MECHANISMS OF SUCCESS: PLANT-HERBIVORE INTERACTIONS AND THE INVASION OF NON-NATIVE LONICERA SPECIES IN NORTH AMERICA

A dissertation submitted in partial fulfillment of the Requirements for the degree of Doctor of Philosophy

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I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY Deah Lieurance ENTITLED Plant-Herbivore Interactions and the Invasion of Non-native Lonicera Species in North America BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Doctor of Philosophy

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ABSTRACT


Invasion by non-native species is a complicated process and many hypotheses have been proposed to explain how invasive plant species are often poor competitors in their native range, but dominant in their novel range including the enemy release and novel weapons hypotheses. Additionally, many invasive species are characterized as being tolerant and/or resistant to both damage and limitations in abiotic resources. These hypotheses are based on plant-plant, plant-microbial, and plant-herbivore interactions in the invaders novel range and are not mutually exclusive. The genus Lonicera (Caprifoliaceae) includes approximately 200 species worldwide, with 18 native and 16 introduced species in North America. Some Asiatic species like Lonicera maackii, L. tatarica, and L. japonica are particularly successful invaders in North America, while North American natives are relatively uncommon or not abundant where they are found. I investigated the plant-insect interactions and defensive strategies of non-native Lonicera species, with particular focus on L. maackii in Ohio. I first quantified the amounts of arthropod herbivore damage occurring on L. maackii across two seasons. I expanded this
assessment to include a co-occurring native congener *L. reticulata* and the confamiliar *Viburnum prunifolium*. Additionally, I included feeding bioassays to assess the performance of a specialist and generalist herbivore on native and non-native *Lonicera* species. Tolerance of mature shrubs was evaluated through measures of growth responses after repeated clipping. Greenhouse experiments with real and simulated herbivory were completed to determine the tolerance of juvenile *L. maackii* plants to herbivory and how this may be affected by changes in resource availability. Resistance traits were also evaluated in this experiment through measures of secondary metabolites with and without herbivory. Finally, resistance traits were further evaluated through a common garden experiment including multiple native and non-native *Lonicera* species, where herbivore damage, generalist herbivore performance, and both qualitative and quantitative analyses of defensive chemistry were evaluated in high and low nutrient treatments.

*Lonicera maackii* and other non-native *Lonicera* species receive insignificant amounts of arthropod herbivore damage in the field and the damage they receive is much less than amounts incurred on native *Lonicera* and confamiliar *V. prunifolium*. Mature *L. maackii* shrubs are highly tolerant to large amounts of simulated herbivore damage, juvenile *L. maackii* is both tolerant and resistant to high amounts of real and artificial damage, and limiting light and soil nutrients did not limit their ability to tolerate herbivory. A honeysuckle specialist avoids *L. maackii* in the field, but can develop on *L. maackii* in the laboratory. Plants in the *Lonicera* genus that display resistance to arthropod herbivores can be characterized as being chemically well defended, and are generally poor hosts to generalist herbivores. Although native/non-native origin did not explain the chemical profiles of species, native *Lonicera* tended to produce more iridoid
glycosides and non-native *Lonicera* produced more phenolic compounds. *Lonicera maackii* and other non-natives appear to not only escape damage from arthropod herbivores, they are also able to tolerate and resist the damage they do incur suggesting that a combination of mechanisms contribute to the success of these non-native *Lonicera* in North America.
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1. INTRODUCTION

1.1 INVASIVE SPECIES

As the economical and ecological consequences associated with the invasion of non-native species continue to mount, invasive species have become a global concern (Mack and D'Antonio 1998; Pimentel et al. 2005). It has been suggested that biotic invasions are second only to habitat destruction as the cause of depleted biodiversity worldwide (Vitousek et al. 1997). The presence of an established population of invasive species often results in a reduction of biodiversity across trophic levels as a result of competitive effects and changes in resource availability (Kourtev et al. 2002; Mack and D'Antonio 1998; Vitousek et al. 1996). Additionally, invaders can alter biogeochemical cycling, hydrology, and disturbance regimes and are estimated to cost the United States upwards of $120 billion in environmental and agricultural damage per year (Gordon 1998; Pimentel et al. 2005). As the ecological and economic costs of biological invasion continue to accelerate, we must refine our understanding of the mechanisms that make invaders successful in their new habitats to prevent new invasions and to combat those invasions already established in novel habitats.

When a species is introduced to a new habitat, they encounter a number of physiological, ecological, and evolutionary filters that prevent every species that reaches a new habitat from becoming naturalized and/or invasive (Mack et al. 2000). But many invasive species have limited distribution and are often rare in their native range and what
remains unclear is how these species are often poor competitors in their native range, but dominant in their novel range. Many hypotheses have been proposed, often overlap, and may apply to different stages of invasion to explain the establishment, persistence, and potential failure of invasive plants in their new habitats. These hypotheses include the novel weapons, enemy release, and evolution of increased competitive ability (Blossey and Notzold 1995; Keane and Crawley 2002; Callaway and Ridenour 2004; Catford et al. 2009). There is no universal theory that applies to all invasions and mechanisms for success are species dependent and multifaceted. With continued research, the attributes contributing to invasion success will become clearer and this knowledge will help prevent the release of new invasive species and help manage those already established.

1.2 ENEMY RELEASE HYPOTHESIS

The Enemy Release Hypothesis (ERH) posits that when exotic plant species are introduced to a novel habitat, they experience a release from regulation by natural enemies, especially specialist herbivores often resulting in rapid increase in distribution, abundance, and vigor (Colautti, et al., 2004; Keane and Crawley, 2002). Keane and Crawley (2002) summarized three main assumptions of the Enemy Release Hypothesis; 1) specialist enemies of the exotic invader will be absent in the novel region, 2) it is rare for specialist herbivores to switch hosts from native congeners, and 3) generalists will feed on both exotic and native species, but impact will be greater on native plants. Exotic plants may not be able to support growth and reproduction of specialist insect herbivores from the introduced range because of characteristics such as novel defenses or elevated amounts of common chemical defenses (Callaway and Ridenour 2004; Cappuccino and
Arnason 2006) or the lack of specific oviposition cues (Jahner et al. 2011). In contrast, generalist herbivores are better equipped to cope with a variety of defensive strategies and therefore may be able to include exotic plants in their diet, and in some cases, show a preference for some invasive species (e.g. Bernays and Minkenberg 1997; Morrison and Hay 2011; Parker and Hay 2005). Previous studies have suggested that enemy release has provided a competitive advantage to such invaders as Norway maple (Acer platanoides) in northeastern United States, butterfly bush (Buddleja davidii) in Europe, soapbush (Clidemia hirta) in Hawaii, and whitetop (Lepidium draba) in the western United States (DeWalt et al. 2004; Cripps et al. 2006; Morrison and Mauck 2007; Ebeling et al. 2008; Adams et al. 2009; Cincotta et al. 2009). Results supporting this hypothesis commonly report that plants in their introduced range exhibit increased plant vigor, are often taller and thicker, demonstrate increased reproduction, and show reduced herbivore damage (e.g. Colautti et al. 2004; Ebeling et al. 2008; Adams et al. 2009).

Direct comparisons of ecologically similar species, biogeographic genotypes, and congeners have resulted in some of the more compelling evidence regarding ERH. In side-by-side comparisons of the European invasive Acer platanoides and native congener A. saccharum, herbivore and pathogen damage was greater for the native trees in both autumn of 2005 (2.5% vs. 1.3%) and the summer of 2006 (1.7% vs. 0.4%) even though a survey showed that insect assemblages were nearly identical (Cincotta et al. 2009). Additionally, when herbivore damage on Acer platanoides was assessed in the native and invasive ranges, herbivore damage was 4 times higher in the native range of Europe than in North America (Adams et al. 2009). Clidema hirta, a perennial shrub native to Costa Rica that escaped cultivation in Hawaii and Australia was also observed in both the
native and introduced ranges. Results revealed that specialist herbivores including stem borers, gall formers, weevils and leaf rollers were absent in the introduced range and the plant only sustained 0.9% damage as compared to 4.4% in the native range. (DeWalt et al. 2004). *Buddleja davidii* plants in their invasive range of Germany had increased reproduction, 79% greater stem size, and had no sign of herbivory compared to plants in the native range of China that suffered an average 15% leaf area loss (Ebeling et al. 2008). Results such as these illustrate that lower herbivory on non-native species can result in moderate to substantial reductions in damage and may lead to competitive advantages in their introduced range.

1.3 RESISTANCE AND TOLERANCE TO HERBIVORY

A plant’s growth, survival, fecundity, and competitive success can be compromised by relatively small amounts of herbivory. There is no consensus for how much damage is required to affect the fitness of a plant as this varies by taxon, but research on various woody trees indicate between 6 and 12% leaf area loss reduced growth and reproductive output (Crawley 1985; Poorter et al. 2004; Whittaker and Warrington 1985). The way plants respond to foliar damage incurred from herbivores and pathogens can be classified as either resistance or tolerance to herbivory. Tolerance is exhibited by mechanisms that maintain the overall fitness of a plant following damage including increases in physiological performance (i.e. increased photosynthesis and improved plant water status), reallocation of resources to compensate for tissue removal, enhanced nutrient uptake resulting from increased root-to-shoot ratios, and maintenance of reproductive success following damage (McNaughton 1983; Strauss and Agrawal
Resistance is the ability of a plant to reduce the preference or performance of the herbivore often through mechanical defenses such as increased leaf toughness, physical barriers such as thorns or trichomes, or chemical defenses including the production of secondary defensive compounds (Choong 1996; Strauss and Agrawal 1999; Gadd et al. 2001; Ashton and Lerdau 2008). When considering that non-native species experience some herbivory in their invasive range, and in some cases, the non-native plant is preferred by native fauna (Morrison and Hay 2011), understanding the strategies employed to contend with herbivory can yield valuable information about the persistence and proliferation of that species in a given habitat.

Research addressing tolerance to herbivory and plant invasions typically involve comparisons of growth including changes in leaf area and plant mass, effects on relative growth rates, altered biomass allocation patterns, and differences in fecundity (Strauss and Agrawal 1999; Hawkes and Sullivan 2001). Ashton and Lerdau (2008) found that invasive vines were more tolerant to simulated herbivory and quickly replaced aboveground tissues allowing them to maintain root-to-shoot ratios similar to what they had before clipping, while native vines had higher root-to-shoot ratios after herbivory simulation, reflecting the loss of aboveground biomass. When Lonicera japonica and L. sempervirens were evaluated for growth responses to natural herbivory (both insect and mammal), invasive L. japonica compensated for damage by increasing allocation to leaves and producing higher overall biomass than native L. sempervirens (Schierenbeck et al. 1994). Research on Triadica sebifera (formerly Sapium sebiferum) indicates that invasive ecotypes exhibit decreased resistance and an increased tolerance to herbivory,
specifically from specialist herbivores (e.g. Rogers and Siemann 2004; Rogers and Siemann 2005; Zou et al. 2008). For these studies of *Triadica*, ERH and tolerance were simultaneously evaluated and it appears that greater tolerance to herbivory may contribute more to the persistence of these invasive trees than the lack of herbivory.

Plants produce a suite of secondary metabolites that serve a range of functions including resistance to microbes and herbivores (e.g. San Francisco and Cooper-Driver 1984; Cipollini et al. 2008). Because they have been linked (but not limited) to allelopathic interactions, two classes of compounds of particular interest are phenolics and iridoid glycosides. Phenolic compounds such as chlorogenic acid, and apigenin have been implicated in inhibition of seed germination, herbivore deterrence, and reduced herbivore performance (Felton et al. 1992; Chaves et al. 2001; Cipollini et al. 2008). Aqueous extracts containing various phenolic compounds from the leaves of *Citrus ladanifer* inhibited and delayed germination for some Mediterranean shrub species (Herranz et al. 2006). *Spodoptera littoralis* larvae preferred undamaged alfalfa plants over damaged ones that were characterized with increases in induced chemical defenses including saponins and apigenin (Agrell et al. 2003). Irioid glycosides such as loganin, aucubin and catapol are phenolic-based compounds with an attached glucose molecule that can act as a feeding deterrent, inhibit larval development, and can be toxic (Puttick and Bowers 1988). When generalist lepidopteran *Spodoptera eridania* larvae were reared on artificial diets containing the iridoid glycosides aucubin, loganin, or catapol, survivorship was reduced between 24 and 40%, growth rates were reduced by half or more, and when given a choice, larvae consistently avoided diet containing the iridoid glycosides (Puttick and Bowers 1988).
Resistance traits of non-native species, specifically those targeting generalist herbivores, may act independently or in combination with tolerance mechanisms to help invasive species cope with herbivore damage, however great or small in their invasive ranges. Differences in abiotic factors such as light and nutrient availability can alter the tolerance and/or resistance of a plant to herbivore damage. Reducing light availability to plants limits carbon through reduction in photosynthesis, and may influence its ability to recover from herbivory. In a study evaluating 10 species in the Dipterocarpaceae family, removal of 35% of the leaf area from these species reduced relative growth rate by 22% for seedlings grown in deep shade, but only by 9% for those grown in high light (Paine et al. 2012). Additionally, woody plants growing in high nutrient conditions favor growth and place higher allocation to above ground components at the expense of below-ground carbon stores (Hawkes and Sullivan, 2001). This may result in reduced tolerance in high nutrient treatments as belowground storage is often tapped to compensate for losses from aboveground damage. Alternatively, high nutrient availability may provide plants with adequate resources to rapidly compensate for leaf loss to herbivory (Maschinski and Whitham 1989).

Production of secondary metabolites can vary with resource availability and may depend on the class of compound produced, with carbon-rich compounds such as phenolics and tannins often responding to light limitation and nitrogen-rich compounds such as pyrrolizidine alkaloids, cyanogenic glycosides and glucosinolates responding to fertilization (Bryant et al. 1983; Koricheva et al. 1998; de Boer 1999; Burns et al. 2002; Herms 2002; Lambdon et al. 2003). Phenolics typically increase with light availability and not only offer defense against herbivores, but some also protect against photodamage.
(Dudt and Shure, 1994; Close and McArthur, 2002). It has been suggested that a trade-off between growth and resistance may determine how a plant responds to herbivory and these responses may vary with resource availability (Herms and Mattson, 1992). However, it is possible that plants could respond to herbivore damage with a mixed response in both tolerance and resistance to herbivory (Koricheva et al. 2004; Núñez-Farfán et al. 2007). Perhaps a trait of highly successful invasive plants is being a “jack of all trades” in the face of herbivore pressure (Koricheva et al. 2004).

1.4 LONICERA SPECIES

The genus *Lonicera* (Caprifoliaceae) includes approximately 200 species worldwide, with 18 native and 16 introduced species in North America (Kartesz and Meacham, 1999; Zheng et al. 2006). Several exotic *Lonicera* species have become established and often dominate the landscape in their invaded range (e.g. *L. japonica*, *L. tatarica*, and *L. maackii*) (Woods 1993; Luken and Thieret 1996; Schierenbeck 2004). In contrast, native *Lonicera* species, including *L. reticulata*, *L. dioica*, and *L. flava*, are relatively uncommon across their range and when found, are not abundant. In some states they are listed as rare, threatened, or endangered (e.g. Hill 2003a; 2003b; http://plants.usda.gov). Invasive traits possessed by non-native *Lonicera* include an apparent release from natural enemies (Schierenbeck et al. 1994; Waipara et al. 2007; Lieurance and Cipollini 2012; 2013), evidence of allelopathic suppression of other plants (Skulman et al. 2004; Dorning and Cipollini 2006; Cipollini et al. 2008; McEwan et al. 2010), high aboveground growth rates (Luken et al. 1997; Lieurance 2004), extended leaf phenology (Schierenbeck and Marshall 1993; Trisel 1997; McEwan et al. 2009),
abundant fruits that are dispersed by birds, deer, and mice (Vellend 2002; Drummond 2005; Bartuszevige and Gorchov 2006; McCay et al. 2009), and the production of secondary metabolites associated with anti-herbivore defense (Cipollini et al. 2008). The majority of research on these species has been conducted in their invaded ranges, and little information about the ecology of these species in their native habitat exists. Secondary metabolites associated with herbivore resistance have been identified in *Lonicera* species, including the identification of two major flavones- apigenin and luteolin and their glycoside derivatives, chlorogenic acid, and other phenolics (Cipollini et al. 2008; Ochmian et al. 2009; Ren et al. 2008), as have several iridoid glycosides including secologanic acids, which have been associated with deterrence of generalist herbivores and feeding stimulation or oviposition cues for specialist herbivores (Song et al. 2006; Peñuelas et al. 2006).

### 1.5 GOALS OF RESEARCH

The goal of this research was to determine the extent to which the successful invasion and persistence of *Lonicera maackii* (Rupr.) Maxim (Caprifoliaceae) and other non-native *Lonicera* species in the Midwest can be attributed to escape from regulation by arthropod herbivores, tolerance and/or resistance to herbivory, and/or composition of defensive chemistry profiles. First, I quantified the amount of damage to *L. maackii* that can be attributed to arthropod herbivores in the field. I also tested predictions of the Enemy Release Hypothesis by quantifying differences in herbivore damage between native *Lonicera reticulata*, confamilial *Viburnum prunifolium*, and non-native *L. maackii* in the field. I used simulated herbivory experiments to test whether *L. maackii* plants
respond to large amounts of defoliation through tolerance and/or resistance mechanisms by measuring differences in growth and quantifying the production of secondary metabolites as a response to herbivore damage. To further test if non-native *Lonicera* are more resistant than natives to arthropod herbivore damage, the performance of a generalist and specialist arthropod herbivore on native and non-native *Lonicera* species was assessed through no-choice feeding bioassays. Finally, the phenolic and iridoid glycoside profiles of 3 native and 5 non-native *Lonicera* species was analyzed quantitatively and qualitatively to investigate the possibility that non-native *Lonicera* possess different chemical profiles than their native congeners. This arm of research was to clarify understanding of mechanisms of competitive success of invasive *Lonicera* species in particular, and non-native, invasive species in general. This knowledge can be applied to the management of current invasions (biological control) and in the prevention of future invasions (identify traits to target in risk assessment).
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2. DAMAGE LEVELS FROM ARTHROPOD HERBIVORES ON LONICERA MAACKII SUGGEST ENEMY RELEASE IN ITS INTRODUCED RANGE

2.1 INTRODUCTION

There are many studies that have tried to determine what characteristics (i.e. efficient resource utilization, high relative growth rates, and phenotypic plasticity) make an invasive species successful (eg. Bazzaz et al. 1986; Feng et al. 2007; Osunkoya et al. 2010a; Osunkoya et al. 2010b). One prominent hypothesis that attempts to explains the success of invasive plants in their new habitats is the ‘enemy release hypothesis’ (ERH) which suggests that when a plant invader is introduced to a new habitat it will experience a reduction in regulation by specialist herbivores or pathogens that are present in the native range, resulting in increased distribution and abundance (Keane and Crawley 2002). Research has suggested that enemy release has provided a competitive advantage to such invaders as Norway maple (Acer platanoides) in northeastern United States, butterfly bush (Buddleja davidii) in Europe, soapbush (Clidemia hirta) in Hawaii, and whitetop (Lepidium draba) in western United States (Adams et al. 2008; Cincotta et al. 2009; Cripps et al. 2006; DeWalt et al. 2004; Ebeling et al. 2008; Morrison and Mauck 2007). Studies supporting this hypothesis commonly report increased growth and reproduction of plants in the invasive range compared to the native range (eg. Colautti et al. 2004; Crawley, 1983; Ebeling et al. 2008).
**Lonicera maackii** (Rupr.) Maxim (Amur honeysuckle) is one of the most important and prominent invasive plant species in the Midwestern United States.

*Lonicera maackii*, a deciduous woody shrub native to China, Japan, Korea, and southeastern Russia, was originally introduced in the late 1880s for habitat improvement, erosion control, and horticultural landscaping (Luken and Thieret 1996). Traits that *L. maackii* shares with many other invasive plants include extended leaf phenology, rapid aboveground growth rates, high fecundity, broad phenotypic plasticity, and tolerance to a variety of habitats (Lieurance 2004; Luken et al. 1995a; Luken et al. 1995b; Luken et al. 1997a; Luken et al. 1997b; Trisel 1997; McEwan et al. 2009b). Additionally, *L. maackii* responds to increases in light availability with a plastic response in branch architecture, producing two distinct branch types, ‘long’ and ‘short’ (Luken et al. 1995a; Luken et al. 1995b). Long branches extend the shrub height in response to increased light and are presumably cheap in construction, poorly defended, and expendable, while short branches have leaves that are thicker, tougher, and more resistant to herbivory (Ballare 2009; Guerra et al. 2010; Ishii and Shoko 2010).

