CONSEQUENCES OF GLOBAL REDISTRIBUTION ON THE ECOLOGY AND EVOLUTION OF THE INVASIVE WEED *PASTINACA SATIVA* AND ITS ASSOCIATED INSECT FAUNA

BY

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DISSERTATION

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Abstract

Biological invasions can provide useful insights on how communities persist and change over space and time. The wild parsnip (*Pastinaca sativa* L., Apiaceae) and its coevolved specialist florivore, the parsnip webworm (*Depressaria pastinacella* Duponchel, Lepidoptera: Oecophoridae), are native to Europe but they occur as an invasive association throughout the temperate world. New Zealand (NZ) populations of wild parsnips were free from webworm herbivory for 140 years until 2004, when webworms were discovered, presumably having been accidentally introduced. This escape and subsequent reassociation of wild parsnips with parsnip webworms in NZ presented a unique opportunity to study herbivore-mediated evolution as it occurs. In Chapter 1, I examined the chemical mediation of wild parsnip pollination to understand selection pressures imposed by both florivores and pollinators. In Chapter 2, I evaluated phenotypic evolution in wild parsnips after six years of webworm infestation (2004 – 2009) in a large-scale common garden experiment. In Chapter 3, I identified leaf volatiles that mediate oviposition by webworms to determine whether plants can experience selection to deter oviposition. In Chapter 4, I used ribosomal internally transcribed spacers (ITS) and chloroplast markers to determine the colonization history of wild and cultivated parsnips in three geographic regions: Eastern North America, Western North America and New Zealand. Results from these studies showed that volatile chemical signals mediate pollination by flies and webworm oviposition in wild parsnips. Floral volatile compounds positively associated with pollinator visitation were produced in higher proportions in NZ flowers, suggesting that in the absence of specialized florivores, NZ flowers may be chemically better constituted to attract pollinators. In the common garden, defensive chemistry remained unchanged in both infested
and uninfested New Zealand populations; however, plants in infested populations were larger after three to six years of webworm florivory. Evolution of large size as a component of florivore tolerance may occur more rapidly than evolution of enhanced chemical defense. While adult moths were deterred by leaf volatiles, oviposition counts did not correspond with lower fitness and, consistent with this finding, infested NZ parsnips showed no phenotypic change in leaf volatiles after reassociation with parsnip webworms. However, leaf volatiles implicated as oviposition deterrents were positively correlated with floral defenses, suggesting that webworm adults choose hosts based on larval suitability. Finally, the analysis of molecular markers in wild parsnips showed high genetic diversity in all three geographic regions of invasion. The haplotype distribution from chloroplast markers suggests that parsnips in North America originated from wild and cultivated haplotypes, whereas NZ parsnips are primarily escapees from cultivation. These findings illustrate the complexity of chemically mediated biotic interactions that are lost and regained by multiple invasions, the potential for rapid adaptive evolution in response to shifting biotic selection pressures, and the availability of genetic variation from agricultural or accidental introductions that can provide the raw material for evolution.
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Dedication

For Art Zangerl

Who was an incredible mentor and to whom I owe so much of this dissertation.

His warmth and laughter are dearly missed
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Introduction

Globalization in trade has resulted in a dramatic increase in the number of exotic species worldwide (Mack et al. 2000; Palumbi 2001). Exotic invasions can be extremely detrimental to native ecosystems (e.g. Mooney and Cleland 2001; Pimentel et al. 2005), but they also provide a large-scale experiment in evolutionary biology (Sax et al. 2007). The altered genetic structure of founding populations along with novel selection pressures pave the way for rapid evolution in invasive populations. Recently documented invasions offer a unique opportunity to study the fundamental ecological and evolutionary processes of colonization and adaptation.

A remarkable diversity of plant traits have been attributed to insect-mediated evolution. Many plants require insects for pollination and also contend with detrimental insect herbivory. Both mutualistic pollinators and antagonistic herbivores can have a strong impact on plant fitness and can thus impose selection on defense, morphological, and life-history traits. The role of herbivores and pollinators in the evolution of plants has been inferred via correlations between phenotypes and fitness and recently through long-term herbivore exclusion experiments (Agrawal et al. 2012; Turley et al 2013). Exotic invasions provide an opportunity to observe herbivore- and pollinator-mediated evolution as it occurs. When a plant is introduced to a new range, it generally leaves behind its coevolved specialist herbivores (Keane and Crawley 2002). Escape from natural enemies might result in relaxed defenses and increased pollinator attraction, especially if herbivores and pollinators impose conflicting selection pressures. On rare occasions, coevolved specialist herbivores are co-introduced, either
deliberately as biological control agents or accidentally. These plant-herbivore reassociations provide an opportunity to observe how plants respond to selection by herbivores and how herbivore-mediated selection alters interactions with pollinators.

For evolution to occur in an invasive species, adequate genetic variation must exist for selection to act upon. It has been widely assumed that invasive populations suffer genetic bottlenecks upon introduction because of small founding populations (Bossdorf et al. 2005). However, recent evidence suggests that multiple introductions, which can result in high genetic diversity, are frequently the norm in introduced populations (Bossdorf et al. 2005). Thus, examining the population genetic structure and the colonization history of invasive populations is germane to predicting the potential for evolutionary change.

In my dissertation, I examined evolutionary changes in the invasive wild parsnip Pastinaca sativa L. (Apiaceae) after escaping and subsequently reassociating with its coevolved specialist florivore, the parsnip webworm, Depressaria pastinacella Duponchel (Lepidoptera: Oecophoridae), in New Zealand. Wild parsnips and parsnip webworms are both native to Europe. Wild parsnips were introduced to North America in the 1600s and New Zealand in the 1800s; however, parsnip webworms were only discovered in New Zealand in 2004, thus providing a unique opportunity to examine trait evolution in plants in response to herbivory (Zangerl et al. 2008).

**Literature review of Pastinaca sativa - Depressaria pastinacella interactions**

The interaction between wild parsnips and parsnip webworms is an exemplary model for the coevolutionary arms race between a plant and its specialized herbivore. Potent chemical
defenses render wild parsnips relatively free from herbivory with the exception of the parsnip webworm, an oligophagous florivore that specializes on wild parsnips, *Pastinaca sativa*, and a few species in the genus *Heracleum* (Berenbaum 1981; Berenbaum and Zangerl 2006). Physiological and behavioral adaptations allow parsnip webworms to overcome wild parsnip defenses but webworms are still not entirely immune to the repertoire of secondary compounds present in their host (Berenbaum et al. 1986; Berenbaum et al. 1989). Substantial variation exists in both the defensive chemistry of individual plants and the detoxification capacity of individual insects. Given that both sets of traits are heritable, reciprocal selection frequently results in phenotype matching (Berenbaum et al. 1986; Berenbaum and Zangerl 1992; Berenbaum and Zangerl 1998; Zangerl et al. 1997; Zangerl and Berenbaum 2003). The simplicity of this reciprocal interaction as well as a strong foundation of prior research makes this system amenable to further investigation.

**Wild parsnip defensive chemistry**

Plants in the Apiaceae have a diverse and unique array of secondary compounds and *P. sativa*, a member of this large family, is no exception (Berenbaum 1990). Wild parsnips produce a plethora of terpenoids, aliphatic esters, and phenylpropanoid compounds as well as linear and angular furanocoumarins. While many of these secondary compounds mediate interactions with insects, furanocoumarins are by far the best characterized in this system. Furanocoumarins are notorious for their ability to cause dermatitis in humans by intercalating DNA in the presence of UV light, and this phototoxicity is also responsible for deterring many herbivores (Berenbaum and Zangerl 1996). Furanocoumarins are benz-2-pyrone derivatives
with either a furan ring attached at the 6, 7 position, in linear furanocoumarins, or at the 7, 8 position in angular furanocoumarins (Berenbaum and Zangerl 1996). Linear and angular forms are derived from the phenylpropanoid and the mevalonic acid pathways and the split between the two forms occurs early in the biosynthetic pathway, after the synthesis of umbelliferone from phenylalanine. Furanocoumarins are abundant in all above-ground tissues of wild parsnips, with one angular (sphondin) and five linear furanocoumarins (psoralen, xanthotoxin, bergapten, isopimpinellin, imperatorin) commonly detected in floral tissues (Berenbaum and Zangerl 1996). Furanocoumarins differ structurally based on the presence and the placement of methoxy groups and other moieties. Structurally different furanocoumarins also differ in their toxicity to insects. The production of many structural forms is thought to be adaptive because mixtures have higher toxicity compared to individual compounds (Berenbaum et al. 1991, Berenbaum and Zangerl 1996). All furanocoumarins are stored in oil ducts called vittae and are released upon damage (Berenbaum et al. 1990). The number and surface area of oil tubes are strongly correlated with the furanocoumarin content and all of these traits are heritable in wild parsnips (Zangerl et al. 1989).

Furanocoumarin production varies with developmental stage, tissue type, and among individuals in a population (Berenbaum 1981; Zangerl and Berenbaum 1993). Reproductive parts (buds, flowers, fruits and seeds), which are associated with the highest fitness cost if lost or damaged, also produce highest quantities of furanocoumarins compared to all other tissues (Berenbaum 1981). Leaves contain lower constitutive levels of furanocoumarins but, in contrast with floral tissues in which defenses are not inducible, herbivore damage can increase furanocoumarin production (Zangerl 1990; Zangerl and Rutledge 1996). Individuals can vary
dramatically in the quantity of furanocoumarins produced and some furanocoumarins, such as sphondin, are polymorphic within populations (Zangerl and Berenbaum 1993). The underlying physiological and genetic basis for tissue-specific and individual level variation has not been fully examined. However, many of the biosynthetic steps of furanocoumarin production are known to be catalyzed by cytochrome P450 enzymes and a small subset of these P450 genes, psoralen synthases (CYP71AJ1, CYP71AJ2, CYP71AJ3 and - CYP71AJ4), have been identified from three plants in the Apiaceae: Ammi majus, Apium graveolens and P. sativa. At least one these genes, CYP71AJ4, is implicated in the production of angular furanocoumarins from umbelliferone (Larbat et al. 2009).

Aliphatic esters, which occur abundantly in floral tissues of P. sativa, also mediate interactions with webworms. Aliphatic esters as floral volatiles are limited in distribution, only occurring in a few other closely related Heracleum species (Katz-Downie et al. 1999). These compounds have been associated with larval attraction (octyl acetate) and deterrence (octyl butyrate) in laboratory Y-tube choice assays (Carroll and Berenbaum 2002) and may increase the toxicity of furanocoumarins to webworms by enhancing the absorption of furanocoumarins into the insect’s gut (Carroll et al. 2000). While the physiological effect of these compounds on webworm performance has not been examined, esterase enzymes from parsnip webworm midguts can effectively break down these compounds to alcohols (Zangerl et al. 2012). Both octyl acetate and octyl butyrate are phenotypically correlated with furanocoumarins, possibly as a consequence of co-occurring in the vittae; however, they are not genetically correlated, suggesting that ester production may also be under webworm selection (Carroll et al. 2000).
Parsnip webworm adaptations to parsnip defenses

Furanocoumarin detoxification occurs via cytochrome P450-mediated metabolism (Berenbaum and Zangerl 1992; Mao et al. 2006; Nitao 1989). One furanocoumarin, xanthotoxin, is metabolized 10 to 300 times faster in this insect compared to more polyphagous species, reflecting a physiological adaptation for host utilization (Zangerl and Berenbaum 1993). Bergapten and sphondin are metabolized more slowly than xanthotoxin and are key resistance factors to webworm herbivory (Berenbaum et al. 1986; Berenbaum and Zangerl 1992). The efficacy of furanocoumarin detoxification is heritable and metabolism rates can vary up to threefold between individuals (Berenbaum and Zangerl 1992), indicating that plant chemistry can act as a selective force on insect physiology. Approximately 70 P450 enzymes have been identified from parsnip webworms (Johnson, R. et al. unpublished data) of which four P450 enzymes have been characterized: CYP6AB3, CYP6AE1, CYP9A6 and CYP9A7 (Li et al. 2004). Both CYP6AB3 and CYP6AE1 are related to the xanthotoxin-metabolizing CYP6B from the butterfly Papilio polyxenes and CYP6AB3 is highly specialized for the metabolism of imperatorin, the furanocoumarin produced in greatest abundance in host plants of webworms (Mao et al. 2006). Two allelic variants of CYP6AB3, differing in five amino-acids, have been recovered from parsnip webworms; CYP6AB3v2 metabolizes imperatorin five times faster than CYP6AB3v1, allowing parsnip webworms to adapt to variation in host furanocoumarin concentrations.

Even though parsnip webworms are efficient at metabolizing furanocoumarins, unmetabolized furanocoumarins create oxygen free radicals and can be toxic in the presence of ultraviolet light. Parsnip webworms actively avoid exposure to UV light by constructing and feeding exclusively inside silken webs. Parsnip webworms also have two- to four-fold more
antioxidant enzyme activity compared to the polyphagous moth *Trichoplusia ni*, which may serve to reduce phototoxicity from unmetabolized furanocoumarins (Lee and Berenbaum 1990). Caterpillars also sequester an antioxidant, lutein, from flowers (Carroll et al. 1997). In a laboratory assay, caterpillars that were not fed lutein actively avoided UV light (Carroll et al. 1997), suggesting that physiological adaptations are coupled with behavioral responses to overcome toxicity.

Evidence for reciprocal selection

Because the webworm feeds on parsnip reproductive tissue, herbivory can substantially reduce fitness, thereby exerting selection for chemically based resistance in plant populations (Berenbaum et al. 1986). Damage by webworms selects for increased concentrations of xanthotoxin, bergapten, and sphondin (Berenbaum et al. 1986; Zangerl and Berenbaum 1993). Within a population, plants with the highest concentrations of these furanocoumarins are most frequently free from herbivory but also experience lower fitness in the absence of herbivores (Zangerl and Berenbaum 1997) and populations of wild parsnip experiencing lower risk of herbivory produce lower levels of constitutive furanocoumarins (Berenbaum and Zangerl 2006; Zangerl and Berenbaum 1990), indicative of a cost of producing these compounds.

That reciprocal selection acts on plant defenses and webworm detoxification capacity has been demonstrated by phenotype matching across landscapes where the frequency of plant defense phenotypes matches the frequency of insect detoxification phenotypes in a population (Berenbaum and Zangerl 1998). Twelve out of twenty wild parsnip populations in the midwestern US had chemical profiles that corresponded with webworm detoxification capacities (Zangerl and Berenbaum 2003). Differential larval mortality and increased fitness of
well-defended plant genotypes can cause phenotype matching to occur rapidly. However, reciprocal selection for increased plant defenses and webworm metabolism does not result in fixation of alleles because gene flow and trait mixing can occur frequently between populations (Zangerl and Berenbaum 2003). Moreover, the cost of maintaining defensive compounds in the absence of herbivory is high and many defensive traits (and possibly life-history traits) are genetically correlated, impeding the fixation of alleles (Berenbaum and Zangerl 1998; Zangerl and Berenbaum 2003). Phenotype matching does not consistently occur in all populations; populations where reciprocal selection is strong are coevolutionary “hotspots” while other populations where plant defense and herbivore metabolism are mismatched are coevolutionary “coldspots” (Zangerl and Berenbaum 2003). In the parsnip-webworm system, coevolutionary cold-spots are often associated with the presence of alternate webworm hosts - *Hercleum sphondylium* in Europe or *H. maximum* in the midwestern and western US (Berenbaum and Zangerl 2006; Zangerl and Berenbaum 2003), suggesting that other ecological factors can alter the strength of selection.

Reciprocal selection is also evident from chronological studies of invasive parsnip populations, before and after the introduction of webworms. Furanocoumarin allocation in invasive wild parsnip populations in the US has been altered by re-association with webworms (Zangerl and Berenbaum 2005; Zangerl et al. 2008). Parsnip seeds from herbarium specimens collected in North America before the documented arrival of parsnip webworm in 1869 contained significantly lower levels of furanocoumarins than seeds of European specimens collected during the same period (Zangerl and Berenbaum 2005). Seeds collected after the arrival of webworms had significantly higher furanocoumarin content. Moreover, New Zealand
populations of *P. sativa*, historically free from webworm herbivory for 140 years until its introduction in 2004, experienced altered selection for remixing of defensive chemical traits in populations re-associated with webworms (Zangerl et al. 2008).

While the interaction between parsnips and webworms has been extensively studied, our understanding of herbivore-mediated evolution in this system has been either retrospective (Zangerl and Berenbaum 2005) or prospective (Zangerl et al. 2008) and no studies have investigated the combined effect of webworms and pollinators on the real-time evolution of wild parsnips. The recent well-documented invasion of webworms in New Zealand provides an extraordinary opportunity to examine herbivore- and pollinator-mediated evolution over the course of six years (2004 – 2009). In my first three chapters I focus on the interaction between wild parsnips, parsnip webworms and pollinators in North America and New Zealand. In my final chapter, I examine source and genetic diversity of wild parsnips in North America and New Zealand, an important component of this system that has not previously been investigated.

**Chapter 1 - Implications of enemy escape on chemically mediated interactions with mutualists: wild parsnip pollination in two hemispheres**

Chapter 1 examines the pollination biology of wild parsnips with the goal of identifying floral volatiles that are involved in wild parsnip pollination in North America and in New Zealand. Observational studies have long suggested that parsnips are promiscuously pollinated by many insects, especially dipterans (Bell 1971; Lohman et al. 1996, Tooker et al. 2006), and floral volatiles have also been previously identified from *P. sativa* (Borg-Karlson 1998); however, the link between floral emissions and pollination has not been examined. I monitored
insect visitation, quantified floral volatiles and measured reproductive success for parsnip flowers in both countries to determine which insects effectively pollinate wild parsnips, which chemical signals are attractive to pollinators, and if changes in floral volatile chemistry in New Zealand parsnips reflect increased pollinator attraction in the absence of herbivory. Evolutionary changes in New Zealand wild parsnips after six years of webworm reassociation might be constrained by the interactions between wild parsnips and their pollinators. Thus, understanding the chemical signals that mediate pollination and the impact of pollinator-mediated selection is important for interpreting the evolutionary response of wild parsnips to webworm herbivory.

Chapter 2 - Real-time evolution of tolerance in an invasive weed after reassociation with its specialist herbivore

Chapter 2 is a comprehensive examination of herbivore-mediated trait evolution in wild parsnips following six years of reassociation with parsnip webworms in New Zealand. In a reciprocal common garden experiment planted in the US and in New Zealand, I compared the overall susceptibility of US and New Zealand (NZ) parsnip genotypes to webworm florivory and documented phenotypic changes in infested NZ populations relative to uninfested NZ populations to determine if rapid adaptive evolution in response to webworms has occurred, and if rapid evolution has altered parsnip-pollinator interactions.

Chapter 3 - Impact of reassociation with a coevolved herbivore on oviposition deterrence in a hostplant: *Depressaria pastinacella* and *Pastinaca sativa*

Chapter 3 examines whether parsnips are under selection to decrease oviposition by parsnip webworm adults. While parsnip webworm larvae feed exclusively on floral tissues,
parsnip webworm adults oviposit only on rosette leaves of pre-bolting plants. The use of
different plant parts for oviposition and larval feeding suggests that wild parsnips should be
under dual selection for increased production of feeding deterrents as well as increased
production of oviposition deterrents. The chemical mediation of oviposition has not been well
characterized in this system. I examined volatile compounds volatiles that can be detected by
female moths via gas chromatography coupled with electroantennographic detection (GC-EAD).
In a common garden experiment, I collected leaves for chemical analysis, counted eggs laid on
all plants, and correlated leaf chemistry with oviposition to determine whether detectable
compounds are attractive or deterrent in the field. I compared populations of NZ parsnips with
and without a history of infestation to determine if parsnip populations have experienced
predictable changes in their foliar chemistry in response webworm infestation. The impact of
oviposition on plant fitness and the potential for ovipositing insects to impose selection on
plant traits have not been previously examined, even though it is a significant aspect of many
plant-insect interactions.

Chapter 4 - Population genetic structure and colonization history of the globally-invasive weed
wild parsnip, *Pastinaca sativa*

Chapter 4 examines the genetic variability, the origin, and the colonization history of
wild parsnips in North America and New Zealand. I used ribosomal nuclear and chloroplast DNA
markers to investigate haplotypic variation in parsnip populations from North America, Europe
and New Zealand. It is unknown whether the invasive wild parsnip escaped from cultivated
parsnips, *P. sativa* subsp. *sativa*, or if the wild parsnip is a separate subspecies, *P. sativa* subsp.
*sylvestris*, that was accidentally introduced throughout the world. It is also not known whether
one introduction or multiple introductions of parsnips were made in each of these areas. The mode of introduction can substantially influence the success of an invasive weed. Single or multiple introductions can determine the amount of genetic variability in introduced populations and the potential for adaptive evolution. The source of invasive populations (wild versus cultivars) can also have an impact on the ecology of the weed. While many studies have documented the migration patterns of invasive species, few have examined weeds that have been associated with cultivation.
REFERENCES


Chapter 1 - Implications of enemy escape on chemically mediated interactions with mutualists: wild parsnip pollination in two hemispheres

ABSTRACT

When plant species invade new areas, they can escape from specialist enemies and thereby reduce investment in chemical defense. Enemy release may have other impacts on plant chemistry; in the absence of specialists, plants may be able to increase production of volatiles that enhance attractiveness to pollinators. In the United States (US), the introduced European wild parsnip, *Pastinaca sativa*, has long been subject to attack by an introduced coevolved florivore, *Depressaria pastinacella*, the parsnip webworm. In 2004, webworms were found for the first time attacking parsnips in New Zealand (NZ). Relative to US *P. sativa*, NZ *P. sativa* produces lower levels of defenses but higher levels of certain floral volatiles, suggesting that escape from its specialist florivore may have resulted in changes in volatile profiles to increase pollinator attraction. In this study, I examined the influence of wild parsnip floral volatiles on pollinator attraction and seed production in NZ and the US. While insects of many taxonomic groups were observed on parsnip umbels, the percentage of flowers that set seed could significantly be predicted by the occurrence of large calyptrate flies and small syrphids in both localities. In the US, β-pinene, γ-terpinene, hexyl butyrate, octyl butyrate, germacrene D and an unknown monoterpene were all positively correlated with visitation by large calyptrates and small syrphids. In NZ, *trans*-ocimene, carene and octyl butyrate were positively correlated with visitation. Remarkably, most compounds positively associated with visitation are produced in
significantly higher proportions in NZ flowers, suggesting that NZ flowers, in the absence of specialized florivores, may be chemically better constituted to attract pollinators.

