

Evaluating threats to the rare butterfly, *Pieris  
virginiensis*

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Samantha L. Davis ENTITLED Evaluating threats to the rare butterfly, *Pieris virginiensis* BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy.

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## ABSTRACT

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*Evaluating threats to the rare butterfly, *Pieris virginiensis*.*

Humans have caused drastic changes in ecosystems and communities through their modification of the natural landscape. Rare species, often highly specialized, are more impacted by these changes. *Pieris virginiensis* is a rare butterfly native to eastern North America that is a species of concern due to negative influences from habitat loss and plant invasion. This thesis discusses several threats to *P. virginiensis*, including habitat loss, climate change, competition, and the cascading effects of a novel European invasive plant, *Alliaria petiolata*, that attracts oviposition but does not allow for larval survival.

First, I examined a local extinction event and attributed it primarily to several seasons of poor weather and extreme climatic events, but with contributions by an increasing deer population and the introduction of *A. petiolata*. Second, I found that *A. petiolata* attracts approximately two-thirds of total eggs, but no larvae survive on the novel host. I tested several chemical causes of larval death and identified two potential contributors: sinigrin, which delays growth, and alliarinoside, which reduces survival.

I also examined competition between *P. virginiensis*, its host plants, and novel competitors in the habitats. First, I looked at shared habitat use between *P. virginiensis* and another, exotic Pierid butterfly *P. rapae*. Although habitats are occasionally shared, *P. rapae* is most likely not a large influence on the success or failure of *P. virginiensis*. Second, I examined the influence of *A. petiolata* when it competes with two native host plants of *P. virginiensis*, and found differential effects of each life stage of *A. petiolata* on the native host plants.

Finally, I used a combination of species distribution modeling and genetic sequencing to determine the current and future states of *P. virginiensis* given the changing climate and other stressors on *P. virginiensis* populations. Although secure currently, future stressors will most likely cause a range contraction and local extinctions.

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Bradley Sarson, who has cleaned up more animal discharge than necessary in my pursuit of a degree; my parents, Mary and Rick Davis, who encouraged me to read and let me play in the woods as a child; my non-science friends, who keep me grounded; and my many countless other supporters, colleagues, and family.

# Introduction

More than three-quarters of the earth's ice-free land has been modified by humans, often at the expense of global biodiversity (Thomas et al., 2004b; Ellis and Ramankutty, 2008). Although human contributions to ecosystem changes are complex and hard to quantify, they generally include changes in land-use through urbanization, agriculture, and logging (Czech et al., 2000; McKinney, 2002); drastic changes in ecosystems and communities resulting from exotic species introduction and escape (Pimentel et al., 2005; Zavaleta and Hulvey, 2004); non-target and cascading effects of pest-control chemicals and biological agents (Howarth, 1991; Louda et al., 2003); and species extinctions and range shifts as a result of anthropogenic climate change (Thomas et al., 2004a).

Rare species are more impacted by environmental changes as a result of their narrow distributions and highly specialized niches (Lawton, 1993; Gaston, 1998; Johnson, 1998). Insects are expected to bear the brunt of the current (sixth) world extinction event, as they often are narrowly defined habitat or host specialists (Thomas et al., 2004b; Dunn, 2005). Wilcove et al. (1998) found that 87% of surveyed invertebrates ( $n = 331$ ) were imperiled by habitat loss or degradation, 45% by pollution, and 27% by associations with alien species. Arguably the most dynamic insects, 97% of surveyed butterflies and skippers ( $n = 33$ ) were impacted by habitat loss, followed by species invasion (36%), overexploitation (30%), and pollution at 24% (New, 1997; Wilcove et al., 1998). Lepidoptera (butterflies and moths) have served well in the past as “umbrella” taxa, species of concern



Figure 1.1: Photo of a female *Pieris virginiensis* in 2011 in Morrow Co., OH. *Pieris virginiensis* are differentiated from the more common *P. rapae* by their mostly white color, relatively small size, diffuse shading of wing veins and absence of dark dorsal eye spots and ventral yellow shading.

that act as surrogates for all species in a given area or habitat, as well as “flagship” taxa – species, usually imperiled, that are popular and used to draw attention and funding towards conservation efforts (New, 1997; Guiney and Oberhauser, 2008). Examples of flagship taxa include the well known Monarch butterfly (*Danaus plexippus* L.), and the endangered Karner blue butterfly (*Lycaedes melissa samuelis*), both North American butterflies with bright colors and unique life-history that help them appeal to the public (Guiney and Oberhauser, 2008).

*Pieris virginiensis* Edwards is a rare univoltine Pierid butterfly endemic to mature forests in Eastern North America (Fig 1.1 and 1.2). Held up as a flagship species by the Lake Erie Allegheny Partnership for Biodiversity (LEAPbio), *P. virginiensis* has suffered in recent times from its changing environment (Finnell and Lehn, 2007). From historical logging and urbanization (Klots, 1935; Toronto Entomologists’ Association, 1975; Finnell and Lehn, 2007) to more recent issues with mammalian herbivory (Davis and Cipollini, 2014a), climate change, and species invasion (Courant et al., 1994; Porter, 1994; Davis and Cipollini, 2014b), *P. virginiensis* has been anecdotally noted as “in decline” for most

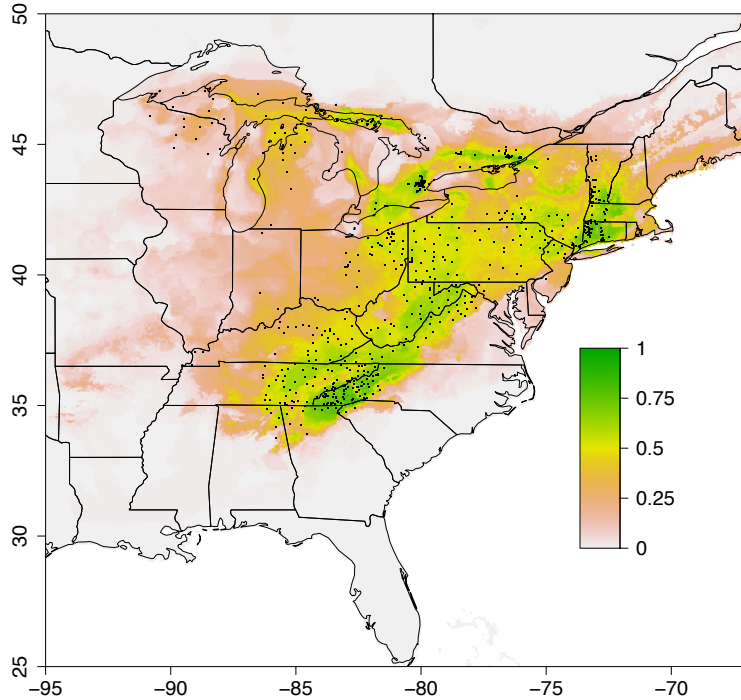


Figure 1.2: Known locations (points) and estimated current distribution of *P. virginien-sis*. Distribution was estimated using WorldClim predictors and maximum entropy species distribution modeling (Chapter 7). Scale represents probability of presence (0-1).

of the twentieth and twenty-first centuries. Like many rare organisms, this rare insect has a narrow host range (Dunn, 2005). It oviposits primarily on *Cardamine diphylla* and other spring ephemeral mustards like *C. angustata*, *Boechera laevigata*, and occasionally *C. concatenata* when other, more suitable hosts are unavailable (Cappuccino and Kareiva, 1985; Calhoun and Ifter, 1988; Shuey and Peacock, 1989). *P. virginien-sis* also feeds from and pollinates non-brassicaceous spring wildflowers, including *Trillium* spp., violets (*Vi-ola* spp.), spring beauties (*Claytonia virginica*), trout lilies (*Erythronium* spp.), wild geranium (*Geranium maculatum*), Virginia bluebells (*Mertensia virginica*) and woodland phlox (*Phlox divaricata*) (Bess, 2005).

As a univoltine butterfly, *P. virginien-sis* flies for only a few weeks each year in the springtime, and is strongly influenced by unsuitable weather, with low temperatures, high

wind, and precipitation all unacceptable for flight (Cappuccino and Kareiva, 1985). In 2011, just 33% of days were acceptable for flight in the month of April for *P. virginiensis* butterflies at a site north of Columbus, OH (Davis and Cipollini, 2014a), and Doak et al. (2006) estimated that only 60% of total available days and 28% of daytime hours were suitable for *P. virginiensis* flight. These butterflies seem unwilling to fly outside of the forest canopy, which makes long-distance dispersal and recolonization of abandoned sites unlikely (Cappuccino and Kareiva, 1985). Finally, *P. virginiensis* populations persist outside of recognized Lepidopteran hot-spot areas such as old-growth fields and meadows, which means that *P. virginiensis*, like other rare species, are often missed by long-term butterfly monitoring transects.

Although historical logging and urbanization may have caused initial habitat loss and fragmentation for *P. virginiensis*, current threats to this flagship species include climate change, exotic species, and competition. As the earth's climate changes, there will be a global rise in temperature and an increase in extreme weather events, which may lead 15-27% of butterfly species towards extinction by 2050 (Thomas et al., 2004a). The increasing temperature may advance butterfly emergence, and in some cases, cause butterfly range expansions and increases in yearly generations (Sparks and Yates, 1997), but not all butterflies will benefit under climate change predictions (Murphy and Weiss, 1992). As a time-limited butterfly, increases in extreme weather events and precipitation may reduce or eliminate *P. virginiensis* populations especially at the periphery of its range (Forister and Fordyce, 2011; Davis and Cipollini, 2014a). The effects of climate change on *P. virginiensis* are discussed in detail in Chapters 2 and 7 (Davis and Cipollini, 2014a).

Exotic species invasion is another troublesome influence on *P. virginiensis* populations. With the increase in worldwide travel, there has been an influx of exotic organisms into the United States from Europe and Asia (Pimentel et al., 2005). Although not all exotic organisms successfully establish, those that do can cause remarkable changes in natural habitats. Exotic plants decrease community diversity, alter soil biogeochemical cycles, and



Figure 1.3: Photographic evidence of an egg laid by *P. virginiensis* on flowering *Alliaria petiolata*, an invasive mustard, in Allegany State Park, NY in 2013.

compete against established plants for nutrients, light and resources (Gordon, 1998). Exotic insect invaders can disrupt established plant and herbivore communities, and in some cases, may cause exceptional economic damage. Examples include the currently invading Emerald Ash Borer, *Agrilus planipennis* (Buprestidae), which is systematically destroying native ash trees (Wang et al., 2010); the established *Pieris rapae* (Pieridae), which is believed to have caused local extinctions of the native *P. oleracea* (Scudder, 1889); and the Africanized “killer” bees (*Apis mellifera*), which inflict damage on any creature thought to pose a threat (Bresolin et al., 2002).

*Pieris virginiensis* seems to be primarily influenced by two exotic invaders: *Alliaria petiolata* Bieb. (Cavara & Grande), a European biennial mustard that now flourishes in North American forest understories (Nuzzo, 1993), and *Pieris rapae*, the European cabbageworm, a generalist Pierid butterfly that ravaged North American crops after its initial



Figure 1.4: Male *P. rapae* butterfly resting in a patch of *C. diphylla* in Wooster, OH on April 15, 2012. *P. rapae* differs morphologically from *P. virginiensis* by spots on the dorsal wing surfaces (pictured), shading of the upper wing tip (pictured) and yellow coloration on the ventral wing surfaces (not pictured).

introduction in Quebec in the 1800's (Scudder, 1889). The former poses a serious threat to *P. virginiensis*, as it seemingly attracts females to oviposit on its cauline leaves when flowering, but cannot support successful larval development (Fig 1.3; Bowden, 1971; Courant et al., 1994; Porter, 1994). This "oviposition mistake" behavior is a major concern for organizations working to conserve this rare species (Bess, 2005; Finnell and Lehn, 2007). With its unique assortment of flavonoids, glucosinolates, and other chemicals, including cyanide, and its already documented effects on other native Pierids, *A. petiolata* is a serious threat to the understory health of North American forests (Renwick et al., 2001; Keeler and Chew, 2008; Barto et al., 2010a). Oviposition mistakes on *A. petiolata*, their frequency, and possible chemical mechanisms are discussed in Chapters 3 and 4 (Davis and Cipollini,



2014b).

Two other chapters of this dissertation deal with competitive interactions with the two invaders described above. *Pieris rapae* occupies habitats where *P. virginiensis* occurs, and uses the same host plants, nectar sources, and flight period as the native congener (Fig 1.4). In Chapter 5, the extent of resource use by *P. rapae* in forest habitats with and without *P. virginiensis* is examined. In Chapter 6, the direct competitive effects of the invasive plant, *A. petiolata*, with two native mustard hosts of *P. virginiensis*, *C. diphylla* and *B. laevigata* are investigated. Although *A. petiolata* has been previously investigated for its allelopathic effects on mycorrhizal plants, neither *C. diphylla* nor *B. laevigata* have fungal mutualists, and so *A. petiolata* must compete without its documented novel weapons (Vaughn and Berhow, 1999; Prati and Bossdorf, 2004; Cipollini et al., 2008; Barto et al., 2010a; Wixted and McGraw, 2010). The sheer size of *A. petiolata* may influence its ability to compete against *B. laevigata* and *C. diphylla* for nutrients and light (Fig 1.5).

The final chapter of this dissertation examines the current state of *P. virginiensis*, its distribution, and its genetic diversity within cytochrome oxidase subunit I, a mitochondrial gene, and the internal transcribed spacer I region, a nuclear locus. A species distribution model is constructed and used to determine the future distribution of *P. virginiensis* in light of climate change. The genetic information is used to evaluate the dispersal ability and genetic structure between populations of *P. virginiensis*.

Although a seemingly unimportant contributor to forest ecosystems, this flagship species contributes to public awareness of ecosystem function and ecology. It also serves as a model of how a rare, threatened species interacts with its changing environment. Perhaps most importantly, *P. virginiensis* and its relationship with *A. petiolata* inform the scientific community about the cascading chemical and ecological effects of exotic invaders within novel ecosystems.

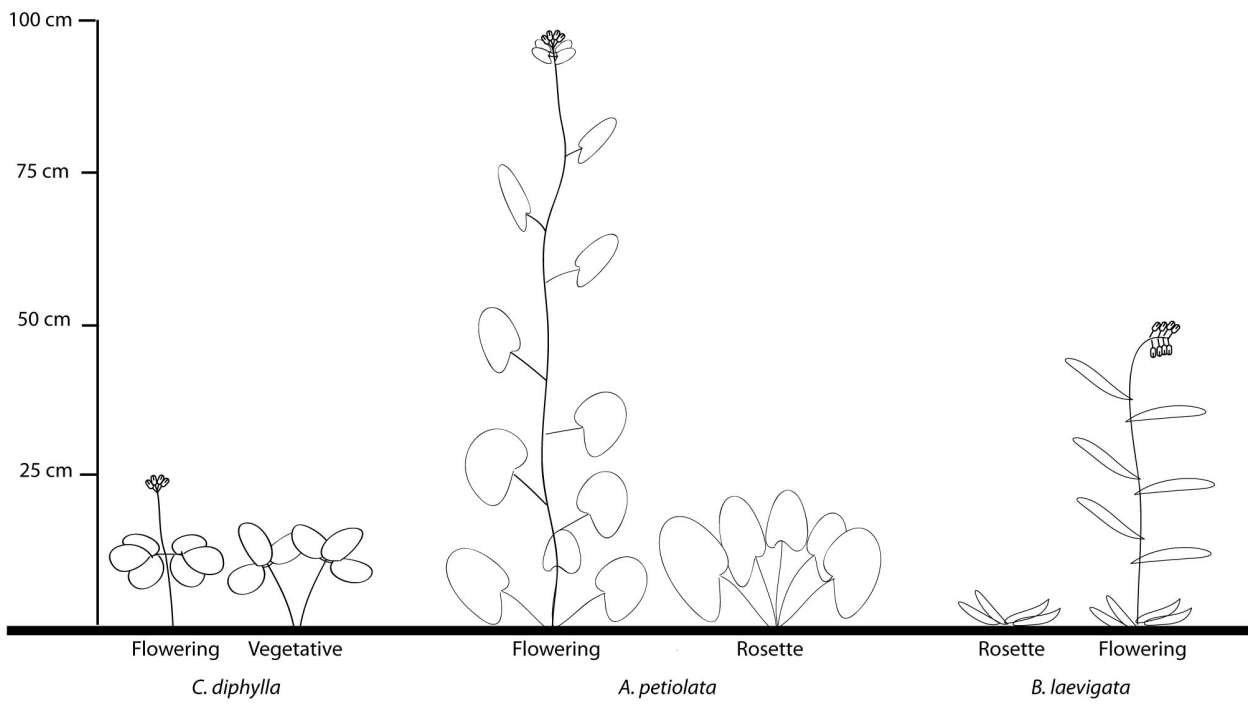


Figure 1.5: Drawings of *B. laevigata*, *A. petiolata*, and *C. diphylla*, representing their respective height and leaf characteristics that may influence competitive outcomes.

# **How environmental conditions and changing landscapes influence the survival and reproduction of a rare butterfly, *Pieris virginiensis* (Pieridae).**

## **2.1 Introduction**

Rare species are often narrowly distributed and survive by occupying unique niches in the ecosystem (Gaston, 1998; Zavaleta and Hulvey, 2004). Rare or extremely specialized native herbivores may suffer population reduction or local extinction after a major disturbance or loss of habitat (Dunn, 2005). Under pressures such as exotic plant invasion and climate change, many rare species are in danger of extinction if migration is not feasible (Roy and Sparks, 2000; Jump and Penuelas, 2005; Neilson et al., 2005).

*Pieris virginiensis* Edwards, the West Virginia White butterfly, is a rare, univoltine butterfly native to riparian areas of mature forests in North America, where it completes its lifecycle on native spring ephemeral crucifers. *Pieris virginiensis* can be found along the northern border of the United States, from Wisconsin to Vermont and Massachusetts,

and as far south as northern Georgia and Alabama (Finnell and Lehn, 2007). *Pieris virginiensis* has been anecdotally considered in decline due to forest disturbance via logging, fragmentation, deer grazing pressure, and plant invasion (Finnell and Lehn, 2007). It is considered rare, but has not yet been evaluated by the International Union for Conservation of Nature's Red List, and there are no long-term studies of *P. virginiensis* populations to confirm this anecdotal observation of continual decline (IUCN, 2012). Although there are excellent butterfly monitoring organizations, such as the Ohio Lepidopterists' Society, *P. virginiensis* is frequently overlooked as it flies early in the spring in forested areas, which are not major sources of butterfly diversity, and are not often regularly monitored.

*Pieris virginiensis* primarily uses the spring ephemeral mustard, *Cardamine diphylla* as its larval host plant, but also occasionally uses *Boechera laevigata*, a spring ephemeral biennial mustard. Sparsely distributed, *B. laevigata* is not an ideal host, but is the primary host of *P. virginiensis* in a site in Marengo, OH, where *C. diphylla* does not occur. An alternative host, *C. concatenata* can be used but is not preferred due to its small size and early senescence (Shuey and Peacock, 1989).

Courant et al. (1994) and Porter (1994) observed *P. virginiensis* females ovipositing on *Alliaria petiolata*, an invasive shade-tolerant biennial mustard that is most likely toxic to emerging offspring. In a closely related native Pierine butterfly, *Pieris oleracea*, sinigrin is believed to be the primary oviposition stimulant in *A. petiolata*- this may hold true also for *P. virginiensis*, although it has not yet been tested (Huang and Renwick, 1994). Previous studies have shown full larval mortality after consumption (Bowden, 1971; Courant et al., 1994; Porter, 1994). Several chemical constituents of *A. petiolata* leaves have been shown to deter feeding and reduce survival in 1st and 4th instars of *P. oleracea*, although *P. oleracea* populations that have been exposed to *A. petiolata* for 60-100 generations may be adapting to its chemical arsenal (Renwick et al., 2001; Keeler and Chew, 2008). If *A. petiolata* is similarly toxic to young *P. virginiensis* caterpillars, adults may be wasting eggs on the plant; if *A. petiolata* deters feeding in older *P. virginiensis* caterpillars, caterpill-

lars searching for a new host plant after consuming their previous host may starve before reaching an appropriate native food source (Cappuccino and Kareiva, 1985; Porter, 1994).

Shuey and Peacock (1989) examined a population of *P. virginiensis* reproducing entirely on the alternative hosts, *B. laevigata* and *C. concatenata*. The study site is surrounded by agricultural fields, adjacent to Alum Creek in Morrow Co., OH. They examined plants in three locations along a roughly 150 meter section of woodlands: a ridge above a shale embankment, the shale embankment, and bottom-lands below. They found that of the two hosts, *B. laevigata* was strongly preferred, perhaps because *B. laevigata* senesces much later than *C. concatenata*, increasing time available for larval development. In addition, more eggs were laid on the south-facing shale embankment than in the other two regions examined; perhaps because it warmed more quickly during the day, which in turn would decrease caterpillar development time. During the study period in 1988, Shuey and Peacock (1989) found 102 eggs on 52 *B. laevigata* plants and only 21 eggs on 57 *C. concatenata* plants. Shuey and Peacock (1989) completed their study before the conversion of nearby agricultural areas to fallow fields, increases in deer population, and the introduction of *A. petiolata* (Porter, 1994; Côté et al., 2004; Stinson et al., 2006; Finnell and Lehn, 2007; Ripple et al., 2010). Each of these changes in the study location may have influenced the survival and reproduction of this isolated population of *P. virginiensis*.

Deer populations in Ohio have been steadily increasing, and may negatively influence the presence or quality of nectar sources and host plants for this rare butterfly (Ripple et al., 2010). *Pieris virginiensis* adults feed on a variety of nectar sources, including members of *Claytonia*, *Trillium* and *Viola* genera (Bess, 2005). Increased deer browsing may change the plant community, and in turn, alter the habitat quality for *P. virginiensis*, although the effects of deer on butterflies are complex and life-history dependent (Feber et al., 2001).

Introduction of the invasive *A. petiolata* may have also had profound effects on this location. As noted above, *P. virginiensis* oviposits on *A. petiolata*, although the frequency of this maladaptive egg-laying behavior is unknown, and caterpillars experience moder-

ate to complete mortality when it is used as a foodplant (Bowden, 1971; Porter, 1994). Poor oviposition choices could severely reduce this site's population that, in 1988, had only 14.8 % of eggs survive to fourth instar even on its native hosts (Shuey and Peacock, 1989). *Alliaria petiolata* may also host potential egg/caterpillar predators, such as spiders or predatory ants, reducing survival of *P. virginiensis* on a potentially novel host. In addition, *A. petiolata* is known to negatively influence plants around it through direct competition and allelopathy, reducing the frequency or quality of nearby nectar or host plants (Stinson et al., 2006). In addition to these direct effects, *A. petiolata* is occasionally used as both an oviposition substrate and a nectar source by *Pieris rapae*, the European cabbage white butterfly.

Although *P. rapae* were noted adjacent to the study area in 1988, Shuey and Peacock (1989) documented no instances of *P. rapae* entering the forested area to use nectar sources or oviposition sites; in 2012, however, *P. rapae* butterflies were flying through the wooded areas at this location (SD and DC, pers. obs.). The conversion of agricultural fields to fallow fields at this location may have increased resident *P. rapae* populations, perhaps increasing competition for nectar and/or oviposition sites within the forest and edges.

Finally, changing climatic conditions may influence *P. virginiensis* populations. Although many butterfly species are expected to increase under warming temperatures, butterflies that have strict habitat requirements or fly at the edge of their range may be at risk for population reduction and eventual extinction (Forister and Shapiro, 2003; Forister and Fordyce, 2011). *Pieris virginiensis* flies best in winds under 25 km/h and in temperatures between 19 – 30 °C (Cappuccino and Kareiva, 1985). To complicate matters, it is often the previous year's weather that has the most effect on butterfly population in the following year (Roy et al., 2001).

We investigated *P. virginiensis* survival and reproduction over two field seasons (2011-2012) in a habitat which previously hosted a robust population of *P. virginiensis*, to answer the following questions: Does successful reproduction occur at this location? Does *P.*

*virginiensis* differentially use *A. petiolata* and *B. laevigata*? How frequently does non-caterpillar damage (e.g. deer herbivory) occur to host plants? What is the frequency of potential predators on all possible host plants? Have climatic conditions relevant to suitable flying conditions changed over time at this site?

## 2.2 Methods

The study site, in Morrow County, Ohio, is a privately owned forest fragment bordering Alum Creek adjacent to two fallow fields, which was originally surveyed by Shuey and Peacock (1989). On April 21, 2011, we surveyed the site and found 5 flying adult *P. virginiensis* individuals, of which two were collected for identification and further study in the laboratory. This was the only occasion that we observed flying adults in 2011, but this confirmed that *P. virginiensis* still persisted in this location.

In 2011, mimicking Shuey and Peacock (1989), we systematically searched for and tagged flowering stalks of both the native *B. laevigata* ( $n = 64$ ) and the invasive *A. petiolata* ( $n = 54$ ) on April 21, 2012, and returned twice to score plants (May 5 and May 11) for the presence of *P. virginiensis* eggs or caterpillars, potential predators (ants, spiders), and herbivore damage (deer or other). We chose not to survey *C. concatenata* because it was a minor host in 1987. Plants were examined at the same ridge and shale embankment zones studied by Shuey and Peacock (1989), but were not systematically examined in the lowland-areas, as only one egg was found during their study in the lowland zone. Casual observations in the lowland zone revealed no eggs or caterpillars. During tagging and scoring events after the initial site visit, we searched visually for flying *P. virginiensis* adults. We only conducted search events on days appropriate for butterfly flight (temperature above 10 °C, wind speed under 25 km/hr) to maximize our chances of witnessing oviposition events.

In 2012, flowering stalks of both the native, *B. laevigata* ( $n = 113.6 \pm 26.85$  plants

searched per visit) and the invasive, *A. petiolata* ( $n = 95 \pm 34.53$  plants searched per visit) were tagged (March 30) and scored weekly (April 6, 13, 20, 27; May 4) using methods identical to 2011. In addition to these tagged plants, any unmarked plants found during repeated random searching were scored, but unmarked. During tagging and scoring events, we searched visually for flying *P. virginiensis* adults, but could confirm none, as the individuals seen may have been *P. rapae* adults.

These 2011-2012 scoring data were converted to presence/absence values and fit to one of several binomial regressions in R (R Development Core Team, 2013). Year (2011 or 2012) and Host Plant (*Boechnera* or *Alliaria*) were used as predictors for the presence or absence of deer damage, other herbivorous damage, and potential predators.