Current land use patterns have resulted in disturbed habitats, fragmentation of forests in rural areas, and creation of suburban woodlots, all of which provide appropriate habitat for *L. maackii* to spread across the landscape. In Ohio and Kentucky, *L. maackii* shrubs comprise up to 50% of the understory species composition in some small woodlots, and near monocultures along the edges of old fields and roadsides (Hartman and McCarthy 2008; Medley 1997; Pennington et al. 2010; Watling and Orrock 2010). As anthropogenic habitat fragmentation increases, the creation of edge habitat insures the persistence and spread of *L. maackii* in the landscape. Abiotic conditions of edge habitats
are often very different than those of the understory with significant microenvironmental
differences in temperature, light, and moisture (Matlack, 1992). The adaptive plasticity of
*L. maackii* allows exploit the increased light availability of edge habitat (Hutchinson and
Vancat 1997; Luken et al. 1995b). However, edge habitats can exhibit increased
arthropod abundance and diversity relative to understory habitats, and presumably
increased herbivore incidence and damage potential (Barbosa et al. 2005; Ozanne et al.
2000).

The extent to which the ERH may explain the success of *L. maackii* in North
America has not been determined, and a first step requires estimates of herbivory levels
on this species in its invasive range. Anecdotal observations indicate that *L. maackii*
escapes meaningful levels of herbivore and pathogen attack in its invasive range (D.
Lieurance and D. Cipollini, personal observations). Trisel (1997) reported damage
amounts from a combination of frost, deer browse, insect herbivory, and drought on *L.
maackii* at Miami University in Oxford, Ohio from 1992-1994 that were well below
various native woody species. Damage levels have never been quantified on this species
in its native range. However, observations of *L. maackii* in its native versus introduced
range indicate that the shrub grows more vigorously and abundantly in its introduced
range (Luken and Thieret 1996), while being much rarer in its native range in China (J.
Ding, personal communication), and endangered in Japan (http://www.biodic.go.jp
/english/rdb/red _plants.csv). In an effort to characterize the natural enemies of *Lonicera*
spp. in their native range and to identify possible biocontrol candidates, 44 insect species
and 21 fungal species were reported on the genus in China including 4 arthropod species
specifically attacking *L. maackii* (Zheng et al., 2006). In contrast, observations of *L.*
maackii growing alongside the native Lonicera reticulata in Ohio revealed that native vines suffered substantial early season damage from the specialist honeysuckle sawfly (Zarea inflata) while exotic shrubs remained undamaged by this insect (D. Lieurance, personal observation).

Enemy escape can result from a combination of this apparent lack of specialist herbivores and resistance to generalists that may be present in the invasive range. Resistance strategies employed by plants include the production of secondary metabolites used to defend against herbivores. Previous research has identified several phenolic metabolites in leaves of L. maackii, including chlorogenic acid, several flavones and their glycoside derivatives, and iridoids (Cipollini et al. 2008a, D. Bowers, personal communication). These compounds may act as deterrents to generalist herbivory and be responsible for some of the allelopathic effects attributed to L. maackii (Cipollini et al. 2008a; Cipollini et al. 2008b; Dorning and Cipollini 2006; Cipollini and Dorning 2008; McEwan et al. 2009a; Trisel 1997). It has been postulated that secondary metabolites effective in herbivore defense are a result of coevolution with herbivores present in their native habitat (Becerra 2007; Erlich and Raven, 1964) and for non-native plants the separation from coevolved herbivores is integral to ERH (Keane and Crawley 2002). However these metabolites may be novel and/or effective against generalist herbivores that may be present in the introduced range.

Sometimes a non-native invader can experience some herbivory from either generalists or from specialists that may attack closely related native plants (Mitchell et al. 2006). However, enemy release can still occur if levels of damage are insufficient to reduce the performance of the plant. Moreover, damage rates experienced by any plant
can be influenced by differences in herbivore diversity and exposure between edge habitat and interior forest (Barbosa et al. 2005; Ozanne et al. 2000), as well as intra-plant variation in host quality (Cornelisson et al. 1997; Cornelisson et al. 2008; Price 1991). Additionally, the timing of herbivory can be influenced by the phenology of the plant. This is true for *L. maackii* as it leafs out before the majority of native competitors making it an available food source for early season generalist herbivores (McEwan et al. 2009b; Trisel 1997).

We conducted a study to quantify the incidence, amount, and type of herbivory occurring on *L. maackii* across several populations over a two-year period in Ohio. We also investigated the role of habitat location, timing of removal, and branch preferences by herbivores. We predicted that 1) overall, damage by arthropod herbivores on *L. maackii* would be minimal; 2) despite the geographic spread of populations sampled, there would be no difference in the amount of damage across sites; 3) due to differences in arthropod diversity and exposure between edge and interior habitats, edge shrubs would have more leaf area removed by arthropod herbivores as well as a higher incidence of herbivory than interior shrubs in the forest; 4) because of possible palatability differences between the two branch morphologies, leaves on long branches would suffer more damage than those growing on short branches; and 5) because the early season phenology of *L. maackii* provides an abundant food source for early spring herbivores, the majority of damage would occur in the spring and then increase through time. Results of this study provide information on the autecology of *L. maackii* in its introduced range and indicate whether enemy release may contribute to the success of this woody invasive.

### 2.2 MATERIALS AND METHODS
2.2.1 Sample collection 2008

Leaf samples of $L. \text{maackii}$ were collected in October, 2008 from eight sites in Ohio (Table 1). Sites were selected throughout the geographic region to cover 7 counties in southwestern and central Ohio. The surrounding area around the sites were either a rural-suburban interface (Shawnee Prairie Preserve, Germantown Metropark, Sharon Woods, Taylorsville Metropark), urban green space (Franklin Park Conservatory, Wright State Woods), or less disturbed natural areas (Hueston Woods State Park, Glen Helen Nature Preserve). Mature shrubs were sampled in both forest edge and interior habitats at each site. Shrubs were considered to be in the interior if they were at least 15 meters from the forest edge and there was no clear evidence of a gap in the canopy. Edge shrubs were located at the interface between forest and open field. Twenty mature shrubs were selected from each habitat. Ten branches with several leaves attached were each taken from the exterior (sun leaves) and interior canopy (shade leaves) of each plant in the edge habitat. Ten branches total were sampled from each plant in the interior habitats. Two leaves from each branch were randomly selected for assessment for a total of 40 leaves from each edge shrub and 20 leaves from each interior shrub. Preliminary analysis of herbivore damage indicated no significant differences in damage amounts between sun and shade leaves of edge plants ($F_{1,3163}=0.59$, $p=0.4406$) and for this reason all edge leaves were pooled for statistical analysis.

A visual assessment of damage was conducted for all leaves. Damage was scored on a percentage leaf area basis as 1, 2, 5, and then in increments of 5% to a maximum 100% for leaf area lost or infected. Based on previous studies of leaf herbivory, categories for leaf damage were established as follows: 1) chewed or skeletonized, 2)
scraped, 3) mined, 4) infected with pathogen (i.e. fungal infection), and 5) mixed (Adams et al. 2008). The percent of total leaves experiencing some amount of damage (# leaves damaged/# total leaves) was also calculated for both years.

2.2.2 Sample collection 2009

In 2009, damage levels were sampled in June, August and September, at 3 sites studied in 2008 (Wright State University Woods, Taylorsville MetroPark, Sharon Woods) to determine the timing of the accumulation of herbivore damage. Because of the difference in morphology and potential for differential damage levels, a distinction was also made between long and short shoots. Long shoots had larger, greener leaves and elongated branches greater than 15cm long (>12 leaves/branch). Leaves on short shoots were smaller, darker in color and the branches ranged between 5 and 15cm (≤12 leaves/branch). Fifteen mature shrubs were selected in both the edge and interior habitats and 5 branches were tagged (3 short and 2 long) on each shrub. Herbivory was assessed on a percentage basis using the same rating scale as 2008.

2.2.3 Data analysis

Nested analysis of variance (ANOVA) was used to test for differences in percent leaf area removal (herbivore damage) and incidence of herbivory among sites and among habitat location types nested within sites for 2008. The effect of site was tested over the location nested within site effect. End of season data from 2009 (October only) for herbivore damage and incidence was analyzed using nested ANOVA, with site, location nested within site, and branch type nested within location as factors in the model. The
effect of site was tested over the location nested with site effect, and the effect of location nested within site was tested over the branch nested within location effect. In both models, site was treated as a random effect. Percent herbivore damage data were transformed using inverse square transformation after adding one to all values \([x+1]^2\) and percent incidence data were arcsine transformed to fulfill assumptions of normality. To determine differences in the type of damage accumulated by \(L.\ maackii\), in 2008, a chi-squared test of independence was performed on data pooled across sites and locations. Repeated measures ANOVA was used to analyze changes in herbivore damage through time for 2009, with time as the within subjects effect, and site, location nested within site, and branch nested with location as between subjects effects. Comparisons of means were made using Tukey post-hoc tests. All statistical analyses were performed using SAS software (Version 9.2, SAS Institute, Cary, North Carolina).

### 2.3 RESULTS

Results of our 2008 survey indicated that the mean amount of damage was 1.83\% ±0.09 across sites and habitat locations. Nested ANOVA revealed no significant differences in herbivory among sites, but significant differences between habitat locations (edge vs. interior) within sites (Table 2.2). Similar results were found for percent incidence in 2008 (Table 2.2). With the exception of the Sharon Woods (SW) site, percent damage and incidence were 64\% and 63\% higher, respectively, in the forest edge habitat than in the forest interior habitat (Figure 1a and 1b). Analysis of data collected in 2009 revealed a mean amount of damage of 3.09\% ±0.16, across all samples but no differences among sites or habitat locations for damage and incidence, but there were
significant differences among branch types within habitat locations (Table 2.2). Percent damage and incidence were 66% and 76% higher, respectively, on long branches than on short branches (Figure 1c and 1d).

Results of the chi-squared test for independence illustrated significant differences in the type of damage that plants received ($P<0.0001$, $\chi^2=2808.55$). Across the 8 sites in 2008, chewing was the most prevalent form of damage with a mean of 76.8% ±11.7 of damaged leaves receiving this type of damage, while scraping (7.06%±3.17), mining (0.13%±0.09), rolling (0.13%±0.09), and mixed herbivory (1.69%±0.42) contributed an insignificant amount of damage (Figure 2.2). A low level of pathogen infection was observed on *L. maackii* sampled at all sites with a mean of only 4.81 ±7.04% of damaged leaves showing symptoms of infection. The main symptom of possible infection was leaf necrosis with the majority of these leaves were located in the edge habitat at Hueston Woods State Park (HW), Oxford, Ohio, and Sharon Woods (SW) in Cincinnati, Ohio.

To address the question of the temporal dynamics of herbivory in 2009, results of the repeated measures ANOVA revealed that herbivore damage varied over time, and that changes through time varied between habitat locations within sites (Table 2.3, Figure 2.3). Herbivore damage occurred early in the season for both locations, increased over time in the edge location, but there was little change through time on shrubs located in the interior habitat. Results of the between subjects effect showed that site and location nested within site had no significant effect on herbivore damage while there was a significant effect of branch type nested within habitat location and site (Table 3). Herbivore damage was higher for long branches for both edge and interior shrubs (Figure 2.3).
2.4 DISCUSSION

Results of two years of observation at multiple sites in Ohio indicate the amount of leaf area removed by arthropod herbivores (and pathogens) from the leaves of *L. maackii* is less than 3% on average, an amount that is likely too low to affect the fitness of these shrubs. Because results were largely consistent across multiple sites, and site was considered a random factor, we can assume that these results are typical for *L. maackii* across the region. Assuming that damage rates are higher in the native range in the presence of specialist herbivores and other generalists capable of feeding on *L. maackii* (J. Ding, personal communication; Zheng et al., 2006), these results indicate that *L. maackii* experiences a release from arthropod herbivory in its invasive range, a necessary assumption of the ERH. Additionally, observations of *L. maackii* co-occurring with the native *L. reticulata* at Kiser Lake State Park in Champagne County, Ohio reveal that the native plants receive extensive early season damage from the specialist *Zaraea inflata* (Honeysuckle sawfly) while *L. maackii* is left untouched (D. Lieurance, personal observation.). We did not examine damage resulting from white-tailed deer, a generalist herbivore, but Trisel (1997) reported lower values of deer browse on *L. maackii* than on native tree species (1.7% vs. 10.6-21.0%), an observation also consistent with the assumptions of ERH. The prevalence of leaf chewing indicates that the small amount of damage that does occur on *L. maackii* can be attributed to some unidentified generalist herbivores, possibly including lepidopteran larvae feeding minimally on early season foliage (J. Stireman, personal communication.). The low values of percent leaf area consumed but with a moderate damage incidence could be due to induced changes in
palatability of the leaves after small amounts of tissue removal forcing herbivores to move to other leaves or other plants, where they continue to remove only small amounts of tissue.

Resistance is the ability of a plant to reduce the preference or health of the herbivore often through mechanical defenses, such leaf toughness, thorns, or trichomes, or chemical defenses including the production of secondary defensive compounds (Ashton and Lerdau 2008; Choong 1996; Strauss and Agrawal 1999). Much research has been conducted on secondary metabolites present in plants from the Lonicera genus, mostly in the context of their pharmaceutical benefits in herbal medicine (Chen et al. 2009; Heinrich et al. 2008; Machida et al. 2002). Many of the identified secondary chemicals found in the Lonicera genus are known to be present in North America and therefore not novel, but a complete profile of leaf chemistry has not been done for Lonicera spp., leaving the question of whether invasive species in this genus possess chemicals novel to the native flora and fauna (Cipollini et al. 2008b). A number of flavonoids and other phenolics have been identified within the genus (Cipollini et al. 2008b; Flamini et al. 1997; Ochmian et al. 2009; Ren et al. 2008). In the context of herbivory, iridoid glycosides have been identified in L. implexa, which deter feeding and decrease growth rates of generalist herbivores (Peñuelas et al. 2006). This same class of compounds has been identified in L. maackii (D. Bowers, personal communication). Two major flavones, apigenin and luteolin, and their glycoside derivatives, as well as chlorogenic acid, were identified in the leaves of L. maackii, and diet plugs dosed with ecologically relevant concentrations of crude leaf extracts from L. maackii, or apigenin itself deterred feeding of a generalist herbivore (Cipollini et al. 2008b). Additionally,
feeding trials indicated that L. maackii was resistant to the generalist gypsy moth (Lymantria dispar) with caterpillars consuming small amounts of L. maackii foliage in choice tests, and either losing weight or dying on foliage in no-choice tests (McEwan et al. 2009a). Secondary metabolite concentrations vary through time and are often produced in higher concentrations early in the season (Scogings et al. 2004). Bud break for L. maackii typically occurs in March (Trisel, 1997) and potentially provides an abundant food source for early season herbivores, but herbivores that do sample this foliage likely suffer deleterious effects on fitness from the higher concentrations of some defensive compounds (Cipollini et al. 2008b; McEwan et al. 2009a).

Results from 2008 supported the prediction that edge shrubs would have a higher incidence and amount of herbivory than interior shrubs and, although comparisons of edge vs. interior plants in 2009 were not statistically significant, the same trend is apparent. Typically, edge habitats are a more dynamic system with higher light levels and temperatures, and higher biodiversity where plants experience increased exposure to potential herbivores, which may help explain increased herbivory in this habitat location (Matlack 1992; Ries et al. 2004). While shrubs experienced higher herbivory in edge habitats, the amount was still very low, and the increased amount of light available to the plant on the edge might facilitate compensatory growth or other tolerance mechanisms, which would counteract any effect the herbivores might have on the health of this shrub. In a study comparing biomass accumulation and allocation patterns of the invasive congener L. japonica with the native L. sempervirens, the exotic exhibited greater compensatory growth in response to natural herbivory (Schierenbeck et al. 1994). These Lonicera species share similar maximum photosynthesis rates in both open and interior
habitats and it is likely that L. maackii responds to herbivory in a similar manner (Lieurance 2004; Schierenbeck and Marshall 1993).

Our results also supported the prediction that leaves growing on long branches should experience more damage than leaves on short branches. The differences in damage and incidence between long and short shoots may be attributable to characteristically more tender and likely more nutritious leaves on long shoots. Leaves on short branches are thicker and tougher indicating leaves are more heavily defended through either mechanical or chemical means (Ballare 2009; Guerra et al. 2010; Herms and Mattson 1992; Ishii and Shoko 2010). Future research will include a comparative analysis of leaf nutrition, secondary metabolite production, and leaf toughness between branch types.

The 2008 data collection was an end of season cumulative assessment of leaf herbivory by arthropod consumers. This year was a dry year in terms of summer precipitation and there was also a major windstorm associated with Hurricane Ike on September 14 that affected all or part of the sample sites included in this study. Damage from the windstorm was consistent at all 8 field sites and included mechanical damage to the leaves, but very little leaf loss. While the end of season assessment did not quantify early leaf abscission due to abiotic or biotic stress, little abscission was observed and we do not believe that abiotic conditions dramatically influenced our results. In addition, herbivory levels in 2009, a season with more favorable abiotic conditions, were strongly comparable to those in 2008, suggesting that results in both years are robust. Results of the season long assessment in 2009 was also consistent with the prediction that L.
*maackii* receives the majority of damage early in the season (June and earlier) with moderate increases thereafter (McEwan et al. 2009b; Trisel 1997).

Overall, our results are consistent with those found in a comparable study of the congener *L. japonica* in its introduced range in New Zealand, where approximately 25% of all plants showed signs of herbivory, the amount of foliage consumed was less than 5%, and the damage was not regarded by the authors as biologically significant (Waipara et al. 2007). There is no consensus for how much damage is required to impact the fitness of a plant as this would vary by species, but research on various woody trees indicate between 6-12% leaf area loss reduced growth and reproductive output (Crawley, 1985; Poorter et al. 2004; Whittaker and Warrington, 1985). While we did not measure the cost of herbivory on the fitness of *L. maackii* in the current study, mechanical removal of 5%, 25% or 50% of the leaf area had no significant effect on the growth of selected branches of mature shrubs in the field. Simulated or arthropod removal of 50% leaf area was capable of impacting growth of first year plants in the greenhouse (D. Lieurance, D. Cipollini, unpublished data). Our herbivory assessment revealed levels of folivory (<3%) that are much lower than what appears to be needed to the affect fitness of *L. maackii* and should indicate that arthropod herbivores are currently having no significant affect on the performance of the plant. In addition, the lack of endophagous herbivore pressure in the form of leaf mining and various forms of galling also indicates enemy release. This type of specialized feeding requires herbivores to adapt specifically to their host plant and it is rare to find generalist endophagous herbivores (Frenzel and Brandl 2003).

### 2.5 CONCLUSIONS
The paucity of herbivores, including specialist and generalist leaf chewing insects and endophagous consumers, coupled with evidence of both resistance and tolerance strategies within this genus (e.g., Cipollini et al. 2008b; McEwan et al. 2009a; Schierenbeck and Marshall 1988; Schierenbeck et al. 1994), suggest that enemy escape may be occurring for *L. maackii* in its introduced range. “Invasive” attributes including extended leaf phenology, rapid growth rates, high fecundity, broad phenotypic plasticity, and tolerance to a variety of habitats (Lieurance 2004; Luken et al. 1995a; Luken et al. 1995b; Luken et al. 1997; Luken, Kuddes, Tholemeier 1997; Trisel 1997), in combination with the lack of regulation by herbivores, may contribute to the rapid spread and persistence of *L. maackii* throughout its introduced range. *Lonicera maackii* will likely not experience significant amounts of herbivory anytime soon without the introduction of a specialist herbivore or pathogen, as is the case with classical biological control programs. Biological control has proven to be effective approach with such invaders as *Melaleuca quinquenervia, Eichhornia crassipes*, and *Lythrum salacaria* (Center and Dray 1992; Franks et al. 2006; Grevstad 2006). While having the potential to be an effective and sustainable control to *L. maackii* invasion, the presence of several *Lonicera* species in the horticultural trade, and the presence of numerous native *Lonicera* species will provide obvious challenges to finding an appropriate biocontrol agent. The research presented in this paper provides the framework for further research we are conducting on mechanisms for why herbivore damage is low (e.g., physical and chemical defenses, oviposition signals, etc.), and how much damage is required to impact the fitness of *L. maackii*. We are also conducting comparative studies of growth responses, arthropod assemblages, herbivory levels and resistance mechanisms of *L. maackii* in relation to a
suite of native and exotic *Lonicera* species. Such information in conjunction with results from this study would further clarify the role of ERH in the invasion biology of *L. maackii*. 
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Table 2.1 List of sites sampled in central and South-western Ohio for 2008 study. SW, TV, and WS sites were sampled again in 2009.

