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## INVASIVENESS OF AN EXOTIC GALL WASP IS INFLUENCED BY INTERACTIONS WITH EXOTIC AND ENDEMIC ORGANISMS

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INVASIVENESS OF AN EXOTIC GALL WASP IS INFLUENCED BY  
INTERACTIONS WITH EXOTIC AND ENDEMIC ORGANISMS

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DISSERTATION

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A dissertation submitted in partial fulfillment of the requirements for the degree of  
Doctor of Philosophy in the College of Agriculture, Food, and the Environment at the  
University of Kentucky

By

Ignazio Graziosi

Lexington, Kentucky

Director: Dr. Lynne K Rieske Kinney, Professor of Entomology

Lexington, Kentucky

2015

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## ABSTRACT OF DISSERTATION

### INVASIVENESS OF AN EXOTIC GALL WASP IS INFLUENCED BY INTERACTIONS WITH EXOTIC AND ENDEMIC ORGANISMS

American chestnut was once a crucial component of North American forests, but it was functionally eliminated by the introduction of the chestnut blight fungus. Chestnut is recently experiencing resurgence, but the introduced Asian chestnut gall wasp, a specialist herbivore, threatens chestnut recovery. I characterized this invasion and the interactions developing with host associates as the gall wasp spread in North America. Gall wasp dispersal is attributable to host plant distribution, effects of prevailing winds during adult insect emergence, and to topography. This knowledge may be useful to more accurately predict locations of future gall wasp infestations. Gall wasp invasiveness is also affected by its ability to modulate reproduction and reallocate nutritive resources for body maintenance and egg quality. The gall wasp can form galls of different shape and structure based on population levels and parasitism pressure, in order to optimize fitness. Simple, uni-lobed galls are formed when population pressure is low, and complex, multi-lobed galls are formed when gall wasp populations are high. A fungal plant pathogen, identified as *Colletotrichum* sp., infects galls and acts as an opportunistic entomopathogen, causing gall wasp mortality while sparing the parasitoid. A non-native torymid parasitic wasp has been deployed for biological control. Using a Y-olfactometer I demonstrated that a combination of olfactory and visual cues from chestnut galls and chestnut foliage is required for parasitoid adults to locate hosts. In addition, I evaluated interactions between the gall wasp and stem cankers on chestnut. Using potted Chinese chestnut seedlings with or without gall wasp or a native *Nectria* cankering pathogen, I found that the gall wasp has a negative impact on plant fitness, and a positive impact on fungal fitness. My work helps elucidate ecological mechanisms underlying the success of the exotic Asian chestnut gall wasp in North America, and adds to our knowledge base characterizing evolving ecological interactions between native and introduced species.

**KEY WORDS:** *Castanea*, *Dryocosmus kuriphilus*, invasiveness, host location, community associates

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Student's signature

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Date

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2015

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## Chapter One: Introduction

Exotic species introductions, either intentional or unintentional, represent a complex force in our rapidly changing world. The frequency and amplitude of biological invasions is increasing globally (Elton 1958, Meyerson and Mooney 2007, McGeoch et al. 2010) with dramatic impacts on natural ecosystem conservation, agroecosystem productivity, human health, and economic development (Mooney and Hobbs 2000, Pimentel et al. 2005, Holmes et. al 2009, Ehrenfeld, 2010). Non-native organisms can irreversibly and irreparably degrade ecosystems (Elton 1958). They interact unpredictably with native and non-native species as they proliferate through newly invaded regions, thus affecting their invasiveness and magnifying the consequences of the invasion (Simberloff and Von Holle 1999, Johnson et al. 2009). Managing non-natives represents a major challenge for biologists and natural resource managers (Simberloff et al. 2005, Hoffmann 2010).

Forest systems can be vulnerable to invasion by a multitude of plants (Fine 2002), pathogens (Loo 2008), vertebrates (Wardle et al. 2001) and insects (Liebhold et al 2012), which are triggering deep changes in forest composition, structure and function (Liebhold et al. 1995, Lovett et al. 2006, Gandhi and Herms 2010). In many cases the impact of such invasions is dramatic, threatening survivorship and reproduction of key forest tree species (Ellison et al. 2005). Recent examples in the USA include laurel wilt disease impacting red bay, *Persea borbonia*, in the southeast (Fraedrich et al. 2008), and thousand cankers disease affecting black walnut, *Juglans nigra*, in many states (Tisserat et al. 2009). One of the most striking examples of a biological invasion impacting a foundation species, however, is the functional elimination of American chestnut,

*Castanea dentata* Marshall Borkh., from North American forests by the chestnut blight fungus, *Cryphonectria parasitica* (Ascomycota: Diaporthales), a stem cankering fungus of Asian origin.

American chestnut historically played an integral role in the economic development, sustainability, and culture of the eastern USA, particularly in the Appalachian region (Youngs 2000). American chestnut was a dominant component of mixed mesophytic eastern forests (Braun 1950), especially in Central Appalachia (McCormick and Platt 1980), and was an important nutritional resource for humans and animals. The accidental introduction of the chestnut blight fungus at the turn of the twentieth century functionally eliminated American chestnut from its native range within decades (Anagnostakis 1987, Griffin 2000). In recent years chestnut is experiencing a resurgence in popularity, due in part to heightened awareness of agricultural sustainability issues and to human health concerns (Gold *et al.* 2005). Efforts to restore American chestnut are coming to fruition (Jacobs 2007), as American × Chinese hybrids and transgenic chestnuts are successfully developed (Hebard 2006, Zhang *et al.* 2013). Chinese, *C. mollissima*, Japanese, *C. crenata*, and European, *C. sativa*, chestnuts are increasingly cultivated for nut production (Gold *et al.* 2005, Fulbright *et al.* 2009), and blight resistant hybrids are widely used in reclamation projects (McCarthy *et al.* 2010).

Although several arthropods are thought to have been extirpated with the loss of American chestnut (Opler 1978), insect herbivory remains a significant factor in chestnut propagation and cultivation in North America (Payne 1983, Anagnostakis 2001). Native and non-native herbivores readily utilize chestnut; most noticeably the polyphagous ambrosia beetles (Oliver and Mannion 2001), gypsy moth, *Lymantria dispar*, Japanese

beetle, *Popillia japonica*, and fall webworm, *Hyphantria cunea* (Kellogg et al. 2005).

The resurgence of chestnut in North America, however, is potentially endangered by an additional invader, the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae).

The Asian chestnut gall wasp is a global pest of chestnut included in the regulated pest list compiled by the Animal and Plant Health Inspection Service of the US Department of Agriculture (APHIS 2000) and is classified as a quarantine pest by the European and Mediterranean Plant Protection Organization (EPPO 2005). The wasp is native to China and invaded Japan in 1941, where it was described as a new species (Yasumatsu 1951). It arrived in Korea in 1958 (Cho and Lee 1963), the USA in 1974 (Payne et al. 1975), Nepal in 1999 (Ueno 2006) and Italy in 2002 (Graziosi and Santi 2008), and rapidly spread throughout Europe (Avtzis and Matošević 2013, EPPO 2014a, EPPO 2014b).

Gall inducing herbivores are extreme specialists (Shorthouse et al. 2005) which benefit from manipulating the growth and physiology of their plant hosts (Hartley 1998). Galls are neo-plastic growths induced in response to insect stimuli (Mani 1992), and function as physiologic sinks to provide the developing insect with nutrition (Larson and Whitham 1991) and protection (Hartley 1999, Stone and Schönrogge 2003). Members of Cynipidae are among the most specialized gall makers (Stone and Cook 1998); their life cycle is finely tuned with their host plants, showing high host specificity, extensive variation of gall traits, and various reproductive strategies, including host alternation and parthenogenesis (Stone et al. 2002).

*Dryocosmus kuriphilus* is a univoltine and non heterogonic gall wasp, and reproduces by thelytokous parthenogenesis (Zhu et al. 2007). Larvae develop rapidly within the galls throughout the spring, pupate, and adults emerge in early summer, ovipositing in chestnut buds (Itô et al. 1962, Ôtake 1980). Eggs hatch and first instar larvae overwinter within dormant buds until the following season (Itô et al. 1967), but detection within buds is impossible by external plant inspection (Sartor et al. 2012). The gall wasp induces formation of spherical, 1-3 cm galls on new vegetation, thus disrupting tree growth (Kato and Hijii 1997, Panzavolta et al. 2012), leading to plant decline and drastic yield reductions (Payne et al. 1983, Battisti et al. 2014).

The first report of *D. kuriphilus* in the USA was in 1974, when an infestation was identified in Chinese chestnut orchards in Peach County, Georgia (Payne et al. 1975); it rapidly spread beyond cultivated chestnut orchards to horticultural plantings and forest settings. During subsequent decades the gall wasp moved in a north-northeasterly direction, nearly encompassing the former range of American chestnut (Rieske 2007). Long distance dispersal of the gall wasp is clearly due to movement of infested plant material (Rieske 2007), but dispersal of winged adults likely plays an important role in local spread (Oho and Shimura 1970, EFSA 2010). The occurrence of sporadic lingering American chestnut and Chinese and hybrid chestnut planted for horticultural purposes presumably facilitated the movement of *D. kuriphilus* through its invaded North American range (Anagnostakis 2001, Rieske 2007).

Insecticides are impractical against the gall wasp (Bosio 2010), and the efficacy of resistant cultivars, which were initially successful in Asia, is now limited (Shimura 1972, Dini et al. 2012). Classical biological control using the ectoparasitoid *Torymus*



*sinensis* Kamijo (Hymenoptera: Torymidae) has provided some population regulation. *Torymus sinensis* releases were initiated in the US (Payne 1978) and Japan (Moriya et al. 2003), and more recently in Europe (Quacchia et al. 2008, Gibbs et al. 2011). In North America extensive parasitization of the gall wasp is attributable to *T. sinensis*, and also to native hymenopterans associated with oak gall makers (Cooper and Rieske 2007, 2011). Nevertheless, *D. kuriphilus* still undergoes periodic outbreaks that affects chestnut production and threatens restoration and reclamation efforts, and naturally occurring American chestnut (Cooper and Rieske 2007).

As the prevalence of chestnut across the eastern North American landscape increases, the risks associated with native and non-native pests increases. An invader's ability to disperse is impacted by the prevalence and occurrence of its host (Tschardt and Brandl 2004), which is highly sporadic for *Castanea* resources in North America. Asian chestnut gall wasp performance depends on physiological and chemical interactions with its chestnut host (Cooper and Rieske 2011), other herbivores (Kellogg et al. 2005, Cooper and Rieske 2009), and with native and non-native natural enemies and microorganisms (Cooper and Rieske 2007). Developing interactions between the gall wasp and associated organisms has yet to be fully characterized.

My overall objective is to evaluate the interactions developing with abiotic and biotic components of the environment as *D. kuriphilus* invades and establishes in new regions of the eastern USA, and to determine their effect on gall wasp invasiveness. I used remotely sensed data and Geographic Information Systems to describe the dispersal of the gall wasp, and tested the hypotheses that geomorphology, host occurrence, and prevailing winds influence local proliferation (Chapter 2). Next I evaluated potential

fecundity of the gall wasp by comparing ovarian, egg, and body metrics of adult wasps of different ages (Chapter 3) to better understand its invasiveness. I then turned my attention to the morphology of galls induced by *D. kuriphilus*, and evaluated how gall morphology relates to population pressure and natural enemy pressure (Chapter 4). My next focus was to investigate the occurrence of necrotic lesions formed by an unknown microbe on *D. kuriphilus* galls and to elucidate the effects of this Plant-Microbe-Insect interaction on gall wasp invasiveness (Chapter 5). Then I evaluated how the parasitic *T. sinensis* detects wasp odor and visual cues in order to successfully locate and utilize its host (Chapter 6). Finally, I assessed the interaction between the gall wasp and stem cankering fungi, and measured the impacts of such interactions on plant, insect and fungal performance (Chapter 7).

My research approach involves different techniques at various spatial and temporal scales as I seek to elucidate the key components contributing to the invasion process of this non-native specialist herbivore in North America, and provide insights on how invasiveness of an exotic species colonizing non-native ranges is affected by interactions developing between biotic and abiotic components of the environment.

## **Chapter Two: Local spread of an exotic invader: using remote sensing and spatial analysis to document proliferation of the invasive Asian chestnut gall wasp**

Exotic species invasions pose a substantial threat to biological integrity and sustainability. In our increasingly global economy, the rate of species' invasions is increasing exponentially, compromising biodiversity and altering ecosystem function. Non-native invasive species can affect plant, animal, and human health, and these invasions can have devastating economic impacts (Schnase et al. 2002). A critical challenge for invasive species management is gaining a greater understanding of the invasion process. Predicting the spatial and temporal dynamics of newly established invaders is crucial to understanding their proliferation and mitigating their impacts.

Emerging technologies such as remote sensing improve our ability to better understand factors influencing an invasion, including the invasability of an area, the dynamics of an invasion, predictions about invasiveness (Schnase et al. 2002, Holcombe et al. 2007), and mitigation of these invasions. The combined use of Global Positioning Systems (GPS) and Geographic Information System (GIS) offers a powerful set of tools to record movement and describe the behavior of invasive organisms. In the last few decades GIS-based analyses have been used effectively to investigate the pattern of dispersal of many diverse organisms. Hyperspectral images, GPS collected data and GIS have been used to locate and map invasive plants in California (Underwood et al. 2003), and combined with spatial regression analysis, identify the parameters that affect their spread (Dark 2004). Remotely sensed data and GIS have also been used to understand invasions by naturalized horticultural imports (Lemke et al. 2011) and woody plants (Rouget et al. 2004).

To predict the impacts of the invasive hemlock woolly adelgid, *Adelges tsugae* Annand (Hemiptera: Adelgidae), in eastern North America, remote sensing and spatial analysis has been used to map the occurrence of the highly susceptible eastern hemlock, *Tsuga canadensis* (L.) Carr. (Clark et al. 2012). These technologies have also proven effective in modeling the spread of windborne and flying insects (Riley 1989, Reynolds and Riley 2002). Remote sensing and GIS have been used to locate bark beetle (Coleoptera: Curculionidae) populations, characterize the scope and magnitude of infestations, and predict their spread (Wulder et al. 2006). Similarly, gypsy moth, *Lymantria dispar* L. (Lepidoptera: Lymantriidae), distribution in North America has been described and predicted using GIS and spatial analysis (Liebhold et al. 1992). These technologies have also been used to monitor pests in cropping systems. Carriere et al. (2006) modeled the movement of the lygus bug, *Lygus hesperus* (Knight) (Heteroptera: Myridae), through diverse settings. They tracked lygus bug populations within different crops over time, and were able to distinguish between source and sink locations. Clearly GIS-based spatial analyses allow a greater understanding of the biology, behavior and ecology of insects and how they interact with biotic and abiotic factors, and represent a powerful tool to mitigate the impacts of species' invasions. I use these tools here to address local proliferation of an exotic insect pest attacking a sporadically occurring tree genus containing both native and non-native members, and to understand how local conditions influence its spread.

American chestnut, *Castanea dentata* (Marshall) Borkh, was historically a dominant component of forests of eastern North America (Braun 1950), but was functionally eliminated by the 1904 introduction of the exotic chestnut blight fungus,

*Cryphonectria parasitica* (Murr.) Barr (Griffin 2000). Lingering American chestnuts persist across the landscape as woody shrubs that reach 1-2 m in height before dying back, and Chinese chestnut, *C. mollissima* Blume, is widely planted as a landscape and horticultural tree.

However, chestnut is again under threat from an additional exotic invader, the Asian chestnut gall wasp (ACGW), *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). The gall wasp induces formation of spherical, 1-3 cm galls on new spring shoots, thus disrupting tree growth, leading to plant decline and drastic yield reductions (Payne et al. 1983). *D. kuriphilus* is univoltine, and reproduces by thelytokous parthenogenesis (EPPO 2005). Larvae develop within the multi-chambered galls throughout the spring, pupate, and adults emerge in summer (late May to late July). Adult flight occurs over 10-15 d, depending on latitude (EPPO 2005). Each adult produces 100-150 eggs, and oviposits up to 20 eggs in a single developing chestnut bud (EPPO 2005), which hatch in 30-40 d. First instar larvae overwinter within dormant chestnut buds until the following spring; gall development occurs concurrently with budbreak and leaf expansion. Detection within dormant buds is impossible by external plant inspection.

Since its initial introduction into North America in Peach County, Georgia, in 1974, the gall wasp has spread throughout the natural range of American chestnut (Figure 2.1). Initially (1970s and 1980s) range expansion was fairly localized. However, beginning in the late 1990s there was a rapid range expansion in a north-northeasterly direction.

Because of its cryptic nature gall wasp infestations spread easily due to movement of infested plant material, evidenced by the appearance of disjunct satellite populations in Ohio and Maryland (Rieske 2007). However dispersal by flight, influenced by winds, likely also plays a role (Anagnostakis 2001, Rieske 2007, Graziosi and Santi 2008, EFSA 2010). Chestnut resources occur sporadically across the landscape, either as lingering American chestnut or as Chinese chestnut for horticultural purposes. Presumably *D. kuriphilus* utilized these resources, facilitated by wind, to spread through forested regions from northern Georgia to Virginia in the 1990's and early 2000's (Anagnostakis 2001, Rieske 2007). The Asian chestnut gall wasp is currently established in 11 states (Figure 2.1), with infestations proliferating throughout the historic range of American chestnut.

On a localized scale, gall wasps disperse by active flight. Winds play a crucial role in influencing adult cynipid flight, with the direction of dispersal being consistent with that of prevailing winds (Hough 1951). Wind speed is critical in determining the mechanism of this dispersal (Oho and Shimura 1970, ESFA 2010). Low wind speeds stimulate adult flight (0.15 - 0.45 m/s), after which flying wasps are carried on prevailing winds. While higher wind speeds inhibit flight ( $\geq 0.73$  m/s), these will also result in wasps being passively carried with the prevailing winds.

In 2010 the Asian chestnut gall wasp was discovered on a single Chinese chestnut in Lexington, Kentucky, USA. Lexington lies in the heart of the Bluegrass Region (Braun 1950), in an area where suitable host plants occur only very sporadically. Chestnut was historically absent from the Bluegrass (Wharton and Barbour 1973), but Chinese or hybrid chestnut trees planted in farmland and urban settings occur. Because the gall wasp is highly invasive in eastern North America and *Castanea* host plants occur only

infrequently in the Bluegrass Region, I sought to understand its establishment and proliferation through the area. I used GIS and spatial analysis to map the distribution and dispersal of the gall wasp on a local scale (Lexington, Kentucky USA), and to evaluate factors influencing that spread. I evaluated local distribution of the gall wasp in relation to 1) chestnut distribution, 2) landscape features, and 3) prevailing winds. I hypothesized that the local gall wasp population originated from a single source tree, and dispersed locally from the source tree through active flight by adult wasps, facilitated by prevailing winds, since movement of infested plant material within this locale is unlikely.

### **Materials and methods**

The city of Lexington, Kentucky was systematically surveyed for *Castanea* in 2010 and 2011 in the area encompassed within New Circle Rd. (Figure 2.2). An additional area extending 1 km beyond New Circle Rd. was also surveyed. Each located chestnut was geo-positioned using a portable GPS device (Garmin Ltd, Olathe KS, USA), characterized (height, diameter, crown condition), and the presence or absence of the gall wasp was noted (Table 1). An  $8 \times 5 \text{ km}^2$  area with the highest concentration of *Castanea* delineates my study area (Figure 2.3). Elevation ranges from 240 to 330 m.

Data on chestnut location and condition were geo-referred and included in a GIS using ArcGIS10 (ESRI Redlands CA, USA). Additional data obtained for spatial analysis included Fayette County boundaries, major roads, topographic information and digital ortho images from the Kentucky Geographic Network website (Commonwealth of Kentucky 2011). I also obtained a digital elevation model (DEM) with 9.1 m (30 ft) resolution from the US Geological Survey National Elevation Dataset (USGS 2011) and

a second digital elevation model with 1.5 m (5 ft) resolution from the Geographic Network (Commonwealth of Kentucky 2011). These additional data were combined with the results of my survey using ArcGis10 to produce maps. Digital elevation models were used to visualize slope and aspect (9.1 m (30 ft) DEM) and land shape (high resolution 1.5 m (5 ft) DEM). Land shape was visualized by applying a dynamic range adjustment to the DEM (Bolstad 2005). The slope was represented by using natural breaks (Jenks optimization method) (Jenks 1967).

Wind direction and speed were extracted from the monthly climatological data of the Kentucky Climate Center (Western Kentucky University 2011) for the time period corresponding to the emergence and adult flight period for those wasps causing the infestation observed in 2011 (June 23 to July 4 2010). These measurements were generated at the University of Kentucky South Farm (Lat. 37.98°; Lon. -84.53°), located 13.5 km south-southwest of the initial gall wasp find.

## **Results and discussion**

### Chestnut distribution

My survey revealed the presence of 27 *Castanea* trees in a 40 km<sup>2</sup> area (Figure 2.3) within the greater surveyed area. Twenty trees were discovered in 2010 and seven additional trees were located in 2011 (Table 2.1). The majority (81%) are Chinese chestnut planted in residential yards or as street trees. Two (7%) are American chestnuts,



two are *C. dentata* × *C. mollissima* hybrids, and one (4%) is a chinquapin (*C. pumila*). Chestnut resources in Fayette County are clustered (Figure 2.3); nine (33%) *C. mollissima* are growing in the Cassidy – Cooper area. Seven (26%) are growing in the University of Kentucky Arboretum and State Botanical Garden, including three Chinese chestnuts, both hybrids, a single American and a chinquapin. Land shape clearly influences chestnut occurrence in the study area, but chestnut occurrence is confounded by human influence. All trees are growing at elevations of 303 – 319 m (Table 2.1, Figure 2.4). The majority are located on low (3-15%) slopes (Table 2.1, Appendix 1), but their aspect is variable (Table 2.1, Appendix 2).

#### Gall wasp presence and landscape features

In 2010 the gall wasp was found only on a single tree (L17) facing southeast (Table 2.1, Figure 2.3), while the other chestnuts surveyed were gall-free. The initial gall wasp discovery was well established and the infestation level was high; this tree is considered the source tree for subsequent gall wasp infestations. After the initial gall wasp find in 2010, 17 additional hosts were found to be infested in 2011, primarily located in a south southwesterly direction. Thirteen of those trees were gall free during the initial 2010 survey, thus indicating that the 2011 infestations were due to dispersal of the wasp during the adult flight in summer 2010. The remaining four trees were surveyed for the first time in 2011 and found to be already infested. However, their very low galling rate suggests those trees were initially infested in summer 2010 as well. Seven trees remain gall wasp free. Thus 67% of the chestnut in the study area (18 trees) harbor confirmed gall wasp populations (Table 2.1, Figure 2.3). The trees newly infested in 2011 had very low gall wasp populations, and were 700 – 2700 m from the source tree in a

primarily south-southwest direction (Figure 2.3). Aspect may be playing a role in gall wasp occurrence since many of these newly infested trees were oriented northeast or east, essentially facing the source tree. Collectively this information suggests that the source tree and the infested chestnut are situated in positions exposed to the same wind flows: higher elevation (ridges), source tree facing newly infested trees, and low slopes.

### Prevailing winds

There was a 12 d period of adult gall wasp flight in 2010 (June 23 – July 4) during which ambient conditions were relevant. The average daily temperature during this period was 24.3 °C, well within the acceptable temperature range for adult wasp flight (Table 2.2). Measureable precipitation, which clearly could impede flight, was recorded on only two days (24 and 28 June), during which solar radiation was less than 20 MJ/m<sup>2</sup> and below the mean for the 12 d flight period (19.9 MJ/m<sup>2</sup>). The average relative humidity, 66%, did not deviate from normal (Table 2.2). On four days (June 25, June 30, July 1, and July 2) prevailing winds were south westerly. On one day (June 29) prevailing winds were directed easterly (Table 2.2). During the remaining days winds were directed mainly northeasterly at 1.8 – 4 m/s (mean = 3 m/s) (Table 2.2), appropriate for passive adult transport (>2.1m/s) rather than active flight.

In 2010 I located a single heavily gall–infested source tree. In 2011, sixteen of the 21 trees (76%) located southwest and east of the source tree were infested. Prevailing winds during adult flight (June 23 – July 4 2010) were sufficient to account for the pattern of gall wasp–infested chestnuts documented in my surveys (Figure 2.5). Nevertheless I must use caution in interpreting my result. The majority of the chestnut I

located were southwest of the source tree, so it's conceivable that the geographic distribution of these hosts could in and of itself be the cause of the infestation pattern I observed. However, if prevailing southwesterly winds were lacking during the critical window of time for adult gall wasp flight in 2010, or were primarily in a different direction, I would expect to see little or no gall wasp present on trees located southwest of the source, providing support for my hypothesis. To fully test my hypothesis would require a more evenly distributed host plant base than what is available in Lexington.