INTRODUCTION

When plant species invade new areas, they often escape from coevolved specialist enemies; escape in certain circumstances can be accompanied by reduced investment in chemical defense. This relationship in fact forms the basis of multiple hypotheses aimed at predicting the success of invasive plants (Maron and Vila 2001; Keane and Crawley 2002; Joshi and Vrieling 2005; Liu and Stiling 2006). Escape from coevolved specialists, however, may have impacts on the chemistry of plants other than reducing investment in defense. Some plants avoid herbivores by reducing the production of attractants used by adapted specialists to find host plants (Feeny 1977). Colonizing new habitats where coevolved enemies are absent may result in altered selection on volatile attractants, particularly if these chemicals mediate interactions with mutualists such as pollinators. While floral volatiles render plants attractive to beneficial pollinators, they may also make them conspicuous to detrimental specialist herbivores that have evolved the ability to recognize host-specific volatiles (Theis 2006, Theis et al. 2007, Raguso 2008, Kessler and Baldwin 2011, Kessler et al. 2012, Theis and Adler 2012).

In the presence of coevolved specialist herbivores, plant species may experience considerable selection pressure to alter their floral profiles to reduce their apparency (Baldwin et al. 1997). A potential cost of this alteration is a reduction in pollinator attraction, especially if the same components of the floral blend are attractive to both the herbivores and pollinators. For example, although benzyl acetone is a pollinator attractant for the flowers of wild tobacco,
Nicotiana attenuata, it is emitted at low levels because plants with elevated production are subject to greater herbivory (Kessler et al. 2008). As well, benzaldehyde and phenylacetaldehyde emitted by flowers of the thistle, Cirsium arvense, are attractive to both florivores and pollinators (Theis 2006). Increased scent production in Curcurbita pepo results in greater herbivore damage and reduced fitness (Theis and Adler 2012). Understanding how volatiles mediate both antagonistic and mutualistic interactions is thus a prerequisite for predicting the phytochemical and ecological consequences of enemy release.

A system in which the chemical consequences of herbivore escape for pollinator attractiveness may be examined is the European weed Pastinaca sativa L. (Apiaceae), wild parsnip, and its European florivorous specialist, Depressaria pastinacella Duponchel (Lepidoptera: Oecophoridae), the parsnip webworm. The defensive chemistry of this interaction has been well characterized both in its native Europe and in the introduced range in North America, where the wild parsnip has been part of the flora for over 300 years and where it has been reassociated with the parsnip webworm for more than 150 years (Bethune 1869; Riley 1888).

In 2004, webworms were discovered on wild parsnips in New Zealand (NZ), where the plants had grown free from significant herbivory since the introduction and establishment of the weed in the mid-nineteenth century (Zangerl et al. 2008). This accidental introduction provided a unique opportunity for a real-time analysis of evolution in this interaction. Extracts of parsnip buds and flowers in NZ contained lower amounts of certain chemicals, including linear furanocoumarins, octyl butyrate, and myristicin, which function in defense against webworms in the US (Berenbaum and Zangerl 1996; Carroll and Berenbaum 2002, Mao et al.
2008). NZ flower and fruit parsnip extracts also contained higher amounts of octyl acetate, an olfactory attractant for larval webworms (Carroll and Berenbaum 2002). Reduced levels of costly defensive compounds, such as the furanocoumarins, are a predictable consequence of enemy escape, but increased production of other compounds is consistent with a response to other agents of selection, such as pollinators.

Throughout its native and invasive range, *P. sativa* is thought to be “promiscuously pollinated” by a wide range of insects (Bell 1971), including, in central Illinois, dipterans in nine families (Lohman et al. 1996; Tooker et al. 2006). Nectar is secreted by a stylopodium that is easily accessed by generalist visitors, particularly flies. Many of the volatile constituents of parsnip flowers, including octyl esters and sesquiterpenes (Borg-Karlson et al. 1993), are attractive to saprophilic pollinating flies (Jürgens et al. 2006). Visitation by a wide variety of generalist pollinators has been suggested to contribute to the ability of parsnips and other apiaceous species to establish a broad geographic distribution and to succeed in new habitats after introduction (Bell 1971). Yet, despite an abundance of potential pollinator species, pollination success is rarely 100% in US populations. In many populations, a substantial proportion of ovules go unfertilized, as evidenced by a high frequency of parthenocarpy (the production of normal-sized seeds lacking endosperm or embryo) (Zangerl et al. 1991). Parsnips prevent selfing by protandry, so seed set is heavily limited by visitation. Thus, pollinator visitation can impose strong selection on reproductive fitness; release from its specialist enemy in a newly invaded area may free *P. sativa* from constraints on production of volatile attractants and enhance its ability to attract pollinators.
The invasion of webworms in NZ allows us to compare floral chemical traits of wild
parsnips in habitats with and without a coevolved specialist. Tradeoffs between
conspicuousness to antagonists and to mutualists should be particularly striking in interactions
between florivores and their hostplants. Although the interaction between parsnips and
webworms has been studied in detail for over 30 years, little information is available on the
interaction between parsnips and their pollinators and how this interaction might shape floral
traits. I hypothesized that volatiles are important in mediating interactions between parsnips
and their pollinators and that changes in volatile chemistry accompanying range expansions
may reflect differences in the composition of the pollinator community. Thus, the primary
goals of this study were to: 1) evaluate how variation in floral volatiles influences pollinator
visitation and seed production; and 2) determine if differences in parsnip floral chemistry
associated with introduction into NZ are consistent with a role in increasing pollinator
attraction. In this study, I examined the floral volatile bouquet of wild parsnips in NZ and US and
its influence on pollinator attraction and pollination success. Volatiles and floral visitors were
collected, identified, and quantified from parsnips in the US and NZ, and I utilized the natural
variation in the volatile profiles to determine which chemical differences among individual
plants explained differences in pollination success and consequently in fitness gained through
seed production.

**MATERIALS AND METHODS**

Experiments were conducted in June 2009 in the “Overpass” parsnip population (N
40.121173, W 88.143400) in Champaign County, Illinois, USA, which has long hosted webworm
populations (Zangerl and Berenbaum 2009) and in December 2009 in the “Cottage” population in Seacliff, Otago, New Zealand (S 45.678541, E 170.625247), known to be infested by webworms only since 2007 (Zangerl et al. 2008). Identical methods were used in both localities to measure insect visitation, volatile production, and pollination success.

**Insect visitation and volatile collection**

Flowering in *P. sativa* commences with the maturation of the primary apical umbel followed by flowering in the secondary umbels, which branch out laterally from the main stalk. Insect visitation and volatile production were monitored on separate secondary umbels on the same plant. *P. sativa* is protandrous and all measurements were made when the flowers were receptive to pollen (i.e., when most of the inflorescence had matured into the female stage). For all plants in this study, I removed primary umbels to ensure that sufficient resources were allocated to the full development of secondary umbels and that fruit development was not resource-limited.

*P. sativa* flowers develop from male to female within the span of a few days. I secured fine nylon mesh bags over secondary umbels with male flowers that were beginning to lose their anthers and develop stigmal structures but were still unreceptive to pollen. I identified 4 to 5 plants per day that were intermediate in size and uninfested by webworms and bagged two secondary umbels per plant to prevent insect visitation. Bagged plants were checked daily for the appearance of mature female flowers (with bifurcated stigmas). On maturation, bags were removed from both umbels. A camera was positioned above one of the umbels to photograph visitors and volatiles were collected from the other umbel. Photographs and volatiles were collected from a total of 52 plants in Illinois and 32 plants in NZ.
The camera and the volatile collection apparatus were affixed to a customized aluminum pole that was inserted into the ground next to the plant. Four adjustable arms that extended from the pole held the camera, the volatile collection apparatus and two umbels in place securely. The camera (Canon Powershot S31S, 6 megapixels) was placed directly over one of the umbels and programmed to take high-resolution photographs every 3 minutes for 5 hours (100 pictures per plant). All experiments were conducted over a standardized time period within a day, 9 am to 2 pm for the US component and 10 am to 3 pm for the NZ component. A small picture-card containing printed text was placed on the surface of the umbel and used to focus manually on the flowers to obtain sufficient image resolution of floral visitors for identification. The card was removed prior to the experiment. The height of the camera over the umbel was adjusted to obtain the best resolution of the picture-card.

Volatiles were collected from the second umbel on the same plant over the same time period by dynamic headspace sampling. An inverted Erlenmeyer flask was secured around the umbel. Air entering the flask was purified with a small tube filled with activated charcoal. Air from the flask was drawn out at the rate of 0.5L/minute through an adsorbent Porapak Q cartridge with the aid of a vacuum pump (Airchek XR5000, Eighty four, PA). The cartridge consisted of 150 mg of Porapak Q packed inside stainless steel tubing, 5 cm in length and 2.5 mm in diameter. Both the charcoal filter and the Porapak Q were held in place by insertion into a foam stopper that plugged the opening of the Erlenmeyer flask. The flask was covered with an aluminum sun-shield (Zangerl and Berenbaum, 2009) to keep temperature inside the flask close to ambient temperature. At the end of the 5-hour period, the umbels were re-bagged to
prevent further pollination and remained enclosed for 2-3 weeks until fruits completed development.

All photographs were scored for the presence and identity of visitors. The number of visits per morphospecies was generally very low so I combined visitor groups based on taxonomy and size (Table 1). The detail in the photographs allowed me to identify certain insects to species (e.g. *Apis mellifera, Chauliognathus pennsylvanicus*), whereas others were identifiable to family (Diptera: Syrphidae, Bibionidae, Stratiomyidae; Hymenoptera: Vespidae, Formicidae; Coleoptera: Coccinellidae, Lampyridae, Cerambycidae), groups of families (calyptrate Diptera, acalyptate Diptera), or order (Thysanoptera). In addition, I used the photographs to count number of flowers as an estimate of umbel size and the amount of nectar available per umbel. A glossy sheen, generally clearly visible in the photographs, indicates the presence of nectar on the flower; the last photograph for each plant (of the 100 taken) was scored for the proportion of flowers bearing nectar. *P. sativa* flowers do not age synchronously within an umbel but age centripetally. Owing to the non-synchronous maturation of flowers in an umbel, I counted the number of receptive stigmas from the photograph to measure pollination success, the percentage of receptive flowers that had set seed. The number of receptive stigmas was obtained from the final photograph for each umbel following the protocols in Zangerl and Berenbaum (2009).

Porapak Q cartridges from the volatile apparatus were extracted with 0.5 ml of hexane containing a 0.0001 % tridecane as an internal standard and the contents were eluted into 2 ml 11 mm clear glass crimp top vials (Thermo Fisher Scientific, Waltham, MA). They were then cleaned with pentane and dried with nitrogen gas. Eluents were analyzed by gas
chromatography with flame ionization detection and subjected to gas chromatography-mass spectrometry (GC-MS) to identify the volatile compounds. US samples were analyzed by GC-MS on the date of collection and NZ samples were stored in a –20 °C freezer and shipped to the US for analysis. A small number of the NZ samples unavoidably volatilized in transit, leaving a total of 19 NZ samples to be analyzed for floral volatiles.

One µL per sample was injected in the GC-MS (Shimadzu QP2010 Plus, SHRXI-5MS capillary column, 30m X 2.5mm X 0.25u) in splitless mode for 1.5 minutes with an inlet temperature of 250 °C and helium as the carrier. The initial oven temperature was set at 60 °C, held for 30 seconds, increased at a rate of 10 °C/minute until 80 °C, at a rate of 30 °C/minute until 120 °C, followed by 3 °C/minute until 150 °C and finally at a rate of 30 °C/minute for 1 minute. The spectra were recorded in Single Ion Mode (SIM) and the ions detected were m/z 93, 56, 71, 161, 192. Volatile compounds from P. sativa flowers were identified in this study from a small sample of flowers after completing a full-scan of total ions; these have also been identified in a prior study (Zangerl and Berenbaum, 2009). I used SIM to increase the sensitivity of peak detection and selected five ions because of their relative abundance. Peaks were identified by comparison to the NIST08 library, to Kovat indexes obtained from an alkane ladder, and to standards when available. Amounts were quantified based on peak area integrations of total ion chromatograms. Areas were adjusted with the peak area of the internal standard (tridecane). The absolute areas of each compound were converted to a proportion of the total emission. Using proportions, I was able to standardize floral emissions and compare the composition of floral volatiles between US and NZ populations.
Bagged umbels with fully developed fruit were clipped and brought to the laboratory, where all fruits were evaluated for the presence of endosperm. The parsnip fruit is a schizocarp; each of the two mericarps of an individual fruit was placed over a light source to determine whether it was “empty” or filled with endosperm (Zangerl et al. 1991). The presence of an endosperm on each side of the mericarp fruit indicates a successful pollination of the corresponding stigma on the flower.

**Pollen analysis**

On days when experiments were in progress, insect visitors were collected from female secondary umbels that were not photographed in this study. Insect were collected with an aspirator and placed in a 2-ml tube with 70% ethanol. Pollen counts for all captured insects were estimated following the protocols in Zangerl and Berenbaum (2009), as follows: vials with insects were sonicated for two minutes, insects were removed, and the remaining ethanol was centrifuged at 15,000X for 30 seconds. Five µL of fuchsin dye solution (in glycerol) were mixed with the pellet and pipetted onto a glass slide, which was then covered with a cover slip. Parsnip pollen grains were counted in three microscope fields (109 objective, field width = 1.9 mm) of an Olympus CH-2 compound microscope. Pollen counts determined if an insect visitor was carrying parsnip pollen when captured and accordingly had the potential to be a successful pollinator.

**Statistical analysis**

Statistical analyses were conducted in R version 2.13.0 (R Development Core Team 2011) and PASW Statistics version 18. Data sets from the US and NZ study were analyzed separately using Generalized Linear Models. Pollination success was analyzed as a binomial
variable with successes measured as the number of fertilized fruit and failures as the number of receptive flowers that were not fertilized (total number of receptive flowers minus the number of fertilized fruit). Pollination success was fitted using a Generalized Linear Model with a quasi-binomial error structure, with visitor pollinator potential (frequency of visitation*average pollen load) as the explanatory variable. I included only frequency of visitation for NZ thrips and acalyptrate flies as I did not have pollen load data for these visitor groups. Visitors categorized as “very small insects” were frequently observed but their very small size precluded both taxonomic identification and quantification of pollen load.

I used the “drop1“ function in R to determine the significance of each explanatory variable in the model. The drop1 function drops each explanatory variable in turn and each time compares the difference between the partial model deviance and the full model to a Chi-square distribution (Zuur et al., 2009). Based on the results, I could identify visitor groups that are most likely to be important in predicting pollination success. Insect groups with very rare visits (less than an average of 5 visits per umbel per 5 hours) were considered unimportant pollinators and thus were not included in the analysis. After calculating correlations and Variance inflation factor (VIF) values for all explanatory variables, I found that two groups, large flies and small syrphids, were highly correlated in the US data set (VIF: Large flies = 6.26, small syrphids = 4.68, Pearson’s r = 0.83, p<0.001). Because of the difficulty in separating out the individual effects of each group, I ran the model once with large flies and once with small syrphids.

Absolute peak areas for each volatile were converted to a relative proportion of the total and proportions were compared between countries using multivariate analysis of variance
(MANOVA). I used a multivariate distance-based (square root transformed, Bray-Curtis
distances) approach DISTLM in Permanova+ for Primer 6.1 to identify the relative contribution
of individual volatiles to pollinator visitation. Absolute values, adjusted with the internal
standard, were used in the Permanova+ analysis. A stepwise selection procedure based on AIC
measures of fit was used to determine which compounds in the volatile bouquet could best
predict large fly and small syrphid visitation. In US plants, cis-ocimene and trans- ocimene were
highly correlated and I excluded trans-ocimene from the stepwise selection procedure. In the
NZ data, isomers of alpha-farnesene were highly correlated and only (E,E) alpha-farnesene was
included in the model. Individual volatiles deemed important from the DISTLM analysis were
plotted against total visitation to discern the overall direction of the relationship.

I also fitted a GLM model with a negative binomial error structure to determine if
visitation counts of insect groups were dependent on nectar (proportion of flowers bearing
nectar) and umbel size (total number of flowers per umbel).

**RESULTS**

**Visitation and pollination success**

Pollen loads were correlated with insect size (by weight) in both the US and NZ (US
Pearson’s r = 0.45, p<0.001, NZ, Pearson’s r = 0.22, p<0.001), but certain groups of large insects,
such as honey bees and large syrphid flies, were observed too infrequently to have contributed
significantly to pollination success in this study irrespective of pollen load size (Table 1). In both
the US and NZ, wild parsnip flowers were pollinated primarily by large calyptrate and syrphid
flies, but the identity of dipteran visitors varied by country (Table 1). In both countries, small
syrphids and large calyptrate flies (Calliphoridae, Muscidae, Sarcophagidae, Tachinidae) were likely the most important pollinators as they carried relatively large pollen loads and were also frequent visitors (Table 1). In contrast with the US, wild parsnips received proportionately fewer visits from syrphids in NZ, where large calyptrate flies carried large amounts of pollen and were also frequent visitors. Despite striking geographic differences in visitation frequency, in both the US and NZ the percentage of flowers that set seed could significantly be predicted by the occurrence of large calyptrate flies and small syrphids (Table 2).

Insects with high pollen loads did not necessarily predict pollination success. In the US, soldier beetles (*C. pennsylvanicus*) carried large amounts of pollen and were also frequent visitors but their visitation was not significantly associated with pollination success (Table 2). Soldier beetles appeared to utilize the large, flat umbels of *P. sativa* as a mating site and movements between umbels were not observed. Similarly, NZ umbels were visited primarily by bibionid flies, which carried relatively large pollen loads but were not predictive of pollination success (Table 1, 2). Like *C. pennsylvanicus* in the US, they aggregate and mate in large numbers on the umbels without moving often from flower to flower.

I did not directly compare pollinator visitation rates between the US and NZ because of local differences in insect diversity and because visitation rate is not necessarily correlated with effective pollination. Although pollination success in NZ appeared to be higher (32.1% of plants experienced >50% fertilization) than in the US (20% of plants experienced >50% fertilization), this difference was only marginally significant (*p* = 0.056, as determined by a binomial GLM model).
Volatile chemistry

The floral volatile bouquet of US parsnips comprised 16 distinct compounds, 14 of which could be identified by my methods and two monoterpenes could not be definitively characterized. The most abundant constituents were cis- (30.81%) and trans-ocimenes (23.69%) (Figure 1.1). As in the US, the floral bouquet of P. sativa in NZ was dominated by cis- (32.27%) and trans- (49.12%) ocimenes and octyl butyrate. NZ umbels emitted low quantities (<1%) of five compounds absent in the US floral bouquet: linalool, carene, allo-ocimene and two isomers of α-farnesene (Figure 1.1). I found a total of 20 identifiable constituents but I was unable to identify two compounds present in low concentrations. A comparison of US and NZ floral volatiles revealed significant differences in the proportional representation of seven compounds (Wilks λ F13,57 = 680.64, p<0.01); NZ flowers emit a greater proportion of beta-pinene, trans-ocimene, gamma-terpinene, octyl butyrate and germacrene, whereas US flowers emit a greater proportion of octanol and octyl acetate.

Association between pollination and volatile chemistry

In the US, volatiles that best explain the variation in the pollinator matrix were β-pinene, γ-terpinene, hexyl butyrate, octyl butyrate, germacrene D and an unknown monoterpen e. These compounds were all positively associated with pollinator visitation (large calyptrate flies and small syrphids, Figure 1.2). In NZ, volatiles that best explain the variation in the pollinator matrix were trans-ocimene, carene, octyl butyrate and octyl acetate. This model had the lowest AICc score (AICc = 103.95, R² = 0.69). Trans-ocimene, carene and octyl butyrate were positively associated with pollinator visitation while octyl acetate was negatively associated with visitation (large calyptrate flies and small syrphids) (Figure 1.3). Most compounds
positively associated with visitation (β-pinene, γ-terpinene, octyl butyrate, germacrene D, trans-ocimene and carene) were emitted in higher proportions in the NZ plants, whereas octyl acetate, which is negatively associated with visitation, is emitted in lower proportions in NZ plants.

Associated between pollination and nectar availability

Large calyptrate and small syrphid visitation was significantly associated with nectar availability in the US but not in NZ (Tables 3a and b, Figures 1.4a and b). The number of visitors was positively associated with umbel size for large calyptrate and anthomyiid flies in the US and large calyptrate, small syrphid and bibionid flies in NZ (Tables 1.3a and b)

DISCUSSION

Wild parsnips depend on insect visitation for reproduction, indicating that seed production in this monocarpic biennial is highly contingent on the plant’s ability to attract effective pollinators. Easily accessible nectar and large conspicuous inflorescences render wild parsnips a target for visitation by a wide range of insects. Many (possibly most) frequent visitors, however, do not appear to be effective pollinators. Frequency of visitation and pollen loads did not prove to be reliable indicators of pollinator efficacy in this study; C. pennsylvanicus in the US and bibionid flies in NZ, for example, were extremely abundant and carried substantial amounts of pollen but were poor predictors of pollination success, possibly because of their tendency to aggregate and remain on the inflorescence for long periods (personal observations). Increased duration of visits per flower likely reduces the frequency with which other flowers are visited, an important determinant of pollinator effectiveness
(Madjidian et al. 2008). Similarly, large ants, although common on flowers, do not appear to contribute proportionately to pollination success.

Despite an apparent “promiscuous” strategy (sensu Bell 1971), in both the US and NZ the insects chiefly responsible for parsnip pollination in this study were species in the order Diptera. In fact, this so-called “promiscuously pollinated” plant may be somewhat specialized for pollination by flies. Visits by large calyptrate flies and small syrphids were significantly predictive of seed set in both the US and NZ. Moreover, the composition of floral volatiles of *P. sativa* is suggestive of some degree of specialization for dipteran pollinators. Although containing representatives from phytochemical classes widespread among angiosperms, the floral volatiles of *P. sativa* and closely related species in the genus *Heracleum* (Katz-Downie et al. 1999) are essentially unique in containing octyl esters (Borg-Karlson et al. 1993, Knudsen et al. 2006). Evidence suggests that some dipterans may rely on aliphatic esters as cues for locating floral hosts (Knudsen and Stahl, 1994). Similarly, attractiveness to flies among some plant species has been linked to production of *trans*-ocimene (e.g., *Prestoea schultzeana*, Arecaceae, Knudsen et al. 2001; *Silene otitis*, Caryophyllaceae, Dotterl et al. 2012). Indeed, I found that many of these floral volatiles have a significant role in attracting dipteran pollinators to *P. sativa*. Pollinators appear to respond mainly to monoterpenes, sesquiterpenes and esters in the floral bouquet. *Trans*-ocimene and octyl butyrate dominate the floral bouquets of both NZ and US flowers (Figure 1.1) and are positively associated with visitation. In addition to floral volatiles, pollinator visitation is also significantly driven by nectar rewards (Figure 1.4a) and the conspicuousness of the umbels (umbel size, Tables 1.3a and 3b).
Remarkably, most compounds positively associated with visitation are produced in significantly higher proportions in NZ flowers. In the absence of herbivory, NZ plants might be under selection to increase fitness by producing more pollinator attractants. However, in this study, the tradeoff between compounds that mediate pollinator and herbivore attraction/deterrence may be constrained or otherwise influenced by biosynthetic considerations. Octyl butyrate is a known webworm deterrent but also a pollinator attractant, whereas octyl acetate is a webworm attractant and a pollinator deterrent, suggesting that selection should favor plants with high octyl butyrate and low octyl acetate. Because octyl acetate and octyl butyrate are extremely polymorphic in P. sativa, their production might be genetically constrained via pleiotropic associations with other chemical pathways. In a heritability study conducted on North American wild parsnip mature fruit, Carroll et al. (2000) determined that amounts of each ester are independently phenotypically correlated with linear furanocoumarins; if these phenotypic correlations reflect underlying genetic correlations, each ester may thus respond differently to selection on furanocoumarins or on other defense compounds. Despite absence of direct evidence of conflicting selection on floral chemistry in wild parsnips, the floral bouquet of wild parsnips in NZ reflects overall a profile of volatiles that is more attractive to pollinators.

