7051	0.62 (1,115)	0.4311	0.27 (1,128)	0.6010
Treatment	47.65 (1,127)	< 0.0001	37.95 (1,127)	< 0.0001	3.95 (1,115)	0.0493	3.69 (1,136)	0.0570
Origin*Treatment	0.82 (1,127)	0.3673	0.03 (1,127)	0.8584	0.04 (1,115)	0.8410	0.07 (1,136)	0.7870
PC1	0.99 (1,384)	0.3214	1.37 (1,384)	0.2418	2.13 (1,355)	0.1454	2.16 (1,492)	0.1423
PC1*Origin	0.00 (1,384)	0.9492	0.05 (1,384)	0.8249	4.49 (1,355)	0.0347	0.19 (1,492)	0.6661
PC2	0.26 (1,384)	0.6126	0.04 (1,384)	0.8355	3.78 (1,355)	0.0527	0.04 (1,492)	0.8450
PC2*Origin	0.06 (1,384)	0.8009	0.02 (1,384)	0.9024	4.86 (1,355)	0.0281	1.00 (1,492)	0.3170
PC3	0.69 (1,384)	0.4077	0.94 (1,384)	0.3317	1.18 (1,355)	0.2773	0.20 (1,492)	0.6584
PC3*Origin	0.33 (1,384)	0.5686	0.06 (1,384)	0.8065	0.58 (1,355)	0.4486	0.00 (1,492)	0.9787

Table 1.3 Generalized linear mixed model results for the effect of treatment, origin and climate on resistance. Resistance was defined as $\left(\frac{1}{\text{percent herbivore damage}}\right)$, and measured at the individual plant level. Fixed effects included treatment (reduced or ambient herbivory), range of origin (native or introduced), PC1 (attributed mostly to temperature), PC2 (attributed mostly to precipitation during wet or warm periods), and PC3 (attributed to precipitation during periods of drought), as well as interactions between range of origin and treatment, and between range of origin and each linear bioclimatic component (*i.e.*, PC1, PC2, PC3).

Fixed Effects	Resistance	
	<i>F</i> (df)	<i>P</i>
Origin	0.07 (1,73)	0.7904
Treatment	12.65 (1,73)	0.0007
Origin*Treatment	0.86 (1,73)	0.3570
PC1	2.65 (1,152)	0.1058
PC1*Origin	0.65 (1,152)	0.4200
PC2	0.06 (1,152)	0.8067
PC2*Origin	0.14 (1,152)	0.7103
PC3	1.28 (1,152)	0.2603
PC3*Origin	0.01 (1,152)	0.9348

Table 1.4 Generalized linear mixed model results for the effect of origin and climate on tolerance. Tolerance was measured at the population level, and defined as the slope of log-transformed rosette area (cm²) plotted against percent damage by herbivory. Fixed effects included range of origin, PC1 (attributed mostly to temperature), PC2 (attributed mostly to precipitation during wet or warm periods), and PC3 (attributed to precipitation during periods of drought), as well as interactions between range of origin and each linear bioclimatic component (*i.e.*, PC1, PC2, PC3).

Fixed Effects	Tolerance	
	<i>F</i> (df)	<i>P</i>
Origin	2.32 (1,92)	0.1312
PC1	5.06 (1,92)	0.0269
PC1*Origin	4.05 (1,92)	0.0470
PC2	0.25 (1,92)	0.6185
PC2*Origin	0.84 (1,92)	0.3622
PC3	0.12 (1,92)	0.7293
PC3*Origin	1.68 (1,92)	0.1979

Table 1.5 Generalized linear mixed model results show that the effect of origin and climate on herbivory is similar across gardens. For each garden, we ran analyses of the subset of plants that were exposed to ambient levels of herbivory to confirm that the absence of clinal variation in herbivory was consistent across both gardens. Fixed effects included range of origin, treatment (*i.e.*, reduced or ambient herbivory), PC1 (attributed mostly to temperature), PC2 (attributed mostly to precipitation during wet or warm periods), and PC3 (attributed to precipitation during periods of drought), as well as interactions between range of origin and each linear bioclimatic component (*i.e.*, PC1, PC2, PC3) and an interaction between range of origin and treatment.

Fixed Effects	Binomial Attack Rate (Czech Republic)		Binomial Attack Rate (United States)		Percent Damage (Czech Republic)		Percent Damage (United States)	
	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>	<i>F</i> (df)	<i>P</i>
Origin	0.10 (1,115)	0.7541	1.38 (1,84)	0.2442	0.00 (1,115)	0.9961	0.51 (1,84)	0.4759
PC1	0.25 (1,115)	0.6191	0.44 (1,84)	0.5087	0.86 (1,115)	0.3555	1.46 (1,84)	0.2301
PC1*Origin	0.26 (1,115)	0.6110	0.19 (1,84)	0.6665	0.07 (1,115)	0.7891	0.98 (1,84)	0.3253
PC2	0.00 (1,115)	0.9666	0.12 (1,84)	0.7259	0.01 (1,115)	0.9240	0.05 (1,84)	0.8197
PC2*Origin	0.11 (1,115)	0.7355	0.10 (1,84)	0.7542	0.01 (1,115)	0.9110	0.30 (1,84)	0.5869
PC3	0.14 (1,115)	0.7109	1.03 (1,84)	0.3132	0.55 (1,115)	0.4594	0.52 (1,84)	0.4739
PC3*Origin	0.16 (1,115)	0.6938	1.33 (1,84)	0.2526	0.05 (1,115)	0.8301	0.28 (1,84)	0.5994

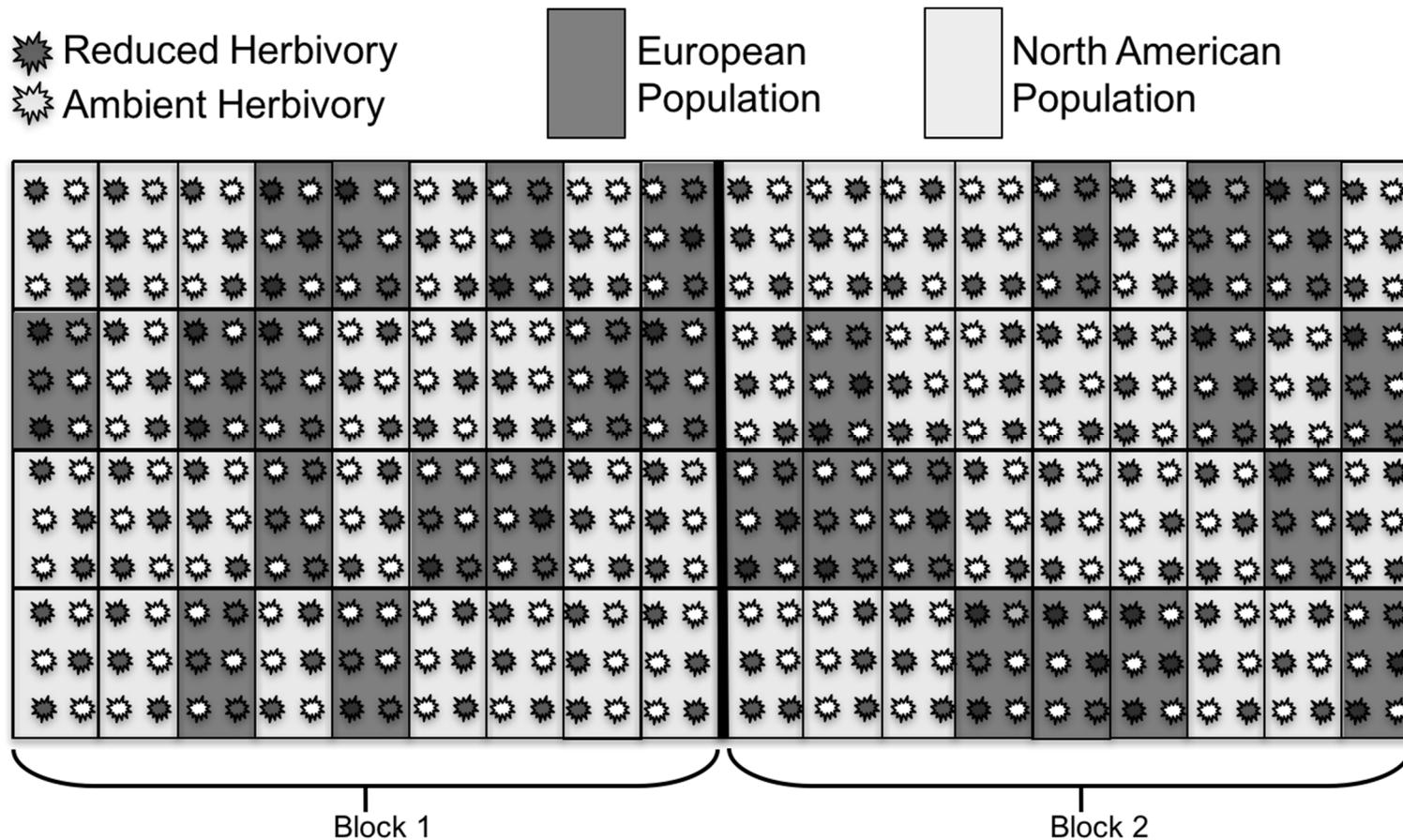


Figure 1.1 A schematic representing the modified split-plot design we used within each common garden. Our experiment was replicated across two spatial blocks, although within each block each population was represented by a different maternal line. Within each block, each of the 36 populations were randomly assigned to the whole-plot level in groups of 6 individuals. Within each of these whole-plots, three plants were randomly assigned to a reduced herbivory treatment and three plants were randomly assigned to an ambient herbivory treatment.

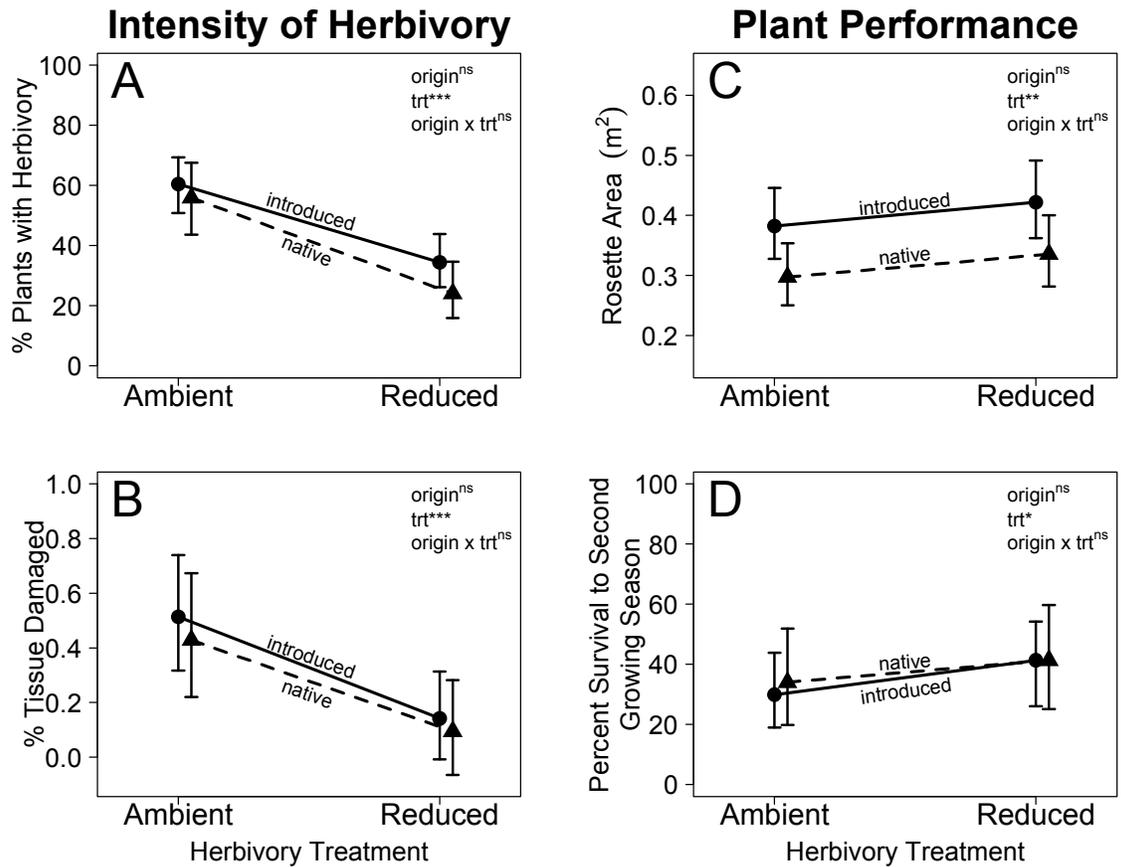


Figure 1.2 Least-squares means and 95% confidence intervals for generalized linear mixed models of the effect of the reduced herbivory treatment on herbivory (A, B) and plant performance (C, D). We report whether the effect of range of origin, treatment (trt), and the interaction between origin and treatment is nonsignificant^{ns} ($P > 0.1$), marginally significant^{*} ($P < 0.1$), significant^{**} ($P < 0.05$), or highly significant^{***} ($P < 0.0001$). In Table 1.2 we report fixed effects in greater detail, including the additional fixed effects of each linear bioclimatic component (*i.e.*, PC1, PC2, PC3), as well as the interaction between origin and each linear bioclimatic component.

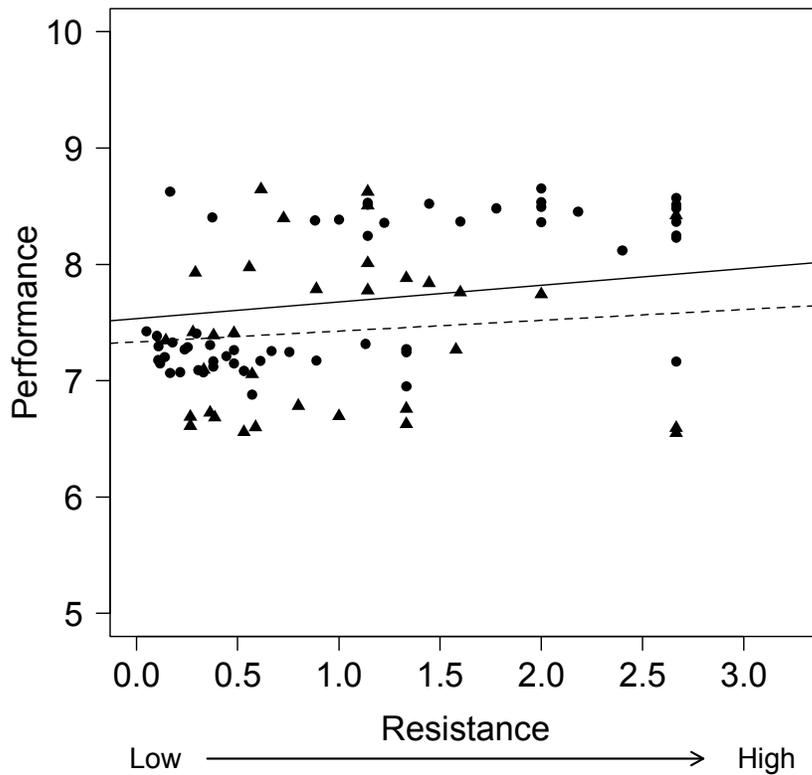


Figure 1.3 The linear regression model (dotted line for native populations, and solid line for introduced populations) and predicted values (triangles for native populations, circles for introduced populations) for performance under reduced herbivory given the resistance of that population as measured under ambient levels of herbivory. Performance was measured as the log-transformed rosette area for each population, while resistance was measured as $\left(\frac{1}{\text{percent herbivore damage}}\right)$. Each data point represents a single maternal line.

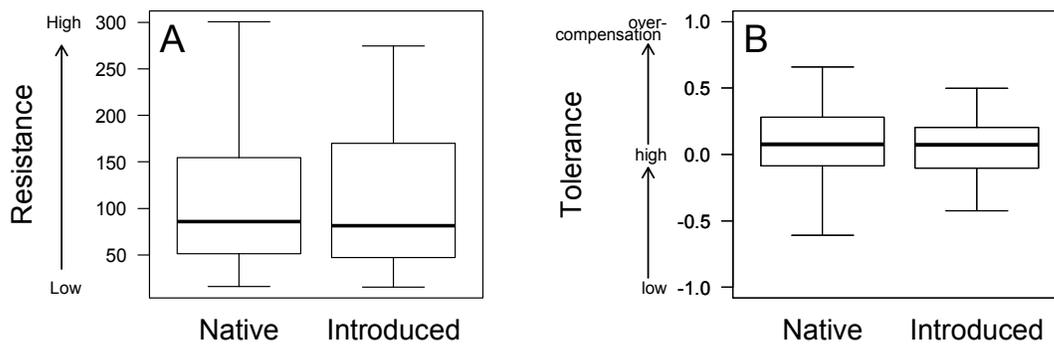


Figure 1.4 Both native and introduced populations of *V. thapsus* were resistant against herbivory (A). Resistance was measured at the plant level as $\left(\frac{1}{\text{percent herbivore damage}}\right)$, which means that 0 on the y-axis is equivalent to total defoliation. Tolerance to herbivory was measured at the population level, and defined as the slope of log-transformed rosette area (cm^2) plotted against percent damage by herbivory (B). A negative coefficient implies that herbivory reduces plant size, while a positive coefficient implies that plants overcompensate in response to herbivory. All boxplots depict model-adjusted values.

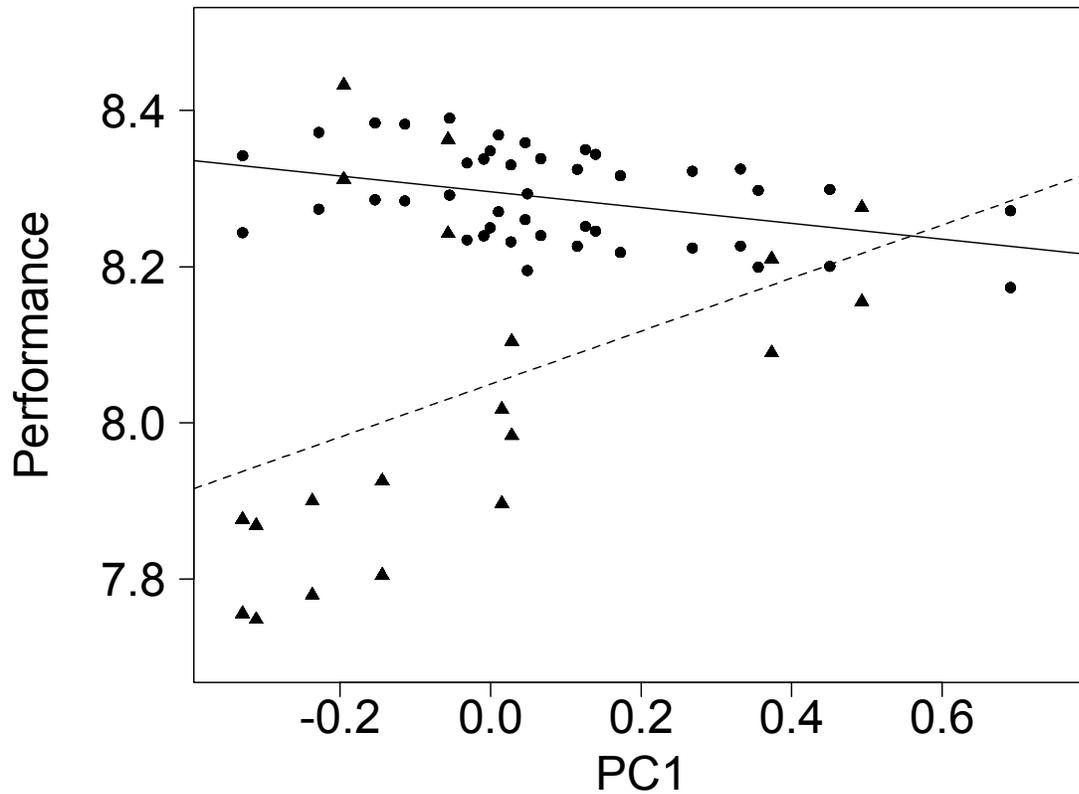


Figure 1.5 The linear regression model (dotted line for native populations, solid line for introduced populations) and predicted values (triangles for native populations, circles for introduced populations) for performance (log-transformed rosette area) by PC1, which is attributed mostly to temperature. Predicted values were calculated using the lsmeans of the model without the climate predictors. Each population is represented by two data points: one for ambient herbivory, and one for reduced herbivory. While rosette size decreases with cooler temperatures within the native range, introduced populations overall produced large rosette irrespective of precipitation or temperature. We detect a similar pattern of variation for the interaction between rosette area and PC2, which is attributed mostly to precipitation during wet or warm periods.

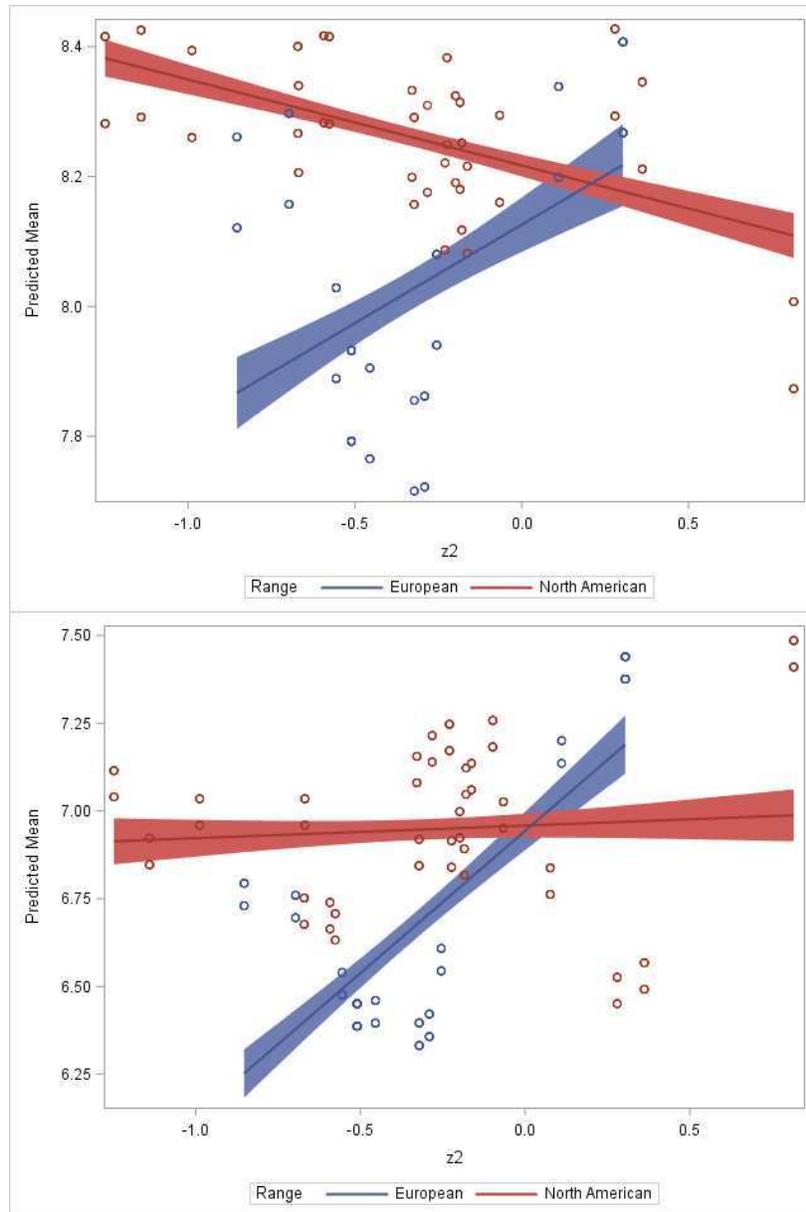


Figure 1.6 The interaction between PC2 (attributed mostly to precipitation during wet or warm periods) and range of origin was the most significant predictor of rosette size (Table 1.2). Here, we show that plants grown in the Czech Republic garden (left) and the United States garden (right) exhibit similar range-level patterns of clinal variation.