In 2011, the captured adult butterflies were kept together in a  $0.216m^3$  enclosure and allowed to feed from a 10%(v/v) sugar: water solution, and placed on a 16:8 hr light/dark cycle under fluorescent lights. These butterflies were given the choice of individual flowering *C. diphylla* (collected from Pennsylvania), *C. concatenata* (collected from Dayton, OH), or *A. petiolata* (collected from Dayton, OH) as oviposition substrate. We examined each plant daily for eggs until the butterflies died.

In 2011, emerging caterpillars ( $n = 4$ ) were allowed to hatch and feed on *C. diphylla*, *B. laevigata* (collected from Yellow Springs, OH), or *A. petiolata*. All four caterpillars were initially fed on *C. diphylla*, but were divided evenly and transferred to either *B. laevigata* or *A. petiolata* at the 4th instar for a no-choice survival test.

In addition to these field-collected variables, we examined weather data from the Port Columbus International Airport Weather Station (about 40 km from research location) to evaluate if there were increasing trends in weather during the month of April (*P. virginiensis* flight season) between 1987, the year preceding the Shuey and Peacock (1989) study, and 2012. We analyzed climatic trends from 1987 to present using simple linear regression. All statistical analyses were completed in R 2.15 (R Development Core Team, 2013).



## 2.3 Results

### 2011 Results.

Despite multiple visits to the study site, we recovered no *Pieris virginiensis* eggs, no caterpillars, and found little damage that could be attributed to caterpillar herbivory (Table 2.1). Furthermore, we witnessed no flying adults after April 21, 2011. In addition to these direct observations, indirect observations of host-plant conditions suggested no *Pieris*-related herbivory, although there was occasional incidence of leaf or stem damage from deer (*Boechnera*: 5.47%, *Alliaria*: 4.63% ) or other organisms (*Boechnera*: 5.47%, *Alliaria*: 12.96%). Ants and spiders (*Boechnera*: 4.69%, *Alliaria*: 17.5%) were observed on both study species.

Table 2.1: Number of eggs located on plants in Morrow Co., OH, in 1988 (Shuey and Peacock (1989), 2011 and 2012.

	<i>C. concatenata</i>	<i>B. laevigata</i>	<i>A. petiolata</i>
<b>1988</b>	21 (n=68)	102 (n=52)	-
<b>2011</b>	-	0 (n=64)	0 (n=54)
<b>2012</b>	-	1(n=113.6)	0 (n=81)

*Footnotes.* *C. concatenata* was not searched in 2011 and 2012 due to low incidence of egg deposition in 1988. Numbers are as follows: Eggs found (n=total plants searched).

When captured adult butterflies were given the choice between three potential host plants, all eleven eggs were oviposited on *A. petiolata*, the invasive mustard. These data were pooled, as the adult butterflies were not separated. The four surviving caterpillars readily consumed native mustard tissue, but those placed on *A. petiolata* only consumed a small amount of tissue, then would enter a quiescent state during which they refused to eat *A. petiolata*, but would resume eating when placed on *C. diphylla*.

## 2012 Results.

In 2012, we began our search in March when unusually warm weather facilitated early plant and butterfly emergence. We found one egg on an *Boechnera* plant, but saw no confirmed *P. virginiensis* butterflies, and occasionally witnessed *P. rapae* individuals flying through the woodlands. The egg was not removed for identification, and a week later, although there was minor herbivory to the *Boechnera* plant where the egg was found, no larva was recovered (Table 1). In addition, there was one Pierid caterpillar recovered, from a second-year (flowering) *Alliaria* individual, however, the caterpillar was small and we were unable to confirm its identity as either *P. virginiensis* or *P. rapae*. The incidence of leaf or stem damage from deer (*Boechnera*: 2.29%, *Alliaria*: 0.63%) or other organisms (*Boechnera*: 8.27%, *Alliaria*: 3.58%), as well as ants and spiders (*Boechnera*: 7.75%, *Alliaria*: 9.68%) was low on both study plant species.

Combined scoring for 2011 and 2012. The presence of deer damage was affected by both host plant species and year, with a model:

$$DeerDamage = -1.0529 * Year - 1.0066 * PlantSpecies - 2.7648 + Error \quad (2.1)$$

All factors in the model were significant, and it was more likely for us to find deer-browsed *Boechnera* plants than *Alliaria* plants ( $p < 0.05$  for Year, Plant).

The presence of other herbivorous damage was predicted by host plant species, but not year, with *Boechnera* having a higher incidence of damage being present ( $p < 0.01$ ):

$$OtherDamage = -0.6712 * PlantSpecies - 2.2026 + Error \quad (2.2)$$

Finally, predator presence or absence could not be predicted by either host plant species or year.

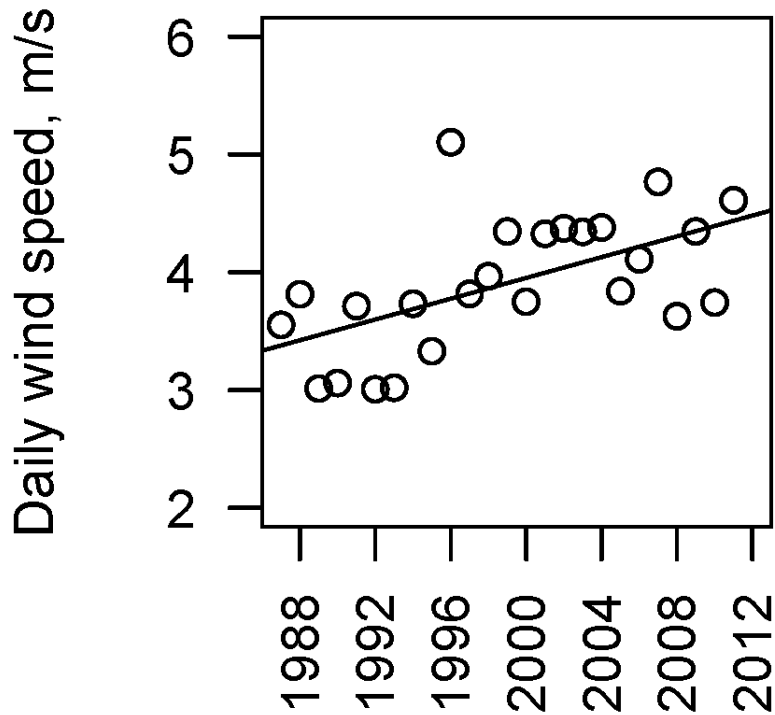


Figure 2.1: Average wind speed (m/s) at Port Columbus International Airport in April, 1987-2012 ( $p < 0.01$ ).

### **Weather Analysis.**

Linear regressions across all years (1987–2012) indicate significant increases through time in average wind speed, maximum temperature, and minimum temperature in the month of April (Figures 2.1 - 2.3).

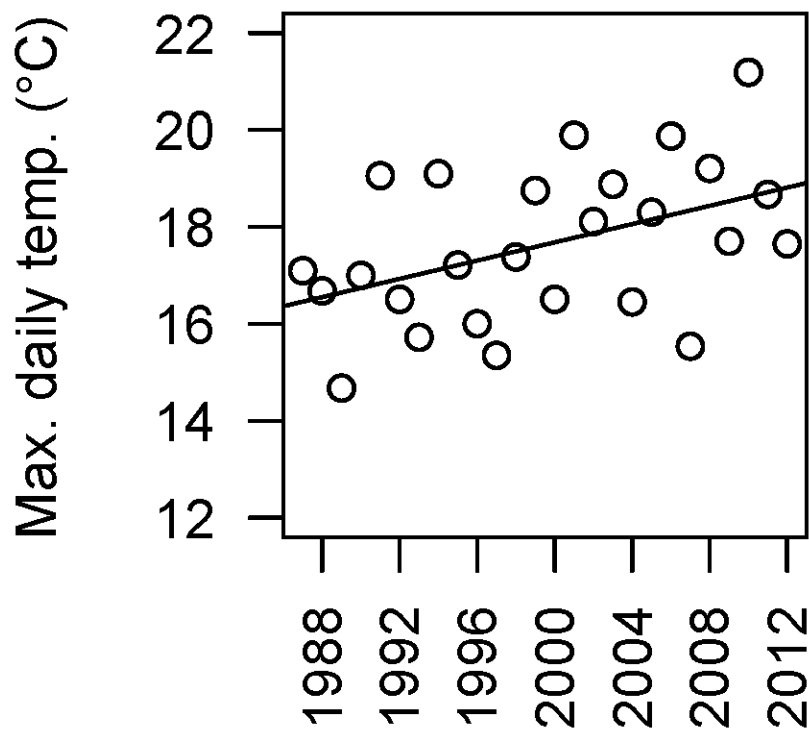


Figure 2.2: Average maximum daily temperature (Celsius) in April, 1987-2012 ( $p < 0.05$ ).

## 2.4 Discussion

In this study, we investigated the survival and reproductive success of *P. virginiensis* at a site last evaluated in 1988 (Shuey and Peacock, 1989). It is clear that at this site, *P. virginiensis* is not successfully using either a native host, *B. laevigata*, or an invasive host, *A. petiolata*. In 1988, 102 eggs were found across 52 marked *Boechera* plants in contrast, we found only one egg on over 150 plants repeatedly searched in two years at this location (Shuey and Peacock, 1989). This suggests that *P. virginiensis* at this site has faced severe population reduction and may, in the near future, face local extinction.

It is possible that sometime in the intervening 23 years, *P. virginiensis* may have attempted to shift to *A. petiolata*. A shift to *A. petiolata* could result in one of three outcomes:

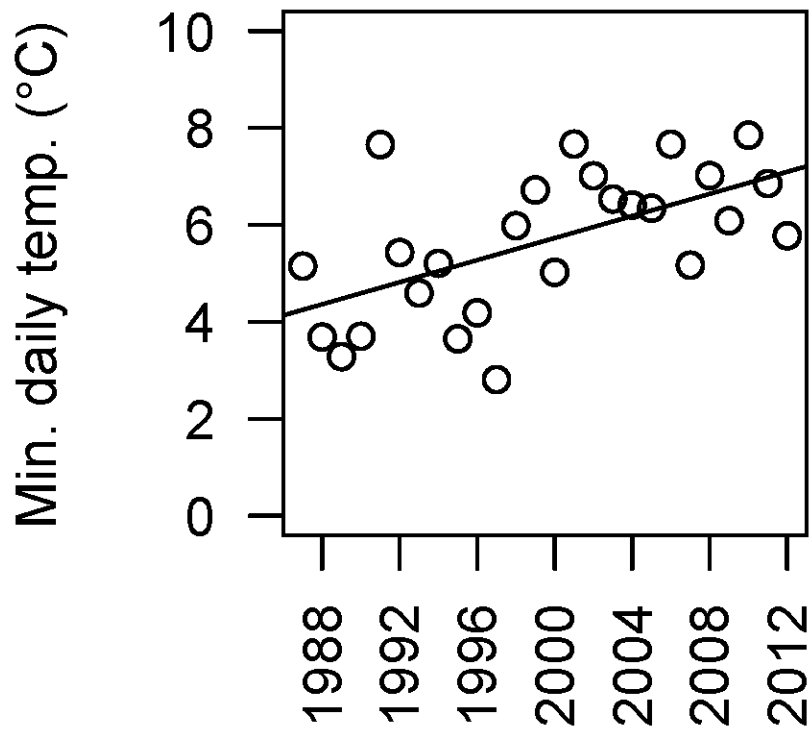


Figure 2.3: Average minimum daily temperature (Celsius) in April, 1987-2012 ( $p < 0.01$ ).

the population could respond neutrally (e.g., no population growth), positively (a full shift causes an increase in population), or negatively (the population declines because of increased mortality on *A. petiolata*) (Porter, 1994). In a scenario where *A. petiolata* was the only novel introduction to this location, we would perhaps conclude that *A. petiolata* had a detrimental effect, but the clear decline in this population could be due to any number of other factors, including changes in nectar source or host plant quality, deer damage, or climate.

A reduction in nectar plant or host plant quality has been shown to strongly influence what constitutes an acceptable habitat for butterflies (Holl, 1995; Mevi-Schutz and Erhardt, 2005; Severns et al., 2006). We only examined host plant identity in this study,

and found evidence of low to moderate damage from both deer and other sources, as well as a significant presence of potential predators, like ants and spiders. Although there were no differences in predator presence by host plant species, *Boechera* plants had a higher incidence of both deer browsing and other, non-caterpillar herbivory.

This difference in herbivory incidence between native and exotic plants could be attributed to both the enemy release and the novel weapons hypotheses (Keane and Crawley, 2002; Callaway and Ridenour, 2004). The enemy release hypothesis posits that an invasive plant will do well in a novel environment because it is released from its native range specialist herbivores, specialist herbivores in its introduced range do not switch hosts, and generalists attack the introduced plant at a much lower frequency than its nearby native neighbors. Lewis et al. (2006) show that *A. petiolata* indeed receives less damage in its introduced range than in its native range, there is only minor evidence of native specialist herbivores switching to *A. petiolata*, and our observations suggest lower herbivory on *A. petiolata* when compared to native crucifers like *B. laevigata* (Keeler et al., 2006). The novel weapons hypothesis posits that a plant in a novel environment has a unique chemical arsenal that can prevent herbivory in its introduced range. Previous studies indicate that *A. petiolata* contains several chemicals that affect native North American herbivores, and again, our herbivory incidence observations support this hypothesis (Haribal and Renwick, 1998; Haribal et al., 2001; Renwick et al., 2001).

Although the authors have witnessed *P. rapae* using the same nectar and oviposition resources as *P. virginiensis* in nearby populations (Wooster, OH, SD, pers. obs.), there is no evidence to suggest that *P. rapae* presence has directly reduced *P. virginiensis* population in this location. Instead, it may be that *P. rapae* uses this site occasionally, but primarily subsists in open fields adjacent to the study area.

We believe aberrant weather in 2011 and 2012 caused two recent years of failed *P. virginiensis* reproduction at this location. If it is too cool, wet, or windy, the univoltine *P. virginiensis* cannot fly, mate, or reproduce. These recent unusual weather patterns may

soon become a chronic issue for this butterfly under predictions of global climate change.

Evidence from other butterfly population studies indicates that although some butterflies benefit from warming global temperatures, others may suffer (Sparks and Yates, 1997; Roy et al., 2001; Forister and Shapiro, 2003). In four different scenarios for the Bay Checkerspot Butterfly, Murphy and Weiss (1992) found that only one of the climate scenarios was beneficial for the organism: warmer, wetter summers. Severe weather could have particularly strong effects on butterfly populations at the edge of their acceptable weather ranges. Furthermore, butterflies with low population numbers are more at risk for local extinction events when faced with multiple bad years and the lack of carry-over pupae (Forister and Fordyce, 2011).

It is clear that there are differences in temperature and average wind speed, and in some instances, precipitation and cloud cover between decades at this location. In addition, climate data demonstrate a linear increase in temperature and wind speed over the last 23 years that may have influenced these butterflies. Cappuccino and Kareiva (1985) showed that *P. virginiensis* has a difficult time flying in strong wind speeds, or in cool weather. As ectotherms, many butterflies are reliant on sunshine to bask and prepare for flight. Many days in a *P. virginiensis* adult lifespan were not ideal for flight in the 1980s, and although warming springs may facilitate population growth, an increase of windiness and in some cases, cloud cover at this location may ameliorate any benefits of climate change for *P. virginiensis* (Cappuccino and Kareiva, 1985; Doak et al., 2006). 2011 was a remarkable year for rainy, poor weather in Columbus, OH, with a record of 18.1cm of precipitation. The month of April 2012 was equally remarkable in its excessively warm temperatures that facilitated early plant and butterfly emergence. As the probability of extreme or unusual climate events increases, we expect further decline in *P. virginiensis* populations across its range.

*Alliaria petiolata* may further contribute to *P. virginiensis* decline by serving as a population sink, however, despite the observations made by Courant et al. (1994) and Porter

(1994), no one has yet determined how frequently this occurs and how risky it is for *P. virginensis* to exist in *A. petiolata* invaded habitats. Our limited lab data suggest that *P. virginensis* adults will oviposit on *A. petiolata*, but caterpillars refuse to feed on *A. petiolata* in the fourth instar. Continued contact with *A. petiolata* may increase the use of *A. petiolata* by *P. virginensis* through time, as was seen in populations of *P. oleracea* by Keeler and Chew (2008). However, *P. virginensis* populations are small, and migration is limited, which may reduce *P. virginensis* genetic diversity and consequently, populations' ability to adapt to *A. petiolata*. This particular population of *P. virginensis* is already low in number and may soon face local extinction. *P. virginensis* has limited dispersal potential due to an observed aversion to flying in open spaces, and so, recolonization of this site is unlikely (Cappuccino and Kareiva, 1985). We are unable to confirm the role of *A. petiolata* in *P. virginensis* decline at this site, but we believe that severe or chronic weather anomalies, like the cool and wet spring of 2011 may negatively influence butterfly population, as was seen in other studies. In addition, selective herbivory of nectar and larval host plants by deer may directly and indirectly contribute to *P. virginensis* decline. Future studies will include more observation of this location, as well as expansion into other locations to investigate the direct impacts of deer, predators, climate, and *A. petiolata* on the *P. virginensis* life cycle.



# **Do mothers always know best?**

## **Oviposition mistakes and resulting**

### **larval failure of *Pieris virginiensis* on**

### ***Alliaria petiolata*, a novel, toxic host.**

#### **3.1 Introduction**

Invasive plants often have direct negative effects on native species that occupy the same habitat. Exotic plant invaders are known to alter biogeochemical cycles, decrease community diversity, and compete against established plants for nutrients, light, and pollinators (Gordon, 1998). Exotic organisms not only damage neighboring plants, but also can damage native plant-herbivore communities through novel interactions, occasionally threatening rare and endangered species (Pimentel et al., 2005).

Novel plant-insect interactions occur when a plant or insect species moves into a novel environment and begins to interact with the surrounding community. Novel plant-insect interactions have one of three outcomes: the native insect adopts the novel plant and benefits through increased population size; the native insect fails to recognize the plant as a poten-

tial host or there are no fitness effects; and finally, mismatches occur when native insects incorrectly recognize the novel plant as a host but larvae cannot develop. Successful adoption of sweet fennel (*Foeniculum vulgare* Miller) by *Papilio zelicaon* resulted in a transition from univoltinism to multivoltinism, decreasing generation time and increasing population size and health (Tong and Shapiro, 1989). In other cases, the insect fails to recognize a potential host that can support larval development, as in the interaction of the Clouded Sulphur butterfly (*Colias philodice*) and Crown Vetch (*Securigera varia*; Karowe 1990), and the West Virginia White butterfly (*Pieris virginiensis*) and watercress (*Nasturtium officinale*; Bowden 1971). In a “worst case” scenario for native butterflies, the insect incorrectly accepts an ill-suited host, wasting eggs and threatening population stability. These oviposition mistakes are well documented in Lepidoptera and include members of Papilionidae (Berenbaum, 1981), Nymphalidae (Straatman, 1962), and Pieridae (Chew, 1977).

*Alliaria petiolata* Bieb. (garlic mustard) is a European invasive biennial herb that was introduced to the United States in the 1800’s and was recognized as a major invasive plant in the mid-to-late 1900’s. *A. petiolata* reduces native seed germination through allelopathy, and directly competes against native plants for nutrients and light (Meekins and McCarthy, 1999; Prati and Bossdorf, 2004). In addition to these direct effects, *A. petiolata* can indirectly affect native plant health through negative effects on beneficial soil microbes, including bacteria, arbuscular mycorrhizal fungi (AMF) and ectomycorrhizal fungi (EMF) (Roberts and Anderson, 2001; Burke, 2008; Callaway et al., 2008; Wolfe and Rodgers, 2008).

*Alliaria petiolata* has been implicated in the decline of two native butterfly species, *Pieris oleracea* and *Pieris virginiensis* (Pieridae). Both of these species are springtime forest butterflies that normally use native crucifer hosts, most frequently *Cardamine* and *Boechera* (*Arabis*) species (Shuey and Peacock, 1989; Finnell and Lehn, 2007; Keeler and Chew, 2008). Since the introduction of *A. petiolata*, both butterfly species have been observed occasionally ovipositing on *A. petiolata*, though the frequency and effects of these

events are unknown, and no long-term studies have been undertaken (Courant et al., 1994; Porter, 1994). Although it seems that *P. oleracea* may be adapting to using *A. petiolata* as a novel host (Keeler and Chew, 2008), there is nothing known about how *P. virginiensis* populations are responding to mistake oviposition events, other than that populations appear to be declining (Finnell and Lehn, 2007).

*Pieris virginiensis*, the focus of this study, emerges in the early spring (March-May) to mate and lay eggs on native crucifers, most commonly *C. diphylla* (Michx.), although there are occasional small populations that use *C. concatenata* (Michx.), *C. dissecta* (Leavenw.) or *Boechera laevigata* (Muhl. ex Willd.) when *C. diphylla* is absent (Calhoun and Ifter, 1988; Shuey and Peacock, 1989; Finnell and Lehn, 2007). In addition to interactions with larval host plants, *P. virginiensis* pollinates early springtime herbs in the genera *Claytonia*, *Erythronium*, *Mertensia*, *Phlox*, *Trillium* and *Viola* (Bess, 2005). Finnell and Lehn (2007) suggest that the perceived decline of *P. virginiensis* may be due to habitat loss and fragmentation, poor environmental conditions and exotic plant invasion. The distribution of *P. virginiensis* (Wisconsin to Vermont, south to Georgia and Alabama) overlaps strongly with *A. petiolata* distribution and if *A. petiolata* commonly elicits mistake oviposition events fatal to hatching caterpillars, *P. virginiensis* populations may soon be reduced and even eliminated from heavily invaded areas. Bowden (1971) demonstrated that *P. virginiensis* caterpillars could not survive on *A. petiolata*, but his sample sizes were small and these experiments occurred before widespread contact between *P. virginiensis* and *A. petiolata*. Porter (1994) conducted similar trials, but terminated his experiment before results became conclusive. As a result, the fate of *P. virginiensis* caterpillars on the novel host is not conclusively known, and may have changed as contact increased between the two species.

Although we know that mistake oviposition events happen, we do not know how frequently these events occur, nor do we know if there is a fitness cost to *P. virginiensis* when they oviposit on *A. petiolata*. Furthermore, we do not know if populations vary in their ability to successfully utilize the novel host *A. petiolata* as young caterpillars. We investigated

oviposition preference and larval performance of *P. virginensis* through a combination of field observations and laboratory manipulations to answer the questions: Does *P. virginensis* oviposit on *A. petiolata* in the field, and how frequently does this occur? Does *P. virginensis* show an oviposition preference for *A. petiolata*, its native host *C. diphylla*, or neither? Can *P. virginensis* neonates consume and survive on *A. petiolata* leaves? And finally, can potentially toxic or deterrent chemicals extracted from *A. petiolata* leaves mimic the effect of whole leaves on larval performance?

## 3.2 Methods

### Field surveys of oviposition preferences.

To investigate how frequently oviposition events occur on the novel host plant (*A. petiolata*) and the native host plant (*C. diphylla*), we surveyed known populations of *P. virginensis* in Ohio, Pennsylvania, and New York. *C. diphylla* grows and spreads through rhizomes underneath the soil and rarely produces fertile seeds (Sweeney and Price, 2001). For each survey of *C. diphylla*, we considered a section of rhizome with leaves clustered near each other (usually 1-3 leaves) as an individual plant. Only flowering *A. petiolata* plants were searched for eggs and caterpillars, as to our knowledge, *P. virginensis* does not oviposit on rosette *A. petiolata*.

We surveyed linear transects in three locations in 2012 and 2013. Holden Arboretum near Cleveland, OH (HA), a Beech/Maple old-growth forest that is uninvaded by *A. petiolata*, was surveyed in April 2012 for eggs on *C. diphylla* ( $n = 227$  plants). We haphazardly selected *C. diphylla* plants from within 2 meters of the boardwalk trail area for examination. *Pieris virginensis* uses *C. diphylla* in this location exclusively as its larval host plant, which can be found mostly along riparian areas in mature, old growth forest. Allegany State Park (ASP, Salamanca, NY), an old-growth Black Cherry/Oak/Hemlock habitat with

occasional riparian zones, was systematically surveyed in April 2012 and May 2013 for eggs on both *C. diphylla* ( $n = 173$ ) and *A. petiolata* ( $n = 411$ ). Every possible host plant (all *C. diphylla* and second-year *A. petiolata*) within 2 meters of the road edge was examined for eggs and caterpillars. Roaring Run Recreational Area near Apollo, PA (RR) was surveyed in May 2013 for eggs only on flowering *A. petiolata* ( $n = 265$ ), although *C. diphylla* is the primary host plant at this site. Second year *A. petiolata* within 5 meters of a 300-meter transect were haphazardly chosen for examination. This site is also dominated by Black Cherry, Oak, and Hemlock and the sampled areas included the riparian area along Roaring Run and uplands bordering a shaded gravel bike trail.

*Alliaria petiolata* was surveyed through destructive harvesting. We pulled second-year flowering *A. petiolata* from the ground and examined all sides of the leaves and stems for eggs and neonates (eggs are typically placed on the undersides of leaves on the upper third of the plant). Eggs and neonates were removed from *A. petiolata* for subsequent larval survival assays. We surveyed *Cardamine diphylla* non-destructively by flipping leaves and searching for eggs and neonates. Variation in egg deposition among sites (ASP, RR, and HOL) and years (2012 vs. 2013 for ASP only) was analyzed, then data were pooled by species for comparisons between plant species. We used a test of equal or given proportions ( $X^2$  statistic) of eggs laid on each host plant to analyze variation in each of these categories.