My data document how proliferation of the gall wasp through the city of Lexington was determined by host plant distribution and was strongly affected by prevailing winds. Wind is a well-documented factor influencing insect dispersal (Johnson 1966, Gressitt and Yoshimoto 1974, Moser et al. 2009, Eagle et al. 2011), including that of a cynipid gallmaker on oak (*Quercus* sp.) (Hough 1951), and is an important aspect of pest dispersal in agro forest settings (Epila 1988). My data suggests that adult Asian chestnut gall wasps were transported by wind in a southwesterly direction from the source tree to suitable hosts on four dates (Figure 2.5): June 25, June 30, July 1 and July 2. Evidence also suggests that winds blowing northwesterly on June 29 facilitated movement of wasps from the source tree to the tree L16, located south southeast of the source tree (Figure 2.5). Furthermore, winds on July 3 and 4 were of suitable speed and an appropriate direction to passively transport adults to two trees in the northeastern edge of the study area (L18 and L19) (Figure 2.5).

Not all hosts southwest of the source tree were infested. The gall-free trees L08 and L09 were heavily pruned in winter 2010, potentially removing infested buds and locally extinguishing the gall wasp population. Trees L1, L2, L3 and L20 are surrounded

by vegetation that may have perturbed wind flow, disrupting exposure to prevailing winds that would carry adult wasps and lead to gall wasp infestations. Vegetation, buildings, and other windbreaks do impact local insect movement (Hough 1951, Pasek 1988), and likely play a role in the patterns of gall wasp proliferation I observed.

The maximum distance the gall wasp dispersed in Lexington during the 2010 flight period was the distance between the source tree L17 and the L4 L5 L6 cluster, approximately 3 km (Figure 2.3, Figure 2.5). The gall wasp did not colonize trees L25 and L26, located 6 km southwest of the source, but those are very isolated hosts at twice the distance. My findings on local gall wasp proliferation are comparable with data from Europe, where gall wasp dispersal by flight has been reported at 8 km per year (EFSA 2010). Dispersal rates of 15-25 km per year were reported in the USA in the years immediately following the gall wasp introduction in Georgia in 1974, but the movement of infested plant material was likely involved (Rieske 2007).

## **Conclusions**

I used remotely sensed data and GIS technology to characterize local proliferation of a newly arrived exotic insect, the Asian chestnut gall wasp, attacking a sporadically occurring tree in the Bluegrass Region of Kentucky. I sought to understand how host plant distribution, landscape characters, and prevailing winds might influence the gall wasp's spread in the formative stages of the invasion and barring movement of infested plant material by humans.

This is the first use of remotely sensed data and GIS analysis to document movement of the invasive Asian chestnut gall wasp. My analysis provides insight into

factors that affect the local spread and proliferation of the gall wasp in a newly invaded area. The gall wasp colonized 17 sporadically distributed hosts found at a very low density within a 40 km<sup>2</sup> area. My analysis demonstrates how the invasiveness of the gall wasp is enhanced by abiotic factors. This work will provide a useful tool and model system to test hypotheses and to make predictions about the spread of this invasive pest on local scale, leading to appropriate mitigation efforts such as delineation of quarantine areas.

Table 2.1. Location and characteristics of *Castanea* resources and status of infestation by the Asian chestnut gall wasp in Fayette County, KY (USA), determined during a systematic survey (2010-2011). The **bolded** tree indicates the presumed infestation source.

No.	Chestnut species	Location	Setting	Elev (m)	Slope (%)	Aspect	Height (m)	Diameter <sup>1</sup> (cm)	Crown condition <sup>2</sup>	ACGW infestation <sup>3</sup>	Initial ACGW report (yr)
L01	<i>C. dentata</i>	Arboretum	park	317.6	5.8	S	2.0	5.0	1	none	---
L02	Hybrid	Arboretum	park	318.3	3.7	S	4.5	14.0	1	none	---
L03	Hybrid	Arboretum	park	318.3	3.4	S	3.0	7.0	1	none	---
L04	<i>C. mollissima</i>	Arboretum	park	318.0	1.5	W	5.0	12.0	1	low	2011
L05	<i>C. mollissima</i>	Arboretum	park	318.2	1.4	W	4.0	9.0	1	low	2011
L06	<i>C. mollissima</i>	Arboretum	park	317.9	1.1	W	8.5	26.0	2	low	2011
L07	<i>C. mollissima</i>	Cooper	yard	313.2	4.5	NE	10.0	46.0	1	low	2011
L08	<i>C. mollissima</i>	Cooper	yard	318.3	2.9	NW	5.5	25.0	1	none	---
L09	<i>C. mollissima</i>	Cooper	yard	319.1	1.3	W	5.5	26.0	1	none	---
L10	<i>C. mollissima</i>	Cassidy	street	316.6	0.2	NE	8.0	32.0	1	low	2011
L11	<i>C. mollissima</i>	Cassidy	street	316.4	0.6	SE	8.0	38.0	2	low	2011
L12	<i>C. mollissima</i>	Cassidy	street	316.4	0.6	SE	8.5	42.0	2	low	2011
L13	<i>C. mollissima</i>	Cassidy	street	316.2	1.3	NE	9.0	38.0	2	low	2011
L14	<i>C. mollissima</i>	Cassidy	street	316.1	2.2	NW	9.5	44.0	2	low	2011
L15	<i>C. mollissima</i>	Cassidy	street	316.1	2.4	NW	9.5	37.0	2	low	2011

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continued on page 19

continued from page 18

L16	<i>C. mollissima</i>	Fontaine	yard	315.7	3.5	NE	8.5	32.0	1	low	2011
<b>L17</b>	<b><i>C. mollissima</i></b>	<b>Fincastle</b>	<b>yard</b>	<b>310.3</b>	<b>3.0</b>	<b>SW</b>	<b>8.5</b>	<b>31.0</b>	<b>2</b>	<b>high</b>	<b>2010</b>
L18	<i>C. mollissima</i>	Oldham	yard	303.7	0.5	NW	13.5	38.0	2	low	2011
L19	<i>C. mollissima</i>	Oldham	yard	304.4	0.6	W	8.5	22.0	1	low	2011
L20	<i>C. pumila</i>	Arboretum	park	316.7	6.4	SW	1.5	4.0	1	none	- - -
L21	<i>C. mollissima</i>	Tateswood	yard	308.5	2.9	S	11.0	39.0	1	low	2011*
L22	<i>C. mollissima</i>	Garden	yard	314.1	2.0	SW	3.5	6.0	1	low	2011*
L23	<i>C. mollissima</i>	Garden	yard	313.9	2.0	SW	6.5	22.0	2	low	2011*
L24	<i>C. mollissima</i>	Garden	yard	314.0	2.0	SW	7.5	22.0	2	low	2011*
19 L25	<i>C. mollissima</i>	Wilson	yard	307.0	5.5	NE	8.5	30.0	2	none	- - -
L26	<i>C. mollissima</i>	Wilson	yard	306.5	4.0	NE	7.5	30.0	3	none	- - -
L27	<i>C. dentata</i>	McConnell	woods	282.4	13.2	N	4.0	7.0	1	none	- - -

<sup>1</sup>diameter at 1.4 m above ground level

<sup>2</sup>crown condition – 1: live canopy 90-100%; 2: live canopy 75-90%; 3: live canopy <75%

<sup>3</sup>ACGW infestation – low: <30% shoots galled, high: >30% shoots galled (Gyoutoku and Uemura 1985, EFSA 2010)

\*estimated year of infestation

Table 2.2. Ambient conditions in Fayette Co., KY (USA), during the adult flight period of *Dryocosmus kuriphilus* (23 Jun – 4 Jul 2010)

Date	Mean T (°C)	Precipitation (mm)	Solar radiation (MJ/m <sup>2</sup> )	Humidity		Wind	
				Minimum (%)	Maximum (%)	Direction	Speed (m/s)
23 June	27.3	0	27.5	47	90	SW	3.2
24 June	25.8	4.6	17.5	48	88	W	3.3
25 June	24.8	0	29.5	40	86	NNE	1.8
26 June	25.4	0	27.4	50	95	SW	2.5
27 June	27.6	0	25.7	57	89	SW	4
28 June	24.2	3.8	15.9	63	96	WSW	3.4
29 June	24.9	0	24.6	39	98	NW	3.1
30 June	21.3	0	30	38	83	NE	3.5
1 July	20.1	0	31.2	32	77	NE	3.9
2 July	20.8	0	31.5	31	78	ENE	3.1
3 July	23.7	0	27.8	37	75	SSW	2.3
4 July	25.7	0	24.8	47	93	SSW	2.5



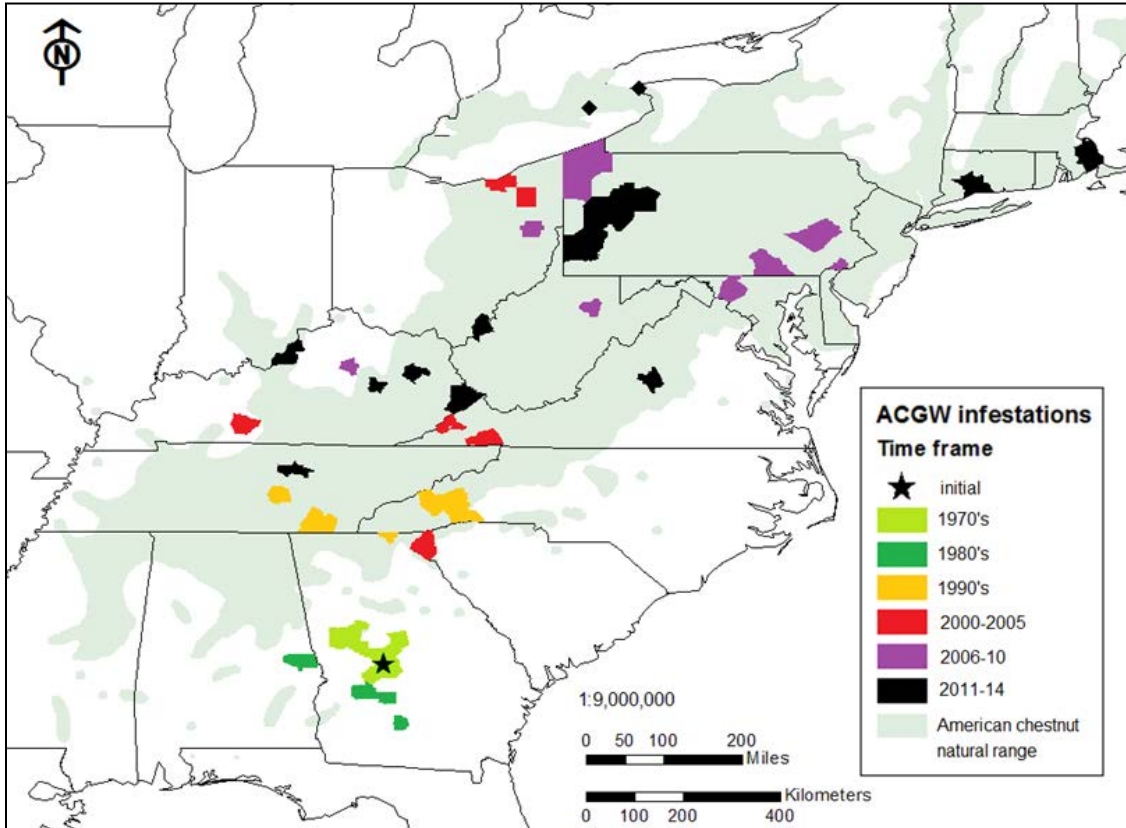


Figure 2.1. Distribution and spread of the Asian chestnut gall wasp in North America since its introduction in 1974 (updated December 2014).

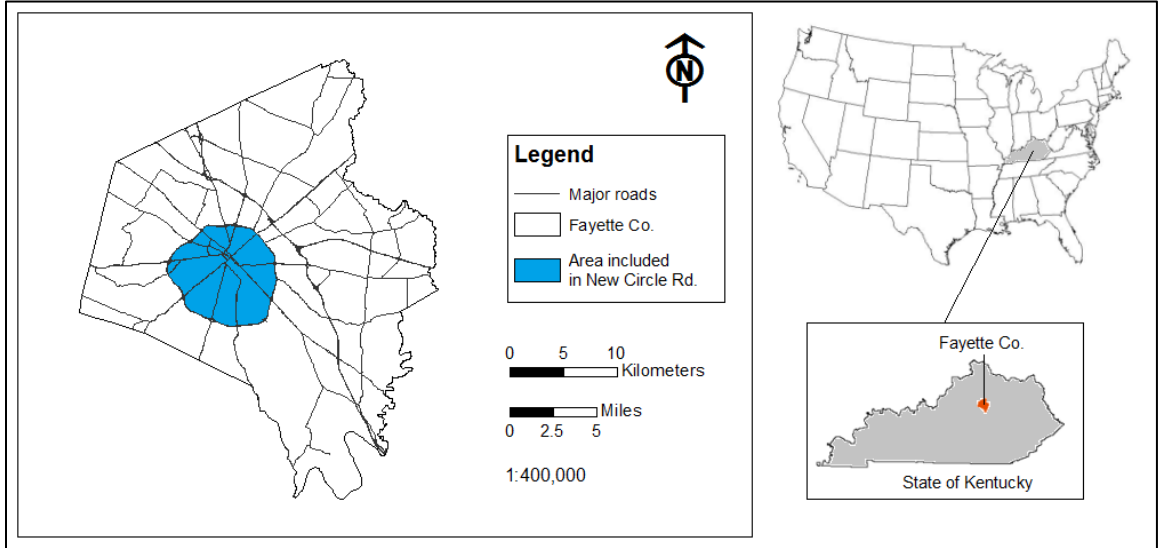


Figure 2.2. Area in Lexington, KY, USA, surveyed for *Castanea*.

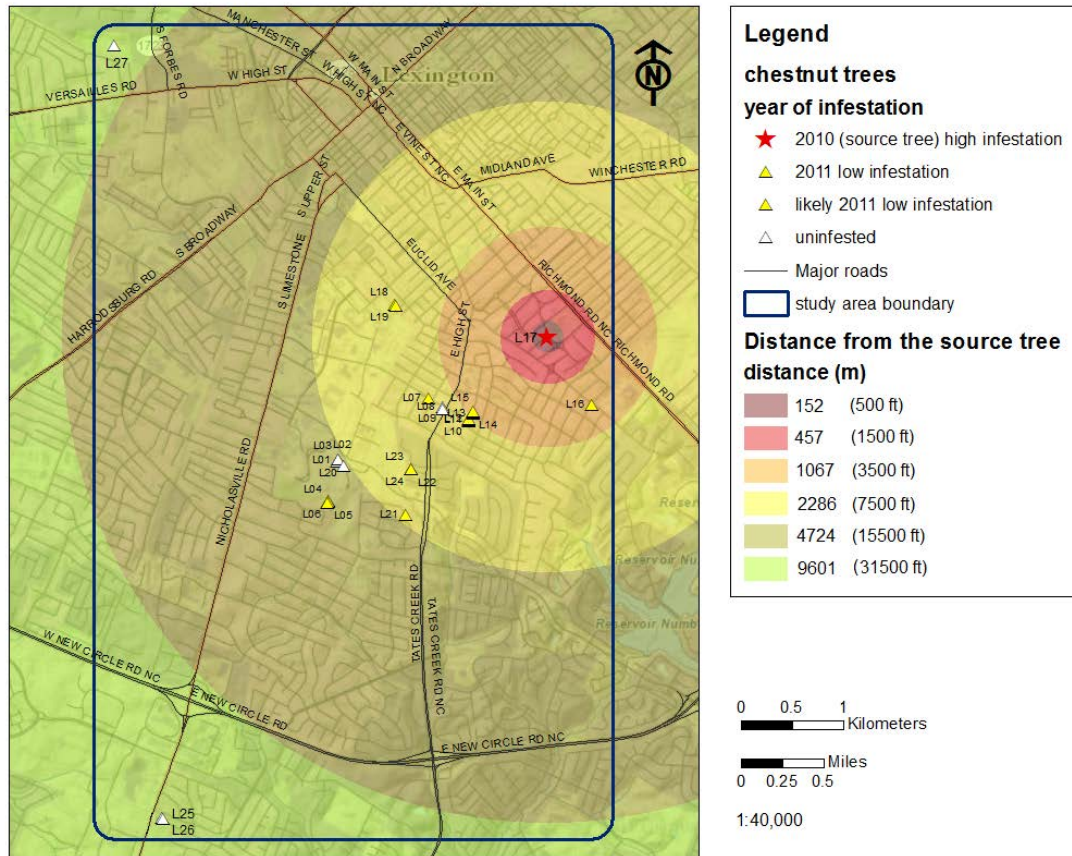


Figure 2.3. Location of *Castanea* trees and the relative incidence and location of gall wasp infested trees within the study area of Fayette Co., KY, USA.

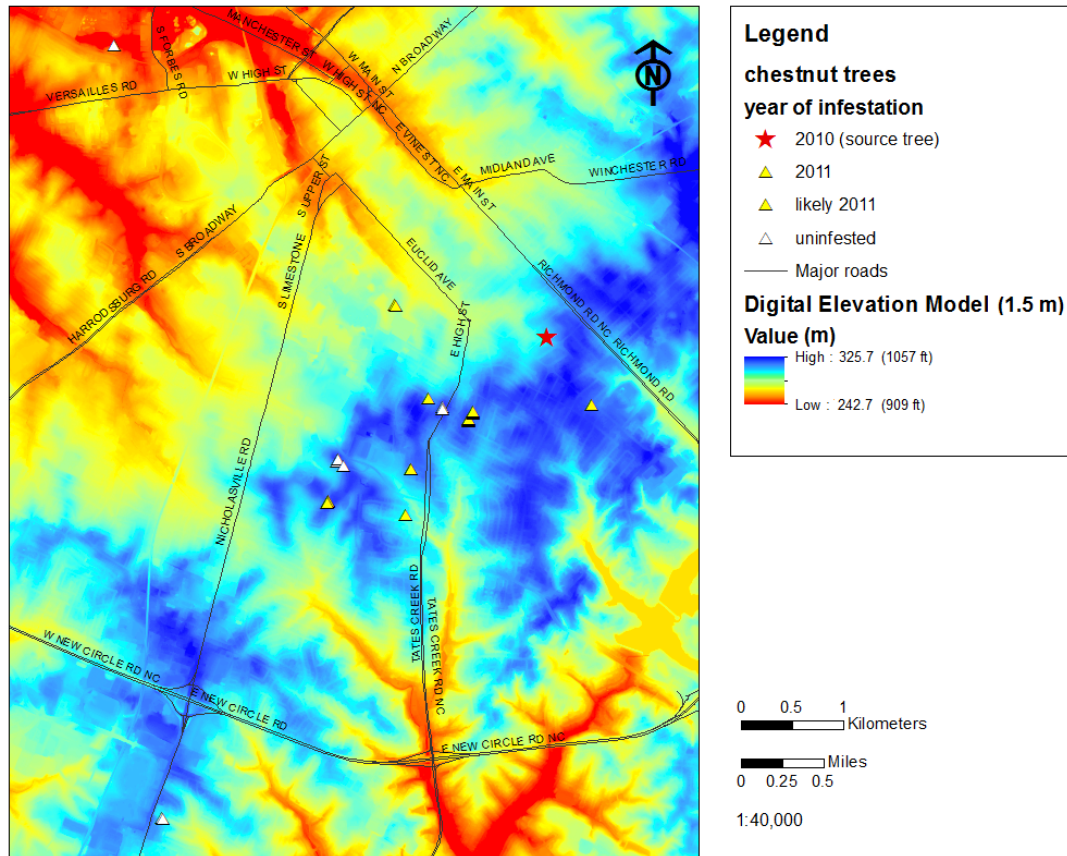


Figure 2.4. Digital elevation modeling (1.5 m resolution) demonstrates that chestnut occurred at relatively consistent elevations in the study area. See also Table 2.1.

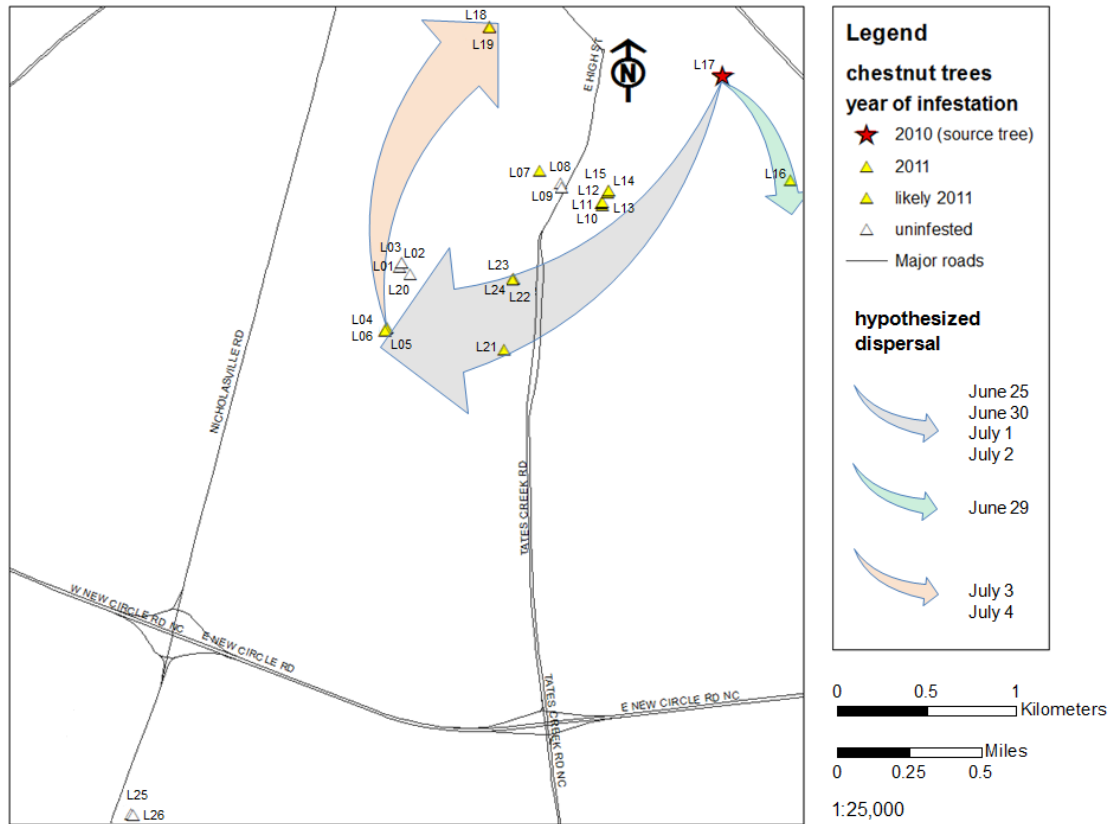


Figure 2.5. Hypothesized movement of the chestnut gall wasp in Lexington, KY, USA, in the period of June 23 – July 4 2010, resulting in newly infested trees in 2011.

**Chapter Three: Potential fecundity of a highly invasive gallmaker, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae)**

The dynamics of biological invasions are strongly influenced by life history traits of the species in question (Liebhold and Tobin 2008). Life-history traits can promote species' invasiveness by enhancing phenotypic plasticity, increasing the potential for evolutionary changes, or by triggering rapid population expansion (Sakai et al. 2001). Establishment and spread in newly colonized areas are critical steps in the invasion process, and are affected by population growth rate (Lockwood et al. 2007).

Fecundity is a key factor defining population growth, and can be used as a predictor of species' invasiveness (Keller et al. 2007). In insects, fecundity is generally a function of time, increasing after egg maturation and decreasing as females' age, and is strongly correlated with body size, food quality and ambient temperatures (Kindlmann et al. 2001). Prevailing dogma suggests that large, heavy insects are more fecund than smaller individuals (Honěk 1993, Tammaru et al. 1996), but this assumption has been questioned due to the lack of validity across taxa (Berrigan 1991, Blackburn 1991), and the realization that weight is more closely correlated to egg load than to total fecundity (Leather 1988). Fecundity is also affected by temperature, which regulates egg maturation and oviposition activity (Berger et al. 2008). In herbivorous insects, host plant quality may also affect insect fecundity by influencing allocation to reproductive resources, including egg quality and size, and by shaping oviposition strategies (Awmack and Leather 2002). Presumably the relationship between host plant quality and insect fecundity falls along a continuum, and is stronger for highly specialized insects and weaker for extreme generalists (Mooney et al. 2012).

I sought to investigate linkages between insect fecundity and invasiveness in a specialist insect herbivore, the Asian chestnut gall wasp (ACGW) *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). The gall wasp is native to China; it has invaded Japan (Yasumatsu 1951), Korea (Pak 1970) North America (Payne et al. 1975), Nepal (Ueno 2006) and Europe (Melika et al. 2003), and is a global pest of all *Castanea* species (Fagales: Fagaceae). It was first reported in the United States in 1974 in Georgia and it has since spread quickly throughout the former range of American chestnut (Rieske 2007, Rieske and Cooper 2011) due to larval movement in infested plants and adults' ability to colonize sporadically occurring host plants using prevailing winds (Graziosi and Rieske 2012). Gall wasp larvae overwinter inside host buds and induce formation of spherical galls on new vegetation the following spring (Yasumatsu 1951, Payne et al. 1975). Galling disrupts tree growth and alters floral development leading to drastic yield loss and plant decline (Payne et al. 1983, Kato and Hijii 1997, Battisti et al. 2013). While the gall wasps' cryptic nature has contributed to its invasiveness and subsequent global distribution, its potential fecundity in relation to its invasiveness has not been fully evaluated.