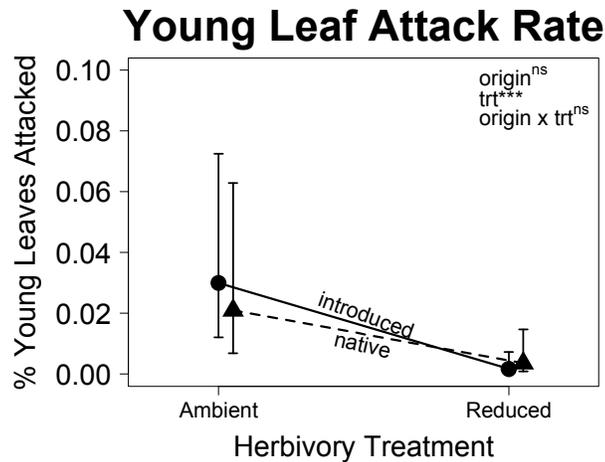


Figure 1.7 Least-squares means and 95% confidence intervals for generalized linear mixed models of the effect of the reduced herbivory treatment on young leaf herbivory. We report whether the effect of range of origin, treatment (trt), and the interaction between origin and treatment is nonsignificant ^{ns} ($P > 0.1$), marginally significant * ($P < 0.1$), significant ** ($P < 0.05$), or highly significant *** ($P < 0.0001$).

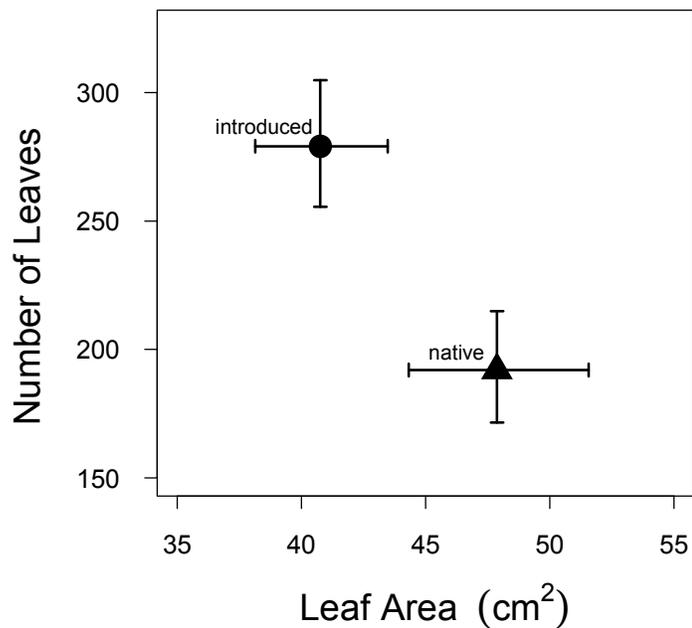


Figure 1.8 Native and introduced populations of *V. thapsus* differ in allocation strategies. Specifically, introduced populations invest in fewer, but larger leaves than native populations of *V. thapsus*. Here we depict the least-squares means and 95% confidence intervals for generalized linear mixed models of the effect of range, treatment, and climate on both the number of leaves per rosette, as well as leaf area.

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CHAPTER II: PREDICTIONS OF OPTIMAL DEFENSE FOR NON-ANNUAL, TEMPERATE, SEMELPAROUS PLANTS

DEFENSE ALLOCATION AS AN ADAPTATION TO HERBIVORY

Plant-herbivore interactions are fundamental to community functioning within most ecosystems (Schoenly *et al.* 1991; Hobbs 1996; Augustine and McNaughton 1998; Adler *et al.* 2001; Schemske *et al.* 2009; Larios *et al.* 2017). Understanding how plants invest in and allocate the defenses that mediate these interactions is therefore an important, long-standing goal of ecology. Current understanding of defense investment is built upon the assumptions that defense against herbivory or pathogens is costly to produce and maintain and that plant resources are limited, which leads to a trade-off between growth and defense (Herms and Mattson 1992; Strauss *et al.* 2002; Stamp 2003a; Huot *et al.* 2014; but see, Neilson *et al.* 2013). As a result of this trade-off, a plant's level of defense, if it is genetically variable, should reflect the ecological context of its ancestors. For example, plant populations that have historically faced high attack should have high defense, as better defended plants are better able to escape from damaging herbivory. In contrast, plant populations that have historically faced low attack should have low defense, as defense represents a costly investment that provides little benefit in the absence of herbivores.

Herbivory, however, is highly variable through space and time (Givnish 1999; Vehvilainen *et al.* 2007; Basset *et al.* 2015; Abdala-Roberts *et al.* 2016; Hahn *et al.* 2017). In addition, costs of defense against herbivory are notoriously difficult to detect (Cipollini *et al.* 2003; Zangerl 2003; Agrawal 2011), especially ecological costs that represent shifting trade-offs across variable environments (Strauss *et al.* 2002). As a result, we are often unsure of the exact

selective pressure herbivores exert on plants, or whether this pressure remains consistent enough to elicit a predictable evolutionary response from the plants they feed upon. Testing predictions regarding how herbivores drive evolution of plant investment in defense can therefore be difficult and prohibitively time consuming, as it requires experimentally altering herbivore regimes over many generations of plant populations (for one such elegant design, see Agrawal *et al.* 2012). Optimal defense theory, however, argues that we can still create useful, easily testable, predictions by placing the trade-off between growth and defense within the context of a second trade-off: investing more of a limited resource in one tissue means investing less in another (McKey 1974; Rhoades and Cates 1976). Specifically, optimal defense theory predicts that because defense is a costly, limited resource, plants should evolve allocation strategies that best defend tissues that are most valuable to future plant reproduction as well as at highest risk of being attacked if undefended.

One notable prediction of the optimal defense hypothesis is that young leaves should be better defended than old leaves (van Dam *et al.* 1995; Iwasa *et al.* 1996; Barto and Cipollini 2005). Young leaves are typically more valuable and, if undefended, more vulnerable than old leaves, as they bring in more energy for future reproduction and are more attractive to herbivores due to their higher nitrogen content (Bazzaz 1984; Field and Mooney 1986; Aerts 1996; Mediavilla and Escudero 2003). In line with these predictions, a recent meta-analysis found that young leaves are consistently better defended than old leaves across diverse plant taxa (McCall and Fordyce 2010). Interestingly, however, reproductive tissue has the potential to respond even more strongly than leaves to selection by herbivory as, unlike leaves, a plant's reproductive tissue is directly linked to its ability to contribute to offspring in the following generation. Given its high fitness value, optimal defense would therefore predict that reproductive tissue should be

better defended than leaf tissue. Yet this same meta-analysis found that reproductive tissue was similarly defended to leaf tissue, rather than better defended as optimal defense would predict (McCall and Fordyce 2010). Thus, at least in some instances, processes other than adaptation to herbivory or pathogens appear to either drive or constrain within-plant allocation of defense.

Tests that investigate the evolution of within-plant allocation of defense should therefore be designed to reflect that mechanisms other than herbivory may also drive observed patterns in defense. For example, defense may reflect source-sink dynamics; thus, because young tissues are often the strongest carbohydrate sink, defenses will be high in them (*e.g.* Kozlowski 1992; Honkanen *et al.* 1999). In addition, the concentration of defenses may decrease as a leaf expands and ages, such that although old leaves possess the same total amount of defenses as young leaves, they are distributed over a greater area (*e.g.* Wallace & Eigenbrode 2002; Brunt *et al.* 2006). Source-sink dynamics and dilution of defense with growth are both regularly documented, but rarely incorporated into plant defense theory (Berenbaum 1995).

The need to develop a more rigorous test of optimal defense theory is clearly stated within the literature (McKey 1974; Stamp 2003b; McCall and Fordyce 2010). Here we refine predictions of optimal defense using our understanding of tissue phenology and plant physiology for a small, but important, subset of plant species: non-annual, temperate, herbaceous plants with semelparous reproduction. These species are important to study for two main reasons. First, non-annual (*i.e.*, biennial and perennial) plant species comprise almost 25% of the known plant diversity within North America (Silvertown 1983), and although semelparity is relatively rare among these long-lived species, it still spans a wide diversity of taxa across at least 20 families (Young and Augspurger 1991). The evolutionary ecology of non-annual, semelparous plants is therefore of broad ecological interest, especially in the context of understanding the evolution of

life history and bet-hedging strategies (Vitalis *et al.* 2004; Verdu and Traveset 2005; Childs *et al.* 2010).

Second, the unique biology of these plants allows us to make clear predictions about how value of young and old leaves should vary over the course of their first growing season. For example, whereas annual plants or non-annual iteroparous plants may form reproductive tissue towards the end of their growing season, non-annual semelparous plants still only have young and old leaf tissue, but not reproductive tissue, at the end of their first growing season. Thus, investigating seasonal variation in defense allocation to young versus old leaves is much more straightforward for non-annual semelparous plants (at least in their first growing season), as this comparison does not need to account for the additional complication of reproductive tissue.

Here, we therefore propose that using non-annual, semelparous plants to incorporate predictions of tissue value phenology with those of adaptation to herbivory can help to refine predictions of optimal defense. This refined framework would recognize that adaptation to herbivory is driven not only by herbivory itself, but also by tissue value, which regulates the strength of the selection that herbivory exerts upon plant populations.

THE PHENOLOGY OF TISSUE VALUE

Variation in tissue value, or the contribution of a specific tissue to reproductive output, is best measured by calculating the difference in fitness between an undamaged control and a plant for which the tissue of interest removed. Tissue value plays an important role in mediating the strength of selection by herbivory, as tissues that contribute most strongly to fitness should be under the strongest selection in response to herbivore damage. To better illustrate the effect of tissue value on adaptation to herbivory, we can therefore represent the predictions of optimal

defense quantitatively as a tissue's value multiplied by its risk of attack should predict its level of defense.

Current tests of optimal defense, however, often implicitly assume that both tissue value and risk of attack are static (Fig. 2.1A), as they do not typically test or make predictions about how plants vary their defense allocation over the course of the growing season (*e.g.*, McCall and Fordyce 2010; Alba *et al.* 2012; Kooyers *et al.* 2017; but see, Diezel *et al.* 2011; Heath *et al.* 2014). Yet defenses are well known to vary through time and ontogeny (Boege and Marquis 2005; Barton and Koricheva 2010) and the few studies of optimal defense that directly measure tissue value all show significant within-season variation in the relative value of young versus old leaves (Ohnmeiss and Baldwin 2000; Barto & Cipollini 2005; Traw and Feeny 2008; Heath *et al.* 2014). Of these studies, all but Barto and Cipollini (2005) showed that variation in tissue value reflected how plants allocated at least one line of defense against herbivory; contrary to predictions of optimal defense, Barto and Cipollini (2005) instead found that for *Arabidopsis thaliana* old leaves, not young leaves, had higher levels of defense, even though young leaves contributed more to plant fitness. Directly addressing whether variation in tissue value may be driving observed variation in defense allocation is therefore critical when testing predictions of the optimal defense hypothesis, but to our knowledge, has never been explicitly incorporated into the optimal defense framework.

Here, we propose that we can make informed predictions about how tissue value will change over the course of the growing season for non-annual, temperate, herbaceous plants. Specifically, we capitalize on the idea that as a plant prepares to overwinter, the value of root tissue should increase as the value of leaf tissue begins to decrease. In addition, while leaves, overall, are valuable at the beginning of the growing season, young leaves should be especially

valuable, as they bring in the most energy for future reproduction due to their long life expectancy and high photosynthetic capacity (Harper 1989; Iwasa *et al.* 1996). In contrast, as leaves become less valuable towards the end of the growing season, the value of young and old leaves should decrease, especially given that part of the value of young leaves is that they eventually become old leaves, and young leaves produced towards the end of the growing season may not have the time to age into old leaves before the arrival of winter.

If, as optimal defense theory predicts, defense allocation tracks tissue value, these assumptions about seasonality lead to two logical conclusions. First, a plant should decrease its defense investment in leaves as it prepares to overwinter and its roots become most valuable; although leaves may retain some of their value by resorbing and remobilizing resources during leaf senescence, even the most efficient of plants typically don't recover more than 50% of the energy and nutrients they invest in their leaves (Brant and Chen 2015), which suggests that roots should still be significantly more valuable than leaves as plants prepare to overwinter. Second, young leaves should be especially well defended compared to old leaves early in the season when leaves are overall most valuable, but should become less differentiated from old leaves as the plant approaches overwintering, as roots, not leaves, become most valuable (Fig. 2.1B). Thus, if adaptation to herbivory is the strongest driver of defense allocation we would expect plants to evolve predictable variation in how they shift their defense investment and allocation over the course of the growing season. Importantly, however, if plant physiology is the strongest driver of defense, we would also expect predictable, yet different, variation in how plants shift their defense allocation through the growing season. By comparing how predictions concerning how seasonal variation in defense should adapt in response to selection to herbivory versus

respond to innate plant physiology, we can therefore better disentangle whether herbivory or how the plant grows is a stronger driver of within-plant allocation of defense.

Here, we propose a conceptual model that compares seasonal variation in investment in defense when this investment is driven by adaptation to herbivory versus when it is driven by plant physiology. We first compare how these two potential drivers of defense allocation may shape defense allocation to young compared to old leaves. We then present a different set of predictions for immobile defenses, which cannot be reallocated once invested, and mobile defenses, which can be reallocated once invested (Coley *et al.* 1985; Basey and Jenkins 1993). We take this approach, as we recognize that different physiological processes and constraints act upon these two different categories of defense. Immobile defenses, such as lignins, spines, or other mechanical defenses, represent initially high construction costs, but once made cannot be easily broken down, even upon leaf senescence (Coley *et al.* 1985). Mobile defense, such as alkaloids or phenolic glycosides, represent initially low construction costs, but rapidly break down and must be continually synthesized (Coley *et al.* 1985).

ASSUMPTIONS OF THE CONCEPTUAL MODEL

Our conceptual model best applies to plants in their first-year of growth from temperate populations, as in later growing seasons roots likely contain energy stores from previous growing seasons that would increase the relative value of belowground to aboveground tissue in ways that are difficult to predict. Importantly, we also assume that the value of root tissue increases as a plant prepares to overwinter, which leads to a decrease in the relative value of leaf tissue.

Although our predictions extend to belowground tissue, for clarity we choose to focus our graphical depictions on leaf tissue, which is the most commonly sampled tissue in tests of plant

defense theory. To better illustrate our predictions, we assume that leaves turn over, such that young leaves eventually become old leaves, and old leaves eventually senesce and abscise from the plant. In addition, we assume that plants become larger throughout the course of the growing season and, as plants become larger, their old leaves, at least initially, grow exponentially larger (Fig. 2.2). This assumption is based on our understanding of leaf growth within rosettes, which is the typically growth form of non-annual, semelparous, herbaceous plants (Li *et al.* 1998; Pantin *et al.* 1998; Gonzalez *et al.* 2010). Overall, these assumptions play a particularly important role in our predictions of how the role of plant physiology in allocating defense might lead to predictable variation over the course of the growing season.

In practice, plants likely exist along a continuum between the two extremes represented by Fig. 2.1A&B. For example, many temperate plants are able to resorb and remobilize some of the nutrients and defenses they invest in their leaves (Brant and Chen 2015), which may mitigate the decline in leaf value towards the end of the growing season, and shift predictions more towards the traditional assumptions depicted by Fig. 2.1A. However, even highly efficient plants typically only recover about 50% of the energy and nutrients they invest in their leaves (Brant and Chen 2015), which means that the predicted direction of these relationships should hold true, even if the strength of these relationships varies due to differences in nutrient resorption rates. Fig. 2.1A thus represents an idealized scenario, but should still provide a useful framework for exploring patterns of defense that may be common across diverse plant taxa.

The models we present also assume semelparity, as plants with the potential to reproduce in their first year of growth may evolve bet-hedging strategies, such as prolonged dormancy (Gremer *et al.* 2012) or flexibility in reproductive timing (Wilbur *et al.* 2006), that would alter predictions of how relative tissue value shifts through the course of the growing season. Plants

that live for multiple years and reproduce only once represent less than 5% of global plant diversity (Silvertown 1983), but are spread across at least 20 families (Young and Augspurger 1991) and represent an evolutionary strategy that provides important evolutionary insight into plant community dynamics (Vitalis *et al.* 2004; Verdu and Traveset 2005; Childs *et al.* 2010). Importantly, the utility of this conceptual model is not restricted to this strict category. Instead we hope to present a framework for testing patterns of defense allocation that are likely ubiquitous across plant species, regardless of their life history.

Finally, our predictions are meant to reflect defense concentration, as opposed to the total amount of defense invested in each leaf. Here, we assume that defense concentration encompasses both constitutive defense, which is expressed independent of damage, and induced defense, which is expressed in response to environmental cues, including herbivory. Constitutive defense should evolve in response to predictable variation in herbivory, while induced defense, by its very nature, is thought to evolve when herbivory is more ephemeral and unpredictable, as well as when costs of alternate defense strategies are high (Karban and Baldwin 1997). Thus, while the relationship between the strength of selection by herbivory and the evolution of constitutive defense is relatively straightforward, how the allocation of induced defense evolves in response to shifts in herbivore pressure remains less well understood. Evidence, however, does seem to suggest that induced defense decreases with plant maturity (Karban and Baldwin 1997; Barton and Koricheva 2012), which aligns with our predictions. In addition, herbivory often does not measurably induce resistance (Karban and Baldwin 1997; Nykanen and Koricheva 2004), and when it does, defense induction is often a whole-plant, systemic response (Schilmiller and Howe 2005; Gozzo and Faoro 2013), with induced defenses often matching predictions of optimal defense (*i.e.*, young leaves are more strongly induced than old leaves, or flowers are

more strongly induced than leaves) (Euler and Baldwin 1996; Zeier 2005; Eisenring *et al.* 2017). For example, Baldwin and Karb (1995) found that when leaves of *Nicotiana attenuata* are exposed to herbivory, nicotine is induced in all aboveground tissue, but, following predictions of optimal defense theory, more so within the reproductive tissue that is most valuable to plant fitness. Importantly, both chemical and physical defenses, such as trichomes or leaf toughness, may be induced in response to herbivory (Barton 2016). Thus, while we present predictions of within-plant variation in defense allocation within categories of defense, we do not focus on predictions on how the relative contribution to mobile versus immobile defense may change throughout the course of the season, or among populations with different herbivore compositions.

ASSUMPTIONS REGARDING MOBILE DEFENSES

Whether or not a defense can be broken down or reallocated once invested in a leaf has important implications for how physiology may drive seasonal variation in allocation of that defense. Mobile defenses, such as secondary metabolites, are often highly effective at low concentrations and may have initially low construction costs (Cornell and Hawkins 2003). However, secondary metabolites have high turnover rates. The continued metabolic cost associated with this constant turnover may end up being higher than defenses that have a higher construction cost, but lower turnover rate (Endara and Coley 2010). However, high turnover of these compounds is typically used as evidence that these compounds are low cost, as this breakdown allows compounds to be recovered from the leaf during senescence (Coley *et al.* 1985). Secondary compounds therefore represent a reversible commitment to plant defense (Coley *et al.* 1985), as while there is a construction cost, the cost associated with reallocating

these resources within the plant is actually considered quite low (Schultz *et al.* 2013). In addition, Neilson *et al.* (2013) recently argued that the cost of this type of defense may actually be able to be completely offset, as the plants are often able to recycle these compounds, once broken down, into compounds directly responsible for the plant's survival or reproduction, or other compounds related to plant defense against herbivory.

Here, we therefore assume that there is a low construction cost associated with mobile defenses, and that there is even less cost associated with the redistribution of defenses within the plant itself. Thus, unlike immobile defenses, the evolutionary pressure acts directly upon the biological end result (the concentration of these defenses, which is what the herbivore will perceive), instead of having to rely on indirect mechanisms for altering these concentrations, as is the case for immobile defenses (Fig. 2.3). As a result, if defense allocation reflects the predictions of optimal defense, we would expect adaptation to be the product of two distinct responses (Fig. 2.4). First, investment in mobile defenses should decrease in leaf tissue over the course of the growing season; as plants prepare to overwinter, leaves should become less valuable both because of an increase in the relative value of roots, and additionally because plants are growing larger, and should thus be more tolerant of herbivore damage (Trumble *et al.* 1993). Second, plants should evolve to disproportionately better defend young leaves as compared old leaves early in the growing season when leaf tissue is overall most valuable.

ASSUMPTIONS REGARDING IMMOBILE DEFENSES

Immobile defenses, such as lignin, fiber, or trichomes, are often perceived to have high initial construction costs (Züst and Agrawal 2017) and, unlike mobile defenses, low rates of turnover. As a consequence, these defenses cannot be easily reallocated within the plant.

Immobile defenses therefore represent a long-term investment on behalf of the plant that cannot be recovered as leaves age and senesce (McKey 1979, 1984; Coley *et al.* 1985).

Thus, although plants can directly increase total defense investment in a leaf, once an investment is committed, it cannot be decreased. To better defend young than old leaves, plants are therefore constrained to two different pathways (Fig. 2.3). First, a plant can increase defense concentrations in young leaves by increasing the total defense invested in that young leaf. Second, a plant can decrease defense concentration in old leaves through leaf expansion, which doesn't change the total amount of defense, but dilutes the concentration that is present as the leaf grows and expands.

With these pathways in mind, we predict that the overall decrease in the relative value of aboveground to belowground tissue will have the strongest selective effect. Thus, according to the predictions of optimal defense, investment in defense, as controlled by investment in young leaves, should decrease over the course of the growing season (Fig. 2.5). In addition, optimal defense predicts a secondary selective effect of herbivory: that young leaves should be better defended than old leaves. However, the amount of defense invested in an old leaf is constrained by how much defense was committed to that leaf as a young leaf earlier in the season, as once invested, these defenses cannot be broken down. Thus, if plants decrease investment in defense in new leaves over the course of the growing season, old leaves will always have a greater total amount of defense as compared to the current suite of young leaves at any one point in time (Fig. 2.5B). If these assumptions hold true, the only way that young leaves are better defended than old leaves would be if old leaves expand as they grow and age (Fig. 2.5D). Interestingly, we see a similar pattern of defense allocation regardless of whether or not allocation is driven by adaptation to herbivory (Fig. 2.5F,H) or merely a result of how leaves expand as they age and

grow (Fig. 2.5E,F). Measuring both a plant's total investment in a leaf, as well as the leaf's concentration is therefore especially critical for disentangling the processes that drive the within-plant allocation of immobile defenses.

Additionally, we see that the physiological constraints that prevent the breakdown of immobile defenses in young leaves should lead to old leaves being better defended as the season progresses, not less well defended as optimal defense predicts. This constraint may imply that mobile defenses, and their ability to reflect the shifting value of young versus old tissue, may become more heavily disinvested in old leaves towards the end of the season to make up for the allocation constraints faced by immobile defenses.

CONCLUSIONS

For temperate plants, under optimal defense, predictable changes in tissue value should lead to predictable allocation to defense against herbivory. Mobile defenses, due to their low cost and high turnover rates, should directly track tissue value, and thus young leaves should be better defended than older leaves, especially early in the season when those young leaves are most valuable. In contrast, the nature of immobile defenses prevents plants from directly disinvesting in old leaves, which should instead lead to young leaves with increasingly high concentrations of immobile defenses as plants progress throughout the growing season. Importantly, the relationship between belowground and aboveground plant tissue is implicit in our predictions, both in terms of its risk of attack if undefended, as well as its relative value to future plant reproduction. Future studies that explicitly evaluate the relationship between defense allocation to aboveground versus belowground allocation of defense, and its seasonality, will provide much-needed insight into the mechanisms driving the evolution of defense and its allocation.

