Risk of invasion by walnut twig beetle throughout eastern North America

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Dedication

To Dr. Debra Ricci, Steven Hefty, and Palmer and Elizabeth Hefty.

Abstract

The walnut twig beetle (*Pityophthorus juglandis* Blackman) is a domestic alien invasive bark beetle in the United States of America (USA) that vectors a phytopathogenic fungus. Together, the beetle and fungus cause thousand cankers disease in species of *Juglans* and *Pterocarya*. Geographic range expansion by *P. juglandis* from its native range in the southwestern USA throughout the western United States and isolated areas of the eastern United States provides evidence for human-mediated movement. The disease is now present in more than 120 counties on naïve native and cultivated hosts in the eastern and western USA and in northern Italy. This research describes the cold mortality and host suitability of *P. juglandis*.

I measured the cold tolerance of *P. juglandis* adults and larvae from a northern California population monthly from January 2013 to May 2014. I found significant seasonal changes in adult supercooling points in fall, winter, and spring. I observed a shift in cold-tolerance strategy in *P. juglandis* adults from freeze-intolerance (December 2013 and January 2014) to partial freeze-tolerance (February 2014). Adults appear to be more cold-hardy than larvae. Predicted winter survivorship in the southeastern USA is higher than in the northeastern USA.

I conducted field and laboratory trials to determine if reproduction by *P. juglandis* varies between two black walnut (*Juglans nigra* L.) parent trees and black walnut and butternut (*Juglans cinerea* L.). Fewer adult offspring developed in branch sections of the black walnut maternal ‘Sparrow’ parent than the paternal ‘Schessler’ parent over three summer months and one winter month in the lab. In the field, *P. juglandis* reproduction in black walnut and butternut did not differ.

In an expanded laboratory study of host suitability, I screened 11 *Juglans* spp., one *Pterocarya* sp., and two *Carya* spp. over two years. Eleven *Juglans* and one *Pterocarya* species supported complete brood development. *Julgans nigra*, *J. californica*, and *J. hindsii* had the greatest levels of reproduction. Less suitable hosts include native southwestern United States hosts (*J. major* and *J. microcarpa*), Eurasian species (*J. regia*), Asian butternuts (*J.*

ailantifolia, *J. mandshurica*, and *J. cathayensis*), and native eastern United States butternut (*J. cinerea*) and Japanese walnut-butternut hybrid (J. *ailantifolia* × *cinerea*). The two *Carya* species were not hosts.

Finally, I present a framework that provides strategies for accessing stakeholder knowledge of unspecified pathways that may move forest insect pests. Using social science, stakeholder analysis, and design principles, the framework provides risk managers a tool to consult stakeholders for pathway information. The result is a list of pathways that can be validated independently.

My research provides biological information of the potentially limiting factors of the spread and establishment of *P. juglandis*. Although the impacts of thousand cankers disease appear variable, the probability of exposure of walnut to *P. juglandis* appears to be limited by cold temperatures and host species. The overall risk of *P. juglandis* to the eastern United States is not determined by this body of work. The national perception of risk or concern over *P. juglandis* to walnut has decreased since I began this dissertation. Despite the shift in national perception, however, the completion of this work provides state and federal regulators information for improved decision-making regarding trapping and monitoring, quarantines, and how to research unspecified pathways of movement.

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Introduction

Pest risk analysis is a systematic way to gather and analyze information, measure and communicate uncertainty, and make recommendations to respond (i.e., accept, reduce, avoid, or eliminate) to the introduction or establishment of an invasive plant pest (Devorshak 2012). Due to the inherent uncertainty of ecological, economic, or social harm that could occur from the invasion of a new organism to a naïve ecosystem, risk analysis provides a way to systematically determine the probability of an invasion (arrival, establishment, integration, and spread potential) and the consequences of invasion (economic, environmental, social, and political impact potential). If the invasion probability or consequences are zero, there would be no risk due to a multiplicative relationship (Devorshak 2012). Tools such as pest risk mapping and pest risk analysis allow management agencies to make decisions when, 1) a new invasive organism is detected and, 2) there is incomplete or conflicting information about the organism (Venette et al. 2010).

The process of risk analysis is iterative and is intended to describe the risks (i.e., risk assessment), manage the risks that are unacceptable (i.e., risk management), and communicate them (i.e., risk communication) (Yoe 2012). Within risk assessment scientific information is necessary to understand the factors that govern the probability of invasion. For example, if arrival is probable, what is the potential that the organism will establish and spread? This question requires careful examination of an invading species' climatic tolerances and host range (see Liebhold and Tobin 2008 for overview of insect invasion biology and management). Global movement of forest pests via known and unspecified pathways is of high concern given the landscape-scale mortality of trees that can result from establishment on naïve plants (Anulewicz et al. 2008; Økland et al. 2011; Flø et al. 2014; Umeda et al. 2016).

One emerging threat is thousand cankers disease on the Juglandaceae (Kolařík et al. 2011; Tisserat et al. 2011; Seybold et al. 2013b). The walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Scolytidae, *sensu* Bright 2014), is a domestic alien invasive bark beetle within the United States of America and vectors a phytopathogenic fungus,

Geosmithia morbida Kolařík et al. (Ascomycota, Hypocreales) in *Juglans* spp. and *Pterocarya* spp. (Kolařík et al. 2011, Hishinuma et al. 2016). The walnut twig beetle and *G. morbida* are native to the southwestern United States of America (USA) and appear to have coevolved in a native host, Arizona walnut, *Juglans major* (Torrey) Heller (Zerillo et al. 2014, Rugman-Jones et al. 2015). The disease is named for the large number of small cankers that develop on a tree as multiple walnut twig beetles penetrate the outer bark. The abundance of the cankers is a consequence of the aggregation behavior of the beetles on host trees. Coalescence of these cankers on the main stem and intensive feeding by walnut twig beetle adults and larvae can cause crown dieback and tree death (Seybold et al. 2013b).

Thousand cankers disease threatens native *Juglans* species across the USA in forests (Graves et al. 2011, Wiggins et al. 2014), urban landscapes (Tisserat et al. 2011), and orchards (Flint et al. 2010, Yaghmour et al. 2014). Widespread tree mortality due to thousand cankers disease would lead to significant economic losses in the timber and walnut industries (Newton and Fowler 2009) and threaten genetic resources of *Juglans* spp. in the USA (Leslie et al. 2010). The current distribution of *P. juglandis* in the USA includes over 120 counties, with individual collection sites located primarily in urban or peri-urban areas (Seybold et al. 2016). *Pityophthorus juglandis* has also been collected from forested areas in Arizona, California, New Mexico, and North Carolina (Flint et al. 2010, Graves et al. 2011, Hadziabdic et al. 2014, Rugman-Jones et al. 2015). The current north-south range of this insect in the USA extends from 47°43’ N (Kootenai Co., Idaho) to 31°24’ N (Cochise Co., Arizona) (Seybold et al. 2012a). The beetle occurs in Mexico (Bright 1981), but has not been detected in Canada to date (Seybold et al. 2016).

Mitochondrial and ribosomal DNA analyses of *P. juglandis* populations across the USA revealed two genetic lineages (L1 and L2) (Rugman-Jones et al. 2015). L1 is found primarily west of the Rocky Mountains and in all populations east of the Mississippi River, whereas L2 is limited to New Mexico, Colorado, Utah, and southern Idaho. Both lineages co-occur in populations from Sierra County, New Mexico and the Wasatch and Front Ranges of the Rocky

Mountains (Rugman-Jones et al. 2015). In addition, walnut twig beetle (L1) was found in northeastern Italy in *J. nigra* and *J. regia* timber plantations and residential gardens, respectively (Montecchio and Faccoli 2014, Montecchio et al. 2014, Faccoli et al. 2016).

In contrast to other anthropogenic pathways, such as shipments of firewood (Jacobi et al. 2012), untreated walnut wood can also move *via* woodworkers and hobbyists on unspecified pathways over long distances. Hobbyist trade has likely contributed to thousand cankers disease spread throughout the USA and to Europe (Campbell and Schlarbaum 2014). In 2009, the United States Department of Agriculture-Animal and Plant Health Inspection Service identified potential pathways that could move thousand cankers disease from western to eastern states (Newton and Fowler 2009). The authors concluded that raw wood (i.e., firewood, logs, burls) is the most likely and most critical pathway of movement. Concerned stakeholder groups, such as The Walnut Council, joined state and federal forest managers to identify and prioritize key biological questions in April 2012 at the Thousand Cankers Disease: Methods and Research Development Needs Assessment (West Lafayette, Indiana). Key research topics for the insect identified by participants included:

1. Number of generations per year (voltinism)
2. Host colonization behavior
3. Origins and association with fungus
4. Host suitability/resistance
5. Cold tolerance
6. Treatment of infested wood and biological control

Of the six identified highest research priorities, this dissertation addresses two: cold tolerance and host suitability. This information will be used in a pest risk assessment to determine the risk of *P. juglandis* to the eastern United States.

In Chapter one, I characterized the effects of low temperatures on larvae and adults of *P. juglandis* to determine if and where cold temperatures might limit spread. Branches from mature trees of a black walnut hybrid, *Juglans hindsii* X (*J. nigra* X *J. hindsii/J. californica*), were

collected monthly (June 2012 to May 2014) in a commercial seed orchard located 5 km south of Yuba City, CA and sent to a Biosafety Level-2 facility in St. Paul, MN. Life stages were peeled from branches and allowed to starve for 48 hours to allow the gut to clear of ice nucleators. Contact thermocouple thermometry was used to measure the supercooling points of adults and larvae, and lower lethal temperatures of adults to assess seasonal trends in cold tolerance. Cold tolerance results inform risk assessment by predicting the extent of insect cold mortality across a geographic range. Areas with high predicted cold mortality (75-90%) are considered less at risk than areas with low predicted cold mortality (<50%).

In Chapter two, I performed field and laboratory trials to determine if reproduction by *P. juglandis* varied between two black walnut (*Juglans nigra* L.) parent trees of a full-sib mapping population of 323 offspring, and between black walnut and butternut (*Juglans cinerea* L.). Host effects on *P. juglandis* colonization and reproduction were tested in the field using pheromone-baited cut branches and beetle populations near Knoxville, TN. I developed a laboratory assay based on previous methods used for other scolytids to measure *P. juglandis* reproduction by artificially infesting cut branch sections with one male and one female parent. Reproduction was measured as the number of brood individuals (adult or immature) per female. I examined differences in *P. juglandis* reproduction between black walnut parent trees or butternut cultivars resulting from field colonization densities or controlled colonization densities in the laboratory. I found that there were intraspecific differences in *P. juglandis* reproduction between black walnut cultivars.

In Chapter three, I characterized potential variation in host suitability and host range among known and suspected hosts of *P. juglandis*. Using artificial infesting methods developed in Chapter two, I tested male-colonization and brood production in no-choice laboratory experiments across 11 species of *Juglans*, one *Pterocarya* sp., and two *Carya* spp. These species were collected from multiple locations across the USA. I repeated the laboratory assays in 2014 and 2015. I measured likelihood of male establishment after one introduction and differences in reproduction per female between hosts. I also measured the cold tolerance

of adult brood to determine the potential spread of *P. juglandis* within host ranges. For risk assessment, the effect of host on the insect's ability to establish and spread can be combined with cold mortality to predict areas of high risk (i.e., low cold mortality, high reproduction in host X) or low risk (high cold mortality, low reproduction in host Y).

In Chapter four, I developed a framework that relies on stakeholder knowledge to identify pathways through which forest pests may be moved. Methods to rank pathways and test their validity are outside of the scope of this chapter; therefore, only the stakeholder participation process is discussed in detail. The framework is intended to reveal specific pathways that may not be evident from a national or regional perspective. Within pest risk assessment, this framework may be utilized if human-mediated spread is determined by risk managers to be a likely pathway of a destructive organism.

Chapters one through three of this dissertation were prepared for publication in peer-reviewed journals. Although I am the lead author, the work presented is the effort of a number of authors. Hence I present these sections in the plural voice. Chapter one is being prepared for submission to *Environmental Entomology*. Chapter two was accepted for publication in *HortTechnology*. Chapter three is being prepared for submission to the *Oecologia*. Chapter four is an example framework developed for identifying unspecified pathways of non-native forest pests via stakeholder knowledge and is designed for inclusion in a pest risk management text and for use by forest managers. Appendix 1 is being prepared for submission to *Environmental Entomology* by the first author, Aubree Wilke. To maintain the integrity of each chapter as a stand-alone unit, there may be a small degree of redundancy between chapters.

1 Chapter 1 - Cold tolerance of *Pityophthorus juglandis* (Coleoptera: Scolytidae) from northern California

1.1 Summary

Winter survivorship of insects is determined by a combination of physiological, behavioral, and micro-habitat characteristics. We characterized the cold tolerance of the walnut twig beetle, *Pityophthorus juglandis* Blackman, a domestic alien invasive bark beetle that vectors a phytopathogenic fungus. Together, the beetle and fungus cause thousand cankers disease in species of *Juglans* and *Pterocarya*. The disease is now present in more than 120 counties in the eastern and western United States of America (USA) and in northern Italy. Contact thermocouple thermometry was used to measure the supercooling points of adults and larvae, and lower lethal temperatures of adults from a population from northern California. We found significant seasonal trends in adult supercooling points in fall, winter, and spring. The supercooling point for males was 0.5°C colder than for females over all months and 1°C colder in the winter than in other seasons. We observed a shift in cold-tolerance strategy in *P. juglandis* adults from freeze-intolerance (December 2013 and January 2014) to partial freeze-tolerance (February 2014). An intermediate level of cold-tolerance in combination with a plastic response to cold partially explains survival of *P. juglandis* outside of its native range in the southwestern USA. In addition, we characterize the relationship between minimum-phloem and minimum-air temperatures in two *Juglans* spp in northern California and Colorado and characterize portions of the native geographic range of eastern black walnut, *J. nigra*, that may be too cold currently for this insect to persist.

1.2 Introduction

The walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Scolytidae, *sensu* Bright 2014), is a domestic alien invasive bark beetle that vectors a phytopathogenic fungus, *Geosmithia morbida* Kolařík et al. (Ascomycota, Hypocreales) in *Juglans* spp. and *Pterocarya* spp. (Kolařík et al. 2011, Hishinuma et al. 2016). *Pityophthorus juglandis* and *G. morbida* are native to the southwestern United States of America (USA) and appear to have coevolved in a

native host, Arizona walnut, *Juglans major* (Torrey) Heller (Zerillo et al. 2014, Rugman-Jones et al. 2015). This insect-pathogen complex is the primary cause of thousand cankers disease, first described in Colorado (Tisserat et al. 2009). The disease is named for the large number of small cankers that develop at or near the point where beetles penetrate the outer bark. The abundance of the cankers is a consequence of the aggregation behavior of the beetles on host trees. Coalescence of these cankers on the main stem and intensive feeding by *P. juglandis* adults and larvae can cause tree death (Seybold et al. 2013b). The typical decline pattern of an originally healthy tree is a slowly developing die back of the crown. As a result, thousand cankers disease threatens native *Juglans* species across the USA in forests (Graves et al. 2011, Wiggins et al. 2014), urban landscapes (Tisserat et al. 2011), and orchards (Flint et al. 2010, Yaghmour et al. 2014). Widespread tree mortality due to thousand cankers disease would lead to significant economic losses in the timber and nut industries (Newton and Fowler 2009) and threaten genetic resources of *Juglans* spp. in the USA (Leslie et al. 2010).

The current distribution of *P. juglandis* in the USA includes over 120 counties, with individual collection sites located primarily in urban or peri-urban areas (Seybold et al. 2016). *Pityophthorus juglandis* also has been collected from forested areas in Arizona, California, and New Mexico (Flint et al. 2010, Graves et al. 2011, Rugman-Jones et al. 2015). One of the first finds of *P. juglandis* in a forested area in the eastern USA was near a campground in Great Smoky Mountains National Park in North Carolina (Hadziabdic et al. 2014). The current north-south range of *P. juglandis* extends from 47°43' N (Kootenai Co., Idaho) to 31°24' N (Cochise Co., Arizona) (Seybold et al. 2012a). The beetle occurs in Mexico (Bright 1981), but has not been detected in Canada to date (Seybold et al. 2016).

Mitochondrial and ribosomal DNA analyses of *P. juglandis* populations across the USA revealed that *P. juglandis* appears to have two genetic lineages (L1 and L2) (Rugman-Jones et al. 2015). L1 is found primarily west of the Rocky Mountains and in all populations east of the Mississippi River, whereas L2 is limited to New Mexico, Colorado, Utah, and southern Idaho. Both lineages co-occur in populations from Sierra County, New Mexico and the Wasatch and

Front Ranges of the Rocky Mountains (Rugman-Jones et al. 2015). In addition, *P. juglandis* (L1) was found in northeastern Italy in *J. nigra* and *J. regia* timber plantations and residential gardens, respectively (Montecchio and Faccoli 2014, Montecchio et al. 2014, Faccoli et al. 2016).

Laboratory cold-tolerance measurements for insects include supercooling point (i.e., the temperature at which insect body fluids begin to freeze; Lee 2010), lower lethal temperature (i.e., changes in mortality after instantaneous exposure to sub-zero temperatures), and lower lethal time (i.e., changes in mortality over time during exposure to constant, low temperatures; Salt 1950). Functions that characterize the probability of freezing and the probability of dying at a particular temperature can be compared in the laboratory to determine cold-tolerance strategy (Slabber et al. 2007, Hanson et al. 2013). If mortality occurs before individuals begin to freeze, the population would be chill intolerant, though technically this is not a cold-tolerance strategy. If mortality coincides with the onset of freezing, the population would be chill tolerant/freeze intolerant. If mortality occurs after equilibrium ice formation, the population would be freeze tolerant (Lee 2010). The extent of cold-induced mortality in the field can also be affected by an insect's behavior and/or its micro-habitat.

The effect of cold on bark and ambrosia beetles is important for modeling potential spread (e.g., Hansen and Sømme 1994, Koch and Smith 2008) and/or population dynamics (e.g., Trân et al. 2007). Plasticity in cold tolerance (i.e., seasonal changes in supercooling point or lower lethal temperature resulting in changes in cold-tolerance strategy) of invasive bark and ambrosia beetles can explain how they establish or spread successfully in the introduced range (Migeon et al. 2015) (or, conversely, be limited by a lack of plasticity, c.f., Sobek-Swant et al. 2012). Historically, cold temperature mortality has been observed in many species of bark beetles when air temperatures reach extreme lows (Chansler 1966, Berryman 1970, Frye et al. 1974, Ragenovich 1980). As climate change continues to shift winter minimum temperatures, beetle outbreaks at higher elevations and latitudes that will negatively impact forests in North America become more likely (Venette et al. 2009, Bentz et al. 2010, Sambaraju et al. 2012).

Relatively little is known about the overwintering ecology of *P. juglandis*. "Winter" for these insects likely begins when temperatures remain below the flight threshold (17 – 18°C; Seybold et al. 2012b, Chen and Seybold 2014) or the developmental threshold [an as-yet unidentified temperature between 0 – 10°C; see Seybold and Downing 2009 for a discussion of this concept with the Mediterranean pine engraver, *Orthotomicus erosus* (Wollaston)]. Live *P. juglandis* larvae, pupae, and adults have been observed under the bark of northern California black walnut, *Juglans hindsii* (Jeps.) Jeps. ex R.E. Sm., during the winter in Alameda County, CA. (P. L. Dallara, personal communication). Both larvae and adults have been found during winter months in Colorado (Luna et al. 2013) and Tennessee (Nix 2013). No evidence suggests that *P. juglandis* overwinters at the base of the tree, in aggregation, or in the litter layer of the forest or orchard floor as other bark and ambrosia beetles do (Kinghorn and Chapman 1959, Annila 1969, Weber and McPherson 1983, Lombardero et al. 2000). Luna et al. (2013) described the cold tolerance of *P. juglandis* from the Front Range in Colorado, and reported that -23°C is lethal to approximately 99% of *P. juglandis* adults. They also suggested that larvae and adults are freeze intolerant. This population was likely composed of the L2 lineage or a hybrid of L1 and L2 individuals (Rugman-Jones et al. 2015). Little is known about the cold tolerance of the L1 lineage.

We are interested in the effects of low temperatures on larvae and adults of northern Californian *P. juglandis*, representative of the L1 lineage that has invaded the eastern USA and Italy, to determine if and where cold temperatures might limit spread. The objective of our study is to measure potential seasonal changes in the lower lethal temperature of adults and in the supercooling points of larvae and adults. We test three hypotheses. For both life stages, we hypothesize that supercooling points change seasonally, and for adults supercooling points may differ by sex. We predict that larval and adult supercooling points decrease in the fall, remain low in the winter, and increase in the spring. Second, we hypothesize that L1 adults are chill tolerant/freeze intolerant. Finally, we characterize the relationship between ambient air temperature and under-bark temperatures for *Juglans* spp. trees in California and Colorado

through the winter. We conjecture that temperatures under the bark of walnut tree stems and branches buffer against minimum air temperatures.

1.3 Materials and Methods

1.3.1 Insects

Branches from mature trees of a black walnut hybrid, *Juglans hindsii* X (*J. nigra* X *J. hindsii/J. californica*), were collected monthly (June 2012 to May 2014) in a commercial seed orchard located 5 km south of Yuba City, CA, USA (Sutter Co., 39°03.681'N, 121°36.818'W, 19.2 m elevation). *Pityophthorus juglandis* was generally in flight at this site during all months of the year except December and January when it flew sporadically when air temperatures warmed (S.J.S., unpublished data). Branches with entrance/emergence holes and/or sap staining on the bark surface were cut from trees and shipped overnight to a biosafety level-2 facility in St. Paul, MN, USA under the terms and conditions specified in Permits P526P-12-01650 and P526P-12-02498 from the U.S. Department of Agriculture, Animal and Plant Health Inspection Service. Adults and larvae were excised from branches on the day of arrival by using a #22 blade X-ACTO knife (Elmer's Products, Inc., Westerville, OH) and fine paintbrush. Insects were held in sealed Petri dishes on moist Kimwipes (Kimberly Clark, Roswell, GA) at room temperature (≈22°C) for 48 h to allow for gut evacuation. Preliminary starvation assays indicated that gut contents could elevate supercooling points (Supp. 1.10.1).

1.3.2 Cold tolerance assays

To ensure that adult and larval *P. juglandis* were undamaged from the removal process and starvation period, we measured the cold-tolerance of individuals that could walk normally when placed on a Kimwipe or, if in the larval stage, were white and moved when probed. For both life stages, we removed external frass or phoretic mites with a fine paint brush under a dissecting microscope (30-60X) to avoid external ice-nucleation. The sex of each adult was determined based on morphological features described in Bright (1981) and Seybold et al. (2013a). All cold-tolerance trials were completed within 7 d after infested branches arrived in

St. Paul. A separate analysis showed that supercooling points did not change significantly after a week at room temperature (i.e., there was no evidence of de-acclimation) (Supp. Figure 1.7).

1.3.2.1 Supercooling point determination

The supercooling points of adult *P. juglandis* were measured once per month from December 2012-March 2013, and May 2013-May 2014. Larval supercooling points were measured November 2012-February 2013, June 2013, December 2013, and February-May 2014. For both life stages, between 4 to 43 individuals were tested per month. Females and males were sampled arbitrarily from cut branches. After starvation, one active insect was placed in a 5 x 7 mm gelatin capsule (Capsuline, Pompano Beach, FL) and secured to a copper-constantan cradle thermocouple with a small amount of high vacuum grease (Dow Corning, Auburn, MI) per Hanson and Venette (2013). Females and males were placed on separate thermocouples in random order. Each thermocouple was placed in a calibrated polystyrene cube and cooled at ~1°C min^{-1} when placed in a -80°C freezer (Carrillo et al. 2004). Eight thermocouples were attached to each of two multi-channel data loggers (USB-TC, Measurement Computing, Norton, MA) for a total of 16 thermocouples. Temperatures were recorded once per second. The supercooling point was recorded as the lowest temperature before a spontaneous release of heat (i.e., an exotherm) due to the formation of ice within the body.

1.3.2.2 Supercooling point analyses

All analyses were done in R, v2.15.1 (R Core Team 2013). Analytical assumptions of homoscedasticity and normality of errors were assessed by graphical inspection of residual plots. We tested the effects of sex, sampling period (i.e., month and year), and the interaction between sex and sampling period on adult supercooling points with ANOVA. We also used ANOVA to examine the effect of sampling period on larval supercooling points. Differences among the mean supercooling points of adults and larvae on the same collection date were analyzed by using a two-sample independent *t*-test with a Bonferonni correction for multiple mean comparisons (critical $\alpha = 0.006$ to maintain an overall $\alpha = 0.05$).

To examine seasonal changes in larval and adult supercooling points, each climatological season was analyzed separately. We used ANCOVA to examine the effect of month, included as a continuous variable, on supercooling point. A term for sex (adult data) was included as a categorical variable in each model. If sex was not significant ($P \geq 0.05$), we removed it, and refit the model as a linear regression. Simple linear regression or mixed-effects models were used to evaluate changes in supercooling point over time within each season, and in each case, month was treated as a fixed effect and year (when applicable) was treated as a random effect. Supercooling points of adults in summer and fall and larvae in spring were analyzed by using simple linear regression models. A square root transformation was applied before analysis of supercooling points for adults in spring to satisfy assumptions of homoscedasticity and normality of errors; these data were analyzed with a linear mixed effects model. Supercooling points for adults in winter were analyzed with a polynomial mixed effects model because exploratory data analysis indicated a curvilinear trend in supercooling points through time that could be described by a quadratic function. Larval supercooling points in winter were analyzed with a linear mixed effects model. R packages lme4 and lmerTest were used to fit mixed-effects models (Bates et al. 2015, Kuznetsova et al. 2013).

1.3.2.3 Lower lethal temperature (LLT) determination

The lower lethal temperature of adults was measured once per month from June-December 2013 and January-May 2014. The experiment followed a randomized complete block design. One adult was placed in a gel capsule, onto a thermocouple, and into a polystyrene cube as described for supercooling point determinations. Each insect was assigned randomly to one of five treatment temperatures: 21°C, -5°C, -15°C, -20°C, and -25°C. This method allowed up to four insects to be exposed to each treatment temperature per block. An insect was removed from the freezer after a brief (<10 sec) exposure to the treatment temperature as per Bale et al. (1988), Pullin and Bale (1989), and Lencioni et al. (2015), regardless of whether an exotherm was detected. Individuals were allowed to warm to room temperature while in the gel capsule. Survival was determined 1 h after cold exposure. An

individual was considered alive if it moved when probed with a fine paint brush. Depending on how many insects were available in a month, two to six blocks were performed.

1.3.2.4 Lower lethal temperature analysis

We examined the relationship between brief exposures to cold and mortality using logistic regression as per Hanson et al. (2013) and Stephens et al. (2015). Modifications to glm.control in R, such as increasing the number of iterations to 50 in the parameter estimation routine, were made when fitted probabilities did not initially converge. From these functions, a lower lethal temperature for 50% of individuals (LLT_{50}) or 90% of individuals (LLT_{90}) and associated standard errors of the mean (SEMs) were estimated each month using the command dose.p() in the MASS package (Venables and Ripley 2002).

1.3.2.5 Cold-tolerance strategy analysis

We compared the proportion of individuals that froze and the proportion that died as a function of exposure temperature in each month (June 2013-May 2014) by using analysis of covariance (ANCOVA) logistic regression as per Slabber et al. (2007) and Stephens et al. (2015). We incorporated terms for treatment (categorical variable for supercooling point or lower lethal temperature), temperature as a continuous variable, and an interaction between temperature and treatment. If model terms were not significant ($P \geq 0.05$), they were removed, and the model was refit.

Using ANCOVA logistic regression, we are testing for initial differences between the estimated proportion of individuals that froze or died at 0°C (i.e., a test of different intercepts) and differences between the rates of change in the proportion of individuals that froze or died with each degree change in temperature (i.e., a test of different slopes). The cold-tolerance strategy can be interpreted from significant differences found between the proportions of mortality and freezing (Slabber et al. 2007). At cold temperatures (below 0°C), if the proportion of individuals that die is significantly greater than those that froze, than this pattern suggests that the population is chill-intolerant. If the proportion of individuals that froze is not statistically different than the proportion that die, than this pattern suggests a freeze-intolerant population. If the rate of freezing varies significantly from the rate of dying, above or below a cold

temperature, than this pattern suggests a population with a mixed-strategy (i.e., freeze-intolerance and partial freeze-tolerance). If the proportion of individuals that die, at low temperatures, is significantly less than those that froze, than this suggests a partial freeze tolerant or freeze tolerant population.