A previous examination of wild parsnip floral chemistry in the US and NZ compared the composition of ethyl acetate extracts of buds and flowers (Zangerl et al. 2008). However, my focus in this study was attraction of pollinators and I compared headspace volatiles (Knudsen et al. 1993) as more ecologically relevant. Accordingly, the content and composition of floral volatiles differ between studies, with greater proportional representation of monoterpenes and
lower representation, or absence, of compounds of higher molecular weight (e.g., palmitolactone) in this study.

The presence of herbivores can also have a direct impact on pollination. In *P. sativa*, florivory by webworms can greatly reduce the number of floral visitors to *P. sativa* (Lohman et al. 1996). Even a single webworm can reduce visitation; webworms not only web together and consume flowers, reducing the size of the floral display and restricting access of pollinators to flowers, they also deposit frass in their webs, altering the composition of the floral bouquet. Zangerl et al. (2009) demonstrated that webworm frass contributes octanol to the floral bouquet, through webworm detoxification of the esters octyl acetate and octyl butyrate. Conversion of octyl esters to octanol may thus contribute to reductions in pollinator visitation via reducing the abundance of a potential pollinator attractant and increasing the abundance of an otherwise rare volatile component.

In undertaking this study, I found evidence consistent with the hypothesis that floral chemical traits in wild parsnips free from webworm florivory will reflect a volatile profile that maximizes fitness via the attraction of pollinators. My findings suggest that future studies of phytochemical impacts of enemy release should address interactions with mutualists as well as interactions with enemies, in order to obtain a more comprehensive understanding of both the ecology and evolution of plant-insect interactions in non-indigenous environments.

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REFERENCES


FIGURES AND TABLES

Table 1.1: Frequency of insect visitation on *Pastinaca sativa* umbels. Observations were made from 100 photographs (5 hours) for each umbel and the frequencies were averaged over all umbels. Visitation frequencies were calculated as the average number of times the insect was observed in the span of 5 hours (in 100 photographs). Pollen counts were divided by the weight of the insect to obtain a pollen count per mg measure, which was averaged over all specimens collected.

<table>
<thead>
<tr>
<th>Pollinators</th>
<th>Frequency of visitation/5 hours</th>
<th>Average pollen count/mg (US/NZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>US</td>
<td>NZ</td>
</tr>
<tr>
<td><strong>COLEOPTERA</strong></td>
<td></td>
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<tr>
<td><em>Chauliognathus pennsylvanicus</em></td>
<td>8.94</td>
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<td>Coccinellidae</td>
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<td><strong>DIPTERA</strong></td>
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<tr>
<td>Anthomyiidae</td>
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<td>Bibionidae</td>
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<td>0.97</td>
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<tr>
<td><em>Apis mellifera</em></td>
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<td>671.47</td>
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<td>&lt; 1mm, unidentified</td>
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Table 1.2: Deviance (Chi-sq) tests for GLMs (quasi-binomial model) predicting pollination success by insect visitation frequency x average pollen load in US and NZ parsnips. Significant differences at p<0.001 are indicated by *** and p<0.05 by *

<table>
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<tr>
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<th>Df</th>
<th>Deviance</th>
<th>Scaled deviance</th>
<th>p-value</th>
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<td><strong>US: Pollination success: with large calyptrate Diptera</strong></td>
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<td></td>
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<tr>
<td>Full model</td>
<td></td>
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<td>Full model</td>
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<td><strong>NZ: Pollination success</strong></td>
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<td>Full model</td>
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Table 1.3: Log-likelihood ratio (Chi-sq) tests for GLMs (negative binomial model) predicting large fly and small syrphid visitation by nectar availability in US and NZ parsnips. Significant differences at p < 0.001 are indicated by *** and p<0.05 by *

a) US

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<tr>
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<td>309.33</td>
<td>16.91</td>
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</tr>
</tbody>
</table>

| **Small Syrphidae**          |    |          |       |      |         |
| Full Model                   |    | 51.95    | 409.18|      |         |
| Nectar                       | 1  | 62.89    | 418.12| 10.94| <0.001  |
| Umbel size                   | 1  | 55.48    | 410.71| 3.53 | 0.06    |

| **Anthomyiidae**             |    |          |       |      |         |
| Full Model                   |    | 49.80    | 266.16|      |         |
| Nectar                       | 1  | 56.03    | 269.84| 5.67 | 0.01    |
| Umbel size                   | 1  | 55.48    | 270.38| 6.22 | 0.01    |

| *Chauliognathus pennsylvanicus* |    |          |       |      |         |
| Full Model                   |    | 50.07    | 240.93|      |         |
| Nectar                       | 1  | 50.91    | 270.49| 0.84 | 0.35    |
| Umbel size                   | 1  | 50.74    | 270.31| 0.67 | 0.41    |

| **Small Formicidae**         |    |          |       |      |         |
| Full Model                   |    | 43.90    | 240.93|      |         |

Table 3a (continued)

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| **Large Formicidae**         |    |          |       |      |         |
| Full Model                   |    | 43.49    | 228.94|      |         |
| Nectar                       | 1  | 46.10    | 262.06| 2.60 | 0.10    |
| Umbel size                   | 1  | 43.52    | 259.48| 0.03 | 0.86    |
Table 1.3 (continued)

b.) NZ

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<td>Umbel size</td>
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<td>413.78</td>
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Figure 1.1: A comparison of the composition of volatile emitted from US and NZ flowers. Error bars represent standard error. Significant differences between countries (MANOVA) indicated by *.
Figure 1.2: Compounds that best explain variation in the US pollinator matrix (DISTLM analysis: AICc = 336.23, $R^2 = 0.34$). Graphs represent the direction of relationship between pollinator visitation (counts of large calyptrate flies and small syrphids) and individual volatile compounds in US parsnip flowers. The Kovat index for the unknown monoterpane (monoterpane 1) calculated from C9 and C10 alkanes at 80°C is 916 and m/z ion fragments in order of relative abundance are 93, 56, 71, 192, 161.
Figure 1.3: Compounds that best explain variation in the NZ pollinator matrix (DISTLM analysis: AICc = 103.95, $R^2 = 0.69$). Graphs represent the direction of relationship between pollinator visitation (counts of large calyptrate flies and small syrphids) and individual volatile compounds in NZ parsnip flowers.
Figure 1.4: Relationship between the number of visits and nectar availability (percentage of flowers per umbel bearing nectar on the day’s last photograph) on a) US parsnips and b) NZ parsnips for large calyptrate flies (US: p<0.001*, NZ: p=0.75, GLM negative binomial) and small syrphids (US: p=0.01*, NZ: p=0.93 GLM negative binomial).
Chapter 2 - Real-time evolution of tolerance in an invasive weed after reassociation with its specialist herbivore

ABSTRACT

The interaction between the European wild parsnip *Pastinaca sativa* and its coevolved florivore the parsnip webworm *Depressaria pastinacella*, established in North America for over 150 years, has resulted in evolution of local chemical phenotype matching. The recent invasion of New Zealand by webworms, exposing parsnips there to florivore selection for the first time, provided an opportunity to assess rates of adaptive response in a real-time experiment. I planted reciprocal common gardens in the US and NZ with seeds from: 1) US populations with a long history of webworm association; 2) NZ populations that had never been infested and 3) NZ populations infested for 3 years (since 2007) or 6 years (since 2004). I measured impacts of florivory on realized fitness, reproductive effort, and pollination success, compared overall susceptibility of US and NZ genotypes to webworms, and measured phenotypic changes in infested NZ populations relative to uninfested NZ populations to determine if rapid adaptive evolution in response to florivory occurred. Irrespective of country of origin or location, webworms significantly reduced plant fitness, but the cost of damage was lower for larger plants. Webworms also reduced pollination success in small plants but not in larger plants, irrespective of country of origin or location. While defense chemistry remained unchanged, plants in infested populations were larger after three to six years of webworm florivory. As plant size is a strong predictor of realized fitness, evolution of large size as a component of florivore tolerance may occur more rapidly than evolution of enhanced chemical defense.
INTRODUCTION

Selection pressure exerted by herbivory is thought to have shaped a wide range of plant defense traits over macroevolutionary time and may have been the principal driving force behind the spectacular diversification of angiosperms (Futuyma and Agrawal, 2009). Herbivores may also cause rapid adaptive evolution in plants over contemporary timescales. Documenting microevolutionary change in a particular plant species in real time is challenging in view of the fact that response to selection can be influenced by other ecological processes within the community in which plant-insect interactions are embedded. Only a handful of studies have quantified real-time ecological and evolutionary change in plants in response to selection by herbivores. These studies have generally relied on synthetic plant populations and controlled access by herbivores to these populations (Agrawal et al., 2012; Zust et al., 2012; Uesugi and Kessler, 2013). Recent common-garden herbivore exclusion studies have shown that, in the absence of herbivory, rapid adaptive evolution in defense and life history traits can occur. For example, when protected from herbivory, *Oenothera biennis* (Onagraceae) evolved earlier flowering time, higher seed defenses and greater competitive ability in four generations (Agrawal et al., 2012). Herbivore exclusion also resulted in altered *O. biennis* genotype frequencies and community composition. In a longer-term exclusion study, after protection from rabbit herbivory for 26 years (Turley et al., 2013), *Rumex acetosella* in garden plots demonstrated reduced growth rate with no concomitant change in chemical defense (oxalate content) or competitive ability. By contrast, competitive ability and allelopathic compound production increased in *Solidago altissima* genotypes experiencing 13 years of herbivore exclusion (Uesugi and Kessler, 2013). Studies documenting rapid responses to selection
imposed by the introduction, rather than exclusion, of herbivores are fewer in number. One such study, Zust et al., (2012), demonstrated rapid evolution (within five generations) in *Arabidopsis thaliana* of specific glucosinolate defenses in the presence of different species of aphids (Zust et al., 2012).

Because evolution occurs in an ecological context, phenotypic changes predicted by manipulative experiments may not follow in real populations. To date, no studies have examined rapid evolution in natural populations in response to a single herbivore or the impact of rapid adaptive evolution on other ecological processes such as plant-pollinator interactions. Determining rates of evolutionary response to herbivory in real time is of importance not only for understanding basic mechanisms underlying plant-insect interactions, but also for evaluating the potential for the evolution of resistance in weeds to introduced biocontrol agents. Invasive plants long separated from their coevolved specialist herbivores are often reunited as part of classical weed biological control programs. Evidence suggests that as plant species invade and colonize new communities they undergo rapid microevolutionary change, including, in some cases, increased competitive ability and reduced defense investment (Zou et al., 2008). Such evolutionary changes may well influence the efficacy of biological control programs in a diversity of ways, particularly via influencing the rate at which populations reassociated with their coevolved herbivores respond to selection (Mueller-Scharer et al., 2004; Huffbauer and Roderick, 2005).

The invasion by the European weed, wild parsnip, *Pastinaca sativa* L. (Apiaceae) and its subsequent reassociation with its coevolved specialist herbivore, the parsnip webworm, *Depressaria pastinacella* Duponchel (Lepidoptera: Oecophoridae), in both North America and
New Zealand (NZ) have provided an opportunity to quantify ecological and evolutionary responses to herbivory in real time. *P. sativa* has been established as an invasive weed in North America for at least 400 years. The presence of phototoxic furanocoumarins in all aboveground tissues of the plant renders it relatively free from herbivory (Berenbaum, 1981; Berenbaum and Zangerl, 1996), with the notable exception of the parsnip webworm, a coevolved European florivore that feeds on buds, flowers and fruit, which was accidentally introduced into Canada in 1869 (Bethune, 1869) and which now is found on wild parsnips across the North American continent. Wild parsnips are invasive in many other regions of the world, including in NZ since 1867 (earliest record). In contrast to US populations, NZ wild parsnip populations were free from significant herbivory until 2004, when parsnip webworms were discovered in Port Chalmers, Otago, on the South Island (Zangerl et al., 2008).

Reciprocal selection between parsnips and webworms has been examined in its invasive North American and native European ranges (Zangerl and Berenbaum, 2003; Zangerl and Berenbaum, 2005). High levels of three furanocoumarins--bergapten, xanthotoxin and sphondin--are associated with resistance to webworms (Berenbaum et al., 1986; Zangerl and Berenbaum, 1993); these phytochemicals are metabolized in the webworms by cytochrome P450 monooxygenases with differing efficiency. Webworm florivory, which both directly and indirectly reduces fitness of this monocarpic plant, exerts selection pressure on furanocoumarin content and composition (Berenbaum et al., 1986) and phenotype matching over small spatial scales in the midwestern US strongly suggests that reciprocal selection between parsnips and webworms takes place (Berenbaum and Zangerl, 1998; Zangerl and Berenbaum, 2003). Chemical analysis of North American and European herbarium specimens has also shown
retrospectively that, prior to webworm introduction, wild furanocoumarin production in North American seeds was lower relative to plants in Europe; after 1890, once webworms were present in North America, furanocoumarins levels increased in seeds to levels equal to or higher than those in Europe (Zangerl and Berenbaum, 2005).

In New Zealand, Zangerl et al. (2008) found that webworm florivory resulted in an average reduction in seed numbers of 75%, with some plants in many populations producing no viable seeds at all. A comparison of US and NZ parsnips collected in 2006 and 2007 showed that NZ parsnips were chemically distinct from US parsnips, with lower levels of imperatorin, isopimpinellin and bergapten but much higher levels of xanthotoxin and sphondin. NZ parsnips also differed in having lower octyl butyrate levels and higher octyl acetate levels compared to US populations. Multivariate selection analysis on these chemical traits revealed that reassocation with webworms led to a complex pattern of non-linear or correlated selection that differed among populations. Thus, parsnip webworms have had a significant negative impact on plant fitness and have exerted strong selection pressure on chemical traits in NZ parsnip populations. Given that phenotypic variation in chemical defense traits was high in these populations (Zangerl et al., 2008), that these traits are characterized by high additive genetic variance (Zangerl and Berenbaum, 2005) and that plant response to selection by florivores should be especially pronounced (Wise and Rausher, 2013), phenotypic changes in infested NZ wild parsnips in response to webworm selection should occur rapidly.

Selection imposed by webworm florivory also has the potential to alter interactions between wild parsnip and its pollinators. Florivorous insects can directly or indirectly disrupt pollination by reducing the visual and chemical attractiveness of the floral display (McCall and
Irwin, 2006). Alternatively, selection on resistance traits may be balanced by pollinator selection on floral traits, especially if either the same compounds mediate interactions with florivores and pollinators or if pollinator attractants and florivore defenses are genetically correlated. Kessler et al. (2013), e.g. showed that the floral bouquet in Petunia x hybrida consists of compounds that both attract pollinators and deter herbivores. Parsnip plants are obligate outcrossers and rely on large calyptrate flies and syrphids for effective pollination (Jogesh et al., 2013). Parsnip flowers damaged by webworms are visited by fewer pollinators (Lohman et al., 1996), in part because they emit a disproportionately high amount of octanol, a component of webworm frass, the presence of which can result in lower pollination success (Zangerl and Berenbaum, 2009). That parsnip plants in an uninfested NZ population emitted a floral bouquet that was more attractive to pollinators compared to a US population with a long history of infestation (Jogesh et al., 2013) suggests that floral chemistry may be under selection by both florivores and pollinators.

In view of the genetic, chemical, and ecological conditions prevailing in NZ, I hypothesized that the six years of reassociation of parsnips and parsnip webworms should have been sufficient to result in rapid evolution of enhanced plant defense against webworms. With the dual function of certain volatiles in deterring webworms and attracting pollinators (Jogesh et al., 2013), I also hypothesized that six years of reassociation should have resulted in reduced attractiveness to pollinators. To test these hypotheses, I planted reciprocal common gardens in the US and in NZ, consisting of US parsnips with a long history of infestation as well as infested and uninfested NZ populations collected from 2004 to 2009, after two to six years of webworm infestation. My specific objectives were to compare the overall susceptibility of US and NZ
parsnip genotypes to webworm florivory and to document phenotypic changes in infested NZ populations relative to uninfested NZ populations to determine if rapid adaptive evolution in response to herbivory has occurred and if that rapid evolution has altered parsnip-pollinator interactions.

**MATERIALS AND METHODS**

**Life histories of the herbivore and plant in the US and NZ**

Wild parsnips are biennial and overwinter as rosettes. In the US, after overwintering, parsnips bolt in early May, flower in June and set seed in mid-late July. In mid-April, prior to hostplant bolting, parsnip webworms adults oviposit on rosette leaves. After bolting, the largest inflorescence, the primary umbel, unfurls at the apex of the main shoot, followed by the secondary umbels that unfurl at the tips of branches, and tertiary umbels on the stalks of secondary umbels. Webworms larvae, upon hatching, move up to unopened buds and feed almost exclusively on floral tissues. When umbels are open, the larvae continue to feed on flowers and developing fruit and subsequently burrow and pupate within the hollow parsnip stems. In NZ, parsnip rosettes bolt in late November, flower throughout December and set seed in mid-late January; however, in contrast with the US, webworms oviposit in early December, after the plants have already bolted.

**Reciprocal common gardens**

Reciprocal common gardens were planted in the US and in NZ. In the US, the common garden was established at the University of Illinois Phillips Tract research area (N 40.13205, W 88.143826) (Champaign Co., IL) in an area that has been tilled every other year for the last 21 years. In NZ, the garden was planted on agricultural land in Sawyers Bay, Otago, previously used
to grow potatoes (S 45.821413, E 170.598428). I planted parsnips grown from seeds collected from nine NZ populations in 2006, 2007, 2008 and 2009 (Table 2.1). At the time of experimental setup, four of these populations had been infested for at least 6 years (CRIMP, TOWNLEY, LUMBER, WARRINGTON), two were infested for 3 years (since 2007) (OCEAN, ROCK) and three population had no or minimal (<1% damage) history of infestation (COTTAGE, BUSHY, HERBERT). Nearly 100 percent of all plants with 6 years of infestation were damaged (Table 2.1). Populations infested for 4 years were larger but >50% of all plants were damaged. No webworms were found in BUSHY or HERBERT populations from 2006 until 2009 and, with fewer than 1% of plants experiencing damage, the COTTAGE population was considered uninfested for this study. In 2008, seeds were collected from five US populations in central Illinois (DEA, KAN, PIO, OP and RAN), which are at least 5 miles apart and are known to have been infested with webworms for many years (Zangerl et al., 2008).

Gardens were planted with seeds from all nine NZ populations and five US populations. At least 40 seeds from 20 maternal plants in each population (two maternal half-sibs per parent plant), for each seed collection year (2006-2009), were randomly planted in each garden (a total of 40x4x9 NZ plants and 5x40 US plants). In the US, seeds were germinated in the University of Illinois Urbana-Champaign (UIUC) Entomology greenhouse in early March 2009 in plastic pots filled with 1:1:1 peat:perlite:Drummer soil and grown at 24°C at 16h day length. The seedlings were transplanted to the garden in early July 2009. In NZ, however, with no access to a greenhouse nearby, I planted seeds directly into the ground in September, 2010. Individuals were planted 0.5 m apart in 21 long rows in the US and 29 long rows in NZ, with rows 1 m apart in both locations. The parsnips were regularly watered and weeded to reduce mortality.
In the spring of the year following planting, I scored all plants that had overwintered successfully. In the two weeks between oviposition and egg hatch, one member of each maternal half-sib pair grown in each garden was sprayed with insecticide. The decision as to which half-sib was sprayed was made randomly. Plants were sprayed with acephate (US: Orthene, Ortho, Columbus, OH; NZ: Orthene WSG), a systemic organophosphate insecticide, to create an herbivore-free treatment (=spray treatment). Acephate was purchased as a soluble powder and dissolved in water as per the manufacturer instructions (0.6g/L) and sprayed at the base of the plant. As female flowers matured on secondary umbels, ten flowers were collected for chemical analysis in a pre-weighed Eppendorf centrifuge tubes containing a glass bead. The tubes were immediately placed over dry ice to prevent the loss of volatile components and subsequently stored in -20 or -80°C in the laboratory. When all webworms had pupated, I estimated the percentage (to the nearest 10%) of florets in each umbel sustaining damage. I also recorded umbel diameter with Vernier calipers for each primary (stem apex), secondary (branch apex), and tertiary (secondary branch apex) umbel on the plant. The diameter is an approximate predictor of the number of potential seeds (determined empirically, number of seeds = 91.6 × umbel stalk diameter + 98.3, n = 10, R² = 0.80, p= 0.0005) (Zangerl et al., 2008). Using the umbel diameter and the estimate of percentage of florets damaged, I calculated the total damage (number of seeds eaten = number of potential seeds x percentage of flowers damaged). Seeds from all plants were collected as they matured on stalks; after sifting to remove debris, seeds were weighed to obtain a measure of fitness (=realized fitness). After all seeds had been collected, the plants were cut at the base, dried in an oven at 60°C for 4-5 days, and weighed to measure total biomass (=plant size).
Chemical analysis

Frozen flower tissues collected in 1.5 ml Eppendorf tubes were pulverized with a glass bead in a Wig-L Bug Amalgamator (Crescent Dental Manufacturing, Chicago IL) for 1 minute. The pulverized tissue was refrozen, to reduce volatizing of compounds, and the frozen material was vortexed with 750 µl of 100% HPLC-grade ethyl acetate containing 0.0001% tridecane, an internal standard. The tubes were centrifuged at 9000X for 3 minutes and the supernatant was pipetted into a 2-ml GC-vial. The pellets were dried at 50°C for 48 h and weighed to estimate tissue dry weight. Chemical extracts obtained in this manner were analyzed by gas chromatography with flame ionization detection and subjected to gas chromatography-mass spectrometry (GC-MS).