<table>
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<tr>
<th>Site</th>
<th>Symbol</th>
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<th>GPS Coordinates</th>
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<tr>
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<td>DK</td>
<td>Darke</td>
<td>N 40.09863 W 84.64526</td>
</tr>
<tr>
<td>Germantown MetroPark</td>
<td>GR</td>
<td>Montgomery</td>
<td>N 39.64136 W 84.40111</td>
</tr>
<tr>
<td>Hueston Woods State Park</td>
<td>HW</td>
<td>Butler</td>
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Table 2.2 Nested ANOVA analyses evaluating the effect of habitat location (edge vs. forest interior) in 2008 and habitat location and branch type (long vs. short branches) in 2009 on percent herbivory (% leaf area lost) and incidence (# leaves damaged/total leaves observed) of herbivory on *Lonicera maackii* in central and South-western Ohio.

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<th>DF</th>
<th>F Value</th>
<th>P Value</th>
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<td><strong>Percent Damage 2009</strong></td>
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Table 2.3 Repeated measures ANOVA evaluating the effect of habitat location (edge vs. forest interior) and branch type (long vs. short branches) on percent herbivory (% leaf area lost) on *Lonicera maackii* through the 2009 growing season. Results of within subjects effects presented from Wilks’ Lambda test for multivariate analysis.

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Figure 2.1 Herbivory and incidence of herbivory on *Lonicera maackii* growing in edge and interior forest habitats at eight sites in 2008 (a & b), and at three sites in 2009 (c & d) in central and southwestern Ohio. Herbivory was estimated as a percentage of leaf area removed. Incidence was calculated as percentage of leaves damaged. E and I designate edge and forest interior habitats respectively (b). Means ± standard error (SE) are shown. E and I represent edge and interior habitat types.
Figure 2.2 Percentage of herbivore damage type observed on *Lonicera maackii* in 2008. Percentages were calculated by the number of leaves of a damage type/total number of damaged leaves. Means ± standard error (SE) are shown and letters indicate significant difference in Tukey post hoc tests ($P<0.0001$).
Figure 2.3 Herbivory on two distinct morphological branch types of *Lonicera maackii* through time for the 2009 growing season. Herbivory was estimated on a percentage of leaf area removed. Means ± standard error are shown. Mean percent damage presented on pooled data (Site and location).
3. EXOTIC LONICERA SPECIES BOTH ESCAPE AND RESIST SPECIALIST AND GENERALIST HERBIVORES IN THE INTRODUCED RANGE IN NORTH AMERICA

3.1 INTRODUCTION

As the ecological and economic costs of biological invasion accelerate, we must refine our understanding of the mechanisms that make invaders successful in their new habitats in order to prevent new invasions and to combat those that are already established in their novel habitats. Accordingly, numerous hypotheses have been raised to explain the invasive success of introduced species (Catford et al. 2009). One of the leading hypotheses to explain species invasions is the ‘Enemy Release Hypothesis’. This hypothesis posits that when introduced to a new range, non-native species may benefit from a release from top-down control by co-evolved herbivores and pathogens, which results in increased growth and fecundity in the introduced range (Keane and Crawley 2002; Joshi and Vrieling 2005; Liu and Stiling 2006; Mitchell et al. 2006). Observations of plants in their native versus novel ranges often detail accounts of larger plants in the novel habitat and evidence of reduced herbivore and pathogen loads (DeWalt, et al., 2004; Cripps, et al., 2006; Zou et al. 2007; Ebeling, et al., 2008; Adams et al., 2009; Hartley et al. 2010).
Keane and Crawley (2002) summarized three main assumptions of the Enemy Release Hypothesis; 1) specialist enemies of the exotic invader will be absent in the novel region, 2) it is rare for specialist herbivores to switch hosts from native congeners, and 3) generalists will feed on both exotic and native species, but impact will be greater on native plants. Exotic plants may not be able to support growth and reproduction of specialist insect herbivores from the introduced range because of characteristics such as novel defenses or elevated amounts of common chemical defenses (Callaway and Ridenour 2004; Cappuccino and Arnason 2006), or the lack of specific oviposition cues (Jahner et al. 2011). In contrast, generalist herbivores are better equipped to cope with a variety of defensive strategies and therefore may be able to include exotic plants in their diet, and in some cases, show a preference for some invasive species (e.g. Bernays and Minkenberg 1997; Parker and Hay 2005; Morrison and Hay 2011). However, when faced with the choice between native and the exotic plants, generalist herbivores may choose native plants over exotics because the exotics are better defended against attack (Schaffner et al. 2011), or because they do not recognize the new food source as suitable for consumption (Lankau et al. 2004). For example, in field enclosures and laboratory feeding trials, generalist grasshoppers fed heavily on non-native Triadica sebifera (formerly Sapium sebiferum), but avoided this plant in the field (Lankau et al. 2004). Therefore, a successful plant invasion could be the result of the absence of co-evolved specialist herbivores, the lack of recognition by potential herbivores, and/or resistance to specialist and generalist herbivores in the introduced habitat.

The genus *Lonicera* (Caprifoliaceae) includes approximately 200 species worldwide, with 18 native and 16 introduced species in North America (Kartesz and
Meacham, 1999; Zheng et al., 2006). Several introduced *Lonicera* species have become established and often dominate the landscape (e.g. *Lonicera japonica, Lonicera tatarica,* and *Lonicera maackii*) (Woods 1993; Luken and Thieret 1996; Hutchinson and Vancat 1998; Schierenbeck 2004). In contrast, native *Lonicera* species, including *Lonicera reticulata, Lonicera dioica,* and *Lonicera flava*, are relatively uncommon across their range or not abundant where they are found, and are listed as rare, threatened, or endangered in parts of their range (e.g. Hill, 2003a; Hill, 2003b; http://plants.usda.gov).

*Lonicera maackii*, a shrub, and *L. japonica*, a vine, are two of the most prominent invasive plant species in the eastern United States. Invasive traits possessed by *L. maackii* include evidence of the allelopathic suppression of other plants (Dorning and Cipollini 2006; Cipollini et al. 2008a; McEwan et al. 2010), high growth rates and long leafing seasons (Trisel 1997; McEwan et al. 2009), abundant red fruits that are dispersed by birds (Ingold and Craycraft 1983; Lieurance 2004), and the production of secondary metabolites associated with anti-herbivore defense (Cipollini et al. 2008b). Similar traits have been detected in *L. japonica* (Schierenbeck et al. 1994; Skullman et al. 2004; Ashton and Lerdau 2008; Shang et al. 2011). The majority of research on these species has been conducted in their invaded ranges, and little information about the ecology of these species in their native habitat exists. Herbivore damage has never been quantified in their native range, but 44 arthropod species were reported on the genus in China with 7 species observed on *L. maackii* and 37 on *L. japonica* (Zheng et al. 2006). In addition, both species appear to be much rarer in their native ranges than in their introduced ranges (J. Ding, personal communication; Luken and Thieret 1996; http://www.biodic.go.jp/english/rdb/red_plants.csv). Studies in the invasive range indicate
reduced damage levels on exotic *Lonicera* species as compared to congeners and sympatric species (Trisel 1997; Schierenbeck et al. 1994). In a companion study, we measured herbivory on *L. maackii* for two years across several sites in Ohio and found that arthropod herbivores typically removed less than 3% of the leaf area from these shrubs (Lieurance and Cipollini 2012). Similar amounts of herbivory were observed on *L. japonica* in its introduced ranges in both New Zealand (Waipara et al. 2007) and South Carolina (Schierenbeck et al. 1994). These damage levels in the introduced range are likely too low to affect the fitness of these shrubs. While this information supports the possibility of enemy release for exotic *Lonicera* species, few comparisons of natural herbivory rates and herbivore resistance have been made between invasive *Lonicera* species and their native relatives (but see Schierenbeck et al. 1994). If herbivory rates and levels of resistance in invasive species are no different than in their native relatives, then some mechanism other than low herbivory must be responsible for the differential growth and abundance of these species.

Of the species of *Lonicera* native to the midwestern and northeastern United States, *L. reticulata* is one of the more common (pers. obs.). This species is a twining, woody vine found in moist forest understories and along streams (http://plants.usda.gov). Additionally, many relatives in the Caprifoliaceae, including *Viburnum prunifolium*, inhabit the forests of the Northeast in more abundance than native *Lonicera* species and often compete directly with exotic *Lonicera* species for resources (Luken et al. 1997; Schmidt and Whelan 2001). Using two field sites where *L. maackii* co-occurs with the native *L. reticulata* and *V. prunifolium*, we addressed the question of whether these species receive differential herbivore damage in a phylogenetically and ecologically
relevant setting. We also examined the laboratory preference and performance of a North American specialist, the honeysuckle sawfly, *Zaraea inflata* (Cimbicidae), and the performance of a widespread generalist caterpillar native to the Western Hemisphere, the fall armyworm, *Spodoptera frugiperda* (Noctuidae) fed cut foliage of non-native *L. maackii* and *L. japonica* and native *L. reticulata* and *L. sempervirens*. Although these species were not observed in our field study, we included *L. japonica* and *L. sempervirens* in laboratory trials because they have overlapping distribution in North America, a similar growth habit to each other, and vary in their invasive and native status (http://plants.usda.gov/java/profile?symbol=LONIC). Based on the assumptions of the enemy release hypothesis and our previous research, we predicted that 1) non-native *L. maackii* will receive some herbivore damage in the field, but damage levels would be lower than those observed on both native species and would vary through time for all species, 2) the specialist *Z. inflata* will perform better on native *Lonicera* species compared to exotic *Lonicera* species, and 3) the generalist *S. frugiperda* will perform better on native *Lonicera* species than on non-native *Lonicera*, but will develop on both native and non-native foliage.

### 3.2 MATERIALS AND METHODS

#### 3.2.1 Herbivory Assessment 2009-2011

In 2009, herbivore damage was quantified in June and August at Kiser Lake State Park in Champaign County, Ohio on the non-native *L. maackii* (n=10) and the native *L. reticulata* (n=24). The study site was a relatively undisturbed mixed mesophytic forest
understory. Because each species had a patchy distribution within the forest, plants were haphazardly selected within 10m of a trail that borders the lake. To account for differences in growth habit and assure equivalent comparisons of foliar herbivory, we scored percent herbivore damage on the whole plant for *L. reticulata* and on 20 leaves per plant for *L. maackii*. Percent leaf area removed was scored as 1, 2, 5, and then in increments of 5% to a maximum of 100% leaf area removed, as in Lieurance and Cipollini (2012). Since different plants were observed in June and August, Student’s T-tests were used on square root transformed data to compare mean damage levels among species separately for each sampling date.

In 2010, we returned to Kiser Lake State Park and also included a second site, Cedar Bog Nature Preserve in Champaign County, Ohio. Cedar Bog Nature Preserve is a calcareous fen with many rare and endangered plant species intermixed with stands of white cedar and mixed mesophytic forest. In 2010, we also quantified herbivory on *Viburnum prunifolium*, a related plant of similar stature to *L. maackii* that directly competes with both *L. maackii* and *L. reticulata* in forest edge and understory habitats (pers. obs.). Fifteen plants of each species (*L. maackii*, *L. reticulata*, and *V. prunifolium*) were selected as in 2009 from small patches dispersed throughout the forest understory and sampled in 20 May, 20 August, and 23 October at both sites. Arthropod herbivore damage was scored as in Lieurance and Cipollini (2012) on three branches per plant for *L. maackii* and *V. prunifolium*, while damage was observed on the whole plant for *L. reticulata*.

Because no significant differences in percent damage between the two sites (\(F_{1,67}=0.71, p=0.404\)) were observed in 2010, we conducted observations only at Kiser
Lake State Park in 2011. However, we increased the number of sampling dates to include 5 May, 16 June, 18 July, 15 September, and 17 October to better capture temporal changes in herbivory. Because all plants selected from the previous year could not be re-sampled (weathering of tags and plant mortality), we measured 15 different individuals from each species. Percent herbivore damage data from 2010 and 2011 were square root transformed and repeated measures ANOVA was used to analyze changes in herbivore damage through time, with time as the within subjects effect, and species, site, and species*site (2010) and species (2011) as between subjects effects. Comparisons of means were made using Tukey post-hoc tests. All statistical analyses were performed using SAS software (Version 9.2, SAS Institute, Cary, North Carolina).

3.2.2 Feeding Bioassays

On May 5, 2011, we collected similarly sized (8.2 ± 0.5 mg) larvae of *Zaraea inflata* from *L. reticulata* plants at Kiser Lake State Park. The larvae were brought back to the laboratory on *L. reticulata* foliage, starved for 24h, weighed, and placed in individual 4x6x6 cm plastic feeding arenas. The day they eclosed was unknown as was the stage of larval development, but the mean mass of larvae exposed to different host species was equivalent (F_{3,59}=0.36, P=0.78). Each arena was randomly assigned one of four *Lonicera* species: *L. reticulata* (native), *L. sempervirens* (native), *L. maackii* (non-native), and *L. japonica* (non-native) with 15 replicates per species. Leaves of *L. maackii* and *L. reticulata* were collected and replaced as needed from Kiser Lake State Park and *L. japonica* and *L. sempervirens* were collected from 2-3 year old *Lonicera* plants grown in full sunlight 1.5m apart from one another in a common garden on the campus of
Wright State University. The feeding arenas were maintained in an incubator at 22°C with a photoperiod of 16:8 (L:D), and weighed every 2-3 days. Insects reaching pupation were removed from the arena and weighed. Larval mass, survivorship, and days to pupation were recorded through time, and pupal mass was recorded for those that reached pupation. Also, we calculated relative growth rate as \[\frac{\ln(\text{mass}_{\text{day}14})-\ln(\text{mass}_{\text{initial}})}{\# \text{ of days on day 14 when larvae reached their peak mass}}.\] Larvae on *L. japonica* did not survive to this point. We followed the performance bioassay with a choice-feeding assay in 2012 using *L. maackii* and *L. reticulata*, the two species the larvae were able to pupate on. Larvae were collected from *L. reticulata* plants at Kiser Lake State Park as in 2011 and were brought back to the laboratory where they were starved for 12 hours and placed in the center of 150 X 15 mm Petri dishes with one similarly sized leaf of each of *L. maackii* and *L. reticulata*, which were also collected from Kiser Lake State Park. Larvae were allowed to feed for 24 hours. Leaf area consumed was determined by measuring leaf area before and after the bioassay using image analysis (ImageJ, National Institutes of Health, Bethesda, MD). Choice index was calculated as \[\frac{\text{Leaf area removed}_{L. Reticulata}-\text{Leaf area removed}_{L. Maackii}}{\text{Total leaf area removed}}\] *100.

Eggs of the generalist *Spodoptera frugiperda* were purchased from Benzon Research (Carlisle, PA, USA) and larvae were reared on an artificial *Spodoptera* diet (Southland Products, Inc., Lake Village, AK, USA) in an incubator with the same settings as the previous assay for 7 days. At the start of the experiment, there was no difference between the mass of the larvae assigned to different plant species \(F_{14,90}=.48, P=0.94\). Larvae were randomly assigned one of the four *Lonicera* species used in the previous assay with 15 replicates per species. The procedure followed for this bioassay was the
same as above, except that relative growth rate was calculated at day 27 when surviving
larvae reached their peak masses.

Daily larval masses for both no-choice assays were compared using repeated
measures ANOVA on log transformed data (Log (wt +1)), with plant species as the
between subjects effect and time as the within subjects effect. To determine if the choice
feeding index differed from 0, we used a one sample T-test where 0 indicated no choice,
a positive number indicated choice for L. reticulata, and a negative number indicated
choice for L. maackii. Relative growth rates, days to pupation, and pupal mass from both
assays were compared among plant species using ANOVA. Comparisons of means were
made using Tukey post-hoc tests. Survivorship was compared among plant species using
the non-parametric Kruskal-Wallis test. All statistical analyses were performed using

3.3 RESULTS

In 2009, percent herbivore damage by arthropod herbivores was approximately 18
times higher on L. reticulata than on L. maackii in June, and 8.5 times higher in August
(June: t_{42}=6.16, P <0.0001; August: t_{32}=3.70, P=0.0008, Figure 3.1a). In 2010, surveys
revealed no differences between the Cedar Bog and Kiser Lake sites in average herbivore
damage, but herbivory differed between species at each site (Table 3.1). Herbivore
damage varied over time, but there were no significant interactions between time and site,
time and species, or time and site and species (Table 3.1). Damage on native L. reticulata
was 6.75-10 fold greater than damage on non-native L. maackii, and 1.8-3.6 fold greater
than native V. prunifolium. Damage on V. prunifolium was 3.5-4.6 fold greater than L.
maackii in August and October, respectively (Fig 3.1b). Similar patterns were observed in 2011 across more sampling dates at Kiser Lake where significant differences in herbivore damage were documented between species and through time, and there was an interaction between time and species (Table 3.2). Early season damage occurred between May 5 and June 5 for all species. Lonicera reticulata had the most damage at all sampling dates with 10.5 and 4.7 fold more damage than L. maackii and V. prunifolium, respectively. Lonicera maackii and V. prunifolium received similar amounts of damage through time (Fig 3.1c).

In the no-choice bioassay with Zaraea inflata, repeated measures ANOVA revealed differences in larval mass by species, changes through time, and an interaction of time and species (Table 3.2, Fig 3.2a). There was 100% mortality of larvae feeding on L. japonica by day 3. Relative growth rates of larvae were 17.5 and 9.7% higher on native L. reticulata than on native L. sempervirens and non-native L. maackii, respectively. Relative growth rates of larvae reared on L. maackii were 7.2% higher than those fed native L. sempervirens (Fig 3.2b). There were significant differences in survivorship (H₃=45.12, P<0.0001) of larvae by species. Larvae pupated on both L. reticulata and L. maackii with similarly high survivorship (Fig 3.2c). While larvae feeding on L. sempervirens survived longer than on L. japonica, there was complete mortality just prior to pupation. Pupal mass was lower on L. maackii than on L. reticulata (F₁,24=15.29, P=0.0007, Figure 3.2d), but days to pupation did not differ (F₁,24=0.03, P=0.87). Zaraea inflata larvae preferred L. reticulata over L. maackii with a mean choice index of 66.01±16 (t₁₆=4.04, P=0.0009).
In the no-choice bioassay with the generalist *Spodoptera frugiperda*, repeated measures ANOVA revealed that larval masses varied over time, but with no significant variation among species (Table 3.2, Figure 3.3a). There was no statistical difference in RGR for *S. frugiperda* larvae feeding on each species (Fig. 3.3b). Survival was generally poor on all species, and although differences in survivorship were not statistically significant ($H_3=4.46, P=0.22$), larvae tended to survive better on *L. maackii* and *L. sempervirens* than on *L. japonica* and *L. reticulata* (Fig. 3.3c). Additionally, there were no differences in pupal mass or days to pupation for larvae fed each species ($F_{3,12}=1.20$, $P=0.35$; $F_{3,12}=2.71$, $P=0.09$, Figure 3.3d).