1.3.3 Measurement of phloem and air temperatures

1.3.3.1 California

In winter of 2013-2014, we monitored temperatures beneath the bark of four hybrid black walnut trees at the same location where beetles had been collected for our laboratory studies. The four trees were selected because of their proximity to other trees that had served as the sources for *P. juglandis*. A 0.3 cm diameter hole was drilled at an oblique angle and into the phloem on the north and south side of each tree. Thermistors attached to temperature recorders (HOBO U23 Pro v2 2x external temperature data logger-U23-003, Onset Computer Corp., Bourne, MA) were placed into the holes to record temperature near the phloem every 15 min at 1.8 m above the orchard floor. Recorders were placed in a weather proof box with an additional temperature probe that logged air temperatures every 15 min (TidbiT v2 Water Temperature Data Logger - UTBI-001, Onset Computer Corp.); the weatherproof box was secured to the tree trunk. Installation was completed on 24 November 2013 and probes were removed on 18 March 2014.

1.3.3.2 Colorado

In the same winter, we collected phloem temperatures from three *J. nigra* trees at three separate sites in Fort Collins, Colorado (N 39.71970° W 105.11380°, N 40.57515° W 105.12852°, and N 40.57530° W 105.12826°, 1519-1565 m elevation). These trees were selected because they were positive for thousand cankers disease and located in low-traffic sites to avoid vandalism. Phloem and air probes (iButton Thermochron, Maxim Integrated, San Jose, CA) logged temperatures every 15 min and were installed on trees as in California. Monitoring began 18 December 2013 and ended 4 March 2014.

California and Colorado phloem temperature analysis. We used a linear mixed effects model to examine the effect of daily minimum air temperature (°C) on daily minimum mean

phloem temperature (°C) at each location. The phloem temperature was based on the mean temperature beneath the north and south sides of the tree. Minimum daily air temperature was modeled as a fixed effect, and tree was modeled as a random effect. Separate analyses were conducted for California and Colorado.

1.3.4 Estimated cold mortality of *Pityophthorus juglandis* in the USA

To characterize spatial variation in the potential extent of *P. juglandis* mortality due to cold in the conterminous USA, we first identified the month (February 2014) in which *P. juglandis* adults were most cold tolerant (i.e., had greatest survival during lower-lethal temperature trials; Fig. 1.3). We relied on USDA Plant Hardiness Zones (1976-2005) (USDA 2012) to provide estimates of the average extreme low air temperature across the conterminous USA. We estimated the range of mortality in each hardiness zone by substituting the upper and lower temperature limit for each zone into the logistic regression model for lower lethal temperature in February 2014. We rounded the estimate to the nearest five hundredths, and classified each zone based on whether the minimum overwintering mortality was forecasted to be <50%, 50-75%, 75-90%, or >90%. We used ArcMap 10.1 (ESRI, Redlands, CA) to map areas of predicted beetle mortality and U.S. counties with *P. juglandis* (Seybold et al. 2016). The forecasts assume that *P. juglandis* have achieved their most cold tolerant condition before the winter extreme low actually occurred and that adults were exposed to those temperatures. The model does not account for other sources of winter mortality such as starvation or desiccation nor does it account for the effects of prolonged or repeated exposures to low temperatures. We further assume that all insects have voided their guts of ice nucleating agents. All of these assumptions are intended to provide a risk averse model (i.e., realized mortality should be greater than or equal to our forecasted mortality).

1.4 Results

1.4.1 Cold tolerance

1.4.1.1 Adult supercooling point.

On average over the entire study period, male *P. juglandis* had a mean supercooling point (± SEM) of -17.5 ± 0.16°C, which was half a degree lower than the mean supercooling

point for females ($F_{1, 364}$ = 5.67, P = 0.0178, n = 398). However, the difference in supercooling points between sexes changed depending on month collected ($F_{16, 364}$ = 2.25, P = 0.0040, n = 398). Females, though not significantly so, had a higher mean supercooling point than males in December 2013, January 2014, and March 2014. Males had a higher mean supercooling point than females in March 2013 and February 2014, but the difference was not significant. Mean supercooling points for both sexes were similar in February 2013. Overall, supercooling points for adult *P. juglandis* differed across the 18 months of the study ($F_{16,364}$ = 7.68, P < 0.001, n = 398; Fig. 1.1A).

We found significant within-season changes in adult supercooling points collected in climatological fall, winter, and spring (Fig. 1.1A). In fall, adult supercooling points decreased with month (y = -14.9 – 1.1x, where y is supercooling point and x is month 1-3, where month 1 is September; $F_{1, 77}$ = 16.9, P < 0.001). In winter, on average males had a slightly colder supercooling point (≈1°C) than females. During this period, supercooling points initially decreased then increased (males, $y = 3.7 - 9x + x^2$; females, $y = 4.7 - 9x + x^2$; month 1 in this model is December; for statistical results, see Supp. Table 1.3). In spring, male and female supercooling points decreased with month ($\sqrt{y}$ = -3.3 - 0.11x; month 1 in this model is March; $F_{1, 106}$ = 17.07, P < 0.001). In the summer, we did not find a significant trend in supercooling points for either sex ($F_{1, 70}$ = 2.15, P = 0.15).

1.4.1.2 Larval supercooling point

Larval supercooling points did not differ across eight months ($F_{7, 125}$ = 1.21, P = 0.30, n = 136), and the range of larval supercooling points (-13.6°C to -23.5°C; Fig. 1.1B) was generally within the range of supercooling points that we measured for adults (-12.2°C to -25.0°C; Fig. 1.1A). However, in January 2013, the mean supercooling point for larvae (-16.7 ± 0.41°C) was significantly greater than for adults (-18.7 ± 0.51°C, t = -3.1, d.f. = 37, P = 0.004, critical α = 0.006). Mean supercooling points for adults and larvae were not significantly different in all other months (t < 0.55, d.f. = 11-38, P ≥ 0.05, critical α = 0.006). We found no significant seasonal trends for larvae in winter ($F_{1, 66}$ = 1.75, P = 0.19) or spring ($F_{1, 47}$ = 0.02, P = 0.89).

1.4.1.3 Adult lower lethal temperature and overwintering strategy. We found that the LLT_{50} and LLT_{90} were relatively constant through the year (Fig. 1.2). The likelihood that adult *P. juglandis* would die increased as the temperature declined to -25°C for all insects assayed, regardless of month (Fig. 1.3). From June 2013 to May 2014, all individuals froze between -14°C and -23°C.

In June, August, November, December 2013 and January and April 2014, the cumulative frequency of individuals that froze (i.e., gave an exotherm) and the extent of mortality in *P. juglandis* adults at temperatures <0°C was not different (Table 1.1). This pattern suggests that all individuals survived cooling to near the supercooling point but did not survive freezing, a pattern consistent with freeze-intolerance (Lee 2010).

In July, September, and October 2013 and February, March, and May 2014, freezing was not consistently associated with mortality (Fig. 1.3). In July 2013, as the temperature fell below -5°C, the proportion of individuals that died was significantly and consistently greater than those that froze (Table 1.1). Therefore, in this month, the population was chill-intolerant because mortality occurred before freezing occurred (Lee 2010).

In September and October 2013, at temperatures <0°C, the rate of freezing was significantly different than the rate of mortality (Table 1.1). Above -15°C, a significantly greater proportion of adults had died before freezing. Below -15°C, of the remaining adults, all individuals survived freezing. This result suggests that *P. juglandis* adults in September and October were using a mixed-strategy of freeze-intolerance and partial freeze-tolerance. In this case, we suggest “partial freeze-tolerance” because individuals were removed from treatment temperatures before whole body freezing could have occurred. Our total sample for this period consisted of 28% teneral and 72% fully sclerotized adults.

In February 2014, the proportion of individuals that died was significantly and consistently less than those that froze at low temperatures (Table 1.1). This pattern suggests that the population was the most cold-tolerant and all individuals were able to survive partial freezing.

In March and May 2014, as during fall months in 2013, the rate of freezing was significantly different than the rate of mortality at temperatures <0°C. Above -16°C in March and -17°C in May, a greater proportion of adults died before freezing (Table 1.1). Below -16°C in March and -17°C in May, all remaining adults survived freezing. These results suggest a mixed cold-tolerance strategy, where individuals are chill-intolerant above -16°C in March and -17°C in May and freeze-intolerant below these temperatures, respectively. Our total sample for this period consisted of 4% teneral and 96% fully sclerotized adults.

1.4.2 Micro-habitat

1.4.2.1 California

The lowest minimum daily mean phloem temperature beneath the stem, -2.4°C, during this study was recorded on December 6, 2013. The relationship between phloem temperature and air temperature can be modeled by the equation $y = 3.38 + 0.79x$, where y is the minimum daily mean phloem temperature of a mature tree (1.8 m from the ground) and x is the minimum daily air temperature in degrees Celsius ($F_{1, 458} = 3.5E+3$, $P < 0.001$; Supp. Table 1.4). This relationship holds when daily minimum air temperature is between -7.5°C and 12.8°C. The equation indicates that the buffering effect from the tree (i.e., underbark temperature - air temperature) is at least 3.38°C when air temperatures are ≤0°C and this buffering effect becomes progressively greater as temperatures decrease (Fig. 1.4A).

1.4.2.2 Colorado

The lowest minimum daily mean phloem temperature beneath the stem, -20.6°C, occurred on February 7, 2014. The relationship between phloem temperature and air temperature can be modeled by the equation $y = 2.1 + 0.65x$, where y is the minimum daily mean phloem temperature of a mature tree (1.8 m from the ground) and x is the minimum daily air temperature in degrees Celsius ($F_{1, 194} = 701$, $P < 0.001$; Supp. Table 1.4). This relationship holds for air temperatures between -25.6°C and 4.4°C (Fig. 1.4B).

1.4.3 Estimated cold mortality of *Pityophthorus juglandis* and risk to *Juglans nigra* in the USA

We forecast differing degrees of winter mortality from acute exposure to low temperatures for the L1 lineage of *P. juglandis* across the USA (Fig. 1.5). If the beetle spreads

westward in Pennsylvania and Maryland, the beetle would encounter colder areas where winter mortality could be 50-75%. Approximately half of the current range of *J*. *nigra* has winter temperatures under which *P. juglandis* might survive (i.e., forecasted winter mortality <50%) if it arrived. Substantially fewer infested counties have been reported in the zone with winter temperatures cold enough to cause 50-75% winter mortality than in the <50% winter mortality zone. The majority of those counties occur in Colorado. In more northern latitudes, we forecast that low winter temperatures might cause at least 75-90% mortality annually if *P. juglandis* was introduced to these areas. However, a majority of these areas also appear to be too cold for *J*. *nigra*.

1.5 Discussion

1.5.1 Starvation

Life stages that feed actively are more likely to freeze at warmer temperatures because food in the gut can act as an ice nucleating agent (Salt 1953, Krunic 1971, Salin et al. 2003). For some freeze-intolerant insects, feeding cessation and clearing the gut of food are adapted behaviors to lower the supercooling point in preparation for winter, effectively lowering the risk of freezing (Sømme 1999). The bark beetle *Ips acuminatus* Gyll (Gehrken 1995) utilizes this strategy, for example. Until these behaviors are studied for *P. juglandis*, our data represent conservative estimates of cold temperatures that might cause freezing and mortality of *P. juglandis* in the field (Supp. Figure 1.6).

De-acclimation of overwintering insects occurs at variable rates. De-acclimation can occur in 4 – 7 d for Coleopterans (Fields et al. 1998, Sobek-Swant et al. 2012). Adult *P. juglandis* seem to require >7 d at room temperature (~21°C) for de-acclimation, based on tests of the effects of starvation (Supp. Figure 1.6) and duration at room temperature (Supp. Figure 1.7) on supercooling points. Supercooling points did not begin to increase until insects were held for more than 1 week at room temperature. Further, if de-acclimation to northern California temperatures had occurred during transport or time in the lab during starvation, we would

expect supercooling points across seasons to be relatively constant, but this pattern is not what we observed.

1.5.2 Cold tolerance

1.5.2.1 Adult supercooling point

Generally, the range of adult supercooling points measured in northern California (-12°C to -25°C) fell within the annual range of supercooling points that were reported by Luna et al. (2013) for *P. juglandis* from Colorado (-8.6°C to -23.4°C). An effect of sex on supercooling point was not detected in *P. juglandis* from Colorado (Luna et al. 2013). They also reported slight seasonal differences in adult supercooling points: Supercooling points were lower in the winter than spring or summer, and summer supercooling points were higher than those measured in fall. Supercooling points were studied for one cold season (September 2012 to January 2013), and the results of Rugman-Jones et al. (2015) suggest these individuals were likely from the L2 lineage.

Sexual dimorphism of supercooling ability has not been studied in depth for most insect species. Bark beetles, such as the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, and eastern larch beetle *D. simplex* LeConte, died at a greater rate than females when exposed to cold for prolonged periods (Safranyik 1976, Langor and Raske 1987). Salin et al. (2000) studied the effect of starvation on supercooling points of male and female *Alphitobius diaperinus* Panzer (Coleoptera: Tenebrionidae). Male supercooling points were lower than female supercooling points. This suggests that a variety of conditions specific to male or female beetles (i.e., varying rate of gut evacuation, females retaining more fat due to sexual maturity, females having more ice nucleation sites overall, or sampling more un-fed callow males than females) could effect freezing. For the Mediterranean fruit fly, *Ceratitis capitata* Wiedemann (Diptera: Tephritidae), males tolerated both cold and starvation better than a large portion of females due to efficiently managing body water and metabolism (Carante and Lemaitre, 1990). Any of these mechanisms could contribute to the difference in supercooling points between sexes that we observed for *P. juglandis* and would be appropriate for future investigation.

We expected that supercooling points would respond to seasonal temperatures (e.g., Chansler 1966, Gehrken 1984, Miller and Werner 1987, Koštál et al. 2011, 2014). For northern scolytids, seasonal changes in supercooling point follow a common pattern: supercooling points decrease in the fall, reach a minimum in the winter, and increase in the spring (Gehrken 1984, Miller and Werner 1987, Hansen and Sømme 1994, Koštál et al. 2014). This pattern often reflects the physiological changes that occur during acclimatizion periods (i.e., a reduction in supercooling point in fall or early winter), complete acclimatizion to cold (i.e., maintenance of low supercooling points during winter), and then a deacclimatizion period (i.e., an increase in supercooling point in late winter to spring).

Seasonal trends in supercooling point of adult *P. juglandis* generally followed a northern scolytid pattern, but supercooling points for *P. juglandis* decreased during spring. The lowest supercooling points (approximately -25°C) occurred in April and May 2013. Because we did not evaluate gut filling before trials (Gehrken 1995), these individuals may have been more starved than others when they arrived in St. Paul, resulting in lower supercooling points. We did not observe the same low supercooling points in May 2014 as we did in the previous year. Scolytids that are native to the southern USA, such as the southern pine beetle, *D. frontalis* Zimmermann, appear not to adjust supercooling points to seasonal temperatures (Lombardero et al. 2000). Because *P. juglandis* is not a native northern scolytid, but does respond to seasonal temperatures via changes in supercooling point, adults appear to have an intermediate ability to avoid freezing in cold periods.

1.5.2.2 Larval supercooling point

Supercooling points of *P. juglandis* larvae from northern California typically fell within the annual range of supercooling points (-8.3°C to -22.3°C) reported by Luna et al. (2013). Luna et al. (2013) found a seasonal change in larval supercooling points and a significant difference between adult and larval mean supercooling points in September and October. In northern California, larvae were present in cut branches shipped to St. Paul in all months except January 2014, whereas in Colorado larvae were present in all months tested except April 2012. Other hardwood scolytids in the northern hemisphere overwinter as larvae and have lower

supercooling points than *P. juglandis* (Ring 1977, Barson 1974, Hansen and Sømme 1994). In these species, supercooling ability is correlated with an increased concentration of cryoprotectants (i.e., glycerol) in conjunction with gut evacuation.

1.5.2.3 Adult lower lethal temperature

Increased cold tolerance in winter can also be achieved through decreasing the lower lethal temperature. If the LLT_{50} of the overwintering stage is lower for a population in the winter than summer months than greater survival at cold temperatures is expected. Overwintering adults of the Eurasian spruce engraver, *Pityogenes chalcographus* Linnaeus, dramatically increase survival in January (>75% survival at -15°C for 30 d) when compared to no survival in less than 12 h at the same temperature in August (Koštál et al. 2014). Fourth and fifth instars of the cerambycid, *Monochamus alternatus* Hope, decrease their LLT_{50} from approximately -6°C in the summer to -19°C in the winter (Ma et al. 2006). Soudi and Moharramipour (2011) found that winter LLT_{50}s were lower in winter than in fall or spring in overwintering adults of a chrysomelid defoliator on elm, *Xanthogaleruca luteola* Muller. In our study, whether the relatively low minimum phloem temperatures before February (Fig. 1.2) led to a numerical decrease in lower lethal temperature for *P. juglandis* is unclear.

1.5.2.4 Adult overwintering strategy

During fall 2013 and spring 2014, we found that *P. juglandis* adults used a mixed cold tolerance strategy (Table 1.2). This result is surprising because in a colder than typical winter, beetles and larvae did not experience temperatures near their supercooling points (Fig. 1.1A and 1B), yet most of the population cold-hardened to survive near or below freezing temperatures in both periods. It is possible that the population may have been in transition, where some individuals had acclimated to cold temperatures (partial freeze-tolerance) and others had not. Some of the variation in cold-strategy may have been due to less cold hardy teneral adults. The difference between cold hardiness between teneral and “dark” adults has not been shown to be significant for other bark beetle species (Koštál et al. 2014), although few species have been tested to date.

Plastic responses to cold have been observed in insects, where individuals or populations can shift from one cold-strategy to another depending on environmental cues (e.g., temperature, photoperiod, or moisture; Danks 2005). A cold-strategy shift from freeze-tolerance to freeze-intolerance was found in overwintering larvae of a pyrochroid beetle, *Dendroides canadensis* Latreille, and a cucujid beetle, *Cucujus clavipes* F., in Indiana, USA (Horwarth and Duman 1984, Kukal and Duman 1989). The same shift in cold-strategy was induced at the individual level in one winter *via* a single simulated "freeze-thaw" event in freeze-tolerant larvae of the hoverfly *Syrphus ribesii* L. (Brown et al. 2004). In both cases, they hypothesized that a mixed strategy would lower the risk of death by freezing in variable environments (see Voituron et al. 2002 for discussion of evolution of cold strategies).

Koštál et al. (2007) reported that fluctuating temperature regimes, such as those that might occur under bark (-5°C for 22 h/20°C for 2 h over 11 days), may increase cold tolerance among overwintering insects due to short warm temperature reprieves that facilitate repair of chill injuries (e.g., recovery of ion concentrations in the hemolymph). In northern California, maximum daily mean phloem temperatures in the winter of 2013-2014 did reach 20°C on several days before cold-tolerance increased in February 2014 (Fig. 1.2). These daily maximum phloem temperatures may have provided the population a period of chill-injury recovery.

Luna et al. (2013) suggest that *P. juglandis* adults sampled from central Colorado are freeze-intolerant; however, lower lethal temperature was only measured in April 2011. Nevertheless, this classification agrees with what we found in northern California in April 2013. Freeze-intolerance is common in terrestrial arthropods (Sømme 1982) and found in several scolytids (Barson 1974, Ring 1977, Gehrken 1984, Miller and Werner 1987, Hansen and Sømme 1994, Bentz and Mullins 1999, Lombardero et al. 2000, Koštál et al. 2011, 2014). *Pityophthorus juglandis* in northern Colorado is established and spreading while exposed to the coldest minimum temperatures within its known range in the USA. Although unlikely, if the

population remained freeze-intolerant year-round, at least 50% mortality should be occurring every winter as long as temperatures reach the mean supercooling point.

1.5.2.5 Cold tolerance conclusions

Cold-hardy overwintering stages of insects are characterized by physiological changes that increase the ability to survive low temperatures (Salt 1961). We found that the ability of adults and larvae in northern California to supercool to low temperatures (i.e., lower than temperatures that occur during the winter in that region) was similar. Both larvae and adults of *P. juglandis* appear to be capable of overwintering, as both life stages were detected alive in winter. However, our study suggests adults are the more cold-hardy overwintering stage, and thus are more likely to survive the winter. However, we also collected more measurements on adults.

We found that partial freezing did not cause immediate mortality in all months. For populations that are freeze-intolerant year round, seasonal changes in supercooling point equate to seasonal changes in lower lethal temperature (Bale 1987). *Pityophthorus juglandis* is mainly freeze-intolerant throughout the year, but after a longer exposure to cold, cold-tolerance increased via colder lower lethal temperatures than supercooling points (February 2014). In November, December, and January, when other cold lower lethal temperatures occurred (Fig. 1.2; LT_{50} range = -17.1°C to -18.2°C, LT_{90} = -19.8 to -21.3°C), supercooling points remained fixed. This result suggests that *P. juglandis* has a plastic cold tolerance response and is primarily limited by lower lethal temperature. To further understand the physiological changes that lead to cold-hardening in overwintering *P. juglandis* adults and larvae, fat content (Lombardero et al. 2000), polyols (i.e., glycerol, trehalose), and relative water content/dehydration could be measured over several winters (Sømme 1982). To further study plasticity of *P. juglandis* cold-tolerance, supercooling points and lower lethal temperature should be measured over two or more cold periods in the native range (Arizona, Mexico, and New Mexico) and at the minimum temperature extremes of its current range (e.g., northeastern Colorado, northern Idaho, and Washington) while ensuring that collections are made in locations of the same genetic lineage.

1.5.3 Micro-habitat

We found a significant positive linear relationship between phloem and air temperature in both locations. The linear relationship between phloem and air temperature is different in each location and across a different range of temperatures, where northern Colorado experiences much colder temperatures than northern California. Luna et al. (2013) estimated an LLT_{99} of -23°C for adult *P. juglandis* collected from central Colorado. Because phloem temperatures approached -23°C, in trees of similar size and position, we would expect at least 50% field mortality in Fort Collins from acute cold exposure in the winter of 2013-2014. In northern California, phloem temperatures did not reach lower lethal temperatures; therefore, we would not expect significant field mortality from acute cold exposure in the orchard in Sutter County.

For other deciduous tree hosts, minimum underbark temperatures have been predicted from minimum air temperatures by using a Newtonian cooling model, challenging other predictive models that use a constant degree difference over time and space (Vermunt et al. 2012). In lodgepole pine, *Pinus contorta* Doug. ex Loud., a positive linear relationship was found between daily minimum south phloem temperature (°C) and daily minimum air temperature (°C); north and south daily minimal phloem temperatures averaged 2.1°C and 1.6°C warmer than the daily minimum air temperature (Bolstad et al. 1997). The dark color of black and hybrid walnut bark might allow for greater absorption of solar radiation during the winter. The degree of host bark furrowing, stem/branch diameter and water status of the trees might cause different rates of heat loss during winter nights. From our evaluation of the literature, these factors have been poorly studied. Variations in host location (Bentz and Mullins 1999) and bark qualities could lead to variable mortality of *P. juglandis* in colder areas of its range.

1.5.4 Estimated cold mortality of *Pityophthorus juglandis* and risk to *Juglans nigra* in the USA

Our map suggests that extreme winter cold is unlikely to have a substantial impact on populations of *P. juglandis* in the beetle's native range (i.e., portions of southern Arizona, New

Mexico, and California). Substantial cold mortality is also not expected in a majority of California and portions of Oregon and Washington. In eastern Colorado, however, where genetic lineages, L1 and L2, co-occur (Rugman-Jones et al. 2015) our map predicts 50-75% mortality. The distribution of L1 and L2 in Colorado is unknown and therefore it is difficult to predict if *P. juglandis* populations are persisting via increased cold-hardiness due to genetic differences. In the eastern USA, current populations in Tennessee, Virginia, Pennsylvania and Maryland typical mortality rates from acute cold exposure appear to be low. Overwintering mortality rates of 50 – 75% mortality may slow population growth rates, but *P. juglandis* is still likely to establish if it encounters suitable hosts in these areas.

Forecasted cold mortality is based on assumptions about the beetle, climate, host, and landscape interactions that occur between them. We used a starvation period before measuring lower lethal temperature to avoid underestimating the geographic risk of *P. juglandis* in the U.S. First, our results show that significant acclimation to cold can occur in a population in one winter; in our map we are assuming that adults achieve their maximum level of cold tolerance as we have measured in the laboratory when extreme low temperatures occur. Second, the map is based on the average extreme minimum temperature from 1976 – 2005 (Daly et al. 2012). Many recent winters have been warmer than this average. Third, a suite of biotic interactions that may affect insect overwintering physiology are not included in our model (e.g., host buffering winter temperatures, host effects on *P. juglandis* overwintering physiology, and interactions between *G. morbida* and *P. juglandis*). Finally, across the range of *P. juglandis*, winter warming in different regions may not have the same effect on cold mortality (Weed et al. 2015).

Federal, state, and local governments in the eastern USA can take advantage of preliminary information about *P. juglandis* cold mortality to understand its risk to *J. nigra* resources. Pest risk maps help managers make decisions under high uncertainty and knowledge gaps about an invasive insect's biology (Venette et al. 2010, Yemshanov et al. 2010). Further studies on field mortality and continued trapping and hand-collecting efforts

across the current range of *P. juglandis* would verify if these limits generally confirm the accuracy of our forecasts. Catches of *P.juglandis* in pheromone baited traps were very low during the 2014 growing season in Butler Co., OH (J. Juzwik, personal communication) after a colder than normal winter. If arrival of *P. juglandis* coincides with winter months, *J. nigra* in the Midwest and Northeast may be less at risk than the southern areas of its range (Fig. 1.4). To further understand the risk of *P. juglandis* to *J. nigra* in the USA, future studies might include host effects on cold-tolerance and reproduction among propagated cultivars.

1.6 Acknowledgments

We thank Cliff Beumel, Director of Sierra Gold Product Development, Yuba City, CA for allowing us to collect infested branches and instrument hybrid black walnut trees for this study; Irene Lona, UC-Davis Department of Entomology and Nematology, for assistance with the collections; the staff of the biosafety facility in St. Paul, MN; and University of Minnesota undergraduate research assistants, J. Pohnan and C. Smith. We also thank Stephanie Sky Stephens (USDA FS, Forest Health Management, Lakewood, CO), Rebecca Powell (USFS) and DeNae Cameron (City of Fort Collins) for installing temperature probes in Fort Collins, CO and Yigen Chen (UC Davis Department of Entomology and Nematology) for assistance with the same in northern California. Funding was provided by the NSF-IGERT Risk Analysis of Introduced Species and Genotypes program at the University of Minnesota (DGE-0653827) and a USDA-Forest Service Special Technology Development Program grant (R2-2012-01) that was administered by Jeffrey Witcosky and S. Stephens).

1.7 Tables

Table 1.1 Coefficient estimates (± SEM) for logistic regression models to estimate the cumulative proportion of adult *Pityophthorus juglandis* that were frozen or dead from -25°C to -5°C.

Period	n	Intercept	Temperature	Treatment	Temperature x treatment
June 2013	45	-20.22 ± 1.33***	-1.21 ± 0.08***	—	—
July 2013	54	-15.35 ± 0.97***	-0.98 ± 0.05***	-1.53 ± 0.6*	—
August 2013	39	-22.38 ± -1.38***	-1.37 ± 0.08***	—	—
September 2013	37	-12.52 ± 2.96***	-0.72 ± 0.17***	-14.27 ± 4.84**	-0.92 ± 0.28**
October 2013	42	-8.2 ± 3.1*	-0.45 ± 0.17*	-12.81 ± 3.35***	-0.87 ± 0.18***
November 2013	42	-13.13 ± 1.3***	-0.72 ± 0.07***	—	—
December 2013	42	-13.48 ± 1.01***	-0.79 ± 0.06***	—	
January 2014	42	-15.91 ± 1.28***	-0.91 ± 0.07***	—	—
February 2014	42	-16.75 ± 1.79***	-0.84 ± 0.08***	1.88 ± 0.65*	—
March 2014	30	-6.08 ± 2.47*	-0.39 ± 0.14*	-15.06 ± 3.22***	-0.94 ± 0.19***
April 2014	45	-22 ±1.79***	-1.36 ± 0.11***	—	—
May 2014	46	-12.59 ± 2.73***	-0.73 ± 0.16***	-8.54 ± 3.42*	-0.51 ± 0.2*

The model followed the general equation P(Insect dies or freezes) = 1/[1 + exp(-1*(intercept + b_1*temperature + b_2*treatment + b_3*temperature x treatment)],where treatment = 1 for frozen individuals (i.e., supercooling assays) and treatment = 0 for dead individuals (i.e., lower lethal temperature assays).

*|z| > 2.46; $P \leq 0.05$

**|z| > 3.21; $P \leq 0.001$

***|z| > 4.61; $P \leq 0.001$

Table 1.2 Summary of cold tolerance strategy of adult *Pityophthorus juglandis* by month from June 2013-May 2014 in northern California (Sutter Co.).