One µL per sample was injected in the GC-MS (Shimadzu QP2010 Plus, SHRXI-5MS capillary column, 30m x 2.5mm x 0.25µm) in splitless mode for 1.5 minutes with an injection temperature of 250°C and helium as the carrier. The initial oven temperature was set at 50°C, held for 50 seconds, increased at a rate of 10°C/ minute until 250°C and held at 250°C for 5 minutes. A full scan of ions from 40m/z to 300m/z was recorded. Chemical peaks were identified by comparison to the NIST08 library and to standards when available. Amounts were quantified based on peak area integrations of total ion chromatograms. Areas were adjusted with the peak area of the internal standard (tridecane) and divided by the weight of the dried tissue to obtain a peak area per milligram, a measure that could then be compared across samples.
Statistical analyses

Comparison of US and NZ parsnips—impacts of webworms on fitness

My first analysis was structured to determine whether differences in susceptibility to webworm attack existed between US and NZ parsnips grown in US (2010) and NZ (2011) experimental garden plots. Realized fitness was the dependent variable and country of origin (US, NZ), garden (2010, 2011) and spray treatment were included as fixed effects, population was a random effect, and plant size (stalk weight) was added as a covariate in a linear mixed effects model. Realized fitness was estimated as log-transformed (to ensure equal variance) total seed weight. Thus Log (seed weight+1) = country of origin x spray treatment x garden location, covariate = stalk weight, random factor = ~1|population.

I used this model to determine the effects of:

1) spray treatment (do webworms have an impact on fitness?)
2) country x spray (are NZ parsnips more susceptible to webworms than US parsnips?)
3) garden x spray (does the NZ environment favor webworm susceptibility?)
4) country x garden x spray (is there a genotype-by-environment interaction in webworm susceptibility?)
5) garden x country (is there a genotype-by-environment interaction in realized fitness?)
6) stalk weight (does realized fitness change with plant size?)

Reproductive effort measures the proportion of available resources (biomass) allocated to seed production and as such accounts for variation in realized fitness that may be due to resource availability and thus the relative cost of investing in seeds. To quantify impacts of webworm herbivory on the cost of seed production, I calculated plant reproductive effort as
the ratio of seed weight to stalk weight. I compared US and NZ parsnips grown in US (2010) and NZ (2011) gardens with reproductive effort as dependent variable. Reproductive effort was fitted with a linear mixed model with country of origin, spray treatment and garden location as fixed effects, population as a random effect, and plant size as a covariate.

Models with population as a random effect and random intercept performed significantly better and therefore random effects were included in all models. Likelihood ratio testing comparing AIC values between full and reduced models were used to determine the significance of main and interaction terms in each model. All statistical analyses were conducted in R version 2.14.2 (R Development Core Team 2012) and linear mixed effects models were executed using the package “nlme” (Pinheiro et al., 2013).

In order to determine whether higher levels of herbivore damage were proportionately more costly for parsnips, I examined the relationship between proportional damage (proportion of all seeds that were damaged) and reproductive effort, using only unsprayed plants. Reproductive effort was analyzed as the dependent variable in a general linear mixed effects model, with the interaction between country and proportional damage as the predictor, population as a random effect, and stalk size as a covariate. This analysis was conducted separately for both gardens owing to the large variability in reproductive effort between gardens, and, while the main effect analyzed was whether damage was related to reproductive effort, the model also assessed the effect of country of origin (plants from US vs NZ) on reproductive effort. The relationship between total damage and realized fitness was not analyzed because both measurements are strongly positively associated with plant size (see Results).
I also compared plant size, total damage and proportional damage between US and NZ parsnips grown in different gardens to determine whether these traits: 1) differed between parsnips depending on their country of origin; 2) differed between gardens; and 3) were influenced by genotype-by-environment interaction. For plant size, log-transformed stalk weight was the dependent variable in a linear mixed model, with country of origin and garden location as predictor variables and population as a random effect. I also examined the effect of spray treatment on plant size to ensure that the insecticide spray did not influence plant growth. Total damage was analyzed as a generalized linear model (GLM) with a negative binomial model error structure and proportional damage as a GLM quasibinomial model. Country of origin and garden were included as main effects and plant size as a covariate. The drop1 function in R was used to compare models. Negative binomial GLMs were implemented using the package “MASS” (Venebles and Ripley, 2002). Plants sprayed with insecticide (i.e., webworm-free) were not included in comparisons of damage across countries and gardens.

**Comparison of US and NZ parsnips—pollination success**

I compared pollination success between 1) US and NZ parsnips; 2) sprayed vs unsprayed plants; 3) the two different garden plots; and 4) plant size; to determine if pollination success was influenced by the country of origin (genotype); the webworm, the environment, or plant size, or interactions among these factors. Pollination success was estimated from the weight of fertilized seeds and the total number of available seeds (total potential seeds minus the number of damaged seeds). The number of fertilized seeds was estimated from weight of the fertilized seeds: number of seeds = 329.77*(Seed weight) - 3.85 (n = 27, R² = 0.99). The regression equation was obtained by weighing 2 to 200 seeds. Pollination success (number of
fertilized seeds/total number of available seeds) was analyzed as a GLM with a quasibinomial error structure with country of origin, spray treatment, and garden as main effects and plant size as a covariate.

Evolution of wild parsnips in NZ—impact of duration of infestation

To evaluate the effect of webworms on the evolution of wild parsnips in NZ, and to determine whether susceptibility to webworms decreased in plants associated with webworms over time, I compared NZ parsnips with different infestation histories (infested for 3-6 years and never infested) over the period 2006 to 2009. I analyzed the data using a linear mixed-effects model with log-transformed fitness as the dependent variable, webworm infestation history, year (2006-2009), and spray treatment as main effects, population as a random effect and stalk weight as a covariate. With this model I was interested primarily in the interaction effect of year x infestation history x spray treatment (has susceptibility decreased in parsnips with a history of infestation compared to uninfested parsnips?). I evaluated tolerance to herbivory as a result of size because plant size was very strongly correlated with fitness (see Results) and to determine whether parsnips from populations with a history of infestation become larger over time compared to uninfested populations. I compared plant size in NZ parsnips with different infestation histories from 2006 until 2009 (infested for 6 years, 3 years and never infested) using a linear mixed-effects model. Log-transformed stalk weight was the dependent variable and webworm infestation history and year (2006-2009) were main effects. Population was included as a random factor. I also compared total damage and pollination success in NZ plants with different infestation histories from 2006 to 2009. Damage was compared among unsprayed plants using a negative binomial model with overall damage as the
dependent variable. Webworm infestation history and year (2006-2009) were main effects and plant size was included as a covariate. Furthermore, pollination success was compared in sprayed plants using a binomial model with proportion of seeds pollinated as a dependent variable. Webworm infestation history and year (2006-2009) were main effects and plant size was included as a covariate. With these models I was interested in the interaction effect of year x infestation history (have parsnips with a history of infestation changed over the years compared to uninfested populations?).

Analysis of floral chemistry to determine phenotypic change over time

I analyzed a subset of eight compounds: octyl butyrate, octyl acetate, xanthotoxin, bergapten, isopimpinellin, imperatorin, sphondin and myristicin—those were highly variable among individuals and that have been shown previously to influence webworms either behaviorally (Carroll et al., 2000) or physiologically (Berenbaum and Zangerl, 1992). I compared these eight floral compounds between US and NZ parsnips. Peak areas of individual compounds were dependent variables in independent mixed effects models with country of origin and garden as main effects and population as a random effect.

To detect phenotypic changes in floral chemistry of infested populations from 2006 to 2009, I evaluated changes in floral chemistry in individual populations because the floral chemistry differed among populations. Zangerl et al., (2008) also found that the direction and complexity of selection differed among populations; these differences, along with low survivorship in four populations, limited the statistical power such that only five populations (HERBERT, COTTAGE, CRIMP, WARRINGTON and ROCK) could be analyzed. With no a priori expectation for the direction of change, I used a multivariate distance approach to examine
changes in all compounds simultaneously. The data were square root-transformed and a resemblance matrix was created with Bray-Curtis dissimilarities and used to conduct an analysis of similarity (ANOSIM) among plants from 2006, 2007, 2008 and 2009 in individual populations. ANOSIM tests for differences in rank dissimilarity between a priori defined groups compared to random groups. Much like an ANOVA, the ANOSIM statistic R compares the differences in ranks within years and between years for a population. The analysis was conducted in PRIMER 6 (Clark and Warwick, 2001). Variation in octyl butyrate had a strong environmental component and was not included in this analysis.

Half-sib comparisons to determine environmental effects

I compared eight floral compounds, size, realized fitness, and damage between half-sibs grown in US and NZ gardens to test for trait differences attributable to environmental factors. Half-sibs were compared with a pairwise Wilcoxon sign test.

RESULTS

Of the 1,238 parsnips planted in the US garden, 685 plants bolted, flowered, and set seed. In NZ, 1,603 parsnip seeds were sown and 626 plants overwintered successfully. Of the plants that bolted, some were lost to wind damage and some individuals could not be measured for certain parameters due to losses during processing.

Impact of webworms on parsnip fitness

Irrespective of locality, both realized fitness and reproductive effort were higher in plants without webworms (Figures 2.1 and 2.2, Table 2.2). I did not find a significant country x spray treatment interaction or a garden x spray treatment interaction, suggesting that the
effect of webworm florivory on realized fitness does not differ with environment or genetic background (Table 2.2). However, insecticide treatment increased reproductive effort in the US garden but not in the NZ garden (Table 2.2, significant spray x garden effect), suggesting that environmental factors can mediate the cost of webworm florivory on fitness. A greater proportion of webworm damage on unsprayed plants was correlated with reduced reproductive effort in the US common garden but not in the NZ common garden (US garden, $L=7.54$, $df=1$, $p=0.006$ and NZ garden, $L=1.76$, $df=1$, $p=0.18$).

**Phenotypic differences between US and NZ parsnips in both gardens**

Parsnips grown in NZ were on average 15 times larger than their half-sibs in the US garden. The substantial difference in size is likely a reflection of different abiotic conditions between the two gardens. Plant size was a very strong predictor of realized fitness with larger plants having proportionally higher fitness compared to smaller plants (Fig. 2.1, Table 2.2). Total damage and pollination success were also strongly correlated with plant size (Table 2.2, significant effect of stalk weight); the plant size difference between gardens was reflected in significantly in higher total damage, realized fitness, and pollination success in the NZ garden (Table 2.2, significant garden effects). However, reproductive effort was negatively associated with plant size and the allocation of biomass to floral structures in parsnips grown in NZ was only approximately 1/6th of that allocated by parsnips grown in the US, resulting in an overall lower reproductive effort in the NZ common garden (Fig. 2.2, Table 2.2). The strong garden effect is highlighted by significant differences in plant size, fitness and damage between half-sibs grown in both gardens (Table 2.3).
I found significant garden x country interactions for most phenotypic traits examined in this study. US parsnips were larger and had higher realized fitness and greater pollination success in the US garden while NZ parsnips were more successful in the NZ garden (Figures 2.3, 2.4 and 2.5; Table 2.2, significant country x garden interactions). However, reproductive effort was consistently lower for NZ parsnips grown in both gardens (Fig. 2.3 b). US parsnips also sustained more damage in the US garden whereas NZ parsnips sustained more damage in the NZ garden (Fig. 2.4).

Effect of webworms on pollination success

I did not find an overall significant effect of spray treatment on pollination success but I did find a significant country x garden x spray interaction (Table 2.2). Webworms had a significant effect on pollination success of US parsnips in the US garden but not in the NZ garden (Fig. 2.5). US parsnips without webworms had a higher proportion of flowers fertilized compared to sprayed parsnips in the US common garden. The effect of webworms on pollination success was mediated by plant size; webworms reduced pollination success in smaller plants but not in larger plants (interaction effect of plants size x spray treatment scaled deviance = 25.51, p<0.001).

Phenotypic changes in New Zealand parsnips over time

Reduced realized fitness as a result of webworm florivory was consistent in parsnip populations with no infestation history from 2006 to 2009. However, parsnip populations with 3 and 6 years of webworm infestation showed a reduction in fitness due to florivory in the years 2006 and 2007 and a smaller effect of webworms in 2008 and 2009, suggesting the evolution of tolerance to herbivory (Fig. 2.6), although with small sample sizes and high year-to-
year variation this interaction did not prove to be statistically significant (Fig. 2.6; year x infestation history x spray treatment L=3.26, df=1, p=0.35). The difference between infested and uninfested parsnips is closer to canonical statistical significance in a comparison of just the first and last years of the study, 2006 and 2009 (L=2.65, df=1, p=0.10). Parsnips from infested populations were significantly larger after 3 to 6 years of infestation compared to parsnips from uninfested populations in 2009 (Fig. 2.7; year x infestation history L=10.34, df=1, p=0.01).

Parsnips with a history of infestation did not have lower total damage or higher pollination success in 2009 compared to parsnips with no infestation history (total damage L= 4.53, df=1, p=0.20, proportional damage L= 4.78, df=1, p=0.18 and pollination success SD = 1.62, df=1, p=0.65).

**Floral chemistry traits and phenotypic change over time**

Parsnip chemistry differed according to country of origin, with significantly higher octyl acetate and myristicin in US parsnips and higher xanthotoxin and bergapten in NZ parsnips (Table 2.4; Fig. 2.8). Because these four compounds did not differ between half-sibs grown in the different gardens, their production is not likely environmentally determined (Table 2.3). However, four of the eight compounds—octyl butyrate, sphondin, isopimpinellin and imperatorin—were significantly different between gardens and between half-sibs grown in different gardens (Fig. 2.8; Tables 2.3 and 2.4). The aliphatic ester octyl butyrate and the angular furanocoumarin sphondin were produced in much larger quantities in the NZ garden, whereas two linear furanocoumarins, imperatorin and isopimpinellin, were produced in lower quantities in the NZ garden; these differences are suggestive of some degree of phenotypic plasticity in floral chemistry (Fig. 2.8). Octyl butyrate, myristicin and isopimpinellin production
also had a significant country by garden interaction, indicating that there is a genetic basis underlying the floral chemical profile but response to selection can be dependent on environmental conditions (Table 2.4). The phenotypic plasticity of octyl butyrate, isopimpinellin and imperatorin was partially driven by plant size; large plants produced higher amounts of octyl butyrate and lower amounts of isopimpinellin and imperatorin (octyl butyrate, $\rho = 0.71$, $p < 0.001$, isopimpinellin, $\rho = -0.12$, $p = 0.003$ and imperatorin, $\rho = -0.12$, $p = 0.001$, $n = 739$).

I found no evidence of change in overall floral chemistry in all NZ populations from 2006 to 2009 (ANOSIM tests CRIMP $R = -0.27$, $P = 0.79$; WARRINGTON global $R = 0.033$, $P = 0.25$; ROCK $R = 0.056$, $P = 0.015$; COTTAGE $R = 0.006$, $P = 0.35$; HERBERT $R = -0.003$, $P = 0.50$).

**DISCUSSION**

Consistent with previous research (Berenbaum et al., 1986, Zangerl et al., 2008), I found that parsnip webworms significantly reduced wild parsnip survivorship and reproductive effort. Realized fitness was strongly positively associated with plant size but larger plants invested less of their biomass in seed, resulting in lower reproductive effort for larger plants. Despite the relatively lower reproductive effort, realized fitness is much higher in large plants; thus, even though diverting resources to achieve larger overall size can reduce seed production, plants may benefit from growing larger when resource availability is high, as it may have been in the NZ garden. That webworms had a smaller effect on the reproductive effort of larger plants (in the NZ garden) suggests that large size can mitigate the effect of seed loss via florivory. Larger plants in the NZ garden sustained more florivory and consequently experienced greater total damage but they also achieved higher realized fitness because plant size was very strongly
associated with realized fitness. In the US garden, however, reproductive effort, the proportion of biomass invested in seeds, was negatively associated with total damage, indicating that high levels of damage were costly for the plant. I did not find a negative relationship between reproductive effort and total damage in the NZ garden, where plants were 15 times larger than in the US garden, supporting the inference that losses to florivores are less costly for larger plants.

Plants have evolved a diversity of defense mechanisms against herbivory, including resistance (antibiosis), repellency (antixenosis) and tolerance. Tolerance is the ability of a plant to sustain damage without any demonstrable reductions in fitness (Strauss and Agrawal, 1999, Mauricio et al., 1997) and it often measured as compensation for losses to herbivores via enhanced growth (Nunez-Farfan et al., 2007). Recent studies suggest that plants may invest in mixed defense strategies (resistance vs tolerance) under different ecological conditions (Agrawal, 2011; Turley et al., 2013). For example, Carmona and Fornoni (2013) found that, in Datura stramonium, resistance and tolerance act as complementary defenses against two different folivores, which, when present together, select for intermediate levels of the two strategies. Mixed defense strategies may also evolve under different abiotic conditions; tolerance to herbivory is greater in the absence of competition and under high resource availability (Mutikainen and Walls, 1995; Wise and Abrahamson, 2008). In wild parsnips, populations can evolve enhanced resistance via increased production of furanocoumarins (Berenbaum et al., 1986; Zangerl and Berenbaum, 2005). In this study, however, I found no evidence of change in overall floral chemistry in any of the NZ populations from 2006 to 2009,
so that rapid evolution of chemically-mediated resistance to webworms did not appear to occur over the short duration of infestation in NZ.

Tolerance to webworms has not previously been demonstrated to exist in *P. sativa*. Because the number of inflorescences is predetermined prior to bolting, compensatory growth in response to floral tissue loss cannot occur in this species, but increasing overall size appears to mitigate the effects of webworm florivory, suggesting that growing larger may be an alternate or additional strategy for *P. sativa*. Large plants have much higher pollination success and realized fitness in spite of higher webworm damage and size is likely under strong selection after three to six years of intense florivory. The difference in size between parsnips with and without a history of infestation may become even more evident when environmental factors that can influence plant size, such as competition and resource availability, are controlled, as in a common garden setting.

In addition to having an effect on wild parsnip survivorship and reproductive effort, the presence of webworms had a significant impact on pollination success in this study. Florivores directly influence plant fitness through the consumption of reproductive parts but they can also indirectly influence fitness via a reduction in pollination visitation and efficacy. Florivore damage can change flower morphology, visual appearance (Lohman et al., 1996) and volatile emissions to render flowers less attractive to pollinators (Mothershead and Marquis, 2000; Zangerl and Berenbaum, 2009; Cardel and Kotpur, 2010; Botto-Mahan et al., 2011). Damaged parsnip flowers emit high levels of octanol, a component of webworm frass, and have lower seed set compared to undamaged flowers and the presence of octanol itself can reduce pollination success (Zangerl and Berenbaum, 2009). In this study, webworms reduced
pollination success in small plants but not in larger plants irrespective of where they originated and where they grew. Large plants emitted substantially more octyl butyrate, a putative pollinator attractant (Jogesh et al., 2013), and had higher pollination success. An increase in size may also increase the strength of the visual and volatile signal, attracting more pollinators; umbel size in other species has been linked to pollinator visitation rates (Thomson, 1988; Danderson and Molano-Flores, 2010). Thus, effects of webworm damage in large plants may be moderated by enhanced pollination success. At least in the US, seed set in wild parsnip is strongly pollinator-limited; inadequate pollination is the likely cause of parthenocarpic seed production in this species (Zangerl et al., 1991).

Overall, NZ parsnips did not have lower realized fitness and were not more susceptible to florivory than US parsnips. I found strong evidence for local adaptation of US genotypes to the US garden and NZ genotypes to the NZ garden; US parsnips were larger with higher realized fitness and pollination success in the US garden whereas NZ parsnips were larger with higher realized fitness and pollination success in the NZ garden. However, the cost of seed production for US parsnips (as reflected by reproductive effort) was consistently lower compared to NZ parsnips. I also found local adaptation in US and NZ parsnips in terms of webworm damage and pollination success. Previous studies have shown that coevolved species can display local adaptation in both antagonistic and mutualistic interactions with plants (Kalse et al., 2012). Because both total damage and pollination success were positively correlated with plant size, higher levels of damage and pollination success in the “home” country may have been an artifact of larger size in the “home” country. Webworm moths are known to prefer to oviposit
on larger plants (Zangerl and Berenbaum, 1992), resulting in greater levels of damage and
larger plants may emit larger quantities of floral volatiles.

Local adaptation occurs when resident genotypes have higher fitness in their local
environment compared to exotic genotypes and is a consequence of either strong selection in
one or both populations, genetic drift, and/or geographic isolation over a long period of time
(Kawecki and Ebert, 2004; Blanquart et al., 2013). A meta-analysis of local adaptation in plants
revealed that it is fairly common even over small spatial scales (Leimu and Fischer, 2008).
Where NZ wild parsnip populations originated and the extent to which their gene pool has been
replenished from North American or European wild parsnips is unknown. Higher fitness (both
actual and realized) in the “home country” appears to be driven by size, a phenotypically plastic
trait, which may be driven by genetics or epigenetics (Holeski et al., 2012; Des Marais et al.,
2013). Size is a strong indicator of resource acquisition and individual differences in resource
acquisition determine the ability of a plant to invest resources so as to increase fitness
(Robinson and Beckerman, 2013). That fitness is strongly determined by resource acquisition
and that resource acquisition is genetically and environmental driven suggests that plant size
can be under herbivore selection but the evolutionary trajectories may differ with abiotic
conditions.

A comparison of NZ parsnips with and without a history of webworm infestation
indicated that populations previously associated with webworms may be less susceptible to
herbivory after 3 to 6 years of being infested; this reduction in susceptibility appears to be a
consequence of selection for increased plant size over time. Previous work has shown that
traits associated with plant size (e.g., height or biomass) can have high heritability and can
respond rapidly to selection; for example Johnson et al., (2009), found that biomass has high heritability and is under positive selection by herbivores in *Oenothera biennis*. Herbivore selection on life-history traits might be more common than previously assumed (Carmona et al., 2011). Evolution of increased size has been documented in other invasive species (Siemann and Rogers, 2001; Prentis et al., 2006; Graebner et al., 2012); however, in none of these systems has larger size in response to florivory been examined.

Despite the fact that furanocoumarins are strongly associated with parsnip resistance to webworms in both Europe, where the interaction is native, and North America, where it is invasive, and despite the fact that Zangerl et al., (2008) found that several furanocoumarin traits were under selection by webworm florivory in New Zealand, I did not find any evidence to suggest increased chemical resistance to herbivory has evolved in NZ populations with a 3- to 6-year history of infestation compared to uninfested populations. Response to selection for increased chemical resistance may be constrained by genetic correlations between chemical traits and life-history traits (Johnson et al., 2009) or by negative genetic correlations between chemical resistance traits (Berenbaum et al., 1986). Octyl butyrate levels in this study were positively associated with size whereas imperatorin and isopimpinellin levels were negatively associated with size. Our common garden experiment with half-sib parsnips grown in two different environments (US and NZ) also showed that some of these chemical traits are phenotypically plastic, which has not been previously documented for chemical constituents of wild parsnip reproductive structures. Half-sibs grown in different gardens had strikingly different levels of octyl butyrate, isopimpinellin and imperatorin and I found a significant genotype by environment interaction for these chemicals. Even though these compounds may
be under selection, chemical phenotype is likely to be strongly influenced by the environment. Plasticity of resistance traits may be especially important if the costs of production are high (Coley et al., 1985; Hare et al., 2003), as they are for furanocoumarins (Zangerl and Berenbaum, 1997; Zangerl et al., 1997). That small plants produced higher levels of the defensive compounds imperatorin and isopimpinellin suggests that deterring florivory is more important for small plants; larger plants may have more resources to allocate to flower production and to pollinator attraction to offset losses due to florivory.