### 3.4 DISCUSSION

Results of three years of observation supported our prediction that *Lonicera maackii* receives less arthropod herbivore damage than related co-occurring native species. Herbivory on *Lonicera reticulata* was primarily the result of early season herbivory by the honeysuckle specialist sawfly, *Zaraea inflata*, a species that was never observed on *L. maackii* in the field in this study. Although we did not conduct an extensive arthropod field survey, no specialist arthropod herbivores were observed on *Viburnum prunifolium* or *L. maackii*, but *V. prunifolium* did suffer mid-season fungal damage in 2010 (pers. obs.). Results of our arthropod herbivory assessment were consistent with Trisel’s (1997) observation that *L. maackii* received arthropod herbivory at levels well below native species (including *V. prunifolium*) sharing the same habitat, however Trisel’s comparisons did not investigate temporal variation in damage and did
not include any congeners. *Lonicera japonica* experienced approximately 1.25% leaf area removal across three sites in Ohio and Tennessee when measured at the end of the season in 2011 (unpublished data), levels which were comparable to those observed on *L. maackii* (Lieurance and Cipollini 2012; this study) and on *L. japonica* in its invasive range in New Zealand and South Carolina (Waipara et al. 2007; Schierenbeck et al. 1994). Overall, our results were consistent with many studies that have compared either native vs. exotic congeners, or ecologically similar natives vs. exotics in their invasive range as a method of deducing enemy escape. Liu et al. (2007) showed that native *Eugenia* species sustained higher foliar and seed damage than invasive exotic *Eugenia* species in South Florida. Siemann and Rogers (2003) found significantly less herbivory on the invasive tree, *Triadica sebifera*, than on an ecologically equivalent native, *Celtis laevigata*. In a phylogenetically controlled common garden experiment with 13 species pairs, non-native herbs received 22% less foliar herbivory than native congeners (Agrawal et al. 2005).

Consistent with our predictions, the specialist *Z. inflata* was not observed on *L. maackii* growing alongside the native *L. reticulata*. This was true despite the fact that the early season foliage available to the specialist herbivore by *L. reticulata* was much less than that presented by *L. maackii*. Results of the no-choice feeding assay did not support our prediction that *Z. inflata* larvae could not develop on *L. maackii*. In fact, larvae were able to reach pupation in the same amount of time on both species, but pupal masses were reduced on *L. maackii*. However, results of the choice-feeding assay confirm that *Z. inflata* larvae prefer their native host when given the choice. This indicates that the lack of herbivory on *L. maackii* in the field by this insect could be due to a lack of recognition
of *L. maackii* as a host by ovipositing females, or rejection of it by larvae that are placed on *L. maackii* that can potentially move to other hosts. *Lonicera japonica* was clearly highly toxic to specialist larvae, which is consistent with its lack of herbivory observed in the field. The native *L. sempervirens* was also a surprisingly poor host for the specialist. This could be attributed to a similarity in leaf morphology as both species have tough, semi-evergreen leaves that may be better defended than more deciduous leaves (Coley 1983; Poorter and Bongers 2006). There are some examples of native specialist herbivores preferring or switching to non-native, invasive hosts in field and laboratory settings. The South American lepidopteran specialist, *Utetheisa ornatrix*, preferred the non-native *Crotalaria pallida* (Fabaceae) to their co-evolved host plant, the native congener *Crotalaria incana*, for both oviposition and seed predation (Cogni 2010). *Euphydryas editha*, a specialist butterfly, preferred novel host species in anthropogenically disturbed habitats in California, and even showed evidence of differentiation with some insects refusing their natural hosts (Singer et al. 1993). This leads to the possibility that as the habitat of *L. reticulata* and *L. maackii* continue to overlap, selection may favor a host shift in *Z. inflata* to capitalize on the abundant early season food resource provided by *L. maackii*.

Results of bioassay with the the generalist species supported the general prediction that larvae would develop on multiple *Lonicera* species, but results were not entirely consistent with our prediction that a generalist would perform better on native *Lonicera* species. Overall, survivorship and performance of the generalist was much lower than the specialist on its most suitable hosts, which was not surprising. However, the highest survivorship tended to occur on non-native *L. maackii* and native *L.
*sempervirens*, and the lowest on the exotic *L. japonica* and the native *L. reticulata*. However, there were no species-specific differences in RGR, pupal mass, or days to pupation for larvae that survived throughout the assay. Variation in host plant relationships to herbivores among species may reflect their phylogenetic relatedness, as well as their exotic or native status. In this case, neither phylogenetic relatedness nor invasive status entirely predicts herbivore performance on these *Lonicera* species.

*Lonicera maackii* is more distantly related to the native *Lonicera* species than they are to non-native *L. japonica* (Thies et al. 2008), yet *L. maackii* supported larval development of a specialist and a generalist fairly well. *Lonicera japonica* is more closely related to the native species than to *L. maackii*, yet it did not support development of either herbivore very well.

Results of the feeding assays indicate that the specialist and generalist herbivores tested in this study develop to some extent when fed non-native *Lonicera* species in the laboratory, and yet these species receive little damage in the field. This indicates a behavioral avoidance of some potential novel hosts (Lankau et al. 2004), or an inability to recognize a potential host (Morrison and Hay 2009; Verhoeven et al. 2009). *Zaraea inflata* was chosen as the model specialist because it was the only specialist present on native *Lonicera* at our study sites. Many sawflies are so specialized that they are dependent upon a single genus or even a single species as a host (e.g. Barker et al. 2002; Roininen and Tahvanainen 1989), and *Zaraea* spp. have been reported as occasional pests on ornamental *Lonicera* species (Middlekauff 1956). In contrast, *S. frugiperda* was selected as a model generalist because it is widely distributed and has a wide host range (Luginbill 1928; Sparks 1979). It has not been reported on *Lonicera* species, but is often
used in studies assessing plant resistance to generalist herbivory (e.g. Afkhami and Rudgers 2009; Alves et al. 2007). However, caution should be used when making broad assumptions about the performance of “generalists” and “specialists” on these plants as the results presented here represent one specialist and one generalist and may not reflect the general patterns of each herbivore class. Additionally, assays were carried out using cut foliage from plants known to produce secondary metabolites with anti-herbivore effects (Cipollini et al. 2008b). Whether harvesting leaves induced higher levels of defenses is not known, but the same collection and bioassay procedure was used for all plants. Finally, larvae of the specialist, *Z. inflata* were collected from *L. reticulata* in the field prior to assignment to different hosts, and *S. frugiperda* larvae were initially raised on artificial diet, thus our results do not reflect the initial performance of neonate larvae on novel hosts.

Overall, our current and previous results support the three basic assumptions of the enemy release hypothesis outlined by Keane and Crawley (2002) that *L. maackii* would be released from specialist herbivores, herbivores specializing on native relatives would not “jump” to the invader, and damage incurred by generalist herbivores would be less severe than damage on native congeners. Although less thoroughly examined, these assumptions were also largely supported for *L. japonica*. *Lonicera maackii* and *L. japonica* have relatively short invasion histories with both escaping cultivation roughly 120-150 years ago (Luken and Thieret 1996; Shierenbeck, 2004). As time since introduction increases, the susceptibility of these plants to native specialists and generalists may increase if costly defenses relax (Blossey and Notzhold 1995), or potential herbivores overcome behavioral constraints and begin to utilize these novel
plants. However, escape from or resistance to these species may be part of the explanation for the success of invasive Lonicera species, suggesting that selection may favor maintenance of traits that contribute to resistance despite its costs. Our results indicate that that Z. inflata larvae could already use L. maackii as a host if adult females oviposition preferences expand to include the exotic congener.
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Table 3.1 Results of repeated measures ANOVA evaluating the effect of site and species (2010) and species only (2011) on percent herbivory. Results of within subjects effects presented from Wilks’ Lambda test for multivariate analysis.

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Table 3.2 Results of repeated measures ANOVA evaluating the species of *Lonicera* fed to both *Zarea inflata* and *Spodoptera frugiperda* on larval mass through time. Results of within subjects effects presented from Wilks’ Lambda test for multivariate analysis.

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Figure 3.1 Percent damage by arthropod herbivores in 2009 on native *Lonicera reticulata* and non-native *Lonicera maackii* at Kiser Lake State Park (a). Percent damage by arthropod herbivores through time in 2010 on *L. reticulata, L. maackii*, and the related native shrub, *Viburnum prunifolium* at Cedar Bog Nature Preserve and at Kiser Lake State Park (b), and at Kiser Lake State Park in 2011 (c). Means and standard errors presented. Herbivore damage did not vary by site in 2010 so pooled means are presented. Letters indicate differences in means \(P<0.05\).
Figure 3.2 Mean mass of *Zaraea inflata* larvae, a specialist arthropod herbivore, feeding on the foliage of 2 native and 2 exotic *Lonicera* species (a), relative growth rate (RGR) (b), survivorship (c), and pupal mass (d). RGR was calculated on day 14. Letters indicate differences in means determined through Tukey post hoc testing (*P*<0.05). Larvae fed on species not included in statistical analysis are denoted as “na”.
Figure 3.3 Mean mass of Spodoptera frugiperda larvae, a generalist arthropod herbivore, feeding on the foliage of 2 native and 2 exotic Lonicera species (a), relative growth rate (RGR) (b), survivorship (c), and pupal mass (d). RGR was calculated on day 27. Letters indicate differences in means determined through Tukey post hoc testing ($P<0.05$). The larval mass is shown up to day 27 due to differences in time to pupation and days when larva died.
4 Environmental influences on growth and defense responses of the invasive shrub, *Lonicera maackii*, to simulated and real herbivory

4.1 INTRODUCTION

Invasive plants may succeed in their novel ranges through enemy escape, tolerance, and/or resistance to herbivory. Tolerance to herbivory is exhibited through several mechanisms contributing to the fitness of a plant following damage, including increases in physiological performance (i.e. increasing photosynthesis), reallocation of resources to compensate for tissue removal, and modified root to shoot ratios that lead to improved nutrient uptake (Herms and Mattson 1992; Karban and Baldwin 1997; Strauss and Agrawal 1999; Haukioja and Koricheva 2000). Mechanisms of resistance such as mechanical or chemical defenses may also contribute to the success of an invader in its new habitat (Cipollini et al. 2005; 2008b; Chun et al. 2010). Resistance can be either constitutive (always present), or induced (triggered following herbivore damage), and both are thought to be costly to the plant in terms of resource allocation (Herms and Mattson 1992; Karban and Baldwin 1997; Walters and Heil 2007; Cipollini and Heil 2010).

Differences in abiotic factors such as light and nutrient availability can alter the tolerance and/or resistance of a plant to herbivore damage. Reducing light availability to
plants can limit carbon directly through reduction in photosynthesis and, may influence the ability of some woody plants ability to recover from herbivory. In a study evaluating 10 economically important species in the Dipterocarpaceae, removal of 35% of the leaf area from these species reduced relative growth rate (RGR) by ~22% for seedlings grown in deep shade, but only by ~9% for those in high light, when compared to undamaged plants (Paine et al. 2012). Additionally, woody plants growing in high nutrient conditions grow faster and increase allocation to above ground components at the expense of below-ground carbon stores that can be tapped when plants respond to herbivore damage (Hawkes and Sullivan 2001). This may result in reduced tolerance to herbivory in high nutrient treatments. Alternatively, increases in nutrient availability may provide plants with adequate resources to rapidly compensate for leaf loss to herbivory (Maschinski and Whitham 1989), although evidence for this with woody plants is scarce (Herms 2002).

Secondary metabolite production can vary with resource availability and may depend on the class of compound produced, with carbon-rich compounds such as phenolics and tannins often responding to light limitation and nitrogen-rich compounds such as pyrrolizidine alkaloids, cyanogenic glycosides and glucosinolates responding to fertilization (Bryant et al. 1983; Koricheva et al. 1998; de Boer 1999; Burns et al. 2002; Herms 2002; Lambdon et al. 2003). Phenolics typically increase with light availability and not only offer defense against herbivores, but also protect against photodamage (Dudt and Shure 1994; Close and McArthur 2002). Additionally, the activity of peroxidase, an inducible enzyme linked to defense, was reduced under nitrogen limitation while protein content increased with nitrogen enrichment in Arabidopsis thaliana (Dietrich et al. 2004). It has been suggested that a trade-off between growth and
resistance may determine how a plant responds to herbivory and these responses may vary with resource availability (van der Meijden et al., 1988; Fineblum and Rausher 1995). It is also possible that plants could respond to herbivore damage with a mixed response of both tolerance and resistance to herbivory (Herms and Mattson 1992; Koricheva et al. 2004; Núñez-Farfán et al. 2007). Perhaps a trait of highly successful invasive plants is being a “jack of all trades” in the face of herbivore pressure (Koricheva et al. 2004).

Ontogeny may also affect the response of a plant to damage, as allocation to growth, defense, and reproduction is variable with age (Boege 2005; Boege and Marquis 2006). In a meta-analysis, Barton and Koricheva (2010) showed that variable patterns emerged in tolerance and defense to herbivory by woody species. Trends indicated that plants generally move from a strategy of high investment in growth and increased chemical defense in seedlings to increases in physical defenses in the juvenile and mature stage; tolerance did not vary much with ontogeny according to their analysis. Tannin and phenolic glycoside concentrations were measured through time in seedlings of *Salix sericea*, *S. eriocephala*, and their F1 hybrids by Fritz et al. 2001, who found that initial amounts of these defenses were very low in seedlings and increased linearly over a time period of 6-14 weeks to quantities comparable to those found in mature trees. As plants mature and acquire resources over time, physical defenses such as spines and thicker leaves develop as a response to increases in herbivore pressure (Hanley et al. 2007). Because tolerance is less predictable with ontogenetic stage, high phenotypic plasticity, may be more predictive of tolerance (Heil 2010; Fornoni 2011).
*Lonicera maackii* (Rupr.) Maxim (Amur honeysuckle) is a deciduous woody shrub native to China, Japan, Korea, and southeastern Russia that is highly invasive throughout the Midwestern and Northeastern U.S. (Luken and Thieret 1996). Traits thought to contribute to the success of *L. maackii* include extended leaf longevity, high aboveground growth rates, high fecundity, broad phenotypic plasticity, and tolerance to a variety of habitats (Lieurance 2004; Luken et al. 1995a; 1995b; 1997a; 1997b; Trisel 1997). Previous studies indicate that *L. maackii* loses approximately 3% or less of its leaf area to arthropod herbivore damage in its invasive range in Midwestern United States, receives significantly less damage than native *Lonicera reticulata* and con familial *Viburnum prunifolium* in the same habitats, and escapes damage from the honeysuckle specialist sawfly, *Zarnea inflata*; thus enemy escape seems to have occurred for this species although the amount of herbivory it experiences has not been measured in its native range (Lieurance and Cipollini, 2012; Lieurance and Cipollini, 2013). However, it does experience some browsing by deer in its introduced range, especially in the face of high deer densities (D Lieurance, D Cipollini, personal observation). No direct studies of tolerance to foliar herbivory have been conducted on *L. maackii* to know whether the level of natural herbivory it receives in its invasive range is biologically significant. However, reproductively mature shrubs (7-11 years old) subjected to three years of repeated clipping of all stems resprouted in both open and understory habitats, but showed greater mortality in low light conditions (Luken and Mattimiro 1991). Evidence of tolerance to both simulated and real herbivory has been observed in the invasive congener *Lonicera japonica*. When 50% of the leaves were removed by clipping branches to simulate mammal herbivory, *L. japonica* seedlings responded with no change
in RGR and with a lower root to shoot ratio (R:S) seven weeks after defoliation in a greenhouse experiment (Ashton and Lerdau 2008). When *L. japonica* and the native congener *L. sempervirens* experienced both insect and mammal herbivory in the field, *L. japonica* increased biomass allocation to leaves and had higher total biomass than the vines protected from herbivory, while *L. sempervirens* showed a smaller increase in total biomass (Schierenbeck et al. 1994). While the invasion ecology of *L. maackii* has been generally well studied, its growth responses to foliar herbivory have not been directly examined.

Several secondary metabolites associated with resistance to herbivores have been identified in *L. maackii*, including two major flavones- apigenin and luteolin and their glycoside derivatives, chlorogenic acid, and other phenolics in the leaves (Cipollini et al. 2008b). Such compounds have been implicated in allelopathic effects on other plants, as well as serving an anti-herbivore function (Dorning and Cipollini 2006, Cipollini et al. 2008a; 2008b). While such compounds are known to be constitutively produced, it is not known how components of the defensive chemistry of *L. maackii* may change in response to herbivory, environmental variation, or correlate with growth.

We examined how mature plants respond to varying levels of defoliation, as well as growth and biochemical responses of juvenile *L. maackii* plants to simulated and real herbivory. Additionally, we also examined how abiotic factors such as light and nutrient availability influenced these responses. We first assessed the ability of field grown, mature *L. maackii* plants to tolerate foliar herbivory by following the growth rates of selected branches for two years following removal of a gradient of leaf area through stem clipping. We then evaluated the ability of juvenile *L. maackii* plants to tolerate herbivory
in two greenhouse experiments by measuring the total biomass, root to shoot ratios (R:S), specific leaf area (SLA) and relative growth rates (RGR) of plants with and without herbivore damage (both simulated and real). In the first greenhouse experiment, we manipulated light and nitrogen availability and mechanically removed 50% of individual leaves. In the second experiment, we manipulated total nutrient availability and removed 50% of the leaf area with both simulated and real herbivory. Resistance traits were evaluated in both greenhouse experiments by comparing the leaves of damaged and undamaged plants for total protein content (which should increase with the accumulation of defense proteins and respond to nutrient availability), peroxidase activity (an oxidative enzyme associated with the synthesis of defensive compounds, resistance to insects and pathogens, and the healing of wounds in plants) (Felton 1996; Barbehenn et al. 2010), and total flavonoids (a class of phenolic compounds abundant in L. maackii linked to multiple functions including anti-herbivore effects) (Cipollini et al. 2008b). We predicted that: 1) both juvenile and mature plants would be highly tolerant of herbivory, 2) total foliar protein content, peroxidase activity and total flavonoids would increase with herbivore damage, protein and peroxidase would decrease with nutrient limitation, and flavonoids would decrease with light limitation, 3) total protein, peroxidase activity, and total flavonoid content would increase with time, and 4) responses to simulated and real herbivory treatments would be similar.

4.2 MATERIALS AND METHODS

4.2.1 Field experiment: Effects of simulated herbivory on mature plants
This experiment was conducted on mature *L. maackii* plants in a heavily invaded understory section of the Wright State University Woods, in Dayton, OH. In the spring of 2009, similarly sized shrubs (2-3 m tall) were haphazardly selected from the understory monoculture of shrubs, and were spaced a minimum of 2 m apart from edge to edge. There were four possible one-time defoliation treatments imposed with 15 replicates per treatment. Defoliation treatments were carried out between May 11 and the 15 approximately 2 months after budbreak, at levels of 0, 5, 25, or 50%. *Lonicera maackii* produces two morphologically distinct ‘long’ and ‘short’ branch types. Long branches are arching, hollow stemmed (presumably cheap in construction) branches measuring greater than 15 cm in length (> 12 leaves per branch) that extend the shrub height to reach increased light. Short branches have leaves that are thicker, tougher, and more resistant to herbivory ranging from 5-12 cm (< 12 leaves per branch) (Luken et al. 1995a; 1995b; Lieurance and Cipollini 2012). We removed whole branches of the short branch type and whole leaves from the long-branch type. For example, in the 25% defoliation treatment, short branches were counted and every fourth branch was cut at the base. In the 25% defoliation treatment, every fourth leaf was removed at the petiole from long branches. This resulted in an overall reduction of approximately 25% of leaf area (along with some stem biomass from short branches). Growth was followed on five tagged branches per shrub, including 3 untreated short branches and 2 long branches. Branch length and diameter at the base of the branch (DAB) were measured between May 28 and June 5, and again between October 2 and the 7 in 2009. Relative growth rate (RGR) was calculated as \[ \text{RGR} = \frac{\ln(X_{\text{final}}) - \ln(X_{\text{ini}})}{\# \text{ of days}}, \] where \( X \) = either the diameter at base of branch (DAB) or branch length. From May 13 – 23, 2010, the same shrubs were again
defoliated by the same amounts (0, 5, 25, or 50%) using the same methods, but because some tags fell off over the winter or branches were removed through winter deer browse, new branches (3 short, 2 long) were selected and tracked as before over the 2010 season for RGR. Initial measurements were taken at the time of defoliation, final measurements in October 14 – 16 and RGRs were calculated as above.

4.2.2 Greenhouse Experiment 1: Effects of simulated herbivory across light and nitrogen gradients

Seeds of Lonicera maackii were collected in 2008 from Wright State University Woods and stratified for 5 weeks at 22°C. Germinants were transplanted from Petri dishes to 400 mL plastic round pots in ProMix BX potting soil with mychorrhizae added (Premier Tech Horticulture, Quakertown, PA). Plants were watered with distilled water as needed and fertilized once every two weeks with 125 ml of 1.87 g/L Peters 20-20-20 complete soluble fertilizer plus micronutrients (Grace-Sierra, Milpitas, CA) until the start of the experiment.