Month	Cold strategy
June 2013	Freeze intolerance
July 2013	Chill intolerance
August 2013	Freeze intolerance
September 2013	Mixed strategy (freeze intolerance/partial freeze tolerance)
October 2013	Mixed strategy (freeze intolerance/partial freeze tolerance)
November 2013	Freeze intolerance
December 2013	Freeze intolerance
January 2014	Freeze intolerance
February 2014	Partial freeze-tolerance
March 2014	Mixed strategy (chill intolerance/freeze-intolerance)
April 2014	Freeze intolerance
May 2014	Mixed strategy (chill intolerance/freeze-intolerance)

1.8 Figures

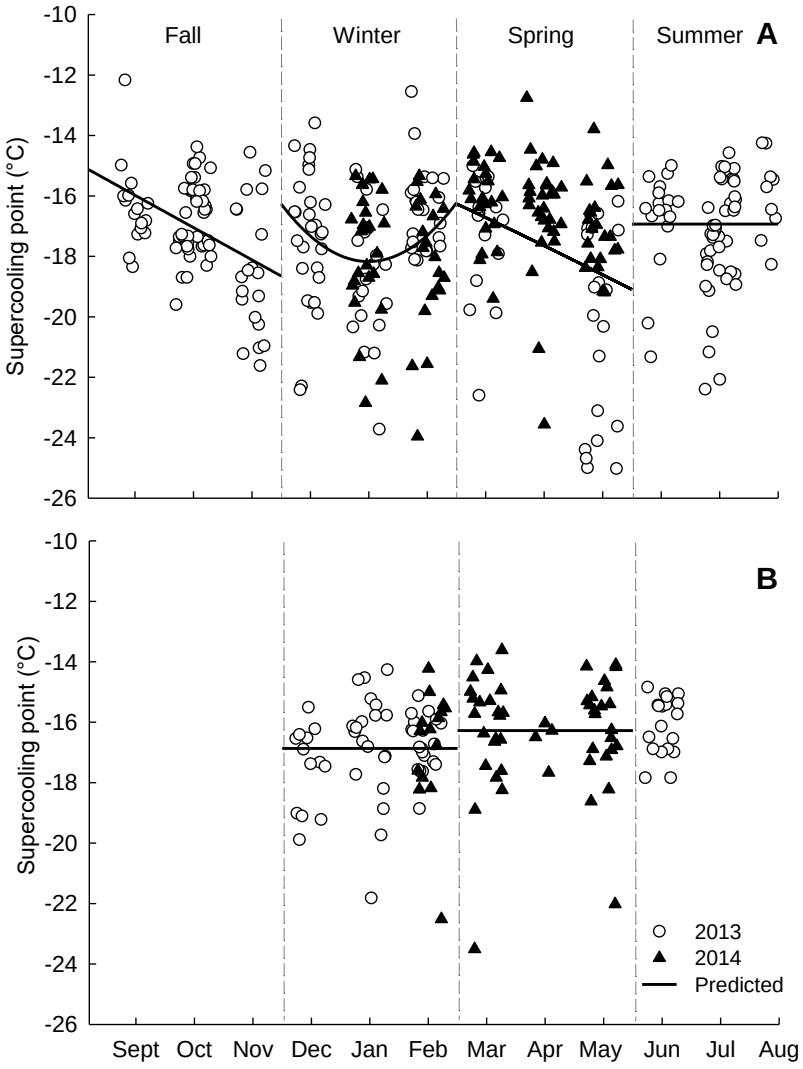

Fig. 1.1 Supercooling point (°C) data of two *Pityophthorus juglandis* life stages (adults and larvae) collected every four weeks from December 2012 to May 2014 (data is jittered on x-axis). (A) Adult supercooling points significantly varied by month (n = 398) and seasonal trends showed a decline in supercooling point from fall to winter (acclimation) but did not show an increase in supercooling points from winter to spring. (B) Larval supercooling points did not differ across months (n = 136). No seasonal trend was found in winter or spring. In January 2013, the larval mean supercooling point was significantly lower than the adult mean supercooling point (P < 0.006).

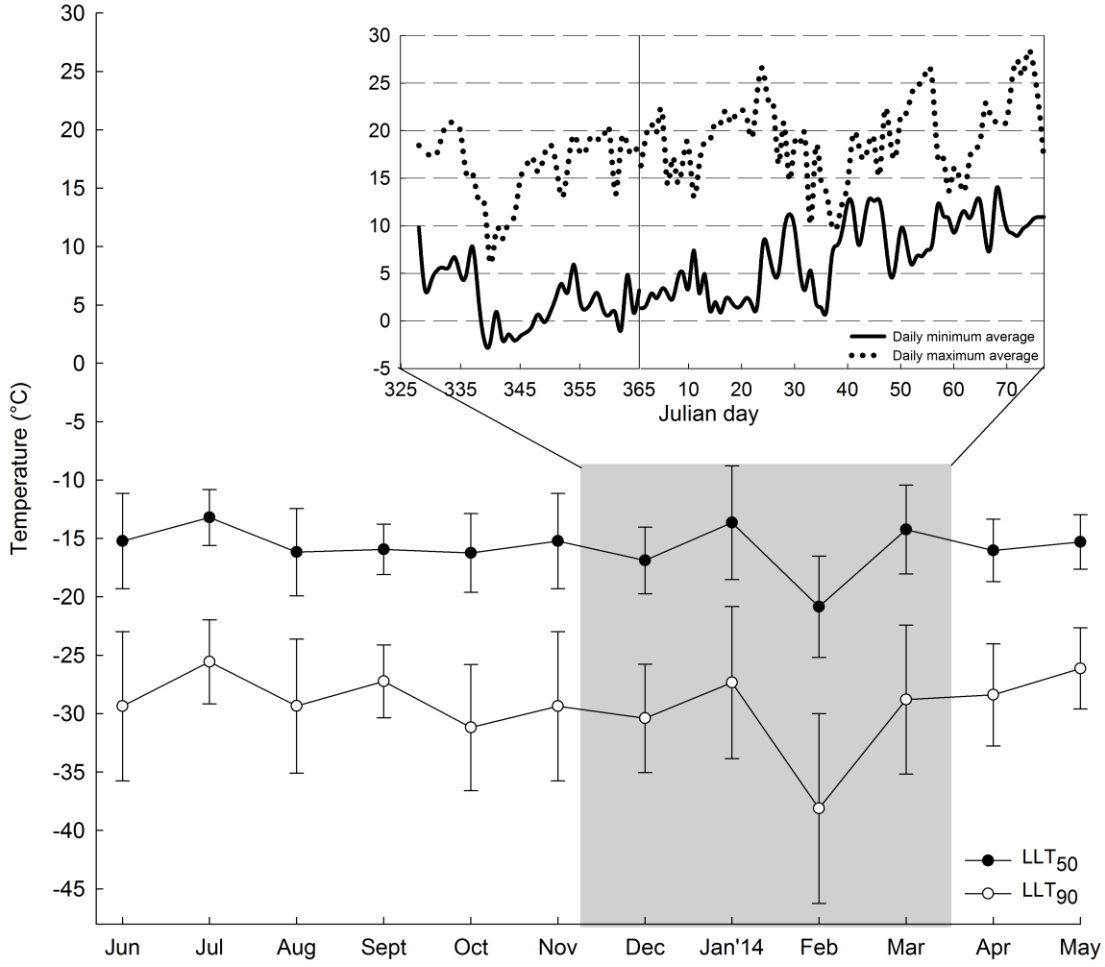

Figure 1.2 Adult *Pityophthorus juglandis* lower lethal temperature (LLT±SEM) measured once per month from June 2013 to May 2014 and minimum and maximum daily mean phloem temperatures (north and south side across four trees) logged from Nov. 24, 2013 to Mar. 18, 2014 in northern California. LLT_{50} is the temperature estimated to cause 50% mortality in a population and LLT_{90} is the temperature estimated to cause 90% mortality.

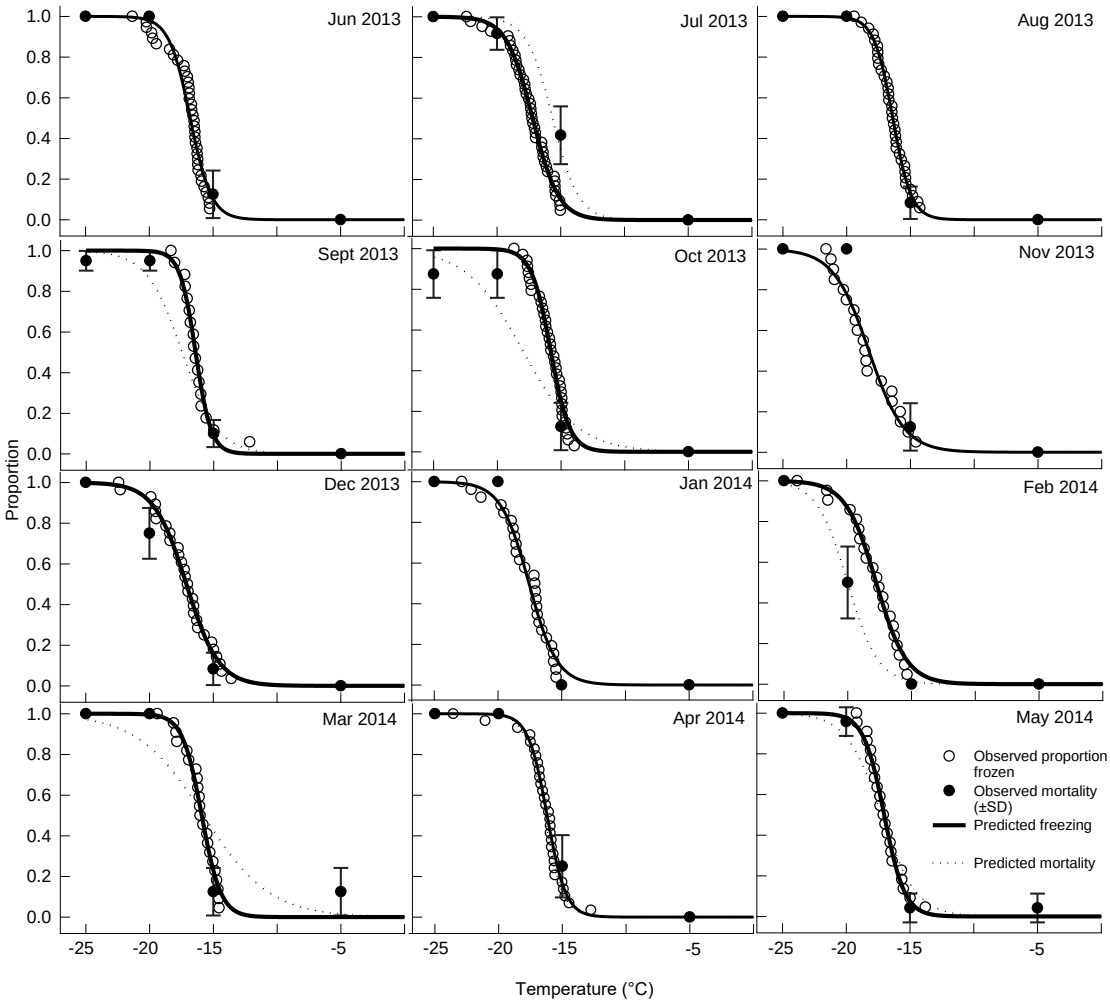

Figure 1.3 Comparison of proportions of frozen (pooled male and female) (○) and lower-lethal temperature mortality (● ± SD) calculated from adult *Pityophthorus juglandis* once per month from June 2013 to May 2014. Adults appear to be mainly freeze-intolerant, but they did show mixed strategies in critical periods (fall and spring) and increased cold-tolerance after a long period of cold (partial freeze tolerance in February).

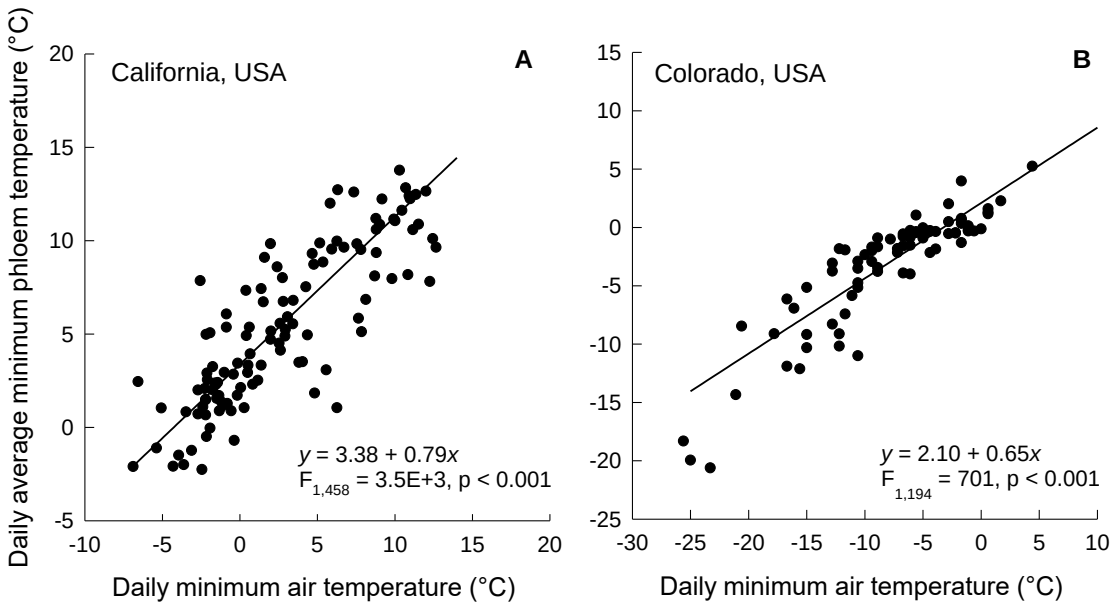

Figure 1.4 Relationship between daily minimum air temperature (°C) and daily average minimum phloem temperature (°C) recorded in (A) four trees in Sutter, Co. CA and (B) three trees in Fort Collins, CO in winter 2013-2014.

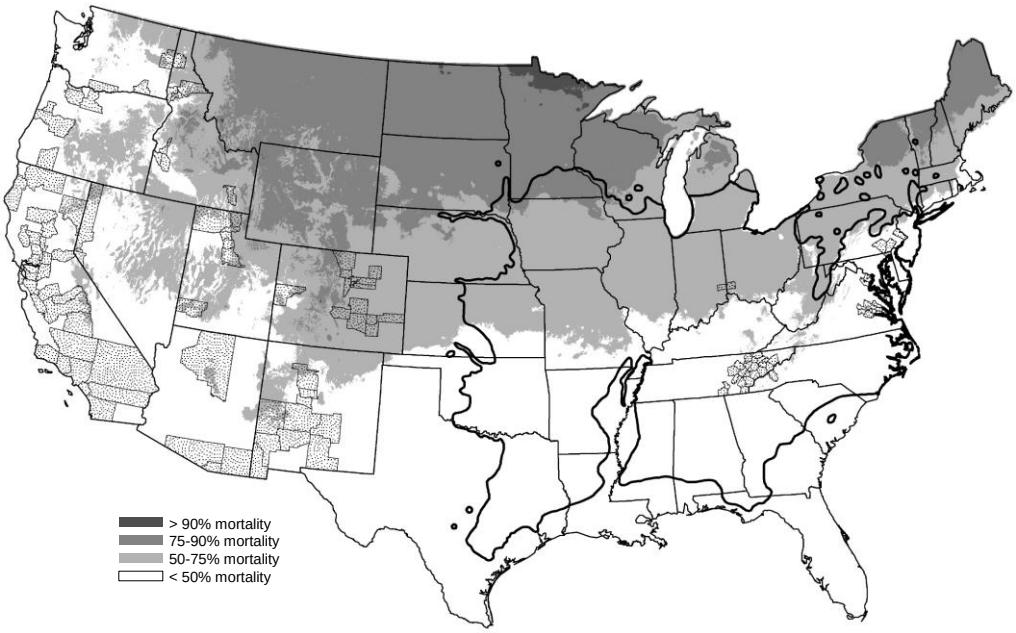

Figure 1.5 Estimated mortality of adult *Pityophthorus juglandis* (L1 lineage) and native host range of *Juglans nigra* (http://esp.cr.usgs.gov/data/little/) in the conterminous United States. Stippled counties have collection records for *P. juglandis* (Seybold et al., 2015). We used February 2014 data to estimate mortality at each zone's upper temperature limit based on average annual extreme minimum temperature (1976-2005).

1.10 Supplemental material -- Methods development

1.10.1 Starvation study

This study was designed to test the effect of starvation on supercooling points of adult *P. juglandis* and to establish a standard starvation period for future studies. In June 2013, eight to ten adults were held for 0, 1, 2, 3, and 4 d at room temperature (~21°C) in sealed Petri dishes on moist Kimwipes. We used ANOVA to examine how the supercooling points changed through time, treating day as a categorical variable, and examining differences among days using a Tukey means comparison procedure. Supercooling points were transformed before statistical analysis by using an inverse transformation to satisfy model assumptions (homoscedasticity and normality of errors). We found a significant reduction in adult supercooling points after 1 d of starvation; however, supercooling points after 2 – 4 d of starvation were not significantly different from the 1 d measurement ($F_{4, 41}$ = 11.5, P < 0.001, n = 46; Figure 1.6). Based on results of this assay, a 48 h starvation protocol was chosen for all cold tolerance trials.

1.10.2 De-acclimation study

In winter 2013 and 2014, it was unclear how quickly adult *P. juglandis* would deacclimate from field-conditions if held in the laboratory at room temperature. This study was designed to test the effect of time in the laboratory (weeks) on supercooling points of adult *P. juglandis*. Bark was peeled to collect adults from cut branches that arrived on November 1, 2013, when beetles had low supercooling points. Individuals were starved for 2 days and then tested as an initial measurement of supercooling point (week 0). Cut branches were held in plastic jars with vented lids at room temperature. Adults were extracted from cut branches and held for 2 days before we measured supercooling points on November 7, 16, 21 and 30, weeks 1 – 4. Using ANOVA, we found no significant difference between mean supercooling points measured in weeks 0 and 1, but supercooling points measured in weeks 2 – 4 were significantly greater than weeks 0 or 1 ($F_{1, 36}$ = 5.48, P = 0.023, n = 38; Figure 1.7). Based on these results, cold tolerance data were only collected within 7 d.

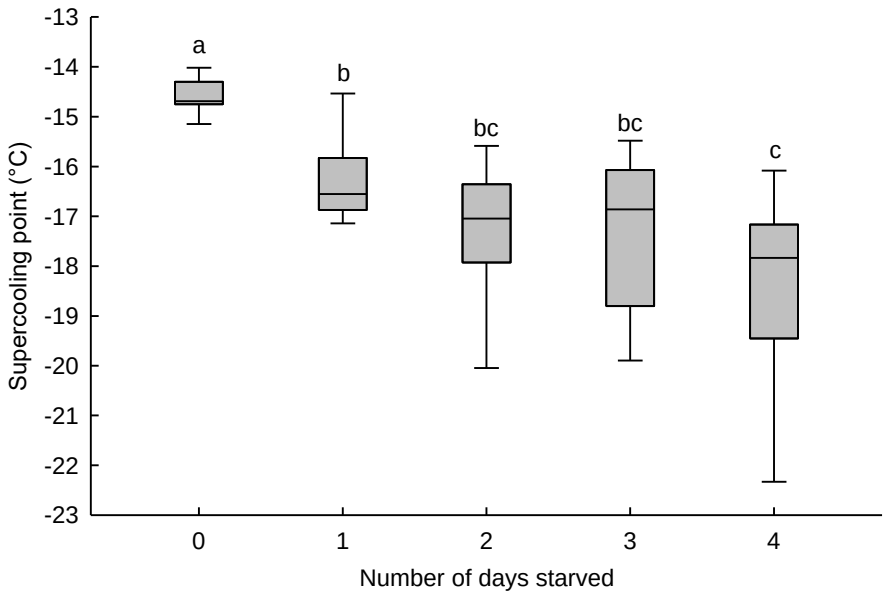

Figure 1.6 Effect of number of days of starvation on supercooling points of adult *Pityophthorus juglandis* in laboratory bioassay, June 2013. Middle bar represents median. The upper and lower portions of the box are 25th and 75th percentiles. Whiskers are 10th and 90th percentiles. Boxplots with the same letters are not significantly different ($P < 0.05$).

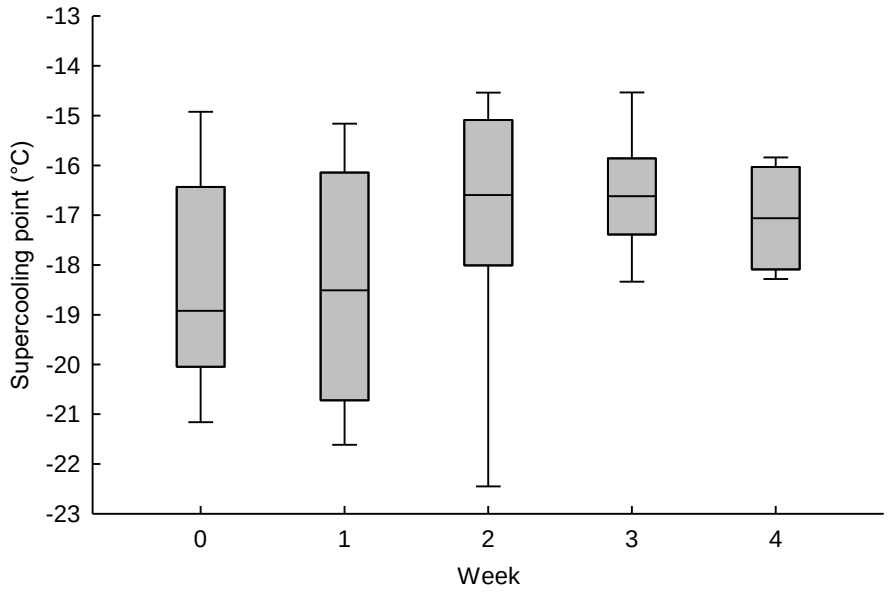

Figure 1.7 Effect of time in the laboratory on supercooling points of adult *Pityophthorus juglandis*, measured in November 2013. Middle bar represents median. The upper and lower portions of the box are 25th and 75th percentiles. Whiskers are 10th and 90th percentiles.

1.11 Supplementary Data

Model results for winter adult supercooling point and California and Colorado phloem versus air temperature data.

Table 1.3 Estimated coefficients for a polynomial mixed effects model to describe monthly changes in supercooling points of adult *Pityophthorus juglandis* in winter.

Predictor	Estimate	SEM	*t* value	d.f.	*P*
intercept	4.72	9.92	0.48	112	0.635
month	-9.08	4.00	-2.27	112	0.025
month^2	0.91	0.40	2.31	112	0.023
sex:male	-1.01	0.40	-2.55	112	0.012

Eq. (1.1) males, $y = (4.72 – 1.01) – 9.08*\text{month} – 0.91*\text{month}^2$, where y is supercooling point and month is 1 – 3 for December – February.

Eq. (1.2) females, $y = 4.72 – 9.08*\text{month} – 0.91*\text{month}^2$

Table 1.4 Estimated coefficients for linear mixed effects models to relate minimum daily mean temperature (°C) in *Juglans* phloem to minimum daily air temperature (°C) in California and Colorado.

State	Predictor	Estimate	SEM	*t* value	d.f.	*P*
CA	Intercept	3.38	0.129	26.21	21	< 0.001
	daily minimum air temperature	0.79	0.013	59.2	21	< 0.001
CO	Intercept	2.10	0.816	2.57	13	0.0233
	daily minimum air temperature	0.65	0.024	26.5	13	< 0.001

2 Chapter 2 - Reproduction of walnut twig beetle in black walnut and butternut

2.1 Summary

The walnut twig beetle (WTB; *Pityophthorus juglandis* Blackman) is the primary insect vector for a pathogen that causes thousand cankers disease (TCD), a disease-complex that leads to mortality in species of walnut (*Juglans* L.). We performed field and laboratory trials to determine if reproduction by WTB varies between two black walnut (*Juglans nigra* L.) parent trees of a full-sib mapping population of 323 offspring, and between black walnut and butternut (*Juglans cinerea* L.). These two tree species are native to eastern North America. In field trials, we found no significant differences in colonization density or mean number of adult offspring per female among branch sections from black walnut parent trees or among branch sections from black walnut and butternut, respectively. In laboratory trials with controlled colonization densities of WTB, we found that significantly fewer adult offspring developed in branch sections of the black walnut maternal 'Sparrow' parent than the paternal 'Schessler' parent over three summer months and one winter month. In the field, high colonization densities likely limited reproduction due to increased intraspecific competition beneath the bark. In the laboratory, where we established a lower colonization density, reproduction was likely influenced by differences in host quality. In laboratory trials, no differences were detected in the number of adult offspring emerging from black walnut and butternut accessions. This finding suggests that butternut is a suitable host for WTB. Future screening of a full-sib mapping population of 323 offspring of black walnut parent trees for WTB resistance is a warranted next step in developing alternative management strategies for TCD in black walnut.

2.2 Introduction

Thousand cankers disease affects walnuts and related species (e.g., wingnut, *Pterocarya* Kunth) and is caused by the interaction between WTB and a phytopathogenic fungus, *Geosmithia morbida* Kolařík et al. (Kolařík et al., 2011). Walnut twig beetle is native to Mexico and the southwestern United States, where the greatest genetic diversity of the species has been measured (Rugman-Jones et al., 2015), but has spread and occurs in 16 U.S. states

(9 western; 7 eastern) as of Sept. 2015 (Seybold et al., 2016). Feeding by WTB in the phloem can inoculate healthy host trees with the canker-causing fungal pathogen. Intensive phloem feeding by larvae and adults coupled with coalescence of the cankers can cause girdling of the host branches and stem, which may lead to host mortality (Seybold et al., 2013b). The extent of TCD in the United States appears to be linked to adventive plantings of highly susceptible black walnut, particularly in the western half of the country (Tisserat et al., 2011; Utley et al., 2013).

In addition to black walnut, colonization of butternut by WTB and infection by *G. morbida* were reported for the first time in Oregon in 2011 (Serdani et al., 2013). Butternut is also highly susceptible to another invasive fungal pathogen (*Ophiognomonia clavigignenti-juglandacearum* Nair, Kostichka & Kuntz) (Broders and Boland, 2011), which has caused butternut canker followed by widespread mortality of butternut across eastern North America (Ross-Davis and Woeste, 2008). As an already threatened hardwood species, remaining butternut populations in North America could be at risk from TCD. Both black walnut and butternut are the only walnut species with native distributions in eastern North America. Resistant cultivars of black walnut and butternut are needed to lower the risk of TCD to regionally important nut and timber industries, germplasm resources, and forests in the eastern United States (Leslie et al., 2010; Newton and Fowler, 2009). The research problem that we investigated, and have described here, is the potential interaction between host selection and reproduction by the WTB and resistance of black walnut and butternut.

Generally, host selection by bark beetles (Scolytidae, *sensu* Bright, 2014) follows discrete behavioral steps that are mediated by host characteristics and physiological cues (Wood, 1972). The process begins when newly emerged adults begin to search for hosts. Adults land on trees, often in response to visual and/or odor cues. Visual, tactile, olfactory, and gustatory cues may elicit boring behavior by the beetle into the outer bark and phloem. If the host is unsuitable, the adult will either reject the host before boring (Walter et al., 2010) or abandon the tree after boring into the outer bark or phloem (Elkinton and Wood, 1980). Sustained feeding in the phloem generally leads to pheromone production and aggregation

(Wood, 1982). Preliminary work on the colonization dynamics of WTB on northern california black walnut (*Juglans hindsii* Jepson, R.E. Smith) led to the development of a male-produced aggregation pheromone, which can be released artificially from a sponge-stabilized bubble cap device (Seybold et al., 2013a, 2015). Experimentally, the lure can be attached to a branch section, whereby pheromone is released to attract males and females to the cut branch.

In *Pityophthorus* Eichhoff, most species are polygynous; a male initiates gallery construction by creating a nuptial chamber in the phloem and is joined by at least two females (Kirkendall, 1983). For WTB, males typically mate with two to four females (P.L. Dallara, personal communication). Females will then lay eggs individually along the walls of an egg gallery as they tunnel away from the nuptial chamber through the phloem. Thereafter, larvae emerge from eggs and continue to mine perpendicularly to the egg gallery in the phloem.

In the United States, WTB reproduce and develop in native and cultivated stands of walnut species and three species of wingnut (Hishinuma et al., 2016). Little is known, however, about comparative reproduction of WTB in different hosts. For other bark and ambrosia beetles, reproduction varies significantly among host species (Eager et al., 2004; Lee et al., 2008; Mayfield et al., 2013; Walter et al., 2010; Zeiri et al., 2015). The number of adult brood per established female (F_1) can be influenced by host resistance (Raffa and Berryman, 1983); interspecific and intraspecific competition (i.e., for food and space); and phloem quality (Ayres et al., 2000; Haack et al., 1987). Methods for artificially infesting cut wood to measure bark beetle reproduction in hosts were developed for mountain pine beetle (*Dendroctonus ponderosae* Hopkins) (Cole and Weenig, 1966), but have not yet been developed for WTB.