*D. kuriphilus* is proovigenic (Nohara 1956) and reproduces by thelytokous parthenogenesis (Zhu et al. 2007). Prior to adult emergence females remain inside galls for 7-10 d for ovarian maturation (Otake 1980, Kato and Hijii 1999, Zhi-yong 2009), and upon emergence are ready to oviposit (Kato and Hijii 1993). Females produce 287-300 eggs. In the field they live 2-3 d (Nohara 1956, Tokuhisa 1981), but in the laboratory they can live 6 d when provided with sugar water (Bosio et al. 2009, Romani et al. 2010).

Considering their high reproductive potential and limited longevity, I hypothesize that insect age is a key factor affecting potential fecundity of *D. kuriphilus*. My objective is to evaluate the effect of age on the egg load of newly emerged *D. kuriphilus* adults, and to relate my findings to egg and body metrics.

## **Materials and methods**

*D. kuriphilus* galls were collected June 23 2012, just prior to adult emergence, from Chinese chestnut (*C. mollissima*) trees in Fayette County, KY (37°54'11.30"N, 84°25'35.80"W) and Portage County, OH (41°19'55.37"N, 81°11'00.12"W). Galls were placed in clear plastic boxes (30 × 23 × 10 cm<sup>3</sup>) in the laboratory (~21 °C with ambient light) and checked for emerging insects daily. Emerging adults were collected and held without feeding in glass vials (20 ml). Wasps were removed from vials at 24 h intervals to generate four age cohorts: 1) 0 d wasps, 2) 1 d wasps, 3) 2 d wasps, and 4) 3 d wasps. Six wasps per cohort were collected and placed in 70% ethanol.

Wasps from each cohort were air-dried for 30 min and weighed. Using a dissecting microscope with a graduated micrometer, I measured total body length (Figure 3.1A), mesosomal length and width, measured at the point where the mesoscutum is widest (Figure 3.1B), metasomal length and width, measured along the median line between the first and second metasomal segments (Figure 3.1C), and length of the left hind femur (Figure 3.1D). Each individual was then placed laterally in a watch glass (67 mm diameter) and supported on paraffin wax. The metasoma was incised longitudinally using a scalpel and the teguments removed. Ovaries were extracted, placed in a clean watch glass, and stained with 200 µl of stain solution (20% of double stain (Bioquip Inc.,



Rancho Dominguez CA) in 70% ethanol). After 10 min. ovaries were placed in glycerol on a microscope slide (Figure 3.1E). Ovarioles were teased apart using a fine pin and the total number of eggs was counted (Figure 3.1F). A subsample of 10 eggs per wasp was used to measure egg length and width (Figure 3.1G). The volume of each individual egg was calculated using the formula for a prolate spheroid (Berrigan 1991):  $V = 1/6 \pi \times W^2 \times L$ , where W and L represent width and length respectively. The number of eggs was assessed using 6 wasps for each age cohort, while body and egg measurements were evaluated using 3 insects per cohort.

The influence of adult age on the number of eggs per female was assessed using a generalized linear model procedure (PROC GENMOD, SAS<sup>®</sup> V. 9.1). Body and egg size data were tested for normality (PROC UNIVAR) and then log-transformed. Analysis of variance (PROC MIXED) on log-transformed data was used to evaluate differences in body morphometrics and egg size. I performed a multiple regression analysis (PROC REG, SAS<sup>®</sup> V. 9.1) with metasomal width, body weight, and egg volume, and used backwards selection to learn the best predictor of fecundity (number of eggs).

## Results

The reproductive potential of *D. kuriphilus* is at a maximum immediately following adult emergence from the gall, with an average egg load of 268 ( $\pm 18.5$ ) for newly emerged individuals. The number of eggs per female declines incrementally over the 4 d period I evaluated (Figure 3.2A), this being confirmed by the highly significant negative correlation between egg number and wasp age ( $R^2 = 0.70$ ;  $F_{1,22} = 52.40$ ;  $p < 0.0001$ ). Similarly, adult *D. kuriphilus* weight was greatest immediately after emergence

and declined significantly with age (Figure 3.2B), with a mean of 0.8 mg across all age cohorts. Metasomal width of newly emerged wasps is also greatest immediately following emergence, and also declines with age (Figure 3.2C). I also found strong positive correlations between the number of eggs and adult wasp weight (Figure 3.3A), and the number of eggs and metasomal width (Figure 3.3B). I found that the best model to determine fecundity is the correlation between number of eggs and wasp weight. Wasps carrying a greater number of eggs weigh more than wasps carrying fewer eggs, and also have their metasoma dilated latitudinally to accommodate their egg load. Wasp weight and metasomal width were similarly positively and significantly correlated ( $R^2 = 0.73$ ;  $F_{1,10} = 26.82$ ;  $p < 0.001$ ).

Not surprisingly, the remaining adult metrics I measured were independent of wasp age (see Appendix III), including body length ( $F_{3,8} = 2.59$ ;  $P = 0.126$ ;  $N = 12$ ; mean 149.5  $\mu\text{m}$  across age cohorts), mesosomal length and width ( $F_{3,8} = 1.71$ ;  $P = 0.242$ ;  $N = 12$ ; mean 1075.0 and ( $F_{3,8} = 2.64$ ,  $P = 0.121$ ;  $N = 12$ ; mean 802.5  $\mu\text{m}$ , respectively, across cohorts), metasomal length ( $F_{3,8} = 2.55$ ,  $P = 0.129$ ;  $N = 12$ ; mean 1325.0  $\mu\text{m}$ ), and hind femur length ( $F_{3,8} = 1.11$ ,  $P = 0.399$ ;  $N = 12$ ; mean 745.0  $\mu\text{m}$ ). I found no significant correlations between the number of eggs and these adult wasp metrics.

Individual egg size also varied with insect age. Egg volume increases following wasp emergence, reaching a maximum at two days, before declining the third day (Figure 3.2D). This variation in area and volume is driven primarily by changes in egg width ( $F_{3,116} = 3.90$ ,  $P = 0.011$ ;  $N = 120$ ; mean 101.1  $\mu\text{m}$  across age cohorts) rather than egg length ( $F_{3,116} = 2.58$ ,  $P = 0.057$ ;  $N = 120$ ; mean 149.5  $\mu\text{m}$ ). I found no correlation between egg volume and egg load (Figure 3.3C).

## Discussion and conclusions

The decline in the number of eggs and the increase in the volume of eggs with wasp age challenges accepted views on reproductive strategies of *D. kuriphilus*. Nohara (1956) describes *D. kuriphilus* as proovigenic, with adult females that emerge with a full complement of eggs and the ability to oviposit immediately, and no need for pre-oviposition feeding or maturation. This is in contrast to species that are synovigenic, where the number of mature eggs within ovaries increases rapidly following adult emergence. *Dryocosmus kuriphilus* is a gall-forming insect, and adults spend several days following eclosion inside chambers within galls in order to mature eggs (Otake 1980, Kato and Hijii 1999, Zhi-yong 2009). This post-eclosion/pre-emergence period appears to create an intermediate approach to egg development and ovarian maturation for *D. kuriphilus*. Ovigyny is a continuum ranging from strictly proovigenic to strictly synovigenic (Jervis et al. 2001), and my data suggest that *D. kuriphilus* may fall on this continuum.

The decline in the number of eggs as adult *D. kuriphilus* age further supports this, and suggests that egg resorption (=oosorption) is occurring. Egg resorption is the ability to dissolve mature oocytes and reallocate nutrients (Bell 1975), and represents a strategy to maintain reproductive capacity when no suitable oviposition hosts are available (Flanders 1942, Rosenheim et al. 2000). Egg resorption occurs in many parasitic Hymenoptera with synovigenic ovarian development (Rosenheim et al. 2000, Jervis 2001). These insects are able to mature additional eggs during the adult stage when hosts are abundant, or resorb eggs when suitable hosts are lacking, whereas egg maturation in proovigenic species occurs only prior to adulthood (Ellers et al. 2000).

Parasitic Hymenoptera are primarily synovigenic (Jervis et al. 2001, Jervis et al. 2008), but within Cynipoidea proovigeny is prevalent (Vårdal et al. 2003). At least some pro-synovigenic cynipids are described, such as *Diplolepis rosae* L., another parthenogenetic gall-former (Vårdal et al. 2003). Egg resorption has also been noted in *Gonatocerus ashmeadi* Girault (Hymenoptera: Mymaridae), a Chalcidoidea previously assumed to be proovigenic and now considered synovigenic (Irvin and Hoddle 2009). My findings similarly suggest that *D. kuriphilus* may be at least partially or facultatively synovigenic, rather than strictly proovigenic. Egg resorption allows reallocation of resources for survival and future reproduction, and is related to nutrient availability during adulthood (Moore and Attisano 2011, Rivero-Lynch and Godfray 1997). Larger eggs have more yolk available for embryonic development (Capinera et al. 1977), suggesting an increase in offspring fitness. The absence of suitable hosts in my experimental conditions may have triggered egg resorption in my experimental *D. kuriphilus*, and the increase in egg volume I observed may be attributable to reallocation of nutrients made available through resorption to remaining eggs, thereby increasing egg volume and quality. This strategy may optimize chances for high quality eggs to generate viable brood. Adult *D. kuriphilus* longevity in the field is a relatively brief 2.5 d (Nohara 1956, Tokuhisa 1981), after which host-deprived adults reallocate nutrients previously invested in eggs to body maintenance, thus explaining the decrease in egg size I observed (Figure 3.2D).

The egg load I report, mean 190 ( $\pm$  13) across age cohorts, is lower than the 287 – 300 egg load previously reported (Nohara 1956, Tokuhisa 1981). This may be due to differences in host plant quality and therefore larval nutrition, but my approach evaluating egg load over several age cohorts suggests another potential explanation. My

newly emerged adults contained a mean of 268 eggs, comparable to previous reports, but in my study egg load declined significantly and incrementally over time. Previously studies of *D. kuriphilus* egg load assumed proovigeny, without measuring or considering egg resorption or the possibility of facultative synovigeny, which my data suggests may be occurring. My findings differ from those of Nohara (1956), who evaluated wasps as a single cohort and reported a positive correlation between adult *D. kuriphilus* size and the associated egg load, discounting the potential influence of wasp age. That approach would obscure the differences I report (Figure 3.2A) and that I attribute to the likelihood of eggs being resorped over time in the absence of suitable host material.

*Dryocosmus kuriphilus* are short-lived with a large egg load. While highly fecund insects often have a short life due to the energy costs of reproduction (Carey et al. 1998), host apparency may also play a role; hymenopteran parasitoids tend to be more fecund but short lived when their chances of successfully locating suitable hosts are high (Price 1973, Blackburn 1991). Similarly, *D. kuriphilus* adults emerge from galls within the host chestnut canopy, where buds suitable for oviposition are likely abundantly available.

I found two morphometric parameters, wasp weight and metasomal width, able to predict egg load. But my data suggest egg resorption, and potentially facultative synovigeny, may be providing *D. kuriphilus* with reproductive plasticity to allow adaptation to environmental conditions, thereby increasing reproductive success and increasing invasiveness.

My findings stress the need for additional work on *D. kuriphilus* to evaluate the effects of feeding and host availability on ovarian development and oviposition behavior,

and to shed further light on reproductive strategies in this cryptic, parthenogenetic, invader.

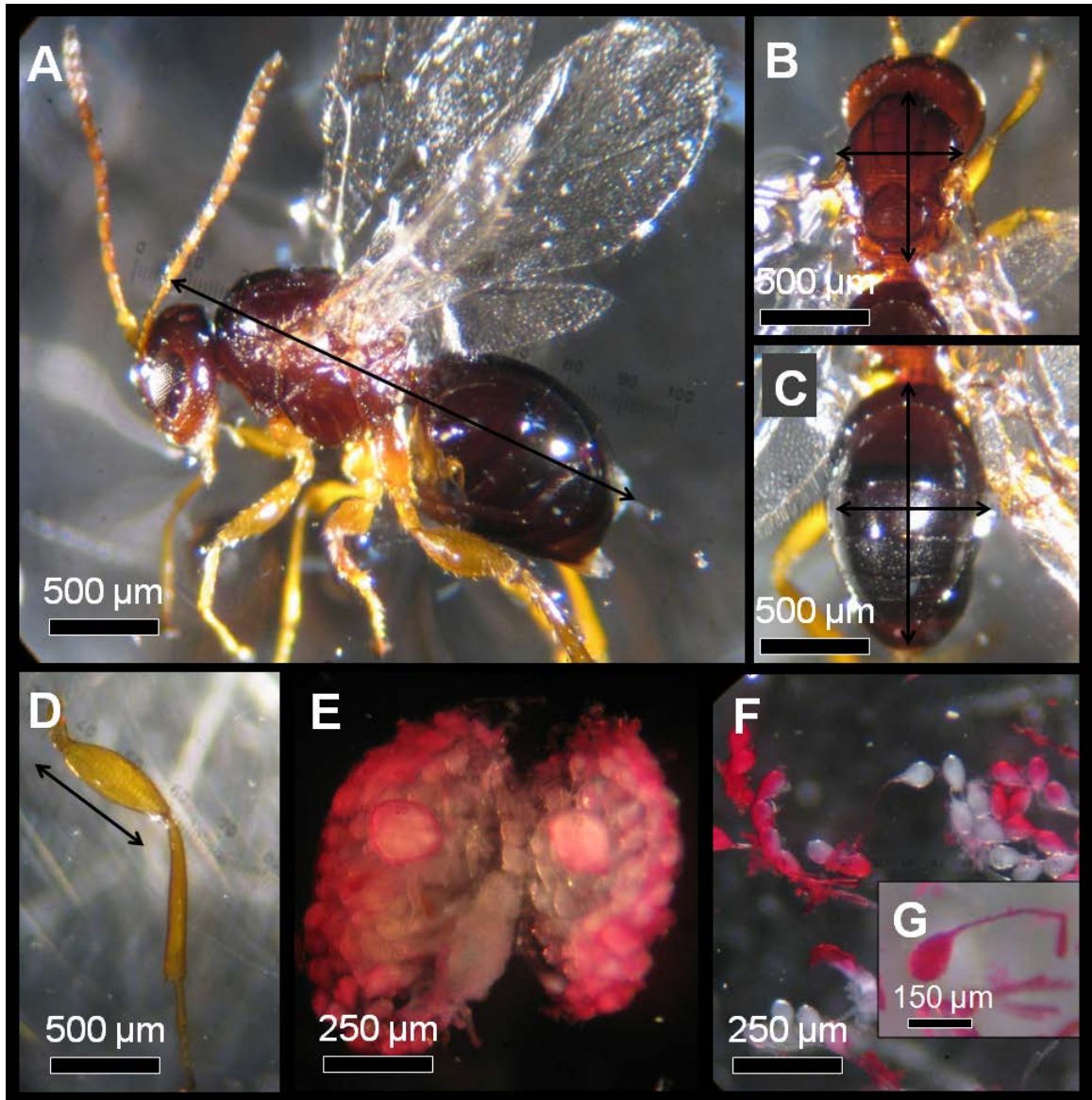


Figure 3.1. Morphometric measurements of *Dryocosmus kuriphilus*: (A) total body length, (B) mesosomal length and width, (C) metasomal length and width, (D) hind femur length, and (E) extracted and stained ovaries, (F) ovarioles teased apart to release (G) individual eggs.

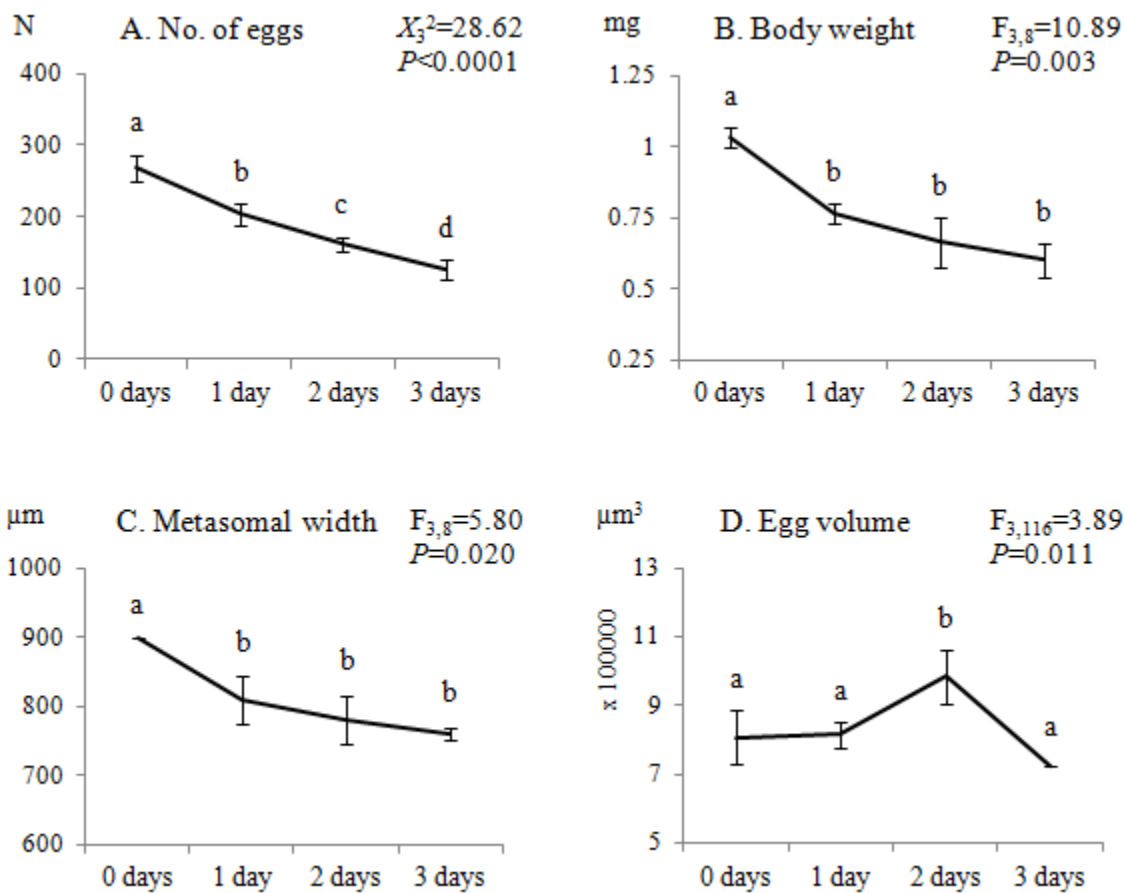


Figure 3.2. Differences among *D. kuriphilus* females of different ages in: (A) number of eggs, (B). body weight, (C). metasomal width, (D) egg volume. Means followed by the same letter do not differ ( $\alpha = 0.05$ ).



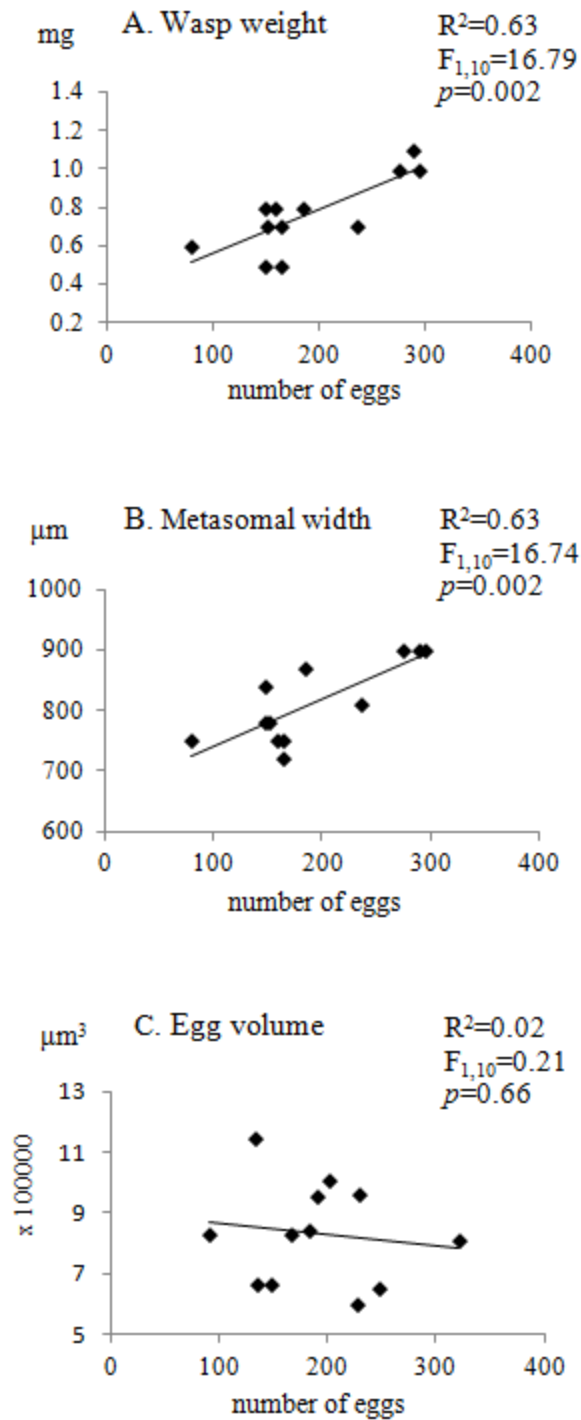


Figure 3.3. Correlations between: (A) wasp weight and number of eggs, (B) metasomal width and number of eggs, (C) egg volume and number of eggs.

## **Chapter Four: Population size and parasitoid pressure influences gall morphology in *Dryocosmus kuriphilus*, a highly invasive pest of chestnut**

Insect-induced plant galls result from interactions between plant phenotype and insect stimuli, and although they consist of plant tissue, they represent an extended phenotype of the inducing insect (Weis et al. 1988, Stone and Cook 1998, Stone and Schonrögge 2003). Oviposition secretions (McCalla et al. 1962), egg exudates, or salivary secretions from newly hatched larvae (Mani 1992, Rohfritsch 1992) induce proliferation of plant tissue surrounding the reactive site, thus triggering gall development. Insect galls show impressive variation among insect taxa (Mani 1964, Dreger-Jauffret and Shorthouse 1992), especially those formed by hymenopteran gall wasps in the family Cynipidae, and their morphology has been shown to be adaptive (Price et al. 1987, Stone et al. 2002, Stone and Schonrögge 2003). Optimization of nutritive resources may be responsible for diversification of some internal structures (Stone and Schonrögge 2003) and chemical composition (Hartley 1998), but escape from natural enemies is thought to be the principal selective force driving inter- and intra-specific variation in gall morphology. For instance, gall size affects parasitization in both goldenrod gall fly (Diptera: Tephritidae) (Abrahamson et al. 1989) and galling sawflies (Hymenoptera: Tenthredinidae) (Price and Clancy 1986). Within the Cynipidae parasitoid and predator pressure relate to gall traits such as size (Stille 1984), shape (Jones 1983) and number of larval cells (László and Tóthmérész 2008, 2013), yet the selective pressures and resulting variation in gall morphologies in this diverse group have not been fully elucidated.

I investigated intra-specific variation in morphology of galls induced by the globally invasive Asian chestnut gall wasp *Dryocosmus kuriphilus* Yasumatsu, a cynipid native to China that has become widespread on *Castanea* species throughout Asia, North America and Europe (Rieske 2007, Avtzis and Matošević 2013). *Dryocosmus kuriphilus* is a non heterogonic wasp; parthenogenetic females oviposit in chestnut buds in early summer, and first instar larvae overwinter within buds until the following spring, when multi-celled galls are induced on new vegetation (Payne et al. 1975, Viggiani and Nugnes 2010). Gallling affects photosynthesis and compromises development of infested organs, thus disrupting tree growth and reproduction (Kato and Hijii 1997) and reducing yield (Battisti et al. 2014). The multi-celled nature of galls is determined at oviposition, when females lay clusters of 1-10 eggs (Nakamura et al. 1964). However, adult females carry large egg loads (Graziosi and Rieske 2014), and multiple oviposition events by the same or different females can occur (Kato and Hijii 2001), resulting in one to multiple galls developing from the same bud. Individual galls contain 1 to over 20 larval chambers or ‘cells’ (Yasumatsu 1951). The average number of eggs per bud and the number of cells per gall is highly variable among years and locations, depending on gall wasp pressure, bud size, and chestnut variety (Miyashita et al. 1965, Kamijo and Tate 1975, Kato and Hijii 1993, Cooper and Rieske 2010, Panzavolta 2011). Cell formation begins in the bud shortly after eggs hatch, when plant tissue differentiates surrounding each larva (Reale et al. 2014). Galls can be classified based on the plant organ from which they develop, including shoot, leaf, or petiole, regular or dormant bud (Maltoni et al. 2012), and simple or compound morphology (Payne 1975, Bernardo et al. 2013). Uni-lobed galls consist of

a single sub-spherical or ellipsoid shape, whereas multi-lobed are formed by multiple lobe-shaped structures.

In North America, *D. kuriphilus* undergoes extreme population fluctuations. Galls are utilized extensively by the exotic *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), a larval ectoparasitoid deployed for biological control and successfully established (Cooper and Rieske 2011, Graziosi and Rieske 2013), as well as by endemic parasitoids (Cooper and Rieske 2007, 2011). In addition galls are colonized by *Colletotrichum* sp., a plant pathogenic fungus that causes extensive gall wasp mortality (Cooper and Rieske 2007, Graziosi and Rieske in review). Based on these observations I investigated the morphology of galls induced by *D. kuriphilus* on chestnut, and evaluated potential factors driving the morphological variability I observed, namely enemy free space and resource competition.