FIGURES

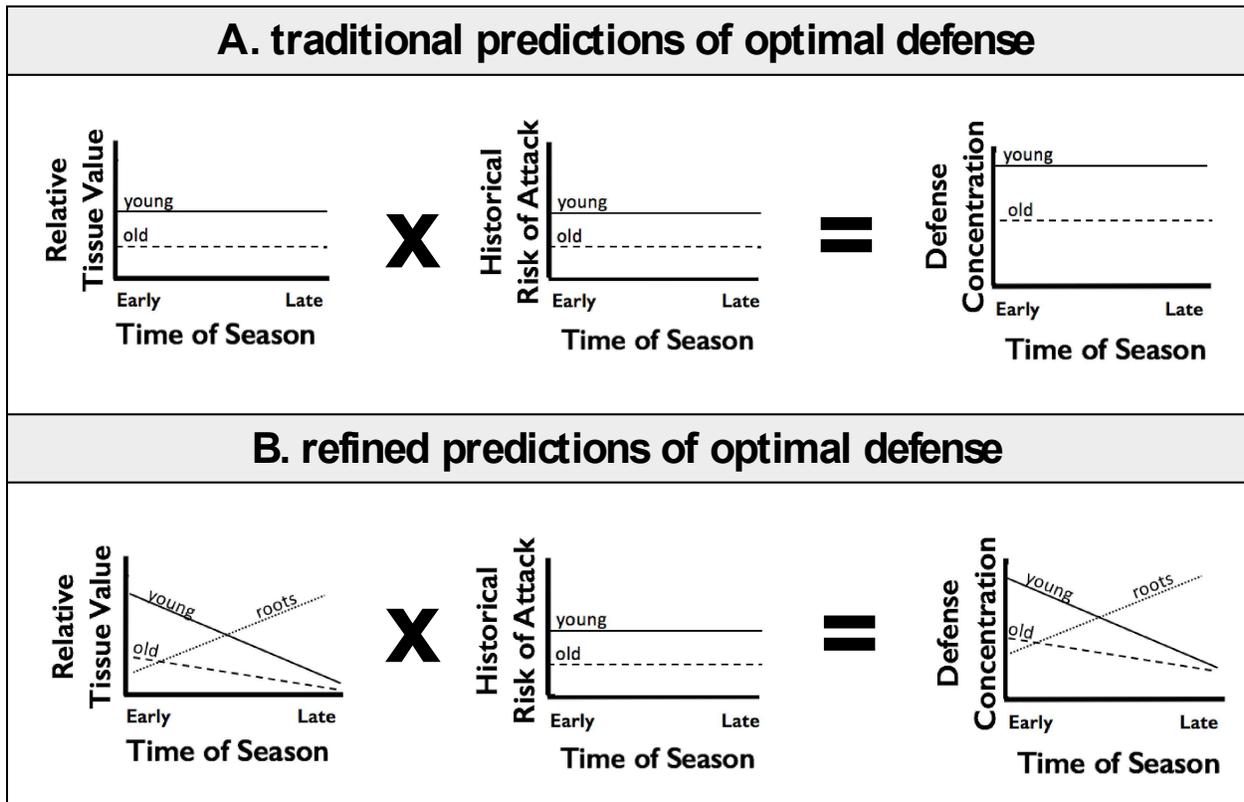
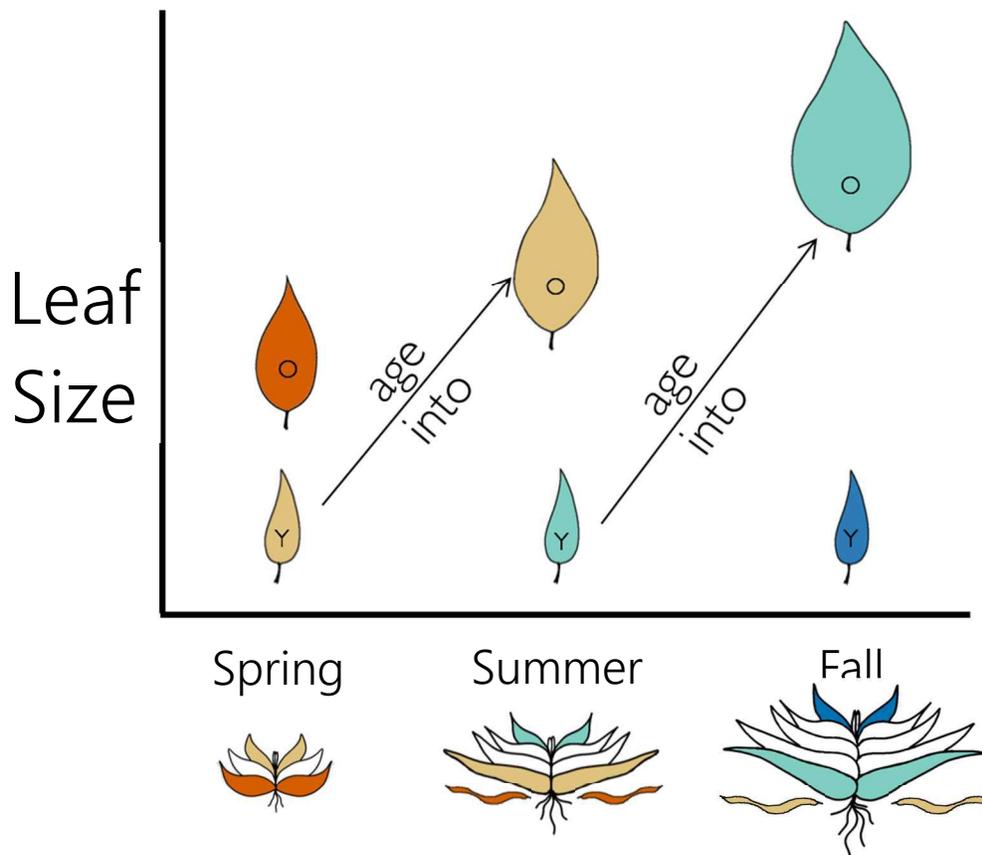


Figure 2.1 Current tests of optimal defense often implicitly assume that tissue value and risk of attack remain constant throughout the growing season (A), which leads to the broad prediction that young leaves should always be better defended than old leaves. We propose that, for semelparous, temperate plants, tissue value shifts over the course of the growing season, such that the value of leaves decreases over the course of the season as root value increases in preparation for overwintering (B). Roots are excluded from the graph representing historical risk of attack; although root damage is known to have strong effects on aboveground tissue (van Dam and Martin 2011; Barber and Soper Gordon 2015), belowground herbivory is cryptic and notoriously hard to study (Preisser and Bastow 2005). Consequently, we have limited understanding about the relative risk of attack of aboveground versus belowground tissue. Here, however, we assume that the risk of root herbivory does not vary across the growing season, which allows us to still make predictions about how defense allocation should shift through time based on tissue value.



Plant Development

Figure 2.2 The above diagram depicts a typical plant in its first year of development. Young leaves are labeled as 'Y' and old leaves are labeled as 'O'. Each unique color represents a unique leaf to better show that old leaves are larger than young leaves, young leaves eventually expand and age into older leaves, and that old leaves eventually senesce. Young leaves typically remain the same size throughout the course of the season, but old leaves often increase in size over the course of the season as the plant itself becomes larger and has more resources to invest into its leaves.

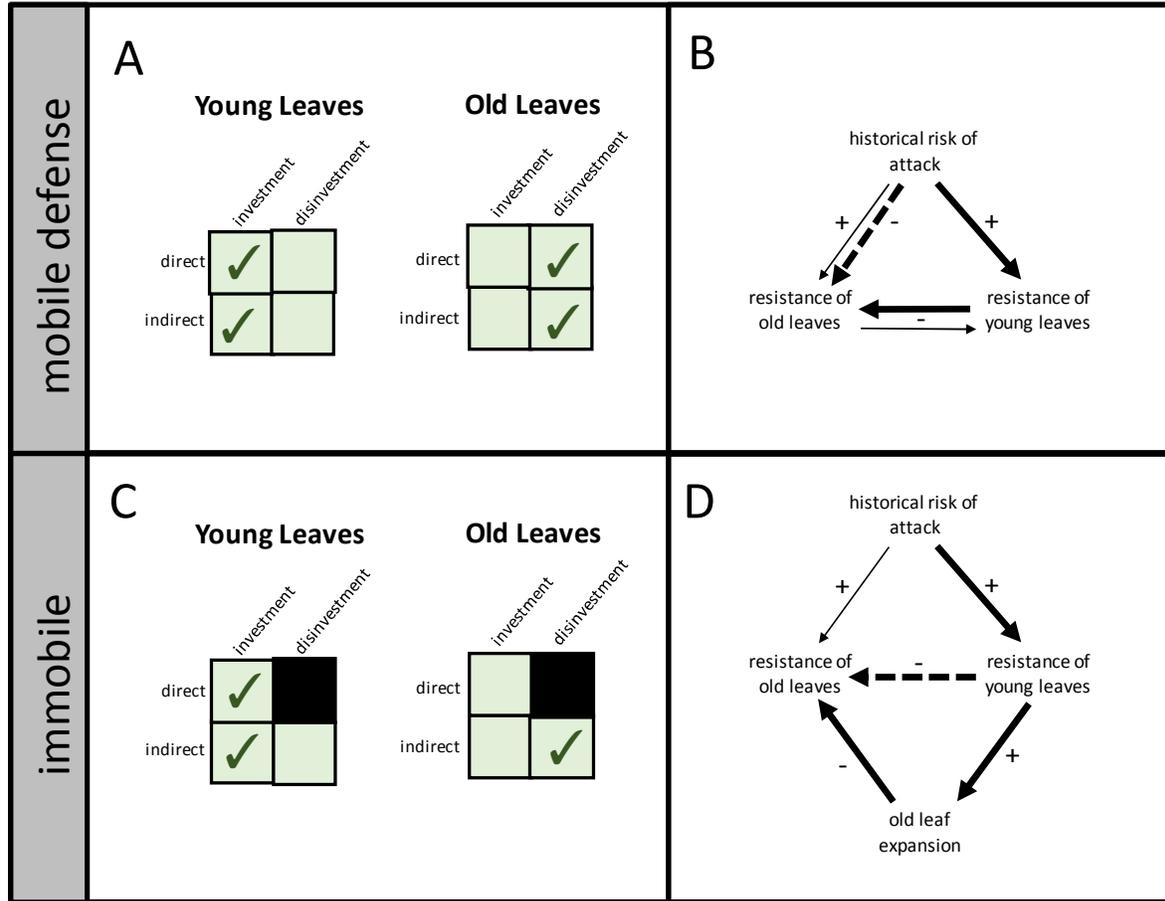


Figure 2.3 Panels A and C visually represent the potential pathways available for plants to invest or disinvest in defense against herbivory for mobile and immobile defenses, directly. Investment of defense in leaves of one age is inherently linked to indirect disinvestment in leaves of a different age. For example, direct investment in defense in young leaves represents an indirect disinvestment in old leaves, as, by comparison, old leaves will be relatively less resistant to herbivory than young leaves. Using mobile defense, plants can therefore manipulate the resistance of their young and old leaves through multiple different pathways (panel A). However, boxes with green checks represent the pathways that optimal defense predicts the plant is most likely to employ. For example, as plants can directly recycle and reallocate mobile defenses at very little cost, they should rely most heavily on directly increasing defense concentration in young leaves (which in turn represents an indirect disinvestment in old leaves) as well as directly decreasing defense concentration in old leaves (which in turn represents an indirect investment in young leaves). For immobile defense, the pathways of investment in young and old leaves are the same as for for mobile defenses, except that old leaves are now constrained to indirect disinvestment (panel C). Although indirect disinvestment in the resistance of old leaves can be achieved by increasing investment in young leaves, and thus making old leaves seem less palatable to herbivores by comparison, old leaves may also disinvest in resistance by expanding, and thus diluting the defense in the leaf to a lower concentration.

Panel B and D respectively depict hypothesized interactions of consistent herbivory on the evolution of mobile and immobile defense within young and old leaves. Direct interactions are depicted by solid lines, and indirect interactions are depicted by dashed lines. Herbivory should select for increased resistance in both young and old leaves, but the strength of this selection should be strongest for young leaves given their higher value. As investment in resistance of young leaves indirectly leads to disinvestment in old leaves, the negative indirect effect of herbivory on resistance of old leaves should outweigh the positive direct effect of herbivory on resistance of old leaves. Importantly, as tolerance increases, selection for resistance will decrease, which will weaken the strength of the interactions depicted above.

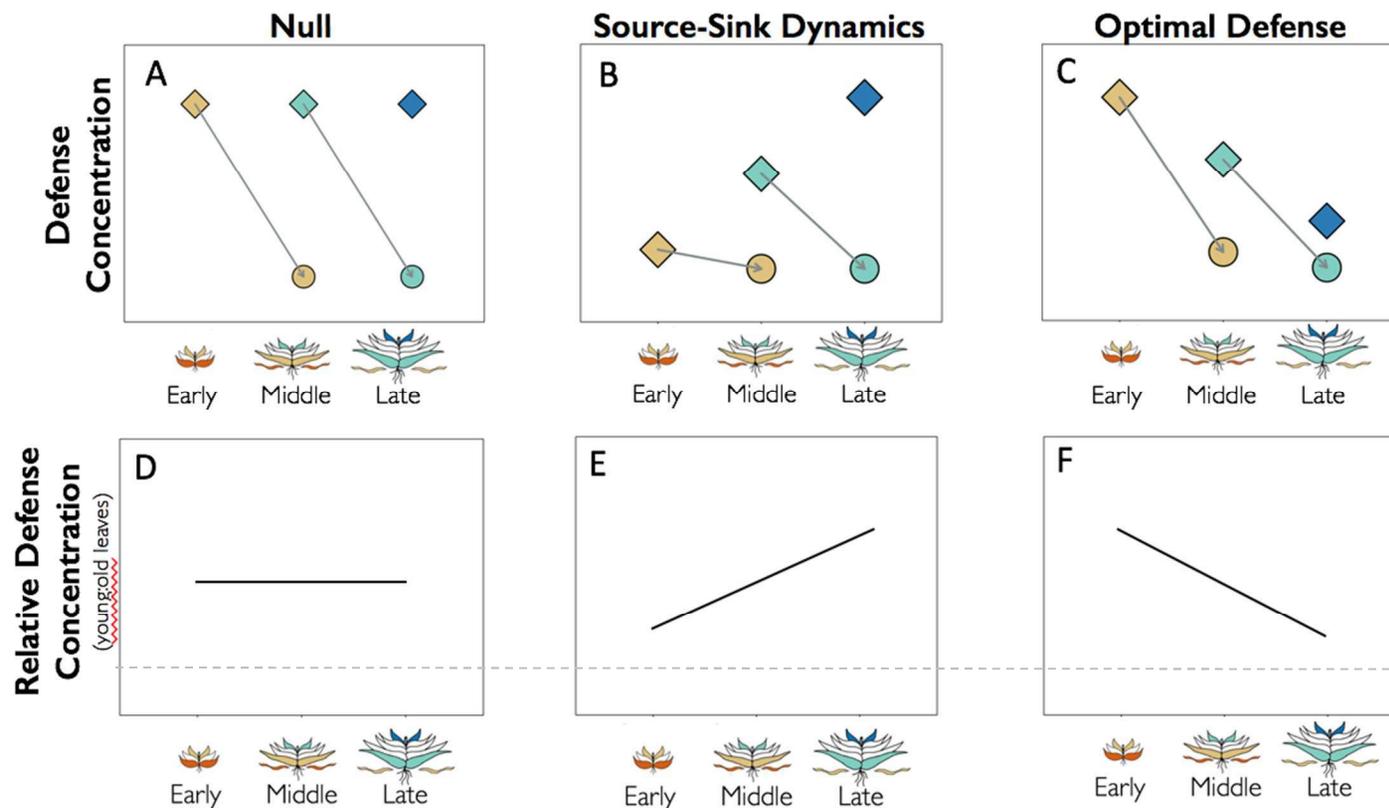


Figure 2.4 Predictions for how immobile defenses against herbivory will change through time based on a null (in which allocation does not vary across the season), source-sink dynamics, and optimal defense. Diamonds represent young leaves and circles represent old leaves. Gray arrows between shapes of the same color depict how young leaves age into old leaves later in the season. This figure depicts three idealized, yet viable, scenarios. In the ‘null’ scenario, young and old leaves are similarly defended over the course of the growing season (A). Alternatively, source-sink dynamics might become stronger over the course of the growing season as plants become larger (B) or, if plants adapt an optimal defense strategy in response to herbivory, defense allocation should reflect both decrease overall defense investment over the course of the growing season, as well as disproportionately better defended young leaves early in the growing season when leaf tissue is most valuable (C). Panels D, E, and F depict the ratio of defense concentration between

young and old leaves based on the corresponding panels immediately above. The dotted horizontal gray line represents when the ratio is equal to one, and young leaves are equally well defended as old leaves. As values increase along the y-axis, young leaves are increasingly better defended than old leaves.

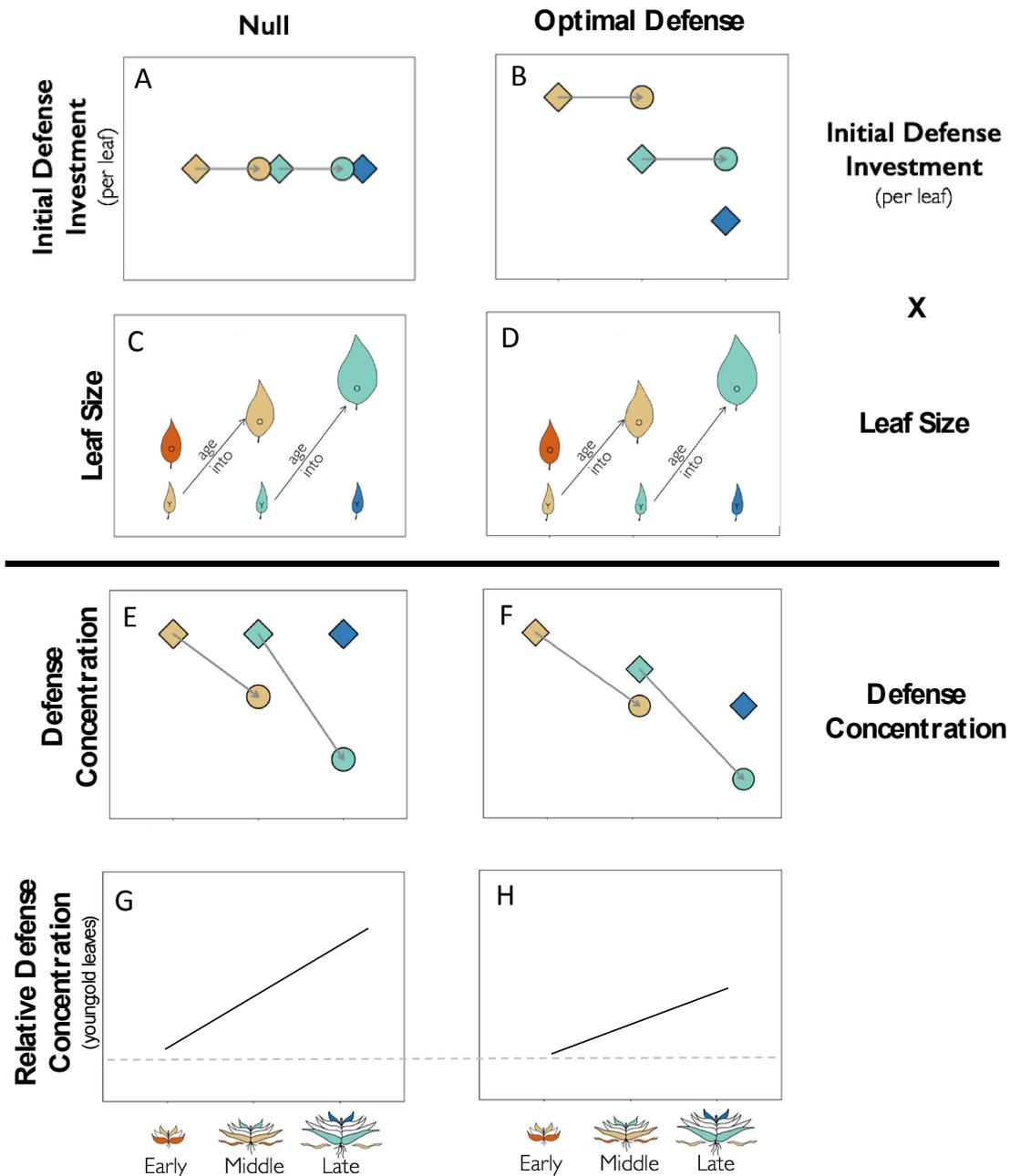


Figure 2.5 Unlike mobile defense, selection cannot act directly upon the concentration of immobile defense, and instead is likely the product of investment in young leaves and leaf size. Diamonds represent young leaves and circles represent old leaves. Gray arrows between shapes of the same color depict how young leaves age into old leaves later in the season. This figure depicts two idealized scenarios. In the ‘null’ scenario, plants consistently invest the same amount of defense in young leaves over the course of the growing season (A). Alternatively, if plants adapt an optimal defense strategy in response to herbivory, defense allocation should reflect both decrease overall defense investment over the course of the growing season, as well as

disproportionately better defended young leaves early in the growing season when leaf tissue is most valuable (B). Panels C and D depict variation in leaf size over the course of the growing season, which effects the final concentration of defense in young and old leaves (panels E and F). Panels G and H depict the ratio of defense concentration between young and old leaves based on the corresponding panels immediately above. The dotted horizontal gray line represents when the ratio is equal to one, and young leaves are equally well defended as old leaves. As values increase along the y-axis, young leaves are increasingly better defended than old leaves.

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CHAPTER III: A TEST OF THE SEASONALITY OF OPTIMAL DEFENSE USING A BIENNIAL, WEEDY INVADER

INTRODUCTION

Plants are sessile, and thus rely on a diverse suite of physical and chemical traits to defend against the herbivores that eat them (Bennet and Wallsgrove 1994; Coley and Barone 1996; Strauss and Agrawal 1999; Walling 2000; Pichersky and Gershenzon 2002). Defense against herbivory, however, is physiologically costly for plants to produce and maintain (Bergelson and Purrington 1996; Strauss *et al.* 2002). As a result, plants face an inherent dilemma: while high defense may reduce herbivory, it may also diminish the amount of resources available for growth and reproduction (Herms & Mattson 1992). Understanding how, and even if, plants adapt to maintain a balance between growth and defense therefore remains an important, long-standing goal in ecology (Strauss and Agrawal 1999; Agrawal and Fishbein 2006; Nunez-Farfan *et al.* 2007; Agrawal 2011; Karban 2011; Hahn and Maron 2016).

The optimal defense hypothesis places this growth-defense trade-off within the context of a second trade-off: investing more defense in one tissue means investing less in another (McKey 1974; Rhoades and Cates 1976). According to this hypothesis, populations should allocate more resources towards defending tissue that is highly valuable to future plant reproduction or highly vulnerable to herbivory if undefended (McKey 1974; Rhoades and Cates 1976). Typically, the optimal defense hypothesis is evaluated using aboveground tissue, with a focus on young versus old leaves. Young leaves are often more valuable than old leaves due to their longer life expectancy and higher photosynthetic capacity (Harper 1989; Iwasa *et al.* 1996), as well as more vulnerable to herbivory due to high nitrogen content (Coley 1980; Bowers and Stamp 1993).

Thus, one recurring prediction within the optimal defense literature is that young leaves should be better defended than old leaves (van Dam *et al.* 1995; Iwasa *et al.* 1996; Barto and Cipollini 2005).