An opportunity to screen two black walnut parent trees for differences in WTB colonization behavior and reproduction was available through an applied breeding program for the improvement of nut cultivars, established in 1996 at the University of Missouri, Center for Agroforestry, Columbia, MO. A total of 57 cultivars were fingerprinted by using 10 microsatellite markers and subsequently confirmed based on seven phenological descriptors for each cultivar over four seed years (Coggeshall and Woeste, 2010). These same markers and phenological

descriptors were used to identify the parents, the maternal tree ('Sparrow') and paternal tree ('Schessler'), of 323 full-sib (F_1) trees (Coggeshall, 2011; M.V. Coggeshall, unpublished data). In our experiments, we focused on comparing reproduction of WTB between parent trees in the field and laboratory. If we were successful in defining that these two "parent" trees in fact differed in their susceptibility to WTB attack (i.e., numbers of F1 offspring produced) then the next step would be to assess the responses to WTB attack among the 323 tree mapping population. If the parents vary in their phenotype, then the offspring will also, which would then allow us to map QTL associated with WTB susceptibility in this species (or in fact any walnut species) for the first time.

In this study, we investigated WTB brood production in black walnut and butternut cultivars in field and laboratory trials. The objectives of our study were to: 1) measure WTB reproduction in cut branch sections that were colonized in the field; and 2) develop a laboratory assay for WTB reproduction by artificially infesting cut branch sections with WTB. If there were differences in WTB reproduction between black walnut parent trees or butternut cultivars resulting from field colonization densities or controlled colonization densities in the laboratory, further study might then identify resistance genes related to female fecundity and beetle development so that the heritability of those traits could be tested. Finally, we conducted the laboratory portion of this study because future host screening assays planned by our project team required a reliable method of infesting hosts with WTB in the laboratory. For these future studies, we also needed to identify the month(s) during the growing season when field-collected cut branches might yield the maximum number of WTB brood.

2.3 Materials and methods

2.3.1 Walnut twig beetle reproduction in the field

Branch sections, approximately 18 inches long and 1.5-3 inch diameter, were cut from the maternal tree ('Sparrow' n = 10) and the paternal tree ('Schessler' n = 10). If these two parents exhibited a differential host response to WTB, then further screening of the full-sib mapping population would be warranted to identify potential quantitative trait loci (QTL) regions

associated with WTB reproductive capacity. Furthermore, there were a limited number of accessions of 'Sparrow' and 'Schessler' to sample from in the collection, so we were not able use cultivar as the unit of replication in our experiment.

Cut branch sections were shipped overnight from the University of Missouri Center for Agroforestry to Knoxville, TN during the week of 22 Apr. 2013. Cut surfaces (ends and large branch stubs) were sealed with paraffin wax. Branch sections were suspended horizontally from stainless steel poles approximately 1.5 m above the ground in a plantation of approximately 140 stems of black walnut (Seymour, TN, 35.876196° N, 83.762168° W, elevation 346 m) known to harbor WTB. The branch sections were installed in a grid (5 m × 5 m spacing) interspaced between plantation trees in a completely randomized design. One lure releasing 1.2 $mg \cdot d^{-1}$ of WTB male-produced aggregation pheromone from a sponge-stabilized bubble cap release device (Scotts Canada, Ltd., Delta, BC, Canada) was centered on the underside of each branch section and attached with push-pins. Branch sections were held outdoors for 5 weeks, collected, and shipped to a Biosafety Level-2 quarantine laboratory in St. Paul, MN for analysis.

Upon arrival in St. Paul, MN, entrance hole density (i.e., number of male colonization attempts per square decimeter) was determined for each branch section. Entrance holes were counted, and the surface area of each branch was determined from the length and mean diameter (average diameter of each end, averaged together). Branches were held on a laboratory benchtop in 1 gal plastic jars (ULINE, Pleasant Prairie, WI) with a modified micro-mesh ("No-see-um" mesh; 625-725 holes per 6.45 square centimeter; Quest Outfitters, Sarasota, FL) top that allowed for air exchange. Broods (F_1) were allowed to develop for 12 weeks at ambient temperatures [14/10 h (light/dark), 30-50% relative humidity, ≈21 °C] in the laboratory. The number of beetles that emerged was counted. Branches were peeled to determine the number of adults that remained under the bark. Because branches were so heavily attacked in Spring 2013, we sampled six 3 × 3-cm square areas: three on the side of the branch where the lure had been placed and three on the opposite side. To estimate the number

of beetles that had not emerged, we calculated the mean number of adults per square centimeter and multiplied by the surface area of the cut branch. The numbers of adults that had and had not emerged were totaled. The number of colonizing (i.e., parent) females was estimated by multiplying the number of entrance holes by two as a conservative estimate based on the polygynous mating behavior (e.g., Langor and Raske, 1987).

For a second field trial, one branch was collected per tree on 28 Aug. 2013 from butternut (n =9) and black walnut (n =10) in a germplasm collection in Rosemount, MN (Dakota Co., University of Minnesota, UMore Park). Branches (1.5-3 inch diameter) were cut to lengths of 18 inches. As before, all cut surfaces with exposed xylem were dipped in paraffin wax. Branch sections were shipped overnight to Knoxville, TN and installed at the same site and in the same manner as the first field trial. Branch sections were left in the field for 5 weeks to be colonized by WTB. On 7 Oct. 2013, branch sections were shipped overnight to the Biosafety Level-2 quarantine lab in St. Paul, MN. Upon arrival, entrance holes on each piece were counted. Brood (F_1) were allowed to develop for 12 weeks in a growth chamber [14/10 h (light/dark), 50% relative humidity, 21 °C], after which branch sections were sampled and the number of adult offspring (F_1) per female was estimated as described for the first field trial.

2.3.2 Statistical analysis of field trial data

For the first field trial, we used analysis of variance (ANOVA; $\alpha = 0.05$) to examine the effect of parent tree (i.e., maternal/paternal) on colonization density (number of entrance holes per square decimeter) and on the number of adult offspring (F_1) per female. We used branch section as the unit of replication. Analytical assumptions (normality of errors, homoscedasticity of variances) were assessed by visual inspection of residual plots. For the second field trial, we also used ANOVA to examine the effect of host species (i.e., black walnut, butternut) on colonization density (number of entrance holes per square decimeter) and on the number of adult offspring (F_1) per female. In this instance, colonization densities and adult offspring per female were transformed by using a square root transformation to satisfy assumptions of normality of errors and homoscedasticity. We report means and standard errors of non-

transformed values for this field trial. All analyses were conducted in R 2.15.1 (R Core Team, 2013).

2.3.3 Walnut twig beetle reproduction in the laboratory

Ten branch sections were cut in Missouri from each of the same trees as in the first field trial, on 30 May, 9 July, and 6 Aug. 2013, and were shipped overnight to St. Paul. Branch sections were cut to 10 inch lengths, and cut surfaces with exposed xylem were dipped in paraffin wax upon arrival. Approximately four holes, spaced on opposite sides of the branch section (density of 2 entrance holes per square decimeter), were pre-drilled to insert WTB. We selected a low colonization density in the laboratory trial for several reasons. First, the higher colonization density that we observed in our field trial was the result of parent colonization that was under the influence of the synthetic aggregation pheromone. Work in California (S.J. Seybold, unpublished data) suggests that colonization densities of WTB on host branch sections or branches on live trees under natural conditions (i.e., in the absence of synthetic pheromone) are generally relatively low. This would especially be the case when walnut twig beetle first begins to invade an area. Thus, the densities we selected have ecological relevance. Second, at the lower density we observed that larval galleries would remain relatively distinct. A higher density would have resulted in several overlapping larval galleries (i.e., competition). Thus, increasing the colonization density would compromise our ability to accurately track parent beetles and data collected from individual galleries. Parent beetles were collected from beneath the bark of naturally-infested hybrid black walnut branch sections [*Juglans hindsii* × (*J. nigra* × *J. hindsiiJ. californica*)] from a commercial seed orchard in Sutter Co., CA (39°03.681' N, 121°36.818' W, 19.2 m elevation). We used this source of beetles because the population density was high at this location and WTB were readily available to us at the site for most of the calendar year. We also used WTB from this site for other laboratory studies involving cold tolerance, so for consistency, we continued to collect from this population. There is no evidence that using WTB reared from species other than black walnut has an effect on successful colonization and reproduction on other host species. Under natural conditions,

switching from its putative native host [arizona walnut (*J. major* Torr., A. Heller)] to walnut species and hybrids in the western and later, eastern United States, did not appear to hinder WTB colonization or reproduction. Infested branches were shipped overnight on 4 June, 2 July, and 7 Aug. 2013 to the Biosafety Level-2 facility in St. Paul, MN to provide a continuous supply of beetles.

Eighty adult WTB (approximately 40 males and 40 females) were collected by removing the outer bark from infested branch sections from California. Males were held in sealed petri dishes with moist tissues (Kimwipes; Kimberly Clark, Roswell, GA) for 2 d to induce feeding when introduced to a new host. To ensure that beetles were healthy, individuals were allowed to walk on a walnut bark surface. If a test beetle could walk normally for 10-15 s on the bark, that individual was used in the breeding trial. If the test beetle did not walk, it was discarded. One male was placed by using a fine paint brush in each drilled hole, and the hole was covered with modelling clay (Craftsmart, Irving, TX) to prevent escape. Males were checked daily until signs of feeding (i.e., production of frass) were visible, and males were replaced if they were dead or inactive. Up to three males were inserted into each drilled hole if males continued to die. After males showed signs of establishment (i.e., frass extrusion, space for a nuptial chamber), one female was introduced by using a fine paint brush to transfer and guide her to the entrance hole. Females also underwent a walking test before they were selected. Holes were re-sealed with modelling clay until signs of feeding were evident. The time from placement of a male or female beetle into a drilled hole to signs of establishment varied from 1 – 5 d. Branch sections were held for 12 weeks inside a growth chamber [14/10 h (light/dark), 50% relative humidity, 21 °C] in 1 gal plastic jars with modified lids (as described above). After this incubation period, branch sections were peeled completely and immature and adult life stages were counted. Adults that had emerged naturally also were counted.

A second laboratory experiment began in Fall 2015. Twenty branch sections were collected in Nov. 2013 from the USDA ARS NCGR facility in Corvallis, OR from four accessions of butternut ['Herrick' = NCGR Accessions JUG 9.002 and JUG 9.001 (Iowa), and 'Craxezy'=

NCGR Accessions JUG 5.001 and JUG 5.002 (Michigan); USDA 2012]. Based on available data in the USDA ARS database, these accessions were considered at the time to be “pure” butternut with limited or no introgression from heartnut (*Juglans ailantifolia* Carrière) (USDA, 2012). Branch sections were shipped to St. Paul, MN on 12 Nov. 2013 and held at -20 °C until Jan. 2014 to kill any associated insects. Ten branch sections of black walnut parent trees (five from each parent) also were acquired at this time from the same Missouri source as described above. The purpose of the latter was to serve as experimental controls. Beetles used to infest the branch sections in this trial were from the same California source and involved the same method of artificial infestation as described above. Branches were held for 12 weeks in plastic jars inside a growth chamber [14/10 h (light/dark), 50% relative humidity, 21 °C], after which branches were peeled completely and the number of immature and adult life stages were counted. In both trials, the number of parents that were initially inserted in a cut branch section was subtracted from the number of adults found to determine the number of adult progeny.

2.3.4 Statistical analysis of laboratory trial data

For the first laboratory trial, we tested the effects of month, parent tree, and their interaction on the number of adults (F_1) per female with a two-way ANOVA. We examined how the number of adults produced varied by month or parent tree using a Tukey means comparison procedure. The numbers of adult offspring (F_1) per female were transformed by using a square root transformation to satisfy assumptions of normality of errors and homoscedasticity. For the second laboratory trial, we tested the effect of butternut cultivar and parent tree on the number of adults (F_1) per female using one-way ANOVA. Results from two trees of ‘Herrick’ and two trees of ‘Craxezy’ were combined, as accession (tree) did not affect mean offspring per female (see Results and discussion). Variation among the numbers of adults produced by butternut cultivar and parent tree were also examined with the Tukey means comparison procedure. Because the errors had a normal distribution and the variances were equal, no data transformation was necessary for the data set from the second laboratory trial. We report means and standard errors of non-transformed values for results of both laboratory trials. All

analyses were limited to adults because immature stage counts were low in field and laboratory trials.

2.4 Results and Discussion

2.4.1 Walnut twig beetle reproduction in the field

2.4.1.1 Colonization density

In the first field trial, mean colonization density (± SE) was 27.5 ± 2.7 WTB entrance holes/100 cm^2 for the maternal 'Sparrow' tree and 28.1 ± 2.6 WTB entrance holes/100 cm^2 for the paternal 'Schessler' tree. The colonization density among branch sections from parent trees did not differ [$F_{1,18} = 0.03$, $P = 0.85$ (Fig. 2.1A)]. In the second field trial, colonization densities also did not differ between butternut and black walnut [$F_{1,18} = 0.05$, $P = 0.83$ (Fig. 2.1B)] but were much lower for both species than in trial 1 (i.e., < 1.0 ± 0.2 WTB entrance holes/100 cm^2). The second field trial, completed in fall, occurred during several days of rain, which likely lowered beetle flight activity when compared to the first field trial, completed in spring. Elevated spring and fall flights are typical in northern California (Chen and Seybold, 2014), and this pattern also likely occurs in Tennessee (S.J. Seybold, personal observation).

Though the flight activity of WTB may have varied seasonally, when compared between hosts tested in spring and fall, we observed that beetle colonization activity was consistent in the field. This suggests that the lure made hosts equally attractive to WTB. Based only on our reproduction results, it appears that both black walnut parent trees, as well as black walnut and butternut, are at equal risk of WTB colonization, and ultimately TCD. Because of our destructive sampling of the branch sections to estimate WTB population density, we were not able to compare development of cankers caused by *G. morbida* at each entrance hole. However, Utley et al. (2013) showed that both black walnut and butternut maternal half-sib families produced medium to large cankers in controlled inoculation greenhouse studies of 1- to 2-year-old trees. Future work on beetle landing rates in the absence of synthetic aggregation pheromone (Wood, 1982) would provide more information on whether or not attraction varies among these host species and cultivars in the field.

2.4.1.2 Adult offspring

In the first field trial, the mean number of adult offspring (F_1) per female did not differ among branch sections from black walnut parent trees [$F_{1,18} = 0.35$, $P = 0.56$ (Fig. 2.2A)]. Similarly, in the second field trial, the mean number of adult offspring (F_1) per female did not differ among branch sections from butternut and black walnut [$F_{1,17} = 1.30$, $P = 0.27$ (Fig. 2.2B)]. Brood production of many scolytids is influenced by several host phloem characteristics, such as nitrogen content, available carbohydrates, inner bark thickness, and moisture (Amman, 1972; Ayres et al., 2000; Webb and Franklin, 1978). Although phloem quality was not directly measured in our assays, the two black walnut parent trees as well as butternut appear to provide sufficient nutrition for development and reproduction of WTB in the field. In general, the high incidence of TCD in urban and rural landscapes in the western USA suggests that black walnut is a very susceptible host (Tisserat et al., 2011).

2.4.2 Walnut twig beetle reproduction in the laboratory

In the first laboratory trial, we found that branch sections of the paternal 'Schessler' tree produced twice as many adults (F_1) per female as the maternal 'Sparrow' tree [$F_{1,62} = 63.61$, $P < 0.001$ (Fig. 2.3)]. Adult (F_1) counts indicate that the paternal tree is a more suitable host than the maternal tree. In the laboratory trials, we used a colonization density that would minimize effects of intraspecific competition on reproduction or development. In our field trials, female parents and developing offspring were competing for space and nutrients in host phloem. For example, in the first field trial, WTB colonization density was relatively high (Fig. 2.1A) and reproduction was low (Fig. 2.2A), whereas in the second field trial, WTB colonization density was relatively low (Fig. 2.1B) and reproduction was high (Fig. 2.2B). The differences in intraspecific competition resulting from the initial differences in colonization density in the two field studies may have been responsible for the higher level of normalized reproduction in the second trial. Furthermore, competition likely limited WTB reproduction overall in the paternal tree branch sections in field trials when compared to laboratory trials because fewer offspring per female were found in the field than in the laboratory trials. It is possible that the Tennessee-based field population of WTB was behaviorally or

reproductively different from the California population used in the laboratory trials, and these differences may have played a role in the differences in WTB reproduction that we observed in the trials. However, populations of WTB from northern California and from several locations in Tennessee did not appear to differ greatly when their mitochondrial COI gene sequences were analyzed (Rugman-Jones et al., 2015), which does not support potential behavioral or physiological differences between our test populations.

Herbivore reproduction also may be limited by juglone, a defensive phenolic compound produced by walnut species, which varies among cultivars of english walnut (*Juglans regia* L.) and is present in the bark and phloem of black walnut and butternut (Gupta et al., 1972; Moore et al., 2015; Solar et al., 2006). Further investigation of resistance traits in the maternal tree or 'Sparrow' should be considered, as juglone or other constitutive defense compounds may differ among cultivars.

We found some evidence ($\alpha = 0.1$) that the mean number of adult offspring (F_1) per female was affected by the month of colonization [$F_{3,62} = 9.09$, $P = 0.05$ (Fig. 2.3)]. There was no interaction between month and parent tree ($F_{3,62} = 0.09$, $P = 0.99$). Water, nitrogen, and carbohydrate concentrations in the phloem can fluctuate seasonally in deciduous trees and juglone levels can fluctuate seasonally in butternut phloem (Moore et al., 2015; Pallardy, 2008; Redmer et al., 2001), but we have limited information on how seasonal changes in phloem chemistry affect WTB. To further understand seasonal changes in host phloem quality, future studies could measure the interactions among *G. morbida*, host tissues, and offspring development over time, as symbiotic fungi have also been shown to alter host nutrition in other bark beetle systems (Ayres et al., 2000; Bentz and Six, 2006; Goodsman et al., 2012).

In the second laboratory trial, we found no difference in mean adult offspring (F_1) per female [$F_{3,26} = 2.35$, $P = 0.09$ (Fig. 2.4)] among black walnut and butternut cultivars tested in Jan. Though high variability obscured significant differences between parent trees, we note that on average branch sections from the paternal 'Schessler' tree produced twice as many adults (F_1) per female than those from the maternal 'Sparrow' tree. This result is consistent with the

results collected in summer months (Fig. 2.3). Results from two trees each of butternut cultivars, 'Herrick' and 'Craxezy,' were combined, as the model term for accession had no effect on adult offspring per female ($F_{1,25} = 0.005$, $P = 0.94$). It appears that black walnut and butternut in winter months are equally suitable for WTB reproduction. These results support our findings from the second field trial. Future work to determine the risk of TCD to butternut, a hardwood species that is already threatened by another pathogen, should examine WTB host colonization, attraction, and establishment rates on additional butternut cultivars and naturally occurring hybrids between butternut and heartnut/Japanese walnut. Genetic analyses were conducted on the specific NCGR butternut accessions used in our experiments after the research was completed, and the analyses revealed that both 'Herrick' and 'Craxezy' showed hybridization with heartnut (J. Romero-Severson, personal correspondence).

2.5 Conclusion

We focused field and laboratory studies on the black walnut parents of 323 full-sibs. Walnut twig beetle reproduction differed substantially between 'Sparrow' and 'Schessler,' and this response was consistent in all months tested. Our field screenings suggested that at high colonization densities, competition may limit WTB reproduction in black walnut and butternut. Our laboratory assays suggested that at low colonization densities, host resistance may limit WTB reproduction in 'Sparrow,' but the underlying mechanisms remain to be determined. Further screening of the full-sib collection in Missouri is justified to determine the source(s) of resistance. Although our results also indicate that black walnut and butternut are equally suitable for WTB reproduction, additional screenings of other walnut species should be completed to identify other potential sources of resistance.

2.6 Figures

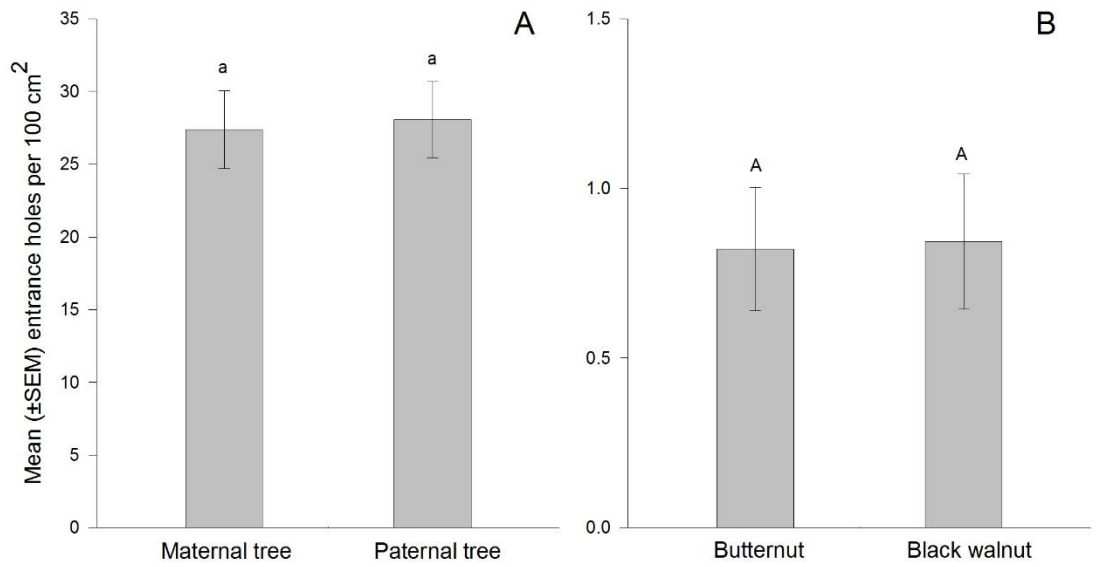

Figure 2.1 Walnut twig beetle (WTB) colonization densities (population from Seymour, Sevier Co., TN) among branch sections from (A) two black walnut parent trees (n=10 branches each), and (B) butternut (n=9 trees) and black walnut (n=10 trees). Note difference in scale for y-axis between panels A and B, representing spring (A) and fall (B) flights of WTB. Bars with the same letter (lower case in panel A and upper case in panel B) are not significantly different by ANOVA ($\alpha = 0.05$; 1 WTB entrance hole/100 cm^2 = 9.2903 WTB entrance holes/ft^2).

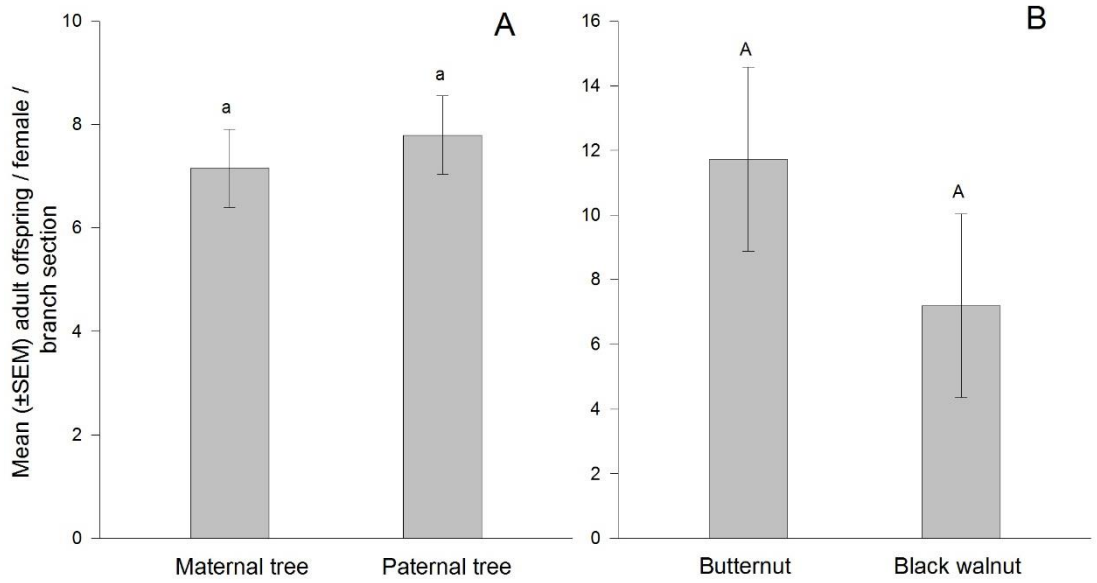

Figure 2.2 Number of walnut twig beetle (WTB) adult offspring (F_1) per female (population from Seymour, Sevier Co., TN) in branch sections from two field trials among (A) black walnut parent trees (n=10 branches each), and (B) butternut (n=9 trees) and black walnut (n=10 trees). Note difference in scale for y-axis between panels A and B, representing spring (A) and fall (B) flights of WTB parents. Bars with the same letter (lower case in panel A and upper case in panel B) are not significantly different by ANOVA ($\alpha = 0.05$).

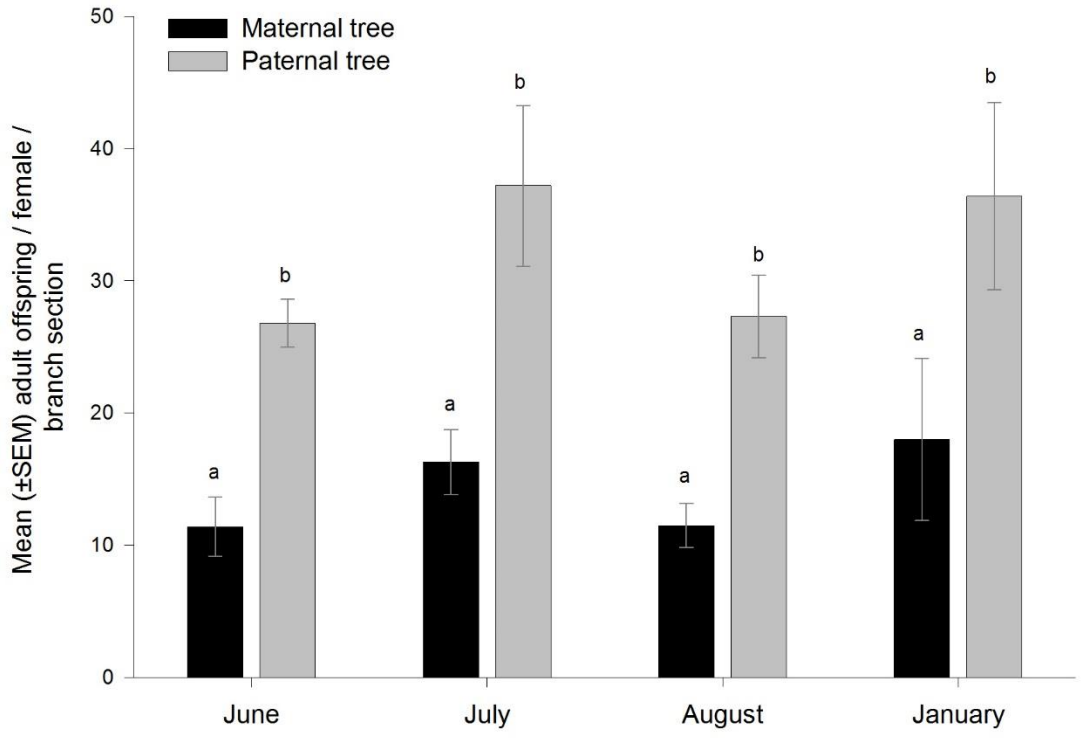

Figure 2.3 Number of walnut twig beetle (WTB) adult offspring (F_1) per female per branch section in black walnut parent trees (n=10 branches in summer months, n=5 branches in January) across all months in laboratory experiments. Bars with the same letter are not significantly different by Tukey's test ($\alpha = 0.05$).