### **Controlled oviposition preference experiments.**

To investigate oviposition preferences of *P. virginensis* under controlled conditions, we conducted oviposition preference assays. Adult butterflies were collected from the Quaker area of ASP in May 2013. Butterflies were collected using a standard butterfly net in forested areas along trails as well as along partially shaded forest roads, most frequently between 10:00-13:00, and 16:00-18:00. After capture, butterflies were sexed and deposited in a shaded communal aquarium with access to freshly picked nectar-producing flowers placed in water (*Claytonia virginica*, *Bellis perennis*, *Taraxacum officinale*, etc.). For each

trial, between one and four mated female butterflies were marked with a pen on the ventral surface of a hind wing and placed in a 106 L glass terrarium with a screen top and sliding side doors. At least 20 wildflowers (freshly picked from ASP forest roads and grassy areas) commonly used by adults as nectar sources (see above for species) were placed in a bottle filled with water in the center of the aquarium; and the experimental choice plants (one potted flowering *A. petiolata* [Dayton, OH] and one potted *C. diphylla* [Hocking Hills State Forest, OH]) were randomly placed to the left and right of the nectar source. Although *A. petiolata* can reach heights of 2 m in natural settings, *A. petiolata* in this experiment were 30-45 cm in height, to fit inside the terrarium and reduce height differences between the species. Eighteen trials were conducted with wild-caught *P. virginienensis* females, and two trials were conducted with one second generation female - a female that had not undergone diapause after being raised on *C. diphylla* at 25 °C in an incubator set to 16:8 L:D. The lab-raised butterfly was fed a 20% sugar:water solution before the oviposition trial. We observed butterflies in oviposition trials for one hour, and each time an oviposition occurred, we recorded the time, butterfly identity, and plant identity. In trials with multiple butterflies, butterflies only interacted with each other during nectaring or resting behaviors, and no butterfly physically interfered with another's oviposition event. At 30 minutes, the plants used as oviposition choices were switched to prevent position bias. Oviposition trials either took place in shaded outdoor areas ( $n=16$ , temp. range 15.5 °C to 26.6 °C) at ASP or at 22 °C with artificial light ( $n = 4$ , 60W standard incandescent bulb and 60W full spectrum daylight / UVA incandescent bulb). Oviposition trials were conducted between 9am and 6pm, when the butterflies were most active in captivity. To reduce impact on this rare butterfly population, butterflies were occasionally used for multiple trials, though never twice on the same day. An oviposition preference index (OPI) was calculated for each individual as the number of eggs laid on *A. petiolata* divided by the total number of eggs laid on both plants. OPI was analyzed using a one-sample t-test. In addition to OPI, the number of eggs laid on each plant by individual butterflies was analyzed using a paired t-test. Cohen's  $d$

was calculated to estimate the magnitude of difference between eggs laid on the two plant species. All statistical analyses were performed in R (R Development Core Team, 2013).

### **No-choice leaf feeding assay.**

After confirming that adults were ovipositing on *A. petiolata*, along with their typical host plant, we used eggs found during field surveys at either ASP or RR, as well as eggs laid by captured adults in the oviposition preference experiments to examine the performance of larvae on native and novel hosts. Freshly hatched neonates were placed in either 100x15mm Petri dishes sealed with parafilm or 236mL plastic containers along with fully expanded stem leaves of either flowering *A. petiolata* ( $n = 36$ , from either ASP or Dayton, OH) or *C. diphylla* ( $n = 42$ , from either ASP or Hocking Hills State Forest, OH) on top of moistened paper. Containers holding caterpillars from RR were held in an incubator at 25 °C with a 16:8 L:D cycle; containers holding other neonates were held in ambient field conditions (6 – 10 °C nights, 25 – 30 °C days, shaded) for up to nine days, depending on when the caterpillars hatched, however, no differences in development time or survival were noted between the two conditions.

We took daily photographs (22-26 hrs apart, depending on field travel and researcher availability) of leaf damage ( $cm^2$ ) to analyze in ImageJ (Abramoff et al., 2004) and assessed the survival of each caterpillar daily. Briefly, we analyzed leaf area difference between daily photographs by setting a standard scale (an included 1cm scale in each picture), transforming images from color to binary (black and white), and measuring the amount of leaf eaten (pixels changes from black to white) in the time elapsed between pictures.

Linear correlations from a subset of plants were performed to establish the relationship between leaf mass (LM) and leaf area (LA) for *A. petiolata* ( $n = 10$ ,  $r^2 = 0.9642$ ,  $LM = 70.325*LA+7.0907$ ) and *C. diphylla* ( $n = 10$ ,  $r^2 = 0.98848$ ,  $LM = 42.278*LA+2.8234$ ). Using these relationships, measures of leaf area consumed were converted to leaf mass consumed. The mean total leaf mass consumption on each host plant (*A. petiolata* or *C.*

*diphylla*) was compared using a t-test in R. We examined caterpillar survival during the course of the assay using a Kaplan-Meier estimator with a log-rank (Mantel-Haenszel) test for differences in survival on the two plant species.

### **Extract preparation and feeding assay**

To examine whether larval performance on leaves of the native and novel host could be mimicked using chemical extracts, we made leaf extracts using a modified procedure from Haribal et al. (2001). Ten g of either stem leaves of flowering *A. petiolata* or leaves of non-flowering *C. diphylla* were extracted in 40mL boiling 95% EtOH. Extracts were evaporated to 10mL using a rotary evaporator, then centrifuged to remove solids. The supernatant was evaporated to dryness and finally brought up with  $H_2O$  to 10mL. The control solution was prepared similarly, without added leaf material. Twenty  $\mu L$  of each extract (*A. petiolata*, *C. diphylla*, or extract control) was applied to the upper surface of 1cm<sup>2</sup> squares of commercial (Meijer, Inc.) cabbage leaves ( $n = 10$  per treatment), as in Haribal and Renwick (1998). After drying, squares were flipped and painted with another 20 $\mu L$  of solution and allowed to dry. Freshly eclosed neonates collected from the oviposition preference experiments ( $n = 10$  per treatment) were placed on the leaf squares in moist filter-paper lined 35x15 mm Petri dishes and allowed to feed for 72 hours in a 16:8 L:D incubator at 25 °C. At least once a day, survival was recorded, and hand drawn estimations were made of the amount of area removed from each cabbage square by larval feeding in a square drawing area. These drawings were later analyzed in ImageJ for leaf area (cm<sup>2</sup>) consumed, as above, and similarly transformed to mass from area. The mean total leaf mass consumption on each host plant (*A. petiolata* or *C. diphylla*) was compared using an ANOVA followed by Tukey's HSD post-hoc testing in R. We performed survival analysis as above. Pairwise chi-squared tests were conducted for post-hoc analysis of the Kaplan-Meier estimators.



### 3.3 Results

#### Oviposition preference.

We examined native and exotic host plants at three sites for *P. virginiensis* eggs. We found on average twice as many eggs per plant on the exotic *A. petiolata* as on the native *C. diphylla* when data were pooled across sites and years ( $X^2 = 5.744$ ,  $df = 1$ ,  $p < 0.05$ , Figure 3.1). There was no difference in the incidence of plants with eggs across years at ASP (2012 vs. 2013:  $X^2 = 0.3897$ ,  $df = 1$ ,  $p > 0.05$ ), however, there was significant variation between sites for both *A. petiolata* (ASP vs. RR:  $X^2 = 9.778$ ,  $df = 1$ ,  $p < 0.01$ ) and *C. diphylla* (ASP vs. HOL:  $X^2 = 5.9585$ ,  $df = 1$ ,  $p < 0.05$ ). We were more likely to find eggs on *A. petiolata* at RR than at ASP, and more likely to find eggs on *C. diphylla* at ASP than at HOL. Since *C. diphylla* was the only species surveyed at HOL, and *A. petiolata* was the only plant species surveyed at RR, a comparison between these two sites was not possible.

We also analyzed oviposition preference in the laboratory, and found that *P. virginiensis* adults prefer to lay their eggs on *A. petiolata* (OPI:  $0.3289 \pm 0.1536$ ,  $t = 2.1416$ ,  $df = 19$ ,  $p < 0.05$ ). We found that individual *P. virginiensis* females laid on average 2.8 more eggs on *A. petiolata* than on *C. diphylla* ( $t = -2.3445$ ,  $df = 19$ ,  $p < 0.05$ , *Cohen's d* = 2.8, Figure 3.2). There was wide variation in the number of eggs laid by individuals, but seventeen of twenty individuals chose to place at least some eggs on *A. petiolata*, and only two individuals laid more eggs on *C. diphylla* when also ovipositing on *A. petiolata*.

#### Larval performance.

Freshly eclosed neonates were fed either *A. petiolata* or *C. diphylla* leaf tissue in a no-choice feeding assay. No differences were found in survival or performance between sites

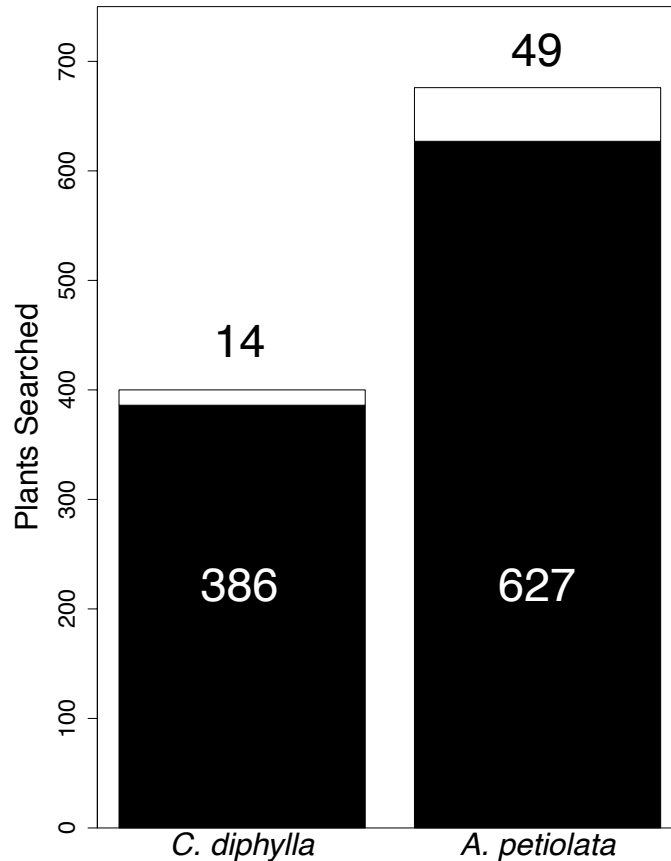


Figure 3.1: The number of native and exotic plant individuals searched with *P. virginiensis* eggs (white) and without *P. virginiensis* eggs (black) in field surveys.

(RR and ASP), so all data were pooled. We found that neonates had significantly higher survival on *C. diphylla* than on *A. petiolata* ( $X^2 = 7.8, df = 1, p < 0.01$ , Figure 3.3). When it occurred, most of the larval failure observed on either host plant occurred during the first 48 hours, however, many caterpillars eating *C. diphylla* were able to develop fully. All but one larva placed on *A. petiolata* leaves died within three days; the final surviving larva died on the fifth day. In contrast, over 30% of larvae survived through pupation (14-17 days) when consuming *C. diphylla*. Neonates also consumed significantly more native leaf tissue (*C. diphylla*:  $0.841 \pm 0.116g$ ) than invasive leaf tissue (*A. petiolata*:  $0.000720 \pm 0.000157g$ ) over the course of the bioassay (Figure 3.4).

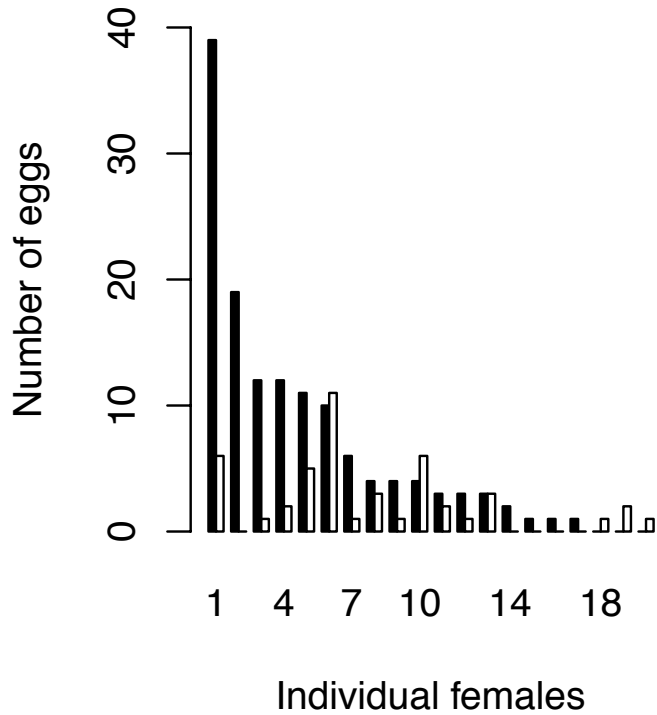


Figure 3.2: The number of eggs laid by adult *P. virginensis* females (n=20) in a two-way choice test on *A. petiolata* (black) or *C. diphylla* (white).

We also examined performance of freshly eclosed neonates fed extracts made from each plant (*A. petiolata* and *C. diphylla*) or a control (EC) solution (Figure 3.5). Pairwise comparisons revealed no differences between caterpillar survival on control and *C. diphylla* treated squares ( $X^2 = 0.629, df = 1, p > 0.25$ ). However, larvae had higher survival on both the control treated squares ( $X^2 = 10.32, df = 1, p < 0.005$ ) and the *C. diphylla* treated squares ( $X^2 = 6.425, df = 1, p < 0.05$ ) than on the *A. petiolata* treated squares. At the conclusion of the experiment, all larvae exposed to the *A. petiolata* treated squares had perished, but at least half of larvae remained alive on the *C. diphylla* (5 of 10) and control (7 of 10) treated squares, respectively. In addition, caterpillars on *A. petiolata* treated leaves consumed significantly less mass ( $5.19 \times 10^{-4} \pm 2.61 \times 10^{-4}g$ ) than either caterpillars on

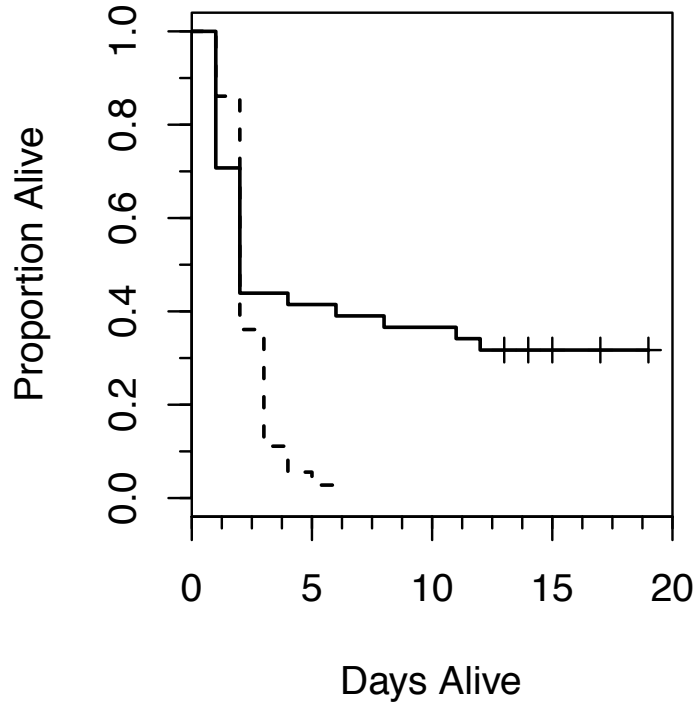


Figure 3.3: Survival of *P. virginiensis* neonates on invasive *A. petiolata* leaf tissue (n = 36, dashed) and native *C. diphylla* leaf tissue (n = 42, solid).

the control ( $1.43 \times 10^{-2} \pm 3.57 \times 10^{-3}g$ ) or *C. diphylla* ( $1.43 \times 10^{-2} \pm 4.78 \times 10^{-3}g$ ) treated leaves, but there was no difference in amount of leaf mass consumed by caterpillars in the control and *C. diphylla* treatments ( $F_{2,27} = 5.3641, p > 0.05$ , means reported as  $g \pm se$ ).

### 3.4 Discussion

Adult *P. virginiensis* often encounter *A. petiolata*, a non-native plant, in both edge and understory habitats (Courant et al., 1994; Porter, 1994). We investigated the frequency of oviposition events of this butterfly on the novel *A. petiolata* relative to its native host *C. diphylla* in both artificial and natural settings; we also examined larval survival on leaves

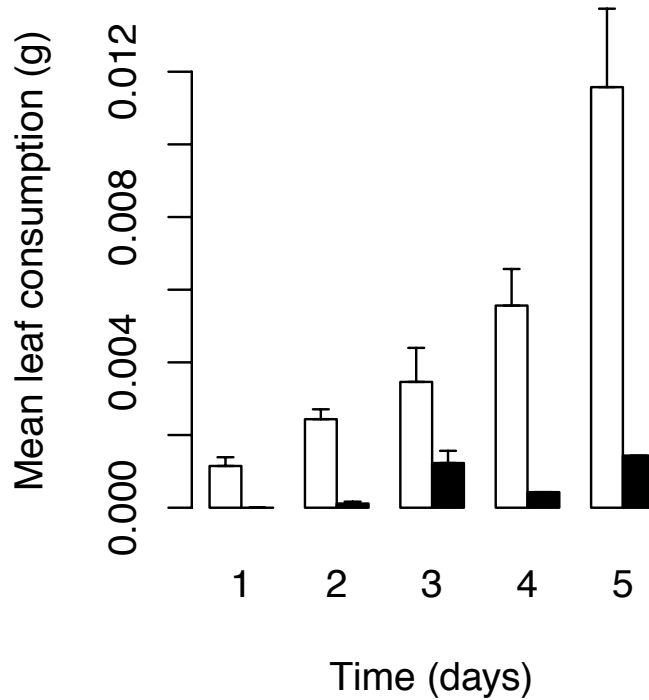


Figure 3.4: Leaf tissue (g) consumed per day by *P. virginienensis* neonates on *C. diphylla* (white) and *A. petiolata* (black) in a no-choice feeding assay. Bars represent mean  $\pm$  1 SE.

of *A. petiolata*, *C. diphylla*, and their ethanol extracts.

We confirmed earlier observations that *P. virginienensis* oviposits on *A. petiolata* (Courant et al., 1994; Porter, 1994). We found that 12.5% of *A. petiolata* plants searched had eggs, but only 3.5% of *C. diphylla* searched had eggs. This, coupled with our laboratory oviposition preference findings, indicates that *A. petiolata* is an important oviposition site for these butterflies. We expected to find some eggs on *A. petiolata*, considering that Courant et al. (1994) and Porter (1994) independently observed *P. virginienensis* females ovipositing on the novel host *A. petiolata*, however, we did not expect to find that *P. virginienensis* actively prefers to oviposit on *A. petiolata*.

If *P. virginienensis* larvae could tolerate consuming *A. petiolata*, this oviposition prefer-

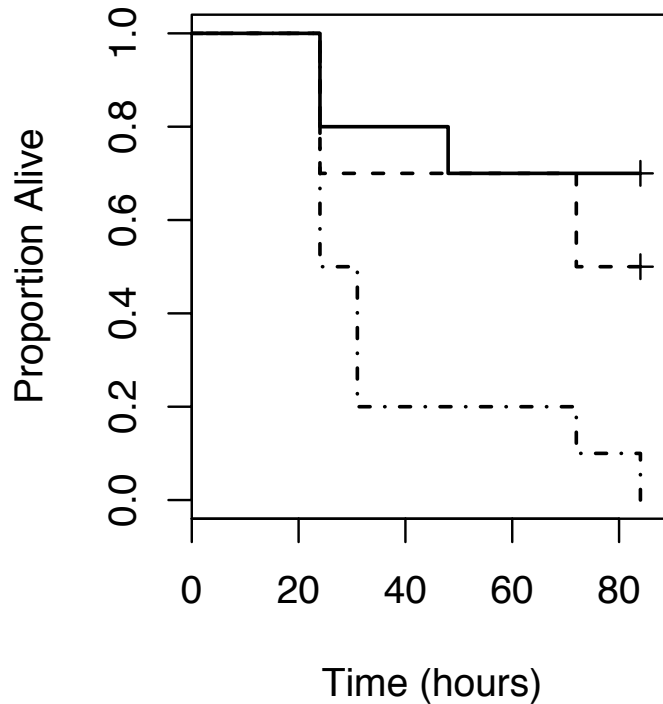


Figure 3.5: Survival of *Pieris virginiensis* neonates (n=10 per treatment) on ethanol extracts of *A. petiolata* (dot-dash), *C. diphylla* (dash) and a control solution (solid).

ence in *Alliaria*-invaded landscapes would perhaps increase populations of *P. virginiensis* across its range, leading to adoption of a new host much like the many novel host shifts in California (Graves and Shapiro, 2003). However, our results suggest that the opposite is true: neonatal *P. virginiensis* do not survive on the novel host plant *A. petiolata* or on cabbage leaves treated with ethanol extracts from its leaves. Survival to fourth instar is low for *P. virginiensis* caterpillars even if the host plant is palatable. Cappuccino and Kareiva (1985) estimate survival on the primary host, *C. diphylla*, as 16% to the fourth instar; and 15% of third instar larvae survive on the alternative host plant *B. laevigata* (Shuey and Peacock, 1989). At the current time, each egg laid on *A. petiolata* in the field is almost certainly wasted, meaning that *A. petiolata* is a population sink. With poor survival rates

on native hosts (Cappuccino and Kareiva, 1985; Shuey and Peacock, 1989) and indications of decline across its range (Finnell and Lehn, 2007), the introduction of *A. petiolata* into *P. virginiensis* habitats may be the final blow to this butterfly (Courant et al., 1994; Porter, 1994).

*Pieris virginiensis* does not exhibit obligatory monophagy on *C. diphylla*, but rather, *C. diphylla* is often the only co-occurring native mustard that persists long enough to support larval development (Hovanitz and Chang, 1963; Shuey and Peacock, 1989; Bess, 2005; Doak et al., 2006). Although *C. concatenata*, *C. dissecta* and biennial *B. laevigata* are alternative hosts, the former two flower and senesce earlier than *C. diphylla*, making them poor oviposition substrates (Bess, 2005); the latter, *B. laevigata*, occurs sporadically in marginally disturbed habitats (cliff edges, rocks, tree bases) and is less abundant in *P. virginiensis* habitats even when it is the primary host (Shuey and Peacock, 1989). Since *P. virginiensis* is not entirely specialized on *C. diphylla*, we hypothesize that as *P. virginiensis* females continue to encounter *A. petiolata*, selection will favor either individuals whose offspring succeed on *A. petiolata* or individuals who actively avoid it in favor of *C. diphylla* or an alternative native host.

Selection has already begun to favor female choice in the closely related *P. oleracea*. Keeler and Chew (2008) found that bivoltine *P. oleracea* exposed to *A. petiolata* for more than fifty years (100 generations) had begun using it as a host, and caterpillars survived despite lower pupal weight and increased development time; in contrast, naïve populations had no demonstrable oviposition preference and poor survival on the novel host. *P. oleracea* could be adapting to *A. petiolata* at a faster rate due to its bivoltinous lifestyle, compared to *P. virginiensis*' univoltinism. In addition, *P. oleracea* naïve to *A. petiolata* still had 7% of larvae survive to pupation in their first generation, suggesting some inherent tolerance for this plant. Unlike *P. oleracea*, there is no evidence that *P. virginiensis* can survive to pupation on *A. petiolata*, and as a result, selection cannot act on larval performance to develop an *A. petiolata*-tolerant phenotype. Given the rarity of this species and the strong

preference for *A. petiolata*, we expect reductions in population sizes and possible local extinctions through *P. virginienensis* habitat invaded by *A. petiolata* until *P. virginienensis* are better able to either find the correct host or tolerate *A. petiolata*.

Although we know that mistake oviposition occurs frequently, we do not yet know the mechanism. There may be two causes of the attraction to *A. petiolata*: visual apparency and chemical apparency. *A. petiolata* can grow close to 2m before flowering and setting seed; in contrast, *C. diphylla* never grows much above 0.5m. *P. virginienensis* have difficulty recognizing *C. diphylla* overtopped by other plants (Cappuccino and Kareiva, 1985). Since *A. petiolata* is so much larger, it may be that *P. virginienensis* encounter *A. petiolata* more frequently than *C. diphylla* in invaded habitats. Our two-way oviposition preference test eliminated some of the height difference, as *A. petiolata* used in those preference tests were no larger than 0.5m, but further work needs to be done to establish how much visual apparency affects *P. virginienensis* oviposition choices.

Chemical apparency may also drive *P. virginienensis* oviposition preference. Future work should include investigations into leaf surface and volatile cues that may induce *P. virginienensis* oviposition on the invasive *A. petiolata* after alighting. The closely related *P. oleracea* responds very strongly to sinigrin as a contact oviposition stimulant, and it is the primary glucosinolate constituent of *A. petiolata* leaves (Vaughn and Berhow, 1999; Huang et al., 1995). However, *Pieris* species respond differentially to oviposition stimulants and host plant quality, and so, a wide range of candidate chemicals must be examined (Renwick and Radke, 1988; Myers, 1985; Renwick et al., 1992; Huang and Renwick, 1993; Huang et al., 1995).

Further work must also be done to examine the toxic and/or deterrent effects of *A. petiolata* on *P. virginienensis* larvae. Most caterpillars in our two feeding assays (tissue and ethanol extract) perished by the third day, supporting previous observations by Bowden (1971) and Porter (1994) of *P. virginienensis* and mirroring trends seen by Renwick et al. (2001) in *P. oleracea*. It is believed that alliarinoside, a compound unique to *A. petiolata*, is



responsible for poor survival of neonate *P. oleracea*, but it is unknown how *P. virginiensis* responds to alliarinose or other chemicals in *A. petiolata* (Renwick et al., 2001). *Pieris virginiensis* neonates are too small to move from the host on which they were placed to find a more acceptable food source, and so, it is imperative that *P. virginiensis* mothers choose correctly. It is only in the last few days of development (4th and 5th instar) when the caterpillars are mobile enough to leave a plant that is either completely consumed or senescing to find another (Cappuccino and Kareiva, 1985). We do not yet know the risk of *A. petiolata* to older, more mobile caterpillars, although another chemical in *A. petiolata*, isovitexin 6 – O –  $\beta$  – D glucopyranoside, deters older instar feeding of *P. oleracea* (Renwick et al., 2001).

The glucosinolates found in *A. petiolata*, sinigrin and glucotropaeolin, may also play a role in poor caterpillar survival Nielsen and Dalgaard (1979). The usual host plants of *P. virginiensis* do not contain sinigrin or glucotropaeolin (Barto et al., 2010b; Montaut et al., 2010). Although Pieridae seem to bypass dangerous isothiocyanate formation (Wittstock et al., 2004), it may be that high levels of sinigrin (13.6mg/g dried leaf, Nielsen and Dalgaard 1979) overwhelm *P. virginiensis* larvae and their nitrile specifier protein (NSP) detoxification system. NSP is a larval gut protein found in Pierids specializing on glucosinolate-containing plants that prevents the plant enzyme myrosinase from changing glucosinolates into isothiocyanates and instead transforms glucosinolates into harmless nitriles. Pierid caterpillars are susceptible to isothiocyanates, but not to glucosinolates or myrosinase alone (Agrawal and Kurashige, 2003). Evidence for NSP activity has been found across the family (Wheat et al., 2007), but more work is needed to determine how effective the NSP system is at forming nitriles, and if *P. virginiensis* has an NSP allele which does not effectively handle sinigrin.