The data available fit this prediction well: young leaves are better defended than old leaves across many plant taxa (McCall and Fordyce 2010). However, this pattern may not result from herbivore-driven adaptation, as optimal defense predicts. Innate plant physiology, or how a plant grows, may also drive young leaves to be better defended than old leaves (Schuman and Baldwin 2016). Young leaves are often a plant's strongest carbohydrate sink, and high defense in young leaves may therefore simply be a byproduct of source-sink dynamics (Kozlowski 1992; Honkanen *et al.* 1999). In addition, as leaves age, they often grow and expand. As a result, even if old leaves possess the same total amount of defense as young leaves, this defense may be distributed over a larger area, and thus present at a lower concentration to potential herbivores (Wallace & Eigenbrode 2002; Brunt *et al.* 2006). Moving forward, tests of optimal defense need to distinguish the predictions of optimal defense from the effects of source-sink dynamics and the dilution of defense with leaf expansion (McKey 1974; Berenbaum 1995; Stamp 2003; McCall and Fordyce 2010).

Plant invasions represent a promising opportunity to tease apart the influence of adaptation to herbivory from the influence of innate plant physiology. First, plants often experience high levels of herbivory in their native range, mostly due to co-evolved specialists, but low levels of herbivory in their introduced range, mostly due to generalists (Keane and Crawley 2002; Colautti *et al.* 2004; Lui and Stiling 2006). Second, rapid evolution is common within introduced populations (Whitney and Gabler 2008; Moran and Alexander 2014). Thus, introduced populations should quickly evolutionarily differentiate from their native congeners in

response to this striking between-range difference in selective pressure (see ‘EICA hypothesis’, Blossey and Nötzold 1995).

Verbascum thapsus L. (Scrophulariaceae), a common weedy invader within North America, is therefore ideal for investigating predictions of optimal defense. Typical of many invaders, *V. thapsus* has consistently experienced less herbivory in its introduced range in North America than in its native range in Europe (Popov 1972; Gross and Werner 1978; Wolfe 2002; Alba *et al.* 2012; Endriss *et al.* 2018). In addition, *V. thapsus* populations were established across the United States by the early 1700s (Wilhelm 1974; Gross and Werner 1978; Mitich 1989), which suggests that populations have had plenty of time to adapt to between-range differences in herbivory (Moran and Alexander 2014). We can therefore use between-range comparisons to investigate two sets of populations from the same species that have historically experienced two very different levels of herbivory: low herbivory in the introduced range, and high herbivory in the native range.

Here, we used a common garden of *V. thapsus* to investigate predictions of optimal defense. Past tests of optimal defense have largely focused on how plants allocate defense against herbivory (McCall and Fordyce 2010). A clear step forward, however, is to rigorously test the driving mechanisms, not just the resulting patterns, of within-plant allocation of defense. We therefore started with the broad hypothesis that young leaves should be better defended than old leaves, but then increasingly developed this hypothesis to incorporate the influence of tissue value and historical risk of herbivory (Fig. 3.1). Specifically, *V. thapsus* is typically biennial (Gross and Werner 1978; Ansari and Daehler 2010), and for non-annual, temperate plants, the value of leaf tissue should decrease towards the end of the first growing season as root value increases in preparation for winter (Endriss, chapter 2). In addition, *V. thapsus* has consistently

experienced higher herbivory in its native than in its introduced range. We therefore integrated predictions of invader evolution and tissue phenology into three increasingly specific hypotheses of optimal defense: (H1) young leaves should be better defended than old leaves, (H2a) especially early in the growing season when leaves are predicted to be most valuable, and (H2b) especially for native populations that have historically experienced a high risk of herbivory. We predicted the influence of tissue value and historical risk of attack to be additive, such that (H3) the difference between native and introduced populations should be most pronounced early in the season when leaves are predicted to be most valuable (Fig. 3.1). Importantly, we also accounted for leaf size, to determine whether (H4) observed differences are driven by dilution with leaf expansion, which may or may not be independent of how plants adapt in response to shifts in historical risk of herbivory.

METHODS

Advantages of using *Verbascum thapsus*

Verbascum thapsus, or common mullein, is native to Eurasia, but is widely naturalized as a result of intentional transport and plantings by humans. *Verbascum thapsus* is thought to have first established in North America, a large part of its introduced range, as a result of multiple introduction events from Europe to the eastern United States in the early 1600s (Wilhelm 1974; Gross and Werner 1978; Mitich 1989). Introduced populations quickly spread westward, with populations established as far as the Pacific Coast by the mid-1870s (Brewer *et al.* 1876; Gross and Werner 1978). Currently, *V. thapsus* is established within all 50 of the United States as well as all of the southern provinces of Canada (Gross and Werner 1978; Ansari and Daehler 2010). In its native Eurasian range, *V. thapsus* occurs throughout the British Isles and Europe (excluding

Iceland and Crete), extending north to 64°N, east into Russia and parts of China, and south to the Western Himalayas and Caucasus Mountains (Gross and Werner 1978). As reflected by its broad distribution, *V. thapsus* tolerates a wide range of climatic conditions (Gross and Werner 1982; Parker *et al.* 2003). Its broad climatic range allows us to account for climate-driven clines in defense that may obscure or interact with herbivore-driven patterns of adaptation.

The typically biennial lifecycle of *V. thapsus* (Gross and Werner 1978; Ansari and Daehler 2010) also allows us test how defense allocation is influenced by tissue value, which optimal defense predicts should mediate adaptation to herbivory, as the value of leaves is expected to decrease towards the end of the first growing season as plants prepare to overwinter. Further, we have strong evidence that introduced populations have evolved since their introduction to North America. North American populations produce more aboveground biomass than their native European congeners (Alba *et al.* 2011, 2012; Kumschick *et al.* 2013), and while European populations express a genetically based cline in performance along climate gradients, North American populations express high performance regardless of their climate of origin (Endriss *et al.* 2018). Finally, enemy escape among introduced populations is well documented. *V. thapsus* experiences more herbivory in its native European than in its introduced North American range (Alba *et al.* 2012; Endriss *et al.* 2018), and while native populations host a large diversity of mainly specialist herbivores (over 40 species), introduced populations are attacked by fewer, mainly generalist herbivores (Popov 1972; Gross and Werner 1978; Wolfe 2002). Reduced herbivore pressure in the introduced range is therefore correlated with a shift in herbivore type. However, both specialists and generalists likely exert selective pressure on populations within both ranges, as a specialist seed-feeding weevil (*Rhinusa tetra* Fabricius) and a specialist thrips (*Haplothrips verbasci* Osborn) are established within both ranges. *Verbascum*

thapsus defends itself against these herbivores with both physical defenses, such as trichomes (Woodman and Fernandes 1991; Alba *et al.* 2014), as well as chemical defenses, including iridoid glycosides and verbascoside (Khuroo *et al.* 1988; Pardo *et al.* 1998; Brownstein *et al.* 2017). *Verbascum thapsus* is therefore a good system for investigating how plants allocate their defense differently in their native and introduced ranges.

Plant propagation

In summer 2015, we grew plants from seed collected from 13 native European populations (*i.e.* have historically experienced a high risk of herbivory) and 15 introduced North American populations (*i.e.* have historically experienced a low risk of herbivory) (Popov 1972; Gross and Werner 1978; Wolfe 2002; Alba *et al.* 2012; Endriss *et al.* 2018). We selected populations within each range to reflect this species' broad global and climatic distribution (Table 3.1). To rigorously test assumptions of optimal defense, we assessed defense allocation at three different time periods during the growing season: early, middle, and late. Our measurements of defense were destructive, and thus required replication not only within populations, but also across time (28 populations_{13 native + 15 introduced} × 1 maternal line × 3 sampling times × 2 individuals = 168 plants).

We seeded six replicates for each maternal line in April 2015, by placing 5–10 seeds on each of six 25-mm-diameter, moistened peat pellets (Jiffy International AS, Kristiansand, Norway). We placed the pellets under a 12-hour light/dark cycle regime at 25/15°C, respectively, and watered as needed. Every two days, we re-randomized the location of peat pellets within the germination chambers. After two weeks, we thinned each pellet down to two individuals.

In May 2015, we thinned each peat pellet down to one individual, and transplanted pellets into 4 x 13.5in tree-pots (2.65L) containing a homogenized mix of 1:1:2 local field soil (collected from Colorado State University's Agricultural Research, Development and Education Center; 40.6526°N, 104.99699°W), sand, and potting soil. This mixture allowed us to maintain a soil environment comparable to natural growing conditions, in which *V. thapsus* is likely nutrient-limited yet exposed to natural soil biota. In addition, plants grown in high-nutrient soils may be highly tolerant, and thus not express variation in resistance against herbivory (Herms & Mattson 1992; Stamp 2003; Fine *et al.* 2006; Strauss and Agrawal 1999), the focus of our study.

Common garden

After transplanting peat pellets into pots, we allowed seedlings one week to acclimate to greenhouse conditions. We then moved plants to an outdoor patio with full exposure to sun as well as local herbivores. We organized plants based on a modified split-plot design. Each whole-plot was represented by three plants from a single population, one each that was destructively sampled in the early, middle, and late part of the growing season. We kept plants grouped by their initial whole-plot assignment (until destructively harvested), but we re-randomized both the location of whole-plots as well as the location of the plants within whole-plots on a biweekly basis. Population was our unit of replication, and each population was represented twice within each harvest (*i.e.* by two whole-plots). We watered plants sparingly (as needed), and timed the watering of each pot to provide a consistent amount of water to each plant during each watering event (~.5L per pot).

Sampling traits related to defense

Early, middle, and late harvests began respectively on July 20th, August 20th, and September 20th, and were each conducted within the span of two days. To determine whether defense allocation tracks tissue value and historical risk of attack, we destructively harvested plants to measure both physical and chemical defenses. For each plant, we cut the aboveground tissue from the roots, and ordered the leaves from youngest to oldest based on their location within the rosette. We then photographed leaves by flattening them between two glass panes. To measure the area of each leaf, we later processed these photographs using the LeafJ plugin (Maloof *et al.* 2013) within ImageJ (Schneider *et al.* 2012) (Fig. 3.1). The youngest unfurled leaf with a width greater than 1.5 cm as well as the oldest leaf without yellowing or browning were used for analyses of trichome length and density as well as leaf toughness. The remaining young and old leaves (the top and bottom 50% of the leaves in the rosette) were lyophilized using a bulk tray dryer with 6-port manifold (Labconco, Kansas City, MO).

i. Physical defense

We measured two traits to evaluate physical defense: trichomes and leaf toughness. To assess trichomes, we measured both the average length and density of the trichome mat on the adaxial surface of each subsampled leaf. To measure trichome density, we took three pictures per leaf: one each of the tip, middle, and base of each leaf blade (Fig. 3.3). We used an EOS 30D Canon DSLR, at shutter speed 1/100 and ISO 1000, attached to a microscope set to 20x magnification. Each photograph was taken to the left of each leaf's midrib, as trichomes are reduced near the midrib, and pictures of these areas would reflect leaf topography rather than among-leaf variation. Photographs were processed using ImageJ (Schneider *et al.* 2012) to

determine what percent of leaf tissue was covered by its overlying mat of trichomes (Fig. 3.2). We then averaged the percent cover across the three photographs from each leaf (hereafter ‘trichome density’).

To assess leaf toughness, we placed each leaf within a vial of distilled water and re-cut the submerged petiole immediately after photographing trichomes. After leaves rehydrated for 24 hours at 40°F, we used a customized Lloyd LF-Plus universal testing instrument (Ametek STC) as a leaf penetrometer. This instrument measured how much force (accurate to within 1% of the force measurement) was required for a blunt, circular probe lowered at a constant speed to puncture each leaf using a 20 N load cell. Before puncturing each leaf, we sliced the leaf from apex to base immediately parallel to the midrib, to allow for the machine to tightly clamp the leaf in place. We measured leaf toughness at three locations: one each towards the tip, middle, and base of each leaf. We then averaged across these three locations to estimate the average force required to puncture each leaf (hereafter ‘leaf toughness’). Leaves were then lyophilized, weighed, and added to our measure of aboveground biomass for each plant.

ii. Chemical defense

We focused on two iridoid glycosides, aucubin and catalpol, as well as a single caffeoyl phenylethanoid glycoside, verbascoside. *Verbascum thapsus* produces high levels of each of these compounds (Alba *et al.* 2012, Mihailović *et al.* 2016), and each likely play important, yet different, roles in mediating herbivory (Bowers and Stamp 1993; Adler *et al.* 1995; Alba *et al.* 2012). In addition, *Verbascum* expresses considerable genetic variation in the expression of these compounds (Alba *et al.* 2014; Mihailović *et al.* 2016), which suggests that *Verbascum* should respond to selection by herbivory.

iii. Sample preparation for detection of iridoid glycosides and verbascoside

After samples were lyophilized, we weighed each leaf to determine dry biomass. We then separated samples into both the youngest leaves and the oldest leaves that yielded 25 mg of dried leaf tissue, placed samples within 50mL Falcon Tubes with six 4.5mm ball bearings, and ground tissue using a 5G-HD 5-gallon shaker (Harbil, Wheeling, IL). We then used a 24-hour extraction using 5 ml of methanol to extract compounds of interest from 25 mg of dried, ground, tissue from each leaf. At the end of the extraction period, we centrifuged samples and removed the supernatant. We then added an additional 1ml of methanol, and vortexed and centrifuged samples for a second time. The resulting 6ml samples of supernatant were evaporated and stored at 1.6°C. Just prior to injection into the HPLC, we re-suspended samples in 1.5ml of 1:1 solution of acetonitrile:H₂O, and vortexed and filtered each sample through .2µm PTFE filters.

ii. HPLC-MS analysis

We adapted our method from Kumar *et al.* (2013) and Sertić *et al.* (2015). We analyzed aucubin, catalpol, and verbascoside using a LC-MS/MS system of a Nexera X2 UHPLC with 2 LC-30AD pumps, a SIL-30AC MP autosampler, a DGU-20A5 Prominence degasser, a CTO-30A column oven, and a SPD-M30A diode array detector coupled to an 8040 model mass spectrometer (Shimadzu). We used a Luna® Omega 3µm Polar C18 100 column (50 x 4.6mm) maintained at 40°C. Solvent A contained water with 0.1% formic acid and solvent B contained acetonitrile with 0.1% formic acid. The solvent gradient for solvent B was: from 0-50% from 0-6min, 50% at 6-10min, and a re-equilibration period from 50-0% from 10-15min. The flow rate was set at 0.4ml/min and each sample was analyzed as a 1µL injection volume. For all three chemicals, we set the MS to negative detection mode. We monitored for an aucubin-formate

adduct (m/z 391.0) as well as three of its breakdown products (m/z 345.15, m/z 183.05, m/z 45.1), a catalpol-formate adduct (m/z 407.0) as well as three of its breakdown products (m/z 361.35, m/z 199.15, m/z 169.10), and verbascoside (m/z 623.1) as well as three of its breakdown products (m/z 161.1, m/z 461.2, m/z 135.0).

At the beginning of each batch analysis we created a calibration curve using a combined stock solution of 5 different known concentrations (10 µg/ml, 25 µg/ml, 50 µg/ml, 100 µg/ml, and 250 µg/ml) of our three compounds of interest. We obtained catalpol and verbascoside standards from Sigma-Aldrich Co. (St Louis, MO, USA), and our aucubin standard from AbovChem LLC (San Diego, CA, USA). We used Shimadzu Corporation's LabSolutions software (version 5.82) for post-run analysis.

Statistical analyses

We fit all statistical models using R version 1.0.143 and the package *lme4* (version 1.1.12). We log-transformed leaf toughness and percent trichome cover to meet model assumptions. P-values for the contrasts reported in the results are Tukey-adjusted to correct for multiple comparisons.

To account for climate-driven variation, which may obscure or interact with between-range differences in herbivore-driven defense (Colautti *et al.* 2009), we used three Principle Components (PCs) described by Dupin *et al.* (2011). These PCs were derived from 19 bioclimatic variables associated with temperature, precipitation, and their seasonality (WorldClim database; www.worldclim.org), and explained nearly 84% of the variation present in the original training set (Dupin *et al.* 2011). To apply these PCs to our data, we first extracted these 19 bioclimatic variables for the location of origin of each of our populations, and then

applied loadings developed by Dupin *et al.* (2011) (see Table 3 of their manuscript). We therefore calculated three Principle Component scores for each of our populations (*sensu* Endriss *et al.* 2018). The first linear component (PC1) is associated mainly with temperature, the second (PC2) with precipitation during the wet or warm season, and the third (PC3) with precipitation during periods of drought.

We evaluated between-range differences and seasonal variation in defense allocation using the same generalized linear mixed model for each measured line of defense. Fixed effects included leaf age (categorical: young or old), origin (categorical: native or introduced), harvest (ordinal factor: early, middle, or late), the numeric values of PC1, PC2, and PC3 (Table 3.1), interactions between origin and each PC, and all possible interactions between origin, harvest, and leaf age. We included population nested within origin as a random effect, as well as plant nested within population nested within origin [defense ~ origin*harvest*leaf age + origin*PC1 + origin*PC2 + origin*PC3 + (1|origin:population) + (1|origin:population:plant)].

To compare variation in physical defense, we used the response variables of trichome length, trichome density, and leaf toughness. To compare variation in chemical defense, we investigated aucubin, catalpol, and verbascoside as independent response variables. However, we also used the percent of aucubin that comprised the total combined amount of iridoid glycosides as a response variable, as aucubin is the biosynthetic precursor to catalpol (Damtoft 1994). In addition, the relative abundance of these two compounds is ecologically significant, as they act differently against different types of insects (Bowers and Stamp 1993; Quintero *et al.* 2014).

To determine whether dilution with leaf expansion explains observed variation in leaf defense, we also reran each of our models with the additional dependent variables of leaf size as

well as an interaction between leaf age and leaf size. If dilution with leaf expansion, at least in part, explains defense allocation, we would expect the effect of leaf size to be significant.

However, a significant interaction between leaf size and leaf age would indicate that leaf size does not fully explain observed differences between young and old leaves, and that a different mechanism also operates to drive variation in defense across leaves of different ages.

RESULTS

Importantly, climate did not appear to drive evolutionary clines in defense allocation. We only found a weak trend for trichomes to become longer as a population's climate of origin becomes hotter (PC1: $p = 0.09$; Table 3.2). We did not find any significant interactions between a population's climate and range of origin (Table 3.2). Thus, any between-range differences we did observe could be more confidently attributed to between-range differences in herbivory rather than to climate-driven patterns of local adaptation.

Q1: are young leaves better defended than old leaves?

i. Physical defense

Young leaves were consistently better defended than old leaves (Table 3.2; Fig. 3.5). Young leaves were on average 74% tougher than old leaves ($p < 0.0001$), and required an additional 1.4 N/m² of force to puncture (95% CI: 1.2 to 1.6 N/m²; Fig. 3.5c). In addition, trichomes were 46% longer ($p < 0.0001$, 95% CI: 39%–52%; Fig. 3.5a) and 102% denser ($p < 0.0001$, 95% CI: 84%–120%; Fig. 3.5b) on young leaves than on old leaves. Specifically, trichomes were on average 0.49mm longer and covered 46% more of the underlying leaf on young leaves than on old leaves.

ii. Chemical defense

For iridoid glycosides, young leaves were consistently better defended than old leaves (Table 3.2, Fig. 3.5). Average concentrations of aucubin and catalpol were respectively 2.3 fold higher (95% CI: 2.1–2.6, $p < 0.0001$) and 18.7 fold higher (95% CI: 17.4–20.2, $p < 0.0001$) for young leaves than for old leaves (Table 3.2; Fig. 3.5d,e). On average, aucubin represented 0.9% of the dry weight of young leaves, but only 0.4% of the dry weight of old leaves, while catalpol represented 4.0% of the dry weight of young leaves, but only 0.2% of the dry weight of old leaves.

Verbascoside, however, was on average 10% more concentrated (95% CI: 1–19%) in old leaves than in young leaves (Table 3.2; Fig. 3.5f; $p = 0.02$), and typically respectively comprised 2.8% and 2.5% of the dry weight of old leaves versus young leaves.

Q2a: are young leaves especially better defended than old leaves early in the season, when young leaves are predicted to be most valuable?

i. Physical defense

The leaves of *V. thapsus* varied both in toughness and trichome density across the growing season but, contrary to the predictions of optimal defense, the difference between young and old leaf defense typically become more, instead of less, pronounced towards the end of the growing season (Fig. 3.5). For example, young leaves became increasingly tougher than old leaves over the growing season (harvest*leaf age interaction: $p < 0.0001$, Fig. 3.5c), as young leaves became 90% tougher between the early and late harvest (95% CI: 76%–104%, $p < 0.0001$), while old leaves only became 32% tougher (95% CI: 22%–43%, $p < 0.0001$).

Temporal variation in the difference in trichome length between young and old leaves was more complex. Trichomes steadily became 115% longer on old leaves between the early and late harvest (95% CI: 98%–132%, $p < 0.0001$, Fig. 3.5a). However, while trichomes on young leaves became 56% longer between the early and middle harvest (95% CI: 46%–66%; $p < 0.0001$), trichome length plateaued in the middle of the season, and remained the same between the middle and late harvest ($p = 0.96$; Fig. 3.5a). As a result, the difference in trichome length between young and old leaves increased between the early and the middle harvest, but then decreased between the middle and the late harvest (harvest*leaf age interaction: $p < 0.0001$). Trichome length was therefore the most similar between young leaves and old leaves late in the growing season, which matched predictions of optimal defense, but was not most pronounced early in the season, which did not match predictions of optimal defense.

Finally, the difference in trichome density between young leaves and old leaves was most pronounced at the middle harvest, but contrary to predictions of optimal defense did not differ between the early and the late harvest (Fig. 3.5b). Specifically, trichome density declined between the early and middle harvest by 21% on young leaves and 80% on old leaves (95% CIs: 10%–31%, 58%–103%, respectively), but trichomes became denser for both young (95% CI: 7%–33%, $p = 0.03$) and old leaves (95% CI: 182%–423%, $p < 0.0001$) between the middle and late harvest (harvest*leaf age interaction: $p < 0.0001$, Fig. 3.5b).

ii. Chemical defense

Contrary to predictions of optimal defense, the difference in iridoid glycoside concentration between young leaves and old leaves was especially pronounced late, instead of early, in the growing season (harvest*leaf age interaction: $p_{\text{aucubin}} < 0.0001$, $p_{\text{catalpol}} < 0.0001$; Fig.

3.5d,e). Young leaves drove variation between young and old leaves: old leaves expressed similar concentrations of both aucubin and catalpol across the growing season (difference between the early and late harvest: $p_{\text{aucubin}} = 0.9$, $p_{\text{catalpol}} = 0.9$), while young leaves became better defended later in the growing season (Fig. 3.5d,e). Specifically, concentrations of aucubin did not increase in young leaves between the early and middle harvest ($p = 1$), but significantly increased between the middle and late harvest ($p < 0.0001$). In contrast, concentrations of catalpol significantly increased in young leaves between each harvest ($p_{\text{early to middle}} < 0.0001$, $p_{\text{middle to late}} = 0.03$). Although we chose to present aucubin and catalpol as separate response variables, it is important to note that the relative concentration of aucubin and catalpol did vary between young and old leaves, as well as across the growing season (Fig. 3.4).

Concentrations of verbascoside also did not follow predictions of optimal defense. Young and old leaves had similar concentrations at the middle and the late harvest ($p_{\text{middle}} = 0.9$, $p_{\text{late}} = 1.0$), while concentrations were actually higher in old leaves than in young leaves at the early harvest ($p = 0.03$, Fig. 3.5f).