Evolution in invasive species has been documented with increasing frequency. Many evolutionary changes are associated with the colonization of a new range or escape from natural enemies (Maron et al., 2004; Prentis et al., 2008; Joshi and Tielbörger, 2012), but these responses are likely short-lived as plants form new associations with native species or reunite with coevolved enemies that also colonize the new range (Siemann et al., 2006; Lu and Ding, 2012). Few studies have quantified responses in invasive plants to reassociation with their coevolved specialist herbivores even though this reassociation is the basis of classical biological control (e.g., Rapo et al., 2010). Wild parsnips in New Zealand have responded remarkably rapidly to strong selection by webworms and this response is even more notable in view of the fact that wild parsnip is a biennial. Whether the evolutionary response is of tolerance or chemically mediated resistance is of less economic consequence than is the fact that coevolved species can evolve rapidly after reassociation. This rapid adaptation and post-establishment evolution may underlie some of the failures in initial weed biocontrol efforts and the long time lags in some of the successful efforts (McFadyen, 2000; Mueller-Schaerer et al., 2004; Atwood and Myerson, 2011).
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REFERENCES


## FIGURES AND TABLES

Table 2.1: New Zealand wild parsnip population sizes and parsnip webworm infestation levels from 2006 to 2009. Infestation level was measured as the percentage of all plants in the population with webworms. Number of plants at each site indicated in brackets.

<table>
<thead>
<tr>
<th>Population</th>
<th>Lat./Long.</th>
<th>2006</th>
<th>2007</th>
<th>2008</th>
<th>2009</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infested for 6 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CRIMP</td>
<td>S 45.89976,E 170.439749</td>
<td>100 (65)</td>
<td>97 (30)</td>
<td>100 (9)</td>
<td>78 (23)</td>
</tr>
<tr>
<td>LUMBER</td>
<td>S 45.900044,E 170.443954</td>
<td>100 (96)</td>
<td>100 (44)</td>
<td>100 (45)</td>
<td>100 (28)</td>
</tr>
<tr>
<td>TOWNLEY</td>
<td>S 45.893159,E 170.459071</td>
<td>100 (116)</td>
<td>100 (79)</td>
<td>100 (26)</td>
<td>100 (3)</td>
</tr>
<tr>
<td>WARRINGTON</td>
<td>S 45.707985,E 170.589459</td>
<td>71 (31)</td>
<td>100 (34)</td>
<td>95 (20)</td>
<td>100 (27)</td>
</tr>
<tr>
<td>Infested for 3 years</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>OCEAN</td>
<td>S 45.699938,E 170.603256</td>
<td>0 (200)</td>
<td>60 (126)</td>
<td>20 (30)</td>
<td>20 (54)</td>
</tr>
<tr>
<td>ROCK</td>
<td>S 45.658185,E 170.641987</td>
<td>0 (200)</td>
<td>76 (144)</td>
<td>100 (82)</td>
<td>67 (200)</td>
</tr>
<tr>
<td>Uninfested</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>COTTAGE</td>
<td>S 45.678391,E 170.625991</td>
<td>0 (200)</td>
<td>0.05 (200)</td>
<td>4 (48)</td>
<td>1 (113)</td>
</tr>
<tr>
<td>BUSHY</td>
<td>S 45.468408,E 170.776248</td>
<td>0 (200)</td>
<td>0 (200)</td>
<td>0 (200)</td>
<td>0 (200)</td>
</tr>
<tr>
<td>HERBERT</td>
<td>S 45.234797,E 170.78011</td>
<td>0 (200)</td>
<td>0 (200)</td>
<td>0 (200)</td>
<td>1 (200)</td>
</tr>
</tbody>
</table>
Table 2.2: Models comparing phenotypic traits between countries and gardens with log likelihood ratio (L) and p-values of main and interaction terms. Difference in AICs in quasibinomial models are estimated with Scaled deviance (SD) instead of L. Significant effects (*) are in bold.

<table>
<thead>
<tr>
<th>Model</th>
<th>Dependent variable</th>
<th>Stalk weight</th>
<th>country</th>
<th>garden</th>
<th>country x garden</th>
<th>spray</th>
<th>spray x country</th>
<th>spray x garden</th>
<th>country x spray x garden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model 1</td>
<td>Realized fitness</td>
<td>L=166.03, p&lt;0.001*</td>
<td>L=14.48, p&lt;0.001*</td>
<td>L=113.22, p&lt;0.001*</td>
<td>L=63.38, p&lt;0.001*</td>
<td>L=7.57, p=0.005*</td>
<td>L=0.05, p=0.81</td>
<td>L=0.60, p=0.43</td>
<td>L=0.07, p=0.78</td>
</tr>
<tr>
<td>Model 2</td>
<td>Reproductive effort</td>
<td>L=40.67, p&lt;0.001*</td>
<td>L=10.19, p=0.006*</td>
<td>L=7.11, p=0.007*</td>
<td>L=7.75, p&lt;0.005*</td>
<td>L=13.47, p&lt;0.001*</td>
<td>L=0.16, p=0.68</td>
<td>L=6.84, p=0.008*</td>
<td>L=0.71, p=0.39</td>
</tr>
<tr>
<td>Model 3</td>
<td>Plant size</td>
<td>-</td>
<td>L=1.83, p=0.17</td>
<td>L=1241.0, p&lt;0.001*</td>
<td>L=179.40, p&lt;0.001*</td>
<td>L=2.66, p=0.10</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model 6</td>
<td>Total damage</td>
<td>L=7.26, p=0.007*</td>
<td>L=1.54, p&lt;0.21</td>
<td>L=31.06, p&lt;0.001*</td>
<td>L=38.22, p&lt;0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model 7</td>
<td>Proportional damage</td>
<td>SD=17.22, p&lt;0.001*</td>
<td>SD=17.33, p&lt;0.001*</td>
<td>SD=20.46, p&lt;0.001*</td>
<td>SD=29.28, p&lt;0.001*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Model 8</td>
<td>Pollination success</td>
<td>SD=8.06, p=0.004*</td>
<td>SD=18.38, p&lt;0.001*</td>
<td>SD=11.74, p&lt;0.001*</td>
<td>SD=11.32, p&lt;0.001*</td>
<td>SD=0.06, p=0.79</td>
<td>SD=0.62, p=0.74</td>
<td>SD=0.04, p=0.82</td>
<td>SD=7.23, p=0.007*</td>
</tr>
</tbody>
</table>
Table 2.3: Differences in damage, fitness, size and floral chemistry between maternal half-sibs grown in the US common garden and NZ common garden. Pair-wise differences were estimated based on the Wilcoxon signed-rank test.

<table>
<thead>
<tr>
<th></th>
<th>W+</th>
<th>W-</th>
<th>N</th>
<th>p</th>
<th>US garden</th>
<th>NZ garden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Damage (proportion)</td>
<td>359</td>
<td>1411</td>
<td>59</td>
<td>&lt;0.001*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Size</td>
<td>9</td>
<td>1369</td>
<td>52</td>
<td>&lt;0.001*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Fitness</td>
<td>33</td>
<td>495</td>
<td>32</td>
<td>&lt;0.001*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Octyl butyrate</td>
<td>1</td>
<td>2484</td>
<td>70</td>
<td>&lt;0.001*</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Octyl acetate</td>
<td>1407</td>
<td>1078</td>
<td>70</td>
<td>0.33</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Xanthotoxin</td>
<td>1226</td>
<td>1259</td>
<td>70</td>
<td>0.92</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Bergapten</td>
<td>1211</td>
<td>1224</td>
<td>70</td>
<td>0.85</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Sphondin</td>
<td>556</td>
<td>1040</td>
<td>56</td>
<td>0.05*</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Myristicin</td>
<td>961</td>
<td>1454</td>
<td>69</td>
<td>0.14</td>
<td>=</td>
<td>=</td>
</tr>
<tr>
<td>Imperatorin</td>
<td>1656</td>
<td>759</td>
<td>69</td>
<td>&lt;0.001*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Isopimpinellin</td>
<td>1855</td>
<td>630</td>
<td>70</td>
<td>&lt;0.001*</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Beta-Farnesene</td>
<td>1967</td>
<td>518</td>
<td>70</td>
<td>&lt;0.001*</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2.4: Models comparing chemical traits between countries or origin and gardens with log likelihood ratio (L) and p-values of main and interaction terms. Significant effects (*) are in bold.

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>country</th>
<th>garden</th>
<th>country x garden</th>
</tr>
</thead>
<tbody>
<tr>
<td>Octyl butyrate</td>
<td>L=37.58, p&lt;0.001*</td>
<td>L=852.44, p&lt;0.001*</td>
<td>L=55.16, p&lt;0.001*</td>
</tr>
<tr>
<td>Octyl acetate</td>
<td>L=4.89, p=0.026*</td>
<td>L=0.11, p=0.73</td>
<td>L&lt;0.01, p=0.99</td>
</tr>
<tr>
<td>Xanthotoxin</td>
<td>L=8.35, p=0.003*</td>
<td>L=0.75, p=0.38</td>
<td>L=0.45, p=0.50</td>
</tr>
<tr>
<td>Bergapten</td>
<td>L=2.89, p=0.08*</td>
<td>L=2.18, p=0.13</td>
<td>L=0.19, p=0.65</td>
</tr>
<tr>
<td>Isopimpnellin</td>
<td>L=1.16, p=0.28</td>
<td>L=60.06, p&lt;0.001*</td>
<td>L=4.72, p=0.02*</td>
</tr>
<tr>
<td>Imperatorin</td>
<td>L=0.05, p=0.81</td>
<td>L=55.16, p&lt;0.001*</td>
<td>L=0.65, p=0.41</td>
</tr>
<tr>
<td>Sphondin</td>
<td>L=1.96, p=0.16</td>
<td>L=25.16, p&lt;0.001*</td>
<td>L=0.004, p=0.94</td>
</tr>
<tr>
<td>Myristicin</td>
<td>L=10.05, p=0.001*</td>
<td>L=26.30, p&lt;0.001*</td>
<td>L=8.91, p=0.002*</td>
</tr>
</tbody>
</table>
Figure 2.1: Total realized fitness of wild parsnips with webworms (no spray, n=495) and without webworms (spray, n=474) in (a) US garden and (b) NZ garden. Fitness is strongly predicted by plant size ($L = 139.79$, df= 1, $p < 0.001$) and significantly higher without webworms ($L=7.57$, df= 1, $p =0.005$).
Figure 2.2: Reproductive effort (total seed weight g/ stalk weight g) of wild parsnips with webworms (no spray, n=495) and without webworms (spray, n=474) in (a) US garden and (b) NZ garden. Reproductive effort is negatively associated with plant size ($L = 139.79$, $df = 1$, $p < 0.001$) and significantly higher without webworms ($L=7.57$, $df=1$, $p =0.005$).
Figure 2.3: Realized fitness (a) and reproductive effort (b) for US and NZ parsnips, with and without webworms (spray treatment) grown in the US common garden and the NZ common garden.
Figure 2.4: Plant size (a) and proportional damage (b) for US and NZ parsnips grown in the US common garden and the NZ common garden. Spray treatment had no effect on plant size (F=0.006, p = 0.93) and only unsprayed plants were analyzed for webworm damage.
Figure 2.5: Pollination success of US and NZ parsnips with and without webworms (spray treatment) grown in the US common garden and the NZ common garden. Pollination success was measured as realized fitness/total number of potential seeds. Only parsnips sprayed with insecticide were analyzed. Effect of country x garden x spray on pollination success, scaled deviance = 7.23, p=0.007
Figure 2.6: Realized fitness of NZ parsnips from populations with a 3-6 year history of webworm infestation compared with populations with no history of infestation in the presence and absence of webworms from 2006 to 2009. Effect of infestation history x year x spray treatment on realized fitness $L=3.26$, $df=1$, $p=0.35$
Figure 2.7: Plant size of NZ parsnips from populations with a 3-6 year history of webworm infestation (black) compared with populations with no history of infestation (grey) collected from 2006 to 2009. Effect of infestation history x year on plant size (L=10.34, df=1, p =0.01).
Figure 2.8: Comparison of floral compounds between US and NZ parsnips grown in US and NZ gardens. Average peak areas per mg of compound are plotted in the x-axes. Error bars represent standard error values.
Chapter 3 - Impact of reassociation with a coevolved herbivore on oviposition deterrence in a hostplant: Depressaria pastinacella and Pastinaca sativa

ABSTRACT

The parsnip webworm, Depressaria pastinacella (Lepidoptera: Oecophoridae) is a highly specialized florivore restricted to feeding on the wild parsnip Pastinaca sativa L. (Apiaceae) throughout much of its range. Webworm adults oviposit on leaves of pre-bolting plants and the larvae feed exclusively on floral tissues. Previous studies have demonstrated that webworm florivory selects for increased production of feeding deterrents in floral tissues; mutually exclusive use of different plant parts for oviposition and larval feeding suggests that oviposition by webworm moths should simultaneously select for increased production of deterrents or decreased production of attractants in webworm foliage. The recent invasion of New Zealand (NZ) by parsnip webworms provided an opportunity to assess phenotypic changes in parsnip chemicals that mediate oviposition in response to the presence of ovipositing moths. I planted a common garden in the United States with seeds from long-infested United States populations, NZ populations that had never been infested, and NZ populations infested for 3 years (since 2007) or 6 years (since 2004) in order to ascertain the relationships between parsnip floral and foliar chemistry and webworm oviposition, larval feeding, and plant reproductive effort (realized fitness/biomass). Using gas chromatography-electroantennogram detection analysis, I identified three parsnip leaf volatiles that elicited a strong and consistent response from female antennae: cis- and trans-ocimene and β-farnesene. All three compounds were negatively associated with oviposition, suggesting that they function as oviposition
deterrents. Leaf β-farnesene content was positively correlated with levels of floral furanocoumarins that serve as defenses against florivores; higher rates of oviposition on plants with low floral furanocoumarin levels indicate that adult moths preferentially oviposit on parsnips that are more suitable for larval growth. Although webworm feeding damage reduces plant reproductive effort, the presence of a greater number of eggs on leaves did not similarly lower reproductive effort for the plant. Consistent with the low fitness impact of oviposition, NZ parsnips showed no phenotypic change in foliar chemistry in response to 3 to 6 years of webworm infestation, suggesting that selection exerted by ovipositing moths on foliar chemistry is not as strong as selection exerted by larvae on floral chemistry.

**INTRODUCTION**

For most lepidopterans, early larval stages are relatively immobile and host choice is determined largely by the ovipositing adult. Thus, according to the preference-performance hypothesis, the ability to differentiate among and then select host individuals that are suitable for offspring has significant fitness implications for the ovipositing female (Gripenberg et al. 2010; Jaenike 1978). The three phases of oviposition—host searching, alighting and substrate acceptance—are generally mediated by physical and chemical attributes of the host plant (Renwick and Chew 1994), and acceptance of the host is often tightly linked to host-plant chemistry (Thompson and Pellmyr 1991).

In many systems, larvae and adults interact with the same tissues and host choice based on larval suitability is predictable because chemical signals are shared by both life-stages. For oligophagous lepidopterans, non-host volatiles can function as oviposition deterrents. Adult
female diamondback moths (*Plutella xylostella*), e.g., which are specialists on Brassicaceae, are repelled by foliar monoterpenes from the asteraceous non-host *Chrysanthemum morifolium* (Wang et al. 2008); similarly, the Brassicaceae specialist *Pieris rapae* recognizes glucosinolates from brassicaceous foliage but is deterred by cardenolides in foliage of *Erysimum cheiranthoides*, a Brassicaceae non-host (Renwick et al. 1989). In addition to relying on chemistry to recognize appropriate host species, narrowly oligophagous or monophagous lepidopterans can detect intraspecific differences in chemistry and choose plants that are chemically more suitable (Reudler Talsma et al. 2008; Prudic et al. 2005), less well-defended (Bruinsma et al. 2007; Khan et al. 1987), nutritionally superior (Heisswolf et al. 2005; Taylor and Forno 1987), or lacking prior damage (Wise and Weinberg 2002). In these systems, the same defense signal may be under selection by both adults and larvae. Glucosinolates in leaves of Brassicaceae, for example, are attractants for both ovipositing pierid butterflies and folivorous pierid larvae (Renwick and Lopez 1998; Renwick and Chew 1994). By contrast, indole-3-acetonitrile, a glucosinolate byproduct, is deterrent to both life-stages and plants in the presence of pierids experience selection to increase its production (de Vos et al. 2008).

For some lepidopterans, however, oviposition is spatially or temporally uncoupled from larval feeding. Recent evidence suggests that plants can defend themselves against oviposition via the release of induced volatile chemicals (Hilker and Meiners 2006; Hilker and Meiners 2002). Egg-laying itself can induce systemic defenses that have an effect on larval growth and survival (Beyaert et al. 2011) or trigger volatile emissions that attract a third trophic level (Colazza 2004). However, few studies have examined the evolution of constitutive defenses against oviposition. While there is evidence that chemical defenses, tolerance and life-history
traits can evolve in response to herbivore feeding (Turley et al. 2013; Agrawal et al. 2012), whether plants can respond to selection exerted by ovipositing females by producing oviposition deterrents has not been extensively examined. Such a selective response seems especially likely if high egg loads result in proportionally greater damage and fitness costs to the plant. The ability to respond to selection imposed by ovipositing females may be most easily detected in systems in which adults and larvae interact with different life-stages of plants and where chemical defenses against larval herbivory and adult oviposition are uncoupled. Encounters with ovipositing females, then, should impose strong selection on plants to reduce production of chemicals that act as oviposition attractants and/or to increase production of chemicals that act as oviposition deterrents or repellents, irrespective of the effects of these chemicals on larval development.

In this study, I investigated the chemical basis of interactions between the wild parsnip, *Pastinaca sativa* L. (Apiaceae), and its coevolved specialist florivore the parsnip webworm, *Depressaria pastinacella* Duponchel (Lepidoptera: Oecophoridae). Both parsnips and webworms are native to Europe and are invasive in North America and New Zealand (NZ). Although parsnips have been recorded in NZ since 1867, webworms were discovered only recently, in 2004, in Port Chalmers on the South Island. The recent invasion of webworms in NZ provided us with a unique opportunity to quantify phenotypic changes in wild parsnips in response to selection by webworms (Zangerl et al. 2008). *D. pastinacella* feeds almost exclusively on floral tissues. High levels of three furanocoumarins—bergapten, xanthotoxin and sphondin— are associated with resistance to webworms (Berenbaum et al. 1986; Zangerl and Berenbaum 1993). Webworm florivory has a direct substantial impact on parsnip fitness,
exerting strong selection for increased furanocoumarin content (Berenbaum et al. 1986).

Phenotype matching over small spatial scales in the midwestern United States (US) suggests that reciprocal selection between parsnips and webworms takes place, whereby increased production in plant defense chemistry results in selection for increased insect detoxification capacity (Berenbaum and Zangerl 1998; Zangerl and Berenbaum 2003).

In contrast to larval interactions with host plants, webworm adults interact solely with parsnip rosette leaves, at a time when no floral structures are present. While floral furanocoumarins (and to a lesser extent aliphatic esters) mediate interactions with larval stages (Berenbaum et al. 1986; Zangerl and Berenbaum 1993; Carroll and Berenbaum 2002), levels of floral furanocoumarins are almost never correlated with foliar furanocoumarin content and esters are completely absent in leaf tissues (Berenbaum and Feeny 2007). Foliar furanocoumarin content is also not correlated with host selection by ovipositing moths (Zangerl and Berenbaum 1992). Given the long coevolutionary association between webworms and wild parsnips, that webworm adults rely on chemical cues in leaves to assess the potential suitability of floral tissues for larval development seems highly likely. If such is the case, ovipositing webworm moths should exert strong selection on parsnip foliar chemistry to decrease production of oviposition attractants and/or increase production of oviposition deterrents. To evaluate this possibility, I examined the headspace of parsnip rosette leaves to determine which compounds are present and detectable by female moths via gas chromatography coupled electroantennographic detection (GC-EAD) and then determined whether these compounds are attractive or deterrent in the field to ovipositing moths. In a common garden experiment, I also compared populations of NZ parsnips with and without a history of
infestation, collected from 2006 to 2009, to determine if parsnip populations have experienced predictable changes in their foliar chemistry after 3 to 6 years of webworm infestation.

**MATERIALS AND METHODS**

**Life history of *P. sativa* and *D. pastinacella***

*P. sativa* is a biennial plant; after germination a rosette is produced and a long taproot overwinters. The following spring, if sufficient reserves have been accumulated the preceding year, the rosette produces a flowering stalk. In central Illinois, parsnips produce rosettes in March, bolt in early May, flower in June, and set seeds in mid- to late July. Parsnip webworms overwinter as adults and oviposit on spring rosette leaves in the first two weeks of April. The larvae emerge after 4 to 5 days, move up to unopened buds of the bolting plants and feed almost exclusively on floral tissues throughout their development, progressively consuming buds, flowers and fruits. Ultimate (sixth) instar larvae move down to the base of the plant, burrow into parsnip stalks, and pupate inside the hollow stalks. In 2 to 3 weeks, adults emerge, overwinter and mate in the spring of the following year. In NZ, flowers bolt in late November, flower in December and set seed in January. In contrast to the US, NZ webworms oviposit in December, after parsnips have already bolted and are in flower.

**GC-EAD analysis of *D. pastinacella* antennae**

A laboratory colony of parsnip webworms was established with third and fourth instar larvae collected from a field site in Urbana, IL (OVERPASS population: N 40.13205, W 88.143826) (Jogesh et al. 2013). Caterpillars were reared on a semi-artificial diet, supplemented with dried parsnip seeds collected from local populations (Nitao and Berenbaum, 1988) at 25°C,
18:6 (L:D) photoperiod. The pupae were sexed and five males and five females were placed in containers over folded paper towels, to ensure normal wing-expansion at eclosion. The adults underwent diapause in a coldroom at 10°C under 6:18 (L:D) photoperiod. After two months under overwintering conditions, adults resume activity, mate and readily oviposit when exposed to 25°C and 18:6 (L:D) photoperiod (Nitao and Berenbaum, 1988). Adults were removed from laboratory overwintering (coldroom at 10°C) in October, sexed, and prepared for GC-EAD analysis.