Our results have important conservation implications. Areas that support large populations of *P. virginiensis*, like Allegany State Park in New York (263km<sup>2</sup>), will be ideal places to allow selection for the use (or avoidance) of *A. petiolata* by *P. virginiensis* to oc-

cur. Not only is ASP large, but invasion by *A. petiolata* is minor and occurs mostly along roadsides, leaving large swaths of intact forest for *P. virginiensis* to inhabit. Unfortunately, most *P. virginiensis* populations occur in small woodland patches susceptible to *A. petiolata* invasion, and many *P. virginiensis* populations have been noted as anecdotally “in decline” since at least the 1970s (Finnell and Lehn, 2007; Shapiro, 1971). One population in particular, in Morrow Co., OH, that was robust at least as late as 1988, is most likely extinct now, an extinction that was coincident with *A. petiolata* introduction (Shuey and Peacock, 1989; Davis and Cipollini, 2014a). It is our fear that small populations may not possess the genetic and phenotypic variation needed for selection to occur, which is further hampered by limited gene flow due to limited dispersal ability of these butterflies. Populations of *P. virginiensis* occupying invaded areas, like Wooster Memorial Park in Wooster, OH, may be at risk for extinction, despite current efforts to remove *A. petiolata* from the area. Because *P. virginiensis* are now known to oviposit more frequently on *A. petiolata*, incomplete removal of garlic mustard is not enough to reduce the harm inflicted on *P. virginiensis* populations. Although management of *A. petiolata* is possible, complete elimination of the species takes years of aggressive control and monitoring (The Nature Conservancy, 2007). At this time, we recommend removing *A. petiolata* from *P. virginiensis* habitats, and removals should be timed to occur before the flight season of *P. virginiensis* in order to reduce the risk of *P. virginiensis* wasting eggs on *A. petiolata*. Although exotic plant invasion may not be the only cause of *P. virginiensis* decline, given the mismatch between oviposition preference and larval performance, it may be a major contributor.

# **How does garlic mustard lure and kill the West Virginia White butterfly?**

## **4.1 Introduction**

The preference-performance hypothesis (PPH, alternatively the ‘mother knows best’ hypothesis) states that female insects will choose oviposition sites that maximize their offspring’s fitness (Gripenberg et al., 2010). As many insects are essentially immobile after hatching due to their small size, there is strong selection for mothers to choose the correct host plant for their vulnerable offspring (Thompson and Pellmyr, 1991). Matching oviposition preference and larval performance maximizes fitness for individuals. Understanding the mechanisms of oviposition preference and larval performance can lead to improvements in pest management, agricultural yields, biocontrol, and conservation of vulnerable species.

The genetic makeup of an insect, alongside environmental cues, usually drives oviposition preference. In some insects, oviposition preference has high heritability, with variation occurring within and between populations (Tabashnik et al., 1981; Singer et al., 1988; Thompson, 1988; Fox, 1993). Oviposition behavior changes through an insect’s lifespan as a response to developmental and environmental triggers, including the age and health of the ovipositing insect, as well as the information they receive from the environment about mi-

crohabitat differences from a variety of visual, volatile, and tactile cues (Thompson, 1988; Honda et al., 2012; Eilers et al., 2013). Although oviposition preference often matches well with larval performance in long-lasting, stable environments and communities, the introduction of novel hosts through range shifts or intentional or accidental introduction can lead to oviposition “mistakes”, instances where eggs are placed on unsuitable hosts, resulting in poor larval performance or mortality.

Oviposition mistakes are well documented in Lepidoptera, including members of Papilionidae (Berenbaum, 1981; Stefanescu et al., 2006), Nymphalidae (Straatman, 1962; Bowers and Schmitt, 2013), and Pieridae (Chew, 1977; Porter, 1994). In cases where some larvae are able to succeed on the novel host, oviposition mistakes can lead to range expansion or possible speciation (Tong and Shapiro, 1989; Beltman et al., 2004; Nylin and Janz, 2007). Where larval mortality is complete, however, oviposition mistakes cause decreased fitness for the individual, and may negatively affect the local insect population (Courant et al., 1994; Porter, 1994; Nakajima et al., 2013). Oviposition mistakes provide a unique opportunity to investigate factors that drive both oviposition preference and larval performance by comparing and contrasting the “normal” hosts with the novel hosts.

*Pieris virginiensis* Edwards (Lepidoptera: Pieridae, the West Virginia White butterfly) is a rare univoltine butterfly native to eastern North America. It feeds on spring ephemeral mustards in the *Cardamine* and *Boechera* genera (Brassicaceae, toothworts and rock cresses) during its larval stage, and provides pollination services for other early spring flowers as an adult (Bess, 2005). *Pieris virginiensis* is a vulnerable species targeted for conservation by groups like the Ohio Lepidopterists’ Society, the Lake Erie Allegheny Partnership for Biodiversity, as well as state departments in New York and Pennsylvania (Finnell and Lehn, 2007).

In 1994, both Courant et al. and Porter noted lethal oviposition mistakes by *P. virginiensis* on the novel invader *Alliaria petiolata* Bieb (Brassicaceae, garlic mustard), although the frequency of mistakes and extent of larval mortality was unknown. Further

investigation revealed both a strong female attraction to the invader in the field and in the laboratory, coupled with complete neonatal mortality on leaves of the novel host (Davis and Cipollini, 2014b).

*Alliaria petiolata* has a chemical profile that drastically differs from its North American brassicaceous relatives. The primary glucosinolate found in *A. petiolata* leaves is sinigrin, or allyl-glucosinolate (Nielsen and Dalgaard, 1979; Barto et al., 2010a; Montaut et al., 2010). Sinigrin has been shown to induce oviposition in another North American pierid, *P. napi oleracea*, despite the lack of sinigrin in its primary springtime host, *Cardamine diphylla* (Huang and Renwick, 1994; Montaut et al., 2010). In addition, an assortment of flavonoids and their derivatives can be found in *A. petiolata* leaves that strongly differ from North American mustard flavonoid profiles (Barto et al., 2010a). Concentrations of these flavonoids vary through time, often peaking in summer (Haribal et al., 2001). There are also two glycosides unique to *A. petiolata*, alliarinoside and isovitexin-6 – O –  $\beta$  – D-glucopyranoside, that have been shown to negatively affect a close relative of *P. virginien-sis*, *P. napi oleracea* (Renwick et al., 2001). Both alliarinoside and sinigrin tend to be concentrated in new leaves, and alliarinoside concentration peaks during mid-summer in rosette leaves (Haribal et al., 2001; Frisch et al., 2014). Finally, *A. petiolata* is unique in its production of other compounds that may be involved in insect resistance, such as cyanide (Cipollini and Gruner, 2007). Davis and Cipollini (2014b) found that ethanolic leaf extracts of *A. petiolata* can cause *P. virginien-sis* larval mortality, indicating the presence of solvent-extractable toxic compounds in *A. petiolata*, but the specific chemical mediation of oviposition and larval performance of *P. virginien-sis* has not been studied.

In this study, we investigated whether sinigrin applied to leaves of an acceptable host was capable of stimulating oviposition of *P. virginien-sis*, and whether sinigrin and alliarinoside applied to leaves of acceptable hosts were capable of inhibiting larval performance of this butterfly.

## 4.2 Methods

### Does sinigrin affect oviposition preference of *P. virginiensis*?

We tested whether or not the application of sinigrin to *Cardamine diphylla* leaves stimulated oviposition by *P. virginiensis* butterflies. Gravid female butterflies were collected from the Quaker area of Allegany State Park (ASP) in Salamanca, NY (42.054146 N, -78.760572 W) as in Davis and Cipollini (2014b). Butterflies were netted between 1000 and 1700 h on clear days, and temporarily stored in an aquarium with access to water and artificial nectar (10% w/v sucrose solution). All butterflies were marked on their hind wings with permanent marker to indicate identity.

*Cardamine diphylla* plants were collected from Hocking County, OH, and transplanted to 79 cm<sup>3</sup> pots with moist Pro-Mix BX soil (BFG Supply, Xenia, OH). The sinigrin solution was prepared by mixing sinigrin (Sigma-Aldrich, Inc.) into water, and the control solution was water alone. Sinigrin was applied to experimental plants at a concentration of 34  $\mu$  mol/g of *C. diphylla* leaf, which was slightly higher than the concentration found naturally occurring in *A. petiolata* leaves by Frisch et al. (2014). Solutions were applied by spraying them evenly across the upper surface of the leaf. Only one leaf (with 3 leaflets) per *C. diphylla* plant was used in a trial, the other leaves were removed by clipping at the soil surface.

Oviposition choice trials were performed as in Davis and Cipollini (2014b). Four trials were conducted with groups of 5 or 6 female butterflies ( $n = 22$  individuals). These groups were placed in a glass aquarium with access to the experimental plants and freshly harvested flowers (*Bellis perennis* and *Taraxacum officinale*) as nectar sources for one hour. Sinigrin-treated and control plants were placed randomly on the left or right side of the aquarium approximately 0.3 m apart and switched after 30 minutes to eliminate positional bias. During each trial, butterflies were observed continuously and any oviposition events were recorded. Female *P. virginiensis* individuals rarely interact with each other and do

not interfere with oviposition or nectaring events of other individuals (Davis and Cipollini, 2014b). Neither nectar plants nor non-experimental plant surfaces (e.g., the aquarium glass) ever received eggs during these trials or during holding periods.

An oviposition preference index (OPI) was calculated as the number of eggs laid on sinigrin-treated plants divided by the total number of eggs laid by an individual times 100. OPI would be equal to 100 if all eggs were laid on sinigrin-treated plants, and equal to 0 if all eggs were laid on control-treated plants. OPI was analyzed using a one-sample t-test at  $\mu = 50$  (no preference).

### **Does a diet with sinigrin affect larval performance of *P. virginiensis*?**

We tested the effects of sinigrin applied to two possible hosts of *P. virginiensis*, the native host *C. diphylla* (collected from Hocking Co., OH) which has no sinigrin present in its glucosinolate profile (Montaut et al., 2010), and a commercial accession of *Brassica juncea* (Sand Mountain Herbs, AL, USA), which has a glucosinolate profile dominated by sinigrin, similar to *A. petiolata* (Nielsen and Dalgaard, 1979; Sang et al., 1984). All plants were transplanted or grown from seed in Pro-Mix BX soil, as in Frisch et al. (2014). Sinigrin was applied to each of these plants as above in the oviposition trials.

Caterpillars used in the *C. diphylla* experiment eclosed from field-collected eggs collected from *A. petiolata* plants in the Roaring Run Natural Area, Apollo, PA. Caterpillars used in the other two larval experiments (*B. juncea*, alliarinoside) eclosed from field-collected eggs or from eggs laid by field-collected females on *A. petiolata* at ASP. As caterpillars eclosed, they were gently transferred with a paintbrush to their experimental treatment. Eggs were checked 3-4 times a day for emergence to prevent caterpillars from consuming their initial larval host. We took our first measurements 24 hr after placement. We eliminated any caterpillars that had died prior to this first measurement from the analysis, because their deaths most likely resulted from the transfer process rather than their host usage.

Sinigrin solutions were prepared and applied as in the oviposition preference test above. After leaves were allowed to dry, newly eclosed *P. virginensis* caterpillars were placed on an experimental leaf (*B. juncea*) or leaflet (*C. diphylla*) in 236mL plastic containers lined with moist filter paper (*C. diphylla* experiment) or in 100x15mm plastic Petri dishes on moist filter paper sealed with Parafilm (*B. juncea* experiment). Containers holding *C. diphylla* leaves were held in ambient field conditions (6-10°C nights, 25-30°C days in shade) for up to 7 days before being transferred to an incubator held at 25°C with a 16:8 L:D cycle, as in Davis and Cipollini (2014b). Caterpillars in the *B. juncea* experiment were held in the incubator continuously from the start of the experiment.

Caterpillars were monitored daily for survival and food availability. Leaves were replaced as they senesced or were totally consumed. In the *B. juncea* experiment, we took photographs of the leaves and caterpillars for analysis of leaf area consumption ( $cm^2$ ), and caterpillar volume ( $\pi * (1/2 * width)^2 * length$ ). All image analyses were performed using ImageJ (Abramoff et al., 2004), as in Davis and Cipollini (2014b).

We tested for differences in survival between treatments using a Kaplan-Meier estimator with a log-rank (Mantel-Haenszel) test for both *C. diphylla* and *B. juncea* experiments. In the *C. diphylla* experiment, we tested for treatment differences in pupal weight (g) and time from eclosure to pupation (days). Overall survival in the *B. juncea* experiment was too low to compare pupal characteristics between treatments. In the *B. juncea* experiment, we tested for differences in the mean of total leaf consumption ( $cm^2$ ) and final caterpillar volume ( $cm^3$ ) on leaves receiving different treatments using t-tests. Finally, differences in consumption and volume of caterpillars fed *B. juncea* were analyzed through time using a two-way ANOVA with days post treatment (DPT), treatment, and their interaction as factors.



## **Does a diet with alliarinoside affect larval performance of *P. virginien-* *sis*?**

We tested the effects of alliarinoside on *P. virginiensis* caterpillars by applying it to leaves of *Brassica oleracea* (cabbage). *Brassica oleracea* is capable of supporting larval development of *P. virginiensis*, has no natural source of alliarinoside, and is not commonly encountered by *P. virginiensis* in the wild. We used cabbage because cabbage has been used previously in larval performance experiments with *P. virginiensis* (Renwick et al., 2001; Davis and Cipollini, 2014b), and there was no *B. juncea* available when these neonates eclosed.

Alliarinoside was chemically synthesized in the laboratory of Mohammed Saddik Motawia as in Olsen et al. (2014). Alliarinoside was applied to cut  $1\text{cm}^2$  squares of *B. oleracea* leaves (Meijer, Inc., cultivar unknown) at a concentration of  $5\text{mg/g}$  FW in distilled water, which is the mean alliarinoside concentration in *A. petiolata* leaves (Frisch et al., 2014). Control leaves were painted with water alone. Leaf squares were allowed to dry before neonate caterpillars were placed on them. Caterpillars and treated leaves were kept as above in petri dishes in a temperature and light controlled incubator.

Caterpillar enclosures were monitored daily for survival and leaf quality as above. Unlike in the sinigrin experiments, the alliarinoside experiment only ran for 7 days due to rapid larval mortality in the alliarinoside treatment. Otherwise, all data were recorded and analyzed as for the sinigrin experiments above.

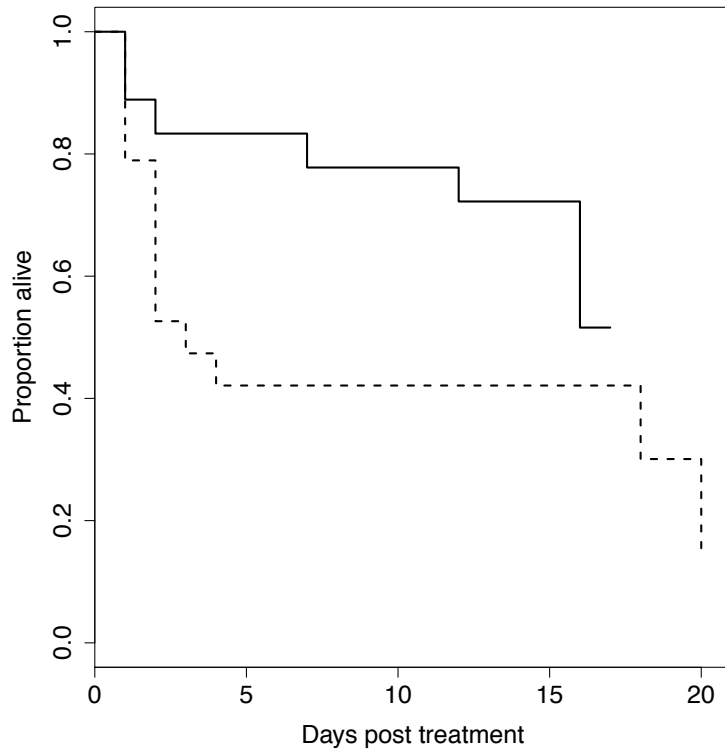


Figure 4.1: Survival of *P. virginensis* caterpillars fed *C. diphylla* without (solid, n=18) and with (dash, n=19) added sinigrin.

### 4.3 Results

#### **Does sinigrin affect oviposition preference of *P. virginensis*?**

Female *P. virginensis* laid an average of 1.9 eggs per individual across all trials, comparable to the amount laid in previous trials with *C. diphylla* as a host (2.3 eggs per butterfly on *C. diphylla* across all trials, Davis and Cipollini 2014b). We found that *P. virginensis* showed no preference for control- or sinigrin-painted *C. diphylla* leaves, indicating that sinigrin did not influence oviposition of *P. virginensis* in our study ( $t = -1.007$ ,  $df = 10$ ,  $P = 0.3406$ ,  $\mu = 0.5$ ).

### **Does a diet with sinigrin affect larval performance of *P. virginiensis*?**

There were no differences in survival between caterpillars feeding on leaves of control and sinigrin-treated *C. diphylla* plants ( $X^2 = 1.06$ ,  $df = 1$ ,  $P = 0.3043$ , Fig 4.1). However, caterpillars fed *C. diphylla* with added sinigrin took 3.2 days longer on average to pupate ( $t = -6.0794$ ,  $df = 12.656$ ,  $p < 0.01$ ) and weighed 19.8% less as pupae ( $t = -3.8477$ ,  $df = 15.779$ ,  $P < 0.01$ ) than those on control plants.

Caterpillars on *B. juncea* with added sinigrin had significantly lower survival than those on *B. juncea* with only the control solution ( $X^2 = 4.96$ ,  $df = 1$ ,  $P < 0.05$ , Fig 4.2 A). Survival in both treatments was not high enough to compare pupation time or mass, but images were analyzed daily for leaf consumption and caterpillar volume. Caterpillars fed sinigrin-treated leaves consumed only 25% of the leaf area consumed by caterpillars fed control leaves (total leaf area consumed:  $t = 5.2426$ ,  $df = 31.974$ ,  $P < 0.01$ ) and were nearly one third the size of those fed control leaves (total caterpillar volume:  $t = 3.5481$ ,  $df = 32.42$ ,  $P < 0.01$ ) between treatments. There was an interaction between DPT and treatment in two-way ANOVAs for both leaf consumption (Fig 4.2B) and caterpillar volume (Fig 4.2C), indicating that caterpillars diverged rapidly on their different treatments.

### **Does a diet with alliarinoside affect larval performance of *P. virginiensis*?**

There was significantly lower survival of *P. virginiensis* caterpillars that consumed alliarinoside-treated *B. oleracea* leaves than consumed control leaves ( $X^2 = 28.29$ ,  $df = 1$ ,  $P < 0.01$ , Fig 4.3A). Caterpillars fed alliarinoside-treated leaves consumed 4.7 times less leaf area (final leaf area consumed:  $t = 4.2197$ ,  $df = 34.27$ ,  $P < 0.01$ ) and were 3.7 times smaller than those fed control leaves (final caterpillar volume:  $t = 3.6523$ ,  $df = 31.765$ ,  $P < 0.01$ ). There was no interaction between DPT and treatment in the two-way ANOVA for leaf con-

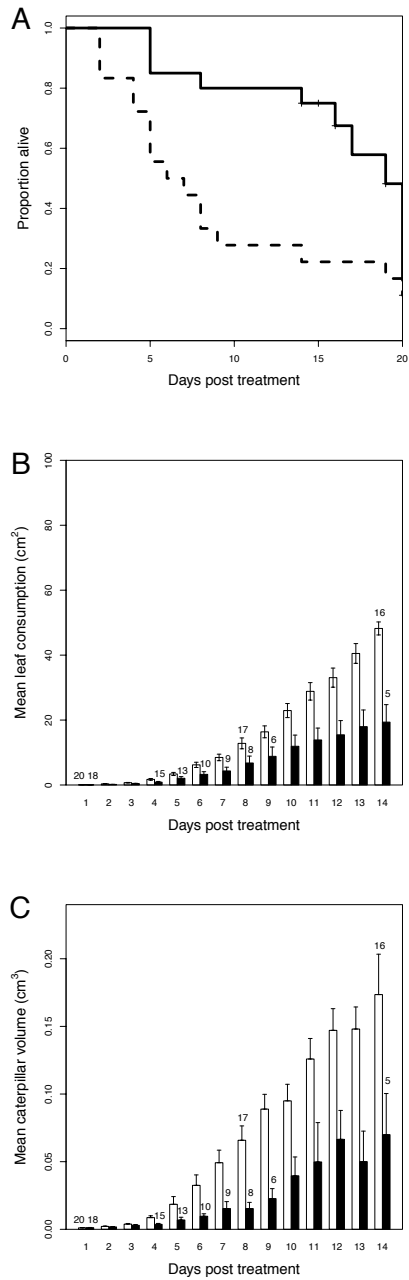


Figure 4.2: **(A)** Survival of *P. virginicensis* caterpillars fed *B. juncea* without (solid, n=20) and with (dash, n=18) added sinigrin. **(B)** Mean maximum leaf consumption ( $\pm 1SEM$ ) of caterpillars fed *B. juncea* without (white) and with (black) added sinigrin. **(C)** Mean maximum caterpillar volume ( $\pm 1SEM$ ) of caterpillars fed *B. juncea* without (white) and with (black) added sinigrin. Numbers above bars indicate number of survivors in that treatment; no numbers indicate no change.

sumption (Fig 4.3B) or caterpillar volume (Fig 4.3C), although each factor was significant separately.

## 4.4 Discussion

As a novel interaction, the relationship between *P. virginiensis* and *A. petiolata* provides a unique opportunity to study the mechanism of oviposition mistakes and resulting larval mortality in an insect herbivore. We examined the role of sinigrin as both an oviposition stimulant and a larval toxin, and we also investigated alliarinoside as a possible larval toxin.

Pierid butterflies have varied responses to glucosinolates as oviposition stimulants, and often oviposition preference is determined by both stimulants and deterrents. In our experiment, we observed that treating the leaves of an acceptable host with the major glucosinolate found within *A. petiolata*, sinigrin, had no effect on oviposition preference in the laboratory. Previous studies have described the role of glucosinolates as oviposition stimulants or deterrents for *Pieris brassicae*, *P. rapae*, and *P. oleracea*. Glucobrassicin, the predominant leaf-surface glucosinolate in cabbage (*B. oleracea*), stimulates oviposition for all three *Pieris* species (Renwick et al., 1992; van Loon et al., 1992; Huang and Renwick, 1994).

Responses to sinigrin, the predominant glucosinolate in *A. petiolata* and *B. juncea* are mixed. Sinigrin only slightly stimulates oviposition of the European butterflies *P. brassicae* and *P. rapae*, but has a more pronounced stimulatory effect on *P. oleracea*, a close relative of *P. virginiensis* (Renwick et al., 1992; Huang and Renwick, 1993, 1994). Huang and Renwick (1994) found that the epimer of glucobarbarin (2S or 2R) stimulated oviposition in *P. oleracea* but not *P. rapae*. While all of these glucosinolates are generally considered chemotactile cues, there is some evidence to suggest that non-glucosinolate chemicals (ferulic acid, p-coumaric acid) may also stimulate oviposition by *P. rapae* (Walker et al., 2014). Non-glucosinolate chemicals may also deter oviposition, but the responses are complex and

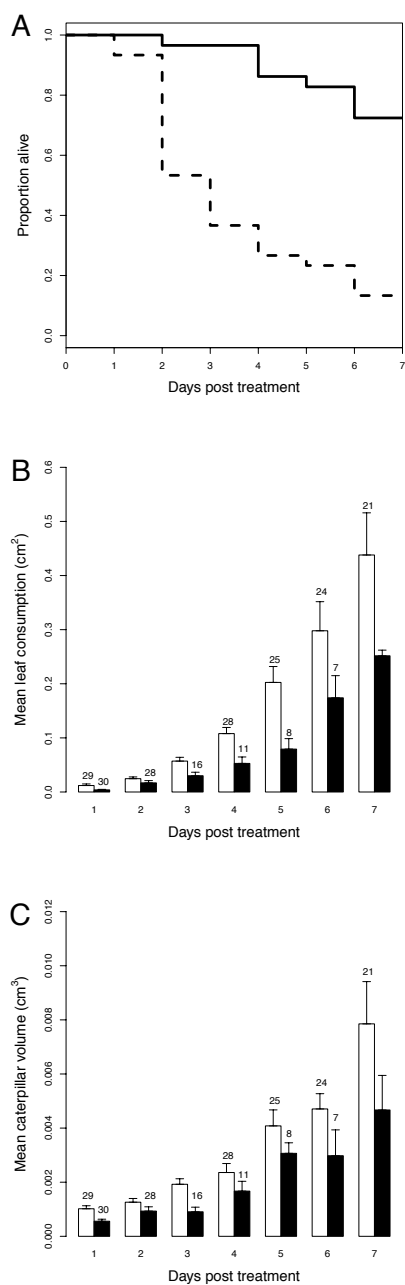


Figure 4.3: **(A)** Survival of *P. virginicensis* caterpillars fed *B. oleracea* without (solid, n=30) and with (dash, n=29) added alliarinoside. **(B)** Mean maximum leaf consumption ( $\pm 1SEM$ ) of caterpillars fed *B. oleracea* without (white) and with (black) added alliarinoside. **(C)** Mean maximum caterpillar volume ( $\pm 1SEM$ ) of caterpillars fed *B. oleracea* without (white) and with (black) added alliarinoside. Numbers above bars indicate number of survivors in that treatment; no numbers indicate no change.

species specific (Huang and Renwick, 1993).

With regard to the oviposition behavior of *P. virginiensis*, *A. petiolata* may produce non-glucosinolate attractants that are a mix of volatile and contact cues. When we place a *P. virginiensis* female directly on a leaf of flowering *A. petiolata*, oviposition almost always occurs, indicating that some contact cues are present (S.L. Davis, personal observation). However, with enough time, *P. virginiensis* females will lay eggs not only on the leaf surface of *A. petiolata*, but also on the nearby glass or plastic pot in an aquarium (S.L. Davis, personal observation). It may be that sinigrin's volatile byproducts activate oviposition by *P. virginiensis*, but our experimental leaves did not degrade the painted sinigrin. Further experiments should investigate volatiles emitted by *A. petiolata* and how they influence *P. virginiensis* oviposition.