Q2b: are young leaves especially better defended than old leaves for native populations that have historically experienced a high risk of attack?

i. Physical defense

Native populations, on average, did not differ in leaf toughness or trichomes from introduced populations (Table 3.2). However, the interaction between origin and leaf age was marginally significant for both trichome length and leaf toughness (for both, $p = 0.07$), as trichomes are about 50% longer for young leaves than old leaves in introduced populations (CI: 42%–60%, $p < 0.0001$), but only about 40% longer for young leaves than old leaves in native

European populations (CI: 31%–50%, $p < 0.0001$; Fig. 3.5a). Similarly, young leaves were an average of 81% tougher than old leaves for introduced populations (CI: 69%–92%, $p < 0.0001$), while young leaves were only an average of 68% tougher than old leaves for native populations (CI: 55%–80%, $p < 0.0001$; Fig. 3.5c). Thus, contrary to predictions, when between-range differences in defense allocation were observed, young leaves were especially well defended for introduced, not native, populations.

ii. Chemical defense

Native and introduced populations did not differ in their concentrations of either iridoid glycosides or verbascoside, or in how they allocated these compounds to their young versus old leaves (Fig. 3.5c,d,e; Table 3.2).

Q3: is the difference between native and introduced populations most pronounced early in the season when leaves are predicted to be most valuable?

i. Physical defense

Investment in trichome length and leaf toughness to young versus old leaves did differ between native and introduced populations, but these differences remained consistent across the growing season (origin*harvest*leaf age interaction: $p_{\text{trichome length}} = 0.2$, $p_{\text{leaf toughness}} = 0.3$; Fig. 3.5a,c). For trichome cover, native and introduced populations did not differ in how they allocated their physical defense to their young versus old leaves (Table 3.2, $p_{\text{origin}} = 0.3$, $p_{\text{origin*harvest*leaf age}} = 0.2$).

ii. Chemical defense

Native and introduced populations did not differ in how they allocated chemical defense to young versus old leaves (main effect of origin: $p_{\text{aucubin}} = 0.9$, $p_{\text{catalpol}} = 0.9$, $p_{\text{verbascoside}} = 1.0$), and their patterns of defense allocation remained consistently similar across the growing season (Table 3.2, no significant origin*harvest*leaf age interactions; Fig. 3.5d,e,f).

Q4: does dilution by leaf expansion account for observed variation between young and old leaves?

Young leaves remained the same size between native and introduced *V. thapsus* populations ($p = 0.7845$), but old leaves were 13% larger for introduced as compared to native populations (CI: 3% to 26%; Table 3.3, origin by leaf age interaction: $p < 0.001$; Fig. 3.6). Interestingly, however, when we included leaf size in our models it explained some, but not all, of the observed variation between young and old leaves (Table 3.4, see significant leaf age by leaf size interactions). Thus, old leaves were better defended than old leaves than would be expected by dilution with leaf expansion alone. The one notable exception is for verbascoside, for which concentrations were not significantly influenced by leaf size (but were also only influenced by leaf age early in the season, and not in the way predicted by optimal defense).

DISCUSSION

In line with past tests of optimal defense (McCall and Fordyce 2010), our data match the prediction that young leaves should be better defended than old leaves. However, adaptation to herbivory is not the only mechanism predicted to drive this pattern of defense allocation. Innate plant physiology, such as dilution of defense with leaf expansion, may also drive young leaves to

be better defended than old leaves. Indeed, upon refining predictions to be more exclusively specific to optimal defense, we found that *V. thapsus* failed to express the more nuanced differences in defense allocation that we expected given variation in tissue phenology and historical risk of attack (Fig. 3.1). Surprisingly, however, we also found that dilution with leaf expansion explained some, but not all, of the observed variation in defense allocation (Table 3.4).

Thus, while dilution with leaf expansion and adaptation to herbivory have historically been posited as the main two alternate explanations for why young leaves are better defended than old leaves (Berenbaum 1995; Stamp 2003), we found that defense allocation was fully explained by neither. Here, we therefore discuss which of our predictions of optimal defense did or did not successfully reflect defense allocation in *V. thapsus*, how these findings refine understanding of defense allocation and evolution, and, moving forward, what this refined understanding means for studies of defense evolution.

Q1: are young leaves better defended than old leaves?

Young leaves are tougher, hairier and better chemically defended by iridoid glycosides than old leaves (Fig. 3.5; Table 3.2). However, investment in verbascoside represents a notable exception to this pattern of defense allocation. Verbascoside concentration does not differ between young and old leaves at the middle and end of the growing season, and early in the season verbascoside is more concentrated in old, not young, leaves (Fig 3.5f; Table 3.2). Verbascoside may be allocated differently than other lines of defense because it serves multiple functions, only one of which is defense against herbivory. For example, verbascoside is known to protect against damaging UV-B radiation (Zhao *et al.* 2000; Alipieva *et al.* 2014), and

McCloud and Berenbaum (1999) showed that verbascoside, but not aucubin or catalpol, is typically induced in *Plantago lanceolata* in response to UV-B exposure. Thus, verbascoside may be under different selective pressures than iridoid glycosides, as protection against herbivory may not be verbascoside's most important contribution to plant fitness.

Further, unlike aucubin and catalpol, verbascoside may represent an irreversible commitment to plant defense (Coley *et al.* 1985); studies of *Plantago lanceolata* show that while aucubin and catalpol are reallocated or broken down as a leaf ages (Bowers and Stamp 1992; Marak *et al.* 2000), verbascoside concentrations remain more consistent across leaves of different ages (McCloud and Berenbaum 1999). Whether or not verbascoside should be classified as a mobile or immobile defense requires further investigation, but our findings may indicate that how much better defended young leaves are than old leaves may be constrained by the mobility of a specific line of defense: while plants are able to reallocate mobile defenses from old to young leaves, once immobile defenses are invested in a leaf, they remain present even as the leaf ages and becomes less valuable (Endriss, chapter 2).

Q2a: are young leaves especially better defended than old leaves early in the season, when young leaves are predicted to be most valuable?

Our predictions of optimal defense state that, because *V. thapsus* is biennial, its young leaves should be especially better defended than its old leaves early in its first growing season when leaves are most predicted to be most valuable (Endriss, chapter 2; Fig. 3.1). Indeed, we found significant seasonal variation in how *V. thapsus* defended its young versus old leaves for every line of defense we measured (Fig. 3.5). Contrary to our predictions, however, we found

that differences in defense were rarely most pronounced early in the season, and that how defense investment varied across the growing season differed for different lines of defense.

For example, we found that leaves, especially young leaves, were most tough and toxic late, not early, in the growing season (Fig. 3.5c). That leaves, especially young leaves, are at their toughest late in the season is perhaps unsurprising, as the compounds that contribute to leaf toughness are often immobile (Westbrook *et al.* 2011). Thus, old leaves are constrained to be at least as tough as they were earlier in the season as young leaves, as they likely cannot disinvest in leaf toughness once it is invested (Endriss, chapter 2).

Iridoid glycosides also increased in young leaves over the course of the season (Fig. 3.5c,d), despite being mobile, which may suggest that their allocation is best explained by ontogenetic shifts rather than by defense mobility (reviewed by Barton 2017). In fact, Barton and Koricheva (2010) found that herbs and woody plants commonly increase investment in constitutive levels of secondary compounds as they grow older (Barton and Koricheva 2010). What drives this observed pattern remains less well understood, but one reason may be that plants allocate more energy to growth than to defense when they are seedlings, as seedlings are highly vulnerable to plant competition, but less vulnerable to herbivory due to their low apparency (Boege & Marquis 2005). Notably, however, differences between verbascoside concentrations in young and old leaves was most pronounced early in the growing season (Fig. 3.5f), although this still did not match predictions of optimal defense as verbascoside was higher in old, not young, leaves. Further studies are therefore needed to understand ontogenetic variation in growth-defense tradeoffs (Orians *et al.* 2010, Boege *et al.* 2007), especially in the context of within-plant allocation of defense.

Variation in trichomes also did not match predictions of optimal defense. Trichomes on young and old leaves did become more similar in length later in the growing season, but largely because the length of trichomes on young leaves plateaued at the middle harvest (Fig. 3.5a). Similar trichome lengths between young and old leaves at the end of the season may therefore be better explained by a physiological constraint on trichome length rather than adaptation to herbivory. Differences in trichome density were also contrary to predictions of optimal defense, as differences in trichome density between young and old leaves was most pronounced at the middle harvest, with trichomes at their most dense at the early and the late harvest (Fig. 3.5b). In addition to defense against herbivory, trichomes also protect against UV radiation and aid in water retention (Bickford 2016). Although trichomes should be most dense during the middle of the growing season if the main driver of their evolution was UV exposure, future studies are needed to investigate whether trichome variation can be explained by seasonal variation in biotic factors such as herbivore pressure, or abiotic factors such as precipitation.

Our findings therefore provide strong evidence of seasonal variation in how *V. thapsus* defends its young as compared to old leaves, but this variation does not reflect our predictions regarding optimal allocation of defense throughout the growing season. Future studies of defense allocation should carefully investigate shifts in resource allocation with ontogeny, as ontogenetic constraints and trajectories may play a stronger role than herbivory or leaf expansion in driving within-plant allocation of defense. Tests of defense should also measure tissue value to confirm that aboveground leaf tissue becomes less valuable as plants prepare to overwinter.

Q2b: are young leaves especially better defended than old leaves for native populations that have historically experienced a high risk of attack?

According to optimal defense, valuable young leaves should be especially well defended for native populations that have historically experienced a high risk of herbivory, as opposed to introduced populations that have historically experienced a low risk of herbivory. For *V. thapsus*, however, we find that relative investment in chemical defense and trichome density does not differ between native and introduced populations (Fig. 3.5; Table 3.2). One reason that we did not observe this predicted pattern of defense may be that optimal defense assumes a growth-defense tradeoff (McKey 1974; Rhoades and Cates 1976; Herms & Mattson 1992), but defense against herbivory may not always be costly to produce or maintain. Herbivory is known to reduce fitness of *V. thapsus*, whether or not defense is ecologically costly for *V. thapsus* in the face of low herbivory remains less well understood (Endriss *et al.* 2018). Thus, evolution of defense may not be symmetrical in response to an increase versus a decrease risk of herbivory. Although *V. thapsus* populations may adapt to increased herbivory by reallocating more defense to young than to old leaves, decreased herbivory (as experienced by *V. thapsus* upon introduction to a new range) may not exert strong selection for shifts in defense allocation if defense is not costly to produce and maintain.

Alternatively, the similarities in how native and introduced populations defend their leaves may reflect that the selection that results from a shift from specialist-dominated to generalist-dominated herbivory counterbalances the selection that results from reduced herbivore pressure. Specifically, herbivore communities may select for introduced populations to increase defense, especially chemical defense, as they no longer face the risk of attracting specialists in the generalist-dominated introduced range (van der Meijden 1996; Müller-Schärer *et al.* 2004;

Joshi and Vrieling 2005; Alba *et al.* 2012). However, reduced herbivory should select for reduced investment in defense, as this now represents a cost that provides little benefit (Blossey and Nötzold 1995). If these pressures are simultaneously acting upon populations in the introduced range, this might explain why introduced populations have not evolved differences in chemical defense after introduction to their novel range.

Yet we do find that within-plant investment in leaf toughness and trichome length differs between the native and introduced populations (Fig. 3.5a,c; Table 3.2). Importantly, this difference is contrary to the predictions of optimal defense (Fig. 3.1): *V. thapsus* differentially allocates even more defense to its young compared to old leaves in its introduced North American range, where populations have historically experienced low, not high, risk of herbivory. This pattern may indicate that the evolutionary effects of introduction history overwhelm the signal of local adaptation. For example, for many invasive species it may be that only the most robust and well-defended genotypes become successfully introduced or established within their introduced range (te Beest *et al.* 2012, Blackburn *et al.* 2015, Iles *et al.* 2016). Thus, although invasions have the potential to provide greater insight into the evolution of defense allocation, they also present their own caveats for disentangling evolutionary mechanisms of defense allocation.

Q3: is the difference between native and introduced populations most pronounced early in the season when leaves are predicted to be most valuable?

Trichome length and leaf toughness were the only two measured lines of defense that were allocated differently between native and introduced *V. thapsus* populations, and this difference remained consistent across the growing season (Fig. 3.5a,c; Table 3.2, no significant

origin by leaf age by harvest interactions). That introduced populations better defended their young leaves than native populations by a consistent amount across the growing season may suggest that seasonal variation in defense allocation is tightly physiologically constrained, and that future studies need to better pinpoint which physiological mechanism is responsible for these observed differences in defense allocation.

Q4: does dilution by leaf expansion account for observed variation between young and old leaves?

Interestingly, dilution by leaf expansion explained some, but not all, observed variation in defense between young and old leaves (Table 3.4, see significant interactions between leaf size and leaf age). For example, accounting for between-range differences in leaf size also accounted for between-range differences in trichome length and leaf toughness. Importantly, however, leaf size fully explained neither differences in defense between young and old leaves, nor seasonal patterns in defense allocation. Thus, although dilution with leaf expansion seems to play an important role in diluting defense with leaf age for most of our measured lines of defense, but it is not the only mechanism responsible for driving young leaves to be better defended than old leaves.

One such mechanism that may help to explain this unaccounted variation in defense is climate. In addition to herbivory, climate is known to be an important driver of invader evolution (Maron *et al.* 2004; Montague *et al.* 2008; Alexander *et al.* 2012; Zenni *et al.* 2014; Lee and Kotanen 2015) and also typically varies between a plant's native and introduced ranges (Colautti *et al.* 2009; Early and Sax 2014; Bocsí *et al.* 2016). *Verbascum thapsus* expresses a genetically based cline in performance along climate gradients in the native range (Endriss *et al.* 2018), and

field populations express phenotypic clines in defense and performance along climatic gradients within both ranges (Ansari and Daehler 2010; Alba *et al.* 2012; Seipel *et al.* 2015). Thus, genetically based clines in defense seem likely to evolve in response to climate gradients. However, climate is not a major driver of how *V. thapsus* defends its leaves, as we only find a weak trend ($p = 0.09$) for trichomes to become longer as temperature increases, and no significant interactions between climate and range of origin. Thus, climate does not appear to be the unaccounted-for mechanism that drives young leaves to be better defended than old even after accounting for variation in leaf size.

Conclusions

V. thapsus overwhelmingly better defends its young than old leaves. Yet how the magnitude of this difference varies across the growing season and between native and introduced populations does not match our increasingly specific predictions of optimal defense. Defense allocation was also not fully explained by dilution with leaf expansion. Adaptation to herbivory and dilution with leaf expansion have traditionally been presented as the two alternate explanations for why young leaves are better defended than old leaves. Here, however, we found that the enormous seasonal variation we observed in defense allocation was explained by neither. Our novel test of optimal defense therefore challenges our understanding of defense allocation in two main ways. First, it underscores the importance of testing the predicted mechanisms alongside the predicted outcomes of the optimal defense hypothesis, as adaptation to herbivory may not be the only mechanism that drives young leaves to be better defended than old leaves. Second, it suggests that we need to rethink what physiological traits or environmental variables may drive within-plant allocation of defense, as understanding what drives these seasonal

patterns of variation, and why patterns vary across different lines of defense, represents an important step forward in understanding plant-herbivore interactions.

TABLES AND FIGURES

Table 3.1 Climate and geographic origin of the 15 low-risk (*i.e.* introduced) and 13 high-risk (*i.e.* native) populations represented within our common garden. To account for climate-driven variation in defense, we used three linear combinations of 19 bioclimatic variables extracted from the WorldClim database (www.worldclim.org). The first linear component (PC1) was attributed mainly to temperature, the second (PC2) to precipitation during the wet or warm periods, and the third (PC3) to precipitation during periods of drought. PCs and elevation are based on GPS approximations for population 11.

Population ID	Historical Risk of Attack	Location	Latitude	Longitude	Elevation (m)	PC1	PC2	PC3
1	low	Tennessee, USA	35.55754	-84.01096	273	0.33217898	-0.22985937	1.60471988
2	low	North Carolina, USA	35.86973	-82.04298	833	0.12609689	-0.09818333	1.79809868
3	low	Maryland, USA	38.90028	-76.55556	18	0.26821721	-0.17950794	1.21854198
4	low	Illinois, USA	40.01650	-88.25696	236	-0.03121997	0.07596859	0.45731336
5	low	Pennsylvania, USA	40.43699	-79.94445	310	0.06672297	-0.06627461	1.02537143
6	low	Colorado, USA	40.66529	-105.21944	1603	0.11539105	-0.67091089	-0.12658414
7	low	Ohio, USA	41.41744	-81.36583	346	-0.00059035	-0.19873257	0.93243963
8	low	Massachusetts, USA	42.20102	-71.77729	134	-0.11379627	-0.28258729	1.73770452
9	low	Oregon, USA	42.66380	42.66380	467	0.35570750	-0.32210922	0.41347238
10	low	New Hampshire, USA	42.84138	-71.67622	78	-0.15351161	-0.32902113	1.53949368
11	low	Wyoming, USA	43.46667	-110.75000	2073	-0.010596000	-0.98862100	0.84529200
12	low	Toronto, Canada	44.02920	-79.53742	295	-0.22833000	-0.22378641	0.76478267
13	low	Wisconsin, USA	44.53314	-92.05947	217	-0.32944143	0.27930400	-0.41213733
14	low	Oregon, USA	45.53100	-121.09190	289	0.17250167	-0.18506013	0.46223226
15	low	Montana, USA	47.55414	-116.91631	1078	0.13967800	-0.66917014	0.85385013
16	high	France	45.94939	1.01531	268	0.37359545	-0.85359466	1.59350574
17	high	Switzerland	46.84713	7.17373	584	-0.19494238	0.30356354	1.88201439
18	high	Switzerland	46.98717	7.14019	453	-0.19494238	0.30356354	1.88201439
19	high	Romania	47.14608	27.63928	54	0.02820559	-0.25488710	0.27429718
20	high	Germany	47.88756	7.58136	217	-0.05630821	0.11035503	1.36094439
21	high	Czech Republic	50.17970	13.37999	343	0.01495890	-0.55586785	0.78348118
22	high	Finland	60.20610	25.13333	17	-0.23681052	-0.45496073	0.75757152
23	high	Sweden	60.22960	17.70548	33	-0.14395426	-0.51036072	0.69397432
24	high	Sweden	60.25826	17.71917	53	-0.14395426	-0.51036072	0.69397432
25	high	Sweden	60.25868	17.63611	40	-0.14395426	-0.51036072	0.69397432
26	high	Sweden	60.25931	17.66047	40	-0.14395426	-0.51036072	0.69397432
27	high	Finland	61.00024	24.41610	121	-0.31137472	-0.32167828	0.39155805
28	high	Finland	61.35197	24.83544	100	-0.32953167	-0.29102200	0.4294889

Table 3.2 Results of a generalized linear mixed model testing the effects of tissue phenology (*i.e.* harvest), historical risk of attack (*i.e.* origin), leaf age, climate (*i.e.* PC1, PC2, PC3), and their interactions on defense investment in trichomes, leaf toughness, and secondary metabolites. PC1 was attributed mainly to temperature, PC2 to precipitation during the wet or warm periods, and PC3 to precipitation during periods of drought. P-values that are marginally (< 0.1) to highly significant (< 0.0001) are in bold.

Fixed Effects	trichome length		trichome % cover		leaf toughness		aucubin		catalpol		verbascoside	
	Chisq(df)	p-value	Chisq(df)	p-value								
Harvest	291.71(2)	< 0.0001	113.15(2)	< 0.0001	224.59(2)	< 0.0001	71.38(2)	< 0.0001	55.47(2)	< 0.0001	3.14(2)	0.21
Origin	0.00(1)	0.99	0.97(1)	0.33	0.65(1)	0.42	0.01(1)	0.93	0.01(1)	0.91	0.00(1)	0.98
Leaf Age	196.52(1)	< 0.0001	324.75(1)	< 0.0001	471.64(1)	< 0.0001	154.35(1)	< 0.0001	603.21(1)	< 0.0001	5.13(1)	0.02
PC1	2.87(1)	0.09	2.11(1)	0.15	0.96(1)	0.33	0.07(1)	0.79	0.42(1)	0.52	0.25(1)	0.62
PC2	0.12(1)	0.73	1.17(1)	0.28	0.47(1)	0.49	0.09(1)	0.77	0.19(1)	0.66	0.30(1)	0.58
PC3	2.06(1)	0.15	0.21(1)	0.64	0.25(1)	0.61	0.05(1)	0.83	1.03(1)	0.31	0.03(1)	0.85
Harvest x Origin	1.58(2)	0.45	2.14(2)	0.34	0.46(2)	0.80	0.52(2)	0.78	0.00(2)	1.00	2.91(2)	0.23
Harvest x Leaf Age	26.00(2)	< 0.0001	24.57(2)	< 0.0001	46.42(2)	< 0.0001	47.03(2)	< 0.0001	80.79(2)	< 0.0001	7.94(2)	0.02
Origin x Leaf Age	3.21(1)	0.07	0.01(1)	0.90	3.25(1)	0.07	0.41(1)	0.52	0.31(1)	0.58	0.06(1)	0.81
Origin x PC1	0.00(1)	1.00	0.09(1)	0.76	1.00(1)	0.32	0.00(1)	0.99	0.65(1)	0.42	0.61(1)	0.44
Origin x PC2	0.04(1)	0.85	0.26(1)	0.61	0.00(1)	0.95	0.09(1)	0.77	0.05(1)	0.82	0.46(1)	0.50
Origin x PC3	0.72(1)	0.40	2.29(1)	0.13	0.49(1)	0.49	0.16(1)	0.69	0.12(1)	0.73	0.15(1)	0.70
Harvest x Origin x Leaf Age	3.19(2)	0.20	3.17(2)	0.20	2.69(2)	0.26	0.88(2)	0.64	0.72(2)	0.70	2.71(2)	0.26

Table 3.3 Results of a generalized linear mixed model testing the effects of the phenology of tissue value (*i.e.* harvest), historical risk of attack (*i.e.* origin), leaf age, climate (*i.e.* PC1, PC2, PC3), and their interactions on leaf size (cm²). PC1 was attributed mainly to temperature, PC2 to precipitation during the wet or warm periods, and PC3 to precipitation during periods of drought. P-values that are marginally (< 0.1) to highly significant (< 0.0001) are represented in bold.

	leaf area	
Fixed Effects	Chisq(df)	p-value
Harvest	1.92(2)	0.38
Origin	0.86(1)	0.35
Leaf Age	1398.50(1)	< 0.0001
PC1	7.16(1)	< 0.01
PC2	0.51(1)	0.48
PC3	0.49(1)	0.48
Harvest x Origin	0.24(2)	0.89
Harvest x Leaf Age	18.82(2)	< 0.0001
Origin x Leaf Age	14.69(1)	< 0.001
Origin x PC1	1.39(1)	0.24
Origin x PC2	0.02(1)	0.88
Origin x PC3	0.91(1)	0.34
Harvest x Origin x Leaf Age	1.79(2)	0.41

Table 3.4 Results of a generalized linear mixed model with the addition of leaf size and an interaction between leaf size and leaf age as independent variables. Other independent variables remained unchanged, and included: harvest (*i.e.* early, middle, late), historical risk of attack (*i.e.* origin), leaf age (*i.e.* young, old), climate (*i.e.* PC1, PC2, PC3), and their interactions. PC1 was attributed mainly to temperature, PC2 to precipitation during the wet or warm periods, and PC3 to precipitation during periods of drought. P-values that are marginally (< 0.1) to highly significant (< 0.0001) are bolded.