## **Materials and methods**

In late June 2013, after adult wasp emergence, four *D. kuriphilus* infested Chinese chestnut trees (*C. mollissima* Blume) were located in Lexington KY, and the severity of gall wasp infestation was assessed by counting the number of galls on an accessible, randomly chosen branch ~1 m in length. Gallings pressure ranged from very low ( $\leq 5$  galls per 1 m branch), low (6-10), medium (11-15), to high ( $\geq 15$  galls per 1 m branch). I collected 5 galls per branch from three branches per tree (N = 50 total), except the tree with very low galling, where only one galled branch was found. Galls were excised, returned to the laboratory, and stored at 4 °C until further analysis. The morphology of each gall was scored as either uni- or multi-lobed (Figures 4.1A and 4.1B). The number

of lobes per gall was counted and each lobe was separated, weighed and measured using a precision electronic balance and a caliper. Lobe volume was calculated using the formula for an ellipsoid,  $V = 4/3 \times \pi \times L \times D_1 \times D_2$ , where L,  $D_1$  and  $D_2$  are length, diameter 1 and diameter 2 of each lobe, respectively. Gall weight and volume were calculated as the sums of the individual lobes. I then dissected individual lobes to evaluate gall cell contents and individual gall cell morphology. The presence of gall wasp and parasitoid larvae was noted (Figures 4.1C, D), and the parasitization rate was calculated as  $N_p / N_c$ , where  $N_p$  and  $N_c$  are the number of parasitoid larvae and the number of cells per lobe, respectively. Similarly, I determined the presence of necrotic tissue surrounding the larval cell and calculated necrotization as  $N_n / N_c$ , where  $N_n$  is the number of necrotic cells. The thickness of the vacuolated parenchyma, destined to become nutritive tissue lining (Stone and Schönrogge 2003, Cooper and Rieske 2009), was measured on one cell per lobe, and the average thickness was calculated using  $(T_1 + T_2) / 2$ , where  $T_1$  and  $T_2$  are thickness values measured at two perpendicular points along the cell (Figure 4.1E).

Data were tested for normality (PROC UNIVAR, SAS<sup>®</sup> V. 9.3), and when necessary transformed using a logarithmic transformation for the number of cells per gall, cells per lobe, volume per gall, volume per lobe and weight per lobe; a square root + 0.5 transformation for the weight per gall and the number of lobes per gall; and an arcsin transformation for the parasitoid attack rate. I used an analysis of variance (PROC GLM) to test the effect of gall morphology (uni-lobed versus multi-lobed) on the number of cells per gall and per lobe; gall volume and weight; lobe volume and weight; parasitization rate; and thickness of nutritive tissue. I also evaluated the variation in the

number of lobes per gall among trees and among branches within each tree (PROC GLM).

## Results

Of the 50 galls collected from four trees, 33 (66%) were uni-lobed and 17 (34%) were multi-lobed (2 – 6 lobes, average 2.9). Multi-lobed galls contain significantly more cells (range 2 – 26) than uni-lobed galls (range 1 – 6), but there are no difference in the number of cells per individual lobe (Table 4.1). Multi-lobed galls are larger and heavier, but volume and weight of individual lobes does not vary between the two morphologies, and no differences in nutritive lining thickness were detected between the two gall morphologies (Table 4.1). Parasitization is significantly lower in multi-lobed galls than uni-lobed galls, while necrotization caused by fungal infection was reported exclusively on multi-lobed galls (Table 4.1).

Of the galls sampled from the most heavily infested tree, 60% were multi-lobed ( $2.4 \pm 0.42$  lobes), whereas trees with medium and low galling had only 33% and 14% of galls with more than one lobe ( $1.5 \pm 0.2$  and  $1.3 \pm 0.11$  lobes, respectively). Only simple uni-lobed galls were found on the tree with the lowest infestation. (Figure 4.2A). Among branches the variation in the number of lobes per gall was high (Figure 4.2B), and the effect of branch and the interaction between branch and tree were significant ( $F_{9, 41} = 6.40$ ;  $P = 0.0004$ , and Figure 4.2B).

## Discussion and conclusions

Natural enemy pressure is frequently cited as a primary factor driving gall morphology (Stone and Schönrogge 2003); here I evaluate its relevance in the *Castanea* – *Dryocosmus* system. I evaluated occurrence and structure, and assessed interactions with natural enemies, of *D. kuriphilus*-induced galls with complex morphologies on Chinese chestnut. Galls with simple and compound morphologies have been reported on *C. sativa* (Bernardo et al. 2012), but here I evaluate gall morphology on *C. mollissima*, focusing on the number and complexity of gall lobes.

*Dryocosmus kuriphilus* galls that harbor multiple cells are thought to be the result of multiple oviposition events that occur when gall wasp populations are high (Itô 1967, Kato and Hijii 2001, Bernardo 2012). In a similar system, Atkinson et al. (2002) showed that multi-celled galls induced by the cynipid *Andricus* spp. on European *Quercus* contain brood from different females.

I found that multi-lobed galls harbor more larval cells than uni-lobed galls, but individual lobes contain the same number of cells regardless of gall morphology, and hypothesize that multi-lobed galls form in response to multiple *D. kuriphilus* oviposition events in a single bud. Furthermore, my findings suggest that each lobe of a multi-lobed gall is determined by an independent oviposition event. *Dryocosmus kuriphilus* eggs are laid in clusters (Nakamura et al. 1964), and each cluster appears to produce a single lobe in the developing gall (Figure 4.3). Consequently, buds containing multiple egg clusters, as occurs when gall wasp populations are high, will develop galls with multiple lobes, generating galls with greater volume that harbor more cells.

However, the host plant may also influence gall morphology, since the number of larval cells per gall differs among species (Cooper and Rieske 2010), among chestnut varieties (Kamijo and Tate 1975, Panzavolta et al. 2011), among trees (Figure 4.2A), and also among branches within a given tree (Figure 4.2B). Adult wasps preferentially oviposit in large buds (Kato and Hijii 2001, Panzavolta et al. 2011), and both bud size and gall wasp pressure likely contribute to the occurrence of multi-lobed *D. kuriphilus* galls. When gall wasp populations are high the availability of optimal size buds for ovipositing female is reduced (Kato and Hijii 2001); multiple females may oviposit in the larger, viable buds, thus driving a high occurrence of multi-lobed galls the following year.

I found that larger, multi-celled, multi-lobed galls are less parasitized than smaller, uni-lobed galls, perhaps due to a thicker sclerenchyma (Cooper and Rieske 2010), or because larger, multi-lobed galls contain a greater number of larval cells. Parasitic *Pteromalus* wasps select small galls among the ones produced by its tenthredinid host, *Euura lasiolepis* (Price and Clancy 1986). Within the Cynipidae parasitism also decreases with increasing gall size; but this is often an artifact of clutch size, as the size of galls is driven by the number of larval cells. This is the case of galls induced on *Rosa* sp. (Rosales: Rosaceae) by the parthenogenetic and univoltine *Diplolepis rosae*, in which parasitism decreases with increasing number of larvae per gall (Stille 1984, László and Tóthmérész 2008, 2010). In cases of high population pressure, the univoltine *Diastrophus kincaidii* reduces gall parasitism by ovipositing multiple times in the same site of its rosaceous host, producing large and multi-celled galls (Jones 1983, Kraft and Erbsch,



1990); larvae located in the innermost part of the galls are protected by surrounding larval cells, and escape parasitism.

Parasitism may similarly be shaping *D. kuriphilus* gall morphology when gall wasp density is high, thus selecting multi-lobed galls generated by multiple oviposition events in the same bud. In general, *D. kuriphilus* females optimize fitness by laying small egg clutches (Kato and Hijii 1993), similar to other cynipids (László and Tóthmérész 2008). Gall wasps may be optimizing fitness by distributing their egg load among several buds within a tree or across multiple trees, rather than concentrating their reproductive potential in only a few buds, triggering formation of larger galls containing most of their brood. Laying small egg clutches in multiple buds within and among trees may increase brood survival by reducing the risk to founder wasps of losing an entire brood due to localized gall predation, especially by vertebrates such as birds and rodents, or due to damage to a given branch or tree bearing galls. In light of this, females would avoid laying large egg clutches or ovipositing multiple times in the same bud, and uni-lobed galls with a low number of cells should be favored (Figure 4.3A). However, with high gall wasp pressure the bud to female ratio decreases, and suitable oviposition sites become scarce. Wasps are then forced to oviposit in small, suboptimal buds, or in buds already utilized by other females, thus increasing clutch size per bud. My data suggest that *D. kuriphilus* opts for the latter, inducing multi-lobed galls, and this represents a trade-off between the reduced fitness due to large clutch sizes, and increased fitness due to low parasite pressure (Figure 4.3B). Within multi-lobed galls some larval cells are located in the inner part of the gall, protected by surrounding cells from the relatively short ovipositor of *T. sinensis* (Figure 4.3B). Consequently, *D. kuriphilus* emergence rate

from less parasitized multi-lobed galls will be higher compared to heavily parasitized, simple, uni-lobed galls, and this positively affects fitness.

The plant pathogenic fungus, *Colletotrichum* spp. (Graziosi and Rieske, Chapter 5), occurred exclusively on multi-lobed galls, suggesting that the more complex gall morphology is more conducive to fungal infection and growth than are simple, uni-lobed galls. *Colletotrichum* infection could represent a selective force on *D. kuriphilus* gall morphology, similar to the gall wasp, *Diplolepis rosae*, on *Rosa* spp., and the gall fly, *Eurosta solidaginis*, on *Solidago* spp., where predation and parasitism act in opposite directions shaping gall size (Abrahamson et al. 1989, László et al. 2014), This hypothesis is yet to be tested and may represent ground for future work.

My data support the hypothesis that *D. kuriphilus* gall morphology is influenced both by population pressure and by natural enemies. Complex, multi-lobed galls are produced through multiple oviposition events by one or more females in highly suitable buds when population pressure is high (Figure 4.3B). Gall wasp larvae developing within these complex galls are provided enemy free space in which to develop. My findings add to the rapidly accumulating knowledge of the biology, reproductive behavior, and population dynamics of the globally invasive Asian chestnut gall wasp. My data suggests that gall wasp population pressure may be assessed by evaluating gall morphology in a relatively small sample. Furthermore, my findings provide additional evidence in support of the enemy hypothesis as a driving force in the observed variation of gall traits.

Table 4.1. Aspects of gall morphology from single lobed (uni-) and multiple (multi-) lobed galls induced by the invasive *D. kuriphilus* on *C. mollissima*.

	Uni-lobed galls	Multi-lobed galls	Test statistic
	mean (SE)	mean (SE)	
N of cells per gall	2.3 (0.2)	7.5 (1.5)	$F_{1,49} = 39.04$ ; $P < 0.0001$
N of cells per lobe	2.3 (0.2)	2.4 (0.2)	$F_{1,49} = 0.22$ ; $P = 0.6422$
Volume per gall (mm <sup>3</sup> )	291.3 (28.9)	928.6 (211)	$F_{1,49} = 16.69$ ; $P = 0.0002$
Weight per gall (g)	0.27 (0.02)	0.76 (0.16)	$F_{1,49} = 25.98$ ; $P < 0.0001$
Volume per lobe (mm <sup>3</sup> )	290.8 (30)	310.5 (42)	$F_{1,83} = 1.06$ ; $P = 0.3056$
Weight per lobe (g)	0.26 (0.02)	0.25 (0.03)	$F_{1,83} = 2.14$ ; $P = 0.1476$
Nutritive lining (mm)	0.31 (0.006)	0.30 (0.006)	$F_{1,81} = 1.61$ ; $P = 0.2081$
Parasitization ( $N_p/N_c$ ) <sup>a</sup>	0.89 (0.05)	0.75 (0.05)	$F_{1,49} = 5.87$ ; $P = 0.0192$
Necrotization ( $N_n/N_c$ ) <sup>b</sup>	0.0 (0.0)	0.39 (0.15)	-

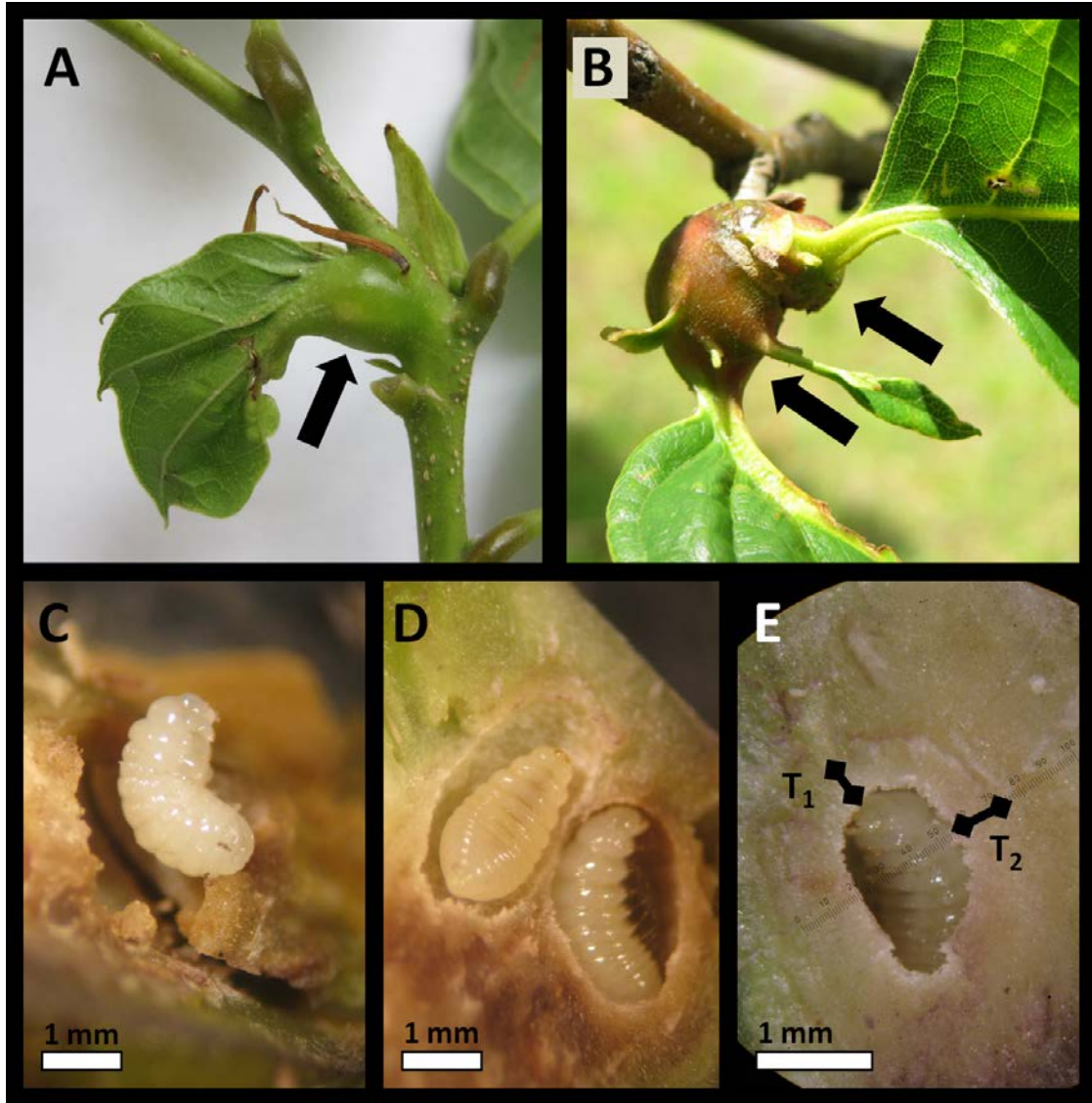


Figure 4.1. Morphological evaluation of *Dryocosmus kuriphilus* galls and gall contents: (A) uni-lobed gall, (B) arrows show two lobes of a multi-lobed gall, (C) gall containing *D. kuriphilus* larval cell, (D) gall cells revealing parasitoid larvae, (E) layer of nutritive tissue (cell lining) surrounding gall cell.

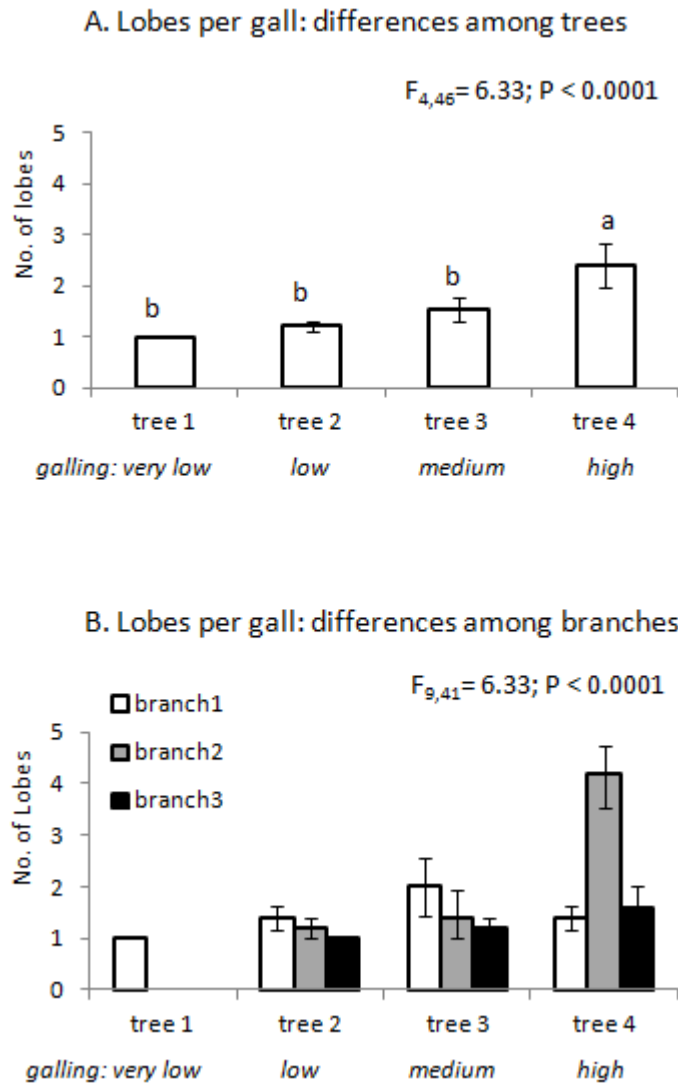


Figure 4.2. Differences in number of lobes per gall among: (A) sampled trees, (B) sampled branches. Galling pressure assessed as number of galls on the terminal 1m length of branch. Means followed by the same letter do not differ ( $\alpha = 0.05$ ).

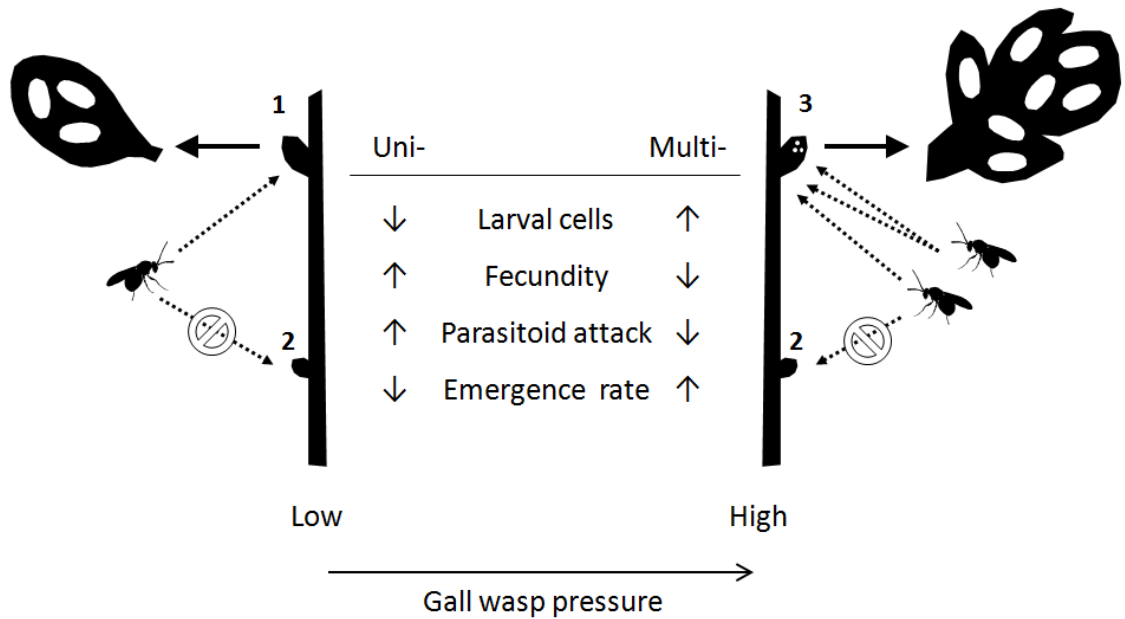


Figure 4.3. Schematic demonstrating potential relationship between gall wasp population pressure, gall wasp morphology, and parasitoid attack. When gall wasp pressure is low (A) ovipositing *D. kuriphilus* select large buds with no prior oviposition activity (1), avoiding small sub-optimal buds (2), and lay relatively small egg clutches that favor formation of uni-lobed galls, which are then subjected to high parasite pressure. In cases of high gall wasp pressure (B), fewer buds per female are available; females tend to avoid small buds (2) and select larger viable buds, and multiple oviposition events may occur (3), thus triggering formation of multi-lobed galls with lower parasite pressure.

## **Chapter Five: Can plant pathogens advantageously utilize insects as resources?**

Introduced organisms colonizing new areas can produce multiple effects on invaded habitats. Effects may be direct, such as the detrimental impact of an exotic herbivore on native plants, or indirect, by producing cascading effects within ecosystems (Lovett et al. 2006). However, while population level impacts are documented (Parker et al. 1999, Cooper and Rieske 2007, 2011, Loo 2009), the method and extent to which invaders trigger changes at community and ecosystem levels is exceedingly complex and poorly understood (Lovett et al. 2006, Johnson et al. 2009, Kenis et al. 2009, Gandhi and Herms 2010, Adkins and Rieske 2013). Regardless of spatial or temporal scale, microbes are increasingly recognized as playing integral roles in invasion dynamics in avian (O'Brien et al. 2011), mammalian (Tompkins et al. 2002), plant (Flory and Clay 2013) and insect (Bennett 2013) invasions.

Microorganisms often mediate insect – host plant interactions, affecting both herbivore and natural enemy performance (Omacini et al. 2001, Hartley and Gange 2009, Biere and Bennet 2013). Through host plant-mediated interactions, plant pathogens play crucial roles in plant-based ecological communities, indirectly affecting herbivore population dynamics and ultimately influencing arthropod communities (Moran 1998, Bennett 2013, Biere and Bennett 2013, Tack and Dicke 2013).

Reports of plant disease-causing microorganisms utilizing alternate resources such as insects are rare, but recent work suggests that phytopathogens may advantageously target arthropods (Cesnik et al. 1996, Marcelino et al. 2008). In light of this I investigated the nature of a microbial associate that colonizes plant galls induced by

an invasive insect specializing on *Castanea* species in North America, and evaluated the extent to which it affects gall-inducer success.

The globally invasive Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), initially invaded the US in the 1970's and has subsequently spread throughout the former range of American chestnut, *Castanea dentata* (Rieske 2007). The gall wasp is parthenogenetic and univoltine. Adult females oviposit in chestnut buds in early summer. Newly hatched larvae overwinter, and when buds expand the following spring, galls grow very rapidly. Galls are single to many-chambered, and each chamber contains a developing gall wasp larva. Larvae feed inside galls, pupate, and emerge in summer (Payne 1978). Gallings causes leaf, petiole and twig deformities that have detrimental effects on plant growth and fruit production (Payne et al. 1983, Kato and Hijii 1997).

A native of China, the gall wasp was initially described as a new pest species in Japan in 1941, and was subsequently reported in Korea in 1958 (Yasumatsu 1951, Cho and Lee 1963, Murakami et al. 1995, Moriya 2010), and in the USA in 1974 (Payne et al. 1975). More recently it was introduced and established in Nepal (1999), and has become highly invasive in Europe, including Italy (2002), France (2005), Slovenia (2005), Hungary (2009), Switzerland (2009), Croatia (2010), and Spain (2012) (Graziosi and Santi 2008, Zhi-yong et al. 2009, Bosio et al. 2010, Matosevic et al. 2010, Pujade-Villar et al. 2013). Clearly it poses a major threat to chestnut cultivation globally (EFSA 2010).

A parasitic wasp, *Torymus sinensis* Kamijo (Hymenoptera: Torymidae), has been deployed as a biological control agent to manage gall wasp populations in North America



(Payne et al. 1975, Cooper and Rieske 2007), Japan (Moriya et al. 2002) and Europe (Quacchia et al. 2008). Female parasitoids locate *D. kuriphilus* galls in early spring using visual and olfactory cues (Graziosi and Rieske 2013), and oviposit into chambers containing developing gall wasp larvae; parasitoid larvae feed on gall wasp larvae and remain inside galls until emergence the following spring (Piao and Moriya 1999). Gall wasp population regulation using *T. sinensis* can be successful (Moriya et al. 2002), but its effectiveness in recently invaded areas and over extended time periods is not consistent (Gibbs et al. 2011, Cooper and Rieske 2011, Rieske and Cooper 2013). In addition, *D. kuriphilus* recruits native parasites as gall wasp populations expand through newly invaded areas (Cooper and Rieske 2007, Rieske and Cooper 2013; Panzavolta et al. 2013, Quacchia et al. 2013), which may affect its pest status and the efficacy of *T. sinensis* as a population regulator (Cooper and Rieske 2010).