Our experiments on larval performance indicated an overall negative effect of sinigrin on *P. virginiensis* caterpillars, including delayed pupation and reduced pupal weight in lower doses (*C. diphylla* trial) and decreased survival, size, and consumption at higher doses (*B. juncea* trial). The difference in survival between trials could be due to the sinigrin that is constitutively present in *B. juncea* at  $4 \pm 2 \mu \text{mol/g}$  FW (Frisch et al., 2014), but was likely caused partly by factors modifying sinigrin-derived defenses, like specifier proteins. Controls in both experiments had similar survival percentages (50%) but those on *B. juncea* with added sinigrin rarely pupated successfully.

Crucifer-feeding Pierid caterpillars have evolved glucosinolate-detoxifying mechanisms (Wittstock et al., 2004; Wheat et al., 2007). When a crucifer is damaged, glucosinolates are brought into contact with the degrading enzymes, myrosinases. The resulting products spontaneously rearrange to highly toxic isothiocyanates, unless modifying factors such as specifier proteins from plant or herbivore promote formation of products with different biological activity (Wittstock and Burow, 2010). Many glucosinolate-feeding Pierids have a nitrile specific protein (NSP) that promotes the formation of nitriles during glucosinolate hydrolysis, which are relatively inert and may be further detoxified (Wheat et al.,

2007; Stauber et al., 2012). Since sinigrin seems to affect *P. virginensis* at high concentrations, it may be that the level of this glucosinolate overwhelms the existing NSP detoxification machinery. Alternatively, *P. virginensis* may have never evolved the ability to detoxify sinigrin, or lost the adaptation after specializing on *C. diphylla*, which does not produce sinigrin. For example, the NSP of *P. rapae* is effective on both benzylglucosinolate and p-hydroxybenzylglucosinolate, but further metabolism of the resulting nitrile byproducts is different, possibly reflecting metabolite patterns of ancient food plants and differential adaptation (Stauber et al., 2012; Agerbirk et al., 2007).

*A. petiolata* contains a specifier protein, thiocyanate-forming protein (TFP), which promotes formation of glucosinolate-derived thiocyanate, epithionitrile and simple nitrile (Kuchernig et al., 2012). As glucosinolate-derived thiocyanates are relatively rare among crucifers (Wittstock and Burow, 2007), *A. petiolata* may exhibit a more diverse sinigrin-derived defense than many other crucifers, including *B. juncea* and *C. diphylla* in our experimental setup. This may contribute to the lethal effects of *A. petiolata* on *P. virginensis*.

Finally, we found substantial negative effects of alliarinoside on the survival, consumption, and size of *P. virginensis* caterpillars. On leaves of *A. petiolata*, *P. virginensis* neonates rarely survive longer than three days (Davis and Cipollini, 2014b). Here, alliarinoside had only slightly more moderate effects on neonates when placed on leaves of an acceptable host. In leaves of *A. petiolata*, the effects of alliarinoside combined with the effects of sinigrin are likely responsible for the lethal effects on neonate *P. virginensis*. Furthermore, alliarinoside, found at high concentrations along with sinigrin in young leaves (Frisch et al., 2014), has been shown to have feeding deterrent effects on *P. oleracea* neonates (Haribal and Renwick, 1998; Renwick et al., 2001). Caterpillars fed sinigrin in this study survived better and occasionally made it to pupation, whereas none of the caterpillars fed alliarinoside progressed beyond the third instar. Alliarinoside may be the primary driver of larval mortality on *A. petiolata*.

Alliarinoside is a  $\lambda$  - *hydroxynitrile* glucoside, closely related to cyanogenic gluco-



sides that are  $\alpha$  - *hydroxynitrile* glucosides. The latter exert their biological activity by release of cyanide, but the mechanism by which  $\lambda$  - *hydroxynitrile* glucosides may participate in plant defense is not understood (Bjarnholt and Mller 2008). Recent work reveals that alliarinoside can be completely degraded, sequestered, or passed through the digestive system by *P. rapae* caterpillars, but comparative chemical work across other *Pieris* species has not yet been done (Frisch et al., 2014). In turn, *P. rapae* caterpillars can utilize *A. petiolata* as a host with relative impunity (Davis & Cipollini, in review). Unfortunately, *P. virginiensis* caterpillars survive so poorly on a diet with alliarinoside that it is difficult to study the fate of alliarinoside in these caterpillars.

The *P. rapae* pathway for metabolism of the benzylglucosinolate derived nitrile proceeds via formation of a cyanogenic intermediate. This indicates that part of its detoxification machinery was developed prior to the shift of an ancestral *Pieris* from the cyanogenic Fabales to the glucosinolate containing crucifers (Stauber et al., 2012). Co-occurrence of glucosinolates and hydroxynitrile glucosides is extremely rare, the only other known example is *Carica papaya* where the occurrence of the cyanogenic glucoside prunasin is extremely low (Olafsdottir et al., 2002). Thus, some crucifer specialist herbivores such as *P. oleracea* and *P. virginiensis* may have lost the ability to detoxify hydroxynitrile glucosides, possibly giving *A. petiolata* an advantage against such species in producing alliarinoside. Co-occurrence of cyanogenic glucosides with  $\lambda$  - *hydroxynitrile* glucosides and other non-cyanogenic hydroxynitrile glucosides is relatively common, and identification of toxicity mechanisms of alliarinoside in sensitive *Pieris* species may also shed light on the function of this type of compounds in cyanogenic plants. Future research should focus on identifying the mechanism of toxicity in *P. oleracea* and *P. virginiensis*, and how it differs from *P. rapae* responses.

Although we found no evidence in our experiments to support the hypothesis that sinigrin stimulates *P. virginiensis* oviposition, we found that both sinigrin and alliarinoside contribute to poor performance and mortality of *P. virginiensis* larvae when fed leaves of

normally acceptable hosts with added sinigrin. There are clearly more experiments that need to be conducted to discover the mechanisms in *P. virginiensis* that create oviposition mistakes, but the novel chemistry of *A. petiolata* appears to have clear direct effects on larval success.

# **Do cabbage white butterflies live in North American forests? Evidence for *Pieris rapae* using invasive *Alliaria petiolata* and implications for native Pierid butterflies.**

## **5.1 Introduction**

Although some of the 50,000 alien species introduced into the United States have economic value, organisms unintentionally introduced to novel habitats have been estimated to cost the United States almost \$120 billion in agricultural and economic damages each year (Pimentel et al., 2005). Invasive species also cause untold damages to natural habitats through changing nutrient cycles, altering resource competition, and affecting the physical landscape structure around them (Gordon, 1998). Where rare species live, invasion by novel plants or animals can cause vulnerable species to become endangered or extinct (Wilcove et al., 1998).

*Pieris rapae* L. (small cabbage white; Lepidoptera: Pieridae) is a multivoltine European butterfly accidentally introduced to Quebec, Canada in 1860. A specialist on glucosinolate-containing Brassicaceae host plants, it soon became a destructive crop pest in North America, moving south and west as far as Kentucky in just 12 years (Scudder, 1889). Now ubiquitous and abundant across the United States and Canada, it is known as a butterfly of open meadows, crop plantings, and sunny areas where its cultivated and wild hosts are typically found (Ohsaki and Sato, 1994; Benson et al., 2003).

Its primary hosts in its native range include *Armoracia rusticana*, *Brassica* spp., *Cardamine* spp., *Crambe maritima*, *Sisymbrium officinale*, and *Tropaeolum majus*, among others, most of which are high light requiring plants (Richards, 1940). In North America, it benefits from habitat fragmentation and disturbance favoring growth of its weedy hosts, such as *Barbarea vulgaris*, introduced *Brassica* species, and *Lepidium* species, many of which are also non-native (Root and Kareiva, 1984; Summerville and Crist, 2001; Woods et al., 2008). A common pest on commercial brassicaceous crops, *P. rapae* is highly visible as an adult, more cryptic in its larval stage, and has been controlled in the past through application of DDT and Bt, along with introductions of *Cotesia glomerata* and *C. rubecula* parasitoid wasps (Dempster, 1968; Benson et al., 2003).

Although occasionally used as a host plant in its native range, *P. rapae* may use the European biennial plant, *Alliaria petiolata* Bieb (Cavara & Grande), more frequently in North America due to the plant's increasing abundance in the understory of forests. Unlike most of its other hosts, *A. petiolata* is shade-tolerant and capable of occupying forest edges and understories. This invasive mustard allelopathically affects mycorrhizal forest plants as well as competes directly with neighboring plants for resources (Meekins and McCarthy, 1999; Callaway et al., 2008). Anecdotal observations suggest that this plant is much more abundant in North America than in Europe (Hierro et al., 2005), and its presence may draw *P. rapae* into forests more often.

There are not many herbivores that use *A. petiolata* as a food source in North America.

Although Yates and Murphy (2008) identified three arthropod herbivores present on *A. petiolata* in Ontario, Canada, they did not observe *P. rapae* consuming *A. petiolata*, and no herbivore eats enough to control its spread or abundance. Even mollusks avoid consuming *A. petiolata*, instead preferring more palatable native plants (Hahn et al., 2011; Hahn and Dornbush, 2012). This suggests that *A. petiolata* is generally well defended from most North American herbivores, and the damage it does accrue rarely reduces fitness. However, *P. rapae* may be able to use the European plant as a host in North America. At present, only anecdotal observations exist of the use of forested habitats by *P. rapae* in North America (Cavers et al., 1979; Chew, 1981; Davis and Cipollini, 2014a).

To investigate how *P. rapae* is using forested habitats and the host plant, *A. petiolata*, in North America, we directly observed *P. rapae* oviposition and nectaring behavior in forested habitats shared with *P. virginensis*, a native congener. We also investigated how *P. rapae* uses *A. petiolata* in forest edge habitats. Finally, we compared the performance of *P. rapae* larvae and adults fed *A. petiolata*, to that observed on its more typical hosts, *Brassica juncea*, and *B. oleracea*.

## 5.2 Methods

### Direct observations of *P. rapae* in forest habitats

Observations of *P. rapae* occurred from April to June in 2011, 2012, and 2013 at three sites known to be occupied by *P. virginensis*: a private site in Morrow Co., OH (MCO), Wooster Memorial Park in Wooster, OH (WMP), and Allegany State Park in Salamanca, NY (ASP). Basic visual observations were recorded using field notebooks and photography.

More detailed behavioral observations were made at WMP. Twenty-five *Pieris rapae* individuals were monitored between 1100-1600 on Apr 15 & 18, 2012 at least 300 meters away from the nearest edge or agricultural habitat. Behaviors of individual butterflies were

recorded in ten second intervals until the butterfly left the area and included flying, gathering nectar, oviposition, and resting. We identified all plants that the butterflies interacted with during oviposition and nectar gathering using the Newcomb (1977) guide to wildflowers. Butterflies were identified as *P. rapae* and not as the native *P. virginiensis* by distinct, dark spots on the dorsal wing surfaces and yellow scales on the ventral wing surfaces. In contrast, *P. virginiensis* is white with occasional wing-vein shading and light spots on the wings (Scudder, 1889).

We also observed herbivory by *P. rapae* caterpillars at WMP during the same observation periods. Although first instar *Pieris* caterpillars are difficult to identify to species, older *P. rapae* caterpillars develop a broken yellow line along the dorsal surface and yellow spots around the spiracles; these characters are missing in native *P. virginiensis* caterpillars (Scudder, 1889).

### **Herbivory by *P. rapae* on *A. petiolata* in edge habitats.**

We examined how frequently *P. rapae* uses *A. petiolata* as a larval host plant in forested habitats by measuring end-of-year herbivory on first-year *A. petiolata* plants in maple-beech-oak forests surrounding Dayton, OH. Although other herbivores occasionally use *A. petiolata*, the only Lepidoptera we have observed in this area consuming *A. petiolata* have been *P. rapae*, and we have observed this through multiple years and across multiple sites. In 2011, we surveyed approximately 9000  $m^2$  of a recreational trail in Beavercreek, OH between Grange Hall Rd. and N. Fairfield Rd (BCT). This trail has grass and unmanaged shrubs on the southern side and a strip of second-growth forest (20-60 m forest perpendicular to the trail) on the northern side. In 2013, we returned to re-survey BCT and also surveyed two other sites: Narrows Reserve in Beavercreek, OH (NAR), and Fairborn Community Park in Fairborn, OH (FCP). Approximately 3000  $m^2$  and 2400  $m^2$  were surveyed at NAR and FCP, respectively. All three sites had parking lots, recreation trails, and forest areas. We walked the perimeter of each study area and systematically examined every

rosette of *A. petiolata*. In patches with more than 10 rosettes clumped together, we randomly chose 10 plants to sample. We surveyed 99 plants at BCT in 2011. In 2013, we surveyed 136 plants at BCT, 53 plants at FCP, and 81 plants at NAR.

Plants with at least one leaf larger than 5cm in diameter were surveyed for chewing damage from caterpillars (asymmetrical, smooth holes away from the leaf edge) on fully expanded leaves. Damage was attributed primarily to *P. rapae* caterpillars for several reasons. First, caterpillar damage is distinct from other causes of damage and disease, including deer herbivory, slug herbivory, and flea beetle damage (SLD & DC, personal observations). Second, we have observed *P. rapae* caterpillars feeding on *A. petiolata* throughout the year at these locations, and *P. rapae* is the only caterpillar that we have ever observed feeding in this area, despite reports of *Plutella xylostella* as another lepidopteran herbivore on *A. petiolata* (Yates and Murphy, 2008). Although some leaf tearing and disease was noted (especially the presence of a powdery mildew fungus, Ciola and Cipollini (2011)), these observations were excluded from herbivory analyses. Each leaf on a chosen plant was scored for leaf area loss by caterpillars from 0 to 5 (undamaged, 1-20%, 21-40%, 41-60%, 61-80%, 81-100% leaf loss). The damage rating was converted to percent leaf loss by weighting each leaf score as follows: 0 (0), 1 (0.1), 2 (0.3), 3 (0.5), 4 (0.7), 5 (0.9). The converted leaf scores for each plant were averaged into a final plant score.

### ***Pieris rapae* larval performance assay.**

In order to determine the suitability of *A. petiolata* as a larval host, we examined *P. rapae* larval performance on both rosette and flowering *A. petiolata* (Wright State Forest, Dayton, OH) and on two commercial brassicaceous crops, *B. juncea* and *B. oleracea* (Meijer, Inc). *Pieris rapae* eggs (Carolina Biological Supply) were raised on either *Brassica oleracea* L. green cabbage' (Meijer, Inc.) or flowering *A. petiolata*, and allowed to emerge as adult butterflies. Adults were placed in 75 L aquaria with artificial nectar (20% sucrose:water solution on delicate task wipes until moist) and allowed to oviposit on flowering *A. peti-*

*olata*. Eggs laid by the adult butterflies were used in the following larval performance experiment, and we distributed the *A. petiolata* and *B. oleracea* neonates evenly among the four treatments below.

After hatching, second generation neonates were placed on either field-collected (June 2014) rosette *Alliaria petiolata*, flowering *A. petiolata*, commercially purchased, non-organic *B. oleracea* (green cabbage, Meijer, Inc.) or *B. juncea* (southern giant curled mustard, Meijer, Inc.) leaves in moist filter-paper lined petri dishes and kept in a 16:8 L:D incubator at 25°C. Commercial plants were rinsed with distilled water before use. We chose *B. oleracea* and *B. juncea* to represent commercial hosts available to *P. rapae* in the wild. After one week of monitoring daily for survival, we took daily measurements of caterpillar mass, until they neared pupation. Pupae were weighed and placed in 75L aquaria according to their larval host plant, with artificial nectar and an oviposition substrate (rosette *A. petiolata*). After eclosion, butterflies were allowed to mate and oviposit freely. When all butterflies died, the number of eggs and the number of females were counted to calculate the mean number of eggs laid per female, an indirect measure of fitness.

## Statistical analysis

All statistical analyses were performed in R (R Development Core Team, 2014). We separated our field herbivory data into two sets: data from BCT alone, and data from 2013 alone. These data were separated because only one site, BCT, was sampled for two years. For both datasets, we used a binomial model with a logit link function followed by Tukey's HSD test (multcomp package in R) to examine how the number of leaves on a plant covaried with location (2013 data) or year (BCT data) to affect the presence or absence of damaged leaves (Hothorn et al., 2008). We also examined the same data sets (2013 and BCT data) for differences in the percent leaf loss score. We removed all zeroes and log-transformed the percent leaf loss scores to meet normality assumptions, then evaluated the data using a general linear model followed by Tukey's HSD post-hoc testing when appro-



appropriate. Plots were constructed with the `gplots` package (Warnes et al., 2014).

For the larval performance experiments, we used the Kaplan-Meier estimator for survival data (`survival` package in R), and one-way ANOVA to compare pupal mass and relative growth rate across host plants (Therneau and Grambsch, 2000). Relative growth rate (RGR) was calculated as larval mass increase divided by the initial larval mass times the number of days of recorded growth. Chi-square testing followed by chi-square tests with Bonferroni correction were used to evaluate differences between the number of eggs laid per treatment.

## 5.3 Results

### Forest observations

At MCO and ASP, we regularly observed *P. rapae* flying in heavily wooded areas, but did not observe any nectar gathering or oviposition behavior. In a given visit to MCO, we would commonly see several *P. rapae* flying through the wooded area; we observed this behavior in all three years of observation. We found an unidentified first instar caterpillar in 2012 at MCO on *A. petiolata* that could have been either *P. rapae* or *P. virginensis*. At ASP, we observed *P. rapae* in wooded areas during regular field visits in 2012 and 2013. One individual was captured and photographed for confirmation (not shown). At WMP, we observed 25 *P. rapae* adults gathering nectar in the understory from several plant species, including *Claytonia*, *Phlox*, and *Viola* species, as well as from *A. petiolata* itself. We also observed 3 female *P. rapae* oviposit on *Cardamine diphylla* in a single observation period, and photographed an older *P. rapae* caterpillar feeding on *A. petiolata* (Figure 5.1).



Figure 5.1: Mature *P. rapae* caterpillar consuming rosette *A. petiolata* in Wooster, OH. Photo taken on May 11, 2012 by SLD.

## Herbivory observations

Although overall percent leaf loss was low, 78.8% of plants were damaged by caterpillars in 2013. Both the number of leaves ( $z = 3.475, P < 0.01$ ) and the location ( $P < 0.01$ ) influenced the probability of plants being damaged. BCT was significantly different from NAR ( $z = 2.614, P < 0.05$ ) and FCP ( $z = 3.631, P < 0.01$ ), but the latter two were not significantly different from each other ( $z = -2.217, P = 0.06$ ). Across years at BCT, only the number of leaves was a significant factor in the model ( $z = 3.622, P < 0.01$ ), indicating no difference in plant damage between years. Evaluating the percent leaf loss score revealed similar results, with BCT being significantly different from both NAR ( $z = 2.387, P < 0.05$ ) and FCP ( $z = 3.697, P < 0.01$ ), but NAR and FCP were not significantly different from each other, and the number of leaves per plant was not correlated with the percent leaf loss score. The model evaluating percent leaf loss score as influenced by date

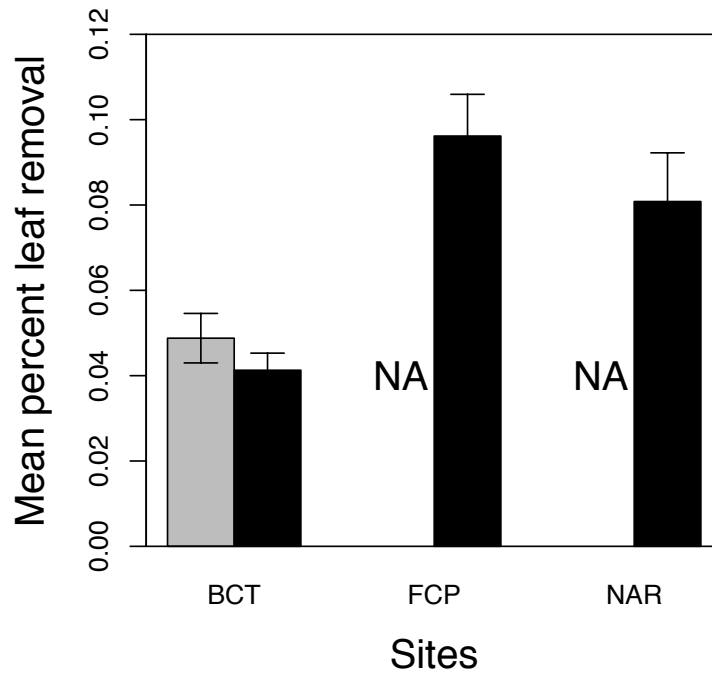


Figure 5.2: Herbivory (percent leaf loss) on *A. petiolata* varied between sites and habitats in 2013. Gray bar represents data from 2011; black bars represent data from 2013.

and number of leaves for the BCT site alone was not significant. Figure 5.2 shows the mean percent leaf loss score for both sites and years.

### ***Pieris rapae* larval performance**

Although there was a trend towards lower survival of *P. rapae* caterpillars feeding on flowering *A. petiolata*, we found no significant differences in survival of *P. rapae* caterpillars on the four hosts that we tested ( $X^2 = 7.4, df = 3, P = 0.0596$ , Figure 5.3). Pupal mass did not vary between treatments ( $F_{3,28} = 2.213, df = 3, P > 0.05$ ), however, there were differences in time to pupation ( $F_{3,28} = 7.897, df = 3, P < 0.01$ ). Caterpillars reared on *B. juncea* pupated significantly earlier than those raised on rosette *A. petiolata* ( $P < 0.05$ , Tukey's HSD) and on commercial *B. oleracea* ( $P < 0.01$ ). Relative growth

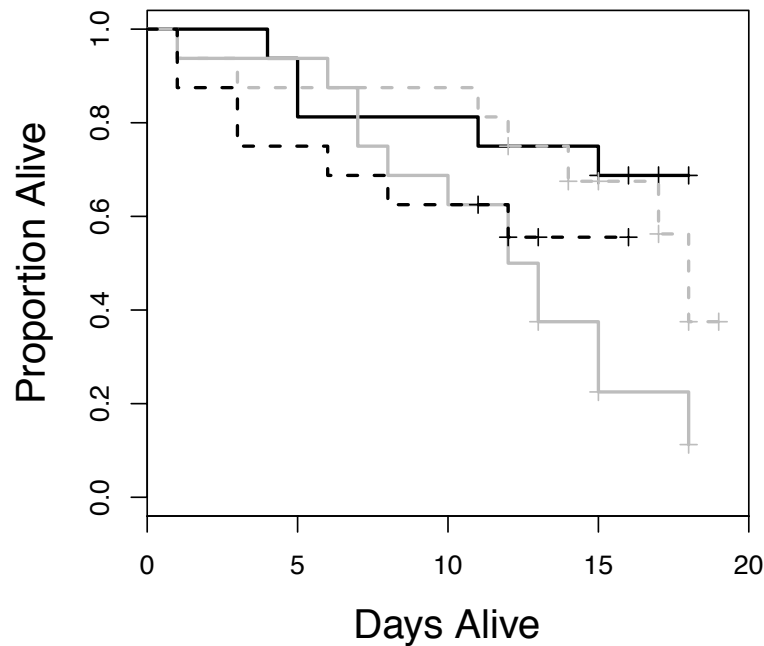


Figure 5.3: Kaplan-Meier survival estimates of *P. rapae* caterpillars fed commercial cabbage (solid black), rosette *A. petiolata* (dash grey), flowering *A. petiolata* (solid grey), or commercial mustard greens (dash black). Cross-marks indicate an event (pupation or death) has occurred.

rates also differed between treatments ( $F_{3,43} = 4.428, df = 3, P < 0.01$ ) because caterpillars on leaves of *B. juncea* grew significantly faster than those on flowering *A. petiolata* ( $P < 0.01$ , Tukey's HSD). To summarize, *P. rapae* caterpillars reared on *B. juncea* grew faster and pupated earlier with no significant loss of pupal mass, whereas caterpillars reared on flowering *A. petiolata* took longer and grew slower than those on *B. juncea* (Table 5.1).

Eclosed butterflies from the larval performance experiment were allowed to freely mate and lay eggs on rosette *A. petiolata*. Butterflies raised on *B. juncea* laid 89.5 eggs per female ( $n = 4$  females, 3 males), those raised on *B. oleracea* laid 176.6 eggs per female ( $n = 3$  females, 3 males), those raised on rosette *A. petiolata* laid 119.5 eggs per female ( $n = 2$  females, 4 males), and the lone female raised on flowering *A. petiolata* laid 147 eggs

( $n = 1$  female, 1 male). A chi-square test for proportions revealed significant differences from the mean of 133 eggs per female ( $X^2 = 31.3284$ ,  $df = 3$ ,  $P < 0.01$ ). Post-hoc testing showed that females laid significantly fewer eggs when raised on *B. juncea* than any of the other groups, and females raised on *B. oleracea* laid significantly more eggs than either *B. juncea* or rosette *A. petiolata* raised butterflies.

Table 5.1: Mean percent survival, days to pupation, pupal weight, and relative growth rate with standard error of *P. rapae* (both sexes) between four host plants ( $n = 16$  per treatment). Different letters indicate significant differences ( $p < 0.05$ ).

	Survival (%)	Days To Pupation (d)	Pupal Mass (mg)	Relative growth rate ( $gg^{-1}d^{-1}$ )
<i>A. petiolata</i> rosette	56	$15.33 \pm 0.85$	$128 \pm 3$	$0.277 \pm 0.046$ ab
<i>A. petiolata</i> flowering	20	$15.33 \pm 1.45$	$112 \pm 8$	$0.182 \pm 0.031$ b
<i>B. oleracea</i> 'cabbage'	69	$16.63 \pm 0.28$	$134 \pm 4$	$0.294 \pm 0.019$ ab
<i>B. juncea</i> 'mustard'	56	$12.78 \pm 0.46$	$132 \pm 6$	$0.380 \pm 0.044$ a

## 5.4 Discussion

We looked for evidence of the non-native butterfly, *Pieris rapae*, using *A. petiolata* in both forest and edge habitats in North America, and also examined larval performance. Previous studies have noted that *P. rapae* occasionally use forests (Cavers et al., 1979; Chew, 1981; Davis and Cipollini, 2014a), but we demonstrate that *P. rapae* frequents forested habitats, using both native and non-native nectar and host plants. We also confirmed that *P. rapae* successfully uses *A. petiolata* as well as its more typical brassicaceous hosts. In forests occupied by *P. virginensis*, *P. rapae* uses the similar nectar and oviposition resources with one exception: *P. rapae* can successfully use *A. petiolata* as a larval host, but the native congener cannot (Bess, 2005; Davis and Cipollini, 2014b; Frisch et al., 2014).