Once the juvenile plants reached 12 weeks of age, treatments including simulated herbivory (0% and 50% removed), light availability (100% and 50% ambient light), and nitrogen availability (full and half strength nitrogen fertilization) were assigned to plants in a 3-way factorial design with 8 replicates per combination for a total of 64 plants. Juvenile plants were an average of 25 cm in height and had approximately 20 leaves. The simulated herbivory treatment was imposed through a one-time manual removal with scissors at the petiole of 50% of the leaves on the plant, alternating every other leaf
(plants generally had 6-10 leaves removed). Plants in the shaded treatment were grown in shade structures (3 structures, 10-11 plants per structure) constructed with PVC pipe and black polypropylene shade cloth (DeWitt Co., Sikeston, MO) that reduced light by 50% while plants in the unshaded treatment received ambient light supplemented with fluorescent lights with photosynthetically active radiation ranging between 0700 and 2100 µmol/m²/sec. The nitrogen treatment was implemented by fertilizing plants bi-weekly with 200 mL of complete nutrient solution with a total of 1500 ppm nitrogen for the high nitrogen treatment and a complete nutrient solution with half the nitrogen concentration (750 ppm) for the low nitrogen treatment. Nutrient solutions were prepared as in Reiss (1994). To avoid possible microclimatic effects in the greenhouse, plants were rotated within the shaded and unshaded treatments every two weeks. All plants were watered with distilled water as needed between fertilization treatments. Stem diameter at base (DAB) and height were measured every two weeks for the duration of the experiment from the initiation of the treatments.

Fourteen weeks after initiation of the treatments, we harvested plants and divided them into roots and shoots. Roots were washed with distilled water to remove soil and biomass was dried to a constant weight at 70°C for a minimum of 72 hours and weighed. Total dry biomass, and the mass of the component parts were recorded. Root-to-shoot ratio (R:S) was calculated as dry mass of roots/dry mass of aboveground biomass (stems and leaves). Relative growth rate in basal stem diameter (RGR) was calculated as
\[ \text{RGR} = \frac{\ln(DAB_{\text{final}}) - \ln(DAB_{\text{ini}})}{\text{number of days}} \]
Mean specific leaf area (SLA) was calculated from three leaves per plant as leaf area (cm²)/g dry leaf mass. At the time of
harvest, whole fresh leaf samples (approximately 500 mg) were collected randomly from
the plant for chemical analysis and immediately placed in the freezer at -20° C.

4.2.3 Greenhouse Experiment 2: Effects of simulated and real herbivory with and
without fertilization

Because there was few pronounced effects of varying nitrogen availability in
experiment 1, we altered the nutrient treatment in experiment 2 to either fertilized or
unfertilized treatments. We added an additional treatment of real herbivory with a
generalist caterpillar to compare the effects of real and simulated herbivory. Plants were
grown as in the previous experiment. Once the plants reached 12 weeks of age (~20
leaves, 25 cm in height), we initiated our treatments, including herbivory (0% removed,
50% leaf area removed through simulated herbivory and 50% leaf area removed through
real herbivory), and nutrient availability (unfertilized and fertilized). A 2-way factorial
design was used with 8 replicates per treatment combination for a total of 48 plants. The
simulated herbivory treatment was carried out as before. Real herbivory was
implemented by caging whole plants in sleeve cages and placing 5-10 Hyphantria cunea
(Arctiidae) larvae collected from L. maackii plants growing in the Wright State
University Woods on each caged plant. These generalist caterpillars will occasionally use
L. maackii in the field. Larvae were allowed to feed for 3 days to achieve ~50%
defoliation on the entire plant. High nutrient plants were fertilized every two weeks with
125 ml of 1.87 g/L Peters 20-20-20 complete soluble fertilizer (240 ppm nitrogen) plus
micronutrients (Grace-Sierra, Milpitas, CA) and watered with distilled water between
treatments. Low nutrient plants were watered with distilled water only. Growth measurements were taken every two weeks and harvest was conducted 10 weeks after initiation of treatments. The same variables were measured as in experiment 1. To capture temporal differences in defense chemistry, leaf samples (approximately 500 mg) were randomly collected as before from each plant for chemical analysis three days after the real herbivory treatments were completed and at the end of the experiment. Initial leaf samples from the real herbivory treatments included leaves damaged by caterpillars; whole leaf samples were taken from simulated and control plants. Leaf samples were immediately placed in the freezer at -20°C until chemical analysis.

4.2.4 Chemical analyses

Soluble proteins were extracted from fresh leaves by homogenizing leaves in ice-cold, 0.01 M sodium phosphate buffer (6.8 pH) containing 5% polyvinylpolypyrrolidone (PVPP). Extracts were centrifuged for 12 minutes at 12,000 rpm and the cleared supernatants were transferred to fresh tubes. Total soluble protein in extracts was measured using bovine serum albumin as a standard and Bio-Rad protein dye reagent, as described in Bradford (1976). We used these estimates to calculate total soluble protein on a fresh mass basis (mg protein/g fresh weight). Peroxidase activity was analyzed in soluble protein extracts as in Cipollini et al. (2004) using guaiacol as the substrate. Peroxidase activities were expressed and statistically analyzed as \( \Delta \text{Abs}_{470\text{nm}} \cdot \text{min}^{-1} \cdot \text{mg extract protein}^{-1} \). Soluble phenolic extracts were made by homogenizing fresh leaf material in 80% methanol and shaking for 30 minutes on ice on an orbital shaker.
Extracts were centrifuged as before and cleared supernatants were transferred to fresh tubes. Total flavonoid content was determined by mixing 0.2 ml of the methanol extracts with 0.01 ml of 1% 2-amino-ethyl-diphenyl borate solution and analyzed spectrophotometrically against a standard curve of apigenin at 404 nm (Hariri et al. 1991). Flavonoid concentration was expressed as mg total flavonoids (apigenin equivalents) per g fresh mass. Apigenin is a major flavonoid found in *L. maackii* leaves (Cipollini et al. 2008b). All assays were performed in duplicate.

### 4.2.5 Statistical Analysis

For the field experiment, RGRs calculated from branch length and DAB of branch were log transformed to meet the assumptions of normality. Nested analysis of variance (ANOVA) was used to test for differences in RGRs among defoliation treatments and branch types nested within defoliation treatments. We removed several shrubs from the analysis that had received either deer browse or vandalism, so an average of 10 shrubs per treatment in 2009 and 12 shrubs per treatment in 2010 were included in the analysis. For the first greenhouse experiment, independent and interactive effects of light availability, nitrogen availability, and simulated herbivory on RGR in stem diameter, biomass and allocation data (total dry biomass, SLA, and R:S), and chemistry data (total protein, peroxidase activity, and total flavonoids) were examined using 3-way factorial ANOVA. For greenhouse experiment 2, independent and interactive effects of fertilization and herbivory treatment on RGR and biomass data were analyzed using a 2-way factorial ANOVA. Chemistry data from experiment 2 were examined using a 3-way
factorial ANOVA with the added factor of sampling time (either three days post treatment, or at the end of the experiment). Tukey post hoc testing was used to compare means. Total biomass, R:S, RGR, SLA, peroxidase activities and total flavonoids were log transformed and protein content was inverse transformed to meet the assumptions of normality. Pearson correlations were performed among all growth and chemical measures separately by each treatment combination in each greenhouse experiment. Root, shoot and total biomass of plants in greenhouse experiments 1 and 2 were significantly positively correlated, thus we only present the statistical results for total biomass. We also estimated the amount of biomass removed during the defoliation treatments based on the percentage of leaf area removed and the average leaf weight (taken from SLA calculations). Adding this biomass back to the shoot biomass of each plant had no effect on statistical patterns, so we show statistical results and means without the removed biomass included. All statistical analyses were performed using SAS (Version 9.2, SAS Institute, Cary, North Carolina).

4.3 RESULTS

4.3.1 Field experiment: Effects of simulated herbivory on mature plants

After a single spring defoliation, RGR in branch length of mature plants in 2009 averaged 6 (±4) µm/mm/day and did not differ among defoliation treatments ($F_{3,56}=0.96$, $P=0.42$) or among branch types nested within defoliation treatments ($F=0.61$, $DF_{4,56}$, $P=0.66$). There were significant differences in RGR in branch diameter among defoliation treatments ($F_{3,72}=3.40$, $P=0.02$), while branch type nested within defoliation
treatment approached significance \(F_{4,72}=2.46, P=0.052\). The mean RGR by diameter ranged between 0.7 to 2 µm/mm/day across treatments. When compared to the controls, there was a 2.3, 1.5, and 2.2 fold increase in RGR by diameter in 5, 25, 50% defoliation treatments, respectively. In 2010, after another round of spring defoliation, there were no differences in RGR in branch length among defoliation treatments \(F_{3,90}=0.97, P=0.41\) or among branch types nested within defoliation \(F_{4,90}=1.12, P=0.35\). RGR in branch diameter also did not differ by defoliation treatment \(F_{3,93}=0.72, P=0.54\) or branch type nested within defoliation \(F_{4,93}=0.33, P=0.85\). The overall mean RGR for 2010 was 2.3 (±0.6) µm/mm/day for length and 0.6 (±0.1) µm/mm/day for diameter.

### 4.3.2 Greenhouse Experiment 1: Effects of simulated herbivory across light and nitrogen gradients

In this experiment, light significantly affected biomass accumulation, RGR and SLA (Table 4.1, Figure 4.1A-D). Overall, total dry biomass was reduced by 55% under light limitation (Figure 4.1A). There was no main effect of nitrogen on growth independently, but the interaction of fertilization, light, and defoliation affected R:S and RGR (Table 4.1, Figure 4.1A-D). With the exception of the high light, low nitrogen treatment, defoliation reduced total biomass between 12 and 33% (Figure 4.1A). Defoliation decreased R:S in low nitrogen/high light treatment but increased R:S by an average of 2.3 fold in all other treatments (Figure 4.1B). Differences in stem diameter RGR were observed with light limitation, defoliation, and the interaction between fertilizer, light, and defoliation. The overall trend was for RGR to decrease with light
limitation, but the greatest difference in RGR was seen between undamaged plants in the high light, high nitrogen treatment and damaged plants in the low light, low nitrogen treatment (Figure 4.1C). Overall, SLA was influenced only by light limitation with higher values under low light conditions (Table 4.1, Figure 4.1D).

Light, fertilization, and defoliation treatments had no significant main effects on total protein, peroxidase activity or total flavonoid concentration in *L. maackii* plants, but the interaction of fertilizer and light significantly influenced protein and peroxidase activity (Table 4.2). In high light, total protein concentrations were much higher in the low nitrogen treatment than in the high nitrogen treatment. In low light, total protein concentrations were lower under low nitrogen than in high nitrogen treatment (Figure 4.2A). Peroxidase activity was much lower in the low nitrogen treatment in low light, than in the other treatment combinations (Figure 4.2B). The interaction of light and defoliation influenced total flavonoid concentration (Figure 4.2C). Defoliation increased flavonoid concentrations under high light conditions, but decreased them under low light conditions, owing partly to the high concentration seen in undamaged plants grown in low light. There were no correlations between total protein, POD, or total flavonoid content with any measure of growth (total biomass, R:S, RGR, or SLA).

4.3.3 *Greenhouse Experiment 2: Effects of simulated and real herbivory across a nutrient gradient*

In the second greenhouse experiment, total biomass was influenced independently by defoliation treatment but not by fertilization (Table 4.3). Total biomass of control
plants was 1.6 times higher than plants receiving real herbivory. Plants receiving simulated herbivory did not differ from controls or those receiving real herbivory (Table 4.3, Figure 4.3A). R:S decreased with fertilization, but the interaction of defoliation treatment and fertilization approached significance (Table 4.3). Differences between unfertilized and fertilized plants were smaller in undamaged plants than in plants receiving damage, and the greatest effect of fertilization was seen in the real herbivory treatment (Table 4.3, Figure 4.3B). There were no significant effects of the treatments on RGR or SLA. (Table 4.3, Figure 4.3 C-D).

Overall, peroxidase activity (11.96±2.23 initial, 5.91±1.28 final ΔAbs470/min/mg protein) and total flavonoid concentration (6.42±0.47 initial, 4.07±0.25 final mg/g fresh weight) decreased through time, but were not significantly affected by defoliation treatment or fertilization (Table 4.4). There was a significant interactive effect between time and defoliation on total protein (Table 4.4). Foliar protein increased through time, but the change was greater in both defoliation treatments than in the control (Figure 4.4). There were no correlations between initial and final total protein, POD, or total flavonoid content with any measure of growth (total biomass, R:S, RGR, or SLA).

4.4 DISCUSSION

Most research considering exotic plants and plant herbivore interactions focuses on the release from herbivore pressure as predicted by the enemy release hypothesis (Liu et al. 2007; Adams et al. 2009; Funk and Throop 2010; Andonian and Hierro, 2012). But responses to naturally occurring amounts of herbivory experienced in the novel habitat,
or effects of potential major defoliation events is not as thoroughly explored. Our results indicate that mature *L. maackii* shrubs growing in the forest understory were very tolerant of large amounts of leaf area and twig removal after two consecutive years of simulated herbivory. However, Luken and Mattimiro (1991) demonstrated that repeated severe clipping (100% stem removal) over three consecutive years had a negative effect on mature shrubs that was greater in the forest understory than in full light. Mature *L. maackii* shrubs exhibit a very large root ball that likely contributes to their tolerance of foliar herbivory. Juvenile *L. maackii* plants were more sensitive to high amounts of herbivory than mature plants, especially to real herbivory by a generalist caterpillar, but were still fairly tolerant of a one-time whole leaf removal. Additional bouts of major defoliation in either age class could eventually lead to chronic growth reductions as belowground resources are exhausted, which would likely be seen in young plants first. Limiting nutrients did not independently affect the growth of *L. maackii* very much, but in combination with defoliation and light limitation, RGR and R:S were affected. Limiting light reduced growth, but neither limited light nor limited nutrients affected the ability to tolerate defoliation. Given that *L. maackii* plants receive significantly lower amounts of arthropod herbivory in their novel range than the experimental levels that we imposed (3% observed vs. up to 50% imposed; Lieurance and Cipollini, 2012), current amounts of natural arthropod herbivory observed on *L. maackii* in the field appear incapable of affecting the performance of plants in most environments where they grow. However, amounts of damage occurring by white-tailed deer has not been assessed since the 1990’s when Trisel (1997) estimated approximately 1.7% damage due to browse and based on personal observations. This estimate is much lower than amounts we observed
(D. Lieurance, pers. obs.). Additionally, we were not able to assess the effect of herbivory on the reproductive output of the plants, which could have been affected more than vegetative growth.

High tolerance to herbivory has been observed in many woody invasive plants. Ashton and Lerdau (2008) found evidence that invasive vines, including *Lonicera japonica, Celastrus orbiculata, Ampelopsis brevipedunculata*, and *Clematis terniflora* were more tolerant than native congeners and con-familials to simulated herbivory treatments in greenhouse conditions. Non-native plants quickly replaced aboveground components that allowed them to maintain a similar R:S to what they had before simulated herbivory. In multiple studies, invasive ecotypes of *Triadica sebifera* (formerly *Sapium sebiferum*) exhibited increased tolerance to simulated and real herbivory compared to native ecotypes. Simulated herbivory did not decrease RGR of *Triadica* seedlings (Rogers and Siemann, 2002), and neither timing nor type of herbivory (low intensity-chronic defoliation, high intensity-acute defoliation) or resource limitations (nitrogen and light) affected growth of seedlings in greenhouse or field experiments (Rogers and Siemann, 2003). Even though we observed undercompensation in total biomass in response to herbivory in both greenhouse experiments, our results tend to be consistent with these findings. Furthermore, R:S increased with simulated herbivory in the first experiment and both real and simulated herbivory and nutrient limitation in the second. Increases in R:S could simply be the result of removing some aboveground biomass. However, when estimated amounts of leaf biomass removed in either experiment were added back to total remaining biomass, the results were the same indicating that changes in R:S were a direct response to defoliation and resource
availability. With increased R:S, plants have a greater allocation of biomass belowground providing more roots for nutrient acquisition and storage reserves necessary for regrowth after defoliation (Karban and Baldwin, 1997; Orians et al. 2011).

Light was the most important limiting resource for biomass accumulation and allocation patterns, especially in combination with defoliation in greenhouse experiment 1. This is consistent with studies illustrating the shade intolerance of *L. maackii* (Luken et al. 1995; 1997). Conversely, a 50% reduction in nitrogen in greenhouse experiment 1 and 100% reduction in all fertilization in greenhouse experiment 2 did not independently affect the growth of *L. maackii* plants, suggesting that they are tolerant of low nutrient habitats. Allocation to roots, as revealed by elevated R:S ratio under low nutrients may have been a compensatory response. This relative lack of response to limited nutrients during the experiment may also be explained by the initial fertilization regime of seedlings in the weeks preceding initiation of the experimental treatments. Overall, our results indicate that even though light reduced total biomass, there was little effect on tolerance. Tolerance to both low light and low nutrient conditions, would suggest that even if future herbivore damage increases with higher herbivore loads, host switching by native herbivores, or increases in mammalian herbivory (e.g. by deer), *L. maackii* plants are well equipped to tolerate herbivory regardless of light and nutrient availability, at least until herbivory becomes severe (Luken and Mattimiro 1991).

Constitutive and induced defense traits in plants can be influenced by resource availability (Cipollini and Bergelson, 2001; Barto et al. 2008) and can vary through time (Gripenberg et al. 2007; Witzell et al. 2007). Results of the chemical analyses did not generally support our prediction that defoliation would increase total protein, POD, and
total flavonoid content, other than an increase in flavonoids noted in defoliated young plants in high light conditions. Since we sampled for chemistry at the end of the experiment in greenhouse experiment 1, we likely could not detect much induction, but in greenhouse experiment 2 we sampled 3 days after damage was inflicted and also did not observe any increases associated with damage. There was also minimal support for our prediction that resource limitation would affect leaf chemistry, but our results supported the prediction that quantities would change through time. In the second greenhouse experiment, temporal changes were larger than any treatment-induced changes in these traits, with total flavonoids and POD declining, and total protein increasing through time. In a previous study, foliar concentrations of luteolin (a flavonoid-derived compound) and its glycoside derivative were shown to decrease from the start to end of season in mature *L. maackii* plants (Cipollini et al. 2008b), paralleling what we observed in foliar total flavonoids in young plants here. However, our analyses of protein, peroxidase, and flavonoids were crude and did not distinguish between types of proteins or flavonoids, thus induced responses or specific defensive responses may not have been fully captured. More detailed studies are required to effectively explore how *L. maackii* plants respond chemically to leaf area loss and resource availability, but our foliar chemical measures of *L. maackii* appear to be seasonally and/or developmentally regulated, but relatively insensitive to environmental variability. Additionally, with no correlations between total protein, peroxidase, and total flavonoids with measures of growth, there appears to be no trade-off between growth and the aspects of plant chemistry that we measured. To further investigate the presence of a trade-off, future experiments should include a range of fertilization concentrations.
Our prediction that simulated and real herbivory would have similar effects was supported for all measured parameters, with the important exception of total biomass. Plants may respond differently to real herbivory than to simulated herbivory due to such factors as a plant responses to enzymes present in herbivore saliva and touch from herbivores (Bown et al. 2002; Musser et al. 2005; Erb et al. 2012; Wu and Baldwin 2012). However, we could not detect a significant chemical response to either simulated or real herbivory in the traits we measured, but other unmeasured variables could have revealed differences. Also, in the simulated herbivory treatment, we removed whole leaves, while real herbivory resulted in partial damage on more leaves and resulted in lower final biomass than simulated herbivory. This difference in effects may be because a sudden one time removal may stimulate the plant to prioritize resources to regrowth rather than costly chemical defenses whereas real herbivory takes more time and other responses may be triggered to compensate or defend against herbivory (Cipollini and Sipe, 2001). We detected no differences in rapid or delayed chemical responses between simulated or real herbivory in the variables that we measured, but other physiological traits associated with resistance or tolerance could have varied among treatments. Strauss and Agrawal (1999) stress that evidence of a plants ability to tolerate herbivory may vary among studies using simulated and real herbivory, and also with the type of herbivory (e.g., leaf or root feeding).