In addition to hymenopteran parasitoids causing *D. kuriphilus* mortality, necrotic lesions on developing galls and associated foliage have been consistently observed in North America and are often associated with gall wasp mortality (Cooper and Rieske 2009, 2010). Necrotic lesions have similarly been associated with gallmaker mortality in Europe (Magro et al. 2010, Addario and Turchetti 2011, Tosi et al. 2014). Insect galls represent an immobile and readily accessible resource and are frequently colonized by microorganisms, especially fungi, with variable effects on gallmaker populations (Wilson 1995). In numerous cynipid gall wasp systems, fungi utilize gall tissue as a nutritive resource, and are a factor in gallmaker mortality (Taper et al. 1986, Butin 1992, Stone et al. 2002, Gange et al. 2008, Cooper and Rieske 2009).

I hypothesize that an obligate plant pathogen is opportunistically functioning entomopathogenically, causing extensive mortality in an obligate herbivore specialist and thereby impeding its invasiveness. I sought to characterize the microorganism – gallmaker – plant interaction by identifying the organism causing necrosis on galls induced by *D. kuriphilus* on *Castanea* trees in the eastern US. I demonstrate that a phytopathogenic microorganism associated with the woody *Castanea* host plant is utilizing and causing mortality of a specialist herbivore, *D. kuriphilus*, while causing virtually no mortality in its functionally specialized parasite, *T. sinensis*.

## **Materials and methods**

### Gall collections and dissections

*Dryocosmus kuriphilus* galls with necrotic lesions were collected on three dates from two Chinese chestnut (*C. mollissima* Blume) located in Fayette County, KY USA (38° 1'39.86"N 84°28'53.22"W and 37°57'21.17"N 84°24'52.77"W respectively), coinciding with late larval-pupal (30 May and 8 June) and post-emergent adults (17 July). Sampling intensity was based on the incidence of necrotic galls. Twenty three galls from two trees were collected randomly from a height of 1.5 – 2.5 m. Galls were placed in sealed plastic bags on ice, returned to the laboratory and stored at 4°C.

Galls were sliced open and internal gall contents were examined under a dissecting microscope. I noted the presence of necrotic tissue associated with gall chambers, and living or dead *D. kuriphilus* or parasitoid larvae, pupae, or adults, as well as fungal mycelia independent of insects or cadavers.

## Isolations

Isolations were conducted from external lesions on 10 galls, 8 from the first tree and 2 from the second tree. Galls were surface sterilized with 70% EtOH and flamed for < 1 sec. Necrotic tissue from the edge of one lesion on each gall was excised using a sterile scalpel, cut into 1 × 2 mm sections, and placed on potato dextrose agar (PDA) in 100 mm Petri dishes. Isolations from insect cadavers and chamber mycelia were conducted using 9 galls from the first tree. Internal gall contents were examined under a dissecting microscope at 2.5× magnification. All insects were removed and 50% of the *D. kuriphilus* cadavers covered with fungal mycelia were placed on PDA. Fungal mycelia independent of insect cadavers was also removed and placed on PDA. Agar plates were sealed and maintained at 24 ±2 °C in a darkened growth chamber, and evaluated daily for fungal growth. After 10 d colonies were sub-cultured on PDA plates maintained at 24 ±2 °C in the dark to obtain pure cultures for use in subsequent assays.

## Inoculations

Pure isolates were used to carry out two sets of inoculations, one in the field and one in the laboratory, to conduct Koch's postulates and evaluate potential pathogenicity to the gall wasp. Galls were surface sterilized using 70% EtOH. A 2 mm diameter cork borer was used to create a superficial hole in the gall surface. A 2 mm disc of each fungal culture was transferred to the hole and the wound was covered with masking tape. After 14 d inoculated galls were excised for evaluation. External necrotic areas were measured, and lesion size was assessed using the equation for the area of an ellipse  $A = \pi \times (W / 2) \times (L / 2)$ , where W and L represent width and length of the necrotic area, respectively.

Chamber contents were evaluated. When symptoms of necrosis were absent, the area of the lesion produced at the inoculation point was measured.

For the field inoculations (June 4), I randomly selected two isolates from the first source tree and one isolate from the second source tree (designated Finc C11, Finc G3, and Old Rich G1). I located five gall wasp infested *C. mollissima* trees in Fayette County, KY USA, and selected three branches from each at 2 – 2.5 m high. I then inoculated 4 galls per branch, randomly assigning the three fungal strains plus a control inoculation (clear PDA disc) to galls on each branch.

For the laboratory inoculations (June 8), I used five gall wasp infested chestnut seedlings (*C. mollissima*) that presented one gall each (five galls total); three were inoculated with one fungal strain (Finc G3) and two were inoculated with clear agar control discs (N = 5). Seedlings were maintained in growth chambers at 20±2 °C with 12L:12D.

After 14 d experimental galls were collected and evaluated for symptoms. Necrotic tissue from inoculated galls was re-isolated as described above.

Analysis of variance was used to evaluate differences in the size of the necrotic lesions among treatments. Field inoculation data were analyzed using an ANOVA (PROC GLM, SAS<sup>®</sup> V. 9.1) to assess fungal strain pathogenicity by evaluating average lesion size per tree.

## Fungal identification

Conidia masses on fungal cultures were observed at 500x magnification and photographed using a microscope-mounted camera. Single spore isolates were obtained from conidia produced on PDA plates. Mycelial DNA was extracted in the Plant Diagnostic Clinic Laboratory at the University of Kentucky using the Cetyl Trimethyl Ammonium Bromide (CTAB) method, and a 650 bp product was amplified using ITS1 and ITS4 primers (White et al. 1990). The amplicon was excised from a 3% agarose gel and Sanger-sequenced at the University of Kentucky Advanced Genetics Technology Center.

## **Results**

### Gall collections and dissections

In the initial gall collection (31 May) necroses appear as small (< 5 mm), irregularly shaped, superficial brown spots that are occasionally associated with mechanical damage. Lesions expand on the surface but did not penetrate internal gall tissue. Dissections revealed no necrotic chambers, and no mortality of gall wasp pupae (Table 5.1). By the second and third collections necrotic areas had coalesced and deepened, eventually affecting the whole gall (Figure 5.1A). Extensively infected galls became dark brown/grey, and fructification structures and conidial masses were visible on the lesion surface (Figure 5.1B). Galls collected on 8 June and 17 August showed extensive internal necrotic tissue (55% and 66% necrotic chambers, respectively), and extensive *D. kuriphilus* mortality. Dead gall wasp larvae, pupae or adults were found inside chambers, appearing as mummified cadavers covered by white and pinkish

mycelium (Figure 5.1C). Parasitization ranged from 15 to 45%, and < 5% (N = 2) parasitoid larvae were dead across the three collection dates (Table 5.1).

### Isolations

After 4 d pink colonies were observed growing from gall tissue fragments and from chamber contents (Figure 5.1D) of field-collected galls. After 7 d orange/pink masses of conidia were evident: conidia were elongate, oblong, measuring 9.7 – 12.08  $\mu\text{m}$  in length and 3.42 – 3.45  $\mu\text{m}$  wide (Figure 5.1G). “Pink” strains were obtained from tissue fragments cultured from 7 out of 8 necrotic galls. Similarly, all the insect cadavers (N = 12) I plated on PDA produced the “pink” morphotype, and two of the three fungal mycelia growing inside the chambers produced the same morphotype. I attempted isolation from one of the two dead parasitoid larvae, but no fungal growth was obtained.

### Inoculations

Among field-inoculated galls, galls treated with the three fungal strains (N = 45) developed necroses, while control galls inoculated with clear agar (N = 15) showed no symptoms. The size of the necrotic area was equivalent among strains, but lesions on treated galls differed significantly from lesions on control galls (Figure 5.2A). No differences in lesion size were found among trees. The high parasitization rate in these field-inoculated galls prevented determination of fungal-associated gall wasp mortality. No *D. kuriphilus* were found in inoculated galls; parasite larvae were present in 89% of the chambers evaluated, while 10% contained only fungal mycelia and 1% were empty. In spite of extensive chamber necrosis, no significant mortality of parasitoid larvae was evident (1 of 151). All inoculations of gall wasp infested seedlings in the laboratory (N =

3) resulted in necrotic galls (Figure 5.1H), and all *D. kuriphilus* found inside necrotic galls were dead and covered by mycelia (Figure 5.1I). The size of the lesions on inoculated galls was greater than lesions on control galls (Figure 5.2B), and other than the inoculation wound, control galls were asymptomatic (Figure 5.1J). Gall wasp mortality in control inoculations was zero; all gall wasp pupae within control galls were living (Figure 5.1K).

Re-isolation from tissue of inoculated galls consistently produced the pink morphotype, with morphology comparable to the isolate used for the artificial inoculation. Re-isolation from control galls produced no fungal growth.

#### Fungal identification

The NCBI BLAST identification of the sequenced amplicon generated strong matches with several accessions of the ascomycete genus *Glomerella* Spauls and Schrenk /*Colletotrichum* Corda (respectively the sexual and asexual morphs). Morphological characterization of conidia (Figure 5.1G) also matches the genus description (Sutton 1980, Barnett and Barry 1998).

#### **Discussion and conclusions**

Here I provide clear evidence demonstrating a complex set of multi-trophic interactions between a woody plant and its pathogen, and a highly specialized herbivore and its parasitoid. My findings support my hypothesis that a plant pathogen can opportunistically function as an entomopathogen, causing extensive mortality in an obligate herbivore specialist. Further, I report that this facultative entomopathogen spares

the parasitic natural enemy of the herbivore specialist. Collectively this suggests that these interactions may be impeding invasiveness of *D. kuriphilus* in the US.

I identified an ascomycete fungus in the *Colletotrichum*/*Glomerella* genus, and fulfilled Koch's postulates to demonstrate it as the agent responsible for necrotic lesions on *D. kuriphilus* galls, and for gall wasp mortality inside gall chambers. In my system *Colletotrichum* invades galls in late May and produces superficial necroses. In less than 14 d necroses proliferates the entire gall; as the fungus penetrates it causes gall wasp mortality by invading insect tissue.

*Colletotrichum* Corda and its teleomorph *Glomerella* Spauld. and Schrenk are cosmopolitan plant pathogens causing anthracnose typified by necrotic lesions on leaves, stems, flowers, and fruits, on a wide range of cultivated and wild plants (Bailey et al. 1992, Prusky et al. 2000, Wharton et al. 2004). They are also occasionally endophytic (Cannon et al. 2012). The exceptionally large host range and extreme morphological variation make classification within the genus uncertain. Maize and sorghum anthracnose are caused by *Co. graminicola* (Vaillancourt and Hanau, 1992), while *Co. acutatum*, *Co. gloeosporioides*, *Co. musae*, *Co. fragariae*, are major post-harvest diseases affecting fruits (Freeman et al. 1998, Wharton et al. 2004, Cannon et al. 2012). *Colletotrichum acutatum* causes stem blight on conifers in nurseries (Griffin et al. 1987), and its teleomorph *Glomerella* produces necrotic lesions on leaf margins of Norway maple, *Acer platanoides*, in the northern US (LoBuglio et al. 2008).

*Colletotrichum gloeosporioides* and its sexual stage *G. cingulata* causes kernel rot, the main post harvest disease affecting Chinese chestnut in China (Yang et al. 1988,



Wang et al. 2000a, Wang et al. 2000b) and on Japanese chestnut (*C. crenata* Siebold and Zucc.) in Japan (Uchida 1981, Weir et al. 2012). In North America *Colletotrichum* has been reported causing blossom end rot on Chinese chestnut (Fowler and Berry 1958), which is a post-harvest disease affecting chestnut production in the US (Miller 2006).

In Europe gall wasp mortality has been associated with *Gnomoniopsis* Berl. and *Fusarium* Link isolated from galls (Magro et al. 2010, Addario and Turchetti 2011). Interestingly, *Colletotrichum* strains have been reported interacting with *D. kuriphilus* in Asia. In Japan and in the gallmaker's native China, galling reportedly aggravates the incidence of kernel rot (Uchida 1982, Yang et al. 1988), and also cause necrosis on *D. kuriphilus* galls (Uchida 1982). But my study is the first to describe an association between *D. kuriphilus* and *Colletotrichum* outside its native Asia, the first to describe the effects of *Colletotrichum* on gallmaker natural enemies, and the first to evaluate the potential role of the fungus in gallmaker invasiveness.

Interestingly, incidences of the plant pathogenic *Colletotrichum* directly impacting arthropods on taxonomically and chemically diverse woody plant hosts have recently come to light. Strains of *Co. acutatum* closely related to phytopathogenic isolates can cause epizootics and eventual mortality in the elongate hemlock scale, *Fiorinia externa* Ferris, feeding on eastern hemlock, *Tsuga canadensis* (L.) Carrière, (Marcelino et al. 2008, 2009). Similarly, the anthracnose and fruit rot pathogen *Colletotrichum* Corda infects citrus scale, *Orthezia praelonga* Douglas, on citrus in Brazil (Cesnik et al. 1996, 2000).

I demonstrate that *Colletotrichum* is a plant pathogen present epiphytically or endophytically on chestnut, that infects galls advantageously and eventually behaves as an opportunistic entomopathogen. This appears compatible with the general biology of *Colletotrichum* species. Anthracnose has an asymptomatic stage in which the fungus colonizes immature fruit, but the fungus remains latent until fruit ripening. At that point the fungus initiates a growing phase on the ripened fruit, causing post-harvest disease (Warton et al. 2004). In my system vegetative or reproductive *Colletotrichum* structures may remain latent on a variety of chestnut tissues, including galls, and necrosis appears only after galls are fully developed. The presence of galls provides the fungus a novel substrate to exploit, resulting in necrotic lesions and eventual gall wasp mortality. It seems unlikely that gall wasp mortality is caused simply by disruption of gall tissue. Insect mortality occurs primarily in the pupal or adult stages, which are non-feeding stages in gall wasp development.

Artificial inoculations in the field and the laboratory resulted in *Colletotrichum* mycelia engulfing insects inside gall chambers, leading to insect mortality. Fungal necrosis of galls has been reported in North America in association with gall wasp mortality (Cooper and Rieske 2010). Along with parasitization by the introduced *T. sinensis* and the native *Ormyrus labotus* Walker (Hymenoptera: Ormyridae) (Cooper and Rieske 2007, 2011), I show here that fungal infection represents an additional significant mortality factor of *D. kuriphilus* in North America. The high mortality rate I describe is compatible with fungal-induced mortality of other gallmakers (Stone 2002), including the cynipid *D. dubiosus* (Taper 1986).

In my woody plant system, parasitic hymenopterans appear resistant to infection by *Colletotrichum*, corroborating results of Cooper and Rieske (2010), who found the occurrence of fungal lesions was positively correlated with gall wasp parasitization. In the current study none of the parasitoids present inside necrotic chambers of field-collected or inoculated galls showed signs of fungal infection, and I found virtually no *T. sinensis* mortality. This is in contrast to findings associated with the conspecific *T. capite*, which is inhibited by the presence of fungi inside the herbaceous perennial goldenrod leaf gall (Weis 1995).

Hymenopteran parasites have evolved multiple resistance mechanisms to help overcome host defenses (Vinson 1974, Strand and Pech 1995) and abiotic extremes. *Torymus sinensis* larvae appear to be protected from secretions produced by the gallmaker host or from degradation of gall tissue due to fungal enzymes. *Torymus sinensis* overwinter in vacated galls as pupae (Piao and Moriya 1999), and so are likely provided with defenses against environmental extremes.

Manipulating the *Colletotrichum* strain I isolated as a biological control agent for managing *D. kuriphilus* populations appears impractical due its potential pathogenicity to *Castanea*. Additional efforts are needed for species-level identification and to evaluate relative pathogenicity to other chestnut tissues and to other chestnut species. In Europe similar concerns are limiting deployment of *Gnomoniopsis*, which reportedly causes gall wasp mortality but is also a known to cause nut rot (Magro 2010, Tosi 2014). But the *Co. gloesporioides* strain isolated from the scale insect *O. praelonga* is deployed in biological control programs to manage this citrus pest in Brazil, demonstrating that such deployment is possible under some circumstances.

In conclusion, I determine that a plant pathogenic microbe, an agent of anthracnose disease, can utilize woody plant galls induced by an invasive specialist herbivore, *D. kuriphilus*, as a resource for nutrition and substrate, and I quantify its impact on gallmaker survival. I also elucidate the role of this interaction on activity and survival of a parasitic wasp utilizing the gallmaker, demonstrating a multi-trophic interaction that affects success of each component. My study is significant in that it increases my understanding of how a plant pathogen can opportunistically colonize an insect herbivore while selectively sparing the herbivore's natural enemy. My work provides additional insights into Plant–Microbe–Insect interactions (Biere and Bennet 2013, Biere and Tack 2013) and to the growing body of literature on how interactions among invasive species may be influencing their invasiveness (Bennet 2013).

Table 5.1. Chamber contents of lesion-bearing galls formed by *D. kuriphilus* field-collected on 31 May (early pupal), 8 June (pupal) and 17 August 2011 (post emergence).

	collection date		
	31 May	8 June	17 August
galls (N)	4	4	15
necrotic chambers (%)	0	55	66
dead <i>D.kuriphilus</i> (%)	0	100	36
parasitization (%)	15	45	35
dead parasitoids (%)	0	11	3

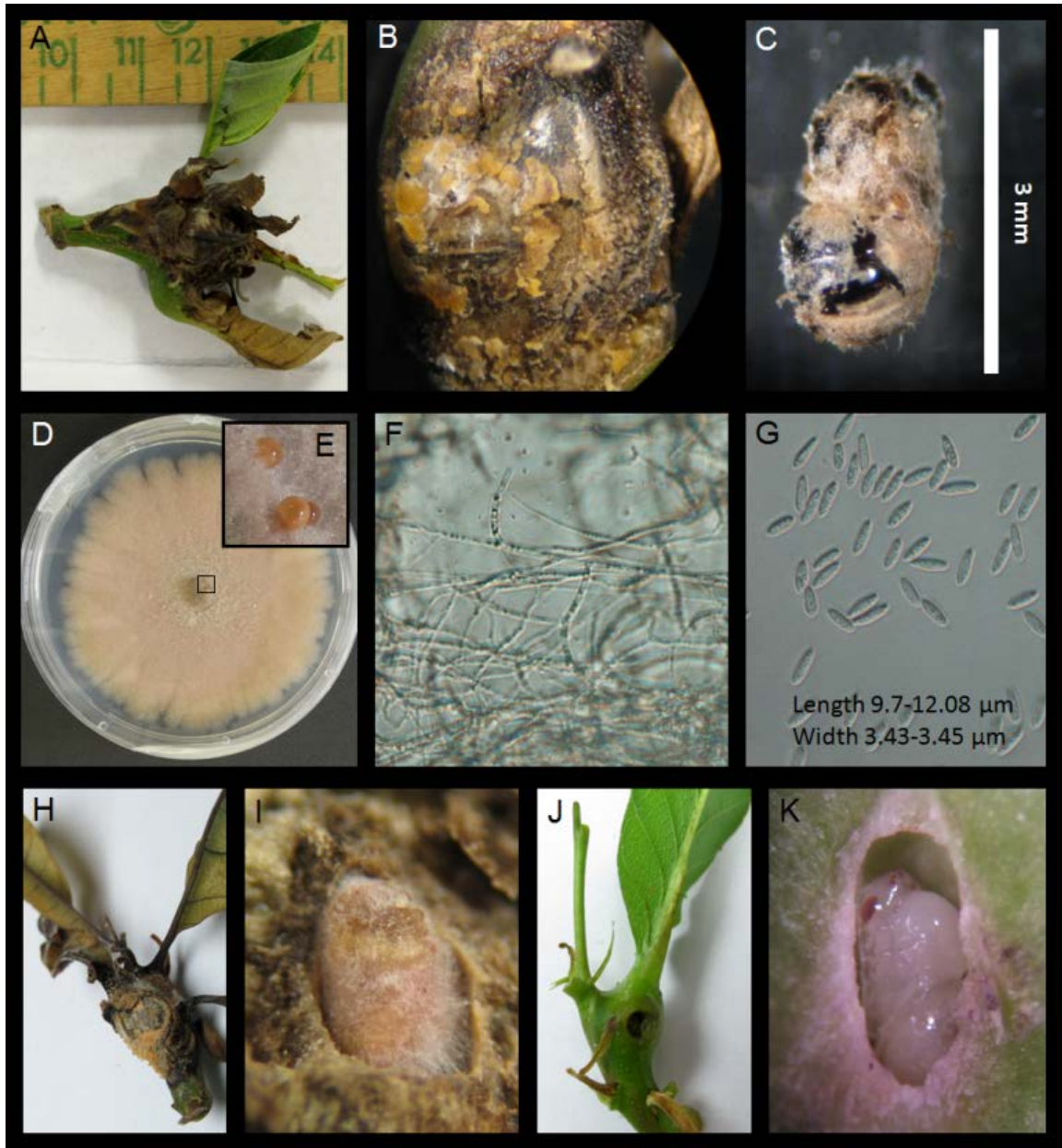


Figure 5.1. A. Necrotic gall, B. fungal fructifications on gall surface, C. cadaver of adult *D. kuriphilus* covered by fungal mycelium, D. 7 days culture plate, E. conidial masses on culture, F. mycelium, G. conidia, H. gall 14 d after artificial inoculation with fungal mycelia, I. cadaver of *D. kuriphilus* pupa, covered by fungal mycelium inside chamber, J. gall 14 d after inoculation with clear PDA, and K. live *D. kuriphilus* pupa inside chamber.

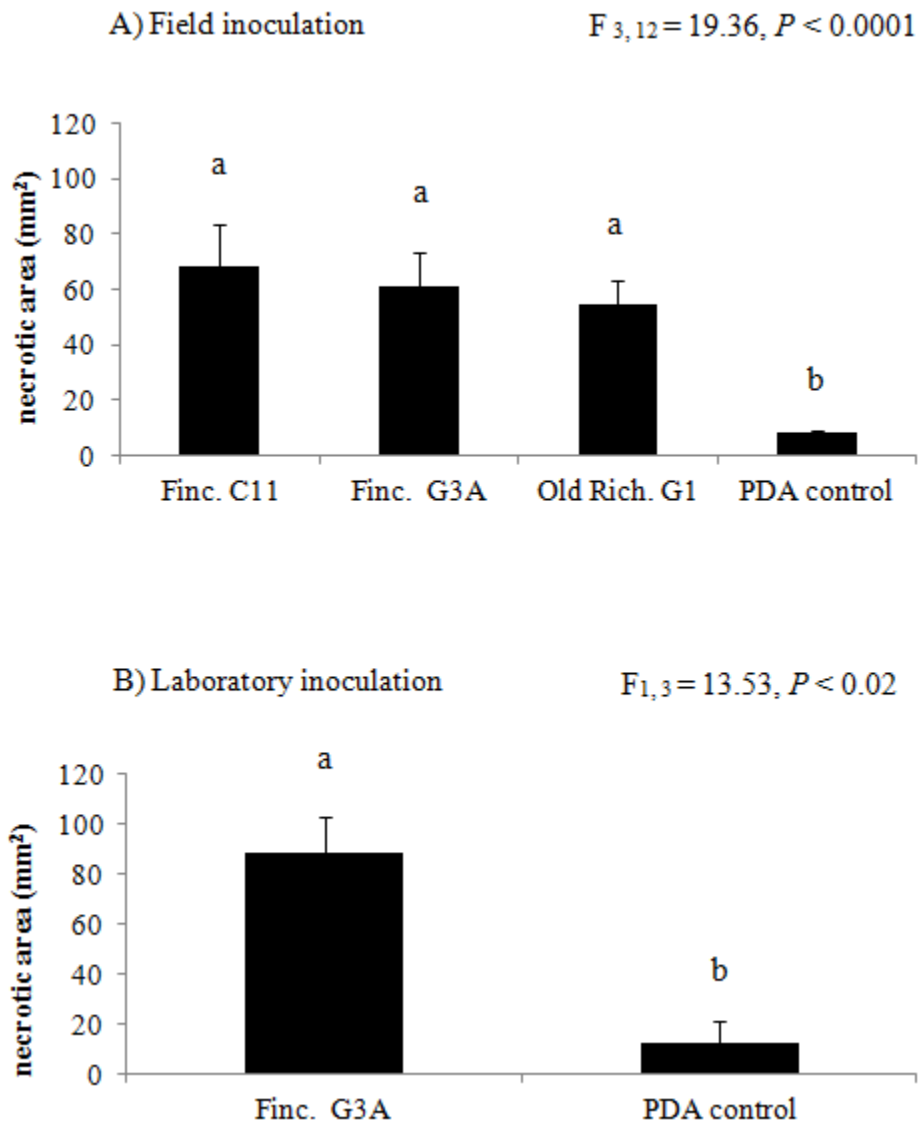


Figure 5.2. Area (mm<sup>2</sup>) of necrotic lesions 14 d after inoculation in: A) a field experiment to evaluate pathogenicity of three fungal isolates, and B) a laboratory experiment to evaluate isolate pathogenicity. Means followed by the same letter do not differ ( $\alpha = 0.05$ ).