One possible implication regarding the use of forested habitats by *P. rapae* is direct competition for oviposition sites and nectar resources with native *Pieris* species. Since both *P. rapae* and native *Pieris* species oviposit on *C. diphylla*, caterpillars may occasionally compete for food, which can be limiting near the end of the larval stage when native ephemeral plant hosts are in decline (Cappuccino and Kareiva, 1985).

However, habitat sharing may benefit *P. virginensis* if *P. virginensis* practices egg avoidance like other congeners, and if *P. rapae* prefers ovipositing on *A. petiolata* instead of on the native *C. diphylla*. Egg avoidance is known not only in *P. rapae*, but also in *P. brassicae* (Schoonhoven et al., 1990). If *P. virginensis* are attracted to *A. petiolata*, but every *A. petiolata* encountered is already “occupied” by *P. rapae* eggs, *P. virginensis* may avoid the lethal host and benefit by being forced to lay its eggs on a suitable host (Davis and Cipollini, 2014a).

The presence of *P. rapae* in forests may have a negative effect on native *Pieris* spp. if nectar is a limiting resource. Nectar resources influence Lepidopteran habitat selection and also contribute to successful egg maturation and oviposition (Wiklund and Ahrberg, 1978; Murphy, 1983; Jervis et al., 2005). In some cases, Lepidoptera compete directly for nectar resources, attempting to dislodge other butterflies occupying desirable flowers (Sourakov, 2009). The initial invasion of *P. rapae* may have caused a severe decline in the abundance of another native butterfly, *P. oleracea*, before the invasion of *A. petiolata* (Scudder, 1889, but cf. Chew, 1981). Further work needs to be done to determine if nectar availability would be limiting for *P. virginensis* or another native Pierid, *P. oleracea*, persisting in forest habitats, and whether competition for nectar with *P. rapae* is important.

Nectar of *A. petiolata* is used by both Lepidoptera and by short-tongued flies and bees, in exchange for the transport of pollen grains to new plants (Courtney et al., 1982; Cruden et al., 1996). In North America, *A. petiolata* is an ideal host for *P. rapae*, providing nectar each spring, as well as plant material year round (rosettes persist through winter before flowering in the spring) for larval development. Although folivory by *P. rapae* may provide

some small ecological benefit by reducing the fitness of *A. petiolata*, it will likely not be substantial. Evans and Landis (2007) found that the minor foliar damage recorded in field observations of *A. petiolata* actually increased *A. petiolata* fecundity. Further work needs to be done to examine how *P. rapae*-inflicted damage affects future fitness of the invasive *A. petiolata*, as well as how the use of *A. petiolata* as an alternative host plant affects *P. rapae* abundance.

In addition to its use as a larval host, the nectar resources offered by *A. petiolata* may draw more *P. rapae* to agricultural fields near forested areas and edges occupied by *A. petiolata* (Courtney et al., 1982). Zhao et al. (1992) found that *P. rapae* were more abundant in broccoli interplanted with nectar-producing plants than in broccoli monocultures. Future experiments should include an examination of *P. rapae* populations in fields with and without nearby woodlands invaded by *A. petiolata*.

There may be an increase in apparent competition for enemy free space when *P. rapae* use forest resources in habitats already occupied by native *Pieris* species (Fryer, 1986). Benson et al. (2003) found no evidence that braconid parasitoids would attack *P. virginien-sis* sentinel caterpillars near meadows; however lab work demonstrates that these wasps readily attack any *Pieris* spp. caterpillar, including *P. virginiensis*. Although not currently a problem, *Cotesia* may be a problem for future generations of *P. virginiensis*, *P. oleracea*, and other native Pierid butterflies if they begin to follow *P. rapae* into nearby forests.

Finally, *P. rapae* may interfere with volunteer-driven conservation efforts for the native *Pieris* species. There are many organizations that track *P. virginiensis* populations over time, but some volunteers estimate unusually high densities of *P. virginiensis* (C. Lehn, unpublished data). Some of these observations may be of *P. rapae* utilizing forest habitat for its nectar and oviposition resources. Differentiating between these Pierids at a distance, by sight or behavior, is difficult (Chew, 1981; Cappuccino and Kareiva, 1985). Volunteers may be overestimating population sizes by misidentifying *P. rapae* as native *Pieris* spp., and consequently missing the signs of declining populations.

In conclusion, *P. rapae* seems to be expanding into North American forest habitats with and without co-occurring native Pierid species, and its use of *A. petiolata* appears to facilitate this movement. *Pieris rapae* may be simultaneously escaping pressure from competition and parasitism, as well as increasing herbivore pressure on the exotic mustard *A. petiolata*. Where *P. rapae* overlaps with native Pierids, there are opportunities for competition. However, more work needs to be done to investigate both the cause of *P. rapae* habitat expansion as well as the ecological implications of moving into forested habitat.



# **Competitive effects of *Alliaria petiolata* (garlic mustard) on the growth of two native mustards, *Cardamine diphylla* and *Boechera laevigata*.**

## **6.1 Introduction**

Invasion by exotic organisms is one of the most serious issues facing our global economy. Exotic organisms are estimated to cost \$120 billion a year in agricultural, forestry, and environmental losses (Pimentel et al., 2005). In addition, exotic organism invasion is only second to habitat loss in causing species decline and threats to global biodiversity (Wilcove et al., 1998). Invasive plants are some of the worst offenders, with economic damages from over 25,000 exotic plants totaling over \$34 billion annually (Pimentel et al., 2005). Invasive plants can radically alter community composition and nutrient cycling, while also having devastating direct and indirect effects on native organisms at different trophic levels (Gordon, 1998; Holdredge and Bertness, 2010; French, 2012).

Often, invasive plant species outcompete native species directly for nutrients, light, or

space. They are capable of modifying the environment around them to maximize their own fitness at the expense of native species (Gordon, 1998). French (2012) found that Bitou bush (*Chrysanthemoides monilifera* spp. *rotundata*) outcompeted native plants as well as other invasive plants in both high- and low- nutrient treatments, and also facilitated the growth of the “secondary invader” asparagus fern (*Asparagus aethiopicus*). Litter from *Phragmites australis*, another prolific invader, effectively shades out wetland plants native to invaded areas (Holdredge and Bertness, 2010). Invasive plants also affect nearby plants through allelopathic interactions with their roots, mycorrhizae, or other microbes (Bais et al., 2003; Hierro and Callaway, 2003).

*Alliaria petiolata* Bieb. Cavara & Grande (garlic mustard) is a prime example of a plant that competes for resources and is allelopathic toward plants and their mutualists in North America. *Alliaria petiolata* is a biennial European forest herb introduced into North America in the late nineteenth century (Nuzzo, 1993). In its first year, it exists as a vegetative rosette, overwinters without dropping its leaves, and then flowers in the springtime, reaching average heights of 1m, but occasionally up to 2m (Cavers et al., 1979; Nuzzo, 1993). Since its initial introduction, it has invaded 37 states in the U.S. and five Canadian provinces (USDA, 2014).

*Alliaria petiolata* directly outcompetes some species, like *Quercus prinus*, in greenhouse experiments (Meekins and McCarthy, 1999), and affects germination and growth of native plants, like *Impatiens capensis*, in both the field and greenhouse experiments (Prati and Bossdorf, 2004; Cipollini and Enright, 2009; Barto et al., 2010a). It also inhibits mycorrhizae and mycorrhizal colonization of surrounding plants (Roberts and Anderson, 2001; Stinson et al., 2006; Burke, 2008; Wolfe and Rodgers, 2008; Barto et al., 2010a), perhaps more in its invasive range in North America than in its native range in Europe (Callaway et al., 2008). Few herbivores appear willing to consume *A. petiolata* in North America, and it seems unaffected by both generalist mammalian herbivores like deer (Rossell et al., 2007; Davis and Cipollini, 2014a), and specialist and generalist insect herbivores (Cavers

et al., 1979; Courant et al., 1994; Porter, 1994; Davis and Cipollini, 2014b).

*Alliaria petiolata* also directly affects the native butterflies *Pieris oleracea* and *P. virginiensis*, as they are attracted to oviposit on the invasive plant, but their larvae either do not survive well (*P. oleracea*, Keeler and Chew, 2008, and refs. within) or at all (Bowden, 1971; Courant et al., 1994; Porter, 1994; Davis and Cipollini, 2014b) on the novel host. *Alliaria petiolata* may also indirectly impact such species by affecting the growth and reproduction of their native ephemeral mustard hosts like *Cardamine diphylla* (A. W. Wood) and *Boechera laevigata* (Al-Shehbaz, formerly *Arabis laevigata*, Kiefer et al., 2009), but this effect has not been studied. *Cardamine diphylla* is a perennial rhizomatous mustard that ranges over much of eastern North America in moist woodlands, and is the preferred host plant for several native Pierids (Chew, 1981). *Boechera laevigata* is a facultative biennial mustard that persists primarily in nutrient poor areas like mossy boulder surfaces and at the base of trees, where there is little leaf litter or competition (Bloom et al., 2001); it is a suitable host for *P. virginiensis* where *C. diphylla* is not present (Calhoun and Ifter, 1988; Shuey and Peacock, 1989).

*Alliaria petiolata* may compete for light and nutrients with these two native hosts of *P. virginiensis*. Not only does *A. petiolata* co-occur spatially, but also temporally with both *B. laevigata* and *C. diphylla* (USDA, 2014). As the taller plant, *A. petiolata* will benefit from increased light availability in competitive situations with these native species (Meekins and McCarthy, 2000). Rodgers et al. (2008) found that rosette *A. petiolata* increased the decomposition of native leaf litter and consequently, increased the nutrient availability of invaded soils and maximized its own fitness, another benefit for the plant when competing against native mustards.

Finally, the extensive chemical arsenal of *A. petiolata* against mycorrhizae has been of much interest in the past (Nuzzo, 1993; Stinson et al., 2006; Burke, 2008; Wolfe and Rodgers, 2008; Barto et al., 2010a), but other members of Brassicaceae, like *B. laevigata* and *C. diphylla*, are non-mycorrhizal (Bloom et al., 2001; Sweeney and Price, 2001). There

have only been a few papers discussing the direct competitive effects of *A. petiolata* on fellow Brassicaceae, and no one has yet examined the potential of *A. petiolata* to affect non-mycorrhizal forest herbs that support *Pieris* species (McCarthy and Hanson, 1998; Cipollini et al., 2008). In this paper, we investigated the competitive effects of *A. petiolata* on these two native mustards, *C. diphylla* and *B. laevigata*, to determine if either lifestage of *A. petiolata* negatively affects their growth and reproduction.

## 6.2 Methods

In 2012, we investigated the effects of *Alliaria petiolata* rosettes on the growth of two native mustard plants, *Boechera laevigata* (rosette form) and *Cardamine diphylla*. To investigate how each responds to heterospecific competition with *A. petiolata* relative to competition with a conspecific, we grew individuals in pots with either another individual of the same species or with an *A. petiolata* rosette. In 2014, we repeated this experiment, but used second year *A. petiolata* in competition with rosette *B. laevigata* and *C. diphylla*.

In both years, we collected *B. laevigata* rosettes (John Bryan State Park, Yellow Springs, OH) and transplanted them in 2.5L pots with Pro Mix BX soil (BFG Supply, Xenia, OH), with either another individual of *B. laevigata* or an *A. petiolata* individual (collected from Wright State University Woods, Dayton, OH). We selected flowering *A. petiolata* that were, on average, smaller in height ( $23.3 \pm 2\text{cm}$ ) and number of leaves ( $12.5 \pm 1$ ) than *A. petiolata* found in natural settings in order to facilitate a conservative estimate of the effects of *A. petiolata*. Rhizomes of *C. diphylla* (collected from Hocking County, OH) were trimmed to approximately 5 cm lengths (1-2 leaves) with scissors before being transplanted as above with either another *C. diphylla* or an *A. petiolata* individual. All plants chosen were similar in size to other members selected of that species, to minimize effects of initial size. All plants were watered regularly, had a biweekly fertilizer application (Plant Marvel Nutriculture 20-20-20), and were treated with pesticide (Safer,

2% potassium salts of fatty acids) for aphid infestation as needed.

After transplant, we monitored plants weekly for individual growth characteristics. Measurements of *B. laevigata* included rosette diameter and leaf number, while *C. diphylla* plants were measured for leaf number and middle leaflet length. All plants were harvested at the end of the experiment for dry mass root and shoot measurements.

In 2012, the experiment with rosette *A. petiolata* ran for six weeks before harvest. Plants were harvested by clipping the stem at the soil surface. Shoot material was placed into paper bags for drying, and rhizomes/roots were removed from the soil and excess soil was removed from them using water and gentle rubbing. Cleaned roots were placed in brown paper bags, and then all samples were dried for 72 hours at 50° C. In 2014, the experiment ran for four weeks before harvest because of aphid infestations primarily on the flowering *A. petiolata* plants. Aphids were only visibly present in the final week of the experiment, and it is unlikely that they affected the outcome. After drying, roots and shoots were weighed for each plant, and root:shoot ratio was calculated.

All statistical analyses were performed in R (R Development Core Team, 2014). The effect of conspecific or heterospecific competition on shoot mass, root mass, and root:shoot ratio was analyzed using MANOVA with the Pillai-M.S. Bartlett trace test statistic, separately for each target species for each year. The effect of conspecific or heterospecific competition on leaf number and rosette diameter of *B. laevigata* and leaf number and middle leaflet length of *C. diphylla* were analyzed through time using using repeated measures ANOVA followed by Tukey's Honestly Significant Difference post-hoc test for each growth variable. Graphs were constructed using the gplots package in R (Warnes et al., 2014).

### **6.3 Results**

When competing with rosette *A. petiolata*, the total mass of *C. diphylla* at harvest was not significantly different from those competing with conspecifics, but there was a slight trend

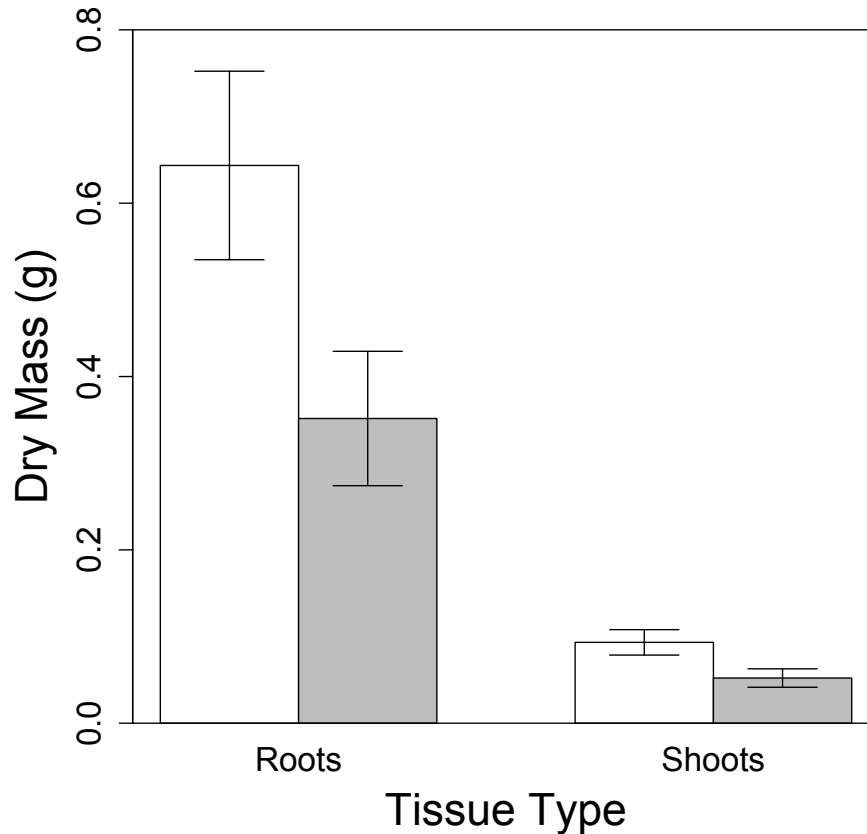


Figure 6.1: Final harvest mass of *C. diphylla* root and shoot tissues after growing in competition with either a conspecific individual (white), or rosette *A. petiolata* (gray). Error bars represent mean  $\pm 1$  S.E.

of more root mass ( $F_{1,34} = 3.167, P = 0.084$ ) and shoot mass ( $F_{1,34} = 3.448, P = 0.072$ ) in plants competing with another *C. diphylla* plant. Root:shoot ratio was not significantly different between treatments (Fig 6.1). Middle leaflet length did not significantly differ between weeks or treatments, and there was no interaction between the two factors.

When *C. diphylla* grew in competition with flowering *A. petiolata*, roots were 45.6% lighter than the control ( $F_{1,34} = 11.248, P < 0.05$ ), and shoots were 51.1% lighter than the control ( $F_{1,34} = 11.313, P < 0.05$ , Fig 6.2). There was a trend towards more mass located in the roots (root:shoot ratio,  $F_{1,34} = 3.9018, P = 0.0564$ ) of plants grown in competition with another *C. diphylla*. The number of leaves on *C. diphylla* plants were

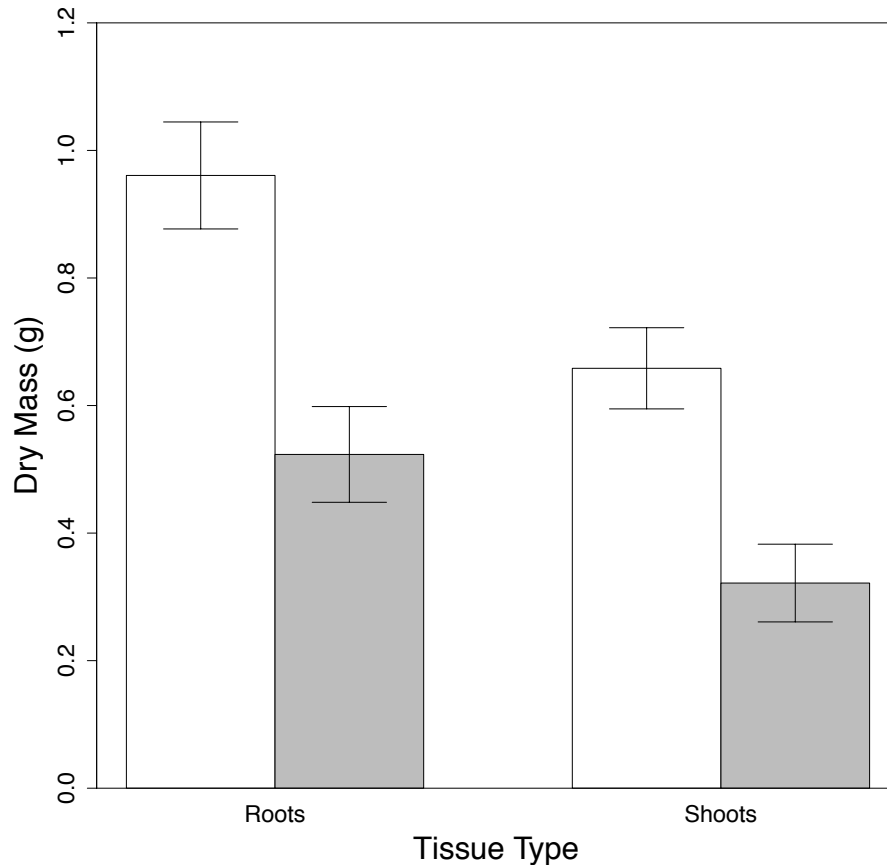


Figure 6.2: Final harvest mass of *C. diphylla* root and shoot tissue when grown in competition with either a conspecific individual (white) or flowering *A. petiolata* (gray). Error bars represent mean  $\pm$  1 S.E.

significantly different by week ( $F_3 = 9.8, P < 0.05$ ) and treatment ( $F_1 = 9.1, P < 0.05$ ) but there was no interaction between the two ( $F_{3,132} = 1.7, P > 0.05$ ). By the fifth week of the experiment, the number of leaves per plant was 35.4% higher in the control (Fig 6.3). Middle leaflet lengths also differed by week ( $F_3 = 9.4, P < 0.05$ ) and treatment ( $F_1 = 30.4, P < 0.05$ ) but there was no interaction between the two ( $F_{3,132} = 0.4, P > 0.05$ ). By the final week of the experiment, middle leaflets were 24.5% longer than on the control plants (Fig 6.3).

We also grew *B. laevigata* in competition with both stages of *A. petiolata*. When grown in competition with rosette *A. petiolata*, root mass was 52% lower ( $F_{1,34} = 5.9002, P <$

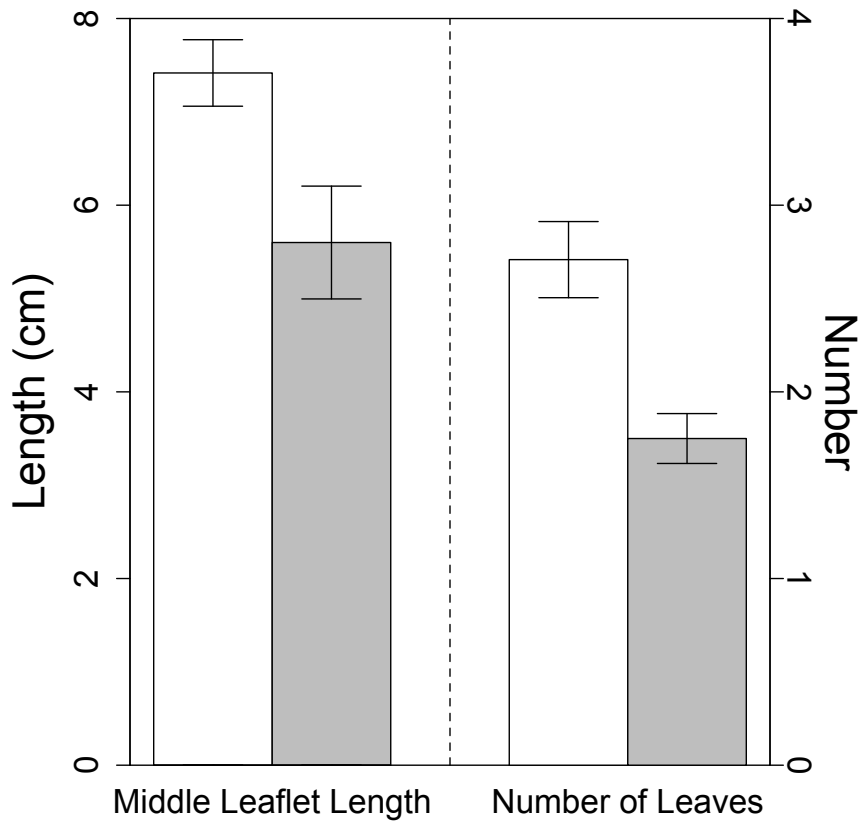


Figure 6.3: Middle leaflet length and average number of leaves of *C. diphylla* in week five after growing in competition with either a conspecific individual (white), or flowering *A. petiolata* (gray). Error bars represent mean  $\pm$  1 S.E.

0.05) and shoot mass was 50% lower ( $F_{1,34} = 12.616, P < 0.01$ ) than when competing with another *B. laevigata*; however, neither the root:shoot ratio nor the rosette diameter differed significantly between treatments (Fig 6.5).

When grown in competition with flowering *A. petiolata*, *B. laevigata* did not differ between treatments in harvested root mass, shoot mass, or root:shoot ratio. Rosette diameter of *B. laevigata* differed through time ( $F_3 = 15, P < 0.05$ ) but not between treatments ( $F_1 = 0.2, P > 0.06$ ) when grown with flowering *A. petiolata* or a conspecific, and there was no interaction between the two variables (Week x Treatment:  $F_{3,135} = 0.3, P > 0.05$ ). The number of leaves on *B. laevigata* differed through time ( $F_3 = 12, P < 0.05$ ) and



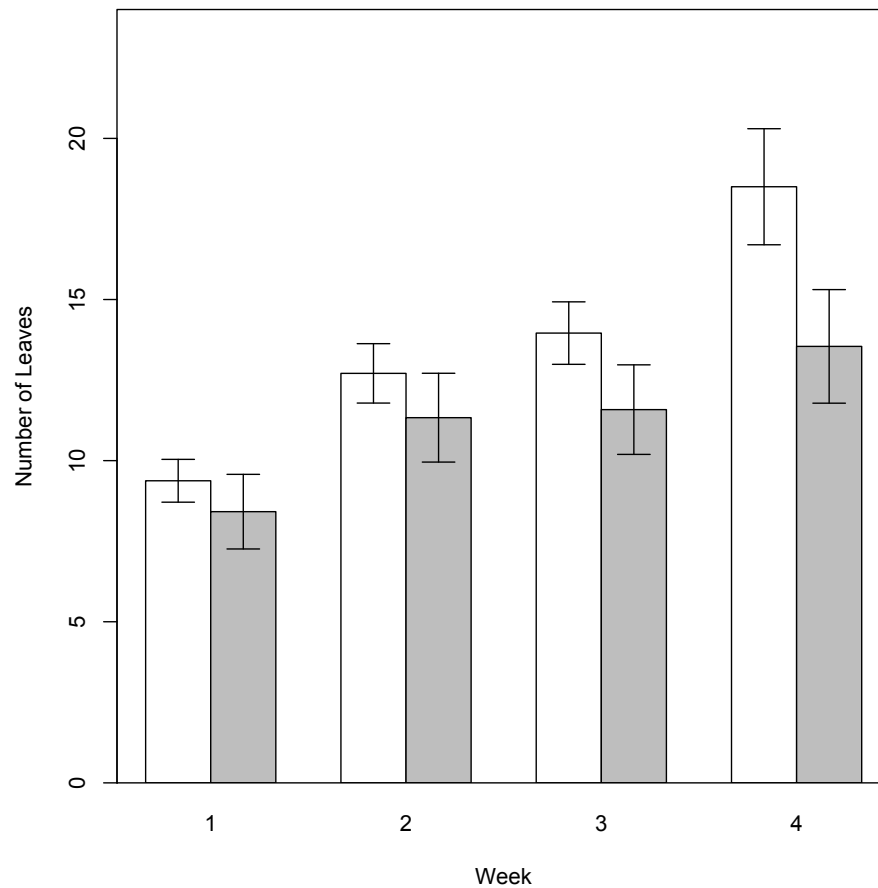


Figure 6.4: Average number of leaves of *B. laevigata* through time after growing in competition with either a conspecific individual (white), or flowering *A. petiolata* (gray). Error bars represent mean  $\pm$  1 S.E.

between treatments ( $F_1 = 5.8, P < 0.05$ ), but there was no interaction between the two ( $F_{3,135} = 0.8, P > 0.05$ ). Post-hoc analysis revealed no true difference between treatments within weeks (adjusted  $P > 0.05$ ), but there was a trend towards more leaves on *B. laevigata* in conspecific competition than in heterospecific competition (Fig 6.4).