4.5 CONCLUSION
Because both tolerance and resistance mechanisms are thought to be costly to a plant, it has been suggested that plants will exhibit a trade-off between the two in response to herbivore damage, especially when limited by resources (van der Meijden *et al.*, 1988; Fineblum and Rausher 1995). *Lonicera maackii* appears to have the capacity to both resist and tolerate herbivory, thus tolerance and resistance are not mutually exclusive in this plant. But, considering the low amounts of herbivory incurred by *L. maackii* in the field, this trade-off is a non-issue as currently observed levels of damage by arthropod herbivores does not appear to be significant enough to impact the performance of the shrub in its invasive range. Chronic or severe damage by browsing animals, such as white-tailed deer, have more potential to affect growth of this shrub in the field, and juvenile plants are more likely to be impacted than mature shrubs.
4.6 References


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Table 4.1 Results of a 3-way ANOVA evaluating the effect of light, nitrogen fertilization, and simulated herbivory on growth and allocation patterns of juvenile *Lonicera maackii* plants in greenhouse experiment 1 evaluating the effects of simulated herbivory across light and nitrogen gradients.
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Table 4.2 Results of a 3-way ANOVA evaluating the effect of light, nitrogen fertilization, and simulated herbivory on foliar chemistry of juvenile *Lonicera maackii* plants in greenhouse experiment 1 evaluating the effects of simulated herbivory across light and nitrogen gradients.
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<tr>
<td>POD</td>
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<td>0.5965</td>
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<tr>
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<td>Light</td>
<td>1</td>
<td>3.07</td>
<td>0.0854</td>
</tr>
<tr>
<td></td>
<td><strong>Fertilizer*Light</strong></td>
<td>1</td>
<td><strong>4.12</strong></td>
<td><strong>0.0475</strong></td>
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<tr>
<td></td>
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<td>0.23</td>
</tr>
<tr>
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<td>Fertilizer*Defoliation</td>
<td>1</td>
<td>0.02</td>
<td>0.892</td>
</tr>
<tr>
<td></td>
<td>Light*Defoliation</td>
<td>1</td>
<td>3.14</td>
<td>0.0821</td>
</tr>
<tr>
<td></td>
<td>Fertilizer<em>Light</em>Defoliation</td>
<td>1</td>
<td>0.72</td>
<td>0.3986</td>
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<tr>
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<td>0.3922</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td></td>
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</tr>
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<td>Defoliation</td>
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</tr>
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<td></td>
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<td>0.3151</td>
</tr>
<tr>
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<tr>
<td></td>
<td>Fertilizer<em>Light</em>Defoliation</td>
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</tr>
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</table>
Table 4.3 Results of a 2-way ANOVA evaluating the effect of defoliation treatment (control, simulated, real) and fertilization on growth and allocation patterns juvenile *Lonicera maackii* plants in Greenhouse Experiment 2 evaluating the effects of simulated and real herbivory across a nutrient gradient.

<table>
<thead>
<tr>
<th>Response</th>
<th>Effect</th>
<th>DF</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Biomass</td>
<td><strong>Defoliation</strong></td>
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<td>8.88</td>
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</tr>
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<td></td>
<td>Fertilizer</td>
<td>1</td>
<td>1.48</td>
<td>0.2310</td>
</tr>
<tr>
<td></td>
<td>Defoliation*Fertilizer</td>
<td>2</td>
<td>1.71</td>
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</tr>
<tr>
<td>R:S</td>
<td><strong>Defoliation</strong></td>
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</tr>
<tr>
<td>RGR</td>
<td><strong>Fertilizer</strong></td>
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<td>27.55</td>
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</tr>
<tr>
<td></td>
<td>Defoliation*Fertilizer</td>
<td>2</td>
<td>2.85</td>
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<tr>
<td>SLA</td>
<td><strong>Defoliation</strong></td>
<td>2</td>
<td>0.77</td>
<td>0.4616</td>
</tr>
<tr>
<td></td>
<td>Fertilizer</td>
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<td>0.5816</td>
</tr>
<tr>
<td></td>
<td>Defoliation*Fertilizer</td>
<td>2</td>
<td>0.1</td>
<td>0.9069</td>
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</table>
Table 4.4 Results of a 3-way ANOVA evaluating the effect of sampling time, defoliation treatment (control, simulated, real) and fertilization on the foliar chemistry of juvenile *Lonicera maackii* plants in Greenhouse Experiment 2 evaluating the effects of simulated and real herbivory across a nutrient gradient.

<table>
<thead>
<tr>
<th>Response</th>
<th>Effect</th>
<th>DF</th>
<th>F Value</th>
<th>P Value</th>
</tr>
</thead>
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<td>time*defoliation</td>
<td>2</td>
<td>3.32</td>
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</tr>
<tr>
<td></td>
<td>fertilization</td>
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<td>0.7566</td>
</tr>
<tr>
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<td>time*fert</td>
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</tr>
<tr>
<td></td>
<td>trt*fert</td>
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<td>0.18</td>
<td>0.8316</td>
</tr>
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<td></td>
<td>time<em>trt</em>fert</td>
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<td>0.97</td>
<td>0.384</td>
</tr>
<tr>
<td>POD</td>
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<td>Defoliation</td>
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</tr>
<tr>
<td></td>
<td>trt*fert</td>
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<tr>
<td></td>
<td>time<em>trt</em>fert</td>
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<td>1.05</td>
<td>0.356</td>
</tr>
<tr>
<td>Total Flavonoid</td>
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<td>12.92</td>
<td>0.0007</td>
</tr>
<tr>
<td></td>
<td>Defoliation</td>
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</tr>
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<td></td>
<td>time*defoliation</td>
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</tr>
<tr>
<td></td>
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<td>3.41</td>
<td>0.0698</td>
</tr>
<tr>
<td></td>
<td>time*fert</td>
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</tr>
<tr>
<td></td>
<td>trt*fert</td>
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<td>0.6596</td>
</tr>
<tr>
<td></td>
<td>time<em>trt</em>fert</td>
<td>2</td>
<td>1.44</td>
<td>0.2453</td>
</tr>
</tbody>
</table>
Figure 4.1 Total dry biomass (A), root to shoot ratios (R:S)(B), stem relative growth rate (RGR)(C), and specific leaf area (SLA)(D) of damaged and undamaged juvenile *L. maackii* plants grown in high (100% ambient) or low (50% ambient) light, and high (HN) or low (LN) nitrogen in greenhouse experiment 1 evaluating the effects of simulated herbivory across light and nitrogen gradients. Means ± 1 standard error are shown.
Figure 4.2 Total protein concentration (A), peroxidase activity (POD)(B), and total flavonoid concentration (C) measured at time of harvest of juvenile *L. maackii* plants from greenhouse experiment 1 evaluating the effects of simulated herbivory across light and nitrogen gradients. HN and LN indicate the high nitrogen and low nitrogen treatments respectively. Means ± 1 standard error of total protein concentrations and POD are presented by fertilization (HN and LN) and light (high and low) treatment, as only their interaction had a significant effect. Means ± 1 standard error of total flavonoid concentrations are presented by light and defoliation (0% and 50% defoliation) treatment, as only their interaction had a significant effect.
Figure 4.3 Total dry biomass (A), root to shoot ratios (R:S)(B), stem relative growth rate (RGR)(C), and specific leaf area (SLA)(D) of damaged and undamaged juvenile *L. maackii* plants grown in full or no fertilization in Greenhouse Experiment 2 evaluating the effects of simulated and real herbivory across a nutrient gradient. Means ± 1 standard error are shown.
Figure 4.4 Total protein concentrations in leaves of damaged and undamaged juvenile *Lonicera maackii* plants measured 3 days after defoliation treatment (initial) and at time of harvest (final) in Greenhouse Experiment 2 evaluating the effects of simulated and real herbivory across a nutrient gradient. Means ± 1 standard error are shown.
5. Secondary defensive chemistry and the resistance of native and non-native Lonicera species grown in high and low nutrient conditions

5.1 INTRODUCTION

Many hypotheses have been proposed to explain how invasive plant species can go from being benign members of the community in their native range, to dominant competitors in their novel range, including enemy release, shifting defense hypothesis, the evolution of increased competitive ability, and novel weapons hypothesis (Blossey and Nötzold 1995; Keane and Crawley 2002; Calloway and Ridenour 2004; Müller-Schärer et al. 2004; Mitchell et al. 2006; Catford et al. 2009). The novel weapons hypothesis suggests that some successful plant invaders possess biochemical novelty in their new environment in either the type or quantity of bioactive compounds, toward which their native plant competitors are unadapted, thus giving the invader a competitive advantage (Calloway and Ridenour 2004; Calloway et al. 2005; Inderjit et al. 2006; Cipollini et al. 2008). Plant invaders may also possess novelty in their overall profile of secondary metabolites, even if some of the individual compounds are not novel in the new habitat (Barto et al. 2010).

The notion of novel weapons has been most often explored in the context of belowground processes. The direct allelopathic suppression of naïve plant competitors by
non-native invaders has been observed in many species resulting in delayed seed germination, inhibited growth, and increased mortality (Dorning and Cipollini 2006; Thorpe et al. 2009; McEwan et al. 2010). These effects have been linked to changes in below ground microbial interactions, specifically the inhibition in viability and infectivity of beneficial mycorrhizal fungi (Callaway et al. 2008; Cipollini et al. 2012). While below ground effects of novel weapons are well documented, they have not been as thoroughly explored for plant-herbivore interactions and current research mainly investigates herbivore performance. For example, results of a laboratory feeding assay indicate North American generalist herbivores had significantly lower relative growth rates than European generalists feeding on the invasive Centaurea stoebe growing in North America illustrating the resistance of a non-native plant to novel herbivores (Schaffner et al. 2011). Studies investigating performance and defensive chemistry rarely connect fitness of herbivores with more than one or two isolated compounds, or focus on limited species comparisons. No studies have investigated novelty of resistance trait profiles through multivariate comparisons of chemical profiles of multiple native and non-native congeners.

Plants produce a suite of secondary metabolites that serve a range of functions, including protection against photodamage, and resistance in the form of microbial inhibition, and herbivore defense (e.g. San Francisco and Cooper-Driver 1984; Close and McArthur 2002; Cipollini et al. 2008). Along with phylogenetic constraints, in most cases, the quantities and profiles of antiherbivore defenses in plants are shaped by co-evolution with herbivores present in their native habitat (Mauricio and Rausher 1997; Rausher 2001; Cornell and Hawkins 2003) and when plants are introduced to new
habitats, their defensive chemistry may differ from profiles present in related species in their novel range. This novelty may allow them to escape or resist herbivores present in their novel range (Lieurance and Cipollini 2013), and may explain why selection may favor maintenance of costly herbivore defense traits in invasive plants even in the absence of specialist herbivores (Cipollini and Lieurance 2012).

There are 18 native and 16 introduced species of the genus *Lonicera* (Caprifoliaceae) in North America (Kartesz and Meacham 1999; Zheng et al. 2006). Non-native *Lonicera* species such as *L. japonica*, *L. tatarica*, and *L. maackii* have become widely established and dominate forest edges and understories, roadsides, and other marginal habitats in North America (Woods 1993; Luken and Thieret 1996; Hutchinson and Vancat 1998; Schierenbeck 2004). Invasive traits possessed by non-native *Lonicera* include an apparent release from natural enemies (Schierenbeck et al. 1994; Waipara et al. 2007; Lieurance and Cipollini 2012; 2013), evidence of allelopathic suppression of other plants (Skulman et al. 2004; Dorning and Cipollini 2006; Cipollini et al. 2008; McEwan et al. 2010), high aboveground growth rates (Luken et al. 1997; Lieurance 2004), extended leaf phenology (Schierenbeck and Marshall 1993; Trisel 1997; McEwan et al. 2009), abundant fruits that are dispersed by birds, deer, and mice (Vellend 2002; Drummond 2005; Bartuszevige and Gorchov 2006; McCay et al. 2009), and the production of secondary metabolites associated with anti-herbivore defense (Cipollini et al. 2008). Less is known about other commercially available non-native *Lonicera* species like *L. fragrantissima* and *L. xylosteum*. In contrast, *Lonicera* species native to North America, including *L. reticulata*, *L. sempervirens*, and *L. flava*, are relatively uncommon or not abundant across their range, and are listed as rare, threatened, or endangered in
parts of their range (e.g. Hill 2003a; Hill 2003b; http://plants.usda.gov). Little ecological research has been conducted on native *Lonicera* species, and most work includes comparisons with non-native congeners (e.g. Schierenbeck et al. 1994; Lieurance and Cipollini 2013). Although no studies have been conducted in the native range of invasive *Lonicera* species, studies in the invasive range in North America indicate reduced damage levels on exotic *Lonicera* species relative to congeners and sympatric species (Trisel 1997; Schierenbeck et al. 1994; Lieurance and Cipollini 2013). This may be due to a release from specialist natural enemies, resistance to herbivores present in the invaded range, or some combination of both.

Because they have been linked (but not limited) to allelopathic interactions and have been detected in the *Lonicera* genus, the classes of compounds of particular interest in this study are phenolics and their glycoside derivatives. Phenolic compounds, including phenolic acids like chlorogenic acid, flavones like apigenin and luteolin and their glycoside derivatives have been linked to the inhibition of seed germination, herbivore deterrence, and reduced herbivore performance (Felton et al. 1992; Chaves et al. 2001; Cipollini et al. 2008). Phenolics can reduce the digestibility of plant material, cause reductions in growth, and increases in mortality for many herbivores (Felton et al. 1992; Cipollini et al. 2008). *Spodoptera littoralis* larvae showed a preference for undamaged over damaged alfalfa plants that were characterized with increases in defensive saponins and the flavone, apigenin (Agrell et al. 2003). Irioid glycosides such as loganin, aucubin and catapol are phenolic-based compounds with an attached glucose molecule that can serve an important role for many oligophagous and monophagous herbivores including sequestration for defense against predators, oviposition cues, and
feeding attractants important for finding plant hosts (Bowers 1991; Reudler Talsma et al. 2008; Lampert and Bowers 2010). In one example, up to 8% of the larval mass of *Calphasia lunula*, a specialist biocontrol agent was composed of the irio id glycoside antirrhinoside that was sequestered from their host plant *Linaria dalmatica* (Jamieson and Bowers 2010). Aucubin was linked to oviposition preference when the specialist *Melitaea cinxia* preferred to oviposit on *Plantago lanceolata* plants producing more aucubin than the plants receiving no oviposition (Reudler Talsma et al. 2008). Iridoid glycosides can have a very different interaction with generalist herbivores, acting as a feeding deterrent, inhibiting larval development, or they can be toxic (Puttick and Bowers 1988). When generalist *Spodoptera eridania* larvae were reared on artificial diets treated with various iridoid glycoside compounds, survivorship and growth rates were reduced, and when given a choice, larvae consistently avoided diet containing the iridoid glycosides (Puttick and Bowers 1988). Clearly, the presence or absence of specific secondary metabolites can be integral in plant-plant, plant-fungal, plant-insect (specialist and generalist) interactions, which may result in a competitive advantage for chemically defended introduced plant species.

Secondary metabolites associated with resistance have been identified in *Lonicera* species, including, apigenin, luteolin, and their glycoside derivatives, chlorogenic acid, and other phenolics (Cipollini et al. 2008; Ochmian et al. 2009; Ren et al. 2008). Additionally, a number of iridoid glycosides have been identified in the *Lonicera* genus including secologanic acids associated with deterrence of generalist herbivores and feeding stimulation or oviposition cues for specialist herbivores (Song et al. 2006; Peñuelas et al. 2006). While many of the identified secondary chemicals found in
*Lonicera* are known to be in North America and therefore not novel, a comprehensive profiling of secondary metabolites of native and non-native *Lonicera* species has not been completed (Cipollini et al. 2008). What remains unanswered is whether non-native invasive species in this genus possess individual compounds or particular combinations of compounds that may affect their resistance to native arthropod herbivores.

Variation in abiotic factors such as light and nutrient availability can alter the production of secondary metabolites in woody plants, with carbon-rich phenolics increasing with increased light availability, but decreasing as nutrient availability increases (Herms 2002; Barber and Marquis 2011; Endara and Coley 2011). Decreasing quantities of secondary metabolites in nutrient rich conditions may seem counterintuitive because increased nutrients might imply that there would be no limitation on growth, and therefore no limitation on secondary metabolite production. However, because it is costly to replace photosynthetically active biomass in low nutrient conditions, plants may increase secondary metabolite production where growth is limited to protect against herbivore and pathogen damage (Bryant et al. 1983). For example, there was an 11% increase in total phenolic concentration in cuttings of *Populus nigra* growing in low nutrient treatments as compared to those grown in high nutrient conditions, and the larval growth of *Lymantria dispar* was 80% higher on plants growing in high nutrient conditions (Glynn et al. 2003). While we know secondary metabolite production can be reduced with fertilization, it is not clear whether nutrient availability affects the diversity of secondary metabolites.

To determine if novel defensive chemistry and increased resistance is one of the mechanisms for the success on non-native *Lonicera* species, we first quantified the
amount of herbivore damage occurring on native and non-native *Lonicera* grown in high and low fertilization in a common garden experiment. Next, we profiled and quantified phenolic acids, flavonoids and the derivatives, and iridoid glycosides produced in the leaves of these plants. We then compared the laboratory performance of the widespread generalist, the Fall Armyworm, *Spodoptera frugiperda* (Noctuidae) feeding on cut foliage of native and non-native *Lonicera* species collected in the same common garden. We predicted that: 1) native *Lonicera* species would incur more arthropod herbivore damage than non-native species and herbivore damage would increase with fertilization, 2) there would be differences in the performance of *Spodoptera* larvae by *Lonicera* species, but not by geographic origin, 3) number of days alive, relative growth rate (RGR), and pupal mass would increase and days to pupation (DTP) would decrease with fertilization, 4) the quantity and diversity of compounds would vary by species and non-native *Lonicera* species will possess phenolic and iridoid glycoside compounds not present in the North American *Lonicera* species, and 5) the quantity and diversity of compounds would increase with fertilization.

### 5.2 MATERIALS AND METHODS

#### 5.2.1 Common Garden design and herbivory assessment

During the first week of June 2010, a common garden experiment was established on the campus of Wright State University, Dayton, Ohio with blocks of high and low fertilization treatment and including 5 non-native and 3 native species of *Lonicera* (Table 5.1). A 30 m by 30 m plot was tilled and a grid was set up and marked every 1.5 m. One
axis was labeled alphabetically A-U, and the other was labeled numerically 1-20 to form a grid where plants were to be planted at each crossing point with a unique id. Species were randomly assigned to grid points using a random number generator within blocks of high and low fertilization with 8 biological replicates per species and treatment combination. Due to the difficulty in acquiring and germinating seeds of multiple Lonicera species, some species were grown from seed, plants of other species were collected from the field, and others were purchased as nursery stock from commercial sources (Table 5.1). The plot was watered as necessary until plants became established. The plot was weeded and mowed as necessary and an exclosure fence was constructed to prevent deer and other mammal damage. Plants that died in the first year were replaced when possible in 2011 and L. tatarica, L. xylosteum, and L. flava were added in 2011. Fertilization treatments were implemented in two ways. Approximately 150 g of time-release fertilizer (Osmacote 14-14-14 NPK, Everris International B.V., the Netherlands) was placed at the base of each plant in October 2010, April 2011, October 2011, and May 2012. The second method of treatment was a monthly application (between May and October) of either 600 ml of water for unfertilized blocks or 600 ml of 3.75 g/L Peters 20-20-20 complete soluble fertilizer plus micronutrients (Grace-Sierra, Milpitas, CA) for fertilized blocks.

A one time visual herbivory assessment was conducted from May 10 to May 16, 2012 on all plants. Three branches were haphazardly selected on each plant for the assessment. Percent leaf area removal was scored as 1, 2, 5 and then in increments of 5% to a maximum of 100% leaf area removed, as in Lieurance and Cipollini (2012). Leaves damaged by aphid herbivory were scored based on the percentage of tissue that appeared
no longer photosynthetically active due to aphid feeding. Because these estimates were more subjective, herbivory estimates were analyzed in separate analyses with and without aphid damage. Percent herbivore damage data were transformed using inverse square transformation after adding one to all values \([x+1]^2\) and data were analyzed using a full factorial ANOVA where independent and interactive effects of species and fertilization were compared in one analysis. Comparisons of means were made using Tukey post-hoc tests and planned contrasts were used to test the influence of origin (native vs. non-native; SAS V9.2, SAS Institute, Cary, North Carolina).