**Chapter Six: Response of *Torymus sinensis*, a parasitoid of the gallforming *Dryocosmus kuriphilus*, to olfactory and visual cues**

Non-native species often become invasive due in part to a lack of population regulators in invaded territories (Elton 1958, Lockwood et al. 2007, Davis 2009). Biotic factors contributing to invasiveness include such things as host plant susceptibility and distribution (Griffin 2000, Orwig 2002), altered competitive interactions (Elton 1958, Ricciardi et al. 1997, Green et al. 2004), and perhaps most importantly, a lack of natural enemies. Enemy free space is often considered a major determinant of invasiveness of a non-native species (Crawley 1987, Keane and Crawley 2002), and manipulation of this is the basis for biological control. Despite criticisms of being oversimplistic (Colautti et al. 2004, Levine et al. 2004) and case-specific (Berdegue et al. 1996), the enemy free space hypothesis remains a standard explanation for invasion success.

Several attributes are essential when considering the effectiveness of a natural enemy for pest population regulation, including synchrony with the target pest's life cycle (Hassell 1985) and the ability to disperse into invaded territory with expanding pest populations (Wright et al. 2005). The ability of a biological control organism to locate suitable host material and disperse is especially important when considering population regulation of an invasive herbivore found on a sporadically occurring host plant across a broad geographic region.

Parasitoid searching and host location is influenced by external stimuli such as olfactory, visual, and tactile cues (Laing 1937, 1938, Vinson 1976, 1998, Broad and Quicke, 2000). Olfactory cues include odors emanating from both the host insect, and



from host associated plants (Price et al. 1980, Vet and Groenewold 1990, Whitman and Eller 1990). The importance of olfactory cues in parasitoid host location behavior is well studied (Weseloh 1981), but the importance of visual cues is less understood (Turlings et al. 1993). Visual cues may function as a primary stimulus for host location behavior in some parasitoids, whereas in others vision appears to function synergistically with additional environmental cues.

*Torymus sinensis* Kamijo (Hymenoptera: Torymidae) is a hymenopteran parasitoid utilized for biological control of the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae), a globally invasive pest of chestnut, *Castanea* spp. *Torymus sinensis* is the dominant parasitoid reared from *D. kuriphilus* galls in its native China (Kamijo 1982), and has been introduced to additional regions of Asia, North America, and Europe for management of Asian chestnut gall wasp populations (Moriya et al. 2003, Cooper and Rieske 2007, Gibbs et al. 2011). *Torymus sinensis* is a univoltine ectoparasitoid that is phenologically synchronized with *D. kuriphilus*, making it a particularly effective biological control agent (Murakami et al. 1995, Moriya et al. 2003, Cooper and Rieske 2007, 2011). Adults emerge from vacated galls in early spring and mate. Females locate newly forming galls, insert their ovipositor, and lay eggs internally on chamber walls or on the surface of the *D. kuriphilus* host larvae (Piao and Moriya 1999, Quacchia et al. 2008).

Long distance dispersal of the gall wasp host occurs through anthropogenic movement of infested plant material, and *T. sinensis* moves with expanding gall wasp populations via infested host material (Cooper and Rieske 2007, Rieske 2007). On a localized scale, gall wasp dispersal occurs by adult flight (Graziosi and Rieske 2012), but

localized movement of *T. sinensis* has not been thoroughly evaluated. Although its oviposition behavior has been described (Piao and Moriya 1999), the environmental cues and behaviors used in host location prior to female oviposition are unknown.

I evaluated olfactory and visual cues contributing to host location behavior of *T. sinensis*, to assess how dispersal of the parasitoid with expanding gall wasp populations is occurring, and to ultimately gain a more complete understanding of the parasitoids' role in gall wasp population regulation. I hypothesized that *T. sinensis* uses host plant cues to detect and orient to galled chestnut shoots.

## **Materials and methods**

Parasitoid responses to olfactory and visual cues were evaluated in the laboratory using a Y-tube olfactometer and excised plant material (Yang et al. 2008). The movement and behavior of parasitoids was observed for the duration of each trial, and grooming behavior, flight attempts, and oviposition attempts were noted.

### Parasitoids

Previous year's *D. kuriphilus* galls were collected in March 2012 from *Castanea* hybrids in Meadowview, VA and Mercer, PA, from established populations of *D. kuriphilus* heavily parasitized by *T. sinensis*. These vacated *D. kuriphilus* galls contain overwintering populations of *T. sinensis* (Cooper and Rieske 2007). Galls were stored in darkened containers at 4 °C until needed. In the laboratory excised galls were placed in darkened boxes (26 × 19 × 9.5 cm) with clear cylindrical collection containers (20 cm × 1.5 cm diameter) inserted on the upper side to collect emerging *T. sinensis* adults. Parasitoids were collected daily, held in 12 ml glass vials plugged with cotton (N = 4

insects per vial, approximately 1:1 sex ratio), and fed drops of honey water. Emergence boxes and vials were maintained at  $20 \pm 3$  °C under ambient lighting for 2-3 d prior to use in assays. Mating was observed in each vial; only mated females were used in assays.

### Olfactometer

The glass olfactometer (Figure 1) consisted of a Y-tube with a 115 mm long central stem (internal diameter 19 mm) and two 135 mm arms set at a 65° angle (internal diameter 25 mm). Air was pumped through silicon tubing (Figure 1a, 1b), purified by passing through an activated charcoal filter and dust filter, humidified by bubbling through distilled water (Figure 6.1c, 6.1d, 6.1e) and then passed through a flow meter (Figure 6.1i). Air flow (flow speed  $600 \text{ ml min}^{-1}$ ; wind speed  $3 \text{ cm sec}^{-1}$ ) was split with a glass Y-connector and passed through two 20 ml flasks (Figure 6.1g), each containing an odor source or blank, and then through the arms of the Y-tube (Figure 6.1g) and the central stem (Figure 6.1h).

The olfactometer was isolated from external stimuli by enclosing it horizontally in a box with the top and internal walls covered with white paper. A hole in the top of the holding box allowed even illumination by two 15 W (47 cm) fluorescent bulbs. Luminosity detected with a light meter (Auto-lumi L-158, Sekonic Japan) at the olfactometer level measured 12000 Lux. Ambient temperature was  $20 \pm 3$  °C, and assays were conducted between 0800 and 1800 hours EDT, 5 April – 23 May.

Parasitoids were introduced into the olfactometer through an entrance in the central stem (Figure 6.1h) and behavioral responses to selected stimuli were monitored. Parasitoids were scored as having made a “choice” when the insect traveled 3 cm up a Y-

tube arm, and the time elapsed was recorded. If parasitoids did not make a choice in 20 min the insect was removed and the trial was recorded as “no choice.” Each insect was used only once. The Y-tube and connectors were washed with acetone and dried at room temperature for 20 min after each trial, and stimuli were switched between arms of the olfactometer every five trials.

### Olfactory cues

Fresh *D. kuriphilus* galls served as my olfactory stimulus, and were collected during the period of larval development and rapid gall expansion (April 2012) from mature Chinese chestnut trees (*C. mollissima*) located in Lexington, KY. Galls were excised, placed in sealed plastic bags, and transported immediately to the laboratory, where they were maintained at 4 °C until use in assays. Galls were collected each morning for use in assays the same day.

### Visual cues

Visual stimuli included 1) *C. mollissima* shoots containing developing galls, 2) *C. mollissima* shoots free of galls but with expanded leaves, and 3) a ‘gall surrogate,’ consisting of an inert, 16 mm diameter, surface-sterilized green marble. Plant material was collected from the same trees and at the same time as described above.

### Dual choice experiments

Olfactory and visual stimuli were manipulated in four dual choice experiments to evaluate female *T. sinensis* response. Each set of stimuli were tested in a series of trials consisting of 20 *T. sinensis* females making a choice.

Experiment 1. Olfactory stimulus. I evaluated the response of *T. sinensis* to host odor by comparing fresh *D. kuriphilus* galls to a blank control (air). A single excised gall was placed in one of the odor source flasks, not visible to the insect, and the second source flask remained empty.

Experiment 2. Visual stimuli. To separate visual and olfactory stimuli to evaluate only visual response, I used a gall surrogate (green marble) inserted into the Y-tube arm 3 cm from the central stem, coupled with a chestnut shoot containing 3 – 5 fully expanded leaves positioned outside the same arm. The opposite arm contained no visual stimulus, and no odor was involved in either arm.

Experiment 3. Olfactory + visual stimuli with fresh gall. A newly excised *D. kuriphilus* gall was inserted in the Y-tube arm and a chestnut shoot with leaves was placed externally next to the same arm as described above.

Experiment 4. Olfactory + visual stimuli with gall surrogate. A visual cue was provided by a surrogate gall coupled with externally placed foliage, and an olfactory stimulus was provided by a fresh gall contained in the flask of the same Y-tube arm, but obscured from the parasitoids' vision.

### Statistical analysis

Preference of *T. sinensis* females for selected stimuli versus blank controls was assessed using a chi-square analysis (Hare, 1998). Analysis of variance was used to evaluate differences in the time elapsed for female wasps to choose (olfactory, visual, olfactory + visual stimuli versus blank control), using SAS v. 9.1 (SAS Institute, 2001).

## Results

Across all experiments, 91% of the parasitoids assayed (80 out of 88) made a choice (traveled  $\geq 3$  cm into one arm of the olfactometer) within 20 min., demonstrating that they were readily able to respond to stimuli. There were no differences in overall response times across the four experiments. The majority of the wasps began grooming immediately after introduction into the olfactometer; the duration of this behavior was highly variable. I did not observe any flight attempts inside the olfactometer.

Experiment 1. Olfactory stimulus. There were no differences in olfactory response of *T. sinensis* between *D. kuriphilus* gall volatiles and air (Figure 6.2a), nor were there differences in the time required for wasps to make a choice (Figure 6.2b).

Experiment 2. Visual stimuli. Only 25% of the *T. sinensis* evaluated responded positively to the visual stimulus of the inert gall surrogate with chestnut leaves, while 75% chose the blank control (Figure 6.2c). When provided only visual stimuli, wasps that chose the stimulus did not attempt contact with the gall surrogate during the 20 min. trial. Similar time elapsed between those wasps choosing the visual stimuli and those choosing the blank control (Figure 6.2d).

Experiment 3. Olfactory + visual stimuli with fresh gall. *Torymus sinensis* responded positively to a combination of host olfactory odor and visual cues; 80% of the wasps assayed chose the olfactory cues associated with a newly excised gall coupled with the visual cues of chestnut foliage placed external to the olfactometer arm (Figure 6.2e). The time elapsed for wasps to choose host cues was significantly longer compared to blank

controls (Figure 6.2f). Those *T. sinensis* choosing the host stimuli made physical contact with the gall surface and initiated oviposition behavior.

Experiment 4. Olfactory + visual stimuli with gall surrogate. Similarly, *T. sinensis* responded positively to the visual cues provided by a surrogate gall and externally placed foliage, coupled with an olfactory stimulus provided by a fresh gall; 75% of assayed wasps chose the stimuli (Figure 6.2g) over a blank control. There were no significant differences in time elapsed between choices (Figure 6.2h), however the trend appears similar to that observed using freshly excised gall material (Figure 6.2f). Those parasitoids that chose the visual stimuli reached the gall surrogate and attempted oviposition on the surface of the glass sphere.

## **Discussion and conclusions**

Here I demonstrate that the simultaneous presence of visual and olfactory stimuli is required to elicit host location behavior in *T. sinensis*, while neither individual stimulus alone triggers this behavior. Parasitoid searching and host location is influenced by a variety of stimuli (Laing 1937, Lewis et al. 1976, Vinson 1976, Broad and Quicke 2000). Olfactory cues are well studied (Weseloh 1981, Meiners et al. 2003, Schurmann et al. 2009), but the importance of visual cues is less understood. Evidence suggests that visual cues may function as a primary stimulus for host location behavior, or such cues may function in combination with additional environmental cues to elicit behavioral changes (Turlings et al. 1993). Egg surrogate studies using rice grains suggest that *Trichogramma evanescens* Westwood relies primarily on visual cues to locate lepidopteran host eggs; host odors alone appear unimportant in perceiving eggs from a distance (Laing 1937,

1938). The braconid *Microplitis croceipes* Cresson locates larval hosts using visual cues from the cotton plant on which the host insect feeds, and odor cues emitted by host insect frass (Wäckers and Lewis 1994). The pupal parasitoid of the greater wax moth, *Pimpla turionellae* (L.), reacts positively to visual or auditory cues emitted by the host, but when these cues are present simultaneously responsiveness is greatly enhanced, demonstrating that a combination of visual and vibrational cues is used to locate hosts (Fischer et al. 2001). ). In other cases the synergistic effect of two different odors is triggering host attraction: the braconid parasitoid *Diachasmimorpha longicaudata* (Ashmead) is not attracted to odors of Asian fruit fly larvae, *Bactrocera dorsalis* (Hendel), unless odors of ripe guava fruits are also present (Jang et al. 2000).

Similarly, a combination of cues appears to stimulate host location behavior in *T. sinensis*. Females demonstrate no response to an odor stimulus alone, and the visual stimulus alone elicits a negative response, but the two presented simultaneously elicit host finding behavior. This pattern of response clearly has adaptive advantages. Adult *T. sinensis* emerge from galls in an exceedingly complex environment and are exposed to a variety of relevant and irrelevant stimuli. Response to two distinctly different, yet simultaneously occurring, stimuli would allow more correct identification of relevant resources, and represents an efficient strategy to overcome environmental complexity to locate suitable hosts. A positive response of females to only one stimulus, either visual or olfactory, could elicit inefficient or incorrect choices for resource location. For *T. sinensis*, visual cues may act as a behavioral switch that allows female parasitoids to detect relevant odors such as developing *D. kuriphilus* galls, which in turn elicits host seeking behavior. This ‘activation hypothesis’ corroborates observations that a single cue



from a host insect may facilitate detection of other cues (Vinson 1976). If there is no exposure to the visual stimulus of developing galls and chestnut foliage (i.e. switch turned 'off'), then host odor is not recognized and there is no response to host stimuli. Further, the visual stimulus of galls on chestnut twigs alone does not produce a positive response, since there is no odor present to trigger the host location behavior. Visual cues appear to activate the responsiveness of *T. sinensis* females to relevant odor cues. My results also indicate that those *T. sinensis* females which respond positively to stimuli require more time to make a choice compared to those not responding to host cues (Figure 6.2.f), suggesting that female parasitoids require a certain amount of time to interpret meaningful cues in order to successfully locate hosts.

While the use of multiple cues to overcome environmental complexity and an activation response may adequately explain my observations of *T. sinensis* host location behavior, associative learning cannot be discounted. Parasitoid host finding behavior leading to oviposition is driven by environmental cues as well by internal parameters such as hunger, age, physiological condition and experience; this is associative learning (Vinson 1998). Experience may produce internal changes that increase sensitivity to certain stimuli (Turlings et al. 1993).

My findings on *T. sinensis* response to environmental stimuli are also interesting when considered in light of the chemical legacy hypothesis (Corbet 1985) and related studies (Herard et al. 1988), which hypothesize that traces of chemical cues associated with immature parasitoids are carried through adult emergence, when they affect the responsiveness of adults to specific cues. *Torymus sinensis* adults emerge in spring from previous year *D. kuriphilus* galls still attached to infested trees, where developing shoots

and galls are expanding at the time of emergence. Emerging parasitoids are immediately exposed to host insect and host plant odors, as well as visual cues associated with developing leaves and galls. These parasites may be associating relevant odors to visual stimuli. My test insects were reared under laboratory conditions and lacked the visual and olfactory cues normally present at emergence, thus resulting in inexperienced wasps that respond poorly to individually presented stimuli. However, I did not directly test for either associative learning or innate response in my study.

Auditory cues appear unimportant for initiation of oviposition behavior in *T. sinensis*. In the absence of auditory cues females were positively attracted to an inert gall surrogate in the presence of fresh gall odor, and test wasps initiated oviposition behavior on inert surrogates. Visual and olfactory cues may override any need for vibrational cues in host location behavior of *T. sinensis*, at least in my assay conditions. Given the importance of tactile, vibrational and auditory cues in other parasitoid–host systems (Wackers et al. 1998; Fischer et al. 2001, 2004, Völkl 2003), this is somewhat surprising. However, I cannot discount the role of vibrational and/or tactile cues in host acceptance leading to actual oviposition, since no eggs were laid during the oviposition attempts on the gall surrogates.

Outside North America, *T. sinensis* has been intensively manipulated in biological control programs targeting the Asian chestnut gall wasp in Japan (Moriya et al. 2003) and Europe (Quacchia et al. 2008; Aebi et al. 2011; Gibbs et al. 2011). In spite of this intensive manipulation, the host location behavior of *T. sinensis* is poorly understood. Following its initial introduction into gall wasp infested orchards in the USA (Payne 1978) and with no additional manipulations, *T. sinensis* moved with expanding gall wasp

populations, often offering effective population regulation and slowing or dampening gall wasp population outbreaks (Cooper and Rieske 2006, 2007, 2011). However, its success in reliably suppressing gall wasp populations in North America is somewhat erratic (Cooper and Rieske 2011). My results provide groundwork to gain a deeper understanding of its host location behavior to enhance the success of any further manipulations of *T. sinensis* for gall wasp management.

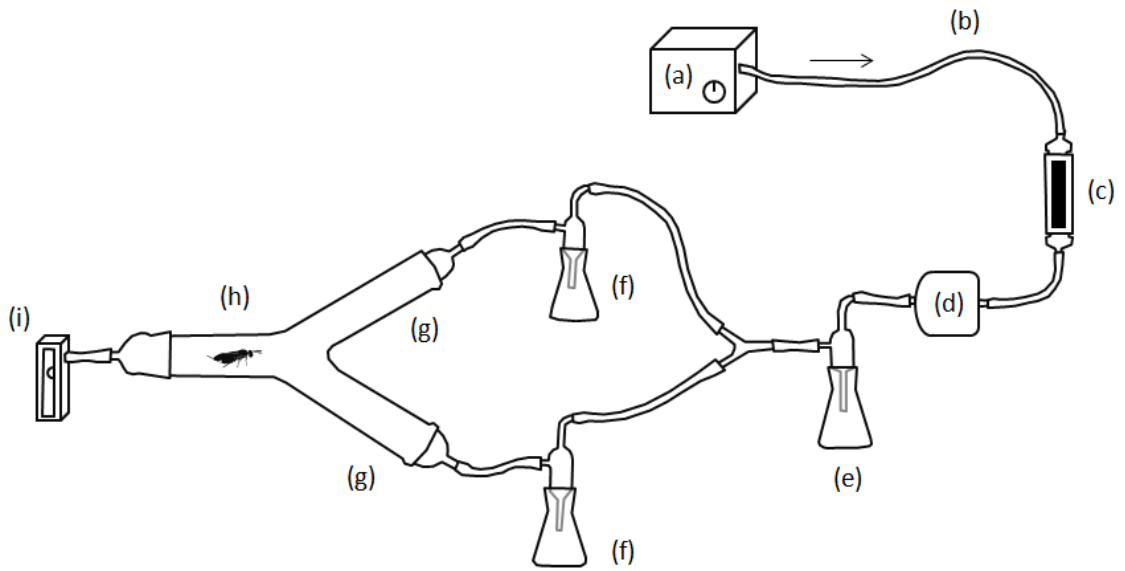


Figure 6.1. Olfactometer configuration consists of: air source (pump) (a), silicon tubing (b), charcoal activated filter (c), dust filter (d), distilled water (e), flasks containing odor source or control (f), side arms (g), central arm (h), flow meter (i).

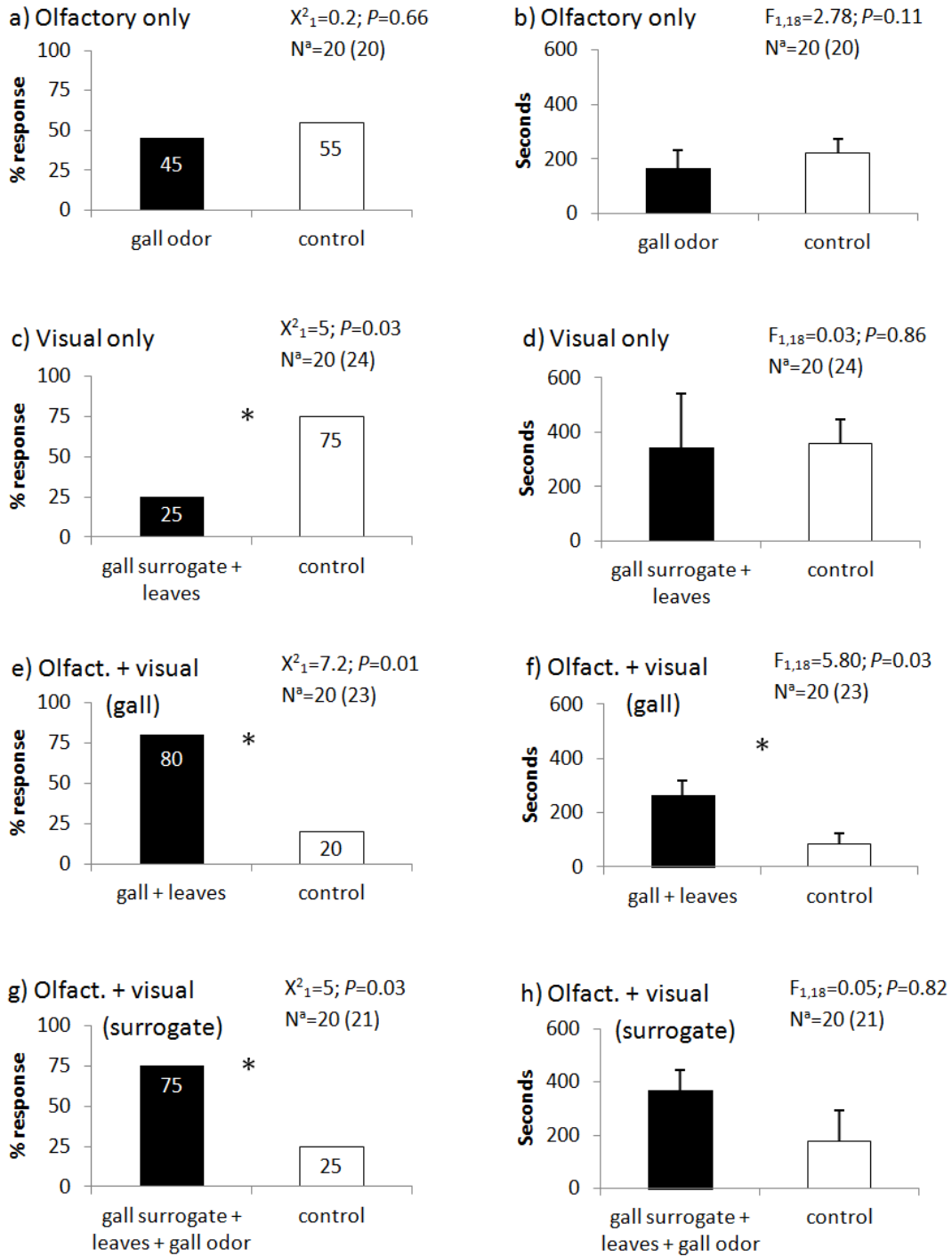


Figure 6.2. Proportion of *T. sinensis* female wasps choosing a stimulus (olfactory and/ or visual) versus a blank control in four dual choice experiments (a, c, e, g), and time elapsed (seconds) for *T. sinensis* female wasps to choose a stimulus (b, d, f, h).

<sup>a</sup>N = number of wasps that chose (total number of wasps evaluated)

\* = indicates significant ( $P < 0.05$ ) difference

## **Chapter Seven: Interactions between *Dryocosmus kuriphilus* and stem-cankering fungi**

The introduction of non-native species into new regions represents a major threat for native ecosystems and biodiversity. North American forests are increasingly targeted by non-native invaders, thus compromising the structure, function and productivity of these systems (Lovett et al. 2006). Forest trees may be impacted by a multitude of organisms which can trigger decline of foundation species and collapse of crucial habitats. Insect herbivores, pathogenic microorganisms, and their interactions play a major role threatening forest resources (Loo 2009, Gandhi and Herms 2010). Oak forests in the western US are rapidly declining following establishment of the highly pathogenic *Phytophthora ramorum*, which causes sudden oak death (Rizzo and Garbelotto 2003). Eastern North America ash are being devastated by the emerald ash borer, *Agilus planipennis*, a buprestid beetle native to China and spreading in the US since 2002 (Poland and McCullough 2006). In some cases novel relationships develop and insects and microorganisms interact (Chapter 5). These interactions can be especially destructive; such is the case with the native walnut twig borer, *Pityophthorus juglandis*, and the novel ascomycete, *Geosmithia morbida*, targeting black walnut, *Juglans nigra*, in many US states (Kolařík et al. 2011). Concerns regarding the impacts of multiple invasions on natural habitats are increasing, as herbivores and pathogens interact with each other and with other components of invaded ecosystems, thus causing a cascading complex of disturbances (Simberloff and Von Holle 1999, Ayres and Lombardero 2000, Anagnostakis 2001). Interactions among herbivorous insects and plant pathogenic microorganisms can be directly or indirectly mediated by the host plant (Hatcher 1995).