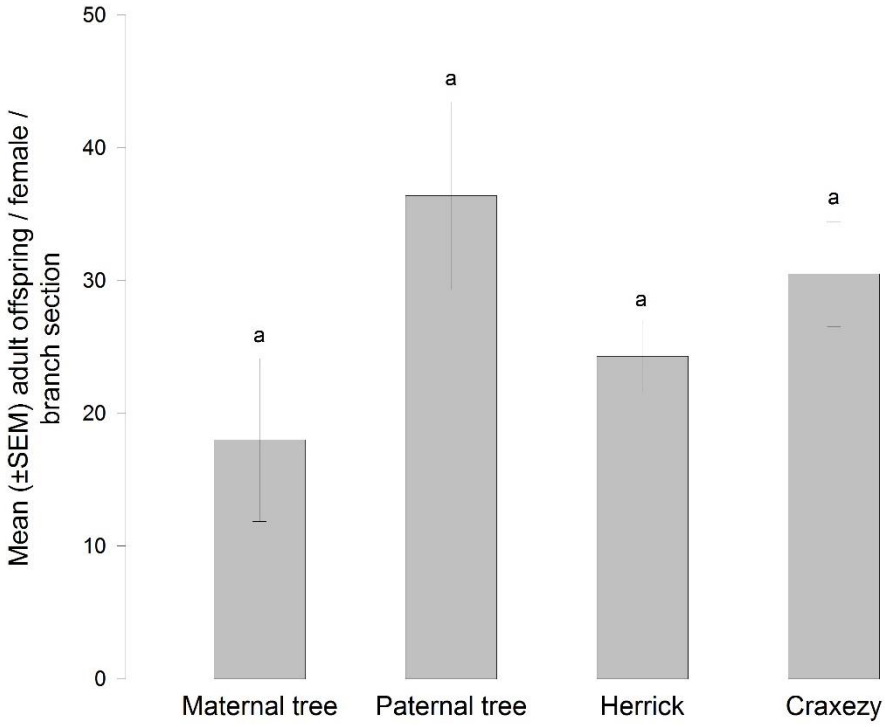

Figure 2.4 Number of walnut twig beetle (WTB) adult offspring (F_1) per female per branch section from black walnut parent trees (n=5 branches each) and two trees each of butternut 'Herrick' and 'Craxezy' (n=10 branches each) in January laboratory experiment. Bars with the same letter are not significantly different by ANOVA ($\alpha = 0.05$).

3 Chapter 3 - Reproduction and cold tolerance of walnut twig beetle across Juglandaceae: Implications for range expansion of a United States domestic invasive

3.1 Summary

Thousand cankers disease is an emerging insect-pathogen complex potentially threatening globally- distributed Juglandaceae species. Feeding by the walnut twig beetle, *Pityopthorus juglandis*, vectors a fungal pathogen, *Geosmithia morbida*, on some species of *Juglans* and *Pterocarya*, but the extent of the beetle's host range is not known. On susceptible hosts, the beetle and fungus can cause phloem necrosis (i.e., cankers), branch dieback, and eventually tree death. Throughout the United States and northeastern Italy, *P. juglandis* has expanded its geographic range among naïve hosts that vary in suitability. We examined colonization and brood production in no-choice laboratory experiments across 11 *Juglans* spp., one *Pterocarya* sp., and two *Carya* spp. over two years. We also examine the cold tolerance of adult brood by measuring supercooling points to help inform potential climatic limits of *P. juglandis* within host ranges. We found that 11 *Juglans* and one *Pterocarya* species supported complete brood development. *Julgans nigra* (from MO and IN), *J. californica* (CA), and *J. hindsii* (CA) supported the greatest levels of reproduction. Less suitable hosts include native southwestern United States hosts (*J. major* and *J. microcarpa*), Eurasian species (*J. regia*), Asian butternuts (*J. ailantifolia*, *J. mandshurica*, and *J. cathayensis*), and native eastern United States butternut (*J. cinerea*) and Japanese walnut-butternut hybrid (J. *ailantifolia* × *cinerea*). The two *Carya* species were not hosts. Males would establish on branches of these species, but often more than one attempt was required by investigators. Brood typically froze and died at temperatures between -15°C and -22°C, although this varied with the species of tree in which they developed. Thousand cankers disease may be another example where escape of an insect herbivore into populations of evolutionary-naïve hosts can facilitate rapid range expansion and even massive mortality to hosts.

3.2 Introduction

Forest insects are important ecological disturbance agents (Gandhi and Herms 2010; Samarasekera et al. 2012). Global movement of forest pests via known and unknown pathways is of high concern given the landscape scale mortality that can result from establishment in naïve hosts (Anulewicz et al. 2008; Økland et al. 2011; Flø et al. 2014; Umeda et al. 2016). Regardless of an insect's native or non-native status, range expansion through colonization of naïve hosts can occur following climatic shifts (Cullingham et al. 2011), human-mediated introductions (Hanula et al. 2008; Herms and McCullough 2014), and widespread use of non-native trees in urban or forested environments (Branco et al. 2015). Predicting where and which species pose the highest risk is a priority because prevention is the least costly form of management of invasive forest pests (Aukema et al. 2011; Koch et al. 2011; Yemshanov et al. 2012).

One emerging threat complex to forestry and agriculture is thousand cankers disease on the Juglandaceae (Kolařík et al. 2011; Tisserat et al. 2011; Seybold et al. 2013). The walnut family is distributed globally and is an important species in forest ecosystems, cultivated nut production, and high quality wood products (Aradhya et al. 2006; Newton and Fowler 2009). In contrast to other anthropogenic pathways, such as firewood (Jacobi et al. 2012), untreated walnut wood moves via woodworkers and hobbyists on unregulated pathways over long distances. Hobbyist trade likely reflects thousand cankers disease spread throughout the United States and to Europe (Campbell and Schlarbaum 2014).

Thousand cankers disease is an insect-pathogen complex. The walnut twig beetle, *Pityophthorus juglandis* Blackman, inoculates the phloem of hosts with a fungal pathogen, *Geosmithia morbida* Kolařík et al. (Kolařík et al. 2011). Colonization of a host is initiated by male beetles, which bore through the outer bark and feed in the phloem; create a nuptial chamber; and attract females *via* an aggregation pheromone (Seybold et al. 2016). After mating, females elongate egg galleries and lay eggs individually along the walls. Intensive feeding by *P. juglandis* adults and larvae and phloem necrosis from the fungal pathogen lead to thousand cankers disease on susceptible hosts.

The beetle is native to northern Mexico and the southwestern United States where its native host, *Juglans major* (Torr.) A. Heller, occurs sympatrically (Rugman-Jones et al. 2015). *Pityophthorus juglandis* has spread out of its native range over the last century and now occurs in 16 U.S. states (9 western; 7 eastern) and northeastern Italy as of September 2015 (Montecchio and Faccoli 2014; Seybold et al. 2016). Mediated by the presence of susceptible hosts, *P. juglandis* is established in both adventive plantings and native stands of *Juglans* spp. and several *Pterocarya* sp. (Tisserat et al. 2009; Flint et al. 2010; Grant et al. 2011; Tisserat et al. 2011; Serdani et al. 2013; Montecchio et al. 2014; Yaghmour et al. 2014; Montecchio and Faccoli 2014; Hishinuma et al. 2016; Seybold et al. 2016). Development from egg through three larval stages to adult is approximately 12 weeks; two overlapping generations occur per year (Dallara et al. 2012; Faccoli et al. 2016). Due to intraspecific variation in development time, at the end of 12 weeks teneral adults, pupae, and larvae are present under the bark. Adult offspring will either emerge or remain in the natal host to reproduce. From field observations, host-switching has contributed to *P. juglandis* range expansions.

Abiotic factors, such as temperature, may also play a role in mediating range expansions. *Pityophthorus juglandis* is a freeze-intolerant insect, which means that ice formation within its body causes mortality (Luna et al. 2013). As temperatures decrease, the beginning of ice formation in the insect body is characterized by the supercooling point (Lee 2010). Cold tolerance can be an excellent predictor of geographic distributions. For example, lower lethal temperature described latitudinal distributions of *Drosphila* spp. (Kellermann et al. 2012; Andersen et al. 2015). Northern range expansions can also be limited by lower lethal temperature (Ungerer et al. 1999). In some insects, supercooling points vary with species of host plant in which they develop or feed (Gash and Bale 1985; Trudeau et al. 2010; Zheng et al. 2014; Feng et al. 2016). If there is variation in *P. juglandis* cold tolerance due to natal host, this may limit or mediate further spread within the range of certain hosts.

Given the high uncertainty of new *P. juglandis* establishments and further spread via unregulated pathways, assays are warranted to (i) test host range within Juglandaceae, (ii)

compare differences in host suitability, and (ii) characterize host effects on cold tolerance. The objective of this study was to screen Juglandaceae species for *P. juglandis* colonization and development to characterize potential host range and variation in host suitability among known hosts. We examined the likelihood of male establishment after one introduction to potential host material in the laboratory under no-choice conditions, and examined differences in reproduction per female between hosts. We also characterized the cold tolerance of adult brood to determine potential climatic range limits of *P. juglandis* in globally distributed Juglandaceae hosts.

3.3 Materials and methods

We examined colonization and brood production in no-choice laboratory experiments across 11 *Juglans* spp., one *Pterocarya* sp., and two *Carya* spp. (Manning 1978). These species were collected from multiple locations across the United States (Table 1). *Juglans* spp. and *Pterocarya stenoptera* de Candolle were included based on *P. juglandis* colonization observed in the field (Tisserat et al. 2011; Serdani et al. 2013; Hishinuma et al. 2016). Multiple cultivars of *J. nigra* were included as positive controls. *Carya ovata* (Mill.) K. Koch was included as a putative negative control. Individual trees from germplasm repositories were selected to avoid clones or trees from the same seed source and ensure a high level of genetic and geographic diversity in our samples.

We defined a tree species as a host if two conditions were satisfied. First, adult offspring had to be produced (i.e., complete growth and development) (Hodkinson and Hughes 1982). Second, the number of female offspring had to equal or exceed the number of female parents (i.e., maternal replacement). Our primary metric of host suitability was the mean total number of offspring produced in cut branches of each tree species.

We used cut branches instead of whole trees as host material for assays for three reasons. First, there are Juglandaceae that either do not occur or are uncommon within the current geographic range of *P. juglandis*. We wanted to screen several known hosts and potential unknown hosts under common conditions simultaneously. Second, a method to study

P. juglandis reproduction in cut branches has recently been developed (Hefty et al. 2016). Host effects have been successfully detected in previous bark beetle assays using cut plant material (Švíhra and Volney 1983; Lee et al. 2008; Walter et al. 2010; Mayfield et al. 2013; McKee et al. 2013).

3.3.1 Insects

Naturally infested hybrid black walnut branch sections [*Juglans hindsii* × (*J. nigra* × *J. hindsii*/*J. californica*] from a commercial seed orchard in Sutter Co., CA, USA (39°03.681' N, 121°36.818' W, 19.2 m elevation) were shipped to a Biosafety Level-2 facility in St. Paul, MN in February, March, April, and May 2014 to provide a source of parent beetles for experiments conducted in 2014. Adult *P. juglandis* emerged from these cut branch sections between July and September 2014. For experiments conducted in 2015, parent beetles were sourced from infested *Juglans californica* branch sections from the *Juglans* collection of the USDA Agricultural Research Service (ARS) National Clonal Germplasm Repository in Winters, California (38°30'10.7"N 121°58'51.5"W) shipped to St. Paul, MN in July 2015. Adult *P. juglandis* emerged daily from those branch sections between September and October 2015. All branch sections, in 2014 and 2015, were held on a laboratory benchtop (20-22°C, 30-50% RH, 14:10 L:D) in 3.8 L plastic jars (ULINE, Pleasant Prairie, WI) with a modified micro-mesh ("No-see-um" mesh; 96-112 holes per cm^2; Quest Outfitters, Sarasota, FL) top that allowed air exchange. Emerged beetles were collected daily, the sexes were separated (Wood 1982), and held in sealed Petri dishes with moist Kimwipes (Kimberly Clark, Roswell, GA) for 2 d before introduction to cut branch sections.

3.3.2 Host range assays

We sampled up to 10 trees per species by removing a branch 25.4 cm in length and 3-5 cm in diameter. For some species, more than one branch was sampled from the same tree because 10 individual trees were not available (Table 1). The lowest replication occurred for *C. ovata*, which had four trees. *Pityophthorus juglandis* preferentially colonizes branches larger than 1.5 cm in the field (Graves et al. 2008). All samples were placed in plastic bags to reduce

desiccation and kept in a cooler. Material from Texas, Missouri, and California was sent via overnight courier to St. Paul, MN. Upon arrival, branches were cut to 25.4 cm, dipped in paraffin wax to seal cut ends, and placed in cold storage (≈4°C) until beetles were available.

Cut branches from California were held in a -80°C freezer for 24 h to kill subcortical insects because these samples originated in counties known to have *P. juglandis*. Once branch sections were thawed, they were dipped in 2-5% bleach solution and allowed to dry to inhibit fungal growth at beetle entry sites (see below). Branches were sealed with Tree Bandage (Forestry Suppliers, Inc., Jackson, MS) because melting paraffin wax was not permitted in the quarantine facility. For the remainder of the text, we refer to tree species (origin of collection).

We followed methods developed by Hefty et al. (2016) to infest cut branches with *P. juglandis*. We calculated surface area from the length and diameter of each branch and drilled two entrance holes/100 cm^2 of surface area. Entrance holes were drilled at a 30° angle to allow easier access to phloem and were approximately 2-4 mm in diameter, extending no further than to the sapwood.

Male *P. juglandis* were placed on bark for 10 – 15 seconds to confirm normal ambulatory behavior. Males that did not walk were discarded. One male was placed in each drilled hole with a fine paint brush. Holes were covered with modelling clay (Craftsmart, Irving, TX) to prevent males from escaping per Hefty et al. (2016). Males were checked daily and replaced until signs of boring were visible (i.e., frass accumulation around hole chewed into phloem). We replaced dead or inactive males up to three times. The number of male introductions was recorded for each hole on each cut branch section. If the first male accepted the branch after one introduction, we recorded the event as a 1. If a second or third male was introduced to a hole due to inactivity or death, we recorded the event as a 0.

Males were introduced to cut branches using a randomized block design of 10 – 12 host species per block per day. If there were not enough male parents for a full block in one day, then that day was skipped. Cut branches from California were received in late summer

after ten other tree species were infested (early summer), so material was infested in sets of ten cut branches as they arrived in St. Paul.

After males showed signs of boring, one female was introduced to each hole with a fine paint brush. Females were also tested for ambulatory viability prior to experimentation. Holes were re-sealed with modelling clay until feeding was evident. The time from placement of a male or female beetle into a drilled hole to signs of boring varied from 1 – 5 days. These procedures resulted in colonization densities of two to six mating pairs per cut branch section. After all mating pairs were established on a cut branch, the branch was placed in a 3.8 L plastic jar with modified lid (as described above), and jars were placed in a growth chamber (14:10 L:D, 50% RH, 21°C) for 12 weeks.

After 12 weeks, all adults that had emerged into the rearing jars were counted. Cut branches were transferred into separate 3.8 L freezer bags and placed in a refrigerator (≈3°C) until branch sections could be sampled. Outer bark was carefully removed using a #22 blade X-ACTO knife (Elmer's Products, Inc., Westerville, OH) to expose maternal and larval galleries. Using a fine paintbrush, larvae, pupae, and adults were removed from galleries and tallied. The total number of parents introduced to each cut branch was subtracted from the number of adults found.

In 2015, the same host materials were screened (Table 1), with a few modifications. Reduced availability of parent beetles limited protocol to one entrance hole/100 cm^2 of surface area and limited competition. Males were replaced up to five times to ensure that reproduction could take place at a lower colonization density. Tree species from California were included with other species in the block design. In 2014, at the end of 12 weeks, we observed that cut branches appeared dry. In 2015, we measured initial percent moisture when the first male was introduced and final percent moisture at the end of 12 weeks with a moisture meter (approximately 1 cm pin length, Delmhorst J-2000, Towaco, NJ).

3.3.3 Supercooling point assays

Brood adults from 2014 and 2015 host screening assays were used for supercooling point assays to determine the temperatures at which insects freeze and die. Sampling days occurred between October and early December 2014 and late January 2016. On a sampling day, one branch section from a set of ten of each host was chosen at random. Four to eight adults were excised from each branch using a #22 blade X-ACTO knife and fine paintbrush. Adults were held in Petri dishes on moist Kimwipes (Kimberly Clark, Roswell, GA) at room temperature (≈22°C) for 48 h to allow for gut evacuation (Hefty et al. 2016, in review). Beetle sex was determined per Bright (1981), and one active insect was placed in a 5 x 7 mm gelatin capsule (Capsuline, Pompano Beach, FL), secured to a copper-constantan thermocouple with a small amount of high vacuum grease (Dow Corning, Auburn, MI) per Hanson and Venette (2013). Each thermocouple was placed in a calibrated polystyrene cube and cooled at ≈1°C min^{-1} when placed in a -80°C freezer (Carrillo et al. 2004). We did not find brood adults in all hosts on all sampling days. A cut branch section from each host was sampled over 5-7 d until 20-30 were tested or all adults were recovered from peeled branches. Sex and natal host were randomized among 16 thermocouples attached to each of two multi-channel data loggers (USB-TC, Measurement Computing, Norton, MA). Temperatures were recorded once per second. The supercooling point was recorded as the lowest temperature before a spontaneous release of heat (i.e., an exotherm) due to the formation of ice within the body.

3.3.4 Data analysis

All analyses were done in R v3.2.3 (R Core Team 2015). Generalized linear mixed effects models with a binomial data distribution were used to examine the effect of tree species on the probability of male establishment after one introduction event, where species was treated as a fixed effect and tree was treated as a random effect to help account for instances where more than one branch was taken from a single tree. After progeny emerged, species were assigned a host status (i.e., host or non-host). Generalized linear mixed effects models (GLMM; family=binomial) were used to examine the effect of host status on the probability of male activity after one introduction where host status was treated as a fixed effect and tree was

treated as a random effect. R package lme4 and lmerTest were used to fit mixed-effects models (Bates et al. 2015; Kuznetsova et al. 2013).

To determine the effect of host on total brood per female, adults per female, and immatures (larvae and pupae) per female (i.e., reproduction), we used separate linear mixed-effect models, where host was treated as a fixed effect and tree was treated as a random effect. In 2014, cut branches collected from California arrived later in the summer and were infested at a later time, so these data were analyzed separately from data collected from branches infested earlier in the summer. In 2015, data from all cut branches were analyzed together. We added two model terms to examine if variation in reproduction was due to a change in moisture: change in percent moisture (initial – final percent moisture) and an interaction term (host × change in percent moisture). We found no effect of change in percent moisture on total brood per female ($F_{1,59} = 1.66$, $P = 0.20$), adults per female ($F_{1,59} = 1.40$, $P = 0.24$), or immatures per female ($F_{1,59} = 0.93$, $P = 0.34$). There was also no effect of an interaction between host and change in percent moisture ($F_{9,59} \geq 0.71$, $P \geq 0.23$). Because we found no significant variation in reproduction due to change in percent moisture, we report model results without a term for change in percent moisture.

We restrict our Results to comparisons among hosts; if *P. juglandis* produced no adult offspring in a tree species, that species was removed from analyses. To determine if the proportion of male to female adult offspring varied among *Juglans* hosts, we used logistic ANOVA and a binomial data distribution. In calculating degrees of freedom for ANOVA models using mixed effects, we used Satterthwaite approximations. To examine the effect of host on supercooling point of adult offspring, we used ANOVA.

We used square root transformations to satisfy model assumptions (normality of errors and homoscedasticity of variances) for analyses of 2014 and 2015 reproduction and supercooling point data. When we found an overall effect of host on a response variable ($\alpha = 0.05$), Tukey's HSD test was used to determine differences among hosts (R package multcomp; Hothorn et al. 2008).

3.4 Results

3.4.1 Male establishment

Male establishment on potential host material is the first step in the colonization sequence. The probability of male establishment immediately after one introduction to a branch did not vary across host species of trees tested in 2014 (χ^2_{16} = 23.24, P = 0.10), although differences emerged in 2015 (χ^2_{11} = 27.75, P = 0.003). In general, we found that males were less likely to colonize material that would not support further brood development, although almost half of them would readily chew exposed phloem at first presentation (Fig. 3.1). This pattern was consistent across both years. In 2014, the likelihood of male establishment after one introduction was 77 – 94% for *Juglans* and one *Pterocarya* spp. Male establishment after one introduction was 69% for *C. illinoensis* (MO) and 64% for *C. ovata* (IN), even though no brood developed in these hosts (see below). In 2015, for *Juglans* species, the likelihood of male establishment ranged from 50 – 89%. In *C. illinoensis* (MO) the likelihood of male establishment after one introduction was 50%, but this was also true for *J. ailantifolia* (IN, MN) and *J. californica* (CA).

3.4.2 Brood production

For tree species assayed in early summer 2014, the number of progeny of *P. juglandis* varied by species ($F_{8,64.3}$ = 11.2, P < 0.001; Table 2). Again, if the number of brood exceeded the number of parents, we considered them to be hosts. We found that all 11 *Juglans* and one *Pterocarya* species are hosts, and two *Carya* species are not hosts. These results are summarized in Table 3.

Among hosts, we found that per capita reproduction varied eightfold (Fig. 3.2), from 39.2 ± 6.2 total brood per female (mean ± SE) in *J. nigra* (IN) to 5.0 ± 2.5 in *J. microcarpa* (TX). We found that *Juglans nigra* (IN and MO) and *J. cinerea* × *ailantifolia* (IN) had the greatest mean number of adult progeny per female (24.3 ± 4.9 and 20.8 ± 4.7, respectively). *Juglans microcarpa* from southwestern Texas had the lowest mean adult brood per female 1.7 ± 1.2. No adult offspring developed in *C. illinoensis* and *C. ovata*. Only one early instar larva developed in *C. illinoensis*, which was successfully colonized by only one male and female.

When we assayed additional species from California in late summer 2014, we again found strong host differences in reproduction ($F_{5,49}$ = 3.55, P = 0.008; Table 2). Mean total brood per female ranged from 23.6 ± 4.48 in *J. hindsii* to 3.71 ± 0.91 in *P. stenoptera* (Fig. 3.3). *Juglans hindsii* and *P. stenoptera* also produced the greatest (10.6 ± 2.56) and lowest (0.59 ± 0.4) mean number of adult brood per female, respectively (Fig. 3.3).

For tree species assayed in 2015, again, strong species-level differences in reproduction emerged ($F_{9,69}$ = 6.37, P = 0.001; Table 2). Among hosts, we found that suitability varied approximately four-fold (Fig. 3.4). Mean total brood per female ranged from 44.8 ± 6.3 in *J. nigra* (MO) to 11.2 ± 2.6 in *J. ailantifolia* (IN and MN). *Juglans nigra* from Missouri and *J. californica* from northern California had the highest mean adult brood per female 15.7 ± 5.39 and 13.8 ± 4.38, respectively. *Juglans ailantifolia* (IN and MN) had the lowest mean adult brood per female, 0.8 ± 0.5. Similar to 2014, no adult offspring developed in *C. illinoensis* and *C. ovata*. In 2015, mean adult brood per female was lower than in 2014 for *J. cinerea* (IN), *J. major* (CA), *J. cinerea* × *ailantifolia* (IN and MN), *J. regia* (IN), and *J. nigra* (IN). All other hosts exhibited similar levels of adult development in 2015 as in 2014. *Juglans* spp. and one *Pterocarya* sp. tested met the two conditions for host status; *Carya* spp. did not meet either condition (Table 3).

In *Juglans* hosts, *P. juglandis* females produced one male offspring to two female offspring (M:F, 0.5 ± 0.02). Sex ratio did not vary by host, however (χ^2_{12} = 18.83, P = 0.09).

3.4.3 Cold tolerance of adult brood in hosts

The temperature at which beetles froze varied with the species of tree in which they had developed in both 2014 (Fig. 3.5a; $F_{12,345}$ = 34.0, P < 0.001) and 2015 (Fig. 3.5b; $F_{7,134}$ = 14.7, P < 0.001). In 2014, mean supercooling points of the insects ranged from -22.0°C ± 0.6 in *J. californica*, CA) to -14.9°C ± 0.3 in *J. ailantifolia* (IN). In 2015, the temperatures at which insects froze ranged from -20.5°C ± 0.7 in *J. californica* (CA) to -15.5°C ± 0.2 in *J. cinerea* × *ailantifolia* (IN).

3.5 Discussion

Our measurements of *P. juglandis* reproduction suggest that geographic range expansion through native and adventive *Juglans* and *Pterocarya* plantings in the United States and Europe would not be hindered by host if appropriate environmental conditions existed for further spread. One native eastern United States species, *J. nigra* (MO and IN) and two western United States species, *J. californica* (CA), and *J. hindsii* (CA), had the greatest levels of reproduction in both years. For the western species, these observations are consistent with high levels of mortality at the Wolfskill NCGR collection and concomitant decline observed in the native range (Seybold et al. 2016). Less suitable hosts include native southwestern United States hosts (*J. major* and *J. microcarpa*), Eurasian species (*J. regia*), Asian butternuts (*J. ailantifolia*, *J. mandshurica*, and *J. cathayensis*), native eastern United States butternut (*J. cinerea*), and Japanese walnut-butternut hybrid (J. *ailantifolia* × *cinerea*). *Juglans* species in the southwestern United States are reported as native hosts of *P. juglandis* (Wood and Bright 1992), so lower levels of reproduction on these hosts suggest that levels of co-evolved resistance to *P. juglandis* may exist (Rugman-Jones et al. 2015). Thousand cankers disease may be another emerging example where escape of an insect herbivore into populations of evolutionary-naïve hosts can facilitate rapid range expansion and widespread mortality to hosts (Haack et al. 2010; Cullingham et al. 2011; Herms and McCullough 2014).

It remains to be seen what the long-term environmental consequences of thousand cankers disease will be on the Juglandaceae resource. Of all species tested, *J. nigra* is the most susceptible to *G. morbida* (Utley et al. 2013). In 2002, the estimated value of *J. nigra* growing stock in the eastern United States was over half a trillion dollars (Newton and Fowler 2009). Aggressive feeding by larvae and adults on *J. nigra*, combined with high susceptibility to the pathogen, and potential host stress induced by local growing conditions, all provide evidence for the rapid decline of *J. nigra* throughout the western United States. Despite this evidence, *J. nigra* in its native range does not appear to be as susceptible to the disease. Initial widespread mortality in the western United States prompted grave concern for all of North America (Newton and Fowler 2009) but in the eastern United States where *J. nigra* is native,

thousand cankers disease appears to progress at a slower rate. In some cases, trees have been able to recover, although recovery varies with site characteristics (Griffin 2015). More research is needed to determine host-selection characteristics of insects on hosts of varying vigor and pathogen interactions across hosts.

Differences in host suitability are readily apparent across several bark beetle and wood-boring insect systems (Svihra and Volney 1983; Holsten and Werner 1990; Veysey et al. 2003; Eager et al. 2004; Lee et al. 2008; Walter et al. 2010; Mayfield et al. 2013; Haavik et al. 2014). Specific mechanisms of insect resistance, such as induced and constitutive defenses, require further study in this system. In our assays, *J. microcarpa*, *J. ailantifolia*, and *P. stenoptera* exhibited the lowest brood production per female, suggesting that potential host resistance to beetle reproduction could be exploited in development of hybrids. In conservation of any resource, it is important to consider its various ecosystem services (Schwenk et al. 2012). *Juglans* spp. are frequently cultivated for commercial nut production, for example, and introducing resistance genes from a species such as *J. microcarpa* that produces small fruit may not be attractive to growers.

In addition to being valuable nut crops, *Juglans* species native to Eurasia and Asia are planted as ornamentals in the United States. *Juglans ailantifolia* Carrière, for example, was introduced from Japan to the eastern states as an ornamental tree (Manning 1978). Its introduced range overlaps with the native North American range of *Juglans cinerea* L. and the two can hybridize (Zhao and Woeste 2011). There are no records of *P. juglandis* colonizing *J. ailantifolia*, *J. cinerea*, or hybrids of *J. cinerea* × *J. ailantifolia* in the eastern United States. If introductions continue to occur however, these species could putatively support *P. juglandis* populations on the landscape (Table 3).

Juglans regia L., native to Eurasia, is widely planted in the western United States and Europe for nut production. *P. juglandis* has colonized commercial *J. regia* orchards throughout California (Yaghmour et al. 2014; Seybold et al. 2016) and northeastern Italy (Montecchio et al. 2014). If *P. juglandis* was introduced from Italy or the United States to the Eurasian native

range of *J. regia* and successful establishment occurs, the eventual spread east could provide a bridge to native ranges of Asian *Juglans*. Previous to our study, it was unknown if *P. juglandis* could develop in Asian butternuts and North American hickories (*Carya*). *Juglans ailantifolia, J. mandshurica, and J. cathayensis* Dode occur in portions of China, Taiwan, Japan, and Korea (Bai et al. 2016). Depending on climate and other abiotic factors in the native range of Asian butternuts, *P. juglandis* establishment in these hosts is possible if introduced to Asia from Europe or North America. Pecan, *Carya illinoensis* (Wangenh.) K. Koch, is native to the southeastern United States and grown widely for nut production (Newton and Fowler 2009). *Carya ovata* (Mill.) K. Koch, also native to eastern North America, is an important food source for wildlife (Grauke). Range expansion via these two *Carya* spp. is unlikely. Our finding that male *P. juglandis* will attempt to colonize *Carya* spp. under no-choice conditions suggests that pathogen transmission could occur, but work by Utley et al. (2013) has indicated that these species are immune to *G. morbida*.

Mate-finding and Allee effects can be important mediators of range expansion for populations of invading insects (Taylor and Hastings 2005). Offspring sex ratio did not vary among *Juglans* hosts, and favored females over males. Sex ratio of offspring may vary with the likelihood of inbreeding among progeny (Kirkendall 1983). For *P. juglandis*, sib-mating (i.e., inbreeding) may occur between brother and sister teneral adults before emergence, or mated females may remain under the bark and extend egg galleries in the natal host before emergence (P.L. Dallara, personal communication). If sib-mating occurs, emergence of mated-females would expedite population growth in nearby hosts.