Fixed Effects	trichome length		trichome % cover		leaf toughness		aucubin		catalpol		verbascoside	
	Chisq(df)	p-value	Chisq(df)	p-value	Chisq(df)	p-value	Chisq(df)	p-value	Chisq(df)	p-value	Chisq(df)	p-value
Harvest	248.61(2)	< 0.0001	100.75(2)	< 0.0001	171.34(2)	< 0.0001	51.89(2)	< 0.0001	28.06(2)	< 0.0001	2.47(2)	0.29
Origin	0.00(1)	0.96	1.31(1)	0.25	0.98(1)	0.32	0.06(1)	0.81	0.00(1)	0.99	0.04(1)	0.84
Leaf Age	9.42(1)	< 0.01	24.38(1)	< 0.0001	31.04(1)	< 0.0001	18.74(1)	< 0.0001	72.38(1)	< 0.0001	0.06(1)	0.80
Leaf Size	2.16(1)	0.14	14.15(1)	< 0.001	23.51(1)	< 0.0001	12.45(1)	< 0.001	29.01(1)	< 0.0001	0.04(1)	0.84
PC1	2.05(1)	0.15	2.68(1)	0.10	1.32(1)	0.25	0.99(1)	0.32	0.07(1)	0.79	0.17(1)	0.68
PC2	0.13(1)	0.71	1.26(1)	0.26	0.40(1)	0.53	0.88(1)	0.35	1.03(1)	0.31	0.73(1)	0.39
PC3	1.50(1)	0.22	0.75(1)	0.39	0.11(1)	0.74	0.01(1)	0.92	0.66(1)	0.42	0.14(1)	0.71
Leaf Age x Leaf Size	2.38(1)	0.12	8.61(1)	< 0.01	9.75(1)	< 0.01	8.73(1)	< 0.01	20.94(1)	< 0.0001	0.48(1)	0.49
Harvest x Origin	1.13(2)	0.57	2.39(2)	0.30	0.84(2)	0.66	0.74(2)	0.69	0.28(2)	0.87	3.98(2)	0.14
Harvest x Leaf Age	25.35(2)	< 0.0001	30.93(2)	< 0.0001	24.99(2)	< 0.0001	29.08(2)	< 0.0001	39.16(2)	< 0.0001	7.85(2)	0.02
Origin x Leaf Age	1.04(1)	0.31	0.01(1)	0.91	2.28(1)	0.13	0.13(1)	0.72	0.71(1)	0.40	0.00(1)	0.97
Origin x PC1	0.01(1)	0.94	0.05(1)	0.82	1.25(1)	0.26	0.28(1)	0.59	1.68(1)	0.20	0.53(1)	0.47
Origin x PC2	0.14(1)	0.71	0.51(1)	0.47	0.10(1)	0.75	0.01(1)	0.92	0.00(1)	0.96	0.81(1)	0.37
Origin x PC3	0.50(1)	0.48	2.52(1)	0.11	0.77(1)	0.38	0.78(1)	0.38	0.08(1)	0.78	0.05(1)	0.82
Harvest x Origin x Leaf Age	2.34(2)	0.31	3.57(2)	0.17	1.77(2)	0.41	1.03(2)	0.60	1.55(2)	0.46	3.95(2)	0.14

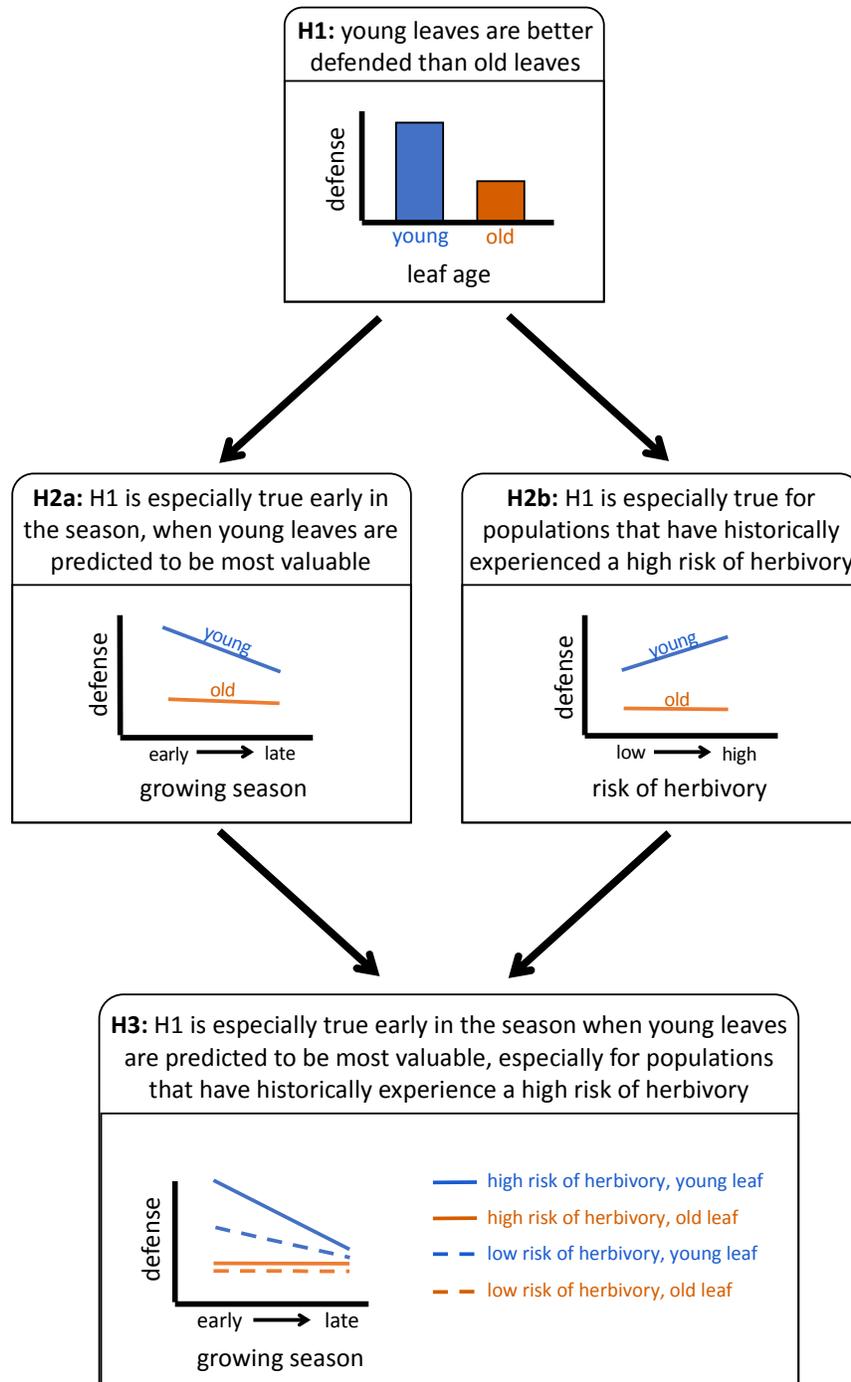


Figure 3.1 Our framework for testing the optimal defense hypothesis. H1 represents a broad prediction of optimal defense: young leaves should be better defended than old leaves (H1). However, many mechanisms might drive this observed pattern, not just optimal defense. To address this issue, we successively developed our original hypothesis to make it increasingly specific to optimal defense. For example, optimal defense predicts that young leaves should be better defended than young leaves as a result of adaptation to herbivory, but this pattern should thus be influenced by the two factors that mediate strength of selection: tissue value and historical risk of herbivory. Young leaves should therefore be better defended than old leaves

(H1), but especially early in the season when leaves are predicted to be most valuable (H2a), and especially for plant populations that have historically experienced high risk of herbivory (H2b). Finally, the influence of tissue value and historical risk of attack should be additive, which means that elevated defense of young leaves in populations that have historically experienced high risk of attack should be most pronounced early in the season when leaves are predicted to be most valuable (H3). If H1 through H3 are true, it allows us to more confidently attribute observed patterns to optimal defense. However, if H1 through H3 are not met, this approach provides us with equally valuable insight: by breaking optimal defense into a series of predictions rather than a single prediction with a binary outcome (*i.e.*, H1), which hypothesis(es) are not met tells us exactly which of the assumption(s) of optimal defense fail to accurately predict real-world processes.

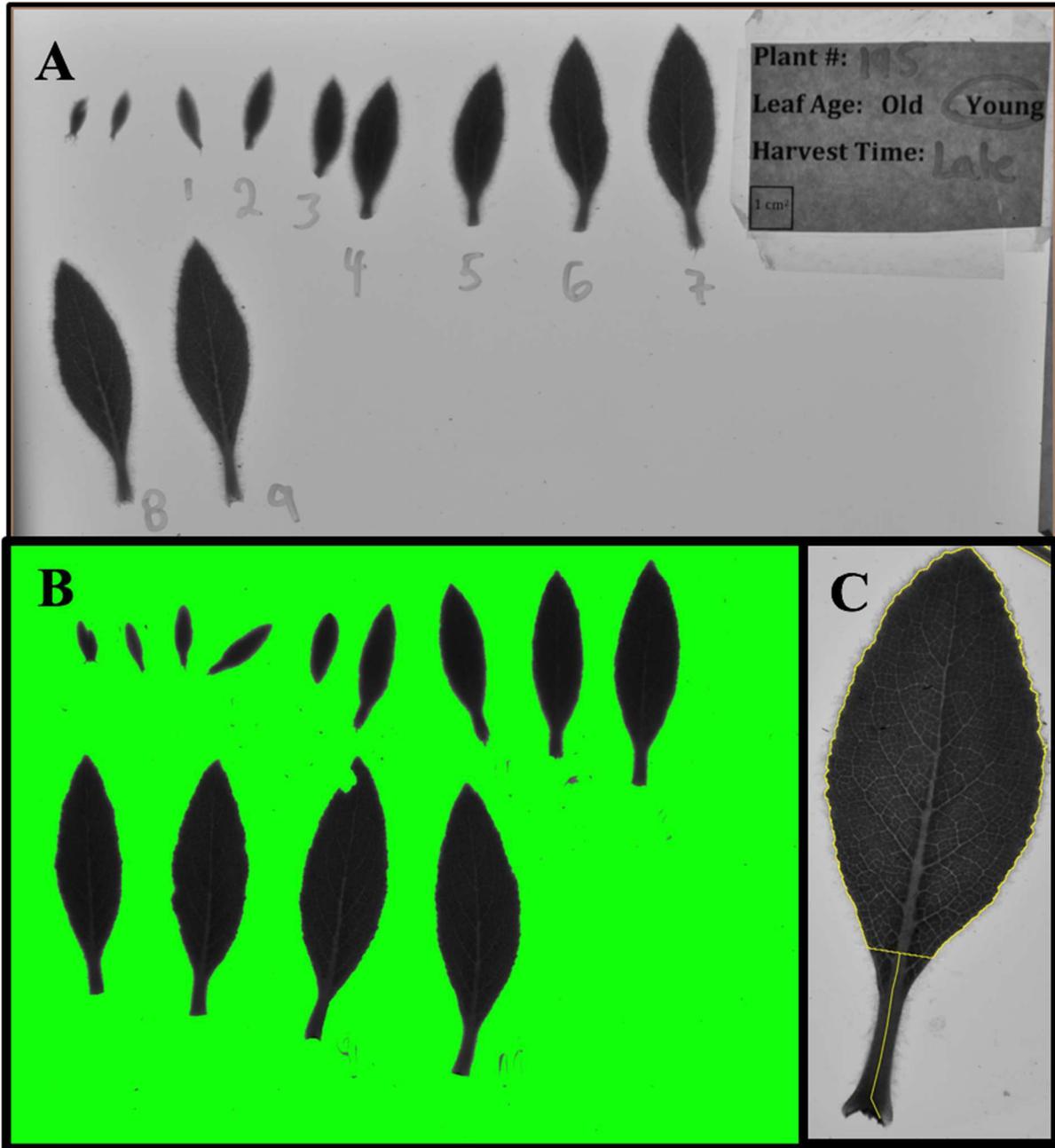


Figure 3.2 Young leaves and old leaves were separately photographed for each plant. For each photograph, leaves were placed on top of a light box and flattened with a glass pane. Within ImageJ, we converted photographs to 8-bit black-and-white images (A), then set the color threshold to ‘over/under’ without a dark background to enhance the contrast (B). We then used LeafJ to measure the area of each leaf (C).

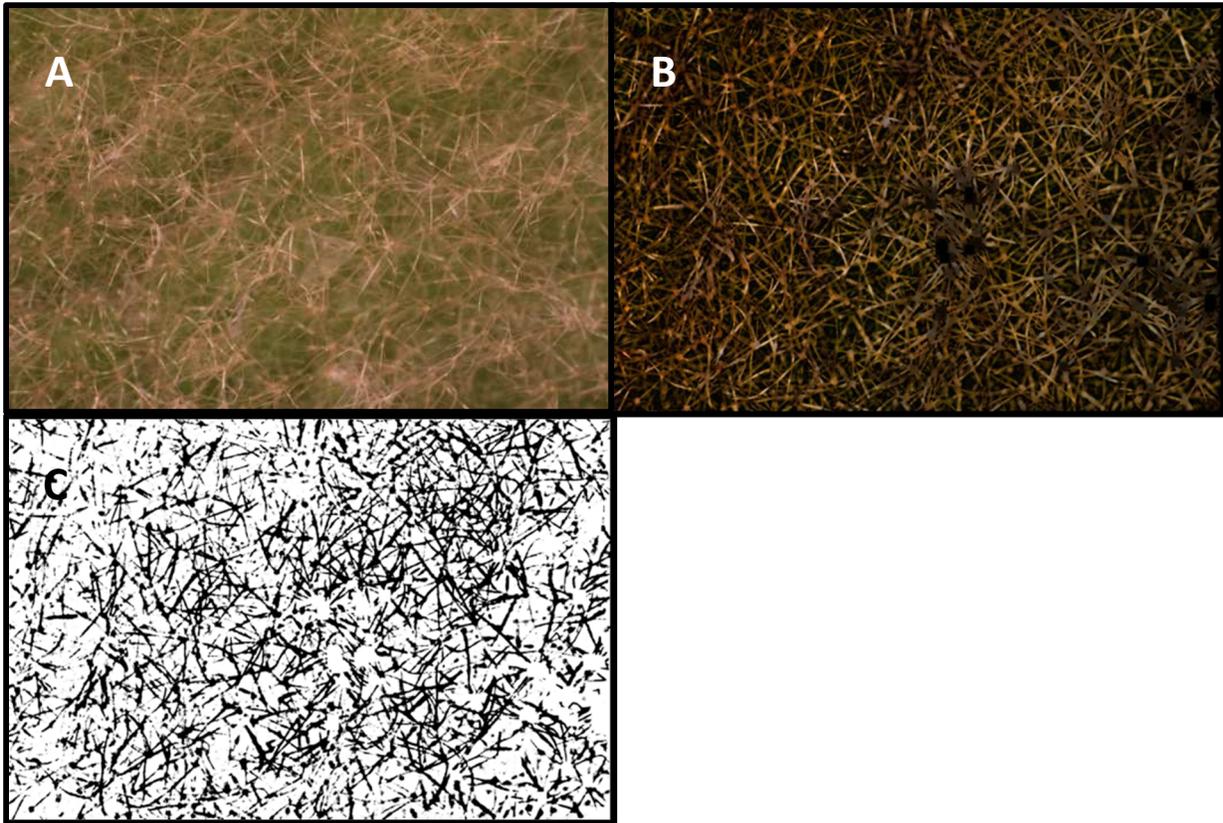


Figure 3.3 After photographing leaves for trichomes (A), we used ImageJ to enhance the contrast (B) and converted each photograph to a binary image (C). We inverted images such that the trichomes were white, and the leaf tissue underneath was black. We then imported a circle selection of known size, and analyzed the percent of white to black particles (pixel size:0-infinity, circularity:0-1, show:‘nothing’) within the circle to measure how much of the underlying leaf was covered by the overlaying mat of trichomes.

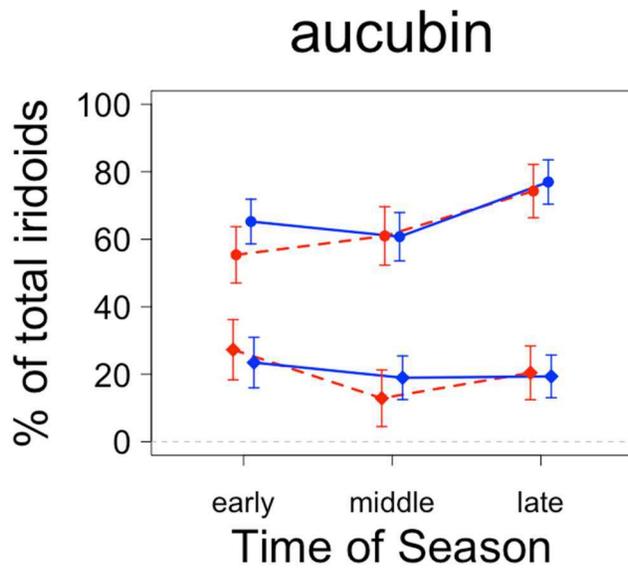


Figure 3.4 The percent of iridoid glycosides that is comprise of aucubin (as opposed to catalpol) in young leaves (diamonds) versus old leaves (circles) of *V. thapsus*. Native populations that have historically experienced high herbivory are shown in red, while introduced populations that have historically experienced low herbivory are shown in blue.

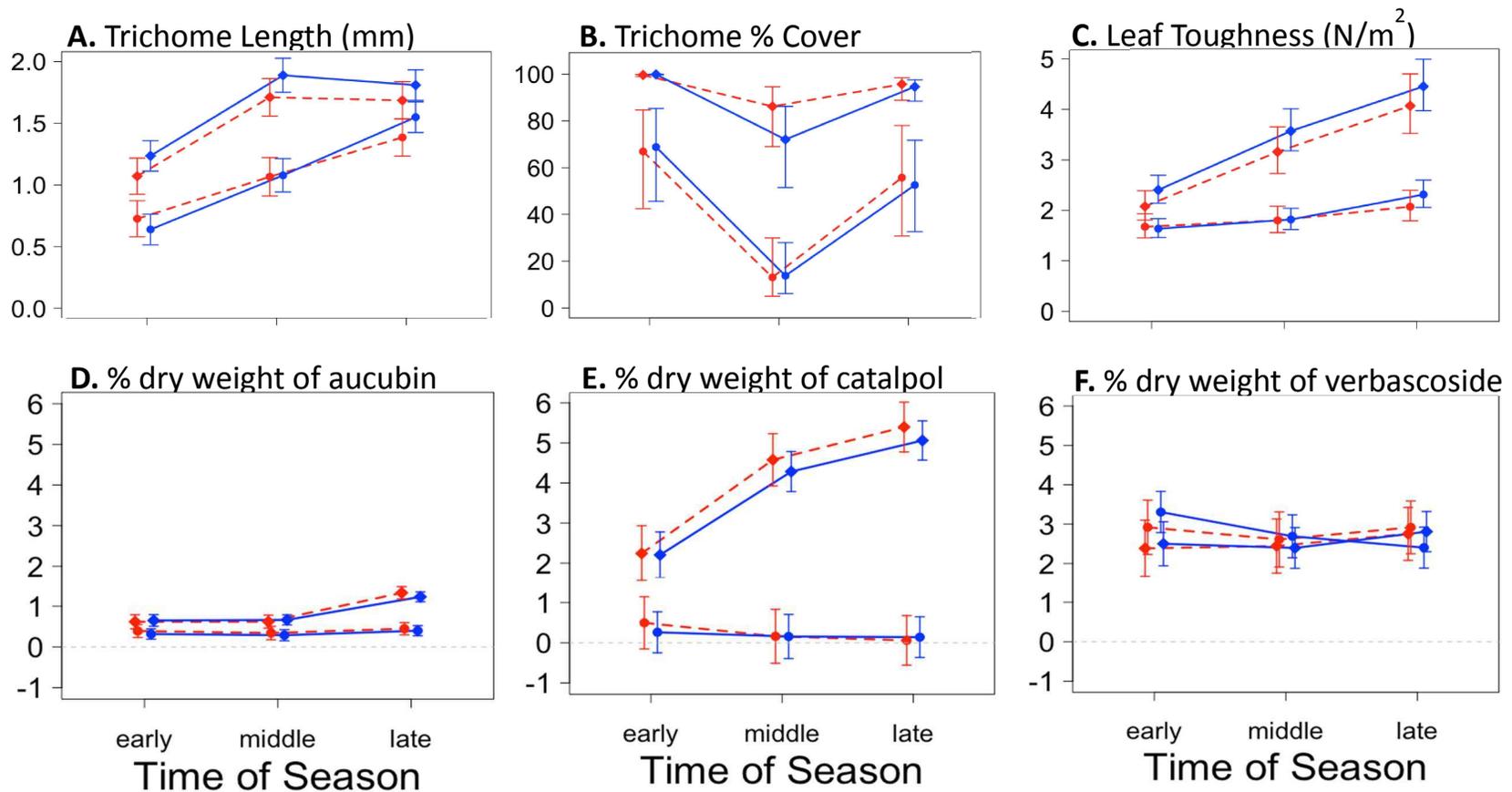


Figure 3.5 Seasonal variation in the concentration of physical (top row) and chemical (bottom row) defense expressed in young leaves (diamonds) versus old leaves (circles) of *V. thapsus*. Native populations that have historically experienced high herbivory are shown by dotted red lines, while introduced populations that have historically experienced low herbivory are shown by solid blue lines. Bars represent 95% confidence intervals. Note: y-axis scales differ for each measurement of physical defense. Bars represent 95% confidence intervals.

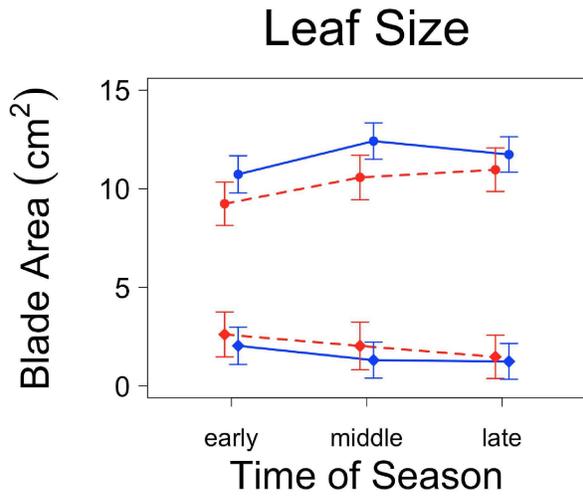


Figure 3.6 Seasonal variation young (diamonds) and old (circles) leaf size of *V. thapsus*. Native populations are shown in red, while introduced populations are shown in blue. Young leaves typically become smaller, and old leaves larger, as the season progresses. In addition, the size difference between young and old leaves is significantly more pronounced for introduced as compared to native *V. thapsus* populations.

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CHAPTER IV: THE IMPORTANCE OF GROWING UP: JUVENILE ENVIRONMENT INFLUENCES DISPERSAL OF INDIVIDUALS AND THEIR NEIGHBORS

SUMMARY

Dispersal is a key ecological process that is strongly influenced by both phenotype and environment. Here, we show that juvenile environment influences dispersal not only by shaping an individual's phenotype, but also by changing the phenotype of its neighbors, who influence how that individual decides to disperse. We used a model system (*Tribolium castaneum*, red flour beetles) to test how an individual's and its neighbors' past environment influence its response to the environment experienced during dispersal. We found that individuals moved especially far when exposed to a poor environment during dispersal if their phenotype (or one-third of their neighbors' phenotypes) were shaped by a poor environment as juveniles. Juvenile environment therefore shapes an individual's dispersal both by influencing its phenotype as well as its external social environment, which suggests that the juvenile environment of even a few individuals can influence the dispersal of an entire population.