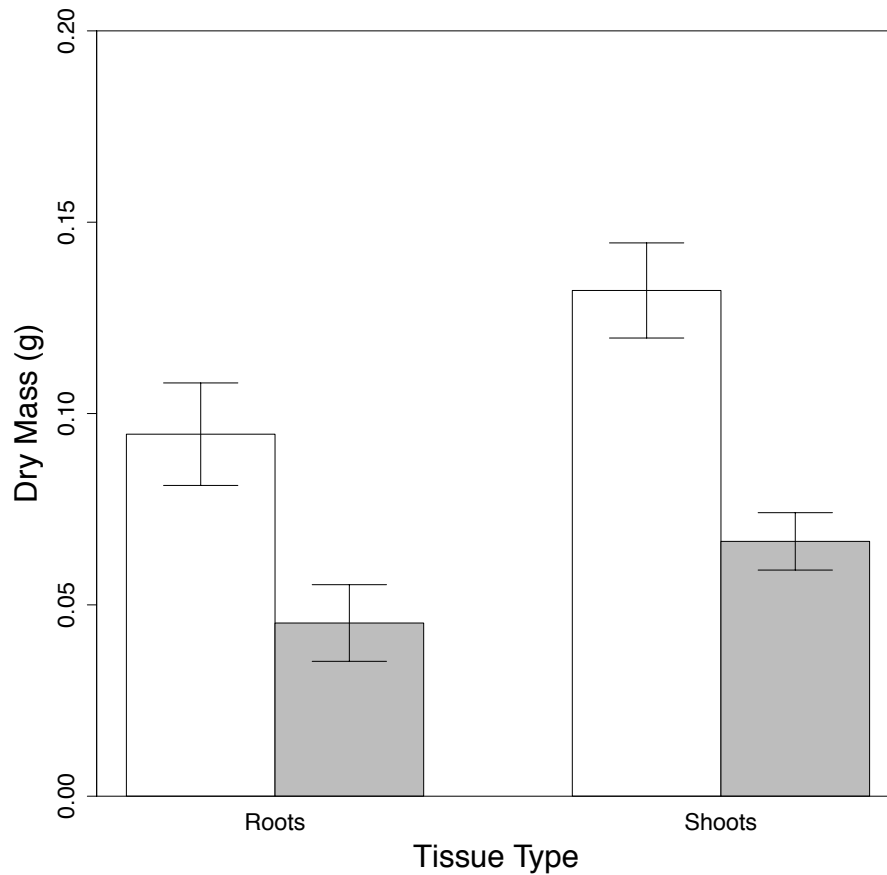


Figure 6.5: Final harvest mass of *B. laevigata* root and shoot tissue when grown in competition with either a conspecific individual (white), or rosette *A. petiolata* (gray). Error bars represent mean  $\pm 1$  S.E.

## 6.4 Discussion

Our results demonstrate that *A. petiolata* reduces growth and success of these two native mustards. The reduced growth of both *B. laevigata* and *C. diphylla* when competing with *A. petiolata* is most likely due to direct competition for nutrients, space, and light. A much smaller plant than *A. petiolata*, rosette *B. laevigata* persists a few centimeters above the soil surface and is adapted to low nutrient levels found on mossy, rocky outcroppings (Bloom et al., 2001). Excessive leaf litter or herbivory (often by deer) both severely reduce its chances of survival and successful reproduction (Bloom et al., 2003; Davis and Cipollini, 2014a). Even large rosette *B. laevigata* individuals are on average much smaller than rosette

or flowering *A. petiolata*, and may be easily shaded out by rosette leaves of *A. petiolata* (Cavers et al., 1979).

Competition for light with flowering *A. petiolata* was most likely minimized because *A. petiolata* rosette leaves senesce after the flowering stalk emerges, and the cauline leaves are smaller on average and relatively far away from the soil surface, reducing the shading effect. In addition, the nutrient requirements of flowering *A. petiolata* may be lower than its rapidly growing rosettes, because of stored resources in the roots available for reproduction. Further work should be done to determine if nutrient requirements vary between *A. petiolata* stages and how the variation would affect competitive outcomes.

*Cardamine diphylla* may have competed similarly with *A. petiolata*, but because *C. diphylla* are larger than *B. laevigata* and their leaves extend a similar distance from the soil surface as *A. petiolata* rosette leaves, they may have more successfully competed for light and nutrients against rosette *A. petiolata* (Meekins and McCarthy, 2000; Sweeney and Price, 2001). However, they may have been shaded by the closer proximity of cauline leaves when competing against flowering *A. petiolata*.

Previous studies on *A. petiolata* have utilized plant extracts, soils previously conditioned by *A. petiolata*, and even direct competition between *A. petiolata* and other plants to examine its invasion potential of native North American woodlands. Many of these studies focused on competition between *A. petiolata* and mycorrhizal plants like *Impatiens pallida*, *Maianthemum racemosum*, and *Acer negundo* (Meekins and McCarthy, 1999; Stinson et al., 2006; Burke, 2008; Wolfe and Rodgers, 2008), while others focus on the effects of *A. petiolata* extracts on seed germination (McCarthy and Hanson, 1998; Prati and Bossdorf, 2004; Barto et al., 2010a).

Only two previous studies have tested the possible effects of *A. petiolata* on another member of Brassicaceae, and neither found an effect of *A. petiolata* extract on seeds (radish, McCarthy and Hanson, 1998) or seedlings (radish, McCarthy and Hanson 1998; *Arabidopsis thaliana*, Cipollini et al. 2008). These previous findings suggest that allelopathy of *A.*

*petiolata* may be primarily directed towards mycorrhizal plants, and resource competition may be the only avenue of influence for *A. petiolata* towards other non-mycorrhizal plants.

If this type of resource competition occurs yearly in areas impacted by *A. petiolata*, both *B. laevigata* and *C. diphylla* may be facing chronic resource limitation. As primarily stationary populations, future generations of *C. diphylla* and *B. laevigata* will be unable to escape the constant presence of spreading *A. petiolata*. The impact may be somewhat reduced by the tendency of *A. petiolata* populations to be dominated by either rosettes or flowering stalks in a given year, however it will also make the effects of competition with *A. petiolata* in field settings difficult to disentangle.

Other invasive plants have been implicated in directly outcompeting native plants for light. Two thirds of Florida's most prolific invaders are thought to outcompete native plants for light (Gordon, 1998). *Lonicera maackii* shades the understory so thoroughly as to increase the mortality of native tree seedlings, and impact overall forest seedling recruitment (Gorchov and Trisel, 2003). The abundant leaf litter produced by *Phragmites australis* can shade and kill native wetland seedlings (Holdredge and Bertness, 2010).

Both of our focal native plants overlap with *A. petiolata* in natural environments where invasion has occurred, alongside other native plants already known to be affected by *A. petiolata* via allelopathy and other mechanisms, such as *Acer negundo*, *Impatiens capensis* and *I. pallida* (Stinson et al., 2006; Barto et al., 2010a). As *A. petiolata* continues to spread, wildflower abundance and overall plant diversity in impacted forests will likely decrease. Although species richness seems unaffected by garlic mustard density, both species diversity and percent cover of native plants were reduced in plots with increased garlic mustard density (Hochstedler et al., 2007; Stinson et al., 2007).

Finally, the competitive effects of *A. petiolata* on other key plants may also have cascading effects on native pollinators and herbivores present in impacted forests. Along with other springtime ephemerals, *C. diphylla* and *B. laevigata* produce nectar and attract visits by springtime pollinators. Under a scenario of chronic resource competition, these two

plants may have weaker relationships with pollinators. Although *C. diphylla* reproduces primarily by rhizome, *B. laevigata* relies on pollinators for its reproduction (Bloom et al., 2001, 2003).

Native herbivores may also be impacted by the interactions that these native plants have with *A. petiolata*. Both *Pieris virginiensis* and *P. oleracea* are mustard specialists residing in *A. petiolata*-impacted forests (Cappuccino and Kareiva, 1985; Shuey and Peacock, 1989; Keeler and Chew, 2008). These butterflies, normally attracted to hosts like *C. diphylla*, often mistakenly oviposit on *A. petiolata* with disastrous results (Courant et al., 1994; Porter, 1994). Caterpillars experience moderate to severe mortality when they begin feeding on *A. petiolata* (Keeler and Chew, 2008; Davis and Cipollini, 2014b). If the competitive effects we observed in the greenhouse occur frequently in nature, then native Pierids may be facing a simultaneous reduction in native host plant density alongside a lethal attraction to the novel host *A. petiolata*. Future research should include investigations into the competitive effects of *A. petiolata* on other non-mycorrhizal plants as well as cascading effects of *A. petiolata* on native insects.

# **Range, genetic diversity, and future of the threatened butterfly, *Pieris virginiensis*.**

## **7.1 Introduction**

With the continuous pressures from exotic organism invasion, habitat loss, and the rapidly shifting global climate, many species are expected to undergo range shifts and possible extinctions in the near future. The resulting biodiversity losses may negatively influence ecosystem services like pollinator services, food production, waste breakdown, and water purification. Wilcove et al. (1998) found that 97% of surveyed Lepidoptera (butterflies and moths) were threatened by loss of appropriate habitat, followed by alien species (36%), overexploitation of natural resources (30%) and pollution (24%). As perhaps the “most loved” insects for their bright colors and non-threatening mouthparts, Lepidoptera are often used as “surrogate” species within conservation biology, as either indicators of environmental conditions, “umbrella” species representative of a guild or habitat, or “flagship” species used to attract attention to a conservation goal (Caro and O’Doherty, 1999).

Umbrella butterfly species have been used with great success in Australia to conserve members of Nymphalidae, Papilionidae, and Castniidae, and also conserve other taxa with

similar habitat requirements (New, 1997). The Monarch butterfly (*Danaus plexippus*) and the endangered Karner Blue butterfly (*Lycaedes melissa samuelis*) are both popular “flagship” species in the United States, and have been used to successfully raise awareness and increase conservation efforts at both the local and global scales (Guiney and Oberhauser, 2008). Often, flagship species are chosen because of their uncertain futures, charismatic nature, or ties to a uniquely local habitat (Caro and O’Doherty, 1999; Guiney and Oberhauser, 2008).

*Pieris virginiensis* Edwards (Lepidoptera:Pieridae) is a flagship species chosen by the Lake Erie Allegheny Partnership for Biodiversity (LEAPbio) that occupies mature, relatively undisturbed deciduous forests in eastern North America (Finnell and Lehn, 2007). As a flagship species, it has not only drawn public interest but also public action, with several private and public groups engaging in long-term monitoring efforts for the butterfly (Bess, 2005; Finnell and Lehn, 2007).

It was noted as early as 1935 that *P. virginiensis* “may well become the first extinct Eastern butterfly” due to its limited range, restricted number of ephemeral host plants, and univoltine lifestyle (Klots, 1935). Local extinctions were documented by Tasker (1975) in Ontario, Canada, and by Davis and Cipollini (2014a) in central Ohio. Tasker (1975) recommended that the species be regarded as endangered in Ontario. Worries of decline and extinction have continued until the present, though now concerns include not only disturbance and habitat loss, but also deer browsing, plant invasion, and climate change (Bess, 2005; Finnell and Lehn, 2007; Davis and Cipollini, 2014a,b). Climate change especially may drive this time-limited butterfly to extinction if it, or its host plants, fail to respond to warming temperatures.

*Pieris virginiensis* fly in early spring, when only 60% of available days and 28% of available daytime hours are suitable for flight (Doak et al., 2006). Although several efforts have been made to identify the potential range and document previously undiscovered populations of *P. virginiensis*, no comprehensive effort has been made to map geographi-

cal and temporal occurrences of *P. virginensis* to examine the possibilities of widespread extinction of populations or possible shifts in response to climate change (Klots, 1935; Tasker, 1975; Shuey and Peacock, 1989; Bess, 2005; Finnell and Lehn, 2007). Little is known about the genetic structure and connectedness of *P. virginensis* populations, but several observers have noted an unwillingness of *P. virginensis* to fly outside of a forest canopy, severely limiting opportunities for migration and recolonization of previously occupied areas (Cappuccino and Kareiva, 1985; Bess, 2005). If this behavior truly limits migration, it may be reflected in genetic differentiation through strong isolation-by-distance effects. Finally, *P. virginensis* is associated with several other rare or endangered invertebrates, including the Six-Banded Longhorn Beetle (*Dryobius sexnotatus*), the American Burying Beetle (*Nicrophorus americanus*), the Diana Fritillary butterfly (*Speyeria diana*), and several other globally imperiled Lepidopterans (Bess, 2005). These associations make *P. virginensis* an umbrella species for its particular habitat, and conservation efforts for places where *P. virginensis* persists may also maximize suitable habitat for other imperiled species.

Here, we used occurrence data to construct a species distribution model for *P. virginensis* in its current climate to determine which climatic and environmental factors correlate strongly with *P. virginensis* presence, and to identify potential areas where previously undiscovered populations of *P. virginensis* may persist. We then used that model to predict where *P. virginensis* would find suitable habitat in the future (2070) under three different climate change scenarios. We then used the dates and times associated with occurrence data to construct temporal species models and identify shifts in emergence day or latitude through time. Finally, we collected specimens from across the current range of *P. virginensis* and sequenced both a mitochondrial and a nuclear “barcoding gene” to examine genetic diversity and structure of *P. virginensis* populations. We hypothesized that overall diversity would be low and the populations would be subject to drift and exhibit geographic differentiation. Together, these studies yield information about how best



to manage *P. virginensis* populations and their habitats now and in the future.

## 7.2 Methods

### **Collection of occurrence data.**

We received occurrence data from the Toronto Entomologists' Association (Jones et al., 2014), the Ohio Lepidopterists' Society, the Butterflies and Moths of North America (BAMONA, Opler et al. 2014), the Connecticut Butterfly Association, and the Butterflies of North Carolina (Legrand and Howard, 2014). In addition, we mined data from individual BugGuide (BugGuide, 2014) and North American Butterfly Association sightings records (NABA, 2014), and also retrieved data from the Global Biodiversity Information Facility (Canadian Biodiversity Information Facility, 2014). Including duplicate records and those with missing fields, there were a total of 1465 records available for analysis. Dates of records ranged from 1905-2014, and are shown in Figure 7.1.

### **Generating the species distribution models.**

We created our initial species distribution model, representing the current distribution of *P. virginensis*, using presence-only maximum entropy (MaxEnt) models in R (Phillips et al., 2006). Briefly, MaxEnt determines environmental constraints on the species given environmental predictors at presence points, and predicts other potential locations of species presence based on these constraints. We used the WorldClim bioclimatic data (2.5 minute resolution) as predictors, which includes 19 temperature and precipitation variables averaged per cell for all years between 1950-2000 (Hijmans et al., 2005). Our occurrence data were trimmed to only unique combinations of latitude and longitude. We used “kfold” testing to split our occurrence data into five groups; four of the groups were used for training the model, and the final group was used for evaluating the model and generating the map.

We used the following R packages to generate these models and their figures: *dismo*, *fields*, *maptools*, *raster*, *rgdal*, *rgeos*, *rJava*, *sp*, and *XML* (Pebesma and Bivand, 2005; Lang, 2013; Urbanek, 2013; Bivand et al., 2014; Hijmans et al., 2014; Nychka et al., 2014; Bivand and Lewin-Koh, 2015; Bivand and Rundel, 2015; Hijmans, 2015).

After generating and evaluating the “current climate” model, we used it to predict the future distribution of *P. virginienensis* under three different representative concentration pathways (RCP), or predictions about global climate change. We generated predictions for RCP 4.5, RCP6, and RCP8.5, which represent carbon dioxide emissions peaking in 2040, 2080, and after 2100, respectively (Field et al., 2014). For future scenario model generation, we used downscaled CMIP5 (Coupled Model Intercomparison Project Phase 5) data calibrated to the WorldClim bioclimatic “current” climate data at 2.5 minute resolution as predicted in the year 2070. We used future climate data generated from several modeling organizations, listed in Table 7.1 (Taylor et al., 2011; Nazarenko et al., 2015).

Table 7.1: Sources of climate data used in modeling the future distribution of *P. virginienensis*.

<b>Center Name</b>	<b>Location</b>	<b>Model Abbreviation</b>
Beijing Climate Center	China Meteorological Administration	BCC-CSM1.1
National Center for Atmospheric Research	Boulder, CO, USA	CCSM4
National Institute of Meteorological Research	Korea Meteorological Administration	HadGEM2-ES
Institut Pierre-Simon Laplace	Paris, France	IPSL-CM5A-LR
Meteorological Research Institute	Japan Meteorological Agency	MRI-CGCM3
Norwegian Climate Centre	EarthClim, Norway	NorESM1-M
NASA Goddard Institute for Space Studies	NASA, USA	GISS-E2-R

Using the “predict” function in R’s *raster* package, we predicted the distribution of *P. virginienensis* in 2070 according to each organization’s prediction of conditions under the three RCP expectations. For each RCP, we averaged the raster predictions of the seven

modeling organizations to produce an average raster prediction of *P. virginiensis* potential habitat.

After prediction rasters were generated for each organization's model, we categorized the predictions into "unacceptable" habitat with probability of occupancy at or below 40%, and "acceptable" habitat with probability of occupancy above 40%, then estimated the area of each region using the area function in the raster package in R. We chose 40% as the acceptable habitat designation in order to provide a conservative estimate of available habitat for these organisms, as relational models will never capture the total variation present in a given habitat. These "acceptable" area values were averaged across each of the seven organizations' predictions to produce an average acceptable area for a given RCP, and analyzed using ANOVA.

### **Generating the temporal species occurrence models.**

We generated the temporal species models using general linear models in R (R Development Core Team, 2014). Data for the temporal models ranged between 1970-2014. We used data at and after 1970 because records before 1970 are sparse. In addition, the data are right-skewed (more records in recent years) due to renewed public interest, and using data at or after 1970 eliminates most of the right-skew bias. For each location, consisting of a unique latitude and longitude combination, the earliest date of appearance was selected as "emergence day" and classified using Julian dates. Although this day is unlikely to be the true emergence day, it is the earliest record of the butterfly at that location in that year. Emergence day was then regressed against both latitude (33-47N) and year (1970-2014) to determine if shifts in emergence are occurring through time or across latitude.

## **Collection of specimens for sequencing**

From 2011-2014, we collected whole specimens or middle tarsi of *P. virginiensis* from locations in NC, NY, OH, PA and TN. After collecting, specimens were stored on ice in field conditions and transferred to  $-20^{\circ}$  C when possible for long term storage. In 2014, we requested that those familiar with *P. virginiensis*, including other researchers, collectors, and photographers, take a middle leg from any confirmed *P. virginiensis* individuals. These legs were shipped overnight on ice for long term storage, extraction, and sequencing at Wright State University, and included specimens from IN, MA, NC, TN, and WV (Figure 7.1). Sequences were grouped by state for IN, MA, and OH, and region (“Allegheny Plateau” or “AP” for NY and PA and “Great Smoky Mountains” or “GSM” for NC, TN, and WV) in analyses.

## **Extraction and sequencing of DNA.**

We extracted total DNA from middle tarsi or pupae (MA populations) of *P. virginiensis* using DNeasy Blood and Tissue kits (Qiagen, USA), in accordance with the manufacturer’s instructions. After extraction, we performed polymerase chain reactions (PCR) with primers representative of two gene coding areas, cytochrome oxidase I (COI, LepF1/LepR1, annealing temperature:  $54^{\circ}$ C) and the first ribosomal internal transcribed spacer region (ITS1, CAS18sF1/CAS5p8sB1d, annealing temperature:  $67^{\circ}$ C). Both of these areas are well studied and used as representative “barcoding” regions for the mitochondrial and nuclear genomes, respectively (Ji et al., 2003; Smith et al., 2012). These regions were amplified using ExTaq polymerase according to the manufacturer’s instructions (ClonTech/-TaKaRa Bio, USA).

After initial amplification, PCR products were sent to the University of Arizona Genetics Core for ExcelsaPure PCR purification and Sanger sequencing with Applied Biosystems 3730 DNA Analyzers (University of Arizona, AZ, USA). The resulting sequence traces

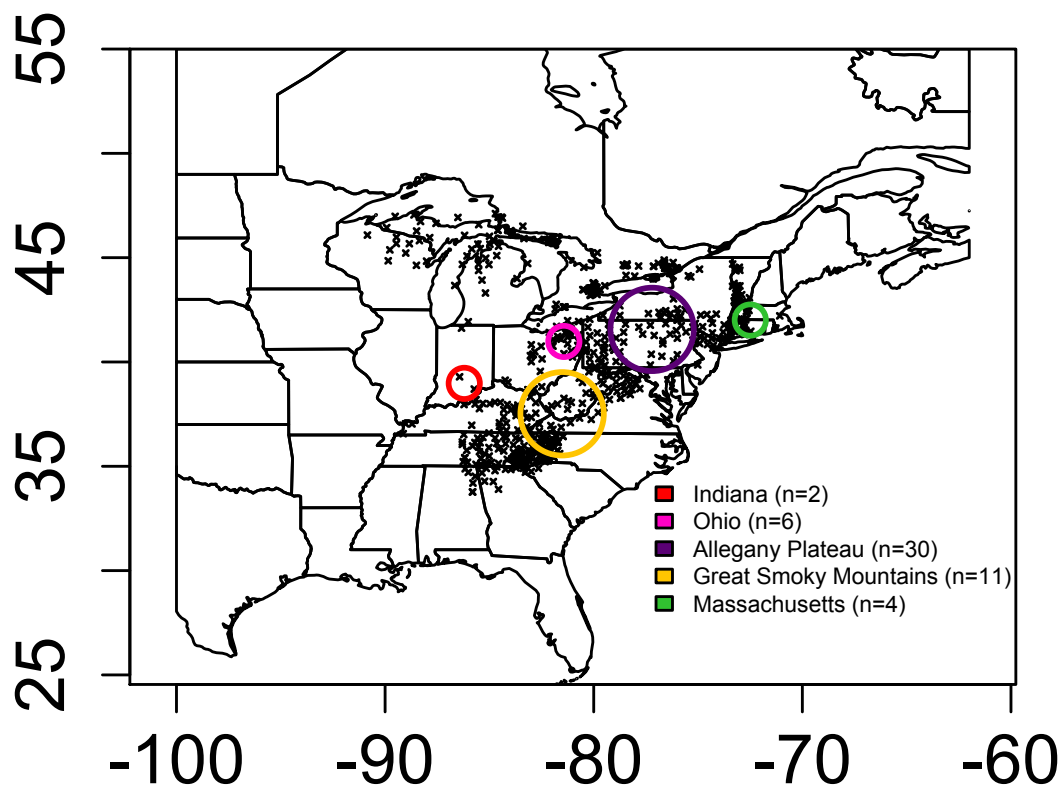


Figure 7.1: Collection locations (colored circles) and presence points (x marks) of *P. virginensis* in this study. Occurrence points presented range from 1905-2014; radii of areas sampled for genetics are approximate. Sample n's in legend represent the total number of individuals sampled in a given region.

were trimmed and edited with Sequencher 5.3 (Gene Codes Corporation, USA). For ITS, we used seqphase to prepare input files and ran PHASE with 5000 iterations, a burn-in of 1000 iterations, and a thinning interval of 10 to probabilistically determine alleles.

### Analyzing selection, diversity, and phylogeny.

After aligning sequences with ClustalW within MEGA 6.0 (Tamura et al., 2013), we used DNAsp to calculate nucleotide diversity ( $\pi$ ),  $F_S T$ , and Tajima's D (Sokal and Rohlf, 1981; Nei, 1987; Tajima, 1989; Fu and Li, 1993; Librado and Rozas, 2009). We estimated Watterson's  $\theta$ , which is an estimate of the population's mutation rate, using Markov chain Monte Carlo simulation algorithms for Bayesian inference (BEAUTi and BEAST 1.7, Drummond

et al., 2012). We used a chain length of three million and logged parameters every 2500 iteration, assuming a uniform prior distribution for both  $\kappa$  and  $\theta$ .

We used estimated mutation rates for *Drosophila* (1.3%/My, Schlötterer et al. (1994)) and *Pieris rapae* (3.54%/My, Jeong et al. (2009)), alongside the simulated  $\theta$  values from BEAST to estimate effective population size from both the nuclear (Equation 7.1) and mitochondrial genes (Equation 7.2), via equations established by Watterson (1975) .

$$\theta = 4 \mu N_e \quad (7.1)$$

$$\theta = 2 \mu N_e \quad (7.2)$$

We used PopART to construct haplotype networks using a median-joining method (Bandelt et al., 1999; Leigh, 2015). We used MEGA 6.0 for phylogenetic cladogram construction (Tamura et al., 2013). Neighbor-joining trees were tested with 500 bootstrap iterations (Saitou and Nei, 1987). Trees were rooted using three *P. rapae* individuals collected from Morrow Co., OH that were sequenced for both ITS1 and COI at the same time as *P. virginienensis*.