Brood that developed in *J. californica* (CA), native to the southwestern United States, had the greatest cold tolerance in both years. This host is not found in northern regions of North America, however. For hosts that experience colder winters in their native ranges, *J. nigra* and *J. cinerea*, we did not find that brood exhibited increased cold tolerance. This result suggests that acute cold temperatures may limit *P. juglandis* populations in the most northerly areas of these hosts where average winter temperatures fall between -18°C and -40°C (USDA

2012). Further research would be helpful to examine differences in phloem nutrient concentrations, as metabolized sugars from different hosts may affect supercooling points (Feng et al. 2014).

Given the global distribution of Juglandacae species, a global distribution of *P. juglandis* via several Juglandaceae hosts appears possible. Although the impacts of thousand cankers disease in the United States and Europe vary, the consequences of *P. juglandis* establishing in Eurasia and Asia are unknown. Certainly, the high incidence of human-mediated movement of *P. juglandis* in infested wood increases the risk of future introductions from the United States and Europe to Eurasia and Asia. The impacts of further spread of the disease are highly uncertain and require continued monitoring in global native and adventive *Juglans* and *Pterocarya* stands.

3.6 Acknowledgements

We thank Charles Leslie, Department of Plant Sciences, UC-Davis, Davis, California, John Preece, USDA ARS NCGR, Davis, California, Andrew D. Graves, USDA Forest Service FHP, Albuquerque, New Mexico, and the UMN Landscape Arboretum, Chaska, Minnesota, for assistance with procuring host material for testing. We thank Cliff Beumel, Director of Product Development, Sierra Gold Nurseries, Yuba City, CA for access to a commercial walnut orchard for collection of walnut twig beetles; and University of Minnesota undergraduate research assistants, Joe Pohnan and Collin Smith. Funding was provided by the NSF-IGERT Risk Analysis of Introduced Species and Genotypes program at the University of Minnesota (DGE-0653827) and a USDA-Forest Service Special Technology Development Program grant (R2-2012-01) that was administered by Jeffrey Witcosky and Stephanie Stephens, USDA Forest Service, Lakewood, CO.

3.7 Tables

Table 3.1 Species, collection month and year, source, and number of trees sampled in 2014 and 2015 from U.S. states to study *Pityophthorus juglandis* host effects of male establishment, suitability, and brood cold-tolerance.

Species	Collection	Source	n
Juglans microcarpa	Jun-2014	Hays County, Texas (between Five Mile Dam: 29°56'26.6"N 97°54'07.2"W and Blanco Shoals: 29°54'24.0"N 97°53'43.4"W)	10
Juglans nigra	Jun-2014	Center for Agroforestry, Missouri	10
Carya illinoensis	Jun-2014	Center for Agroforestry, Missouri	10
Juglans nigra	Jul-2014	HTIRC, Indiana	10
Juglans cinerea	Jul-2014	HTIRC, Indiana	10
Juglans cinerea × *ailantifolia*	Jul-2014	HTIRC, Indiana	9
Juglans ailantifolia	Jul-2014	HTIRC, Indiana	9
Carya ovata	Jul-2014	HTIRC, Indiana	4
Juglans major	Jul-2014	HTIRC, Indiana	9
Juglans regia	Jul-2014	HTIRC, Indiana	5
Juglans ailantifolia	Jul-2014	UMN Landscape Arboretum, Minnesota	1
*Juglans cathayensis**	Jul-2014	UMN Landscape Arboretum, Minnesota	6
Juglans cinerea × *ailantifolia*	Jul-2014	Umore Park, Minnesota	1
Juglans major	Aug-2014	Wolfskill NCGR, California	12
Juglans regia	Aug-2014	Wolfskill NCGR, California	10
Juglans mandshurica	Aug-2014	Wolfskill NCGR, California	9
Juglans hindsii	Aug-2014	Wolfskill NCGR, California	10
Juglans californica	Aug-2014	Wolfskill NCGR, California	10
Pterocarya stenoptera	Sep-2014	Wolfskill NCGR, California	10
Juglans nigra	Jul-2015	Center for Agroforestry, Missouri	10
Carya illinoensis	Jul-2015	Center for Agroforestry, Missouri	10
Juglans nigra	Jul-2015	HTIRC, Indiana	10
Juglans cinerea	Jul-2015	HTIRC, Indiana	10
Juglans cinerea × *ailantifolia*	Jul-2015	HTIRC, Indiana	9
Juglans ailantifolia	Jul-2015	HTIRC, Indiana	9
Carya ovata	Jul-2015	HTIRC, Indiana	5
Juglans regia	Jul-2015	HTIRC, Indiana	5
Juglans major	Jul-2015	Wolfskill NCGR, California	10
Juglans californica	Jul-2015	Wolfskill NCGR, California	10
Juglans ailantifolia	Aug-2015	UMN Landscape Arboretum, Minnesota	1
*Juglans cathayensis**	Aug-2015	UMN Landscape Arboretum, Minnesota	6
Juglans cinerea × *ailantifolia*	Aug-2015	Umore Park, Minnesota	1
Juglans microcarpa	Aug-2015	Chaves County, New Mexico (33.378484°N, 104.763235°W Approx. 1250 m elevation)	8

* Species identity in question. The trees sampled were large, healthy trees. These species are *J. cathayensis* according to records at the UMN Landscape Arboretum; however, this species is not well adapted to grow outside of temperate Mediterranean climates. *Juglans mandshurica* is a more cold-hardy species that would grow well in Minnesota.

Table 3.2 Summary of ANOVA results for tests of species effects on various metrics of reproduction of *Pityophthorus juglandis* across species of Juglandaceae, 2014 and 2015

Year	Response variables	*F*	*df*	*P*
2014 (early summer)	total brood per female	11.2	8,64.34	0.001
	adult brood per female	8.33	8,63.18	0.001
	immature brood per female	9.96	8,63.62	0.001
2014 (late summer)	total brood per female	3.55	5,49	0.008
	adult brood per female	3.07	5,49	0.017
	immature brood per female	3.65	5,41.27	0.008
2015	total brood per female	6.37	9,69	0.001
	adult brood per female	3.43	9,69	0.002
	immature brood per female	3.17	9,69	0.003

Table 3.3 Summary of *Pityophthorus juglandis* host assays, 2014 and 2015. Two definitions were examined for each species: Growth and development to the adult stage and production of female offspring that equaled or exceeded the number of female parents introduced

Species	Growth and development of adult offspring?	Female parent replacement?
Juglans nigra	Yes	Yes
Juglans cinerea	Yes	Yes
Juglans cinerea x *ailantifolia*	Yes	Yes
Juglans ailantifolia	Yes	Yes
Juglans cathayensis	Yes	Yes
Juglans mandshurica	Yes	Yes
Juglans major	Yes	Yes
Juglans regia	Yes	Yes
Juglans hindsii	Yes	Yes
Juglans californica	Yes	Yes
Juglans microcarpa	Yes	Yes
Pterocarya stenoptera	Yes	Yes
Carya illinoensis	No	-
Carya ovata	No	-

3.8 Figures

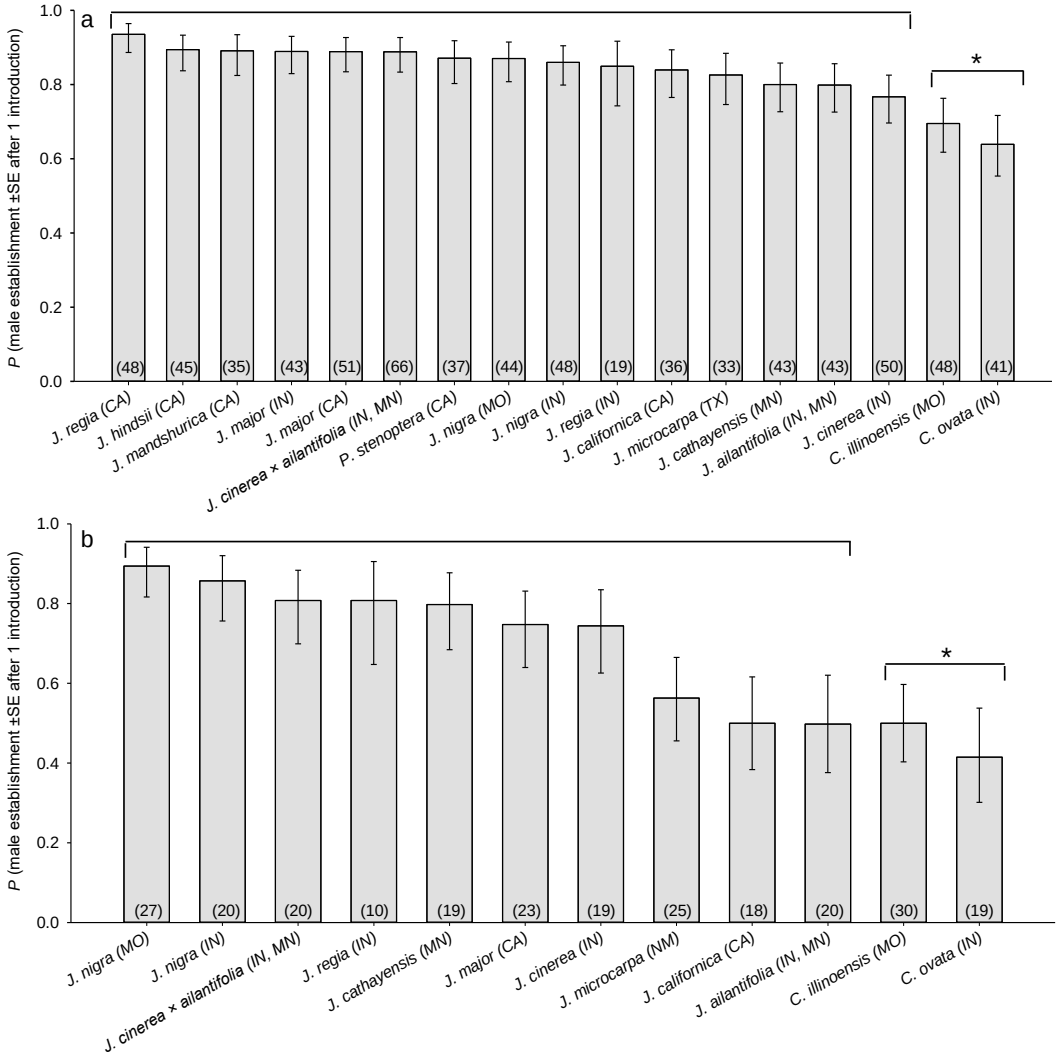

Figure 3.1 Likelihood (± SE) of male establishment after one introduction in species of Juglandaceae (a) in 2014 (significant differences in the likelihood of male establishment after one introduction between hosts and non-hosts; *$\chi 21 = 13.67$, $P < 0.001$) and (b) in 2015 (*$\chi 21 = 9.43$, $P = 0.002$; $\alpha = 0.05$; n is noted at the bottom of each bar in parentheses)

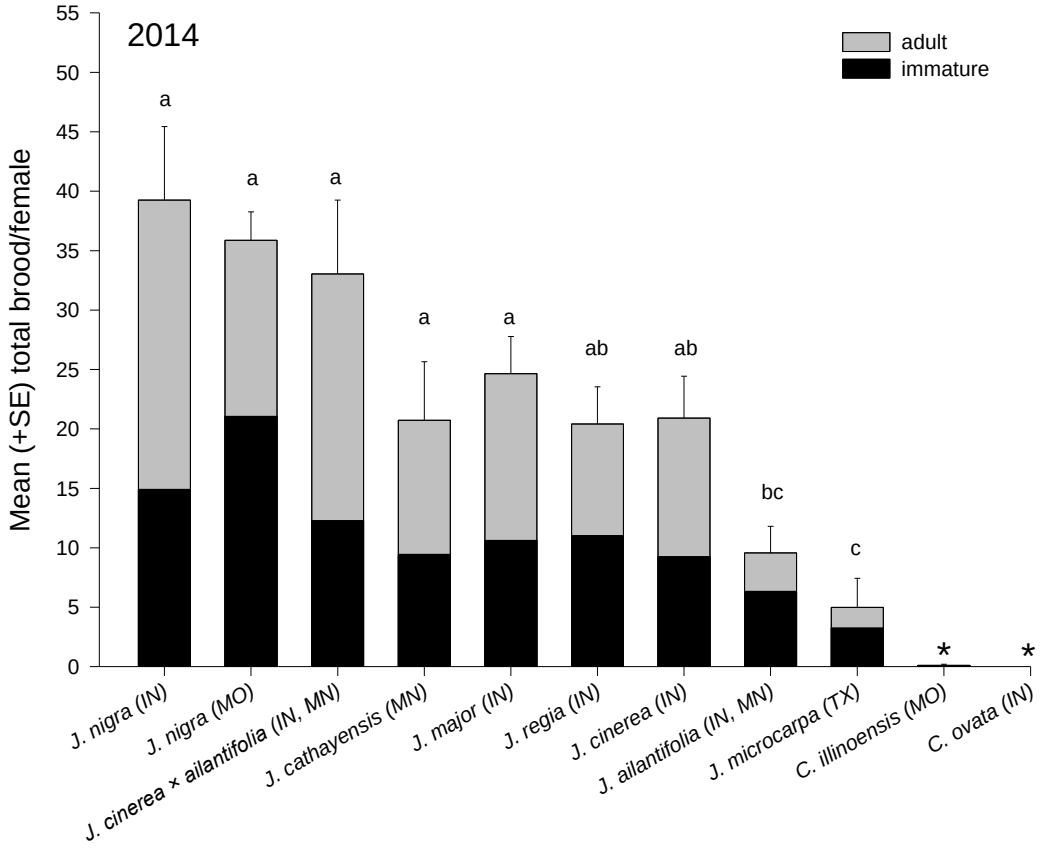

Figure 3.2 Mean (+SE) total offspring per female, adult per female, and immatures per female (larvae and pupae) for species collected in 2014. Means with different letters indicate significant differences at α = 0.05 for total offspring per female

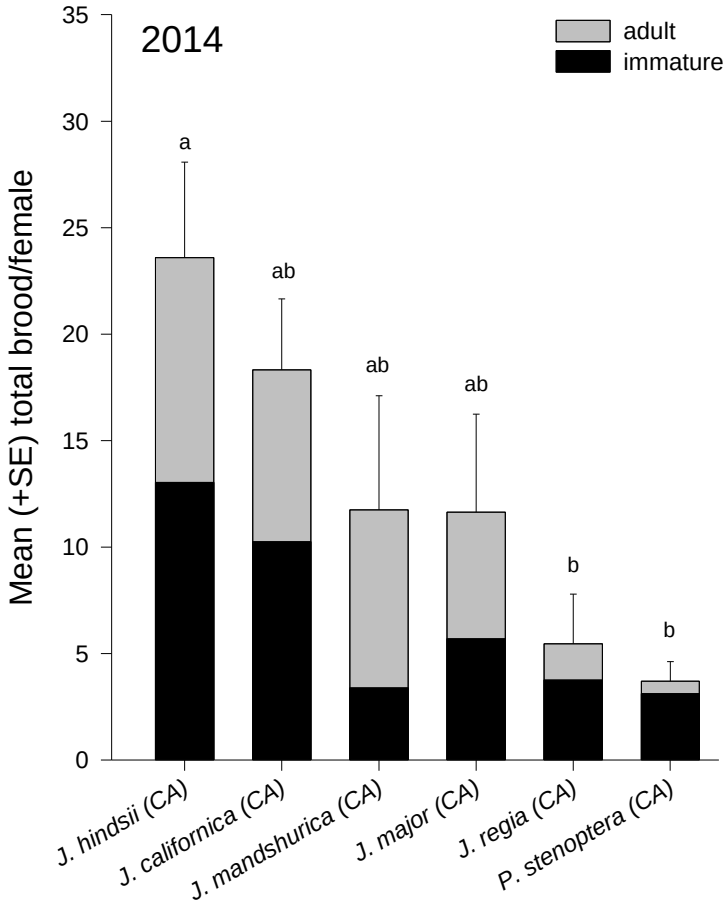

Figure 3.3 Mean (+SE) total offspring per female, adult per female, and immatures per female (larvae plus pupae) for species collected in California in 2014. Means with different letters indicate significant differences at α = 0.05 for total offspring per female

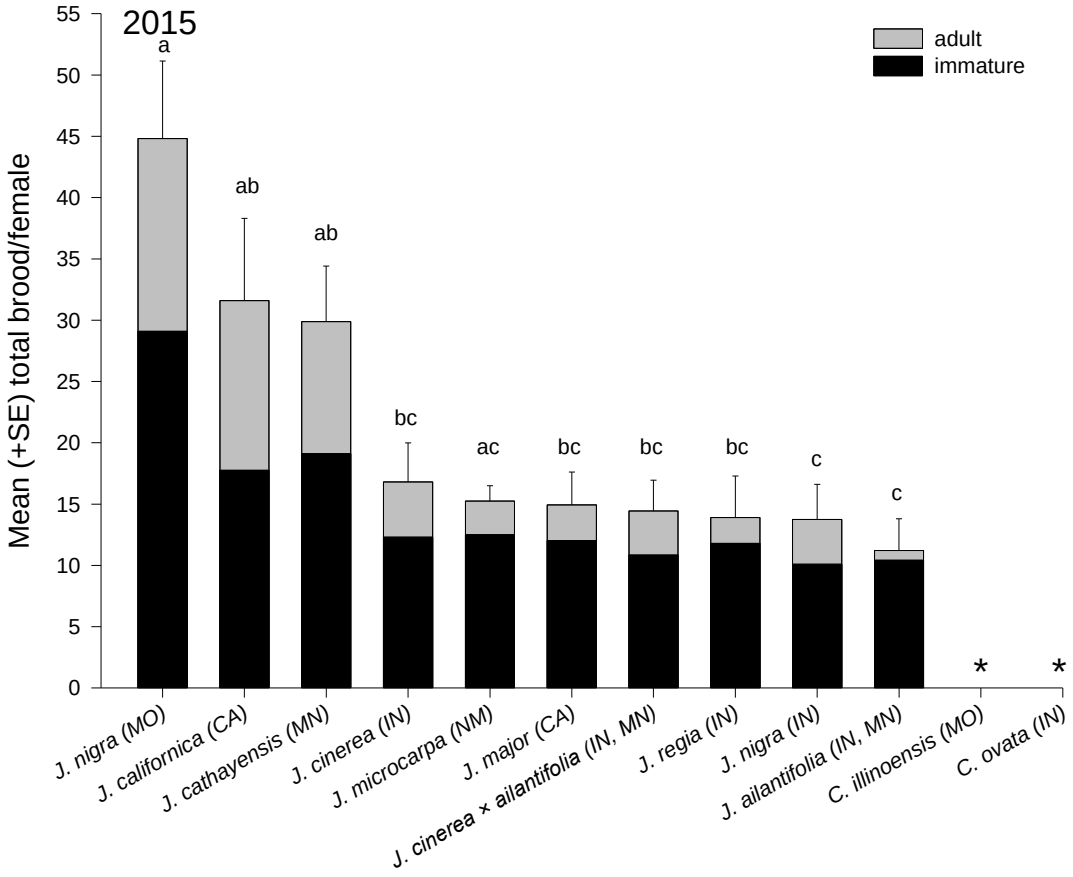

Figure 3.4 Mean (+SE) total offspring per female, adult per female, and immatures per female (larvae plus pupae) for species collected in 2015. Means with different letters indicate significant differences at α = 0.05 for total offspring per female

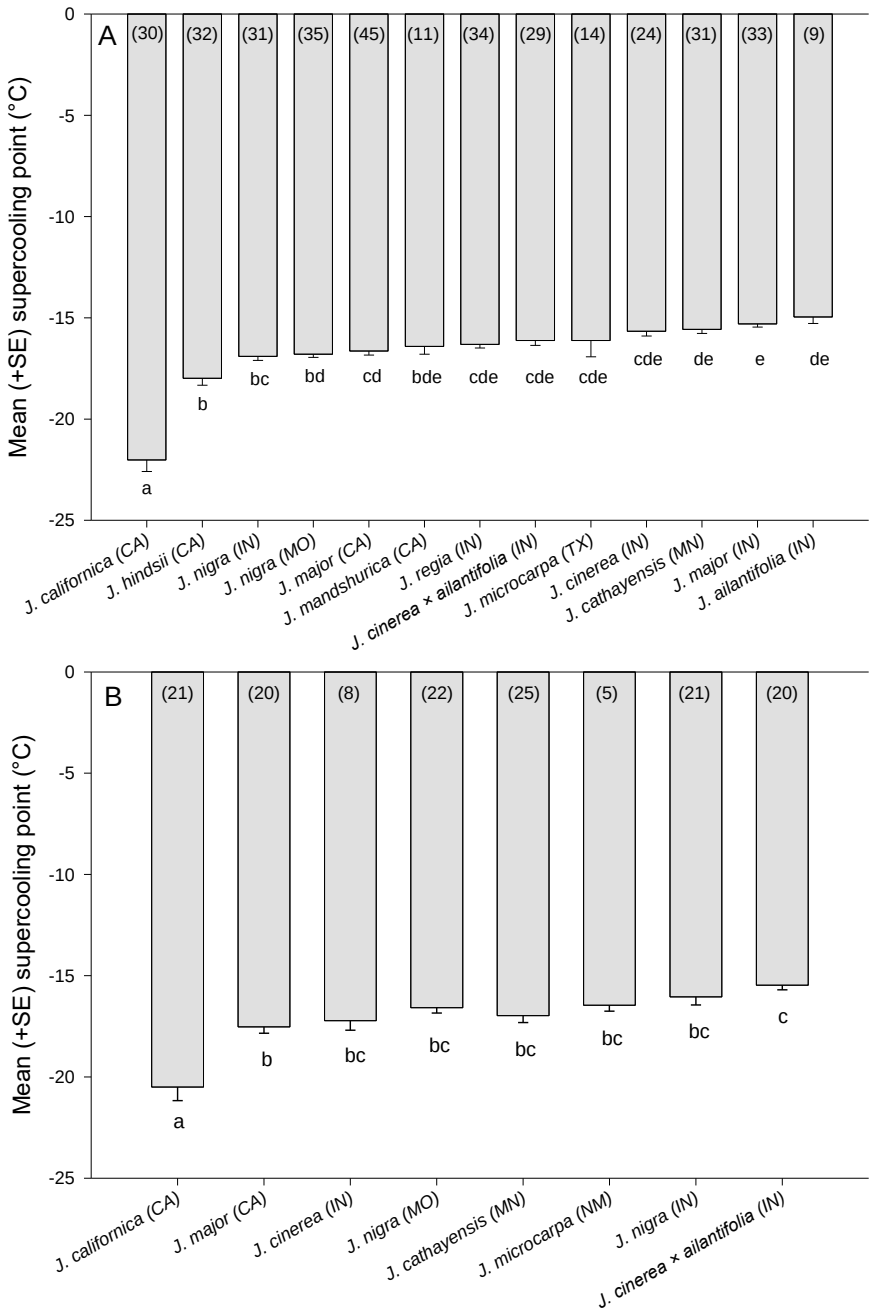

Figure 3.5 Mean (+SE) supercooling point by species infested in (a) 2014 and (b) 2015 (n is noted at the top of each bar in parentheses). Means with different letters indicate significant differences at α = 0.05

4 Chapter 4 - Unspecified pathways of invasive forest insects: A framework for accessing stakeholder knowledge to identify pathways

4.1 Introduction

As a result of increasing global trade and travel, wood-boring pests are being introduced at increasing frequencies to forest ecosystems (Aukema et al. 2010). Throughout the 20th and 21st centuries, international policies and regulatory efforts were implemented in the United States of America (USA) to detect and decrease the entry and establishment of insects and pathogens. Non-profit groups have also attempted to decrease the spread of pest species nationally through public education campaigns. Media encourage key stakeholders to stop moving firewood, for example; a pathway that was once unspecified by state agencies. Decreasing the movement of pest species depends upon managing pathways that present the greatest risk for that particular species' spread. Yet, the pathways may not be well-characterized using traditional assessment methods or well-managed by existing regulatory and educational mechanisms. Therefore, I present a framework that relies on stakeholder knowledge to identify pathways through which forest pests may be moved. Methods to rank pathways and test their validity are outside of the scope of this chapter; therefore, the stakeholder participation process is discussed in detail. The framework is intended to reveal specific pathways that may not be evident from a national or regional perspective. Within pathway risk analysis (i.e., risk of movement of invasive species), this framework may be utilized if human-mediated spread contributes to frequent range expansions and is determined by risk managers to be a likely pathway of a destructive organism (U.S. Environmental Protection Agency 1998). This process assumes that time and resources are available to a risk manager to complete the framework within the timeline of the risk assessment.

Upon detection of a non-native insect population, there are knowledge gaps in life history, host range, genetic transfer, environmental tolerance, spread capability, and future ecological and economic impacts. Given the unknown probability of invasion and the unknown consequences of invasion, pest risk analysis has become a useful tool to characterize the level

of risk (e.g., through gathering information) of an invasive species that leads to policy or action recommendations (Devorshak 2012).

Simply, risk is defined as probability of an unwanted event multiplied by the consequence of that event (Yoe 2012; Fig. 4.1). Risk analysis is used in several industries, such as in the fields of human health and environmental science. The process of risk analysis is iterative and is intended to describe what the risks are (i.e., risk assessment), manage the risks that are unacceptable (i.e., risk management), and communicate them (i.e., risk communication) (Yoe 2012).

4.1.1 How is risk defined in pest risk assessment?

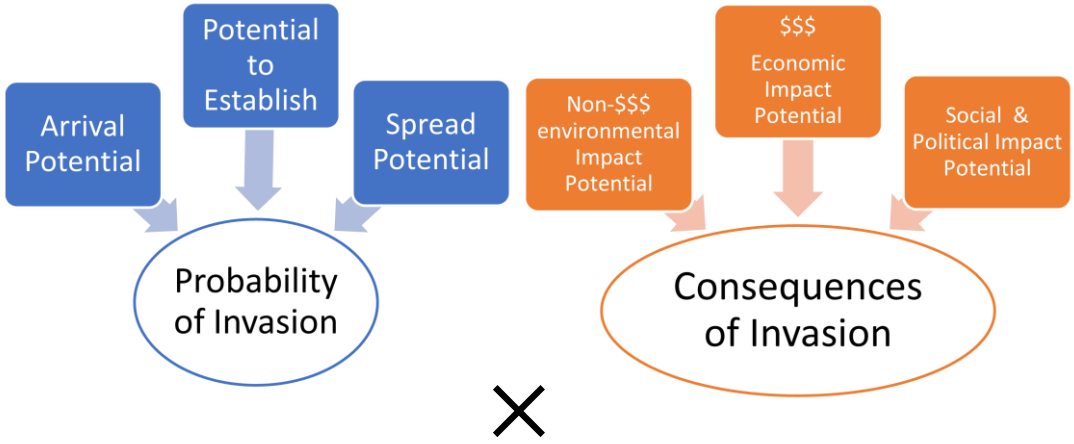

Figure 4.1 Definition of risk used in pest risk assessment. The probability of invasion is influenced by three factors, spread potential, arrival potential, and the potential to establish where the consequences of invasion are the sum of the environmental impact, economic impact, and social and political impact potential. Empirical data, predictive models, and in some cases expert judgement are used to define each component (adapted from Venette, 9/13/11 and Orr et al. 1993).

In pest risk assessment, the unwanted event is invasion of a nonnative species (Venette et al. 2010). Different factors affect the probability and consequence of invasion (Fig. 4.1).

4.1.2 Human-mediated movement of pests

Generally, invasion of an organism (e.g., insect, plant, fungus, bacterium) is initiated by intentional or unintentional transport (via a vector; see definition below) from its native range to release in a novel habitat. This movement can be due to natural colonization or human-mediated movement. Compared to natural colonization, human-mediated movement generally occurs faster and over greater distances than that of which an organism is capable naturally. In the USA, there has been a positive increase in accumulation rate of forest insect pests that result in economic burden (Aukema et al. 2010, 2011). Frequently, however, most introductions do not result in establishment and spread and result in no impact (see Tobin 2015 for discussion of this topic).

4.1.3 Pathways and transport vectors

There are competing uses of the terms ‘pathway’ and ‘transport vector’ in invasion biology literature (Everett 2000; Stanaway et al. 2001; McCullough et al. 2006; Liebhold et al. 2012). In this chapter, I draw definitions and ideas from Lockwood et al. (2013) who defines a transport vector as “the manner in which species are carried along a pathway, and a pathway as the route between the source region of a non-native species and its location of release” (pg. 25). For example, while some authors identify firewood as a pathway, I consider firewood to be the transport vector and the pathway as the means for movement of the firewood from one place to another. For example, the pathway associated with firewood might be a physical route, such as a road or highway, and/or a commercial route, such as an online sale or private exchange.