INTRODUCTION

Dispersal is a key ecological process that can increase fitness by allowing individuals to respond to environmental variation (Ronce 2007; Clobert *et al.* 2012; Matthysen 2012). For example, individuals that disperse may escape from harsh or competitive environments or may be more likely to find mates (Gandon 1999; Palmqvist *et al.* 2000; Leturque and Rousset 2002; Perez-Gonzales & Carranza 2009). However, dispersal is also risky. The mortality rate of individuals that disperse is high, and there is no guarantee that the habitats individuals arrive in

will be more favorable than those they left behind (Bonte *et al.* 2012; Travis *et al.* 2012). How then do individuals decide to disperse when both leaving and staying in their current habitat carries risk? This remains a central question within evolutionary ecology (Cote *et al.* 2010; Bonte *et al.* 2012; McConkey *et al.* 2012; Travis *et al.* 2013; Green *et al.* 2015; Heino *et al.* 2015), especially as individual dispersal decisions have lasting consequences for the evolution, persistence and spread of populations and species (Fisher 1937; Skellam 1951; Levin *et al.* 2003; Kokko and López-Sepulcre 2006; Jongejans *et al.* 2008; Baguette *et al.* 2013; Kubisch *et al.* 2014; Canestrelli *et al.* 2016; Bonte and Doherty 2017).

One way that individuals evaluate the benefits of dispersal relative to the risks is by acquiring information from their surroundings to inform their dispersal decisions (Valone 1989; Danchin *et al.* 2004; Clobert *et al.* 2009; Clutton-Brock and Lukas 2012). For example, the odor of urine can trigger dispersal decisions in mice by communicating information about social environment, such as the relatedness, competitiveness, or mating status of surrounding conspecifics (Isles *et al.* 2002; Latham and Mason 2004). Whether mice ignore this information, or use it to decide to disperse or to stay, depends on their age, sex, and social status (Latham and Mason 2004). Thus, individual dispersal is driven both by an organism's internal physiological and behavioral state (called phenotype dependence) as well as by the information that organism gathers about its external surroundings at the time of dispersal (called condition dependence, Clobert *et al.* 2009, where conditions refers to the individual's external surroundings).

Key to understanding the interplay between phenotype dependence and condition dependence is an individual's juvenile environment. Early development can influence phenotype dependence by changing an individual's dispersal capacity or its dispersal behavior (Clobert 2009). For example, developing at high density increases dispersal capacity of the planthoppers

Prokelisia marginata and *Prokelisia dolus* by triggering production of fully-winged migratory morphs (Denno and Roderick 1992). Early environment also influences dispersal behavior, such as of the western black widow spider, which disperses less via ballooning behavior when reared in isolation in the laboratory than when reared in cohorts under more natural conditions (Johnson *et al.* 2015). Studies such as these have been tremendously informative in revealing factors that alter patterns of dispersal within natural habitats. However, the degree to which individuals disperse as a result of their current environment versus as a result of how their phenotype was shaped by their juvenile environment remains less well understood. Manipulative experiments that separate development from dispersal are therefore necessary to disentangle these two processes moving forward.

In addition, juvenile environment affects not only the phenotype of a specific individual within a population, but also the phenotype of its neighboring conspecifics, whose demography and identities form part of that individual's external environment (Fig. 4.1; Dufty *et al.* 2002; Benard and McCauley 2008; Crean and Marshall 2009; Cote *et al.* 2010). Increasing evidence suggests that nearby conspecifics serve as indicators of habitat quality and competition. For example, Vercken *et al.* (2012) found that the ventral color of neighboring conspecifics motivates dispersal decisions of *Lacerta vivipara* lizards, as the relative abundance of specific colors corresponds with the relative prevalence of different competition strategies. However, in many studies of dispersal, individuals disperse against a backdrop of neighbors who have been raised under the same environmental regimes. For example, Crossman *et al.* (2011) found that the downstream dispersal behaviors of early-stage lake sturgeon larvae were influenced by whether their early development occurred in traditional or stream-side hatcheries. While studies such as these are remarkably useful in management, it is unclear the degree to which individuals

are acting based on their own phenotype, or based on the phenotypes of the individuals around them, as both are likely shaped by their shared environment during development.

Here, we disentangled how juvenile environment influences dispersal using the model system *Tribolium castaneum* (red flour beetles). Specifically, we created two different dispersal phenotypes, which we induced by exposing experimental individuals to either a low or a high density of conspecifics as juveniles. We then allowed experimental individuals to disperse against a background of standardized individuals, which experienced a single intermediate density of conspecifics as juveniles, and thus expressed a standardized phenotype. This novel approach allowed us to disentangle how juvenile environment influences an individual's dispersal via its own phenotype (phenotype dependence) versus via the phenotypes of its conspecific neighbors that form part of its external environment (condition dependence).

We predicted that juvenile environment, due to its effect on phenotype, should determine how strongly an individual is influenced by its external condition (*i.e.* an interaction between phenotype dependence and condition dependence). For example, experimental individuals that experience a high density as juveniles may develop phenotypes that disperse far regardless of external condition; high density could be a reliable indication that future competition will be high (Clobert *et al.* 2009), and thus select for adaptive plasticity that leads juveniles developing at high density to disperse to escape competition as adults. Alternatively, experimental individuals that experience a high density as juveniles may also develop phenotypes that disperse far, but only when conditions are poor, as increased sensitivity to external condition may also be an adaptive response to poor conditions as juveniles.

We also predicted that juvenile environment should influence an individual's dispersal by altering the phenotypes of their surrounding conspecifics, who form part of that individual's

external condition (*i.e.* condition dependence). For example, experimental individuals that experience a high density as juveniles could induce their neighbors to disperse by communicating that patch conditions are poor (Valone 1989; Danchin *et al.* 2004; Clobert *et al.* 2009) or alternatively could reduce dispersal by communicating to their neighbors that they are weak competitors.

MATERIALS AND METHODS

Study system

Tribolium castaneum (red flour beetle) is a well-established system for investigating patterns of dispersal that are likely ubiquitous across many diverse taxa (*e.g.*, Naylor 1961; Campbell and Hagstrum 2002; Melbourne and Hastings 2009; Romero *et al.* 2009; Perez-Mendoza *et al.* 2011; Szűcs *et al.* 2014; Drury *et al.* 2016; Wexler *et al.* 2016). Four aspects of this system make it particularly useful for investigating how phenotype dependence and condition dependence shape patterns of dispersal. First, variation in juvenile environment is known to induce corresponding variation in how beetles disperse as adults (Perez-Mendoza *et al.* 2011; Van Allen and Rudolf 2013; 2016; Van Allen and Bhavsar 2014), which provides a clear mechanism for manipulating phenotype dependence. Second, by manipulating habitat quality and population size, we can control whether *Tribolium* populations are above or below carrying capacity (Stewart *et al.* 2017), providing clear, biologically relevant differences in external conditions. Third, we can standardize the age of individuals, a potentially confounding driver of dispersal (Cote *et al.* 2010). Fourth, we can divide the *Tribolium* life-cycle into two discrete stages: a juvenile stage without dispersal and an adult stage with dispersal. Discrete dispersal stages are a common attribute of many species (Moran 1994; McDougald 2012), and allow

juvenile environment to be independent of the environment experienced during dispersal. Flour beetles therefore represent a promising system for investigating patterns of dispersal that are likely ubiquitous across many diverse taxa.

Rearing environment

Prior to the experiment, beetles were reared in colonies for at least 20 discrete, non-overlapping generations. Colonies were maintained on large, high-quality patches of natal media (95% wheat flour, 5% brewer's yeast) and kept in incubators at 31°C and an average of 54% humidity. We maintained two phenotypically distinct strains of *T. castaneum* (Kramer *et al.* 1984): a wild-type strain with rust-red coloration (hereafter “experimental beetles”), and a mutant strain with distinctive black coloration (hereafter “standardized beetles”).

Maternal effects are strong in *T. castaneum* (Van Allen & Rudolf 2013, Hufbauer *et al.* 2015, Van Allen & Rudolf 2016). Thus, two generations prior to the experiment, we standardized maternal environment by transitioning experimental beetles from their high-quality, natal habitat to a harsher, novel habitat (98.85% corn flour, 1.0925% wheat flour, 0.0575% brewer's yeast; Fig. 4.2A: Generation 0) at a controlled density of 40 adult beetles during oviposition. This harsher habitat was chosen to be intermediate in quality between the low- and high-quality experimental environments described below. One generation prior to the experiment, we created cohorts of individuals reared at low and high juvenile densities, using the same intermediate-quality habitat (98.85% corn flour). We created low juvenile density patches by allowing 18 adult beetles 24 hours to mate and oviposit, and high juvenile density patches by allowing 90 adult beetles 24 hours to mate and oviposit (Fig. 4.2A: Generation 1). We also standardized the maternal environment of standardized beetles one generation prior to the

experiment using the same novel habitat and density as for experimental beetles (Fig. 4.2A: Generation 1).

Experimental design

We allowed populations of *T. castaneum* to disperse across replicated linear arrays, manipulating current density (low = 18 adults, high = 90 adults), current habitat quality (low = 99.5% corn flour, 0.475% wheat flour, 0.025% brewer's yeast; high = 98.2% corn flour, 1.14% wheat flour, 0.06% brewer's yeast), and juvenile density (low and high, as described above) in a fully-factorial design. (Fig. 4.2B). We chose habitat qualities such that for both low-quality and high-quality habitats cohorts established at a low density were likely below carrying capacity (*i.e.* expected population growth rate $\lambda > 1$), while cohorts established at a high density were likely above carrying capacity (*i.e.* $\lambda < 1$) (based on data from Stewart *et al.* 2017). One-third of beetles within each cohort were experimental beetles that experienced either a low or a high juvenile density, while the remaining two-thirds of beetles were standardized beetles that experienced an intermediate juvenile density.

Linear arrays were constructed of at least seven 4 x 4 x 6 cm plastic boxes (hereafter 'patches'), which was enough patches such that dispersal was never limited by the length of the array. Patches were held together by rubber bands and connected by 2mm holes, which were initially blocked by thin plastic sheets. We first allowed beetles to acclimate for 48 hours in the first patch of a linear array, which contained either low- or high-quality habitat. After the acclimation period, we allowed beetles to disperse among patches for 48 hours. At the end of this 48-hour period we halted dispersal and censused all experimental and standardized beetles within each patch of each array (Fig. 4.2C).

Statistical analyses

We fit all statistical models in the package *lme4* (version 1.1.12) in R version 3.2.3 (R core team). We evaluated significance of main effects and interactions for all models using parametric bootstrap methods with 10,000 iterations in the package *pbkrtest* (version 0.4.6). In this method, p-values of interactions or main effects are calculated by comparing deviances of a full model (*i.e.* with interactions of the same order and below) to a model without the interaction or main effect of interest (Halekoh & Højsgaard 2014). We estimated 95% confidence intervals around predicted values from models using the adjusted bootstrap percentile method in the packages *pbkrtest* (version 0.4.6) and *boot* (version 1.3.18).

We assessed the dispersal of the experimental beetles within each dispersal array to assess how phenotype, as induced by juvenile environment, influenced the effect of current density and habitat quality on dispersal. Although dispersal kernels are often used to illustrate how individuals in a population are distributed across a landscape, our smallest population only contained six experimental beetles (18 beetles overall), which is too few to construct a meaningful distribution curve. Within each array we therefore evaluated two metrics of dispersal for experimental beetles: mean dispersal (*i.e.*, how many patches, on average, experimental individuals moved from the initial habitat patch) and maximum dispersal (*i.e.*, the maximum number of patches any experimental individual moved from the initial habitat patch). Similar to dispersal kernels, these two metrics jointly account for both the central tendency and important, but often more variable, longer-distance dispersal events. In addition, how far individuals disperse may be especially sensitive to whether individuals use information about their surroundings to inform their dispersal (Poethke *et al.* 2011).

We evaluated differences in mean dispersal across treatment groups using a standard linear model. Fixed effects were current density (categorical: 18 or 90 adults), current habitat quality (categorical: low or high quality), juvenile density of experimental individuals (categorical: initiated with 18 or 90 adults), and all possible interactions (mean dispersal of experimental individuals ~ current density * current habitat quality * juvenile density of experimental individuals). We evaluated differences in maximum dispersal across treatment groups using a standard linear model that contained the same structure as the mean dispersal model.

We assessed the dispersal of the standardized beetles within each array to address how dispersal is influenced by the phenotype of neighboring conspecifics, as induced by juvenile environment (*i.e.* condition dependence based on social environment). Specifically, we evaluated differences in mean and maximum dispersal of standardized beetles using the same model structure described for experimental beetles above; the fixed effect of juvenile density, in this case, represented the juvenile density treatments of the experimental neighbors, rather than of the standardized beetles used in the model response (mean dispersal of standardized individuals ~ current density * current habitat quality * juvenile density of experimental individuals). An effect of juvenile density (or the interaction between juvenile density and either current density or habitat quality) would indicate that dispersal of standardized beetles is influenced by their neighbors, which differ in their environmentally-induced phenotypes.

RESULTS

Differences between our low-density and high-density juvenile treatments were biologically meaningful. Cohorts from low-density juvenile treatments were, as predicted, below

carrying capacity ($\lambda > 1$), as they were initiated with 18 adults and produced an average of 25 adults in the following generation ($\lambda = 1.39$). Cohorts from high-density juvenile treatments were, also as predicted, above carrying capacity ($\lambda < 1$), as they were initiated with 90 adults and only produced an average of 53 adults ($\lambda = 0.59$) in the following generation.

How far adults dispersed was influenced by their current environment (condition dependence)

Current density strongly influenced mean dispersal (parametric bootstrap, $p < 0.001$): experimental individuals on average dispersed 46.0% further (95% confidence interval: 30.2 – 64.5%) when established at a high density rather than a low density (Fig. 4.3a). This effect was magnified for maximum dispersal (parametric bootstrap, $p < 0.001$). Maximum dispersal was 65.6% further (CI: 47.8 – 86.7%) from high-density patches than from low-density patches (Fig 4.3b).

Habitat quality also effected dispersal. Experimental individuals dispersed, on average, 12.5% further (CI: 0.1 – 25.8%) in low-quality habitats than in high-quality habitats (parametric bootstrap, $p = 0.037$, Fig. 4.3a). This effect was largely driven by individuals established at a low density, which dispersed 35.1% (CI: 11.8 – 64.2%) further in low-quality than in high-quality habitats; in contrast, individuals established at a high density dispersed similar distances across both habitat types (-0.6% change in dispersal, CI: -13.3 – 14.9%; current density by habitat interaction, parametric bootstrap, $p = 0.032$). Maximum dispersal also increased (11.7%; CI: 0.1 – 25.1%) in low-quality habitats as compared to high-quality habitats (parametric bootstrap, $p = 0.061$; Fig. 4.3b). However, unlike mean dispersal, the effect of habitat quality was not altered by differences in current density (current density by habitat quality interaction, parametric bootstrap, $p = 0.641$, Fig. 4.3b).

How far adults dispersed was influenced by their juvenile environment (phenotype dependence)

Juvenile density alone did not influence either mean or maximum dispersal of experimental individuals (parametric bootstrap, $p = 0.793$ or $p = 0.543$, respectively). However, juvenile density strongly mediated the effect of current density on mean dispersal (current density by juvenile density interaction, parametric bootstrap, $p = 0.006$, Fig. 4.4a). Specifically, experimental individuals that experienced a low density as juveniles moved 24.2% (CI: 5.7 – 46.7%) further in response to a high density as compared to a low density during dispersal. This effect was magnified for individuals that experienced a high density as juveniles: these individuals moved 71.6% (CI: 45.5 – 105.4%) in response to a high density rather than a low density during dispersal.

Juvenile density similarly, but not as strongly, mediated the effect of current density on maximum dispersal. As mentioned above, maximum dispersal increased when individuals experienced a high density rather than a low density during dispersal. This difference was again magnified for groups that experienced a high density as juveniles (current density by juvenile density interaction, parametric bootstrap interaction, $p = 0.021$, Fig. 4.4c), in which maximum dispersal from high-density patches was 79.3% further (CI: 48.3 – 120.0%) than from low density patches. In contrast, in groups that experienced a low density as juveniles, maximum dispersal from high-density patches was only 27.4% further (CI: 6.8 – 52.6%) than from low-density patches.

Juvenile density did not mediate the effect of habitat quality on either mean dispersal (habitat quality by juvenile density interaction, parametric bootstrap, $p = 0.154$, Fig. 4.4b) or maximum dispersal of experimental individuals (habitat quality by juvenile density interaction, parametric bootstrap, $p = 0.426$, Fig. 4.4d).

How far adults dispersed was influenced by the phenotype of their neighbors (condition dependence based on social environment)

Dispersal of standardized individuals was mediated by the juvenile environment, and thus the environmentally-induced phenotype, of their conspecific neighbors. Juvenile density of experimental neighbors did not by itself influence dispersal of standardized individuals (parametric bootstrap, $p = 0.840$ and $p = 0.534$, mean and maximum dispersal, respectively). However, juvenile density of experimental individuals did influence the effect of current density on dispersal of their standardized neighbors. When experimental individuals experienced a high density as juveniles, their standardized neighbors on average dispersed 79.3% (CI: 48.3 – 119.9%) further from high-density patches than from low-density patches. However, when experimental individuals experienced a low density as juveniles, their standardized neighbors on average only dispersed 27.4% (CI: 6.8 – 52.6%) further from high-density patches than from low-density patches (juvenile density by current density interaction, parametric bootstrap, $p = 0.011$, Fig. 4.5a).

This pattern was similar for maximum dispersal. If experimental individuals experienced a high density as juveniles, maximum dispersal from high-density patches was 92.1% further (CI: 65.5% – 126.6%) than from low-density patches. However, if experimental individuals experienced a low density as juveniles, maximum dispersal from high-density patches was only 44.4% further (CI: 24.5% – 67.9%) than from low-density patches (juvenile density by current density interaction, parametric bootstrap, $p = 0.021$, Fig. 4.5b).

Juvenile density of experimental neighbors did not mediate the effect of habitat quality on the dispersal of standardized individuals (juvenile density by habitat quality interaction, parametric bootstrap, $p = 0.122$).

DISCUSSION

We found strong evidence that juvenile environment shapes both phenotype and condition dependent dispersal. Individuals typically dispersed away from poor conditions (*i.e.* condition dependence), but moved especially far away from a high density if their phenotype was shaped by a high density as juveniles (*i.e.* an interaction between condition dependence and phenotype dependence). This suggests that external conditions are more likely to influence dispersal if individuals experienced a stressful rather than a benign environment as juveniles. We found that an individual's dispersal also depends on the juvenile environment, and thus phenotype, of their surrounding conspecifics. Specifically, individuals moved especially far when dispersing at a high density if their neighbors' phenotypes were shaped by a high density as juveniles. Importantly, these differences in an individual's dispersal were driven by differences in the environmentally-induced phenotypes of just one-third of that individual's surrounding conspecifics. Therefore, the juvenile environment of even a small portion of a population can have a powerful impact on how the rest of that population decides to disperse.

How far adults dispersed was influenced by their current environment (condition dependence)

Increased dispersal at high densities is common across a variety of taxa, as dispersing individuals often escape costly competition for resources (Bowler and Benton 2005; Matthysen 2005; Kubisch *et al.* 2014). Like many taxa, red flour beetles follow this pattern, as they exhibit negative density-dependent growth (Szűcs *et al.* 2014) and are more likely to disperse (Drury *et al.* 2016) and to disperse further (Szűcs *et al.* 2014) in habitats of low versus high quality, as well as at high versus low population densities (Zeigler 1976).

Here, we confirm that that red flour beetles use both habitat quality and population density to inform their dispersal. For mean dispersal, we found that the effect of population density swamped out effects of habitat quality: individuals exposed to a low density during dispersal did, as expected, disperse further in habitats of low quality than in habitats of high quality, but individuals exposed to a high density during dispersal dispersed far regardless of the quality of their habitat. This could indicate that habitat quality and population density serve as redundant indicators of external condition, and cues about poor habitat quality are ignored if cues about high population density are already present. Alternatively, our specific finding may simply be an artifact of our experimental design, as the difference between our density treatments may have been more challenging (*e.g.* in terms of their absolute effect on λ) than the difference between our habitat quality treatments.

Here, we found that the effects of current density and habitat quality were additive for maximum dispersal: dispersal distance kept increasing when environments were doubly challenging (high density and poor resources), rather than reaching a plateau (Fig. 4.4c). Thus, cues from conspecifics and from resources do appear to serve as independent sources of information about the environment, which makes sense as these two factors often interact synergistically to determine per capita resource availability (French and Travis 2001; Bowler and Benton 2005). Thus, although population density appeared to play a stronger role in driving mean dispersal, both population density and habitat quality played an important role in driving the long-distance dispersal events that often have the strongest consequences for population spread and population-level dynamics.

How far adults dispersed was influenced by their juvenile environment (phenotype dependence)

The effects of environment on dispersal phenotypes can carry-over across discrete life history stages (Arambourou *et al.* 2017), and even across generations (Krug 2009; Crean and Marshall 2009; Meylan *et al.* 2012; Bitume *et al.* 2014; Van Allen and Rudolph 2014; 2016). Yet how juvenile environment influences how an individual disperses later in life remains less well understood, especially for the transient stage of dispersal (Clobert *et al.* 2009; Wey *et al.* 2015).

Here, as predicted, we find that juvenile density has non-additive consequences for dispersal: juvenile density influences dispersal from high-density patches, but not from low-density patches. Juvenile environment therefore has the power to alter how strongly individuals are influenced by their external condition. In addition, this non-additive effect of juvenile environment held true for both mean and maximum dispersal, which suggests that the effect of juvenile environment on individual dispersal likely translates into a strong effect on population spread. Studying juvenile environment is therefore imperative to refining our understanding of dispersal ecology, as it plays a key role in dispersal plasticity. Studying the effects of juvenile density is particularly important since density is already known to have lasting consequences for organismal phenotypes (Sinervo *et al.* 2000; Allen *et al.* 2008; Bitume *et al.* 2014; Betini *et al.* 2015).

Juvenile environment did not, however, mediate how habitat quality influenced dispersal, which may suggest that juvenile environment only induces individuals to better detect the specific conditions that were challenging during their development as juveniles. Alternatively, our density treatments may simply have been more stressful than our treatments for habitat quality. Future studies should manipulate multiple variables during early development across a gradient of treatment levels to gain a better mechanistic understanding of these processes.

How far adults dispersed was influenced by the phenotype of their neighbors (condition dependence based on social environment)

Neighboring conspecifics are increasingly recognized as important sources of information, as their phenotypes may indirectly advertise habitat quality or competitive environment (Valone 1989; Danchin *et al.* 2004; Clobert *et al.* 2009; Vercken *et al.* 2012). For example, Boudjemadi *et al.* (1999) found that for experimental groups of the common lizard *Lacerta vivipara*, ‘frustrated’ dispersers (those that were prevented from dispersing earlier in the season) influenced their conspecific neighbors to disperse later in the season. Thus, individuals might recognize that specific phenotypes are indicative of a poor environment, and disperse further when their neighbors’ phenotypes communicate that they have been induced by a stressful environment. In contrast, individuals may also influence their neighbors if their past environment predisposes them to disperse further than their neighbors, and their neighbors simply follow the furthest disperser.

Here, as predicted, standardized individuals dispersed further when their experimental neighbors’ phenotypes were shaped by a high density rather than a low density during development. The phenotypes of experimental individuals exposed to a high density as juveniles are predisposed to disperse further in response to a high rather than a low density during dispersal, and standardized individuals mimicked their neighbors’ dispersal (Fig. 4.4). Individuals that experienced a high density as juveniles may have directly influenced their neighbors to disperse further, such as through aggressive behavior, or may have indirectly influenced their neighbors to disperse by modifying their shared environment; flour beetles in particular are known to secrete defensive chemicals that may modify their environment (Markarian *et al.* 1978), and beetles may increase secretion in response to developing in a

stressful environment. Regardless of the mechanism, our findings have strong implications for dispersal, as they suggest that the dispersal of an entire population can be driven by the environmental history experienced by a minority of the group; here, for example, we find that two-thirds of the group behave the same as the one-third of the group whose phenotypes are shaped by a stressful environment (*i.e.*, high density) as juveniles.