Antennae were excised with fine scissors, clipped on both ends, and placed into custom-made glass capillaries filled with Ringer’s solution and fitted onto gold wire electrodes. I measured the response of parsnip webworm antennae to headspace extracts of wild parsnip foliar volatiles collected via elution in hexane and via Solid Phase Microextraction (SPME) fibers. For hexane elution, rosette leaves from *P. sativa* were sealed in glass Mason jars (three leaves per jar) and stored at room temperature for 30 minutes. After this time period, a glass tube (6 cm long×9.5 mm o.d.×4 mm i.d.) packed with 100 mg of 80/100 mesh HayesepQ® (Alltech Associates, Deerfield, IL, USA) was fitted over one inlet of a custom-made Mason jar lid with a Teflon® disc equipped with an 8-cm long section of Teflon® tubing. Charcoal-purified air was pulled through the apparatus with a water aspirator (Oakton, Vernon Hills, IL) at a rate of 1 L min⁻¹. Collectors were eluted into silanized glass vials (Cat. #27114, Supelco®, Bellefonte, PA, USA) with three 0.5-ml aliquots of hexane. The resulting extracts were analyzed with a Hewlett-Packard® (Sunnyvale, CA, USA) 6890 gas chromatograph (GC) coupled to a 5973 mass selective detector (MS). For SPME collection, a manual SPME holder and a fiber coated with polydimethylsiloxane PDMS of 100 µm thickness (Supelco®, Bellefonte, PA, USA) were used for
the SPME procedure. Three leaves of P. sativa were sealed with aluminum foil in glass Mason jars and stored at room temperature for 30 minutes. For each extraction, the SPME needle pierced the aluminum foil, and the fiber was then exposed to the headspace above the sample at room temperature. After an extraction time of 10 minutes, the fiber was withdrawn from the sample and inserted directly into the injection port of the GC-MS. The desorption of analytes from the fiber coating was done through the heating of the fiber in the injection port of the GC-MS at 250°C for 5 minute.

The GC-EAD analysis was conducted with a Hewlett Packard® (Sunnyvale, CA, USA) gas chromatograph (GC), model 5890 series II, equipped with an AT-5MS fused silica capillary column (30 m×0.32 mm) coated with polyimide (0.25 µm film thickness) (Grace, Deerfield, IL). I injected 1 µl of sample in splitless mode using an oven temperature program as follows: initial temperature of 50°C and held for 2 minutes raised to 210°C held for 5 minutes at a rate of 10°C/ minute. Temperature of injector and detector were set at 210°C. Helium is used as the carrier gas and compounds eluting from the column were split 1:1 in a two-way splitter and delivered to the FID and to the antenna. Compounds were carried to the antenna through a heated (230°C) transfer line (Syntech, Hilversum, The Netherlands) into a glass tube with humidified air stream at 0.5 m/sec. Signal was amplified with an AC preamplifier (Model p15, Grass Instruments, Quincy, MA) and analyzed with GC-EAD software (PeakSimple, SRI Instruments, Menlo Park, CA). EAD responses to FID peaks were identified as repeatable deflections of the baseline. Each extract was tested on 12 different antennae from 8 females and 4 males. Antennal responses were confirmed with pure compounds (β-farnesene, cis-ocimene, trans-ocimene SAFC Supply solutions, St Louis, MO).
Common garden oviposition experiment

A common garden was established at the University of Illinois Phillips Tract research area (N 40.13205, W 88.143826) (Champaign Co., IL) in an area that has been tilled every other year for the last 21 years (Jogesh et al. submitted). I planted parsnips grown from seeds collected from nine NZ populations in 2006, 2007, 2008 and 2009 and five US populations in central Illinois (DEA, KAN, PIO, OP and RAN), all of which were known to have been infested with webworms for many years (Jogesh et al. submitted; Zangerl et al. 2008; ). At the time of experimental setup, four of these populations had been infested for at least 6 years (CRIMP, TOWNLEY, LUMBER, WARRINGTON), two were infested for 3 years (since 2007) (OCEAN, ROCK) and three population had no or minimal (<1% damage) history of infestation (COTTAGE, BUSHY, HERBERT). At least 40 seeds from 20 maternal plants in each population (two maternal half-sibs per parent plant), for each seed collection year (2006-2009), were randomly planted in the garden (a total of 40x4x9 NZ plants and 5x40 US plants). Seeds were germinated in the UIUC Entomology greenhouse in early March 2009 in plastic pots filled with 1:1:1 peat:perlite:Drummer soil and grown at 24°C at 16 h day length. The seedlings were transplanted to the garden in early July 2009. Individuals were planted 0.5 m apart in 21 long rows, with rows 1 m apart. The parsnips were regularly watered and weeded.

In the spring following planting, I checked parsnip rosettes regularly for webworm eggs. Oviposition counts were made on all plants in the garden over a span of two days and a small piece of leaf tissue was collected for chemical analysis in a pre-weighed Eppendorf tube with a glass bead. The tubes were immediately frozen over dry ice to prevent volatilization of compounds and kept at -80°C in the laboratory. After the plants had bolted, I also collected ten
female flowers for chemical analysis and after all webworms had pupated I measured the proportion of all umbels damaged as outlined in another study (Jogesh et al. submitted). I collected seeds from all plants after they matured on stalks to estimate realized fitness. After all seeds were collected, the stalks were cut at the base, dried in an oven at 65°C and weighed to measure total plant biomass.

Frozen leaf and flower tissues collected in 1.5 ml Eppendorf tubes were pulverized with a glass bead in a Wig-L Bug Amalgamator (Crescent Dental Manufacturing, Chicago IL) for 1 minute. The pulverized tissue was refrozen, to reduce volatilizing of compounds, and the frozen material was vortexed with 750 µl of 100% HPLC-grade ethyl acetate containing 0.0001% tridecane, an internal standard. The tubes were centrifuged at 9000X for 3 minutes and the supernatant was pipetted into a 2-ml GC-vial. The pellets were dried at 50°C for 48 h and weighed to estimate tissue dry weight. Chemical extracts in ethyl acetate were analyzed by gas chromatography with flame ionization detection and subjected to gas chromatography-mass spectrometry (GC-MS).

One µL per sample was injected in the GC-MS (Shimadzu QP2010 Plus, SHRXI-5MS capillary column, 30m x 2.5mm x 0.25 µm) in splitless mode for 1.5 minutes with an injection temperature of 250°C and helium as the carrier. The initial oven temperature was set at 50°C, held for 50 seconds, increased at a rate of 10°C/minute until 250°C and held at 250°C for 5 minutes. A full scan of ions from 40 m/z to 300 m/z was recorded. Chemical peaks were identified by comparison to the NIST08 library and to standards when available. Amounts were quantified based on peak area integrations of total ion chromatograms. Areas were adjusted with the peak area of the internal standard (tridecane) and divided by the weight of the dried
tissue to obtain a peak area per milligram, a measure that could then be compared across samples.

**Statistical analyses**

I used a multivariate distance approach to examine chemical differences between 1) leaves and flowers, 2) US and NZ parsnips, 3) parsnip populations and 4) changes in infested and uninfested NZ parsnips over time. I tested differences in all compounds as well as in the subset of compounds that elicited an antennal response (β-farnesene, trans-ocimene and cis-ocimene). The data were square root-transformed and a resemblance matrix was created with Bray-Curtis dissimilarities and used to conduct an analysis of similarity (ANOSIM). ANOSIM tests for differences in rank dissimilarity between a priori defined groups compared to random groups. Much like an ANOVA, the ANOSIM statistic R compares the differences in ranks within and between groups. The analysis was conducted in PRIMER 6 (Clark and Warwick, 2001). Where significant differences between groups were observed, the chemicals with the greatest contribution to the difference were determined using similarity percentage analysis (SIMPER in PRIMER 6). To compare foliar and floral chemistry, US and NZ chemistry and inter-population chemistry, all data were analyzed; however, to compare changes in infested and uninfested populations over time, only the NZ data were analyzed.

Correlations between foliar compounds that elicited an antennal response and floral compounds were calculated using a non-parametric Spearman’s rank correlation to assess if any detectable leaf constituents could act as indicators for suitability of floral tissues for larvae. Correlations were calculated in IBM SPSS (IBM Corp. 2012).
To determine if webworm oviposition had a fitness impact on wild parsnips, I regressed egg counts against plant damage, to determine if a greater number of eggs resulted in proportionally greater damage; I also regressed egg count against reproductive effort, to determine if oviposition is associated with a higher cost of seed production. Reproductive effort measures the proportion of the plant’s biomass that is allocated to seed production (=Realized fitness/Plant biomass) and is an estimate of the cost of producing seeds. Damage was fitted with a quasibinomial Generalized Linear Model (GLM) whereas reproductive effort was fitted using the non-parametric Spearman’s rank correlation, owing to non-homogeneity of variance. I did not correlate egg counts with total realized fitness because both variables were strongly positively correlated with plant size (see Results). GLM analyses were conducted in R version 2.14.2 (R Development Core Team 2012).

Based on the results of the GC-EAD analysis, I examined the relationship between three compounds detected by antennae of female moths—β-farnesene, trans-ocimene and cis-ocimene—and egg counts in the common garden experiment to determine if these compounds functioned as oviposition attractants or deterrents. I compared peak areas (per mg) of these compounds in parsnip leaves that received no eggs, 1 to 5 eggs, or more than 5 eggs using a one-way ANOVA. Post-hoc pair-wise differences were estimated with Tukey’s HSD test. I also compared floral furanocoumarins, a measure of larval suitability (Berenbaum et al. 1989), in plants that received no eggs, 1 to 5 eggs, or more than 5 eggs with a one-way ANOVA. One-way ANOVA and post-hoc tests were conducted in IBM SPSS (IBM Corp. 2012).
RESULTS

Comparison of leaf and flower chemistry

Eight compounds were detected in the headspace of wild parsnip rosette leaves, including four monoterpenes (myrcene, cis-ocimene, trans-ocimene and alloocimene,) five sesquiterpenes (β-bourbonene, β-caryophyllene, trans-β-farnesene, germacrene D and α-farnesene), and the phenylpropanoid myristicin (Figure 3.1). In addition to compounds present in the headspace, constitutive chemical analysis of leaf tissues indicated the presence of non-volatile furanocoumarins and coumarin derivatives, including psoralen, xanthotoxin, bergapten, γ-palmitolactone, osthol and isopimpinellin. All foliar compounds, except osthol, were strongly positively correlated with each other.

Three compounds--β-farnesene, trans-ocimene and cis-ocimene--in the parsnip leaf headspace elicited a consistent response from antennae of webworm female moths while antennae of male moths did not respond to any of the compounds present in the headspace of parsnip leaves (Figure 3.1).

All compounds present in leaf tissue extracts were also detected in parsnip flower extracts. Additionally, floral chemical profiles were dominated by the aliphatic esters octyl acetate and octyl butyrate as well as two furanocoumarins absent in the leaves – sphondin and imperatorin. Even though many of the chemical constituents of flowers and leaves overlapped, the chemical profiles of flowers and leaves for shared compounds were significantly different from each other (Global R = 0.65, p = 0.001). Cumulatively, xanthotoxin, myristicin and palmitolactone explained 40% of the difference, with floral tissues having significantly higher levels of these compounds. A correlation analysis of leaf compounds and flower compounds
showed that amounts of leaf monoterpenes were correlated with amounts of floral monoterpenes and amounts of leaf myristicin were strongly positively correlated with amounts of floral myristicin and negatively correlated with amounts of floral furanocoumarins (Table 3.1). Only one of the foliar compounds that elicited an antennal response -- β- farnesene--was significantly correlated with floral xanthotoxin, imperatorin and sphondin (Table 3.1, xanthotoxin $p = 0.119$, $p = 0.043$; imperatorin $p = 0.129$, $p = 0.028$; sphondin $p = 0.168$, $p = 0.004$).

**Oviposition, Damage and Chemistry**

Damage to flowers was significantly correlated to the number of eggs (Figure 3.2a, Scaled Deviance = 8.14, df = 1, $p = 0.0004$). However, there was no correlation between egg counts and reproductive effort (Figure 3.2b, Spearman’s $p = 0.01$, $p = 0.94$). The number of eggs laid was strongly predicted by plant size; large plants received significantly higher egg loads compared to small plants (Figure 3.3, LRT = 62.94, $p<0.001$).

Comparison of foliar chemicals to the three egg count groups —no eggs, 1 to 5 eggs and more than 5 eggs-- revealed that plants with no eggs had significantly higher levels of *trans-* and *cis-*ocimene and marginally higher levels of β-farnesene (Figure 3.4; *trans-* ocimene $F_{1,2} = 5.321$, $p =0.005$; *cis-*ocimene $F_{1,2} = 6.106$, $p =0.002$; β-farnesene $F_{1,2} = 2.732$, $p =0.06$). Parsnip rosettes receiving more than 5 eggs also had significantly lower levels of total floral furanocoumarin content, xanthotoxin and γ-palmitolactone (Figure 3.5; total furanocoumarins $F_{1,2} = 3.304$, $p =0.038$; xanthotoxin $F_{1,2} = 3.90$, $p =0.021$ and γ-palmitolactone $F_{1,2} = 3.552$, $p =0.030$).

**Oviposition and chemical differences between US and NZ populations**
US and NZ parsnips did not differ significantly in their content of foliar β-farnesene, *trans*- and *cis*-ocimene (Global R = -0.046, p = 0.922) or in their overall constitutive foliar chemical profile (Global R = -0.046, p = 0.917). However, there were population-level differences in chemistry (Global R = 0.027, p=0.05), to which myristicin was the major contributor. Egg counts also significantly differed between populations (F_{1,13} =3.513, p<0.001) which may be attributable to plant size differences between populations (F_{1,13}=9.399, p<0.001).

Levels of β-farnesene, and *cis*- and *trans*-ocimenes did not differ between populations (Global R = -0.003, p=0.584).

Phenotypic changes in leaf chemistry after 3 to 6 years of infestation

Infested and uninfested NZ populations showed no changed in foliar chemistry from 2006 to 2009 (infested populations: Global R = -0.028, p = 0.885; uninfested populations: Global R = 0.009, p = 0.287).

DISCUSSION

Whereas many studies have revealed correlations between female choice and offspring performance, few have investigated whether female choice and impacts on plant fitness are correlated. I found that egg counts strongly predicted the proportion of damage received by the plant, although this relationship did not translate to a negative association between egg counts and reproductive effort of parsnips. In a related study (Jogesh et al. submitted), I found that larval damage was negatively associated with a cost of reproduction in the plant. In comparison, the number of eggs did not influence fitness cost in the same way that larval damage did. This discrepancy between egg number and larval damage might be attributable to
variation in early instar survivorship after oviposition. Defensive chemistry, physical leaf structures and microclimate have a greater impact on neonates relative to later instars (Zalucki et al. 2002). Wild parsnip buds, on which neonates feed, and fully developed flowers, which are consumed by later instars, also differ in their chemical composition. Buds have higher levels of mono- and sesqui-terpenes but are lower in furanocoumarin content in comparison to flowers (Zangerl et al. 1997; Nitao and Zangerl 1987). While female moths might be able to use chemical cues to assess larval suitability, the fitness impact on the plant may ultimately be determined by neonate survivorship.

Three compounds were consistently detected by female webworm moth antennae, cis- and trans-ocimene and β-farnesene, but none of these compounds are unique to wild parsnips or even to species in the Apiaceae. Ocimenes are widespread monoterpenes, occurring in 63 angiosperm families, and the sesquiterpene β-farnesene occurs in at least 23 families (Schiestl 2010). Oviposition by another Apiaceae specialist, Agonopterix alstroemeriana, was not correlated with host-specific volatiles from its sole host, Conium maculatum (Castell and Berenbaum 2008). As was the case for D. pastinacella, a ubiquitous monoterpene, Z-ocimene, was deterrent to A. alstroemeriana. Host discrimination for parsnip webworms may depend on the ratio of these ubiquitous components rather than just their presence or absence. For many lepidopterans, a blend is a more functional oviposition stimulant than individual components (Berenbaum and Feeny 2007). For example, in the Oriental fruit moth, Cydia molesta, benzaldehyde and benzonitrile must occur with green leaf volatiles to produce an effective oviposition attractant (Pinero and Dorn 2007). Individual components of a synergistic blend can be repellent when presented alone (Collatz and Dorn 2013). Even when moths use host-specific
cues, several compounds may act synergistically to increase attraction; a combination of pyrrolizidine alkaloids from *Senecio jacobaea* is more attractive to the cinnabar moth *Tyria jacobaeae* compared to individual pyrrolizidine alkaloids (Marcel and Vrieling 2003). Even though I could not document a correlation between any of the constitutive foliar compounds and egg number, oviposition can be mediated by many complex non-volatile contact chemical and physical cues that cannot be detected by a GC-EAD analysis. Thus, host-specificity may be determined in ways that were not measured in this study and merit further investigation.

Comparison of egg counts in the garden plot showed that ocimenes and β-farnesene were oviposition deterrents at high concentrations and high levels of β-farnesene were indicative of higher floral furanocoumarin content. On average, plants with higher egg loads were less well defended, suggesting that adult female moths have the capacity to discriminate between poorly-defended and well-defended parsnips, as predicted by the preference-performance hypothesis. These results are not surprising since the ability of females to discriminate is more critical when chemical variation and larval survivorship differ dramatically among individual plants. In wild parsnips, the constitutive levels of xanthotoxin, bergapten and isopimpinellin can vary by almost 50% among plants within a single population (Berenbaum et al. 1989; Berenbaum and Zangerl 1998). Larval survivorship also differs dramatically among plants with different furanocoumarin content (Berenbaum and Zangerl 1998), suggesting that selection for discriminating females should be strong. While there is considerable support for females of herbivorous insects choosing hosts that increase the survivorship of their offspring (Gripenberg et al. 2010), only one other studies has examined the preference-performance
hypothesis in a system in which oviposition is distinctly uncoupled from larval feeding (Clark et al. 2011). How widespread this phenomenon is thus has yet to be determined.

Irrespective of chemical differences, adult female webworms clearly laid more eggs on larger plants. Preference for large size might be attributable to increased larval survivorship, stronger chemical signals, and/or greater surface area available for oviposition with larger plants. Size is an important predictor of herbivore fitness in many plant-Lepidoptera interactions. Host size is a more important oviposition cue than host-plant chemistry in the specialist butterfly Melitaea cinxia, for example (Reudler Talsma et al. 2008). In webworms as well, Zangerl and Berenbaum (1992) found that plant size was a strong predictor of larval success; the fact that females laid eggs in proportion to the size of the substrate, however, suggests that higher oviposition rates on larger plants may be correlated with surface area available in addition to larval suitability.

A comparison of foliar and floral chemistry indicated that leaves were chemically distinct from flowers and only one compound that elicited an antennal response, β-farnesene, was significantly correlated between the two. It is unknown whether the correlation between foliar β-farnesene and floral furanocoumarins is genetically based. Sesquiterpenes and furanocoumarins do not share a common biosynthetic pathway, and, if these traits are not genetically correlated, herbivore-mediated selection for increased furanocoumarin content in wild parsnips may not translate to a greater deterrent signal for ovipositing moths. Thus, webworm adults and larvae may impose independent selective pressures to increase floral defenses and deter oviposition. Nevertheless, I found no evidence to suggest that foliar defenses or putative oviposition deterrents -- cis- and trans-ocimene and β-farnesene -- had
changed in infested NZ populations over the six-year time period investigated. In view of the fact that no fitness impact of oviposition was detectable in this study, the selective pressure imposed by ovipositing adults may be dwarfed in comparison to selective pressures imposed by larval feeding. Additionally, I found that, while floral chemistry had not measurably changed in infested populations during this 3 - 6-year time period, webworm florivory had selected for an increase in plant size, suggesting that, irrespective of the life stage attacking the plant, 6 years may not be adequate to effect a phenotypic change in plant chemistry (Jogesh et al. submitted).

In conclusion, I found support for the preference-performance hypothesis, with adult moths having the capacity to detect deterrent chemicals in leaves and laying a majority of their eggs on plants with lower defenses in tissues that were not directly assessed by the ovipositing individual. To my knowledge, this study is the first to show support for this hypothesis in an interaction in which adult oviposition and larval feeding are uncoupled with respect to plant structure. Such interactions are not uncommon among Lepidoptera and other herbivores and thus these findings may shed light on chemical constraints on host-finding and host utilization in a wide range of taxa.

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REFERENCES


Collatz J, Dorn S (2013) A host-plant-derived volatile blend to attract the apple blossom weevil Anthonomus pomorum - the essential volatiles include a repellent constituent. Pest Manag Sci 69:1092-1098


Pinero JC, Dorn S (2007) Synergism between aromatic compounds and green leaf volatiles derived from the host plant underlies female attraction in the oriental fruit moth Entomol Exp Appl 125:185-194


### FIGURES AND TABLES

Table 3.1: Spearman’s rank correlation coefficients (ρ) between constitutive leaf and flower chemistry (N=291). Significant correlations (at p<0.05) are in bold.

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<tr>
<th>LEAF</th>
<th>trans-ocimene</th>
<th>cis-ocimene</th>
<th>allo-ocimene</th>
<th>β-farnesene</th>
<th>α-farnesene</th>
<th>myristicin</th>
<th>psoralen</th>
<th>xanthotoxin</th>
<th>bergapten</th>
<th>palmitolactone</th>
<th>osthole</th>
<th>isopimpinellin</th>
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Figure 3.1: GC-EAG recordings obtained with antenna of female *D. pastinacella* (in red), and headspace odors from wild parsnip (*P.sativa*) rosette leaves (in black). Identity of peaks confirmed using pure standards.
Figure 3.2: Relationship between egg counts (oviposition) and the (a) total proportion of all flowers damaged (n=234) (b) the reproductive effort (total seed weight g/ stalk weight g) on plants sampled in the common garden (n =221).
Figure 3.3: Relationship between egg counts (oviposition) and the total plant size (g) for plants sampled in the garden plot (n = 337).
Figure 3.4: Comparison of mean foliar cis-ocimene, trans-ocimene and β-farnesene levels (peak areas per mg) between plants that received no eggs (n = 140), 1 to 5 eggs (n=184) or greater than 5 eggs (n =141). Significant post-hoc differences between groups are indicated by “a” and “b”
Figure 3.5: Comparison of mean floral furanocoumarin levels (peak areas per mg) between plants that received no eggs (n = 140), 1 to 5 eggs (n = 184) or greater than 5 eggs (n = 141). Significant post-hoc differences between groups are indicated by “a” and “b”
Chapter 4 - Population genetic structure and colonization history of the globally-invasive weed wild parsnip (*Pastinaca sativa*)

ABSTRACT

Wild parsnip, *Pastinaca sativa* L. (Apiaceae), is an invasive weed native to Europe with a global distribution in temperate climates. Whether invasive wild parsnip populations resulted from escape of the cultivated *P. sativa* subsp. *sativa* or represents a separate subspecies accidentally introduced throughout the world has long been unresolved. In this study, I examined the population genetic structure of the wild parsnip in its native range in Europe and in three distinct geographic regions where this weed has become invasive: eastern North America, western North America and New Zealand. I also compared wild parsnip haplotypes to cultivated parsnips to determine if they are genetically distinct. Based on the global sampling of wild and cultivated parsnips, 27 chloroplast and 14 ITS haplotypes were found. One ITS haplotype was widespread, with the remainder restricted to a few individuals. In contrast, at least six lineages of chloroplast haplotypes were recovered but none of these showed any geographic structure. Cultivated parsnips were not genetically distinct from wild parsnips and several wild parsnip populations shared haplotypes with cultivars. Genetic diversity of wild parsnips in their invasive range was comparable to the diversity in Europe. High genetic diversity in all introduced populations indicates multiple wild/cultivar introductions or a large source population. The haplotype distribution suggested that wild parsnip populations in North America are a combination of cultivar and non-cultivar haplotypes whereas New Zealand parsnips seem to have primarily escaped from cultivation.
INTRODUCTION

One of the major consequences of international trade is the recurrent establishment of invasive weeds on a global scale. Invasive species can be extremely detrimental to native ecosystems and are considered a leading threat to biodiversity worldwide (Pysek et al. 2012; Pysek and Richardson 2011; Vilà et al. 2011). Tracking the source population and invasion routes is the first step to designing effective management strategies (Schaal et al. 2003). Historical documentation and real-time observations of invasions are rare but advances in molecular methods and population genetic tools have made it possible to reconstruct the colonization history of invasive populations (Lombaert et al. 2011). These techniques have been used with increasing frequency to analyze the genetic structure of introduced populations, make inferences about their origin(s), and determine routes of establishment (reviewed in Estoup and Guillmaud 2010). However, few studies have explicitly examined the historical and contemporary role of cultivation in influencing the spread of an invasive weed in spite of the fact that many weeds are closely related to cultivated species.