5.2.2 Generalist caterpillar feeding bioassays

Eggs of the generalist *Spodoptera frugiperda* were purchased from Benzon Research (Carlisle, PA, USA) and larvae were reared on an artificial *Spodoptera* diet (Southland Products, Inc., Lake Village, AK, USA) in an incubator at 22°C with a photoperiod of 16:8 (L:D) for 7 days. At the start of the experiment, larvae were starved for 12h, weighed, and placed in individual 4x6x6 cm plastic feeding arenas. There was no difference in the mean initial mass of larvae exposed to different plant species at the start of the experiment \((F_{6,98}=0.72, P=0.6372)\). Each feeding arena was randomly assigned one of seven *Lonicera* species: *L. reticulata* (native), *L. sempervirens* (native), *L. maackii* (non-native), *L. tatarica* (non-native), *L. japonica* (non-native), *L. fragrantissima* (non-native), and *L. xylosteum* (non-native) with 15 replicates per species for a total of 105 individual arenas. *Lonicera flava* was not included because plants in the garden were not large enough to supply enough cut foliage for this assay. Cut foliage of each *Lonicera* species was collected and replaced as needed from *Lonicera* plants grown in the fertilized treatments in the common garden. The feeding arenas were maintained in an incubator at
22°C with a photoperiod of 16:8 (L:D), and weighed every 2-3 days. Insects reaching pupation were removed from the arena and weighed. Larval mass and number of days alive were recorded through time. Days to pupation and pupal mass were recorded for those that reached pupation. Also, relative growth rate was calculated as ln(mass\textsubscript{day25}) - ln(mass\textsubscript{initial})\/# of days on day 25 when larvae generally reached their peak masses. If larvae pupated before day 25, RGR was calculated using the peak mass before pupation and the number of days was adjusted accordingly in the equation. Larvae that died before RGR could be calculated were included in the model as zero RGR.

To determine the influence of fertilization on the larval performance of *Spodoptera* larvae, we conducted a second feeding assay. *Spodoptera frugiperda* larvae were reared same as above and starved for 12h, weighed, and placed in individual 4x6x6 cm plastic feeding arenas. The mean initial mass of larvae exposed to different plant species at the start of the experiment was equivalent (F\textsubscript{4,95}=1.27, P=0.2862). Each feeding arena was randomly assigned one of five *Lonicera* species grown in high and low fertilization treatments: *L. reticulata* (native), *L. sempervirens* (native), *L. maackii* (non-native), *L. tatarica* (non-native), and *L. fragrantissima* (non-native), with 10 replicates per species for a total of 100 individuals. Due to the poor performance of caterpillars on *L. japonica* and *L. xylosteum* in the previous assay, we excluded theses species for the second experiment. Cut foliage from fertilized and unfertilized plants was collected, supplied to larvae, and replaced as before, and larval performance was followed as before. We calculated relative growth rate as ln(mass\textsubscript{day21}) - ln(mass\textsubscript{initial})\/# of days on day 21 when larvae generally reached their peak masses. Again, RGR for larvae pupating before day 21 was calculated with the peak weight before pupation and the number of
days was adjusted accordingly in the equation. Larvae that died before RGR could be calculated were treated as zeros in the model.

The number of days alive, relative growth rates, days to pupation (DTP), and pupal mass from the first assay were compared among plant species using a 1-way ANOVA. Number of days alive was log transformed and relative growth rates were log transformed after adding 0.1 to all values. Pupal mass and days to pupation had a normal distribution and no transformation was necessary. Data from assay 2 including a fertilization treatment were analyzed using a 2-way ANOVA where independent and interactive effects of plant species and fertilization treatment were compared. The only unfertilized species fed to larvae that resulted in pupation were *L. tatarica* and *L. fragrantissima*. For this reason, we analyzed pupal mass and DTP with a 1-way ANOVA and only included those individuals reaching the pupal stage. Again, the number of days alive was log transformed, RGR was log transformed after adding 0.1 to all numbers, and DTP and pupal mass met the assumptions of normality. Comparisons of means were made using Tukey post-hoc tests and planned comparisons were used to test the influence of origin. All statistical analyses were performed using SAS (Version 9.2, SAS Institute, Cary, North Carolina).

### 5.2.3 Quantification and Identification of Soluble Phenolic Compounds with UPLC

In mid-September 2011, whole leaf samples (2-3 leaves per plant) were taken haphazardly from separate branches on three biological replicates of each species in the common garden in high and low fertilization blocks. Samples were immediately placed
on ice until they could be brought to the laboratory. Once they reached the laboratory, they were placed in the freezer at -20° C for phenolic and iridoid glycoside profiling. Soluble phenolic extracts were made by homogenizing 100 mg of leaf material and extracting it twice in 0.5 ml of 100% HPLC grade methanol for 24 h in the dark at 4° C. Extracts were centrifuged for 5 minutes at 13,400 x g and the supernatants were pooled for a total sample of approximately 1 ml of extract. Extracts were kept at -20 ° C until analysis.

Quantification and identification of phenolic compounds in native and non-native *Lonicera* species was performed using ultra performance liquid chromatography (UPLC) on an Waters Acquity H-class 1200 series (Waters, Milford, MA, USA) equipped with an autosampler, and a Photodiode Array Detector (Waters, Milford, MA, USA). The autosampler and column temperatures were set at 4 and 40 ° C respectively. The binary mobile phase consisted of water/acetic acid (A) (98:2, v/v) and methanol/acetic acid (B) (98:2, v/v), with a flow rate of 1 ml/min. The elution program was translated by Waters Empower 3 (Waters, Milford, MA, USA) to a compatible UPLC method from the Eyles et al. (2007) HPLC elution gradient. The injection volume was 1ml. Samples were passed through a Photodiode Array Detector (PDA) (scanning range, 200–400 nm) and periodic quality checks were conducted by running standards throughout the analysis (% relative standard deviation <5%). Quantities of four compounds (chlorogenic acid, luteolin-7-glucoside, luteolin, and apigenin) were determined using a standard curve of authentic standards. Peak areas of all known and unknown compounds determined at a wavelength of 280 nm were used to compare total phenolics among species and treatments and in the principle components analysis.
5.2.4 Quantification and Identification of Iridoid Glycosides with GC/MS

Methods for extraction of iridoid glycosides from native and non-native *Lonicera* species were modified from previously published studies (Bowers and Stamp, 1993; Gardner and Stermitz, 1988). Approximately 100 mg of fresh leaf sample was ground in liquid nitrogen. Dried ground leaf samples were weighed in approximately 50 mg aliquots, and the exact weight was recorded to the nearest 0.01 mg. Each aliquot was placed in a test tube with 5 mL methanol, vortexed, and left overnight for extraction. The plant material was then filtered, and the remaining extract was evaporated to dryness. Extracts were then re-suspended in 3 mL water, and an internal standard (phenyl-β-D-glucopyranoside) was added to each sample. Samples were then partitioned three times against equal volumes of diethyl ether. The ether fractions were discarded, and the water fraction, containing mostly IGs and sugars, was evaporated to dryness. The residue was then re-suspended in 1 mL methanol, left overnight to allow complete dissolution of IGs into the solvent, vortexed, and 100 µL aliquots were transferred to micro-inserts for GC vials and evaporated to dryness at 50°C.

Aliquots for IG analysis were converted to their TMSi analogs using Tri-Syl-Z derivatizing reagent (Thermo-Scientific) by adding 100 µL of the reagent to the sample and heating for 20 minutes at 70°C in a mineral oil bath. Within 24 hours of derivatization, 0.2 µL of each sample was injected onto an HP Agilent 6890N GC coupled with an Agilent 5975C inert mass selective detector with an ion source of 70eV at 230°C and equipped with a DB-1MS capillary column (15m x 0.25mm i.d. x 0.5 µm film thickness; Agilent Technologies). We used ultra-pure helium as the carrier gas at a flow rate of 2 mL/min, a split flow ratio of 100:1, and a front inlet temperature of 275°C.
Oven conditions were modified from previously described methods (Gardner and Stermitz, 1988) to ensure adequate peak resolution of IGs while minimizing the run time for each sample. The following oven conditions were employed: initial temperature 180°C, initial hold time 1 min; Ramp 1: 5°C/min to 200°C, hold time 11 min; Ramp 2: 2°C/min to 260°C, hold time 0 min; Ramp 3: 30°C/min to 320°C, hold time 0 min; for a total run time of 48 minutes. A blank sample (Tri-Sil-Z only) was run after every five samples to ensure there was no carryover between runs. Data were recorded and processed using MSD ChemStation software (version D.02.00.275).

Iridoid glycosides were identified whenever possible by comparisons of retention times and mass spectral data with authentic reference standards. For unknown peaks that did not match any available standards, mass spectral data were examined for characteristic peaks that distinguish iridoid glycosides from other substances as described in Inouye et al. (1976) and Popov and Handjieva (1983). Quantities of all compounds that were identified as iridoid glycosides were then determined based on peak areas in total ion current chromatograms. A six-point calibration curve was created using authentic reference standards for all available iridoid glycosides: loganin, secologanin, loganic acid, sweroside, secoxyloganin, and morroniside. Concentrations for other compounds were estimated using the internal standard. Peak areas of known and unknown compounds were used to compare profiles in a principle components analysis.

5.2.5 Statistical Analyses of Chemical Profiles
Differences in chemical profiles among honeysuckle species (based on peak areas of compounds) were evaluated with Principal Components Analysis (PCA) in R (Version 2.15.1; R Development Core Team, 2009). Hypothesis tests associated with factors (species, origin, and fertilization) were performed using permutational multivariate analysis of variance (PERMANOVA; Anderson 2001) in the 'vegan' library (version 2.0-4; Oksanen et al. 2012). Two analyses were performed: the first including all 30 phenolic compounds and the second including all 44 iridoid glycoside compounds. Analyses were performed on euclidean distances of Hellinger-transformed data (Legendre & Gallagher 2001). When testing for effects associated with the invasive status of species, permutations were constrained so that replicates within each species were permuted in groups; this accounts for the non-independence among replicates within species and avoids pseudoreplication (Hurlbert, 1984).

To compare the total phenolics among species and treatments, the sums of all peak areas of detected phenolic compounds for each replicate were calculated and then square root transformed. Quantities of chlorogenic acid, luteolin, luteolin-7-glucoside, apigenin were log transformed. Total phenolics and quantities of selected phenolics were analyzed using a 2-way ANOVA on the independent and interactive effects of plant species and fertilization. Comparisons of means were made using Tukey post-hoc tests and planned contrasts were used to test the influence of origin. Total iridoid glycosides were arcsine transformed and loganin, loganic acid, secologanin, secoxyloganin, and swerocide were log transformed. Total IGs and quantities of selected IGs were analyzed as for phenolics with a 2-way ANOVA with Tukey post-hoc tests and planned contrasts to compare means. Planned contrasts for phenolics and iridoid glycosides were
completed with and without *L. japonica* because of its phylogenetic isolation (Theis et al. 2008) and its distinctive profile appeared to heavily influence the PCA results.

5.3 RESULTS

5.3.1 *Common Garden Herbivory Assessment*

Herbivore damage varied by species (*F*<sub>7,203</sub>=6.09, *P*<0.0001), but not with fertilization treatment (*F*<sub>1,203</sub>=1.86, *P*=0.17), but there was a significant interaction between species and fertilization (*F*<sub>7,203</sub>=2.26, *P*=0.03) (Figure 5.1). Of all species, *L. sempervirens* had the highest amount of damage, *L. flava* and *L. reticulata* received intermediate damage, and the non-native *Lonicera* species received the least amount of damage. Damage on *L. flava*, *L. japonica*, *L. fragrantissima* increased by 8.8, 2.5, and 2.3 fold respectively with fertilization, but was 1.4 fold higher under low fertilization treatments for both *L. sempervirens* and *L. maackii*. There was no change in damage with fertilization treatment for *L. reticulata*, *L. tatarica*, or *L. xylosteum*. There were differences in origin (*F*<sub>1,233</sub>=25.52, *P*<0.0001) and overall damage on native *Lonicera* was 9.5 fold higher than damage on non-native *Lonicera*, which received less than 1% foliar damage in both fertilized and unfertilized treatments. When aphid damage was added in, damage varied by species (*F*<sub>7,203</sub>=28.61, *P*<0.0001), fertilization treatment (*F*<sub>1,203</sub>=6.68, *P*=0.01), and there was a significant interaction between species and fertilization (*F*<sub>7,203</sub>=3.30, *P*=0.002). Overall damage was higher in fertilized treatments and aphid damage added 10.25% and 7% foliar damage on *L. sempervirens* and *L. reticulata* respectively.
5.3.2 Generalist Herbivore Feeding Bioassay

In the first no-choice bioassay, the number of days alive varied by *Lonicera* species ($F_{6,98}=6.25$, $P<0.0001$; Figure 5.2A). Larvae feeding on *L. sempervirens* and *L. fragrantissima* survived the longest, while larvae feeding on *L. reticulata*, *L. japonica* and *L. xylosteum* lived the shortest amount of time; *L. maackii* and *L. tatarica* lived an intermediate amount of days. Native/non-native origin of plants fed to larvae did not influence number of days alive ($F_{1,103}=1.42$, $P=0.24$). Relative growth rate (RGR) varied by species ($F_{6,98}=5.29$, $P<0.0001$; Fig 5.2B) and there was no difference relative to the origin of the species fed to larvae ($F_{1,103}=0.04$, $P=0.84$). Larvae grew fastest on *L. fragrantissima*, grew intermediately on *L. maackii*, *L. tatarica*, and *L. japonica*, and grew the slowest on native *L. sempervirens*, *L. reticulata* and *L. xylosteum*. Pupal mass of those larvae reaching pupation did not differ by species ($F_{3,23}=0.99$, $P=0.42$) with an overall mean of $0.125\pm0.004$ mg across species (Figure 5.2C). Origin did no significantly affect pupal mass ($F_{1,25}=0.95$, $P=0.34$). Days to pupation varied by species ($F_{3,25}=21.24$, $P\leq0.0001$). Larvae feeding on *L. fragrantissima* and *L. tatarica* reached pupation approximately 10 days sooner than those feeding on *L. sempervirens* and *L. maackii* (Fig 5.2D). There was a significant effect of origin of *Lonicera* species fed to larvae on days to pupation ($F_{1,30}=16.34$, $P=0.0004$) and larvae reached pupation 5 days faster on non-native *Lonicera*.

When fertilization treatment was included to the second no-choice bioassay, the number of days alive varied by species ($F_{4,90}=3.24$, $P=0.003$), but only tended to be affected by fertilization treatment ($F_{1,90}=2.90$, $P=0.09$) and the interaction between
species and fertilization ($F_{4,90}=2.16$, $P=0.08$). Larvae lived the longest on *L. tatarica* and *L. fragrantissma*, the shortest on *L. sempervirens* and an intermediate number of days on *L. maackii* and *L. reticulata* (Figure 5.3A). Although independent or interactive effects of fertilization were not significant overall, the number of days alive decreased by 19 days and 12 days for larvae fed unfertilized *L. reticulata* and *L. maackii* respectively. There was a significant effect of origin on number of days alive ($F_{1,98}=5.54$, $P=0.02$). Larvae feeding on non-native *Lonicera* species lived an average of 9 days longer than those feeding on native *Lonicera* and no larvae feeding on native *L. reticulata* or *L. sempervirens* or non-native *L. maackii* growing in low fertilization survived to pupation.

There were significant differences in RGR by species ($F_{4,90}=7.90$, $P<0.0001$) and fertilization treatment ($F_{1,90}=8.80$, $P=0.004$), but there was no interaction between species and treatment ($F_{4,90}=0.57$, $P=0.68$). RGR of larvae followed the same pattern by species as number of days alive with highest RGR of larvae fed *L. tatarica* and *L. fragrantissima*, intermediate RGR on *L. maackii* and *L. reticulata*, and the lowest RGR on *L. sempervirens* (Figure 5.3B). Overall, there was a 2 fold increase in RGR for larvae on foliage from fertilized plants, except from *L. tartarica*, and no larvae that were fed unfertilized *L. reticulata*, and *L. maackii* lived long enough to calculate RGR (Figure 5.3B). There was a 2.7 fold increase in RGR for larvae fed non-native compared to native *Lonicera* ($F_{1,98}=9.50$, $P=0.003$). Pupal mass did not differ by species ($F_{4,27}=2.31$, $P=0.08$) or origin ($F_{1,30}=0.95$, $P=0.34$) with an overall mean pupal mass of $0.115±004$ g (Figure 5.3C). Days to pupation (DTP) varied by species ($F_{4,27}=3.76$, $P=0.01$) and origin ($F_{1,30}=16.34$, $P=0.0004$) with the shortest time to pupation for larvae feeding on *L. tatarica*, an intermediate time to pupation on *L. sempervirens*, *L. maackii*, and *L.
fragrantissima and the longest time to pupation on L. reticulata. Surviving larvae on non-native Lonicera species pupated 5 days sooner than on native Lonicera species (Figure 5.3D).

5.3.3 Chemical Analysis and Profiling

Principal component analysis indicated a strong effect of species, explaining 65% of the variation in phenolic profiles of Lonicera species, but fertilization or the interaction between species and fertilization explained an insignificant amount of variation (Figure 5.4A). Species origin (native/non-native) explained only 10% of the variation in profiles and was not significant. With negative loadings, chlorogenic acid and unknown 10, and with positive loading unknown 11 had the strongest effects on the first axis. Luteolin-7-glucoside and unknown 20 had the strongest effects on axis two with negative and positive loadings, respectively. The remaining compounds had either intermediate or indiscernible effects on the analysis. There was separation between profiles of L. japonica, L. fragrantissima, L. xylosteum, L. sempervirens, and L. reticulata, while the profiles of L. maackii, L. tatarica, and L. flava overlapped with each other. Out of the 30 possible compounds detected in our analysis, there were 2 possible unique compounds detected in native Lonicera species and 4 possible unique compounds in non-native Lonicera (Table 5.2). When peak areas of phenolic compounds were combined as a measure of total phenolics, total peak area varied by species ($F_{7,28}=4.22$, P=0.003), but not by fertilization ($F_{1,32}=2.94$, P=0.10), or any interaction between species and fertilization ($F_{7,32}=1.21$, P=0.33). Lonicera maackii had the highest total peak area (indicating the most phenolics), L. flava, L. reticulata, L. tatarica, L. fragrantissima, and
L. xylosteum had intermediate values, and L. japonica and L. sempervirens had the lowest total phenolics. There was no significant difference relative to origin ($F_{1,42}=0.65, P=0.42$) but total peak area of phenolics was 1.3 fold higher in non-native Lonicera species. The individual compounds chlorogenic acid, luteolin-7-glucoside, luteolin, and apigenin followed the same pattern and varied by species only, and not by fertilization or species interacting with fertilization (Table 5.3). Lonicera sempervirens had the lowest amounts of all compounds except for apigenin, for which it had the highest values measured. Quantities of compounds in L. maackii were consistently high. Origin had no effect on any specific compound, but when L. japonica was removed from the model, origin was significant for luteolin ($F_{1,37}=6.85, P=0.01$), which was 3.7 fold higher in non-native Lonicera than in native Lonicera. Concentrations of phenolic compounds tended to be higher in non-native species and varied with fertilization with increases in chlorogenic acid and luteolin in low fertilization and increases in luteolin-7-glucoside and apigenin in high fertilization.