Studies that manipulate the composition of dispersing populations, such that individuals vary in their dispersal phenotypes, therefore present a promising avenue of future research. Ideally, these studies would manipulate the juvenile environment of a single experimental individual, while the rest of their neighboring conspecifics experience a standardized juvenile environment irrespective of treatment. However, such an approach is logistically challenging. A more realistic approach may be to investigate what proportion of the population needs to be comprised of standardized individuals to effectively swamp out the contribution of experimental individuals. This refined understanding of what drives individual dispersal can be used to inform predictive models, allowing them to better capture the individual variation which, in part, makes predicting dispersal so difficult.

Ecological significance of our findings

These findings have broad implications. Individual dispersal is strongly predictive of population spread (Fisher 1937; Skellam 1951; Levin *et al.* 2003; Kokko and López-Sepulcre 2006; Jongejans *et al.* 2008; Kubisch *et al.* 2014; Canestrelli *et al.* 2016), and is therefore key to better managing both for and against rapid population expansion. Here, for example, density and habitat quality have a similar effect on maximum dispersal, a measurement of population spread, as they do on mean dispersal of the group (Fig. 4.3). Patterns of individual dispersal therefore

likely translate into lasting consequences for population-level processes. In addition, the three factors we manipulate here (current environment, habitat quality, and juvenile environment) are all important drivers of rapid population spread (Grevstad 1999; Fagan *et al.* 2002; Theoharides and Dukes 2007; Liebhold and Tobin 2008; Catford *et al.* 2009; Wilson *et al.* 2009; Estrada *et al.* 2016). Promoting population spread is critical to managing or restoring threatened populations (Robinson and Handel 2000; Donald and Evans 2006). In addition, invasive species management relies on both hindering the spread of invaders as well as promoting the spread of agents released to control invaders (Fagan *et al.* 2002; With 2002; Theoharides and Dukes 2007; Liebhold and Tobin 2008). Predicting individual dispersal is therefore critical to better managing population spread, which will only become more pressing in today's world of rapid environmental change.

Conclusions

Social environment is increasingly recognized as an important driver of dispersal. We find that an individual's response to its current surroundings depends on its phenotype. Specifically, individuals disperse away from poor conditions, and how far they disperse depends on both their own juvenile environment, and the juvenile environment experienced by neighboring conspecifics. This finding has strong implications for dispersal ecology, as it suggests that individual's phenotype may influence the dispersal of an entire group, even if that individual's phenotype is in the minority. Incorporating the effect of past social environment into studies of dispersal may therefore greatly strengthen basic understanding of dispersal ecology.

FIGURES

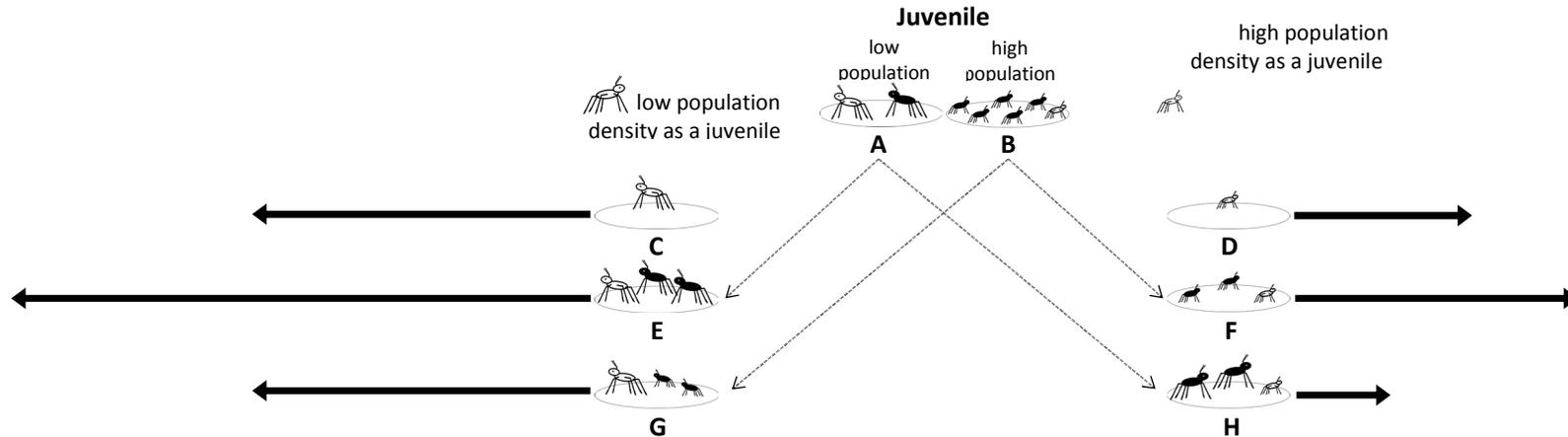


Figure 4.1 The above figure depicts a scenario in which both external condition and phenotype influence dispersal. Individuals of interest (in white) experience either a low (A) or a high (B) population density as juveniles. Juvenile environment, in turn, drives phenotypic differences. In the example above, individuals that experience low population density (a less competitive environment) as juveniles develop larger bodies and comparably longer legs than individuals that experience high population density (a more competitive environment) as juveniles. As adults, individuals are then introduced by themselves or with other individuals (in black) into a habitat of standardized quality. Thin dashed arrows represent the juvenile environment of neighbors, while the juvenile environment of individuals of interest is depicted in the column headings above the dispersal scenarios. Thick black arrows indicate the distance dispersed by the individual of interest.

Here, phenotype influences how far individuals decide to disperse ($C \neq D$), as individuals move further when they are large than small ($C > D$). Dispersal is also influenced by external condition ($C \neq E$, $D \neq F$), as individuals disperse further when they detect neighbors than when dispersing alone ($E > C$, $F > D$). The effects of phenotype and external condition are non-additive ($E \neq F$ indicates an interaction between phenotype and external condition), with individuals dispersing especially far when they detect neighbors if they also experienced a high population density as a juvenile ($[E - C] > [F - D]$). Finally, neighbors influence dispersal not just by their presence, but also by their specific phenotypes, as individuals disperse differently when the same number of neighbors are present, but those neighbors are large, not small ($E \neq G$, $G \neq H$).

Studies of dispersal that investigate juvenile environment typically expose entire populations to the same experimental treatments, which means that individuals and their neighbors have a shared environmental history (such as E or F). Differences

between treatments (E vs. F) may therefore be the result of juvenile environment either altering an individual's dispersal phenotype or altering their neighbors' phenotypes, thus changing an individual's external condition by altering their social environment. Standardizing the juvenile environment of neighbors isolates how juvenile environment influences dispersal phenotypes (E vs. H, G vs. F), by disentangling this from the effect of how juvenile environment alters external condition via neighboring phenotypes. This experimental design thus provides novel insight into how juvenile environment influences dispersal through both external condition and phenotype.

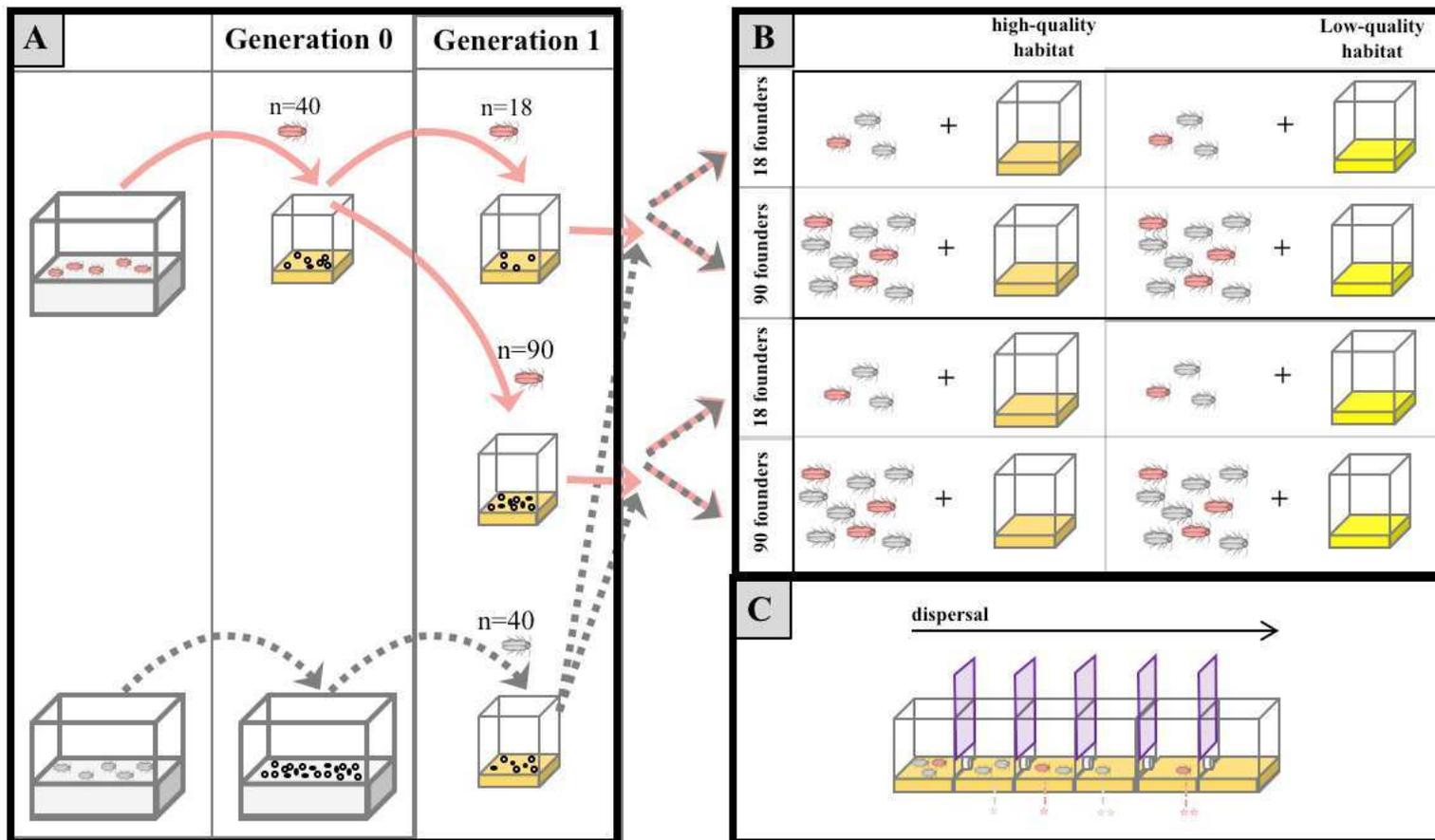


Figure 4.2 Time course and experimental design. We began standardizing maternal effects two generations prior to the experiment; experimental beetles are shown in pink and standardized beetles are shown in grey (A). Pink and dotted grey arrows illustrate how many beetles were allowed to oviposit for 24 hours to establish habitat patches for the following generation. The resulting adults at the end of Generation 1 were used to establish populations at either a low (18 adults) or a high (90 adults) current density on habitat patches of either low or high quality (B). Beetles were given 48 hours to acclimate to the first patch of a dispersal array before we lifted the gates and allowed them to disperse for 48 hours. To investigate how juvenile density influences how current density and habitat quality drive dispersal we measured the mean and maximum distance dispersed (represented in C by pink * and **,

respectively) by experimental beetles within each array. To address how individuals are influenced by the environmental histories of their neighbors, we measured mean and maximum distance dispersed (represented in C by grey * and **, respectively) by standardized beetles within each array.

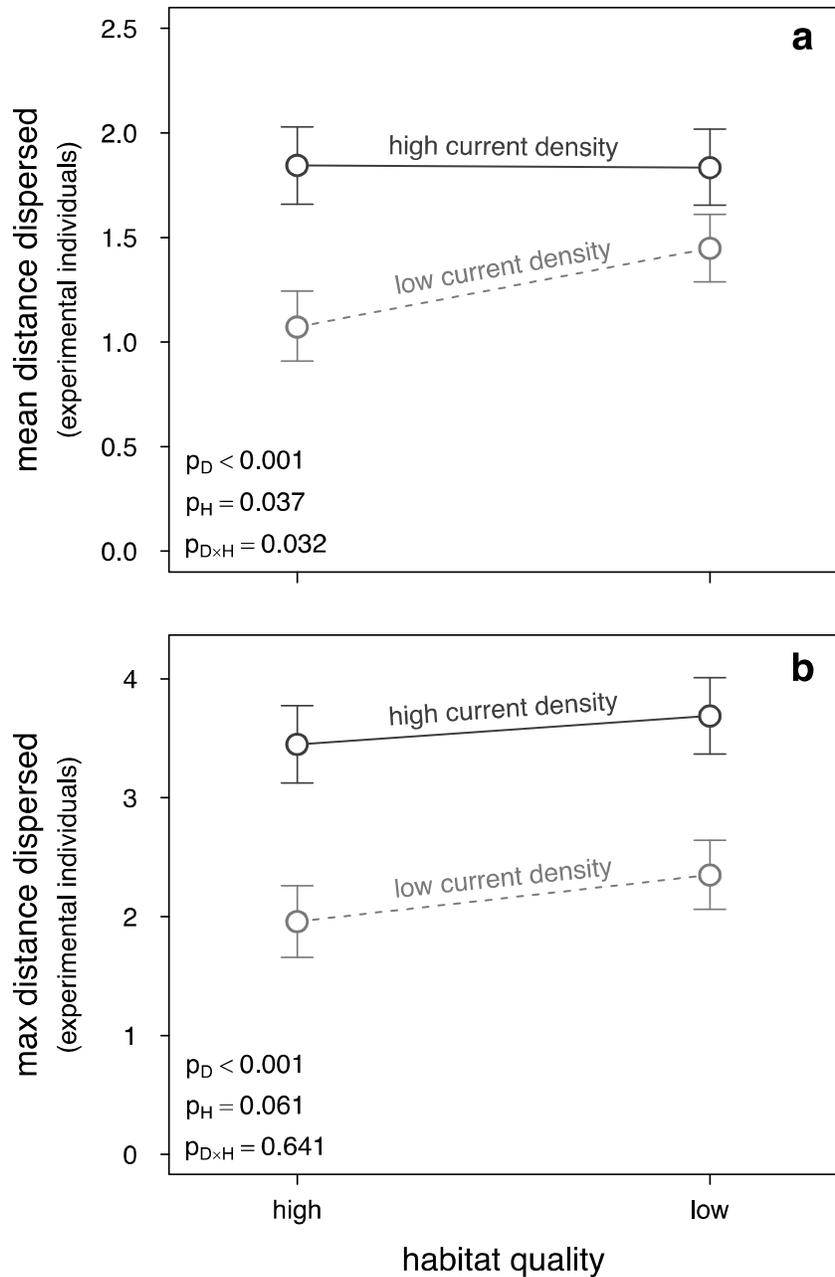


Figure 4.3 Mean (a) and maximum (b) dispersal of experimental beetles for current density and current habitat quality group combinations averaged across juvenile density treatments. Solid, dark-grey lines represent high current density treatments and dotted, light-grey lines represent low current density treatments. Y-axis values represent how many patches individuals moved away from their initial habitat patch (*i.e.* individuals that remained in their initial patch dispersed a distance of 0). P-values indicate significance of the main effect of current density (D), current habitat quality (H), as well as of the two-way interaction term for current density and habitat quality (D×H). Bars are 95% bootstrapped confidence intervals around model means.

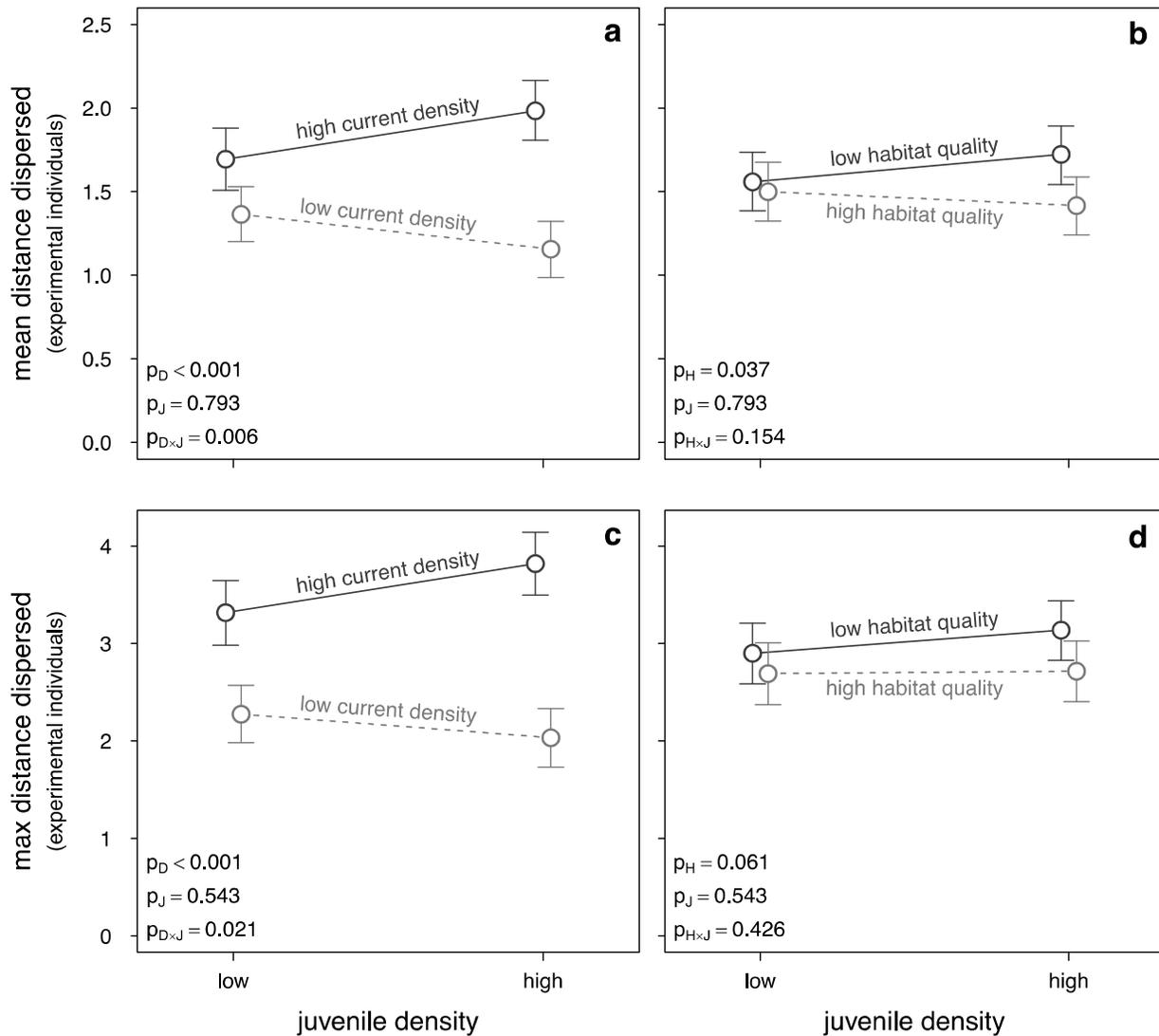


Figure 4.4 Mean (a) and maximum (c) dispersal of experimental beetles for current and juvenile density group combinations averaged across habitat quality treatments, as well as for current habitat quality and juvenile density group combinations averaged across current density treatments. Solid, dark-grey lines represent either high current density or low habitat quality treatments, while dotted, light-grey lines represent either low current density or high habitat quality treatments. Y-axis values represent how many patches individuals dispersed from their initial habitat patch (*i.e.* individuals that remain in their initial patch dispersed a distance of 0). Depending on the panel, p-values indicate significance of the main effects of current density (D), current habitat quality (H), juvenile density (J), as well as the two-way interaction terms for the effect of current density or habitat quality is influenced by juvenile density ($D \times J$ and $H \times J$, respectively). Bars are 95% bootstrapped confidence intervals around model means.

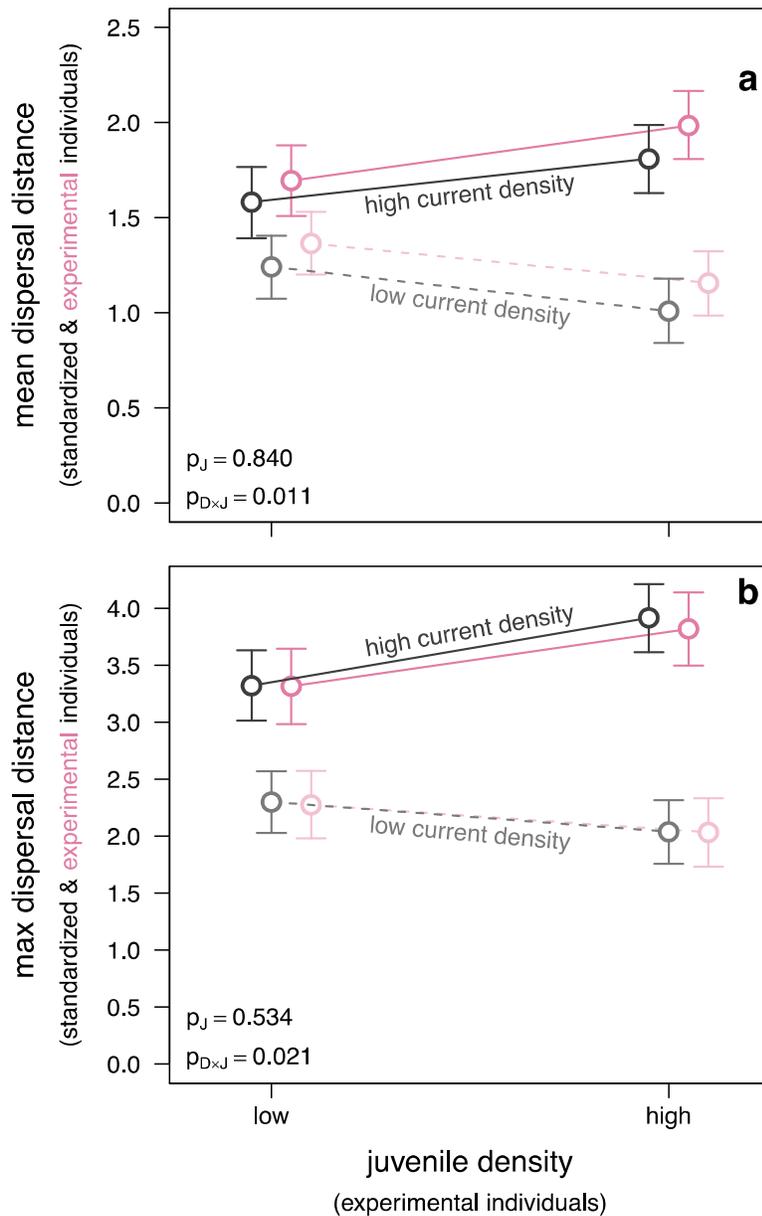


Figure 4.5 Mean (a) and maximum (b) dispersal distances for standardized beetles (light and dark grey) and experimental beetles (light and dark pink) exposed to either a low or a high density as juveniles and as adults (dotted and solid lines, respectively). Bars represent bootstrapped 95% confidence intervals around model means. Juvenile density refers to treatments applied to experimental individuals, as standardized individuals all experienced an intermediate population density as juveniles. P-values refer to standardized beetles, and indicate significance of the effect of the juvenile density of experimental neighbors (J) on dispersal, as well as of the effect of the two-way interaction term for current density and juvenile density (C×J).

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