The mode of introduction can greatly influence the success of an invasive weed in a novel environment. Multiple introductions, for example, can decrease the effect of genetic bottlenecks and drift. Similarly, large initial introductions can maximize propagule pressure and reduce the loss of diversity in founding populations. Greater genetic variation can increase the potential for evolutionary change and facilitate rapid adaptive evolution in invasive populations (Kolbe et al. 2004; Facon et al. 2006; Marrs et al. 2008). Introduced populations might comprise a small subset of founding genotypes or might contain recombinants of vastly different genotypes from multiple introductions of native populations. For example, reed canarygrass
(Phalaris arundinacea) genotypes originating from multiple locations in Europe have recombined to yield novel invasive genotypes in North America (Lavergne and Molofsky 2007). Alternatively, some “invasive” genotypes might be more likely to establish and spread; Macfadyena unguis-cati, native to Central and South America, consists of mostly one haplotype throughout its introduced range (Prentis et al. 2009). Haplotypes from the native range may be augmented with escaped cultivars of the same species. Additionally, invasive weeds may hybridize with cultivated plants and increase the amount of genetic variation, providing a stimulus for evolution and local adaptation. Analyzing the colonization history of invasive species on a global scale can provide insight into how all these processes act individually or collectively to determine the success of independent invasions.

Pastinaca sativa L. (Apiaceae) is an herbaceous biennial native to Europe which now occurs on every continent except Antarctica (Avrill and DiTomasio, 2007). Parsnips are believed to have originated in the Caucasus Mountains, a center of diversity for the genus Pastinaca (Rubatzky et al. 1999). However, P. sativa is the only species in the genus to occur outside Europe and its spread throughout the world may have been linked to its cultivation as a food crop. The long, carbohydrate-rich taproot produced by cultivated varieties of this species is frequently consumed in many parts of the world. Parsnips are currently cultivated commercially in at least three continents: North America, Europe and Oceania; in all three regions, wild parsnips occur as a weed (Averill and DiTomasio, 2007). The distinction between wild and cultivated parsnips is unclear. Some sources cite the cultivated parsnip, Pastinaca sativa subsp. sativa, as a distinct subspecies from its wild counterpart, Pastinaca sativa subsp. sylvestris, owing to differences between the physical and chemical attributes (Averill and DiTomassio,
Roots of wild parsnips are tough and can be poisonous due to their production of large quantities of myristicin, a hallucinogenic phenylpropanoid (Stahl, 1981). Aboveground structures of wild parsnips also contain higher concentrations of the photodermatitis-inducing furanocoumarins compared to cultivated varieties (Berenbaum et al. 1984). In spite of the phenotypic differences, the evolutionary origins of and taxonomic distinction between the two forms has never been fully examined.

Global biogeography and history of cultivation

The genus *Pastinaca* is placed in the tribe Tordylieae within the family Apiaceae (Downie et al. 2000). There are eight species in this genus and all except *P. sativa* are restricted to Europe (Menemen and Jury 2001). There are five described subspecies of *P. sativa* – *sativa, urens, divaricata, sylvestris* and *latifolia*. *P. sativa* subsp. *divaricata* is sometimes considered a separate species, *P. divaricata*. Cultivated parsnips are sometimes classified as a distinct subspecies, *P. sativa* subsp. *sativa*, from the wild form, *P. sativa* subsp. *sylvestris*. The distinction between the different *Pastinaca* subspecies is based on morphological characteristics such as: leaf pubescence, shape of the stem (rounded versus angled), and the shape and size of the primary umbel (Menemen and Jury 2001; Cain et al. 2009). However, Cain et al. (2009) argue that the demarcation of subspecies is artificial since variation in these traits is continuous and may reflect intraspecific variation within one single species *P. sativa*. None of the other putative subspecies occur outside of Europe and some like *P. sativa* subsp. *latifolia*, occur only on the island of Corsica, France (Menemen and Jury 2001).

The history of parsnip domestication from its wild progenitor is obscure. Historically the name *Pastinaca* may have referred to parsnips or carrots (*Daucus carota*) and there is some
evidence that both were cultivated and consumed in ancient Greece (Hedrick 1972). Evidence of what may have been parsnip seeds has been found in a Neolithic Swiss lake dwelling, dating back 4000 years, possibly indicative of the origin of parsnip cultivation (Weaver, 1997). The cultivation of parsnips as a crop distinct from carrot has been documented with some certainty throughout Europe in the fourteenth century, in South America (Venezuela) in 1564, and in 1604 in Peru (Averill and DiTommaso 2007). The earliest reports of parsnip in North America are from 1606 in Virginia and the plant was purportedly used extensively as a medicinal root by Native Americans (Hedrick 1972). Modern varieties of cultivated parsnips can be traced to the *P. sativa* ‘Student’ cultivar selectively bred from wild parsnips in 1849 by Professor James Buckman at the Royal Agricultural College, Cirencester in England (Buckman, 1865). The *P. sativa* ‘Student’ and its derivatives were susceptible to a fungal disease – parsnip canker, *Itersonilia pastinacae*--and all new cultivars have been selected for fungal resistance. At least one parsnip cultivar – *P. sativa* ‘Gladiator’ -- is a known hybrid between a cultivated parsnip and a wild parsnip from Surrey, England.

Historically and taxonomically, the differences between wild and cultivated parsnips are ambiguous. In eastern North America, cultivars may have escaped soon after introduction in the early seventeenth century (Sturtevant 1890). In the western US, although no historical information is available on dates of arrival, earliest herbarium records of *P. sativa* date back to 1879. Relative to the eastern US, wild parsnip is uncommon in the western US, with most populations occurring in disturbed agricultural settings (Paul Ode, pers. obs.). In North America, wild parsnips currently occur in 45 of the 50 US states and in all Canadian provinces and territories except Nunavut and the Northwest Territories (Averill and DiTommasio, 2007).
It also occurs as a weed in southern South America, South Africa, China, Australia and New Zealand. In New Zealand, wild parsnips have been established since the mid-nineteenth century (Zangerl et al. 2008).

In this study, I used molecular markers to investigate the population genetic structure and colonization history of wild parsnip, *Pastinaca sativa*, an invasive weed with a global temperate distribution. Two loci were analyzed for this study: the nuclear ribosomal DNA Internally Transcribed Spacer (ITS) region and the chloroplast DNA (cpDNA) intergenic spacer region, specifically, *psbM-trnT*, to determine if wild *P. sativa* represents a distinct lineage from cultivated *P. sativa* and to investigate the invasion of wild parsnips in three geographic regions, eastern North America, western North America, and New Zealand, which have been independently colonized by this weed. Specifically, I wanted to identify (1) whether North American and New Zealand invasive populations are escapees from cultivars (*P. sativa* subsp. *sativa*) or if European wild parsnips (*P. sativa* subsp. *sylvestris*) have spread globally, (2) if there is any population genetic structure between the four geographic regions and (3) if high levels of genetic diversity are maintained in all three regions of invasion.

**MATERIALS AND METHODS**

**Sample collection**

Samples for DNA analysis were collected from single leaves of individual plants. *P. sativa* samples were collected from 52 sites in western Europe, 21 sites in midwestern and eastern North America, 6 sites in western North America and 9 sites in the South Island of New Zealand (Table 4.1). Each site consisted of a wild parsnip population isolated from other populations by either anthropogenic/geographic barriers or at least 20 kilometers of distance. Ten samples
were collected at each site but a preliminary analysis showed no variation in samples within sites for both loci, so only one sample per site was analyzed. The sampling effort was in proportion to available populations, with more samples in Europe and eastern North America and fewer samples in western North America and New Zealand. One leaf per plant was collected in a standard 10.2 cm x 22.9 cm (4 x 9 inch) office envelope and dried in a field press for 60 days. In NZ, dried seeds were collected by Dr. Margaret Stanley (University of Auckland) and shipped to the University of Illinois at Urbana-Champaign (UIUC). New Zealand seeds were planted in the UIUC Department of Entomology greenhouse and leaf material was collected approximately 4 weeks post-germination and dried. Field collections were supplemented by leaf material from *P. sativa* herbarium vouchers obtained from the UIUC herbarium as well as DNA previously isolated in the laboratory of Prof. Stephen Downie (accession numbers in Table 4.1). In addition, I planted six varieties of cultivated parsnips in the UIUC Entomology greenhouse and collected leaf tissue from rosettes approximately one month after germination. Parsnip cultivars analyzed in this study were acquired from the United States and New Zealand; All American Organic (Todds Seeds, Novi MI, USA), All American Heirloom (Botanical Interest, Broomfield Co, USA) and Excalibur (Thompson and Morgan, Ipswich, England) were bought in the United States and Melbourne Whiteskin (McGregors’s, Auckland, New Zealand) and NZ Supersnip (McGregors’s, Auckland, New Zealand) were bought in New Zealand.

**Molecular Markers**

Two loci, ribosomal ITS and a chloroplast marker (*psbM-trnT*), were used to determine the diversity and distribution of haplotypes across geographic regions and cultivars. The ITS
locus consists of two segments: ITS1, which occurs between the 18S and 5.8S ribosomal genes, and ITS2, which occurs between 5.8S and 28S ribosomal genes. Together, ITS1 and ITS2 are 443 bp in length in *P. sativa* (Downie and Katz-Downie 1996). The ITS locus has been used frequently in lower level phylogenetic analyses in plants because of ease of amplification and high mutation rate. ITS is also useful as a species barcode for Angiosperms (Li et al. 2011) and adequate intra- and interspecific variation exists at this locus to delineate species within the Apiaceae (Liu et al. in press). While the ITS locus has been very useful at infrageneric level analyses, it can be effective at delineating the population genetic structure in some plants (e.g. Gao et al. 2010; Besnard et al. 2007; Lorenz-Lemke et al. 2004). Because it is biparentally inherited and recombines, it has the potential to reveal recent gene flow and hybridization events. From the chloroplast genome, I sequenced the *psbM-trnT* intergenic locus, which has been shown to be a highly variable non-coding region approximately 1400 bp in length (Downie and Jansen, submitted). Chloroplast DNA (cpDNA) is haploid, non-recombinant and maternally inherited and can be informative for intraspecific phylogenetic analysis, especially over a large geographic scale (Ouborg et al. 1999). CpDNA has a different evolutionary rate compared to nuclear DNA and, because it is maternally inherited, measures of gene flow are not confounded by reticulation (McCauley 1995). Both regions have been previously used together (Gao et al. 2010) and they have the potential to elucidate the population genetic structure in a globally invasive weed.

In a preliminary analysis, I also tested the ribosomal External Transcribed Spacer (ETS) region as well as six microsatellites developed for the closely related *Heracleum mantegazzianum* by Henry et al. (2008). In 23 *P. sativa* sequences, I found three single
nucleotide polymorphisms (SNPs) and three indels in the ETS locus, of which only the three SNPs were parsimony-informative. Based on the low variation at this locus, additional samples were not sequenced for ETS. I was unable to amplify any of *H. mantegazzianum* microsatellites in *P. sativa* using the PCR amplification protocols described by Henry et al. (2008), suggesting the *Hercleum* microsatellites might not be transferable to *Pastinaca*.

**DNA extraction, amplification and sequencing**

Whole genomic DNA was amplified from approximately 20 mg of dried leaf material using a Qiagen DNeasy Plant Mini Kit according to the manufacturer's directions (Qiagen Inc., Valencia, California). PCR amplification of the ribosomal ITS locus was conducted for ITS1 (18S to 5.8S) and ITS2 (5.8S to 28S) using established primers (Table 4.2). The conserved 5.8S region that joins ITS1 and ITS2 was not sequenced in this study. For chloroplast DNA, the region from *psbM* to *trnE* and *trnE* to *trnT* was amplified individually and overlapping base-pairs were used to assemble a complete *psbM* – *trnT* contig after sequencing. PCR primers for the chloroplast region are listed in Table 4.2 and were developed in the Downie Lab.

The protocol for PCR amplification was similar for both loci. Each 25-µL reaction included sterile water (9.5 µL in ITS reactions; 10.75 µL in cpDNA reactions), 5.0 µL of 5X of colorless GoTaq Flexi buffer (Promega Corp., Madison, WI), 4.0 µL of dNTPs (each dNTP at 1.25 µM; Invitrogen Corp., Carlsbad, CA), 3.0 µL of MgCl₂ (25 mM), 0.5 µL of each primer (20 µM), GoTaq Flexi DNA polymerase (0.5 µL in ITS reactions; 0.25 µL in cpDNA reactions) (Promega Corp.), and 1.0 µL of unquantified template DNA. DMSO (1.25 µL) was added to the ITS reactions to relax secondary structures. For samples that were difficult to amplify, template DNA was diluted 1:10 or 1:100 to reduce contaminant concentration. For some cpDNA
amplifications, Taq polymerase did not work so I used a high-fidelity Phusion polymerase (New England Biolabs Inc., Ipswich, MA) in 20-µL reactions with protocols as specified by the manufacturer. The thermocycler program used for the ITS locus is outlined in Downie and Katz-Downie (1996) and the program used for cpDNA locus is outlined in Shaw et al. (2007). The thermocycler program used for Phusion reaction was as follows: initial denaturation for 30 sec. at 98°C, followed by 35 cycles of 10 sec. at 98°C, 10 sec. at 58°C, and 30 sec. at 72°C.

PCR products were purified using the Exo-Sap method: 2.25 µL sterile water, 0.25 µL exonuclease I (Exo; 20 units/µL, New England Biolabs, Ipswich, MA), and 0.50 µL shrimp alkaline phosphatase (SAP; 1 unit/µL, Promega Corp.) were combined and added directly to each 25-µL reaction tube, then incubated for 30 minute at 37°C followed by 15 minute at 80°C to inactivate the enzymes. For some samples, SAP was replaced with Antarctic Phosphatase (New England Biolabs, Ipswich, MA).

Sequencing reactions were performed using the ABI Prism BigDye Terminator version 3.1 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Primers used for PCR amplification were also used for forward and reverse sequencing. Each 10-µL reaction included 1 µL ultrapure water, 2 µL sequencing buffer, 4 µL glycerol, 1.5 µL of the forward or reverse primer (10 pM) and 0.5 µL of BigDye. The thermocycler program for the sequencing reaction was as follows: initial denaturation for 1 minute at 95°C, followed by 35 cycles of 15 sec. at 95°C, 5 sec. at 45°C, and 4 minutes at 60°C. Visualization of sequences was carried out using an ABI 3730XL high-throughput DNA capillary sequencer at the UIUC Keck Biotechnology Center.
Data analysis

Forward and reverse sequences were assembled and disagreements manually corrected in Sequencher version 5.2 (Gene Codes Corporation, Ann Arbor, MI, http://www.genecodes.com). Chloroplast sequences psbM-trnE and trnE-trnT were assembled into one contig using default parameters. Exported sequences from all samples were aligned with MUSCLE ver. 3.5 (Edgar 2004) using the default parameters. Sequences for P. pimpinellifolia and P. sativa subsp. divaricata (Downie and Katz-Downie 1996; Logacheva et al. 2008) were included in the data matrices for comparison to wild parsnip sequences. Because the 5.8S region that separates ITS1 and 2 was not sequenced, data matrices for ITS1 and ITS2 were combined in MESQUITE (Maddison and Maddison 2011; http://mesquiteproject.org) after sequence alignment.

Standard molecular diversity indices were calculated for both loci using DnaSP (Rozas 2009; http://www.ub.edu/dnasp/). Diversity calculations included the number of haplotypes, the number of segregating (polymorphic) sites, the gene diversity and average expected heterozygosity of all variable nucleotide sites in each locus. Tajima’s D was calculated to measure the neutrality of both loci. Sequences divergence between geographic regions were inferred using pairwise Exact tests with 10,000 MCMC chains implemented in ARLEQUIN ver. 3.5 (Excoffier and Lischer 2010; http://cmpg.unibe.ch/software/arlequin35/). Exact tests for population differentiation are based on the non-parametric Fisher’s exact test, which is used to determine whether an association exists between the counts of two variables. In the case of populations, Exact tests compare geographic regions based on the occurrence of haplotypes.
(Raymond and Rousset 1995). The Exact test is a useful measure of differentiation when sample sizes are unbalanced (Goudet et al. 1996).

ITS and chloroplast genealogies were inferred using median joining networks implemented in Spiltstree4 (Huson and Bryant, 2004; http://www.splitstree.org/). The median-joining network algorithm generates a consensus of multiple minimum spanning trees to create a network and then infers intermediate haplotypes to reduce tree length. Phylogenetic networks are more useful than bifurcating trees for population-level evolutionary processes owing to the recent divergence of haplotypes and the presence of ancestral haplotypes in extant populations. To compare the topology of the median joining networks to bifurcating trees, trees were constructed using Maximum Parsimony (MP) methods in PAUP* version 4.0b10 (Swofford 2002). In MP analyses for ITS, gaps were treated as missing data and all characters were unordered and equally weighted. For the analyses of cpDNA data, indels were manually coded as present/absent (binary) characters with 0’s and 1’s. Analyses of each dataset involved 1,000 heuristic searches using random stepwise addition and TBR branch swapping. A 50% majority rule consensus tree was obtained for comparison of tree topology to the median joining networks. Bootstrap values were calculated to assess branch support from 1000 replicates using a heuristic search with random stepwise addition and TBR branch swapping.

The chloroplast dataset was additionally used to infer relative divergence times for the different sequence types. I used BEAST version 2.0.3 (Bouckaert et al. 2014), which employs a Bayesian Monte Carlo Markov Chain to infer relative divergence times with an estimated mutation rate. Since no fossils exist for any closely related species, actual divergence times could not be estimated. Intraspecies sequence divergences are expected to be relatively recent
and coalescent priors were specified in *BEAST. Heracleum sphondylium as well as P. armena, P. latifolia and P. pimpinellifolia were used as outgroups. Molecular evolution rates were allowed to vary across branches based on a relaxed exponential clock. Prior to the analysis, I examined models of nuclear substitution in MrModelTest (Nylander 2004) based on Akaike information criterion (AIC) values. MrModelTest identified GTR (General time reversible substitution) as one of the best fitted models and GTR was used to specify the substitution rate in the BEAST analysis. The gamma distribution and substitution rates for each of the four nucleotides were estimated from the data. The prior distribution was estimated from a simple Yule model of speciation. Posterior distribution of parameters were estimated from 10 million runs sampled every 10,000 generations after discarding 10 % of the generations in the burn-in. The software TREE-ANNOTATOR, version 2.0.3 was employed to summarize output results. The consensus tree was generated in FigTree version 1.4.0.

RESULTS

I amplified and sequenced the ITS locus for 93 individuals and the psbM-trnT locus for 81 individuals. Neither locus could be sequenced for all individuals because of unsuccessful PCR amplification. Concatenated ITS1 and ITS2 sequences contained 483 nucleotide positions of which 28 were polymorphic. The polymorphisms were single nucleotide polymorphisms (SNP) and seven of the SNPs were parsimony-informative. Individuals separated into 14 haplotypes (hereby denoted as H1 to H14). The chloroplast intergenic region psbM – trnT contained 1464 nucleotide positions with a total of 14 SNPs, all of which were parsimony-informative. Most of the variation in this region is a result of 48 indels with an average indel length of 11.25 bps.
Combined indel and SNPs polymorphisms separated all 81 individuals into 27 haplotypes (hereby denoted as C1-C27).

Diversity indices for each geographic region are presented in Table 4.3. The majority of all individuals (79%) shared ITS haplotype H1. H11 was the next most common haplotype, shared by 5% of all individuals, and all other observed ITS haplotypes were unique to one or two individuals. Eastern North America had the highest diversity of ITS haplotypes (Table 4.3), with 2 unique haplotypes and two shared with NZ (H11 and H13, Figure 4.1) in addition to the most common haplotype, H1. All 6 Western North American parsnips samples were classified as H1. The two subspecies of wild parsnip from Europe, subsp. urens and subsp. divaricata, had unique ITS haplotypes and, as expected, the ITS sequence for P. pimpinellifolia was different from all P. sativa sequences. Three cultivars, P. sativa ‘All-American Heirloom’, P. sativa ‘Excalibur’ and P. sativa ‘Melbourne Whiteskin’, belonged to the ubiquitous H1. One NZ cultivar (P. sativa ‘NZ Supersnip’) shared haplotype H8 with the European wild parsnips. European parsnips consisted predominantly of the H1 haplotype but had more haplotypes (6) than any other region.

The test for neutrality showed that neither locus locus is under neutral selection in Europe. Tajima’s D was significantly negative owing to the excess of rare alleles (singletons) recovered in Europe.

There were substantially more chloroplast haplotypes (chlorotypes) compared to ITS haplotypes; 27 chlorotypes were present in 81 samples. Gene diversity and expected heterozygosity were very high in all geographic regions and in cultivated parsnips (Table 4.3). Twenty-two chlorotypes occurred in Europe alone, with fifteen being unique. Eastern North
American parsnips shared 86% of their haplotype diversity (7 chlorotypes) with European parsnips and had two unique chlorotypes (Figure 4.2). Western North American parsnips had one unique chlorotype (C9) and shared three chlorotypes (C5, C6 and C7) with Europe and eastern North America. Two haplotypes (C5, C6) were recovered from NZ parsnips, both of which also occurred in Europe, North America and cultivars. Cultivated parsnips shared 80% of their chlorotype diversity with Europe and eastern North America. One cultivar, *P. sativa* ‘Excalibur’, had a unique chlorotype (C27). *P. sativa* subsp. *divaricata* had the ubiquitous C5 chlorotype and *P. sativa* subsp. *urens* shared C4 with European wild parsnips.