The interaction may develop through facilitation: attack from one organism compromises defensive responses and predisposes the host to the disrupting activity of the second organism, thus impacting susceptibility (Schoeneweiss 1975).

American chestnut (*Castanea dentata* Marshall Borkh) represents a dramatic example of a forest foundation species experiencing multiple biological invasions. American chestnut was historically a dominant component of mixed mesophytic forests in Central Appalachia (Braun 1950), but its survival has been compromised by multiple introductions of non-native organisms (Anagnostakis 2001). During the 19<sup>th</sup> century a non-native root rot disease, *Phytophthora cinnamomi*, caused extensive chestnut mortality in the south (Anagnostakis 2001). This was overshadowed in the following century by introduction of the chestnut blight fungus, *Chryphonectria parasitica* Murr. (Barr) (Ascomycota: Diaporthales), which functionally eliminated American chestnut from its native range (Griffin 2000). The blight fungus is of Asian origin; Chinese chestnut (*C. mollissima*) demonstrates significant resistance (Anagnostakis 1992).

Developing an American chestnut resistant to the blight fungus has been the focus of scientific efforts for decades. Researchers have captured the natural resistance inherent in lingering American chestnut (Griffin 2000), have backcrossed *C. mollissima* resistance genes into *C. dentata* genotypes (Hebard 2006, Diskin et al. 2006), and have successfully developed techniques to genetically modify *C. dentata* (Zhang et al. 2013). These efforts are coming to fruition, and resistant ‘American’ chestnut are being deployed (Jacobs 2007, Jacobs et al. 2013). In addition, Asian and European chestnuts, and their hybrids, are increasingly cultivated for nut production (Fulbright et al. 2009). But the resurgence of American chestnut, and the use of chestnut in the US for nut production, is being

threatened by the spread of the Asian chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu (Hymenoptera: Cynipidae). The gall wasp is a specialist herbivore that induces formation of spherical galls on new vegetation. Galls act as physiological sinks that deprive hosts of nutrients (Larson and Whitham 1991), disrupt development of photosynthetic organs, and reduce tree growth, thus triggering decline, and in extreme cases, mortality (Kato and Hijii 1993). Blight resistant cultivars of chestnut are extremely susceptible to galling (Anagnostakis 2012), and field observations suggest that galling may affect the response to blight infection of resistant chestnuts. In light of this I evaluated the interactions between galling induced by *D. kuriphilus* and infection by stem canker fungi; assessing effects on the host plant, and on insect and fungal performance. My overall goal is to evaluate interactions developing among this exotic insect herbivore, pathogenic fungi, and a native host plant of critical importance.

## **Materials and methods**

In June 2011 38 bare-root Chinese chestnut (*C. mollissima*) seedlings, some of which contained *D. kuriphilus* galls (Figure 7.1A), and some of which presented with necrotic stem lesions (Figure 7.1B), were established in the greenhouse in 36 × 12 × 12 cm tree pots. The lesion-causing agent was isolated in pure culture and identified as *Nectria* sp. (Hypocreales: Nectriaceae) (Figure 7.1C). The number of galls and cankers per seedling were counted, and seedlings were assigned to one of four treatments based on their occurrence: seedlings with only galls (N=5), seedlings with only cankers (N=10), seedlings with both galls and cankers (N=15), and seedlings with neither galls nor cankers (untreated controls) (N=8). I measured seedling height, stem diameter at 5 cm, counted the number of leaves and the number of vegetative sprouts produced at the base



of each seedling. Cankers able to girdle and kill the stem above the infection point were classified as “lethal,” while cankers showing proliferation of healing tissue surrounding the necrotic lesion were considered “healing” cankers. External necrotic areas were measured (Figure 7.1B), and lesion size was assessed using the equation for the area of an ellipse  $A = \pi \times (W / 2) \times (L / 2)$ , where W and L represent width and length of the necrotic area, respectively. Similarly, I evaluated the expansion of healing tissue by measuring length and width of the healing callus (Figure 7.1D). Seedlings and cankers were measured initially (June 16) and at the end of the season (August 14), and values for seedling height, diameter, area of the necrosis and healing callus were expressed as Relative Growth Rate, with  $RGR = (Value_{(time\ 2)} - Value_{(time\ 1)}) \times (Value_{(time\ 1)})^{-1} \times (d)^{-1}$ , where  $Value_{(time\ 1)}$  and  $Value_{(time\ 2)}$  are the measured values on June 16 and August 14, respectively, and d is the number of days elapsed. The number of leaves was expressed as a leaf increment,  $I = L_{(time\ 2)} - L_{(time\ 1)}$ , where  $L_{(time\ 1)}$  and  $L_{(time\ 2)}$  are the number of leaves on June 16 and August 14, respectively. Nylon cages (15 × 30 cm) secured over galls on seedling branches were used to collect emerging wasp adults. When the experiment was terminated August 14, galls were dissected to determine the number of emerged wasp and wasps that failed to emerge. I then calculated gall wasp survival as the rate at which gall wasps successfully emerged from galls, ie, emergence rate,  $E = N_w / N_c$ , where  $N_w$  is the number of emerged wasps and  $N_c$  the number of larval cells within each gall.

Data were tested for normality (PROC UNIVAR, SAS<sup>®</sup> V. 9.3) and when necessary transformed using a square root or arcsine transformation. I used an analysis of variance (PROC MIXED) to test the effect of galling and stem cankers on the RGR of

seedling height, diameter, necrosis and healing tissue expansions, on the final size of cankers and healing tissue expansion, and to test if stem cankers affect gall wasp survival (emergence rate).

## **Results**

Differences in seedling height RGR were detected; seedlings with both cankers and galls grew less than those with galls only, which had the greatest height growth among the four seeding treatments (Table 7.1a, Figure 7.2A). Diameter RGR did not differ across seedling treatments (Table 7.1a). Similarly, the increase in number of leaves did not differ significantly, but seedlings with cankers produced significantly more basal sprouts than seedlings without cankers, regardless of galling (Table 7.1a).

The number of cankers affecting each seedling ranged from one to seven (mean 3), but did not differ significantly between seedlings with or without galls. Also, the number of lethal and healing cankers did not differ based on galling (Table 7.1c). Final canker size on galled seedlings was significantly larger than on seedlings free of galls (Table 7.1c, Figure 7.2A), but there were no differences in canker relative growth when galls were present. Finally, both the final size and the relative growth of the healing tissue surrounding cankers were significantly reduced on galled seedlings compared to gall-free seedlings (Table 7.1c, Figure 7.2B, 7.2C).

Among galled seedlings there were one to four galls per seedling (mean 1.7), but there were no differences in galling between seedlings with and without cankers (Table 7.1b), nor were there a differences in gall wasp survival (emergence rate) based on stem cankering (Table 7.1b).

## Discussion and conclusions

Host plants, their insect herbivores, and their microbes can interact directly or indirectly, affecting reciprocal fitness (Hatcher 1995, Schoeneweiss 1975). I evaluate the impact of such an interaction developing among the chestnut gall wasp, *Nectria* stem cankers, and the shared host, *C. mollissima*, and find that asymmetrical effects of these interactions are evident.

I found no meaningful effect of gall wasp infestation or fungal infection on seedling height growth; the lack of logical differences with respect to this parameter is likely due to the architecture of my experimental seedlings; some lacked apical dominance and thus the canopy was excessively branched, obscuring any potential height differences. The most noticeable effect of cankering on seedling growth was in production of basal sprouts, which occurred regardless of galling.

Galling by *D. kuriphilus* disrupts production of healing tissue by the host tree, thus enhancing fungal success and reducing plant performance. Necrotic lesions induced by fungal infection on galled seedlings are larger relative to lesions on gall-free seedlings, and growth of healing tissue was slower.

Although I found no effect of stem cankers on gall wasp survival, expressed as emergence rate from galls, the possibility of a lethal stem canker causing death of the infected organ would directly affect survival of gall wasps developing on the same branch.

My findings demonstrate that the gall wasp can increase the susceptibility of its host to the *Nectria* pathogen (Hatcher 1995), and suggests that a similar interaction may

unfold between *D. kuriphilus* and the chestnut blight fungus (Turchetti et al. 2010), an area of future work. The extent of this interaction is crucial in evaluating the resistance to the blight fungus of improved chestnuts, and in managing chestnut resources in North America.

Table 7.1. Interacting effects (mean (SE)) of stem-cankering *Nectria* sp. and *D. kuriphilus* galling on a) Chinese chestnut seedling performance, b) gall wasp survival, and c) fungal performance.

	Seedling treatment				Test statistic
	Cankers	Gall wasp	G. w. + cankers	Untreated	
<i>a) Seedling performance</i>					
Height RGR	0.004 (0.001)	0.006 (0.002)	0.002 (0.001)	0.003 (0.01)	$F_{1,34} = 5.43$ ; $P = 0.03$
Diameter RGR	0.005 (0.001)	0.006 (0.002)	0.004 (0.001)	0.006 (0.001)	$F_{1,34} = 0.06$ ; $P = 0.81$
Leaf increase	5.3 (8.6)	10.0 (8.9)	-11.7 (8.7)	10.0 (6.5)	$F_{1,34} = 0.70$ ; $P = 0.41$
Basal sprouts	1.2 (0.47)	0.0 (0.0)	1.5 (0.49)	0.1 (0.1)	$\chi^2_{1,34} = 10.5$ ; $P = 0.001$
<i>b) Gall wasp performance</i>					
Galls	-	1.4 (0.4)	1.7 (0.3)	-	$\chi^2_{1,18} = 0.26$ ; $P = 0.60$
Gall wasp emergence rate	-	0.4 (0.2)	0.6 (0.01)	-	$F_{1,32} = 0.47$ ; $P = 0.50$
<i>c) Fungal performance</i>					
Total cankers	2.50 (0.5)	-	3.00 (0.37)	-	$\chi^2_{1,23} = 0.65$ ; $P = 0.42$
Lethal cankers	0.2 (0.13)	-	0.47 (0.13)	-	$\chi^2_{1,23} = 1.28$ ; $P = 0.26$
Healing cankers	2.20 (0.63)	-	1.67 (0.35)	-	$\chi^2_{1,23} = 0.68$ ; $P = 0.41$
Final canker size	215.2 (63.9)	-	416.5 (84.2)	-	$F_{1,68} = 7.03$ ; $P = 0.01$
Canker RGR	0.08 (0.06)	-	0.05 (0.03)	-	$F_{1,68} = 3.5$ ; $P = 0.07$
Final healing tissue size	425.8 (56.4)	-	235.4 (46.9)	-	$F_{1,68} = 3.94$ ; $P = 0.05$
Healing tissue RGR	7.22 (0.96)	-	3.99 (0.80)	-	$F_{1,68} = 9.1$ ; $P = 0.004$

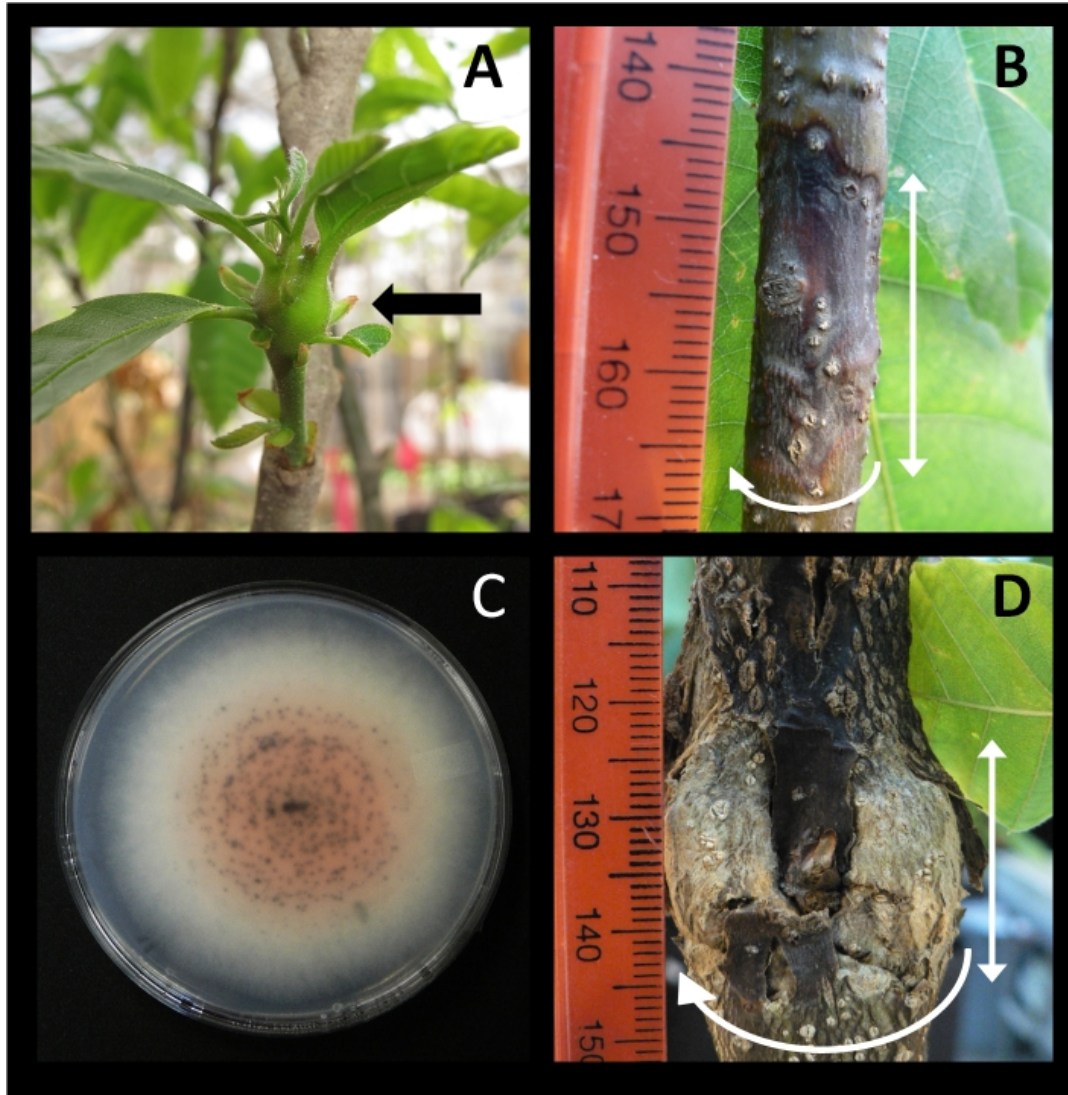


Figure 7.1. A) Gall induced by *D. kuriphilus* on *C. mollissima* seedling, B) canker induced by *Nectria* sp. (June 16), C) culture of *Nectria* sp. and D) measurement of healing callus (August 14).

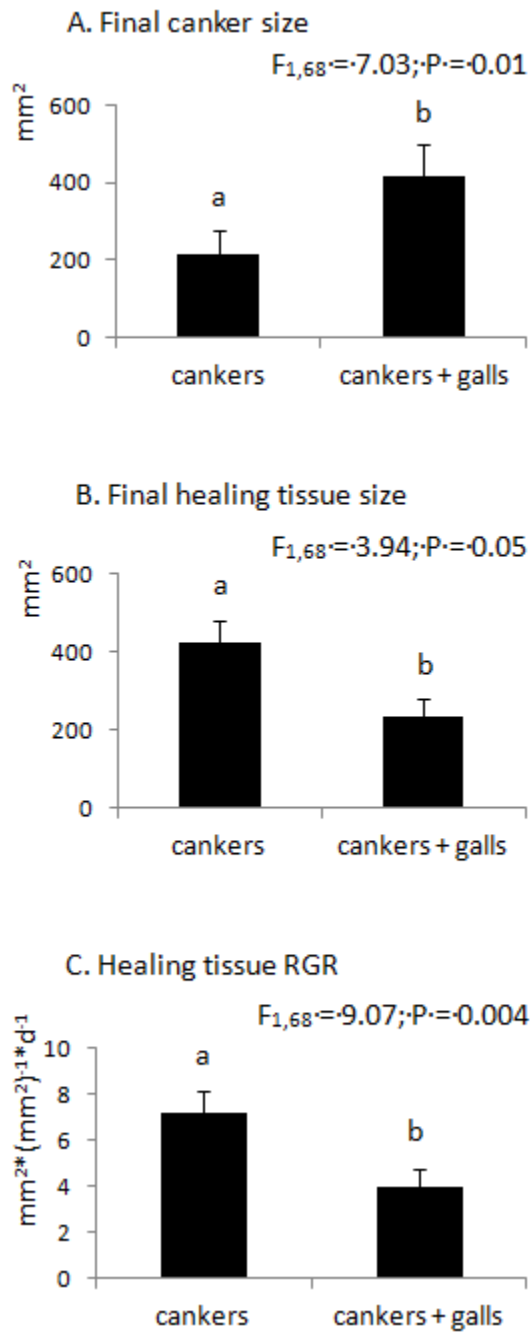


Figure 7.2. Differences among treated seedlings in: A) height RGR (Relative Growth Rate), B) final canker size, C) healing tissue RGR, and D) final healing tissue size. Means followed by the same letter do not differ ( $\alpha = 0.05$ ).

## Chapter Eight: Summary and conclusions

The current North American distribution of *Dryocosmus kuriphilus* throughout the former range of American chestnut is the result of a forty year invasion (Rieske 2007). Since its introduction the gall wasp has interacted with biotic and abiotic components of its invaded environments, including landscape features, exotic and hybrid chestnuts (Cooper and Rieske 2009, Anagnostakis et al. 2011), insect and fungal associates of the host plant, and gall wasp natural enemies (Cooper and Rieske 2007, 2010, 2011). I further elucidated how these evolving interactions have affected its invasiveness and its impacts in North America.

In Chapter 2 (Graziosi and Rieske 2012) I investigated *D. kuriphilus* local dispersal following its initial discovery in Lexington, Kentucky USA in 2010. I used remotely sensed data and Geographic Information Systems to describe the local distribution of the *Castanea* hosts, and the occurrence and dispersal of the gall wasp. I test the hypotheses that geomorphology, *Castanea* occurrence, and prevailing winds influence local proliferation. I found that gall wasp spread is attributable to host plant distribution and to the effects of prevailing winds occurring during a brief period of adult insect emergence, and is influenced by topography. My results suggest that weather data and topographic features can be used to delineate currently infested areas and predict future gall wasp infestations.

In Chapter 3 (Graziosi and Rieske 2014) I investigated potential fecundity of the gall wasp in order to better understand its invasiveness. I found that adult weight and metasomal width were positively correlated with the number of eggs. Egg load decreases



with wasp age, and egg size initially increases before decreasing. My findings suggest that adult *D. kuriphilus*, previously reported as proovigenic, may be resorping eggs in the absence of suitable hosts, and reallocating nutritive resources for body maintenance and egg quality to increase fitness, implicating a plasticity in its reproductive strategy. *Dryocosmus kuriphilus* may be able to vary its potential fecundity in response to nutrition and host availability, thus increasing its invasiveness.

In Chapter 4 I sought to determine the key factors driving *D. kuriphilus* gall morphology, and found that population level and parasite pressure appear to be the major determinants. When gall wasp pressure is low ovipositing females select large chestnut buds with no prior oviposition activity, avoiding sub-optimal buds, and laying relatively small egg clutches that favor formation of simple, uni-lobed galls. These simple galls are readily accessible, with high parasite pressure. When gall wasp population pressure is high, fewer buds per female are available. Females still tend to avoid small buds and continue to select larger viable buds. Multiple oviposition events per bud will occur, triggering formation of more complex, multi-lobed galls. These multi-lobed galls limit parasite pressure because the majority of developing gall wasp larvae are protected by seclusion within the complex gall. This suggests that the gall wasp is able to adjust gall morphology based on population levels and parasite pressure, thus optimizing its fitness. The role of chestnut phenology in influencing gall features and gall morphology should be thoroughly evaluated in order to more fully understand the gall maker-host plant interaction.

In Chapter 5 I elucidated a Plant-Microbe-Insect interaction where a plant pathogenic microorganism is utilizing *D. kuriphilus* as an alternate resource, and using

Koch's postulates, tested the hypothesis that the fungus is affecting the invasion dynamics of the insect on its chestnut host. I isolated and identified *Colletotrichum*, a causal agent of plant-pathogenic anthracnose. Following formation of superficial lesions, the pathogen rapidly penetrates galls, colonizing insect tissue in gall chambers and causing extensive gall wasp larval mortality. Virtually no negative effects were evident on the associated parasitoid, *Torymus sinensis*. Gall wasp invasiveness may be locally reduced by the plant pathogen, which is advantageously utilizing the insect herbivore while sparing its parasite. Future work should focus on elucidating the pathogen mode of action, the apparent immunity of *T. sinensis* to fungal infection, and the native or exotic nature of the fungal pathogen.

In Chapter 6 (Graziosi and Rieske 2013) I investigated host location behavior of *T. sinensis*, the natural enemy used for gall wasp biological control, using a Y-tube olfactometer. I found that both visual and olfactory cues are required to enable the hymenopteran parasite to successfully find suitable hosts. Adult parasitoids were not attracted by the odor of fresh galls alone, and had a negative response to the visual cues of galls and chestnut foliage when odor cues were not provided. However, the combination of olfactory and visual stimuli provided by a fresh gall coupled with chestnut foliage elicited a strongly positive response. My findings improve my understanding of the stimuli that influence *T. sinensis* host location behaviors leading to successful gall wasp parasitization, and may enhance my ability to manipulate *T. sinensis* for gall wasp management.

In Chapter 7 I describe an interaction developing between *D. kuriphilus*, *Nectria* stem cankers, and Chinese chestnut, and found the outcome of this interaction to be

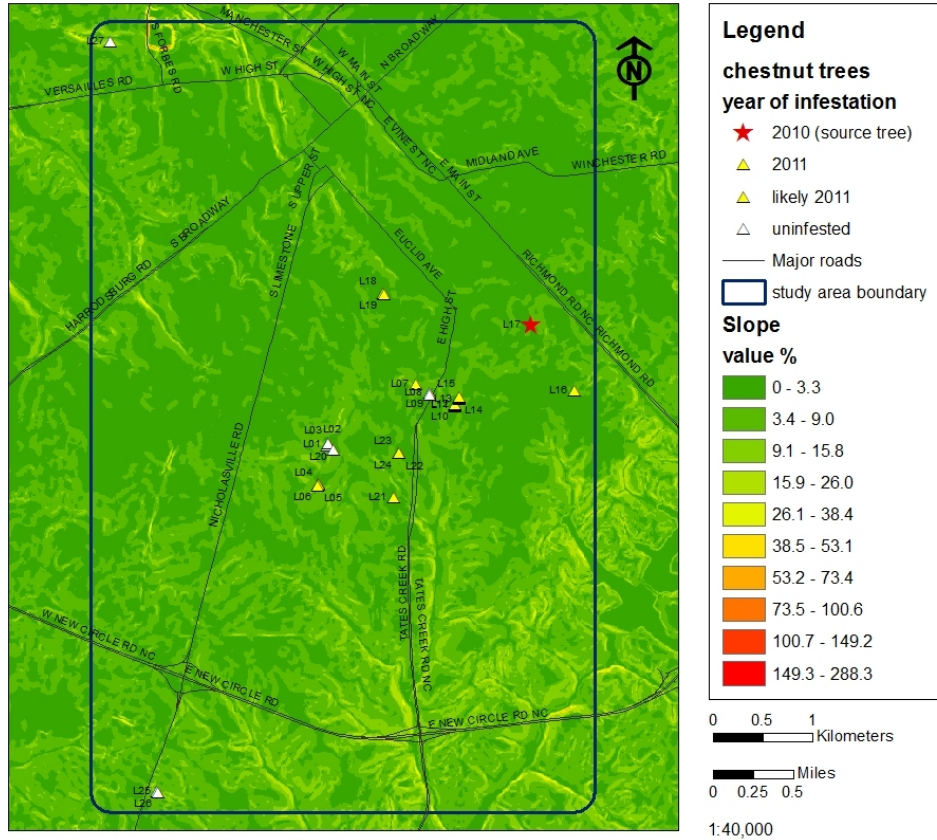
asymmetrical. Galling prevents the host tree from successfully healing stem lesions by inhibiting production of healing tissue on *Nectria* infected seedlings, positively impacting fungal fitness and negatively impacting host plant fitness. In contrast, fungal infection has no effect on gall wasp fitness. I conclude that galling can increase the susceptibility of the chestnut host to this stem cankering fungus. Understanding these interactions may be critical to managing gall wasp populations on blight-resistant chestnut cultivars, and has various implications for chestnut cultivation. My findings suggest that similar asymmetric interactions may establish between the gall wasp and the chestnut blight fungus, and also in other herbivore – microbe – host plant systems.

My work adds to a growing body of knowledge on both basic and applied invasion biology and ecology, insect behavior, and Plant-Microbe-Insect interactions. These findings represent meaningful information to improve my understanding of the invasion process of exotic organisms. In addition, my findings contribute to further development of tools to manage invading Asian chestnut gall wasp populations in light of the invasion history of American chestnut, and support *Castanea* cultivation for restoration, reclamation, and nut production in North America.