4.1.4 Regulated pathways

Here, I define regulated pathways as pathways that are currently under surveillance by a governmental agency or international organization for which the agency/organization has the authority to create and enforce policy related to the pathway itself or to the movement or treatment of goods that move on that pathway. The agency can be state or federal, may cooperate across national borders, and can monitor transport across land, air, and sea. Regulated pathways are also human-mediated pathways because the pathway is often there for

human purposes. For example, a flight path (i.e., pathway) could be traveled by a plane with baggage (i.e., vector). A human, for example could pack a few snails to market as the “latest and greatest” escargot. The snail would not be able to move 2000 miles across an ocean on its own; therefore, the long-distance movement, as is often the case for wood-boring insects, would be human-mediated.

As international trade increased throughout history, governments became responsible for regulating the movement of pest species. For example, nations that participate in international trade of commodities that could move pest species have created policy in response to introductions and impacts of non-native organisms on important crops (i.e., potato and grapes in 18th Century Europe). In 1875, Germany was the first country to regulate the pathway of a serious pest (Colorado potato beetle). In 1878, in Europe, the first international agreement to prevent the spread of a pest was created. This agreement provided the basis for future policy regarding the exchange of information concerning a pest, limiting movement of materials, and certification of treatment or inspection. From the 1800s to the 1900s, globally, laws became more general and broadly addressed the movement of non-native species. In the 20th Century, after several revisions, the International Plant Protection Convention (IPPC), under the Food and Agriculture Organization (FAO) of the United Nations, developed the international language for pest risk analysis (Devorshak 2012). Specifically for the movement of plants and organisms, international standards for phytosanitary measures (ISPMs) (https://www.ippc.int/en/history-of-the-ippc/ accessed 3/23/2016) brought together individual country policies with international standards. This multi-national agreement still stands as 182 countries have agreed to be compliant with IPPC policies (https://www.ippc.int/en/countries/all/list-countries/ accessed 3/23/2016).

The United States Department of Agriculture (USDA) created the Animal and Plant Health Inspection Service (APHIS) in 1972 to make decisions and regulate commodities that pass over the U.S. border to mitigate the number, frequency, and subsequent impacts of pest species. APHIS now has partnerships with several working groups within the USDA Forest

Service to provide more “technical assistance” on “exotic forest pests that may move with logs, chips, and similar green wood products” (http://www.fs.fed.us/foresthealth/management/fhm-invasives-woodimport.shtml accessed 3/22/2016). Specifically, the Forest Service, *via* The Wood Import and Pest Risk Assessment and Mitigation Evaluation Team (WIPRAMET) provides APHIS with risk assessments on any wood product of interest for importation. Another partnership between APHIS and the Forest Service, specifically to produce pest risk maps (PRM), is the Forest Health Technology Enterprise Team (FHTET) and the Eastern Forest Environmental Threat Assessment Center (EFETAC).

While it is straightforward to define the concept of a regulated pathway, it is considerably more challenging to identify and prioritize actual pathways and to regulate them, particularly for newly identified pests. To regulate a pathway, it has to be first identified as a pathway (e.g., international flights, shipping routes, sales delivered by mail). Once the pathway is named, research on the pathway, vector, and pest organism must determine the strength of that pathway (i.e., how many species or individuals are being moved by a vector and at what frequency that occurs). If evidence is found that the pathway is strong, to regulate the pathway agencies must agree that 1) the pathway can transport viable organisms; and 2) those organisms can cause significant economic loss or environmental degradation to resources (i.e., agricultural or natural areas). Only when the pathway is recognized and prioritized can new policies be proposed, adopted, and eventually enforced. The framework proposed here helps to identify and prioritize pathways systematically, so as to regulate them more strategically.

4.1.5 Unregulated pathways

Although APHIS has broad regulatory authority to take necessary action to manage risks posed by invasive insects, I define unregulated pathways as those that are not monitored by an agency that could create policy and enforcement to manage the pathway itself or the vectors that move along the pathway. Unregulated pathways can be known (i.e., identified) or unknown (i.e., unspecified) to a regulatory agency. A systematic framework to address both is needed. There is strong evidence for movement of some forest pests on unspecified pathways

or pathways that were previously identified but unregulated. For example, movement of infested wood was recognized as a potential vector of the pathogen that causes Dutch elm disease as early as the 1950's, yet no regulations were passed until the 1970's (French 2013).

The risk to forests of moving firewood became clearer when the emerald ash borer was discovered in Michigan and Ontario in 2002. As a result of the spread of EAB, millions of ash trees have died, both in urban and natural forests. Satellite populations have been consistently discovered from human-mediated movement of firewood or nursery stock (Herms & McCullough 2014). A surge of research on insects that can be moved by firewood as well as associations between human and emerald ash borer spread and pathways (e.g., road networks, campgrounds, and human population density) resulted from the EAB invasion (Prasad et al. 2010; Jacobi et al. 2011, 2012; Koch & Yemshanov 2012; Peterson & Diss-Torrance 2012; Koch et al. 2014). Engagement with diverse stakeholder groups to educate them about emerald ash borer's pathways and to deliberate with them about management options and their trade-offs has helped regulating agencies hone their strategies to increase buy-in from the key stakeholders whose cooperation is needed to limit emerald ash borer's spread (Dunens et al. 2013; Kovacs et al. 2014). State and federal governments currently manage emerald ash borer via quarantines, inspections of firewood distributors, and certified "clean" wood programs in areas with high camper activity (i.e., state parks) to minimize further movement of emerald ash borer through firewood. The goldspotted oak borer, related to emerald ash borer, is responsible for the widespread decline of three native oak species in southern California. USDA Forest Service researchers predict that long-distance movement from native ranges in Mexico to the United States is likely due to oak firewood movement on roads and highways (Coleman & Seybold 2011). Wood-boring insects like emerald ash borer and goldspotted oak borer were the catalysts for the regulation of firewood nationally.

The detection of wood-boring insects outside of their native range provides evidence that other unspecified pathways exist. For example, a wood-boring insect, the walnut twig beetle (WTB), is native to the southwestern USA, but has expanded its range over multiple

introductions into the northwestern and eastern USA (Rugman-Jones et al. 2015). It is unknown how many introductions have occurred (Tisserat et al. 2009). One new establishment, in Pennsylvania was from a known infested log, left outside and untreated (i.e., no pre-treatment). This log had been sent from an infested area of California (Chico) (Turcotte et al. 2013). In 2013, walnut twig beetle was discovered in Italy in black walnut and has since been found there in English walnut as well (Montecchio & Faccoli 2014; Montecchio et al. 2014). Firewood is a vector for this insect. There is evidence of another stakeholder group, however, namely woodworkers, who have the motivation to move wood for different reasons than people who move it as firewood (Campbell and Schlarbaum 2014).

Participation from stakeholders is critical to both assess and manage a pathway and vector. We are just beginning to understand the motivations and behaviors around people who move firewood, but what about wood workers? Woodworkers are an example of stakeholders who may have knowledge of currently unspecified pathways where the pathways may present significant risk, and are under-recognized. Woodworkers comprise a widespread stakeholder group with 11,000 members of the American Association of Wood Turners throughout the USA and members of the International Wood Collectors Society in 30 countries. They have a strong motivation to move wood, of multiple species, over long distances. They have a different purpose for wood than campers do for firewood (i.e., the relationship with the vector is unique). We do not know the common practices for trading, selling, buying, and exchanging of wood through private networks. Woodworkers can serve as future outreach groups within and outside of their networks, which regulatory agencies are not able to access. Finally, woodworkers are willing to participate in campaigns to stop the movement of destructive forest pests (F. Campbell, personal communication 2011).

Innovative interdisciplinary basic and applied research is needed to characterize such invasion pathways. Accessing stakeholder groups who have a relationship with a vector are key to understanding the overall risk of that pathway. In some cases, these pathways will be previously identified. For example, in the case of walnut twig beetle, a risk assessment for its

movement eastward lists undocumented sales of raw wood sold over the internet as a potential pathway of concern (e.g., Craigslist) (Newton and Fowler 2009). For other organisms, there may be unspecified pathways that can only be revealed *via* stakeholder knowledge. The framework I present here provides strategies for accessing that knowledge.

4.1.6 Why access stakeholder knowledge to characterize invasion pathways?

Assessing environmental risk has routinely been performed by risk professionals whose methods reflect technical expertise and expert knowledge (i.e., ecological modeling). By accessing stakeholder knowledge, evaluation of environmental risk moves away from technocratic values toward democratic values. Lay knowledge is characterized by detailed information about a place, thing, or set of interactions based on experience and/or human qualities such as emotions and values, laying the groundwork for more robust and resilient responses to complex problems like invasive species management (Quick & Feldman 2014). Major arguments for involving stakeholders in evaluating environmental risk are:

1. Substantive argument: “nonexperts see problems, issues, and solutions that experts miss.”
2. Normative argument: “…citizens are the best judge of their own interests,”
3. Instrumental argument: citizen participation can improve the risk decision itself (i.e. reduce error, broader range of values) and increase acceptance of the decision (Fiorino 1990).

Broadly, “Risk characterization involves complex, value-laden judgments and a need for effective dialogue between technical experts and interested and affected citizens who may lack technical expertise, yet have essential information. These citizens often hold strong views and substantial power in our democratic society” (pg. 11, Understanding Risk: Informing Decisions in a Democratic Society, 1996). Consequently, if stakeholders are not involved in environmental risk decisions, what is at stake? First, trust in expert knowledge and expert decision-making (Ozawa 2012). Second, the right to know how decisions are being made in society (i.e. the democratic process), and finally, acceptance and support of the decision/policy being made (i.e., legitimacy).

4.2 Framework for accessing stakeholder knowledge to characterize invasion pathways

Risk managers or specialized teams who work on regulated pathways could design and lead the process laid out in this framework from start to finish. This framework starts with a stakeholder participation process (Phase 1). The result of Phase 1 is a list of pathways that are tested independently in the second phase. If a valid pathway is found, the process ends when pathway management begins (Fig. 4.2). To illustrate the scope and use of the framework, I provide some examples and guidance based upon how an entomologist might use it in the case of the walnut twig beetle.

4.2.1 Criteria

Here, the framework is initiated, for example, for a forest pest organism that has three characteristics:

1. The organism is a forest pest (i.e., non-native or native with the potential to or known to cause harm to forests).
2. The organism is an insect or pathogen.
3. There is empirical evidence that there is movement of the insect or pathogen, unrelated to natural spread.

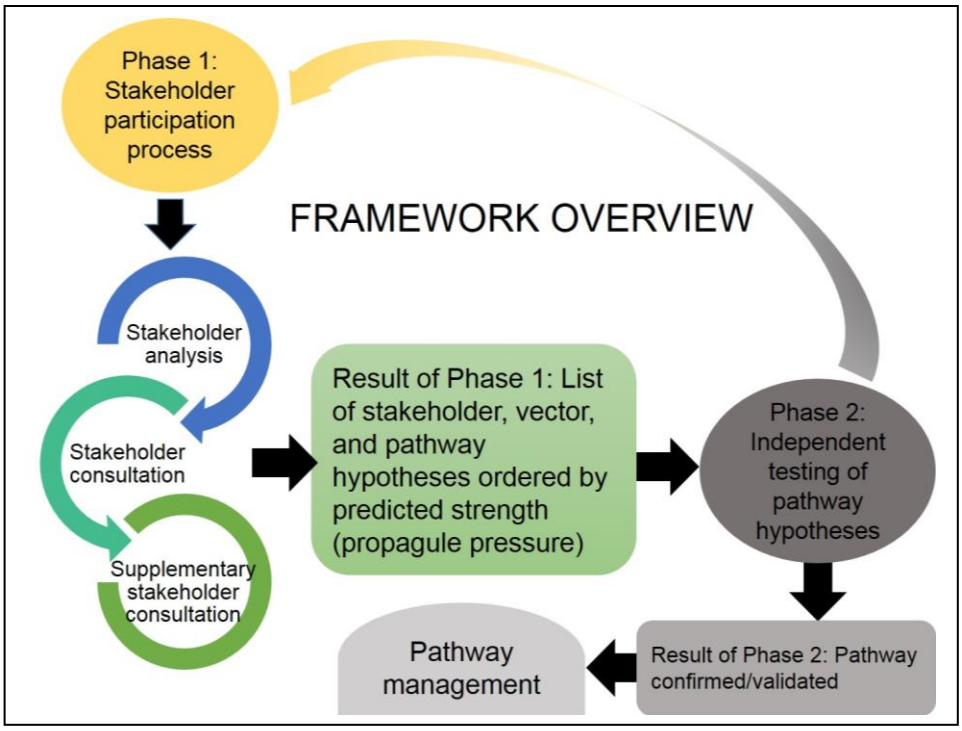

Figure 4.2 Overview of framework for collecting stakeholder-generated pathway hypotheses for validation and management.

4.2.2 Phase 1. Stakeholder analysis

A stakeholder analysis can help focus the purpose for including stakeholders in a decision as well as define what level of participation is required from them (Varvasovszky & Brugha 2000; Bryson 2004). To begin, it is critically important to define the purpose of stakeholder participation (i.e., what do I want to know from stakeholders that I cannot get from other sources? What are some endpoints/goals?). The next step is to consider what kind of influence the stakeholders who participate should have on policy and management, because design of the stakeholder participation process should follow this decision (Bryson et al. 2012). Then, identify stakeholders. Using Bryson (2004), an agency could use a tool (i.e., power versus interest grid) that clearly communicates ideas about who stakeholders are (i.e., identification), how to approach them, and their level of power at each step (i.e.,analysis). Generally, stakeholder categories to examine are:

1. Who might be affected by the decision? Include groups that may be affected but as yet do not know this.
2. Who has information and expertise that might be helpful?
3. Who has been involved in similar situations before?
4. Who has expressed interest in being involved in similar decisions before?
5. Who might be reasonably angered if they are not included?

Before contacting and recruiting stakeholders to participate in the next step, i.e., stakeholder consultations, the team should set the level of participation for involving stakeholders, by revisiting the goals for stakeholder involvement, stakeholder level of influence, available resources, and possible participation modes (i.e., time and funding, viable methods for engaging them, and gather information). This can be done using the IAP2 spectrum (http://www.iap2.org/resource/resmgr/imported/spectrum.pdf, accessed May 27, 2016). Consultation is a common method that facilitates participation to assess unspecified pest pathways. Stakeholders can be involved for the purposes of gathering information and discussing different pathway management options, but they would not be given power to make policy and management decisions. For that purpose, effective participation techniques would include focus groups and surveys to gather information. Consulting the key stakeholders also minimizes promises made by the team, such as listening to concerns and reporting back how the team used their input, as opposed to "we will implement what you decide."

Designing the key stakeholder participation process can be done with the stakeholder analysis or after it is completed (Appendix A). This process is iterative and follows three phases: 1) Assess and design for context and purpose, 2) enlist resources and manage the participation, and 3) evaluate and redesign continuously (Bryson et al. 2012).

4.2.3 Phase 1. Stakeholder consultations and supplementary stakeholder consultation

4.2.3.1 Characterizing invasion pathways

The purpose of this process, summarized in Figure 4.3, is to "enhance understanding of public problems, and explore and generate potential solutions" (Bryson et al. 2012). Here, I define the "public problem" as the movement of forest pests on unspecified pathways and the "potential solution" as a list of invasion pathway hypotheses generated by stakeholders. The

consultation leader would be someone who has a background in ecology and risk assessment. This person would facilitate the stakeholder consultation session or recruit and train additional facilitators if the group is so large that it should be broken into several smaller discussion groups. This person needs to train facilitators to ensure that the facilitator can guide the discussion to suit the desired purpose (i.e., to gather input, or to consult, or to give decision-making authority to the participants), that groups are able to understand the technical issues and the process well enough to have productive discussions, and that they can manage the time to walk through a series of questions and thoroughly document the output. Potential methods are as follows:

1. Whole group, led by a technical expert:
    a. Work through Table 4.1 for each probable vector
    b. Educational component that requires some biological information
        i. For example, what life stage is being moved? How is the life stage surviving the pathway (i.e., is the vector a plant?)
2. In small groups, led by facilitators or technical experts:
    a. Why is the vector moved?
    b. How is the vector moved?
    c. How often is the vector moved?
3. Identify other stakeholders that would want to move the vector
    a. Who else has a specific relationship with the vector?
4. Name the regulated pathways
5. Name the unspecified pathways
6. Group determines how confident they are in their answers (understanding the level of uncertainty can help the risk manager set an overall acceptance level of uncertainty)
    a. Can be done with a scale of “certainty” for all or some judgements made in steps 1-4
7. Finalize list of pathways that will be passed on to researchers

If stakeholders in the first consultation round identify a secondary group of stakeholders who could provide more information, then a supplementary stakeholder consultation can be completed (Fig. 4.3).

Table 4.1. Example list of stakeholder, vector, and pathway relationships that form an invasion pathway. Read from left to right or right to left, vectors associated with a particular stakeholder group move along a regulated or predicted unspecified pathway.

Stakeholder	**Transport Vector**	**Regulated pathway**	**Unspecified pathway**
Campers, homeowners	Firewood	Overland (roads, highways)	Internet sales, private network exchanges
Manufacturers, buyers who use to ship their own goods in WPM	Wood packaging material (WPM)	Shipping routes (land, air, and sea)	Salvage, sales after arrival
Wood turners/woodworkers, hobbyists, artists	Wood with bark on (e.g., burls, logs)	Shipping routes (land, air, and sea)	Trade shows, internet sales, private sales or exchanges
Car owners, truck drivers	Vehicles (personal-use or commercial-use: shipping/commerce)	Shipping routes (land)	?
Fisherpeople, recreators, shipping companies	Boats (recreational-use, shipping/commerce)	Transport to/from lakes (overland), shipping routes (sea)	Dumping bait or ballast water
Landscapers, cities, urban forestry, private citizens	Wood chips	Shipping routes (land, air, sea)	Internet sales

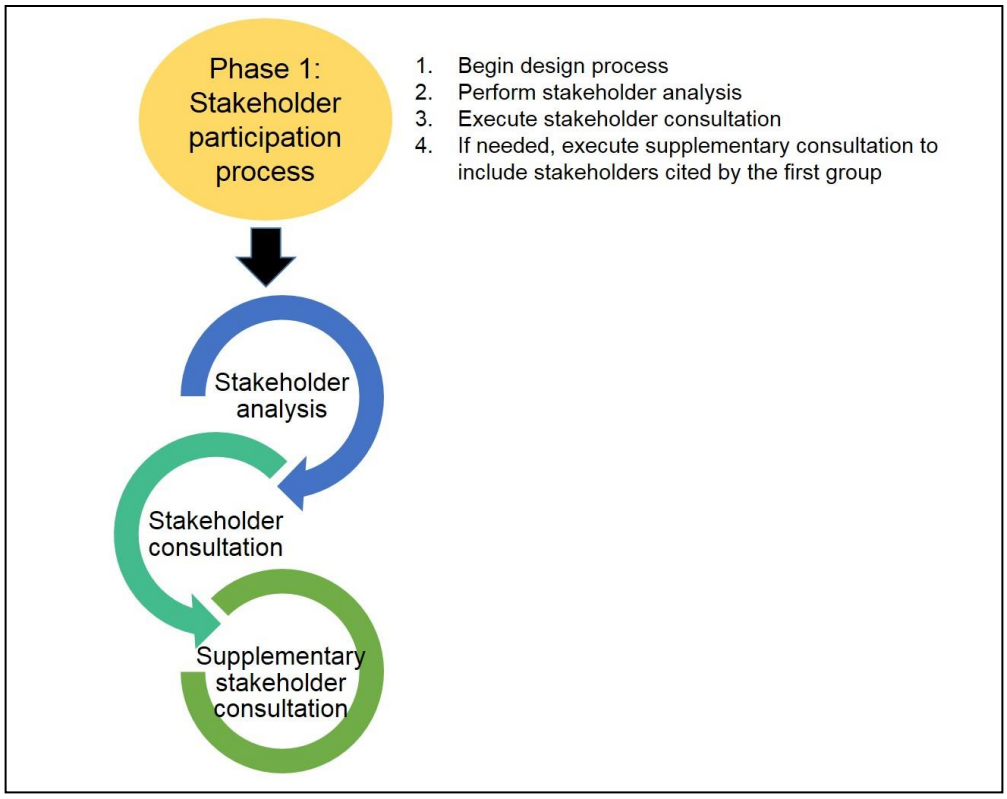

Figure 4.3 Overview of Phase 1 steps. Stakeholder analysis is an important first step to ensure that the process (group sessions 1 and 2) is inclusive so that the desired outcome captures diverse cultural, economic, and social perspectives.

The results of Phase 1 may vary. However, if the design is done well (Appendix A), the result could contribute novel knowledge of invasion pathways and ideally, would lead to stakeholder-supported pathway management. It is important to develop all parts of the process before stakeholders are involved, make it an iterative process, and redesign as needed. A “dry run” of the design may be helpful to work out timing, clarity of purpose, educational components, phrasing questions, and recording ideas (i.e., test all steps, evaluate [did the process produce the desired outcome?], and then re-design).

4.2.4 Phase 2. Independent testing of pathways

The invasion pathways generated by stakeholders have multiple entry points for research to validate:

1. Existence and characteristics of pathway,

2. The propagule pressure of one or multiple species of concern, and
3. Potential management interventions of the pathway, vector, or both for individual pathways

For example, tree burls are frequently used in woodworking. If they were identified as a vector for the species being assessed, researchers could focus on sampling burls for insect species characterizing their diversity, estimating the number of viable individuals (Jacobi et al. 2012). For burls, if artists are the stakeholder and private exchange at trade shows reflects the predicted unspecified pathway, then surveying artists to determine motivation and acceptable interventions would provide pathway characteristics and acceptable management strategies. After devising time and effort of each research entry point, the team might decide to go back to Phase 1 to clarify pathways (Fig. 4.4). The iterative nature of the design process allows new research on the organism of concern to be easily integrated when it becomes available. If an invasion pathway is validated and requires management than the team can make recommendations for management of the pathway, vector, or both (Fig. 4.5).

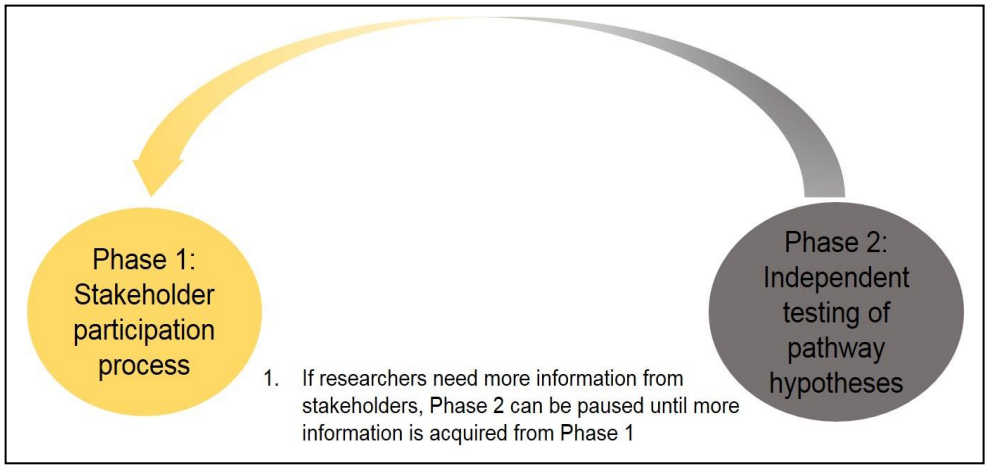

Figure 4.4 If the pathways (i.e., result of Phase 1) are missing information or need more explanation to be investigated than Phase 1 can be modified and repeated as needed.

4.2.5 End: Pathway management

A regulatory agency (state or federal) would be the most likely lead actor for managing the pathway or vector. However, other stakeholders clearly have a central role in effectively managing the spread of the pest. Validating a new invasion pathway for management

generated by a stakeholder group, using this proposed framework, is a significant discovery for several reasons. First, there is a possibility that stakeholders would be more likely to be involved and accepting of new management policies, leading to higher compliance. Second, during the process there could be opportunities to develop dynamic management policies with stakeholders. The goal of these policies would be to adjust management actions to the dynamic nature of the pathway to suppress the level of propagule pressure over time. Finally, the movement of the organism is minimized sooner than if the pathway remained unidentified and unmanaged, leading to a valuable savings of ecological and economic resources.

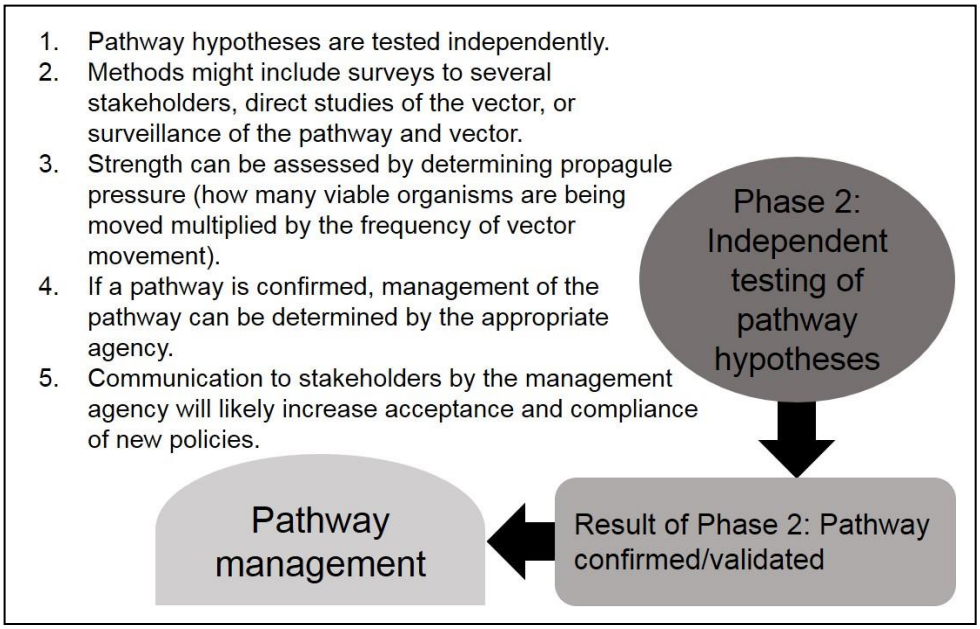

Figure 4.5 Validating pathway hypotheses may lead to confirmation that a pathway exists. Once the pathway is confirmed, a management agency determines the appropriate strategy to communicate and implement new policies.

4.3 Conclusion

Destructive forest insects will continue to threaten valuable forest resources worldwide. The spread of non-native insects has taught us that urban and natural forests are at-risk ecosystems and that knowledge of invasion pathways is important to manage that risk. Working with key stakeholders is critical to identify and prioritize unspecified pathways of invasive forest pests. It allows managers to gain the otherwise inaccessible knowledge that stakeholders have

due to their relationship with a vector. In addition, working with stakeholders at the risk assessment stage provides valuable insights not only about the pathways and their strength, but also about the stakeholders' behavior and preferences. While managers have the primary expertise and responsibility to create policies and management plans to contain invasive species, actually making those strategies effective depends on the participation of the stakeholders involved in those pathways. Therefore, it is important to assess not only what stakeholders are currently doing, but how they would likely respond to different policies and management options as a foundation for designing and mobilizing the most effective options. The framework outlined in this paper is a new tool to gather stakeholders' uniquely valuable knowledge about unspecified pathways. This framework outlines an innovative method that can be used in pest risk analysis that uses stakeholder analysis and stakeholder engagement design principles to strengthen the management of new and continuing spread of non-native species.

4.4 Appendix A

4.4.1 Recommendations for planning the participation process (as per Bryson et al. 2012)

Following the design process for stakeholder participation discussed in Bryson et al. (2012), I outline ideas under each guideline to consider when applying this framework. This outline follows their order, although as the authors state, it is not important that the order be followed but that each guideline be considered carefully and redesigned as the process demands. This is meant to be used as a "quick guide" for what to consider when working through the design process, as well as a justification/purpose for each step.

4.4.2 What is the context of the problem?

In this framework, key stakeholder participation is desired because there is a problem of human-mediated movement of invasive forest insects on unspecified pathways. The definition and understanding of the problem will be further explored and dissected with stakeholders; however, designing the process with a clearly stated problem is important so that,

1. The correct problem is addressed

2. The solutions that are developed do not create the problem that they were meant to solve (i.e., a new pathway is created as a result of fear of increased regulations, etc.)

Ideally, possible solutions can be explored before the “real” problem is understood. That is explore pathway origins before management and educate stakeholders on added risk of human-mediated pathways before management occurs.

The basic design concept is this: different problems will have different solutions. The participation process should be tailored to assist with developing those solutions. Therefore, designing for context and purpose will clarify,

1. What is the nature of the problem?
2. How are stakeholders going to participate in coming up with solutions to the problem?