I found no resolution for the relationship between ITS haplotypes using MP methods. I found weak support for the clustering of H7 and H9 as well as H11 and H14 (Figure 4.1c). MP methods could not resolve the relationship between wild *P. sativa* and the two *P. sativa* subspecies *urens* and *divaricata*. However, the median joining network suggests that the most common haplotype, H1, is ancestral to all other observed haplotypes, which are a few mutational steps away from H1 (Figure 4.1). In contrast, the cpDNA marker separated chlorotypes into six clades with strong support with three clades, A, B and C depicted in the consensus MP tree (Figure 4.2c). The median joining network depicted chlorotypes, C7, C4, C18 and C15 in the center of the network and outlined six clades: clades A, B, C as well as three other clades D, E and F, not supported by the MP tree (Figure 4.2). The central clades contain the subsp. *urens* and chlorotypes that are absent in cultivated parsnips. Clade B occurred in Europe and eastern North America but was not found in cultivars or any other geographic region; clade C was unique to Europe but consisted of only two individuals. Clades D and E contained chlorotypes that occurred in cultivars and in all four geographic regions.
Divergence times for chloroplast sequences could not be estimated with any accuracy owing to extremely large error bars (95% posterior densities) at every node. Clades C and F were well supported based on Bayesian analysis (posterior probabilities greater than 0.95 in Figure 4.3); however, in contrast to MP methods, clade A had weak support and clades B, D and E had no support. Even though chlorotypes clustered in ways similar to the median joining network, relationships between clades were unresolved in the Bayesian tree, making it difficult to interpret the evolutionary origins of the different chlorotypes. However, there was high support for clade F being most closely related to other Pastinaca species which were included as outgroups. The placement of the outgroup species Heracleum sphondylium was also unresolved.

Exact tests showed no overall geographic differentiation at the cpDNA locus between geographic regions and no differentiation between cultivars and wild parsnips (Exact test for overall differentiation, p-value =0.79, pairwise tests in Table 4.4). However, comparison at the ITS locus showed that Europe may be genetically distinct from eastern North America (Exact test for overall differentiation, p-value =0.0007, pairwise tests in Table 4.4). In spite of the extremely high occurrence of haplotype H1 in both regions, eastern North America had two haplotypes, H11 and H13, which were shared with NZ and which did not occur in Europe. However, this finding may be spurious because of the limited nucleotide variation at the ITS locus.
DISCUSSION

14 distinct haplotypes were obtained with the ITS locus and 27 chlorotypes were obtained with the \textit{psbM-trnT} cpDNA marker. The dominance of one ITS haplotype H1 (>70%) and the rare occurrence of others suggests that ITS may not be an appropriate locus for population genetic studies in \textit{P. sativa}. ITS has successfully been used a molecular marker for intraspecific studies in many plants and has shown very little variation in others. For example, Gao et al. (2012) showed 19 distinct ITS haplotypes in an endemic Tibetan plant, \textit{Rhodiola alsia}, with strong branch support for the evolutionary relationship between haplotypes, whereas Soltis and Kuzoff (1993) found very little variation in ITS1 in populations of \textit{Lomatium grayi} and \textit{L. laevigatum}. ITS is used more frequently for infrageneric relationships and been successfully used within the Apiaceae to resolve relationships between species in several genera, including \textit{Heracleum} (Yu et al. 2011), \textit{Pimpinella} (Magee et al. 2010) and \textit{Angelica} (Feng et al. 2009). In this study as well, ITS clearly differentiated \textit{P. sativa} from \textit{P. pimpinellifolia} and the two subspecies \textit{urens} and \textit{divericata}, but variation between \textit{P. sativa} individuals was low. These findings are consistent with its utility as a species DNA barcode within the Apiaceae (Liu et al. in press). In contrast, the cpDNA marker was more variable at the intraspecific level and separated chlorotypes into six clades. Findings from the cpDNA marker will be emphasized in the remainder of the discussion.

I found high levels of genetic diversity (and heterozygosity $H_e$) in all introduced geographic regions, even though western North America and NZ provided disproportionately fewer samples from fewer populations. I found no evidence to suggest that the geographic regions were genetically differentiated. High genetic diversity in introduced populations is
indicative of either multiple introductions of *P. sativa* in all regions or the introduction of a large initial population with the retention of ancestrally polymorphic alleles. Numerous studies comparing neutral genetic variation between native and invasive populations suggest that, compared with that in the native range, genetic diversity is often the same as or higher in the invasive range (Bossdorf et al. 2005). Higher genetic variation is often a consequence of multiple introductions from genetically diverse native populations. For example, the population genetic structure of the brown anole lizard *Anolis sagrei* in Florida shows that at least 8 different introductions from all over the world are responsible for the extant diversity in its invasive range (Kolbe et al. 2004). Similarly, Genton et al. (2005) found that the invasive common ragweed *Ambrosia artemisiifolia* in France originated from multiple sources in North America. For wild parsnips, two possibilities exist in terms of multiple introductions: the weed may have been introduced from what has been traditionally considered the European wild parsnip, *P. sativa* subsp. *sylvestris*, or invasive populations may consist of individuals that have escaped from multiple cultivars. Very few studies have examined weeds that are garden or agricultural escapees and one such study found that high genetic diversity of invasive olive tree, *Olea europaea* subsp. *europaea*, in Australia is consequence of the introduction of multiple cultivars in the invasive range (Besnard et al. 2007).

In this study, I found no evidence to suggest that cultivars were genetically distinct from wild parsnips (Exact tests in Table 4.4). However, the distribution of haplotypes suggests that many chlorotypes occur only in wild parsnip populations. These chlorotypes may represent the original wild form, *P. sativa* subsp. *sylvestris*, while clades A, D and E may represent *P. sativa* subsp. *sativa*— the cultivated parsnip. European parsnips had a much higher diversity of
haplotypes, with at least 15 haplotypes unique to this geographic region, which is expected for the native range. Interestingly, nearly half of Europe’s wild parsnips have chlorotypes shared with cultivars, suggesting that, even in the native range, wild parsnip populations are a combination of escaped cultivars and originally wild *P. sativa* subsp. *sylvestris* chlorotypes. However, I sampled a small subset of modern cultivars and a portion of the European wild parsnip chlorotypes may still have originated from cultivated parsnips. At least two of these non-cultivar chlorotypes (C3 -blue and C7 – purple in Figure 4.2) also occurred with some frequency in North American populations, suggesting that both escape from cultivation and introduction from Europe may have been responsible for the invasion of wild parsnips in North America. In NZ, both haplotypes were shared with cultivars (NZ Supersnip, Hollow Crown and All-American Heirloom), consistent with the interpretation that NZ populations are primarily escapees from cultivation (with the caveat that NZ populations were not comprehensively sampled and additional analyses should be conducted to confirm this finding).

However, it is known with some certainty that some modern cultivars (e.g., *P. sativa* ‘Student’) were bred from wild parsnips and it is reasonable to assume that many other modern cultivars have been frequently created by hybridization with wild forms (e.g., *P. sativa* ‘Gladiator’). It is then entirely possible that wild parsnips in Europe did not escape from cultivation but cultivars have been created from multiple wild chlorotypes. Both hypotheses can explains the sharing of chlorotypes between the two forms and the distinction between the two may only be possibly with a thorough examination of the history of each cultivar.

While divergence times for *P. sativa* chlorotypes could not be estimated, the Bayesian tree provided strong support for clade F being more closely related to the outgroup species.
than any other clades. Clade F contains three chlorotypes, all of which were singletons; C8 was unique to Europe, C26 unique to Eastern North America and C27 was the cultivar *P. sativa* ‘Excalibur’. *P. sativa* ‘Excalibur’ is an F1 hybrid variety potentially between a cultivar and wild form. Chlorotypes in clade F maybe ancestral to all other *P. sativa* chlorotypes.

The differences in morphology and chemistry between wild and cultivated parsnips may be maintained by strong herbivore selection for increased defenses even though wild and cultivated parsnips are not genetically distinct. At least in North America, parsnips are not commercially cultivated on a large scale and these biennial plants are usually harvested for the root prior to flowering, making recent hybrids unlikely in most of the sampled wild populations. In view of the fact that herbivore-mediated selection for chemical traits can act rapidly in wild parsnip populations (Zangerl and Berenbaum 2005; Zangerl et al. 2008), it is likely that strong selection maintains the apparent morphological differences between wild and cultivated parsnips.

The closely related wild carrot, *Daucus carota*, represents a similar system where the species has been cultivated throughout the world and wild forms occur as a globally invasive weed. In comparison to parsnips, wild carrots are genetically distinct from cultivated carrots (Iorizzo et al. 2013; Bradeen et al. 2002; Shim and Jorgensen 2000). In view of the fact that both species were probably domesticated at the same time, the clear genetic distinction between wild and cultivated forms of carrot in contrast to the lack of differentiation in parsnips may reflect differences in the history and manner of domestication. Based on recent evidence from a large SNP dataset, carrots appear to have been domesticated from yellow- and purple-rooted, wild carrots in central Asia which are genetically distinct from European wild carrots (Iorizzo et
al. 2013). The modern cultivars of parsnips, on the other hand, are known to have been bred from European wild parsnips and hybridizations between the two may have occurred at a higher frequency in the process of creating cultivars (e.g., the cultivar *P. sativa* ‘Gladiator’ is a hybrid), reducing the genetic distance between wild and cultivated forms. While the morphological and chemical distinction between wild and cultivated parsnips is a consequence of herbivore-mediated natural selection, the phenotypic differences between wild and cultivated carrot is a result of selection for specific cultivar genotypes (Grebenstein et al. 2011). A recent large-scale study comparing wild and domesticated carrots showed diversifying selection in at least 27 markers (Grezebelus 2014). Wild carrots in North America are more closely related to wild carrots in Europe, suggesting that introduction from Europe, and not escape from cultivation, is primarily responsible for the colonization of this weed. In wild parsnips as well, it appears that accidental introduction from Europe is at least partly responsible for the high diversity of chlorotypes observed in North America.

*P. sativa* subsp. *divaricata* had chlorotype C5, which was shared with wild parsnips found on all continents and in the cultivar *P. sativa* ‘NZ Supersnip’. Similarly *P. sativa* subsp. *urens* had chlorotype C4, which was shared by two European wild parsnips. The inability of cpDNA to separate the morphologically distinct subspecies of *P. sativa* suggests that variation in this marker might be a consequence of an ancient polymorphism or an indication of hybridization between the different subspecies of *P. sativa*. Phenotypically distinct species can share organellar DNA haplotypes and show greater variation among localities than between species (or subspecies) as result of ancestral polymorphisms or introgression between species (Bleaker and Hurka 2001; Palme et al. 2004). For example, in silver birch, *Betula pendula*,
variation in cpDNA was greater between geographic regions than between species owing to large introgression rates among species (Palme et al. 2004). Ancestral polymorphisms, on the other hand, can be retained due to incomplete lineage sorting -- that is, if the time taken for alleles to coalesce to a common ancestor is greater than the divergence between P. sativa subspecies. It is difficult to parse the difference between hybridization and ancestral polymorphisms especially because only one locus is under consideration in this study. Furthermore, because it is maternally inherited and haploid, cpDNA cannot provide any information on the extent of recombination or admixture between populations or species.

Nuclear ribosomal ITS, and the cpDNA psbM-trnT violate the assumptions of neutral variation in Europe. Both regions are non-coding and are not expected to be under selection; negative Tajima’s D caused by the high frequency of rare alleles in Europe may be indicative of a recent demographic expansion in this weed. The high frequency of singleton haplotypes suggest that most of the mutations have accumulated in terminal branches of the haplotype genealogy and are still fairly rare (Excoffier et al. 2009). Demographic and range expansions are usually associated with geological or climatic events (Hewitt 2000) but can also be triggered by anthropogenic factors. Range expansion is typical of most weedy species. Genetic signatures of demographic range expansion of this weed may be indicative of its cultivation and subsequent dispersal as a weed throughout Europe.

The lack of genetic differentiation between isolated geographic regions indicates that either the historical source of all invasive populations is the same and not enough time has passed for the fixation of alleles at this locus, or that contemporary gene-flow between geographic regions occurs with some frequency. Gene-flow between parsnip populations within
A geographic region is expected to be high due to the predominantly roadside distribution of this plant and the ease with which its seeds can be carried long distances by vehicles. It is also possible, although less likely, that the transportation of parsnip seeds occurs with some frequency across continents. Pickering and Mount (2010) found that seeds from 372 exotic plant species have been collected from clothing, equipment, or vehicles, indicating that long-distance human-mediated dispersal can occur on a regular basis. In some species, admixture is associated with global trade. For example, levels of admixture in the Chinese mitten-crab *Eriocheir sinensis* were strongly correlated with shipping volume, an associated expected if human-mediated dispersal was its primary means of gene-flow between continental Europe and the United Kingdom (Herborg et al. 2007). Thus, it is reasonable to hypothesize that high rates of gene-flow via multiple contemporary introductions of *P. sativa* in its invasive range have resulted in the lack of genetic differentiation between continents. Analyses of multiple loci, especially diploid loci with higher mutation rates such as microsatellites, can help elucidate the extent of gene-flow and admixture within and between continents.

The availability of molecular data and advances in population genetic analysis have made it possible to estimate the patterns of species migrations with considerable precision. Recently developed methods have made it possible not only to identify the native sources of haplotypes but also to use rates of evolution to estimate dates of introduction (e.g., Song et al. 2014; Voshell and Hilu 2013). Findings from historical colonization and dispersal studies have the potential to shed light on human-mediated transportation of biological materials and provide a broad understand of how humans may have contributed to the introduction and spread of an invasive species. Evidence obtained in this study indicates that escape from
cultivation led to the globalization of wild parsnips and introduced populations have high genetic diversity. Increased levels of diversity in invasive populations can act as a primer for rapid adaptive evolution in this noxious weed. Future studies with increased sampling and addition loci have the potential elucidate specific patterns of colonization and admixture in this agriculturally entwined invasive species and may serve as a model for investigating the natural and agricultural history of plant species that exist in wild, cultivated, and feral forms.

ACKNOWLEDGMENTS

This work was supported by funds provided by the H.H. Ross Memorial Award for Systematic Biological Research, the Francis M. and Harlie M. Clark Research Support Grant from the School of Integrative Biology, UIUC, and the Tyler Prize for Environmental Achievement to May Berenbaum. Thanks to Yue Xu for help with the molecular work, Maksim Sergeyev for DNA extractions, Rhiannon Perry for troubleshooting and sequence-cleaning, Paul Ode and Margaret Stanley for sample collections as well as Stephen Downie and May Berenbaum for funding, advice and edits on the manuscript.
REFERENCES


### FIGURES AND TABLES

**Table 4.1:** Sampling locations and haplotype information for all *P. sativa* individuals genotyped

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<th>ITS</th>
<th>psbM-trnTR</th>
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<th>Chloroplast Haplotype</th>
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Table 4.1 (cont.)

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<td>N 53°50.652'/E 11°52.814'</td>
<td>x</td>
</tr>
<tr>
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<td>KARI20</td>
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<td>ROC9</td>
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<td>Coordinates</td>
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<td>ROC1</td>
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<td>x</td>
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<tr>
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<td>USA, CO</td>
<td>G</td>
<td>x</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N 39°22.816'/W 107°04.704'</td>
<td>x</td>
</tr>
<tr>
<td><em>P. sativa</em></td>
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<td>USA, CO</td>
<td>AC</td>
<td>x</td>
</tr>
<tr>
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<td>N 40°24.427'/W 106°48.399'</td>
<td>x</td>
</tr>
<tr>
<td><em>P. sativa</em></td>
<td>W. North America</td>
<td>USA, NM</td>
<td>T</td>
<td>H1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>N 36°50.601'/W 106°34.223'</td>
<td>x</td>
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<td>H1</td>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>divaricata</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>urens</td>
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<td>France</td>
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<td>H2</td>
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Table 4.2: DNA fragments and PCR primers for rDNA ITS and cpDNA psbM to trnT

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<th>Reference</th>
<th>Primers</th>
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<td>Feist and Downie 2008</td>
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<td></td>
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<td>5.8S R 3’</td>
<td>Feist and Downie 2008</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>5.8S (ITS-3N) F 5’</td>
<td>Spalik and Downie 2006</td>
<td>CGA TGA AGA ACG TAG CGA AAT</td>
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<tr>
<td></td>
<td></td>
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<td>Wen and Zimmer 1996</td>
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<tr>
<td></td>
<td></td>
<td>trnE R 3’</td>
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<td>TCC TGT AGA GAG AAA GTT CCT G</td>
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<td>trnD F 5’</td>
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<td>ACC AAT TGA ACT ACA ATC CC</td>
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<td></td>
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<td>CTA CCG CTG AGT TAA AAG GG</td>
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Table 4.3: Molecular diversity indices estimated from ribosomal ITS and chloroplast sequences from wild parsnips in three continents and cultivated parsnips. Significant departures from neutrality (Tajima’s D) at p<0.05 are denoted by (*)

<table>
<thead>
<tr>
<th>Sequence region</th>
<th>Geographic Origin</th>
<th>No. sequences</th>
<th>No. haplotypes</th>
<th>Segregating sites(S)</th>
<th>Gene Diversity</th>
<th>Average H_e (Expected Heterozygosity)</th>
<th>Tajima’s D</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS</td>
<td>Europe</td>
<td>52</td>
<td>6</td>
<td>6</td>
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<td>0.044</td>
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<td>7</td>
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<td>1</td>
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<td>0</td>
<td>0.050</td>
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<td>4</td>
<td>4</td>
<td>0.387</td>
<td>0.333</td>
<td>-1.128</td>
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<tr>
<td></td>
<td>Cultivars</td>
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<td>3</td>
<td>2</td>
<td>0.440</td>
<td>0.400</td>
<td>-1.770</td>
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<tr>
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<td>22</td>
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<td>0.941</td>
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<td>45</td>
<td>0.933</td>
<td>0.404</td>
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Table 4.4: Pair-wise comparisons of differentiation (Exact tests) between geographic regions calculated from divergence at the ribosomal ITS and cp DNA locus. P-values derived from 10,000 MCMC runs are depicted in the table and significant differences at $p<0.05$ are denoted by (*) and highlighted in bold. The two subspecies are *divaricata* and *urens*.

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<thead>
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<th>Western NA</th>
<th>NZ</th>
<th>Cultivar</th>
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<td><strong>0.011</strong></td>
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<td>1.000</td>
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Figure 4.1 (a) Distribution of cultivated and wild parsnip ITS haplotypes sampled from three geographic regions (b) median joining network depicting the phylogenetic relationship between haplotypes. The size of each circle is proportional to the number of individuals. Branches between haplotypes are proportional to the number of mutational steps. Shared and unique haplotypes are assigned a color or pattern.
Figure 4.1 (cont.) (c) Maximum parsimony tree typology of all chlorotypes from *P. sativa*; number on branches indicates bootstrap values (1000 replicates). Tree length = 30, CI = 0.93, HI = 0.06, RI = 0.50. The MP tree is rooted with *P. pimpinellifolia*. 
Figure 4.2 (a) Distribution of cultivated and wild parsnip chlorotypes (psbM-trnT) sampled from three geographic regions (b) median joining network depicting the phylogenetic relationship between haplotypes. The size of each circle is proportional to the number of individuals. Branches between haplotypes are proportional to the number of mutational steps. Shared and unique haplotypes are assigned a color or pattern. Boxes outline the six clades delineated by the median joining network.
Figure 4.2 (cont.) (c) Maximum parsimony tree typology of all chlorotypes from *P. sativa*, number on branches indicate bootstrap values (1000 replicates). Tree length = 128, CI = 0.49, HI = 0.50, RI = 0.84. No outgroup is specified. Evolutionary clades are denoted by the alphabets A-F.
Figure 4.3: Consensus phylogenetic tree of the 27 detected *psbM-trnT* sequence types of *Pastinaca sativa* based on the Bayesian inference. Colors of the branches indicate the values of the Bayesian posterior probability. Branches with strong support (>0.90 posterior probability) are emphasized in red. The length of the branches is proportional to mutational steps and the scale indicates the number of substitutions. Evolutionary clades corresponding with the median joining network in Figure 4.2 are denoted by the alphabets A-F.
Conclusions

My dissertation contributes to growing evidence that invasive populations can undergo rapid adaptive evolution and are rarely limited by low genetic variation. In wild parsnips, as is the case in many other invasive weeds, genetic variability in areas of invasion is comparable to the native region. Multiple introductions are one of many ways to mitigate the effects of small founding populations. Inadvertent human-mediated dispersal and escape from cultivation have led to the establishment and spread of highly diverse invasive populations of parsnips in North America, whereas in New Zealand invasive populations are composed primarily of cultivars. In both regions, the availability of high genetic diversity provides the necessary variation required for rapid adaptive evolution to occur.

While many studies have specifically examined rapid adaptive evolution in response to abiotic factors, my research specifically looked at herbivore-mediated evolution. Evolution of weeds in response to herbivory is especially germane considering that the reassociation between a plant and its coevolved natural enemies is the basis of classical biological control. That plant-herbivore reassociation in the invasive range can result in rapid adaptive evolution in the weed suggests that evolutionary response should be a part of management strategies for invasive weeds (Müller-Schärer et al. 2004)

Previous work on the coevolution of parsnip-webworm interactions has focused primarily on chemical defenses and selection pressures from pollinators have not been considered even though the interaction between herbivory and pollination should be particularly relevant for flower-feeders. Evolution of increased defenses is one of many
possible resistance mechanisms against herbivory. In my dissertation, I showed that parsnips can evolve tolerance via increased plant size after only six years of reassociation with a specialist florivore. While I did not see any direct effects of herbivore reassociation on pollination, both herbivory and pollination were mediated by plant size. Large plants experienced reduced herbivore impact and also had higher pollination success compared to small plants. Nonetheless, the absence of herbivores for 140 years in New Zealand significantly altered plant-pollinator evolution as evidenced by increased pollinator attraction in herbivore-free New Zealand populations.

Plants should be under herbivore selection to increase feeding defenses as well as to decrease host-finding from ovipositing adult moths. The selection pressure imposed by ovipositing moths and the evolution of oviposition deterrents have not been thoroughly examined in plant-herbivore interactions. In wild parsnips, oviposition was not negatively correlated with fitness, suggesting that the selection pressure imposed by ovipositing adults is weak -- that is, higher egg counts did not translate into a proportional loss in seeds. Consistent with the lack of selection, I found no changes in leaf chemistry over six years of infestation. However, I did find support for the preference-performance hypothesis, with adult moths having the capacity to detect deterrent chemicals in leaves and lay more eggs on plants with lower defenses.

The global redistribution of two trophic levels has provided us with an experiment that offers an extraordinary opportunity to test hypotheses in a magnitude and scope which would be impossible to simulate. These studies elucidate the complexity of biotic interactions that exist between wild parsnips, its major herbivores, and its pollinators as well as the rapid
evolution of tolerance in response to herbivory. In this ditrophic system, not only is it possible to examine the influence of colonization history on the ecology and evolution of a weed but shifts in antagonistic and mutualistic interactions over continental space provide insights on fundamental aspects of insect-mediated plant evolution.

**REFERENCE**

Appendix: DNA sequences

**P. sativa** concatenated ITS1 and ITS2 haplotype sequences

| H1   | TCGAATCCTGCAATAGCAGAATGACCTGCTAACATGTAAGCACACTGGGCAAGCGTATGGGGGCTTTGGTCCCTTGTTAGCGAAACCCTGGTAGTAGGTG |
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