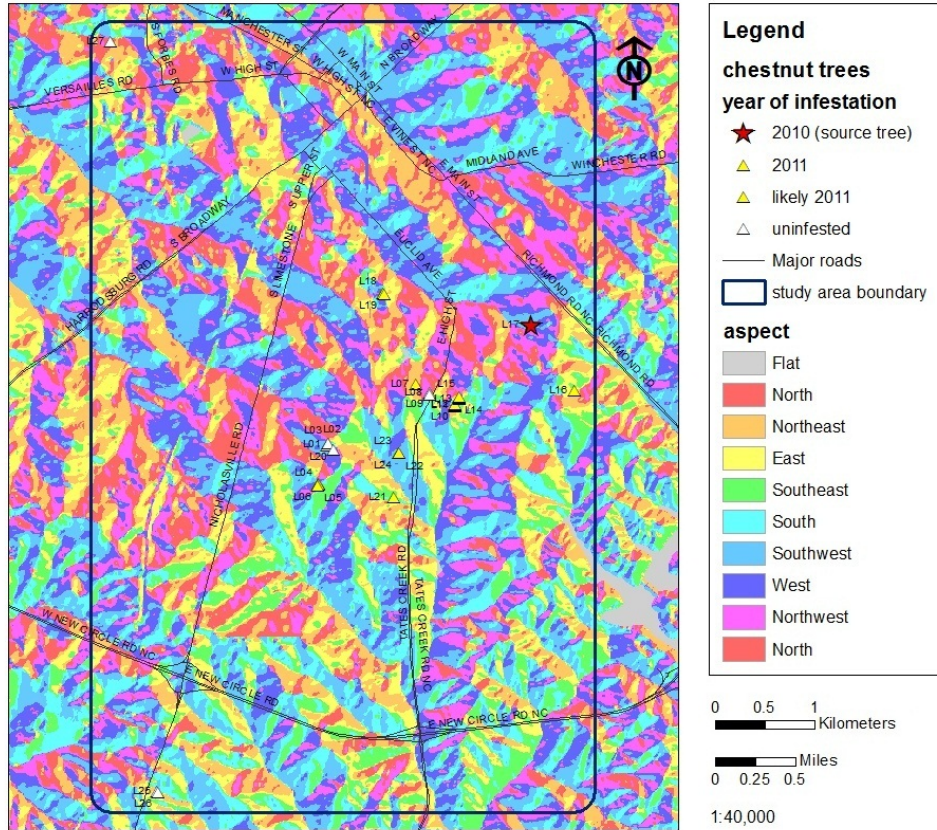
## **Appendices**

Appendix I and II are maps depicting slope and aspect of the study area relative to Chapter 2. Appendix III is a table showing *D. kuriphilus* egg and body metrics relative to the study presented in Chapter 3. Appendix IV is a list of ambrosia beetles trapped in a preliminary experiment conducted in summer 2013. Appendix V resumes a survey assessing gall wasp pressure conducted in early 2014 in a chestnut farm.

**Appendix I.** Slope of the study area in Fayette Co., KY, based on the 9.1 m resolution digital elevation model (Chapter 2).



**Appendix II.** Aspect of the study area in Fayette Co., KY, based on the 9.1 m resolution digital elevation model (Chapter 2).



**Appendix III.** Egg and body metrics for four age cohorts of the globally invasive *D. kuriphilus* (Chapter 3).

	N	Wasp age				Test statistic; P-value
		0 day	1 day	2 days	3 days	
Egg parameters						
number of eggs	24	267.7 (18.5)	203.2 (15.5)	161.2 (8.9)	126.8 (14.0)	$\chi^2=28.62$ ; $P<0.0001$
egg volume $\mu\text{m}^3$	120	807762 (76587)	814477 (39345)	983180 (77317)	722644 (31307)	$F_{3,116}=3.89$ ; $P=0.011$
egg area $\mu\text{m}^2$	120	11598 (662)	11916 (358)	13271.5 (593)	11156.6 (309)	$F_{3,116}=3.71$ ; $P=0.014$
egg length $\mu\text{m}$	120	145.0 (4.0)	149.5 (2.2)	155.5 (2.7)	148.0 (2.7)	$F_{3,116}=2.58$ ; $P=0.057$
egg width $\mu\text{m}$	120	100.0 (2.8)	101 (1.9)	107.5 (2.9)	96.0 (2.0)	$F_{3,116}=3.90$ ; $P=0.011$
Adult wasp parameters						
weight mg	12	1.03 (0.03)	0.77 (0.03)	0.67 (0.09)	0.60 (0.06)	$F_{3,8}=10.89$ ; $P=0.003$
total length $\mu\text{m}$	12	2800 (26.5)	2800 (50.0)	2670 (62.4)	2640 (62.4)	$F_{3,8}=2.59$ ; $P=0.126$
mesosomal length $\mu\text{m}$	12	1090 (10.0)	1060 (13.0)	1090 (20.0)	1060 (10.0)	$F_{3,8}=1.71$ ; $P=0.242$
mesosomal width $\mu\text{m}$	12	820 (36.1)	760 (26.5)	850 (10.0)	780 (17.3)	$F_{3,8}=2.64$ ; $P=0.121$
metasomal length $\mu\text{m}$	12	1360 (43.6)	1360 (26.5)	1290 (34.6)	1290 (17.3)	$F_{3,8}=2.55$ ; $P=0.129$
metasomal width $\mu\text{m}$	12	900 (0.0)	810 (34.6)	780 (34.6)	760 (10.0)	$F_{3,8}=5.80$ ; $P=0.020$
hind femur length $\mu\text{m}$	12	750 (17.3)	730 (10.0)	740 (10.0)	760 (10.0)	$F_{3,8}=1.11$ ; $P=0.399$

**Appendix IV.** Ambrosia beetle associates of *Castanea*.

Introduction. Ambrosia beetles (Coleoptera: Curculionidae) cause extensive mortality of young chestnut in plantation settings. Ambrosia beetles are attracted to conophthorin and ethanol, and repelled by verbenone. To reduce beetle-caused mortality a push-pull technique was evaluated to manipulate ambrosia beetle populations away from susceptible chestnut.

Methods. Three sites were designated where hybrid and backcross chestnut were being grown for evaluation (Table A IV.1). Bottle traps baited with ethanol bags and conophthorin tubes (Contech Inc., Victoria, Canada, emission rate 100 µl/day and 4 µl/day, respectively) were deployed around the perimeter of each chestnut block. Similarly, repellent traps were fitted with Verbenone pouches (Contech Inc., Victoria, Canada, emission rate 50 µl/day), and were deployed in the center of each block. Traps were monitored at 14 d intervals from March 26 to August 13 2013.

Table A IV.1. Sites used to evaluation the push-pull technique for ambrosia beetle management.

Site	County	Location	No. traps deployed
Meades Landing	Oldham	38°26'39.20"N 85°31'9.58"W	10
Wilkins Farm	Shelby	38°10'15.73"N 85°16'50.06"W	9
Spindletop Farm	Fayette	38° 7'37.64"N 84°30'52.85"W	4



Results. Ambrosia beetle pressure was not sufficient to evaluate the push-pull technique at any of the sites. Thirteen species were captured over the course of the monitoring season, nine of which were identified to species, and three to the genus level (Table A IV.2).

Table A IV.2. Ambrosia beetles captured in conophthorin and ethanol baited bottle traps.

	Site		
	N traps (Distance from woodlands)		
	Meades Landing 10 (< 20 m)	Wilkins F. 9 (> 100 m)	Spindletop F. 4 (> 100 m)
<i>Xylosandrus crassiusculus</i>	370	154	102
<i>Xyleborinus saxesenii</i>	61	34	11
<i>Ewallacea validus</i>	52	9	1
<i>Cyclorhipidion spp.</i>	21	7	2
<i>Cyclorhipidion bodoanum</i>	23	0	0
<i>Ambrosiodmus tachygraphus</i>	14	2	1
<i>Phloeotribus sp. 1</i>	12	0	0
<i>X. germanus</i>	5	1	0
Unknown sp.	5	0	0
<i>A. rubricollis</i>	4	1	0
<i>Phloeotribus sp. 2</i>	3	2	0
<i>Dryoxylon onoharaensum</i>	1	2	0
<i>Cnestus mutilatus</i>	1	0	0

**Appendix V.** Assessing *Dryocosmus kuriphilus* population pressure in a chestnut orchard.

Introduction. *Dryocosmus kuriphilus* infestations represent a limiting factor for chestnut cultivation, and its pressure can vary based on several factors included chestnut cultivar and year. I aimed to assess galling pressure on trees of known and unknown cultivar growing in a chestnut farm, and evaluate differences in galling pressure among cultivars and across two years.

Methods. A chestnut farm was located in Mt. Vernon OH (40°21'15.53"N; 82°28'23.47"W), where 90 chestnut trees belonging to several known and unknown cultivars are growing. The planting was visited prior to bud break (04/09/2014) and 50 trees belonging to 16 cultivars were surveyed (Table A V.1). Previous year (2013) galls were counted on four, 50 cm long, branches per tree selected at the four cardinal directions. In addition, I cut one 50 cm branch per tree in order to dissect the buds in the lab and determine the presence of *D. kuriphilus* larvae inside chestnut buds. Current year (2014) infestation was assessed as number of gall wasp infested buds per branch.

Table A V.1. Trees surveyed for chestnut gall wasp pressure in Mt. Vernon OH.

Cultivar Code	Cultivar name	Species	Sampled trees
U	Unknown	Unknown	2
L	Layeroka	Chinese X European	3
C2	Unknown	Chinese	2
C3	Unknown	Chinese	3
C4	Douglas	American X Chinese	2
C5	Unknown	Chinese	3

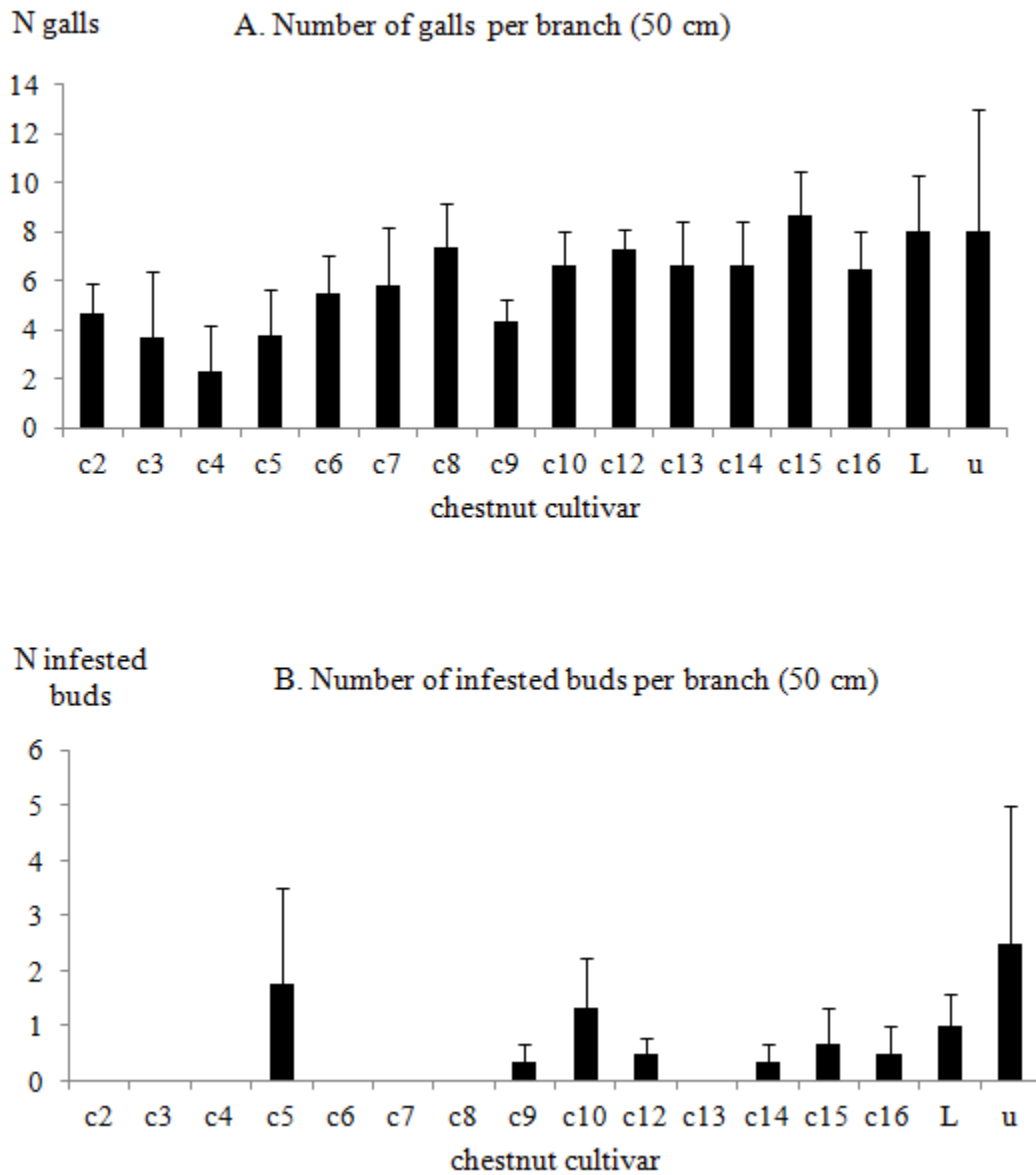
*continued on page 103*

*continued from page 102*

C6	Unknown	Chinese	3
C7	Unknown	Chinese	6
C8	Asia American	Unknown	3
C9	Manchurian	Unknown	3
C10	Sweetheart 1	Unknown	3
C12	Unknown	Chinese	3
C13	Colossal	European X Chinese	3
C14	Simpson	European X Chinese	3
C15	Layeroka	European X Chinese	5
C16	Sweetheart 2	Unknown	3
<hr/>			
Total trees			50

Results. In 2013 *D. kuriphilus* was widespread in the orchard. All the sampled trees were infested (Figure A V.1A), with galling rates ranging from  $3.75 \pm 1.9$  galls of the C2 Chinese cultivar to  $8.7 \pm 1.8$  galls of the C8 “Asia America” cultivar, with no meaningful differences among cultivars. 2104 infestation resulted to be considerably lower; only 13 trees out of 50 sampled were found infested (Figure A V.1B), this suggesting a crash in the gall wasp population.

Figure A V.1. Evaluating gall was pressure by: A) assessing the number of gall per 50 cm branch (describing 2013 infestation), and B) determining the number of infested buds per 50 cm branch (depicting 2014 infestation).



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Zhi-yong Z, Lin Q, Hong-Wen H (2009) Study approaches on the chestnut gall wasp, *Dryocosmus kuriphilus* Yasumatsu in China. *Acta Horticulturae* 844: 425-432

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## VITA

### Education

University of Kentucky, Lexington, KY 2010-2014  
Doctorate: Entomology, December 2014 (expected)

University of Firenze, Firenze, Italy 2000-2005  
Bachelor and Master of Science: Forestry, October 2005

### Professional experience

International Center for Tropical Agriculture, Hanoi, Vietnam Prospected  
Visiting research fellow

University of Kentucky, Lexington, KY 2010-present  
Research assistant  
Instructional Assistant for Forest Entomology (ENT/FOR 402) Fall 2013

Monte Sole Regional Park, Bologna, Italy Jan-July 2010  
Project manager: Management of natural Scots pine (*Pinus silvestris*) stands in the Monte Termine area

Agri2000 Company, Bologna, Italy Sept 2009 -May 2010  
Urban Forester and Arborist assistant

University of Bologna, Italy Feb 2006 -Feb 2009  
Research Assistant: Impact of diseases on chestnut forests in the Apennine Mountains, Italy

The American Chestnut Foundation, Meadowview, VA June-Aug 2007  
Intern (volunteer)

Bologna, Italy Nov 2005-May 2010  
Consultant in forest management, arboriculture, and urban forestry. Main collaborations: Simone Pinzauti professional consultant, Firenze, Italy; “Agristudio” Company, Florence Italy, “Politecnica” Company, Modena, Italy.

## Publications

### *Journal Articles*

- Graziosi I**, Rieske LK (in review). Can plant pathogens advantageously utilize insects as resources? *Agricultural and Forest Entomology* (subm.11/2014)
- Graziosi I**, Rieske LK (2014). Potential fecundity of a highly invasive gallmaker, *Dryocosmus kuriphilus* (Hymenoptera: Cynipidae). *Environmental Entomology* 43, 1053-1058.
- Graziosi I**, Rieske LK (2013). Response of *Torymus sinensis*, a parasitoid of the gallforming *Dryocosmus kuriphilus*, to olfactory and visual cues. *Biological Control* 67, 137-142.
- Graziosi I**, Rieske LK (2012). Local spread of an exotic invader: using remote sensing and spatial analysis to document proliferation of the invasive Asian chestnut gall wasp. *iForest – Biogeosciences and Forestry* 5, 255-261.
- Graziosi I**, Santi F (2008). Chestnut gall wasp (*Dryocosmus kuriphilus*): spreading in Italy and new records in Bologna province. *Bulletin of Insectology* 61, 343-348.

### *Book Chapters*

- Cazzola F (Contributor to) (2013). Emilia Romagna pp. 299-318, in: *Italian Historical Rural Landscapes*, Environmental History I. (Agnolletti M, ed). Springer, Dordrecht. DOI: 10.1007/978-94-007-5354-9\_13.

### *Proceedings*

- Graziosi I**, Rieske LK (2014). “Mapping invasion of the Asian chestnut gall wasp on a local scale using remote sensing and spatial analysis.” Proceeding of the 5<sup>th</sup> International Chestnut Symposium, Shepherdstown WV, 5 September 2012. *Acta Horticulturae* 1019, 113-118.

**Graziosi I, Rieske LK (2013).** “An opportunistic fungus may impact Asian chestnut gall wasp populations in North America.” Southern Forest Insect Work Conference, New Orleans LA, 23-26 July 2013.

<http://www.sfiwc.org/2013/SFIWC2013Proceedings.pdf>

**Graziosi I, Rieske LK (2012).** “Tracking local dispersal of an exotic invader using remote sensing and spatial analysis.” Proceedings of the 54th Southern Forest Insect Work Conference, Charlottesville VA, 25 July 2012. <http://www.sfiwc.org/2012/SFIWC2012Proceedings.pdf>

**Graziosi I, Santi F (2009)** “Indagine sulla diffusione del cinipide galligeno del Castagno in provincia di Bologna.” (Spreading of the Asian chestnut gall wasp in the Bologna province, Italy). Proceeding of the XXII Italian National Congress of Entomology, Ancona, 15-18 June 2009, pp. 222. (In Italian)

<http://www.accademiaentomologia.it/pdf/XXII%20CNIE%20Ancona,%2015-18%20giugno%202008.pdf>

## **Scientific Presentations**

### ***Oral presentations***

Graziosi I: “Invasiveness of an exotic gall wasp is influenced by interactions with exotic and endemic organisms” PhD exit seminar, Department of Entomology, Lexington KY, 18 September 2014.

Graziosi I, Rieske LK: “Can modulating fecundity increase invasiveness?” NE1333 meeting, Lacross WI, 4-6 September 2014.

Graziosi I, Rieske LK: “Can modulating fecundity increase invasiveness?” South Forest Insect Work Conference, Charleston SC, 23 July 2014.

Rieske LK, Graziosi I, Cooper WR, Kellogg SK: “Persistence of American chestnut in the wake of successive invasions.” Invited presentation, Forest Entomology Symposium, North Central Branch of the Entomological Society of America, Des Moines IA, 10 March 2014.

Graziosi I, Rieske LK: “Olfactory and visual cues trigger host location behavior of *Torymus sinensis*, a parasitoid of the globally invasive Asian chestnut gall wasp.” Entomological Society of America, Austin TX, 11 November 2013.

Graziosi I, Rieske LK: “An opportunistic fungus causes mortality of the Asian chestnut gall wasp populations in North America.” NE1333 Meeting, Berea KY, 5-7 September 2013.

Graziosi I, Rieske LK: “An opportunistic fungus may impact Asian chestnut gall wasp populations in North America.” Southern Forest Insect Work Conference, New Orleans LA, 23-26 July 2013.

Graziosi I, Rieske LK: “An endophytic fungus colonizes invasive galls in eastern North America.” 3<sup>rd</sup> Conference on Invasion Biology, Ecology and Management, Lexington KY, 3-4 April 2013.

Graziosi I, Rieske LK: “Mapping dispersal of an invasive gallmaker using GIS and remote sensing: Localized proliferation of the Asian chestnut gall wasp.” Entomological Society of America, Knoxville TN, 12 November 2012.

Graziosi I, Rieske LK: “Tracking the invasion of an exotic pest using GIS and spatial analysis: local dispersal of the Asian chestnut gall wasp.” Ohio Valley Entomological Association, Cincinnati OH, 26 October 2012.

Graziosi I, Rieske LK: “Mapping invasion of the Asian chestnut gall wasp on a local scale using remote sensing and spatial analysis.” International Chestnut Symposium, Shepherdstown WV, 5 September 2012.

Graziosi I, Rieske LK: “Tracking local dispersal of an exotic invader using remote sensing and spatial analysis.” Southern Forest Insect Work Conference, Charlottesville VA, 25 July 2012.

Graziosi I, Rieske LK: “Interaction between an insect and a fungus pest: how American chestnut is impacted by two exotics.” Entomological Society of America, Reno NV, 14 November 2011.

Graziosi I, Rieske LK: "Interaction between a gall making insect and a stem cankering fungus: how American chestnut is again impacted by invasive species." Ohio Valley Entomological Association, Frankfort KY, 28 October 2011.

Graziosi I: "Investigating the dispersal of the Asian chestnut gall wasp and associated parasitoids" PhD proposal seminar, Department of Entomology, Lexington KY, 11 February 2011.

Leonhard S, Schumacher J, Graziosi I, Turchetti T, Wulf A: "Recent observations and results regarding ink disease and chestnut blight in Germany and Italy." Presentation at Julius Kuhn Institut, Federal Centre for Cultivated Plants, Braunschweig, Germany, 15 September 2008.

### ***Poster presentations***

Graziosi I, Rieske LK: "Remote sensing and spatial analysis can be used to track local dispersal of an exotic invader." 3<sup>rd</sup> Conference on Invasion Biology, Ecology and Management, Lexington KY, 3-4 April 2013.

Rieske LK, Dodd LE, Adkins JK, Graziosi I, Nelson L, Sprinkle MA: "Research in Forest Entomology: Building a knowledge base to understand and protect our natural resources." Special symposium, Entomological Society of America, Reno NV, 15 November 2011.

Graziosi I, Rieske LK: "Interactions between two exotic invaders on a native tree: American Chestnut, the chestnut blight fungus and the Asian chestnut gall wasp." 2<sup>nd</sup> Kentucky Invasive Species Conference, Lexington KY, 5 May 2011.

Graziosi I, Rieske LK: "Interactions between an old and new invader: how American chestnut is impacted by two exotics." North American Forest Insect Work Conference, Portland OR, 9-12 May 2011.

Graziosi I., Santi F.: "Indagine sulla diffusione del cinipide galligeno del Castagno in provincia di Bologna." (Spreading of the Asian chestnut gall

wasp in the Bologna province, Italy). Italian National Congress of Entomology, Ancona, 15-18 June 2009.

Graziosi I., Muzzi E., Turchetti T: “Indagine sull’incidenza del cancro della corteccia in castagneti della provincia di Bologna.” (Assessing chestnut blight incidence in the Bologna province, Italy). Italian Society of Silviculture and Forest Ecology (SISEF), Arezzo, 25-27 September 2007.

### **Grants**

Karri Casner Environmental Sciences Grant, University of Kentucky, February 2013.

### **Scholarships**

Fellowship, Center of Ecology, Evolution and Behavior, University of Kentucky, April 2012.

Fulbright Scholarship self placed, US-Italy Fulbright Commission, Rome, Italy, May 2010-present.

### **Awards and Honors**

1<sup>st</sup> place oral presentation, Southern Forest Insect Work Conference, New Orleans LA, July 2013.

1<sup>st</sup> place oral presentation, 3<sup>rd</sup> Conference on Invasion Biology, Ecology and Management, Lexington KY, 3 April 2013.

2<sup>nd</sup> place PhD oral presentation, Ohio Valley Entomological Assoc., Frankfort KY, October 2011.

Vinciguerra Award, Institute of International Education, New York NY, December 2010.

### **Memberships and Affiliations**

Entomological Society of America.

Southern Forest Insect Work Conference.



Ohio Valley Entomological Association.

The American Chestnut Foundation.

H. Garman Entomology Club, University of Kentucky Department of Entomology.

National Council of Agronomists and Foresters of Italy.

### **Community activities and Outreach**

Invasive.org contributing photographer:

<http://www.invasive.org/browse/autthumb.cfm?aut=77634>

Kentucky Bugs Facebook page contributing photographer:

<https://www.facebook.com/pages/Kentucky-Bugs/262237810453730>

Published scientific drawings: <http://www.sisef.it/iforest/contents/?id=ifor0508-002>

<http://www.sisef.it/iforest/contents/?id=ifor1231-008>

Outreach activities with H. Garman Entomology Club: insect Saturday activities at the Explorium Children's Museum of Lexington (since 2011, bimonthly), Insect Night Walk (2011, 2013), insect presentation at the Arbor Day (2012).

AGESCI (Italian Boy and Girl Scout Organization), program leader, Bologna, Italy, 2000-2005.

Volunteer, Guide and custodian at the Abetone Forest and Botanical Garden, Abetone, Pistoia, Italy, summer 2004.