## 7.3 Results

### Current and future climatic species distribution predictions

We constructed a presence-only species distribution model using MaxEnt and *P. virginienensis* occurrence data. We found the potential current distribution to include most states throughout the eastern United states, in an area generally bounded from  $-90^\circ\text{W}$  to  $-70^\circ\text{W}$  longitude, and  $32^\circ\text{N}$  to  $48^\circ\text{N}$  latitude (mean AUC: 0.951, mean COR: 0.682; Fig 7.2A). This current climate potential distribution model has a continuous acceptable habitat corri-

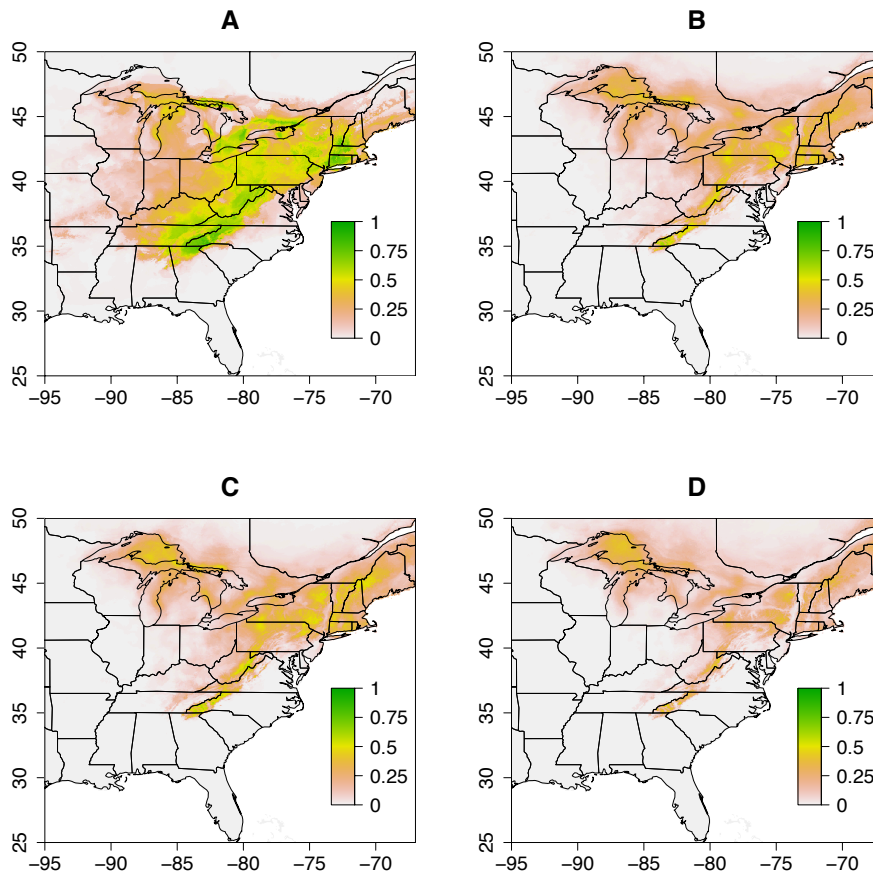


Figure 7.2: The potential distributions of *P. virginiensis* under (A) current climate, or in the year 2070 under predictions of (B) RCP 4.5, (C) RCP 6.0 and (D) RCP 8.5. These distributions are scaled 0-1 as a probability of presence, and are averaged across the seven model sources in Table 7.1.

dor along the Appalachian highlands (Fenneman, 1917).

In the model generated under RCP 4.5, which represents the earliest carbon emissions peak in 2040, there are some areas of reduced potential habitat, including eliminating habitat entirely in Kentucky and Illinois, alongside less acceptable habitat in West Virginia, Ohio, and the edges of the Great Smoky Mountains region in Tennessee and North Carolina (Fig 7.2B). The model generated for RCP 6.0 conditions is very similar to the RCP 4.5 model, and has roughly the same amount of “acceptable” habitat available (Fig 7.2C and 7.3). Finally, the potential habitat model generated for RCP 8.5 is severely reduced,

with much of Ohio, Kentucky, Virginia, and half of Pennsylvania becoming less acceptable (Fig 7.2D and 7.3). All RCP models had significantly less acceptable habitat available to *P. virginiensis* than available under current climatic conditions, but the RCP models were not significantly different from each other (ANOVA followed by Tukey's Honestly Significant Difference test:  $F_{3,22} = 32.931$ ,  $P < 0.01$ ).

All future climate predictions for habitat suitability also demonstrate a shift in northern limits, with acceptable habitat being created in northern New York (Adirondack mountain region), Maine (New England upland), and northern New Hampshire (White and Green mountains). Overall, habitat predictions from RCP 6.0 retained the most "acceptable" area, but was still only 35% of the current distribution (Fig 7.3).

### **Emergence of *P. virginiensis*.**

We examined how emergence date varies over time and latitude, and found a significant negative relationship between year and emergence date ( $P < 0.05$ ;  $r^2 = 0.0281$ ; Fig 7.4). Our model predicts that after one hundred years, the emergence date for *P. virginiensis* has advanced by 26.6 days in the last century; however, it is important to note that this significant relationship had a low Pearson's  $r^2$  and explained little of the observed variation.

We also used latitude as a predictor for emergence date, and found that the two had a significant positive relationship ( $P < 0.05$ ;  $r^2 = 0.2436$ ; Fig 7.5). This model predicts a 21.8 day increase in emergence date for every ten degree increase in latitude, and had a moderate  $r^2$  value an order of magnitude higher than that of the previous year-emergence correlation.



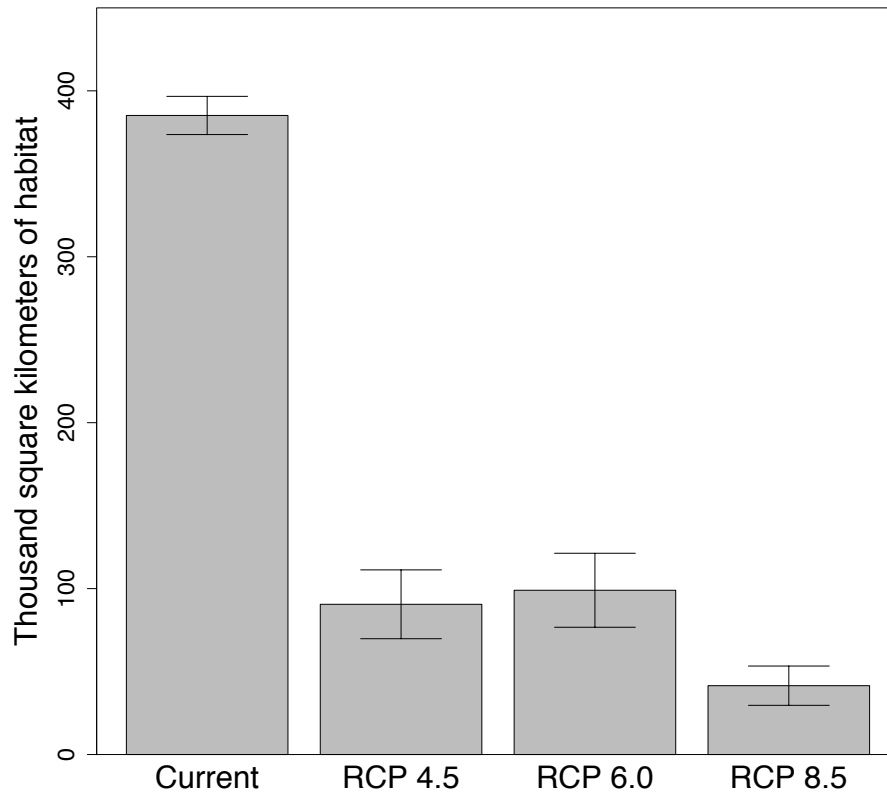


Figure 7.3: The amount of “acceptable” habitat in mapping predictions for (A) current climate, (B) RCP 4.5, (C) RCP 6.0 and (D) RCP 8.5. “Acceptable” habitat is classified as cells where the probability of occurrence was greater than 0.4.

### ***P. virginiensis* genetic diversity and phylogeny**

A total of 52 and 53 sequences successfully amplified for COI and ITS1, respectively.  $F_{ST}$  showed little differentiation between populations in ITS1, but reflect a unique haplotype found in COI (Table 7.2).

In COI, there were 14 unique haplotypes across the range of *P. virginiensis* with 15 polymorphic sites within the sequence. The nucleotide diversity ( $\pi$ ) of COI was  $2.05 \times 10^{-3}$ , Watterson’s estimator ( $\theta$ ) was  $5.53 \times 10^{-3}$  (95% CI:  $2.48 \times 10^{-3}$ ,  $8.4 \times 10^{-3}$ ), and the estimated effective population size was  $1.56 \times 10^5$  (95% CI:  $6.99 \times 10^4$ ,  $2.37 \times 10^5$ ) females. Tajima’s D was -1.93 ( $P < 0.05$ ), indicating a recent population expansion or purifying se-

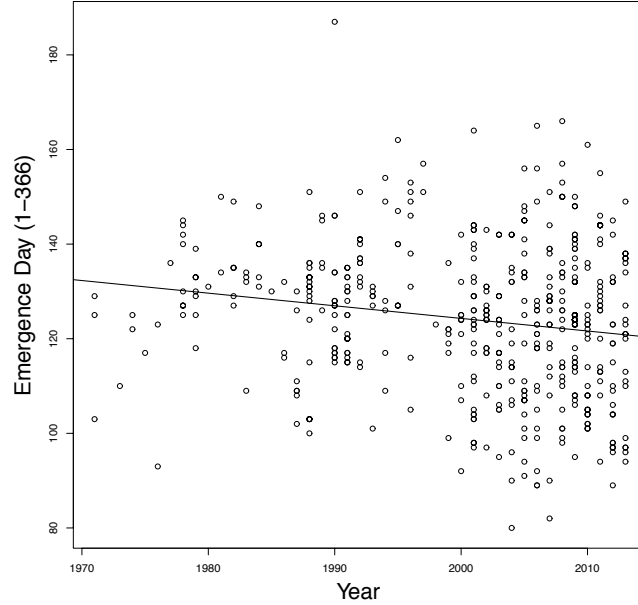


Figure 7.4: The relationship of emergence date with year in *P. virginiensis*.

Table 7.2:  $F_{ST}$  values for cytochrome oxidase subunit I (bottom left) and internal transcribed spacer I region (top right) in *P. virginiensis*.

	AP	GSM	IN	MA	OH
AP		0.085	-0.062	0.036	-0.043
GSM	0.064		0.056	0.229	0.052
IN	-0.006	0.087		0.000	-0.111
MA	0.029	0.114	0.000		0.000
OH	0.851	0.706	0.667	0.800	

lection. The haplotype network and phylogeny constructed reflect phylogenetic intermixing with a unique haplotype present only in OH individuals sampled from Holden Arboretum, Willoughby, OH (Fig 7.6, 7.8). At Holden Arboretum, all 5 individuals had a C instead of a T at position 288 (of 683 total nucleotides). This OH population clustered separately in 75% of all bootstrap iterations. All other deviations from the polytomy were small in branch length and had weak bootstrap support (< 50%), but may indicate impending separation due to isolation and drift.

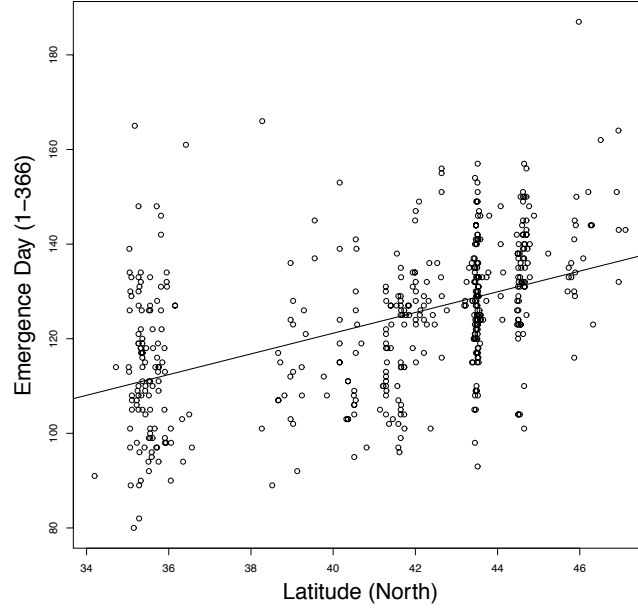


Figure 7.5: The relationship of emergence date with latitude in *P. virginiensis*.

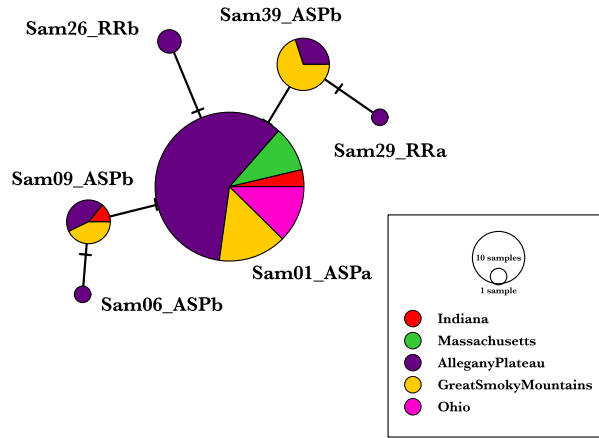


Figure 7.7: Haplotype network constructed by median joining of *P. virginiensis* ITS1 sequences (480 bp).

For ITS1, there were 9 unique haplotypes across the range of *P. virginiensis*, and 5 total polymorphic sites. The nucleotide diversity was  $9.0 \times 10^{-4}$ , Watterson's estimator ( $\theta$ ) was

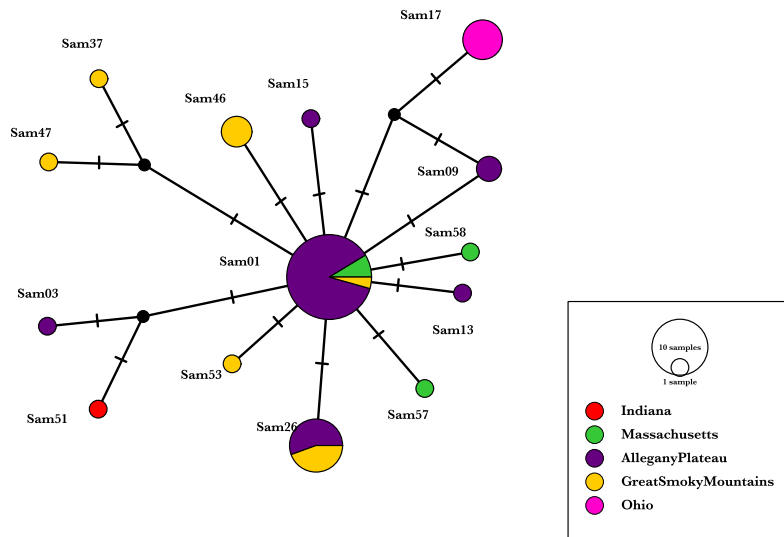


Figure 7.6: Haplotype network constructed by median joining of *P. virginiensis* COI sequences.

$1.49 \times 10^{-3}$  (95% CI:  $4.75 \times 10^{-4}$ ,  $2.75 \times 10^{-3}$ ), and the estimated effective population size was  $2.87 \times 10^5$  (95% CI:  $9.10 \times 10^4$ ,  $5.28 \times 10^5$ ) individuals. Tajima's D was  $-1.15$  ( $P > 0.05$ ). The haplotype network and phylogeny show no discernible genetic structure by location, but does demonstrate an overall lack of genetic diversity (Fig 7.7).

The neighbor-joining tree constructed for ITS1 with *P. rapae* as outgroup showed little genetic structure *P. virginiensis* population with no strongly supported clusters of geographic or other isolation, but instead several outliers with unique genetic make-up. These outliers, in the bottom 3rd of the constructed phylogeny, were heterozygous at several locations within the 100-300 bp region of ITS1 (Fig 7.9).

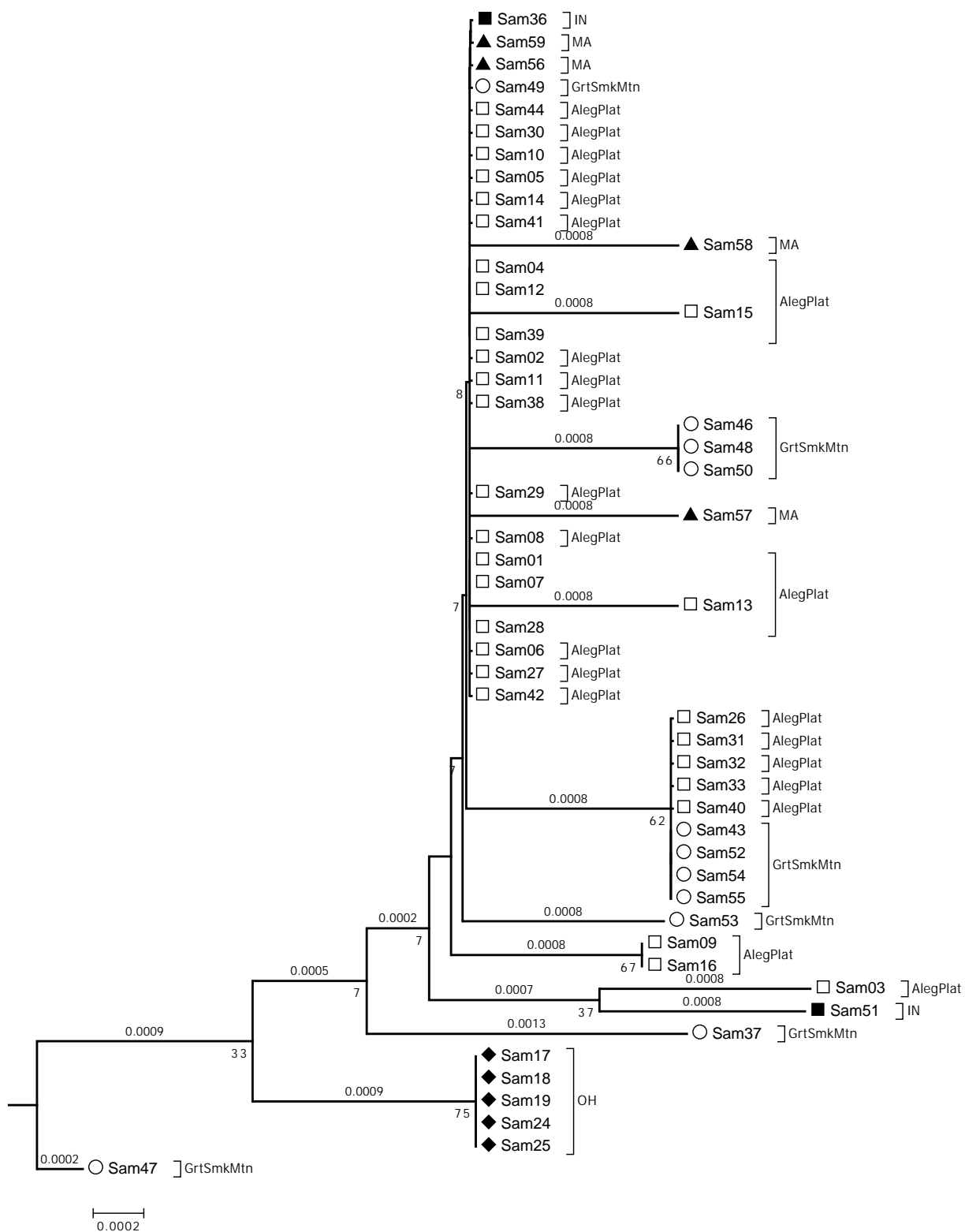


Figure 7.8: Neighbor-joining phylogeny (bootstrap n=500) of *P. virginiensis* with *P. rapae* as outgroup for COI. Populations are shown in Figure 7.1). Bootstrap values are indicated in values greater than 5%, branch length values are indicated in values greater than 0.003.

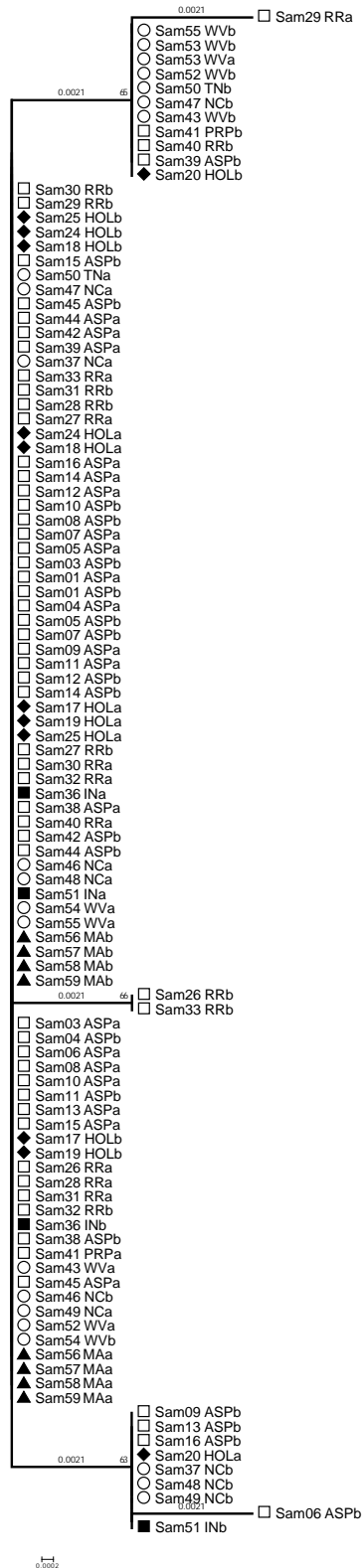


Figure 7.9: Neighbor-joining phylogeny (bootstrap n=500) of *P. virginiensis* with *P. rapae* as outgroup for ITS1. Bracketed abbreviations indicate different populations, as described in Figure 7.1). Bootstrap values are indicated in values greater than 5%, branch lengths are indicated if greater than 0.0002.

## 7.4 Discussion

Using both current and historic records, as well as genetic data gathered from across the range of *P. virginiensis*, we were able to establish several models to describe the current state of this butterfly in the environment. Although most butterflies are expected to prosper under global climate change (Sparks and Yates, 1997; Roy et al., 2001), *P. virginiensis* is facing the possibility of range shifts and population reduction in the near future. Our predictions for 2070 suggest that *P. virginiensis* will lose at least two-thirds of “acceptable” habitat as determined by WorldClim environmental data from the more western parts of the range of *P. virginiensis*, including Kentucky, Ohio, and Tennessee. In addition, the expectation of an increase in severe weather events may disproportionately impact *P. virginiensis*, as it is a springtime butterfly already flying in marginally unsuitable temperatures and wind speeds (Davis and Cipollini, 2014a).

*Pieris virginiensis* demonstrate an advancing yearly emergence date through time and with latitude. Our model estimates that the emergence of *P. virginiensis* has advanced in the last hundred years, and also correlates well with latitude. Like many butterflies, eclosion of *P. virginiensis* pupae seems intimately tied to temperature, and all *P. virginiensis* go through an obligate diapause during the summer, fall, and winter months. Only long days (16+ hrs light/day) induced in laboratory settings can induce a second generation of *P. virginiensis*, but the switch is incomplete and would not occur in the current range of *P. virginiensis* (Shapiro, 1971, SLD unpublished data). Interestingly, the primary host plant of *P. virginiensis*, *Cardamine diphylla*, shows no flowering advancement for the last 100 years, indicating that it is most likely cued for emergence with a variable unaffected by climate change, like photoperiod (Calinger et al., 2013). If the emergence of *P. virginiensis* continues to advance, but *C. diphylla* does not, this may present an additional complication for an already challenged butterfly.

For cytochrome oxidase I, Tajima’s D indicated a recent population expansion or purifying selection. These results, coupled with the relative low diversity and lack of genetic

structure of both phylogenies, were unexpected given the life history of *P. virginiensis*. Others have observed that *P. virginiensis* individuals are hesitant to cross open spaces, suggesting poor dispersal ability (Cappuccino and Kareiva, 1985; Bess, 2005). This supposedly poor dispersal ability, coupled with severe habitat loss in the twentieth century from urbanization and logging (Klots, 1935; Tasker, 1975), led us to hypothesize that *P. virginiensis* sequence data would reveal increased homozygosity and a low effective population size from genetic bottlenecks and drift. We saw the opposite, suggesting that these recent bottlenecks are overshadowed by historic population expansion after the last glacial maximum.

When examining the current potential habitat of *P. virginiensis*, there is contiguous habitat centered around the Appalachian mountains. This large area may be the primary source of genetic diversity reflected in the effective population size estimates.

Individuals from Holden Arboretum (Willoughby, OH) were the only *P. virginiensis* samples that clustered separately from other *P. virginiensis* in the COI phylogeny. This cluster could have been caused by a founder's effect, genetic hitchhiking, or geographic isolation and genetic drift. Some areas in Ohio have already become unsuitable for occupation by *P. virginiensis* (Davis and Cipollini, 2014a), and the region is generally progressing towards unsuitability for *P. virginiensis*. We hypothesize that as time progresses, geographic isolation and resulting genetic drift will cause unmanaged populations to lose diversity and possibly become extinct in the midwestern part of the range.

Although *P. virginiensis* seems relatively secure in the present based on existing genetic data, there are several forces which may negatively influence this butterfly in the future. One major cause of renewed interest in this butterfly has been the introduction of an invasive biennial forest mustard, *Alliaria petiolata* (garlic mustard). Introduced in the 1800s, *A. petiolata* has spread throughout *P. virginiensis* habitat, and recent observations of *P. virginiensis* indicate that in affected habitats, *P. virginiensis* mistakenly place two-thirds of their eggs on the novel mustard *A. petiolata* where their larvae cannot survive (Bowden,



1971; Courant et al., 1994; Porter, 1994; Davis and Cipollini, 2014b). Larval survival on the native hosts, *Boechera laevigata* and *C. diphylla*, is already low (10-15%, Cappuccino and Kareiva, 1985; Shuey and Peacock, 1989). In areas with abundant *A. petiolata*, survival may be reduced to only 3-5%.

Pressures from *A. petiolata* are not yet reflected in the genetic sequence data for *P. virginiensis* presented here, most likely because it is a relatively recent (< 50 years) invader in only part of the range of *P. virginiensis*. Since *P. virginiensis* has a univoltine lifestyle, it will adapt more slowly to *A. petiolata* than multivoltine Pierids, like *P. oleracea* (Keeler and Chew, 2008). There is some variation in oviposition preference, and over time, *P. virginiensis* may be able to overcome the difficulties imposed by *A. petiolata* in invaded sites (Davis and Cipollini, 2014b).

As climate change progresses, *P. virginiensis* may push northward into areas historically occupied by *P. oleracea*, another native Pierid species already in contact with *A. petiolata* (Hovanitz, 1963; Keeler and Chew, 2008). When co-occurring, these two Pierids compete for resources (primarily *C. diphylla*) during the spring, but *P. oleracea* completes another generation in summer. *P. oleracea* has the distinct survival advantage of extra contact with *A. petiolata* to increase the rate of selection, variation in its preference for *A. petiolata* as well as its larval ability to tolerate *A. petiolata*, and the ability to use multiple hosts in the summer generations (Renwick et al., 2001; Keeler and Chew, 2008). All of these factors should enable *P. oleracea* to outcompete *P. virginiensis* in *A. petiolata* impacted habitats, and possibly inhibit northward migration. Impacts of other Pierid butterflies, like the European invasive *P. rapae*, need to be examined in more detail.

Many agencies and conservation groups are already managing *P. virginiensis* habitat for preservation of its native host plant *C. diphylla* as well as elimination of invading *A. petiolata* individuals (Bess, 2005; Finnell and Lehn, 2007). We recommend continued vigilance for *A. petiolata*, especially in areas becoming unsuitable in the near future. Although *P. virginiensis* will likely persist throughout the Appalachian mountains, some populations

at the periphery (IN, OH, etc.) may experience further isolation and population reduction. It is important for land managers to determine if *P. virginiensis* functions as a flagship species for other rare or endangered species like the American Burying Beetle, and assign a priority level to conservation efforts in managed areas.

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