Principal component analysis of iridoid glycoside profiles indicated a strong effect of species, explaining 47% of the variation among Lonicera species, but fertilization or the interaction between species and fertilization explained an insignificant amount of variation (Figure 5.4B). Species origin (native/non-native) explained only 11% of the variation in profiles and was not significant. The compounds secoxyloganin (negative), LBell_D (positive), and loganic acid (positive) had the strongest effects on the first axis and secologanin A and B (both positive) on the second axis. Of the 24 iridoid glycoside compounds included in the analysis, 7 unique compounds were detected in native Lonicera species (with 4 of those present exclusively in L. flava grown in low
fertilizer conditions) and non-native *Lonicera* contained only 2 possible unique iridoid glycoside compounds (Table 5.4). *Lonicera maackii*, *L. tatarica*, and *L. japonica* had no unique iridoid glycosides. There was a distinct separation in the profiles of *L. japonica* and *L. sempervirens*, profiles of *L. flava* and *L. reticulata* overlapped in one cluster, profiles of *L. maackii*, *L. tatarica*, *L. fragrantissima*, and *L. xylosteum* overlapped in a separate cluster. Overall, total iridoid glycosides varied by species (*F*<sub>7,32</sub>=4.55, *P*=0.001), but not with fertilization (*F*<sub>7,32</sub>=0.02, *P*=0.90), and there was no interaction between species or fertilization (*F*<sub>7,32</sub>=1.54, *P*=0.19). *Lonicera flava* contained the most iridoid glycosides, *L. japonica* contained intermediate amounts, and the remaining species had the lowest content of iridoid glycosides. Total iridoid glycosides varied by origin with (*F*<sub>1,46</sub>=4.47, *P*=0.04) and without (*F*<sub>1,40</sub>=8.17, *P*=0.007) inclusion of *L. japonica*. There was a 1.8 fold increase in iridoid glycoside content in native *Lonicera* species compared to non-native species and this increased to 2.3 fold when *L. japonica* was removed from the analysis. Only species had a significant effect on five of the six iridoids (excluding loganin) quantified by standard (Table 5.5) and these tended to increase in low fertilization and native species. *Lonicera flava* had the highest content of secoxyloganin, secologanin, swerocide, and secologanin. *Lonicera reticulata*, *L. maackii*, *L. tatarica*, *L. fragrantissima*, and *L. xylosteum* contained intermediate values for most compounds. *Lonicera sempervirens* contained no secologanin and *L. japonica* contained no secologanin or swerocide and lowest amounts of loganic acid.

### 5.4 DISCUSSION
It is known that non-native *Lonicera* species such as *L. maackii* and *L. japonica* in North America produce various secondary metabolites which may give them a competitive edge over native competitors through microbial defense, inhibition of germination and growth of neighbors, and defense against herbivores (Skulman et al. 2004; Dorning and Cipollini 2006; Cipollini et al. 2008). Although many phenolic and iridoid glycoside compounds have been identified in the genus, a comprehensive analysis investigating the possibility that non-native *Lonicera* possess either a novel secondary chemistry or quantities of known chemicals greater than those of their native competitors has not been done before. In this study, we determined that non-native *Lonicera* received significantly less damage than natives growing in a common garden. In the laboratory, performance of *Spodoptera frugiperda* was generally poor on all species, but larvae performed best on *L. fragrantissima* and *L. tatarica*, and poorest on *L. japonica* and *L. xylosteum*. Performance of larvae was severely reduced when feeding on leaves of unfertilized plants of all natives and from *L. maackii*, but fertilization of the host had little to no effect on larvae fed *L. tatarica* or *L. fragrantissima*. We also determined that even though origin explained little of the variation in phenolic or iridoid glycoside profiles of native/non-native *Lonicera* species as a whole, there were differences in quantity and composition of both groups of compounds among individual species, and notable increases in phenolics in non-native species and increases in iridoid glycosides in native species. To our surprise, even though quantities of secondary metabolites tended to increase, and plants were smaller under low fertilization (D. Lieurance, pers. obs), this treatment had little to no significant effect on plant chemistry overall but some individual compounds (eg. chlorogenic acid and luteolin-7-glucoside) did vary by fertilization.
treatment. Fertilization treatment also influenced herbivore damage in a common garden and the performance of a generalist herbivore in the laboratory.

Results of the herbivore assessment supported our prediction that damage would vary by species, fertilization treatment, and that non-native *Lonicera* would receive significantly less damage than native *Lonicera*. Damage was consistent for non-native species in both fertilization treatments, but natives had more variability in damage and there were pronounced increases in herbivory in high fertilizer treatments on *L. reticulata* and *L. flava*. A significant portion of the high amount of damage observed on *L. sempervirens* and *L. reticulata* can be attributed to damage by aphids, most likely the non-native honeysuckle aphid (*Hyadaphis tataricae*), attacking the tips of the plant (Boisvert et al. 1981). Although *H. tataricae* is considered a pest on many ornamental and often non-native *Lonicera* (Boisvert et al. 1981), they were never observed on non-native *Lonicera* in the common garden, but they were found in low densities on native *L. flava*. Our results are consistent with a similar common garden experiment comparing native, hybrid, and exotic *Senecio* species where non-native *Senecio* species had lower aphid infestation than natives or hybrid species (Hawes et al. 2010). Our results are also consistent with naturally occurring damage levels found in central Ohio where non-native *L. maackii* had much less arthropod herbivore damage than native *L. reticulata* in natural habitats where the species co-occur (Lieurance and Cipollini 2013). Low damage rates on non-native *Lonicera* could indicate a release from natural enemies, increased resistance traits such as elevated or novel chemical defenses, or could be a combination of both.

Results of the first no-choice feeding assays support the prediction that success of larvae would differ by species but not origin of *Lonicera* species. There were also species
differences in the second assay including foliage grown in low fertilization, but contrary to our prediction, there were differences in origin and larvae performed better on non-native species, however *L. japonica* and *L. xylosteum* were not included in the second assay because the larvae performed so poorly. *Lonicera fragrantissima* was the best host for larvae, *L. maackii* and *L. tatarica* were also suitable hosts, and *L. sempervirens* was consistently a poor host. Overall, fertilization affected resistance of most *Lonicera* species with reduced performance of larvae on all unfertilized species except *L. tatarica* and *L. fragrantissima*. These variations by species of larval performance do not reflect the observations of damage in the common garden on all species. For example, *L. sempervirens* and *L. reticulata* were poor hosts in feeding assays, but received the most damage in the field. Conversely, *L. tatarica* and *L. fragrantissima* proved to be highly suitable hosts, but received less than 1% damage in the field. This discrepancy was also noted in a related study testing the performance of a North American specialist sawfly on the same host material (Lieurance and Cipollini 2013) and may be indicative of a behavioral avoidance by herbivores, or an inability to recognize the suitability of a potential host (Lankau et al. 2004; Morrison and Hay 2009; Verhoeven et al. 2009).

Results of the chemical analysis supported our prediction that there would be differences in profiles and quantities of compounds by species. Additionally, we were able to isolate specific compounds influencing the relationship of *Lonicera* species in PCA. Native/non-native origin did not influence profiles overall but total phenolics and chlorogenic acid increased in non-native species and iridoid glycosides increased in native *Lonicera*. Both the phenolic and iridoid glycoside profile of the dominant invader, *L. maackii*, along the total phenolic concentration, were different and higher,
respectively, than co-occurring native species, like *L. reticulata*. Trends indicate unfertilized plants had a higher diversity and quantity of compounds, which was associated with reduced herbivore damage in the field and in reduced performance in laboratory assays on unfertilized plants. Also, high values of compounds like chlorogenic acid, luteolin-7-glucoside, and apigenin seemed to be associated with low damage observed on some non-native *Lonicera* (e.g. *L. maackii* and *L. tatarica*) in the common garden, but not always with the performance of the *Spodoptera* larvae in the laboratory. In this case, higher quantities of phenolics present in *Lonicera* species may have deterred herbivores in the field, but when given no choice, the performance of larvae feeding on the same foliage was not impacted. Cipollini et al. (2008) illustrated in separate feeding assays that larvae of the related *Spodoptera exigua* were deterred from feeding on diet plugs treated with whole leaf extracts from *L. maackii* and the phenolic, apigenin, but when they had no choice but to feed on extracts, their growth rates were not inhibited. Conversely, quantities of iridoid glycosides did not show a relationship with herbivore damage in the common garden for all species, but did associate with the results of the feeding assay where larvae feeding on species with high quantities of compounds like swerocide and secoxyloganin had reduced growth rates and number of days alive.

Overall, we can conclude that plants in the *Lonicera* genus display resistance to arthropod herbivores and can be characterized as being chemically well defended, poor hosts for the generalist *S. frugiperda*, and they receive low amounts of herbivory in the field. Some native species, while better hosts for specialists (Lieurance and Cipollini 2013), are relatively poor hosts for generalists as well. Therefore increased resistance of non-natives compared to common native competitors combined with other traits like high
fecundity and high physiological performance may contribute to the success of non-native *Lonicera*. The resistance of both native and non-native *Lonicera* species may be attributed to various defensive compounds such as the phenolics-chlorogenic acid and luteolin-7-glucoside and iridoid glycosides-secoxyloganin, secologanin, and loganic acid. At this time, our results indicate novelty in the defense profiles of some individual species (both native and non-native), but it is not possible to conclude that non-natives as a whole are more novel than natives species as a whole. However, even though a model generalist herbivore can perform quite well and sometimes even better on non-native *Lonicera* in the laboratory, in some instances, results of our herbivore assessment indicate ‘generalists’ prefer the natives in the field. These resistance traits, escape through behavioral avoidance, a combination of both, or some other resistance or nutritional trait (e.g. leaf toughness, leaf nitrogen) may contribute to the success of non-native *Lonicera* in North America.

This analysis is part of an ongoing study and future efforts will attempt to identify unknown phenolic compounds, particularly unique phenolics detected in non-native *Lonicera* and unknown peaks 10, 11, and 20 determined to be influential in our analysis. Additionally, principle component analysis will be expanded to determine if phylogenetic distance explains profiles better than species. But from our results, differences in the resistance to herbivory may be attributed to the composition and quantity of compounds produced by *Lonicera* species and that in general, plants growing in low fertilization are more resistant to herbivory. Based on these results and the results of previous studies, iridoid glycosides and phenolics may play different roles for specialist and generalist herbivores.
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Table 5.1 Species list of native and non-native *Lonicera* species grown in a common garden experiment on the campus of Wright State University, Dayton, Ohio. Scientific and common names, country of origin, growth habit, preferred habitat in the native range, and planting date are provided.
<table>
<thead>
<tr>
<th>Species</th>
<th>Common Name</th>
<th>Origin</th>
<th>Habit</th>
<th>Native Habitat</th>
<th>Date planted</th>
<th>Source</th>
<th>Citation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Non-native</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>L. maackii</td>
<td>Amur honeysuckle</td>
<td>Asia</td>
<td>Shrub</td>
<td>Riparian areas and forest edges</td>
<td>June, 2010</td>
<td>Seeds field collected various sites in Ohio</td>
<td>(Zheng et al., 2006)</td>
</tr>
<tr>
<td>L. japonica</td>
<td>Japanese honeysuckle</td>
<td>Asia</td>
<td>Vine</td>
<td>Slopes &amp; other marginal habitats</td>
<td>June, 2010</td>
<td>Plants collected McMinn County, TN</td>
<td>(Zheng et al., 2006)</td>
</tr>
<tr>
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<td>Shrub</td>
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<td>June, 2010</td>
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<td>(Zheng et al., 2006)</td>
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<td>Asia</td>
<td>Shrub</td>
<td>Rocky slopes and forest edge</td>
<td>May, 2011</td>
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<td>Shrub</td>
<td>Poor, well drained soils</td>
<td>May, 2011</td>
<td>Seeds purchased B&amp;T World Seeds, Paguignan, France</td>
<td>(<a href="http://www.invasive.org/weedus/subject.html?sub=11564">http://www.invasive.org/weedus/subject.html?sub=11564</a>)</td>
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<td>N. America</td>
<td>Shrub</td>
<td>Upland rocky forests, rocky soils</td>
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<td>Nursery stock purchased North Creek Nurseries, Landenberg, PA</td>
<td>(<a href="http://www.floridata.com/ref/l/loni_sem.cfm">http://www.floridata.com/ref/l/loni_sem.cfm</a>)</td>
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Table 5.2 Retention time and mean peak areas (x $10^{-4}$) of 4 known and 26 unknown phenolic compounds in the leaves of native and non-native *Lonicera* species grown in fertilized (F) and unfertilized (Un) treatments in a common garden at Wright State University, Dayton, Ohio. The 4 known compounds were compared against pure, authentic standards. Samples were analyzed using UPLC.
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<th>F Un</th>
<th>F Un</th>
<th>F Un</th>
<th>F Un</th>
<th>F Un</th>
<th>F Un</th>
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<td>5.47 (2.9)</td>
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Table 5.3 Mean amounts of phenolic compounds quantified using authentic standards. Results are presented by species as there was no significance in fertilization treatment for any compound. Letters indicate significant differences determined through Tukey post hoc testing (P<0.05).

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<td>0 c</td>
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*values are in mg/g fresh weight
Table 5.4 Mean peak areas (x 10^{-5}) of 6 known and 24 unknown iridoid glycoside compounds in the leaves of native and non-native *Lonicera* species grown in fertilized (F) and unfertilized (Un) treatments in a common garden at Wright State University, Dayton, Ohio. The 6 known compounds were compared against pure, authentic standards. Samples were analyzed using GCMS. (*) indicates putative IDs for compounds.
<table>
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<th>L. sempervirens</th>
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<th>L. tatarica</th>
<th>L. japonica</th>
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<th>L. xylosteum</th>
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Table 5.5 Mean amounts of iridoid glycosides quantified using authentic standards. Results are presented by species as there was no significant effect of fertilization treatment for any compound. Letters indicate significant differences determined through Tukey post hoc testing (P<0.05).

<table>
<thead>
<tr>
<th>Species</th>
<th>Swerocide</th>
<th>Secoxyloganin</th>
<th>Loganin</th>
<th>Loganic Acid</th>
<th>Secologanin</th>
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</thead>
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<td>Native</td>
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<td></td>
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<tr>
<td><em>L. flava</em></td>
<td>0.48±0.14 a</td>
<td>7.19±1.25 a</td>
<td>0.23±0.08 a</td>
<td>0.35±0.11 ab</td>
<td>4.97±1.52 a</td>
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<tr>
<td><em>L. reticulata</em></td>
<td>0.23±0.07 ab</td>
<td>2.46±0.70 ab</td>
<td>0.36±0.23 a</td>
<td>0.37±0.16 ab</td>
<td>1.83±1.07 b</td>
</tr>
<tr>
<td><em>L. sempervirens</em></td>
<td>0.12±0.03 ab</td>
<td>3.00±0.70 ab</td>
<td>0.01±0.23 a</td>
<td>0.05±0.02 b</td>
<td>0 b</td>
</tr>
<tr>
<td>Non-Native</td>
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<td></td>
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<td></td>
<td></td>
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<tr>
<td><em>L. maackii</em></td>
<td>0.21±0.03 ab</td>
<td>1.78±0.37 ab</td>
<td>0 a</td>
<td>0.23±0.07 ab</td>
<td>0.38±0.06 b</td>
</tr>
<tr>
<td><em>L. tatarica</em></td>
<td>0.31±0.14 ab</td>
<td>2.29±0.74 ab</td>
<td>0.02±0.01 a</td>
<td>0.18±0.09 ab</td>
<td>0.45±0.38 b</td>
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<td><em>L. japonica</em></td>
<td>0 b</td>
<td>8.12±3.03 a</td>
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<td><em>L. fragantissima</em></td>
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<td>1.39±0.49 b</td>
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<td>0.15±0.15 b</td>
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<td><em>L. xylosteum</em></td>
<td>0.33±0.20 ab</td>
<td>3.51±1.17 ab</td>
<td>0.02±0.01 a</td>
<td>0.22±0.09 ab</td>
<td>0.16±0.14 b</td>
</tr>
</tbody>
</table>

*values are in % dry weight
Figure 5.1 Arthropod herbivore damage on native and non-native *Lonicera* species growing in fertilized and unfertilized treatments in a common garden experiment in Wright State University Woods, Dayton, OH. Herbivory was estimated as a percentage of leaf area removed. Means ± standard error (SE) are shown. Letters indicate significant differences determined through Tukey post-hoc testing (P<0.05).
Figure 5.2 Mean number of days alive, relative growth rate (RGR), pupal mass, and number of days to pupation of *Spodoptera frugiperda* larvae, a generalist arthropod herbivore, feeding on the foliage of 2 native and 5 exotic *Lonicera* species grown in fertilized treatments in a common garden. RGR was calculated on day 25. Letters indicate differences in means determined through Tukey post hoc testing ($P<0.05$).
Figure 5.3 Mean number of days alive, relative growth rate (RGR), pupal mass, and number of days to pupation of *Spodoptera frugiperda* larvae, a generalist arthropod herbivore, feeding on the foliage of 2 native and 5 exotic *Lonicera* species grown in fertilized and unfertilized treatments in a common garden. RGR was calculated on day 21. Means of pupal mass and days to pupation are shown by species due to poor performance on foliage grown in low fertilization treatments. Letters indicate differences in means determined through Tukey post hoc testing ($P<0.05$).
Figure 5.4 Principal components analysis of phenolic compounds (A) and iridoid glycosides (B) in leaf extracts from native (red) and non-native (blue) Lonicera species growing in a common garden. Letters ‘fl’, ‘re’, and ‘se’ indicate natives L. flava, L. reticulata, and L. sempervirens respectively and ‘ma’, ‘ta’, ‘ja’, ‘fr’ and ‘xy’ indicate non-native L. maackii, L. tatartica, L. japonica, L. frantissima, and L. xylosteum respectively. Data were square root transformed after standardized using Hellenger standardization. Grey boxes indicate high fertilization and open boxes indicate low fertilization. Percentages indicate the inertia decomposition associated with each of the two axes.
6. Conclusions, Future Directions, and Applications

In this study, I determined that *Lonicera maackii* receives insignificant amounts of arthropod herbivore damage in the field and that these amounts are much less than amounts observed on native *Lonicera* and confamiliar *V. prunifolium* suggesting an apparent avoidance of herbivory. The honeysuckle specialist, *Zaraea inflata* collected from native *L. reticulata* avoids *L. maackii* in the field, but can develop to pupation on *L. maackii* in the laboratory. I was also able to demonstrate that *L. maackii* is able to both tolerate and resist not only the damage they receive in the field, but also imposed artificial amounts much higher than the amounts recorded. Additionally, I determined that limiting light and soil nutrients did not limit their ability to tolerate herbivory in the juvenile growth stage. Overall, native and non-native plants in the *Lonicera* genus displayed resistance to arthropod herbivores, were chemically well defended, and in general, were poor hosts to generalist herbivores. Native/non-native origin did not explain the chemical profiles of species, but native *Lonicera* tended to produce more iridoid glycosides and non-native *Lonicera* produced more phenolic compounds. *Lonicera maackii* and other non-natives appear to not only escape damage from arthropod herbivores, they are also able to tolerate and resist the damage they do incur suggesting that a combination of mechanisms contribute to the success of these non-native *Lonicera* in North America.
I approached this project with a combination of field studies, greenhouse experiments, controlled feeding assays, and laboratory chemical analyses. With these techniques I was able to answer research questions with a combination of controlled laboratory and greenhouse experiments combined with studies that are subject to the stochastic variability plants experience in the field. While a central focus of this project revolved around questions of the Enemy Release Hypothesis, no definitive conclusions could be made regarding this hypothesis, as we were unable to obtain seeds from the native range of *L. maackii*. However, our findings do indicate *L. maackii* likely benefits from enemy escape and future research should include cross-continental comparisons of herbivore damage in the native and invasive range to yield more conclusive results. Furthermore, questions related to the evolution of increased competitive ability (EICA) hypothesis could also be answered with cross continental studies. For instance, comparisons of growth and secondary metabolite content with and without damage between *L. maackii* either observed in its native and invasive range or grown from seed collected in both ranges would not only elucidate a possible avenue for successful invasion, it might also reveal weakness that may be taken advantage of by an effective biocontrol program. Investigating other forms of herbivory including root feeders, deer, and slugs may expand upon the experiments I conducted related to tolerance and resistance mechanisms employed by *L. maackii* in response to damage. Additionally, results presented in chapter 5 are a part of an ongoing study and my collaborators will attempt to identify unknown phenolic compounds, particularly unique phenolics and the unknown peaks determined to be influential in our analysis. We will also expand out principle component analysis to determine if phylogenetic distance explains profiles
better than species. But to investigate the possibility that novel weapons contributes to the success of non-native *Lonicera*, chemical analyses must include plants in direct competition with these non-natives *Lonicera* species and performance must be evaluated for native and non-native species growing in competition with and without herbivory. However, the results of this study do contribute to the bank of knowledge on problematic invasive species and provide information useful in distinguishing potential invasive species from those species that are not a threat when regulating plants that may be introduced in a novel environment.