4.4.3 Purpose and desired outcomes

The purpose of this process is to gain information from stakeholders and to some degree, manage uncertainty around the spread of an invasive species through space and time. *What is the appropriate time and level of involving stakeholders during this process?*

The strategy for engagement for this process is to inform, but this might shift at the end when management is discussed (if hypotheses are not tested immediately following stakeholder sessions). If stakeholders are interested and are empowered to do so, management could be a collaborative effort between regulatory agencies and stakeholders or citizens. There are several reasons why stakeholders would not be willing to participate in management efforts for invasive species because these are generally restrictive actions or can cost the stakeholder extra time or money to make a vector safe for transport. Actions will likely limit some aspect of the stakeholders freedom. For example, before they participated, they may have been free to partake in a particular behavior, such as shipping wood on Craiglist to family members on the other side of the country. Following participation, that pathway may now considered a strong pathway that is likely to be investigated and restricted. If they are business owner, this might mean a profit loss or expense depending on regulations imposed by the management strategy.

How can we make this process legitimate in the eyes of the stakeholder?

This guideline builds on the one before it. If the form of participation is appropriate, conflict and mistrust can be avoided, and the stakeholder will feel that of the work process they were asked to undertake was fair and balanced. Having internal and external support and resources also helps build legitimacy. Examples of external support might be trusted advocacy groups that stakeholders belong to, or clubs and group certifications related to them.

Legitimacy and trust can be built during interactions among participants and provide the basis for communication throughout the process. Communication is another key to legitimacy. Tell participants the purpose of the process and how their participation will influence outcomes. These three elements can create a stakeholder "buy-in." It is important to communicate with stakeholders so that they care about invasive species and pathways and participate in the work when there is a potential risk that they will fear consequences of restrictions of personal freedom that might cause non-participation, distrust of the process, and/or withholding or contributing false information. The solution is to keep the level of engagement consistent: engage stakeholders as a data source, not promising power at the end.

4.4.4 How to foster effective leadership during the process?

Leadership roles include sponsoring, championing, and facilitating. Sponsoring groups might be APHIS, The Nature Conservency, or the Forest Service. Championing groups might be the research biologists. The facilitating groups, trained by an ecologist with risk analysis training, could be hired and with the ability to work with stakeholders to achieve goals and document important observations generated by stakeholders during the process.

Goal: to design a process that allows the benefits of the participation process to exceed the cost of production (agency seeking information) and participation costs (stakeholder)

For example, if at the end of the participation process an unknown pathway is identified and sometime later invasive forest pests are found to travel on that pathway, management of that pathway can be organized, planned, and executed on a much shorter time period and stakeholders involved in that pathway may already be aware of the problem. Awareness might

also increase communication and decrease the likelihood of conflict and mistrust. Swift management action also minimizes economic and environmental losses.

4.4.5 Set rules and project management team

Before the process begins, it is important for management and research agencies to determine responsibilities, determine rules and guidelines, and who will be making the decision-making. Rules and guidelines for the process might be set by the team of authors writing the risk assessment or the risk manager.

The risk manager or team writing the risk assessment determines the final outcome of the risk rating of a particular invasive species. This stakeholder process would be another element or a separate piece that could contribute to the risk rating, specifically (regarding the probability of spread). The team would have to decide how to integrate the information gained from the stakeholder process depending on the level of uncertainty with which they are comfortable, as more research into each pathway would be needed to include it in the outcome of the risk rating. It might be simpler to add it as a list of pathway hypotheses and then state how they were generated. For example, “there is evidence that pathway x, y, and z, might be strong pathways for the movement of x insect and further research will be done to validate the strength of each starting in month/year.”

4.4.6 Be inclusive!

Increasing diversity among participants increases the chances that unknown pathways will be identified. Making sure that participants in the room are diverse across cultures, practices, occupations, economic and social classes, and uses of the vectors. This practice increases the exchange of information and the depth and span of that information in the real world.

Designing for increased diversity requires a few practical guidelines. Ensure that meeting times and places are accessible to as many participants as possible. Provide child care and language translation services during the process, and keep written and verbal instructions simple and clear. Manage conflict by setting clear ground rules and agendas. Take complaints

seriously and develop a process for resolution. Consensus building is not a goal in this process, so facilitators can work with groups generate the information needed and not waste time with trying to find agreement (i.e., all ideas can be recorded).

4.4.7 Manage power dynamics

"One way to share power more evenly among participants is to engage them in coproducing the agenda and process for decision making as well as weighing in on the policy decisions" (Bryson et al. 2012). Since the goal of this process is to collect data from the participants, the agenda should be set beforehand. Building trust can help manage diversity, conflict, and power dynamics. Building trust throughout the process can be as simple as sharing information and knowledge.

At each phase of participation, power dynamics can be managed. When the groups convene together in large groups and small groups, the seating arrangement within the room should allow everyone to hear, see, and offer feedback. Setting an equal amount of time for each participant to share ideas is important in building trust in the process. Setting rules for disagreement and degree of disagreement, and conflict mediation, will ensure that issues stay "problem centered" and not become "people centered."

4.4.8 Ways of using engagement methods to achieve the purposes of engagement

Telling the story of an ecological problem can be challenging when talking to a diverse audience. However, visual storytelling can be one way to bring everyone to the table with the same understanding of the problem and the goal of the participation process. Facilitators can use maps, photos, and videos to tell the story of an invasive forest pest. The story should include key words that participants are going to be using later to describe relationships between pathways, vectors, and stakeholders of an invasive forest species. To provide information that reaches all learning styles, the process of invasion and all key words can be defined visually, orally, and in writing.

Technology that saves time during the process might be keypad voting that helps the group stay engaged and give quick feedback. To ensure that technology, such as keypad

voting, is inclusive, a test question can be given beforehand to determine if participants agree that it is capturing the groups opinion accurately.

Evaluation: How do we know that participation occurred and that we achieved our goals?

What is the most desirable outcome(s)?

1. A list of hypotheses that are ordered by strength (testable)

To measure a combination of desired outcomes:

1. At the individual-level: Did the participants learn about the impacts (environmental and economic) of invasive forest pests?
2. Do participants understand the role that humans play in the movement of invasive forest pests? Was there a change in understanding?

Process-oriented:

1. Was the process inclusive and fair?

Content-oriented (after process)

1. Were desired outcomes achieved?
2. Did this process lead to novel or more information about human-mediated movement?

Questions for experts or researchers after independent study of pathway hypotheses:

Overall, how well did the participation process match reality/real world pathway?

1. Was an unspecified pathway confirmed after the process?
2. Did the pathway strength match the estimate?
3. What was the resulting strength?
4. How might that change over time?

4.4.9 Coordinate goals, purposes, approaches, promises, methods, techniques, and technologies, steps and resources

Successful participation processes have a plan to avoid miscommunication, misunderstanding, and serious conflicts. There are two types of design or plan that might occur for a participation process. One is emergent design where there is a clear understanding of mission, goals, roles, and action steps that evolve in partnership with stakeholders. This is

typical when the process is not mandated. When stakeholder involvement is mandated, participation might be deliberate because the process is legally necessary to move forward with a decision. In mandated design, case, roles, goals, missions, and action steps are pre-determined before participants are contacted. The framework described in this chapter is related more to emergent design, where stakeholders are consulted to identify pathways of pest species.

5 Dissertation conclusions

1) **Walnut twig beetle adults from northern California have a plastic cold tolerance response and are primarily limited by lower lethal temperature. They are freeze-intolerant throughout the year, but after a longer exposure to cold, cold tolerance increased. This phenomenon was manifested in colder lower lethal temperatures more so than supercooling points.** Based on the lowest lower lethal temperature measurements collected in February 2014, approximately half of the current range of *J. nigra* exhibits winter temperatures under which *P. juglandis* might survive (i.e., forecasted winter mortality <50%) if the insect were to arrive.

2) **We developed a method to infest host material in the laboratory with walnut twig beetle adults to study reproduction. Black walnut and butternut appear equally suitable for walnut twig beetle colonization and reproduction.** In field studies, high colonization densities likely limited walnut twig beetle reproduction due to increased intraspecific competition beneath the bark. In laboratory studies, where we established a lower colonization density, reproduction was likely influenced by differences in host quality. In one parent black walnut tree of 323 full-sibs, at low colonization densities, host resistance may limit walnut twig beetle reproduction. The underlying resistance mechanisms remain to be determined.

3) **Host suitability, defined by reproduction per female, varied across Juglandaceae hosts. Walnut twig beetle does not reproduce in two hickory species that are native to the United States.** Males will chew superficially at the bark surface under no-choice conditions. Host had an effect on brood cold tolerance, measured by freezing temperature (i.e., supercooling point). Brood with the lowest mean freezing temperature was measured from a host whose native range does not extend to the northern United States.

4) **I developed an example framework for accessing stakeholder knowledge to identify pathways of forest pests. Stakeholder analysis and participation process guidelines are used to consult stakeholders on determining unspecified pathways. These pathways might exist because of a specific relationship that a stakeholder has with a vector.** First, a risk management group would perform a stakeholder analysis to determine which stakeholders will be consulted. Second, stakeholder consultations are led by an ecologist with pest risk assessment training. The end goal of the stakeholder consultation is a list of pathways that can be independently tested by the risk management group.

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7 Appendix 1 – Flight capacity of the walnut twig beetle, *Pityophthorus juglandis* (Coleoptera: Scolytidae), on a laboratory flight mill

The data for this section was collected by the first author, Aubree Wilke, and the manuscript was prepared by co-authors, Aubree M. Wilke, Andrea R. Hefty, Robert C. Venette, Steven J. Seybold, and Brian H. Aukema.

7.1 Summary

The walnut twig beetle, *Pityophthorus juglandis* Blackman, and the fungus *Geosmithia morbida* Kolařik et al. comprise thousand cankers disease in walnut and its close relatives (*Juglans* spp and *Pterocarya* spp). Thousand cankers disease is responsible for the decline of *Juglans* species throughout the western United States and more recently, the eastern United States and northeastern Italy. Although recent studies have elucidated several environmental factors associated with fluctuations in flight trap catches of adult *P. juglandis* at daily and weekly time scales, knowledge of factors influencing individual flight capacity is limited. A flight mill study was designed to test the effect of sex and age post-emergence on the flight ability of *P. juglandis* over 24 h in the laboratory. We found no evidence that male and female flight capacities differed, even though males were larger than females based on pronotal width measurements. Age post-emergence had no effect on flight distance, total flight time, or mean flight velocity. The propensity to fly, however, decreased with age. The maximum total flight distance observed was ~3.6 km in 24 h; however, the mean and median distances flown by beetles that initiated flight were ~372m and ~158m respectively. Beetles flew a mean total of 34 minutes within a 24 h flight trial. These flight capacities observed in the laboratory suggest that natural dispersal may only contribute to relatively small scale, incremental spread. The implications of our findings to management and sampling of this hardwood pest are discussed.

7.2 Introduction

The walnut twig beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Scolytidae, *sensu* Bright 2014), is a phloeophagous beetle that is native to the southwestern United States and Mexico (Blackman 1928, Bright and Stark 1973, Bright 1981, Rugman-Jones et al. 2015). This beetle and an associated fungus, *Geosmithia morbida* Kolařik et al., constitute the insect-

fungal complex that causes thousand cankers disease (TCD) (Tisserat et al. 2009, Kolařik et al. 2011, Seybold et al. 2013b). Several native and introduced walnut and wingnut species are hosts for *P. juglandis* and *G. morbida* in the United States (Newton and Fowler 2009, Flint et al. 2010, Hishinuma et al. 2016), and there is a range in susceptibility among these hosts (Flint et al. 2010, Utley et al. 2013). Eastern black walnut, *Juglans nigra* L., appears to be one of the most susceptible species (Tisserat et al. 2009, 2011, Utley et al. 2013).

Widespread ornamental planting of eastern black walnut, *J. nigra,* and English walnut, *J. regia* L., may have facilitated geographic range expansion of *P. juglandis* in the western United States (Graves et al. 2009). In the last few years, *P. juglandis* has also been detected in the eastern states of Tennessee, Virginia, Pennsylvania, North Carolina, Maryland, Ohio, and Indiana, threatening native populations of *J. nigra* in those regions (Newton and Fowler 2009, Cranshaw 2011, Seybold et al. 2012a, 2016; Utley et al. 2013, Wiggins et al. 2014, Rugman-Jones et al. 2015). In 2013 and 2014, *P. juglandis* and *G. morbida* were found in *J. nigra* and *J. regia* in northeastern Italy, the first record of these agents in Europe, generating international concern (Montecchio and Faccoli 2014, Montecchio et al. 2014).

Recent phylogeographic analyses of over 60 populations of *P. juglandis* provide evidence that its range expansions are due, in part, to anthropogenic movement of infested wood (Rugman-Jones et al. 2015). Western walnut is highly prized by woodworkers for crafting furniture, gunstocks, guitars, and other items (Newton and Fowler 2009, Tisserat et al. 2009). *Juglans nigra*, in particular, is one of the most highly valued timber species in North America. Moreover, walnut wood is utilized as firewood and considered good for home heating (Newton and Fowler 2009). Anthropogenic spread *via* wood or wood products is not unique to *P. juglandis*, as the movement of other invasive forest insects such as the emerald ash borer, *Agrilus planipennis* Fairmaire, and gypsy moth, *Lymantria dispar* (L.), provide strong evidence that the movement of firewood is a high-risk pathway for dispersal (Jacobi et al. 2012).

Despite the likelihood that anthropogenic movement of infested black walnut wood fostered the establishment of *P. juglandis* and thus TCD in the eastern United States, it has

been suggested that both natural dispersal and human-mediated movement have fostered the insect's expansion across the western United States (Cranshaw 2011). Little is known about the flight capacity of *P. juglandis,* however. Determining the insect's flight characteristics in its natural environment is extremely challenging due to the insect's minute size (*ca* 1.5-2.0 mm in length) and primarily endophytic life history (Seybold et al. 2013b, 2016).

Various techniques have been used to study the dispersal and movement of insects in the field and laboratory, ranging from mark-release-recapture techniques, implementing tags (Gary 1970), etchings (Klingenberg et al. 2010), radioactive-isotope markers (Godwin et al. 1957) or other non-detrimental labels (Hagler and Jackson 2001) to harmonic radar (Machial et al. 2012). Among bark beetles, study techniques have included mark-release recapture experiments on insects marked with paint or fluorescent powders prior to release in trap arrays (e.g., Dodds and Ross 2002, Costa et al. 2013) sampling experiments netting insects with airplanes (e.g., Jackson et al. 2008) *post hoc* statistical modeling of landscape damage patterns (e.g., de la Giroday et al. 2012) and flight mills (e.g., Atkins et al. 1961, Chen et al. 2010, Evenden et al. 2014, Fahrner et al. 2014, Lopez et al. 2014). Flight chambers and tethered flight apparatuses can measure flight characteristics that are difficult to capture in the insect's natural environment (Stinner et al. 1983, Taylor et al. 2010).

Here, we investigate the flight capacity of *P. juglandis* by using a computer-monitored flight mill in the laboratory. Previous studies have examined diurnal flight patterns of *P. juglandis* in response to aggregation pheromone-baited traps in the field (Seybold et al. 2012b, Chen and Seybold 2014), but knowledge of the basic bioenergetic capabilities of this beetle remains limited. Our objectives are to assess the effect of sex and age on flight characteristics of this insect, including distance flown, flight velocity, and total flight time. Our goal is to characterize the flight potential of *P. juglandis* to improve our understanding of the natural dispersal of this hardwood pest and thus inform assessments of risk posed by the future range expansion of this insect.

7.3 Materials and methods

7.3.1 Insects

All *P. juglandis* were obtained from infested branch sections from hybrid black walnut, *Juglans hindsii* × (*J. nigra* × *J. hindsii*/*Juglans californica*), collected in Sutter County, CA. The branch sections were approximately 5 cm in diameter and 23 cm long, and shipped in February 2014 and March 2014, by overnight courier to the MAES/MDA biosafety level 2 Insect Biocontrol Facility at the University of Minnesota, St. Paul, where flight assays were conducted. Shipment and handling were conducted under the terms and conditions specified in Permits P526P-12-01650 and P526P-12-02498 from the US Department of Agriculture, Animal and Plant Health Inspection Service. Upon receipt, the branch sections were stored at constant room temperature (21°C) and humidity (50% RH) in 3.8 liter, plastic containers (ULINE, Pleasant Prairie, WI). *Pityophthorus juglandis* were allowed to emerge from cut branch sections, and collected and used for flight within 24 h, unless the experimental protocol required otherwise. When preparing a flight mill experiment, the beetles were held briefly in Petri dishes (140 x 20 mm) with moistened Kimwipes (Kimberly-Clark, Irving, TX) prior to attachment to the tether arm of the flight mill.

7.3.2 Flight mill

Twelve computer-monitored flights mills, described by Fahrner et al. (2014), were used to investigate the flight capacity of *P. juglandis*. The tether arm of the flight apparatus was constructed from solid 33 American wire gauge (AWG) (diameter: ~0.171 mm) copper wire, to form a 5.5 cm tether arm to which the insect was attached. For attachment to the tether arm, insects were gently retrieved from Petri dishes with a fine tip paintbrush and placed onto an icepack to slow their activity and limit movement. General attachment procedures were described by Fahrner et al. (2014) and are only briefly summarized here. Once an insect was sufficiently chilled, the tip of the copper tether arm was dipped in a droplet of cyanoacrylate glue (Loctite® Super Glue Gel; Henkel Corporation, Westlake, OH) and lightly pressed against the dorsal surface of the pronotum. Care was taken to ensure full movement of the elytra so as to not inhibit wing movement for flight. This glue was effective at securing the insects, and

previous laboratory studies found no evidence of toxic effects on Warren root collar weevils, *Hylobius warreni* Wood, over the course of up to one year (Machial et al. 2012). The terminal 5 mm of the copper wire was then bent 90° so the insect was facing perpendicular to the tether arm, resulting in a radius of 5 cm for the final tether arm (Fahrner et al. 2014). Due to the small size of the insect, a counterweight was not required to balance the tether arm. Once the insect was successfully attached, the tether arm was placed on the flight mill to begin recording flight. Flight initiation was spontaneous and not instigated by manipulation of any kind.

7.3.3 Defining a flight

An infra-red (IR) sensor recorded all sensitive movements of the tether arm as raw phase change data, including rare but spurious movements due to air currents or accidental bumps during assay initiation; any of which may have been identified as unrealistic flight speeds. Therefore a limit for maximum speed was set at 45 cm/s (1.62 km/h), based on timed observations of *P. juglandis* flight during preliminary assays, to exclude any potential misrecordings, as recommended by Fahrner et al. (2014). Moreover, flights were only included in analyses if a minimum threshold of three full revolutions was met.

7.3.4 Experimental protocols

Newly-emerged beetles (within 24 h at time of flight) were obtained from several host branch sections and randomly chosen for flight experiments. All flight trials occurred at room temperature (21.5°C ± 0.04) and relative humidity (18.9% ± 0.43) (mean ± SE) over a 24-h period in constant light. Insects were not re-used following a 24-hour flight trial. The mortality of each beetle post-flight was determined by using leg movement as an indicator of survival. Survivors were placed in a Petri dish (94 x 16 mm) with a moistened Kimwipe and monitored daily for mortality. Once dead, all insects were stored at -80°C in 0.5 ml microcentrifuge tubes. A subset of sixty insects was chosen at random for pronotal width measurements. Measurements were conducted with a Leica MZ6 microscope (Wetzlar, Germany) connected to a real-time camera and digital micrometer.

Two separate flight experiments were conducted to test the effects of sex (i.e., male and female) and age (i.e., number of days post-emergence) on the flight ability of *P. juglandis*. To determine the effect of sex on flight capacity, newly-emerged beetles were randomly selected and assigned to channels of the flight mill on a given day. Only active (walking) beetles were chosen. The sex of each specimen was determined, prior to flight, under a microscope by the presence of a dense brush of setae on the female frons and the presence of granules on the male elytral declivity (Bright 1981, Seybold et al. 2013a). A total of 282 insects were flown during this experiment.

During the second flight experiment, which characterized how flight capacity changes with age post-emergence, insects of varying ages were assigned randomly to channels of the flight mill on a given day. Beetles between one and five days post-emergence were tested. Newly-emerged beetles were collected and held in Petri dishes with moistened paper towels for up to five days. Active insects were randomly selected from each age group, and used once in a flight trial. In addition, the sex of each beetle was recorded to account for any confounding effect of sex on flight. In this flight trial, a total of 372 insects were flown, with between 67-84 beetles in each age cohort.

7.3.5 Flight mill data

Extraction of flight metrics from the raw phase change data was conducted by using R (R Development Core Team 2014) as described by Fahrner et al. (2014). The flight metrics include total flight time, distance, and velocity for each individual placed on the flight mill. Beetles often fly multiple times instead of one continuous flight, and so the total flight distance and total flight time of all bouts of flight for an individual was used for analysis. Mean flight velocity was calculated by using the total flight distance divided by the total flight time for an individual.

7.3.6 Statistical analyses

All data were analyzed by using R (R Development Core Team 2014). Simple linear regression was used to analyze the relationships between the response variables such as

distance flown, flight velocity, total flight time, and pronotal width with sex and age as independent variables. Graphs of the residual plots were examined to check assumptions of normality of the data and homoscedasticity of the errors. Variance-stabilizing logarithmic transformations were used to transform distance, velocity, and time data. A generalized linear model (glm, family = binomial) was used to analyze the propensity of flight with age post-emergence.

7.3.7 Monte Carlo simulation

A Monte Carlo simulation was used to integrate flight distance with age and estimate flight capacity of a population of beetles over 5 d. The simulation draws a random sample from a binomial distribution parameterized by using day-specific empirical data of the likelihood of an individual chosen at random to fly on a given day if placed on the flight mill (i.e. individuals were less likely to attempt flight as they aged; see Results). This random variable results in a flight or non-flight on a given day, *i*. If this draw results in a flight, a distance value, X_i, is selected from the empirical distribution of flight data on day *i*; if a non-flight was drawn, X_i is equal to zero. These steps are repeated for *i* = 1…5 days and the flight distances are summed for a total flight distance over five days (total flight = $\sum_{i=1}^{5} X_i$) to obtain the expected flight distance of an individual insect in the five days post-emergence. These steps were repeated 100,000 times to estimate the dispersal capacity of a population of beetles emerging from a host.

7.4 Results

A total of 654 *P. juglandis* were placed on the flight mills during these assays (both flight experiments pooled). Of these, 45% initiated flight. The maximum total flight distance was approximately 3.6 km in 24 h of tethered flight; however, the mean and median distances flown by the beetles that initiated flight were approximately 372 m and 158 m, respectively. The longest distance flown in a single bout of flight was 1.2 km. The highest velocity reached in a single bout of flight was 44.8 cm/s. Beetles flew a mean total of 34 minutes within a 24-hour flight trial. The majority of beetles that initiated flight (93%) completed their final flight in the first

10 h of the flight trial (Fig. 5.1). The distribution of last flight recordings was highly skewed, however, such that the mean hour of the last flight event occurred 3 h 58 min into the 24-hour flight trial. Three of 293 beetles performed their last flight after 20 h post trial-initiation (Fig. 5.1). Only nine of the 654 beetles were found to be alive at the conclusion of a one-day experiment.

In determining the effect of sex on flight capacity, a total of 178 *P. juglandis* flew when placed on the flight mill (83 males, 95 females) from an initial cohort of 282 insects (156 males, 126 females). The proportion of beetles that initiated flight versus those that did not fly was not significantly different by sex (z = 0.86, $P = 0.39$). Male *P. juglandis* flew a mean (±SE) distance of 495.0 ± 68.8 m and females flew 331.9 ± 46.5 m ($F_{1, 176} = 2.80$, $P = 0.10$) in our 24 h trials (Fig. 5.2A). Mean flight velocity was similar between sexes (17.7 ± 0.50 cm/s for males, 17.2 ± 0.39 cm/s for females) over a 24-hour flight trial ($F_{1, 176} = 0.51$, $P = 0.48$) (Fig. 5.2B). Males spent 52 percent more time in flight, on average, than females, although this difference was not statistically significant ($F_{1, 176} = 2.62$, $P = 0.11$) (Fig. 5.2C). Male mean pronotal width (0.64 ± 0.01 mm) was significantly larger than female mean pronotal width (0.57 ± 0.01 mm) ($F_{1, 58} = 25.72$, $P < 0.001$) (n=30 for each sex) (Fig. 5.3).

In determining the effect of age following emergence from the host on flight capacity, a total of 372 *P. juglandis,* ages one to five days post-emergence, were placed on the flight mill, of which 115 flew. The propensity to fly decreased with age (Fig. 5.4). Between one and five days following emergence from the host, flight initiation decreased from 60 percent to less than one percent of beetles placed on the flight mill. However, total flight distance ($F_{1, 113} = 2.27$, $P = 0.13$), flight velocity ($F_{1, 113} = 2.247$, $P = 0.14$) and total flight time did not change with age ($F_{1, 113} = 1.46$, $P = 0.23$) (Fig. 5.5).

Results from a Monte Carlo simulation integrating propensity to fly with beetle age indicated that *P. juglandis* adults could potentially fly a mean distance of 491.9 m over 5 d and a median distance of 280 m. Flight frequencies based on Monte Carlo simulation of flight five days post-emergence showed that approximately 33% of the insects fly less than 100 m, whereas 1% of the insects fly more than 2 km (Fig. 5.6).

7.5 Discussion

Our findings are consistent with the hypothesis that natural dispersal may be a small contribution to the incremental spread of this hardwood pest. Once introduction occurs, our findings suggest that insects may remain somewhat localized at the introduction site and will spread slowly from the area if not moved anthropogenically. This appears to be consistent with detection survey data for beetles in Butler Co., Ohio and Bucks Co., Pennsylvania (SJS personal observations). Thus, timely local management strategies might be critical and warranted for this insect. For example, large numbers of pheromone-baited funnel traps could be used at known introduction sites to mass trap and reduce resident populations (Jakuŝ 1998, Schlyter et al. 2001, El-Sayed et al. 2006). Locating local outbreaks, however, may be difficult due to the lag time between infestation and TCD symptom expression (Tisserat et al. 2009). Furthermore, many hundreds if not thousands of funnel traps would need to be deployed in states to initially locate these outbreaks based on the flight activity of the beetle. This is likely not feasible economically.

We did not find significant differences in flight characteristics between males and females. Among bark beetles, the pioneering sex is typically the larger sex when a sexual size dimorphism is present in adults (Foelker and Hofstetter 2014). For example, among several studies of species of *Dendroctonus,* where females are the host selecting sex, larger insects flew farther (Williams and Robertson 2008, Chen et al. 2011, Evenden et al. 2014), perhaps because sustained flight is necessary for pioneering females to successfully locate suitable host trees (Evenden et al. 2014). The absence of sex-specific flight advantages in this system is consistent with other studies (e.g., Forsse and Solbreck 1985) in which males are the pioneering sex, as is the case with *P. juglandis* (Seybold et al. 2016). For example, Botterweg (1982) observed no effect of sex, size, or fat content on the dispersal of the Eurasian spruce bark beetle, *Ips typographus* L., in the field. It is possible that sex-related differences in flight do exist among individual *P. juglandis*, but detection of these differences may only be possible after successive 24 h flight periods (e.g., Chen et al. 2011) as larger-bodied individuals have enhanced lipid reserves to power flight (Lease and Wolf 2011, Kaufmann et al. 2013).

Long-distance dispersal may be most critical to aggressive species of bark beetles that exhaust host resources in as little as one generation when at outbreak levels (Raffa et al. 2005). Less-aggressive species that colonize declining trees may find hosts in this condition to be rare and ephemeral in space and time (Raffa et al. 2005), and such species may complete multiple generations on the same host or find hosts abundant in the face of regional disturbance events such as drought or fire (Botterweg 1982, Raffa et al. 2008). While detailed knowledge of the colonization behavior of *P. juglandis* is limited, it is thought that *P. juglandis* may recolonize lower and lower sections of large natal host trees or attack neighboring trees (Flint et al. 2010). This may occur frequently in urban environments where walnut trees can grow to large diameters and heights, despite being subjected to a variety of stresses that accompany placement along streets or poorly irrigated locations outside of their natural ecological habitats.

We did not find a significant decrease in flight capacity with age, unlike previous laboratory studies of bark beetles (Chen et al. 2011, Evenden et al. 2014) that found negative age-flight correlations associated with depleted lipid stores as a result of starvation during storage prior to placement on the flight mill (Evenden et al. 2014). Some species of bark and wood-boring insects are known to feed after emergence from the host tree. For example, *A. planipennis,* feeds on ash leaves for 2-3 weeks prior to ovipositing (Jennings et al. 2014). *Pityophthorus juglandis* is not thought to feed after emergence before locating a new host tree. Thus, as Evenden et al. (2014) concluded in their study on the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a brief starvation treatment prior to flight may accurately reflect dispersal in field conditions.

Flight studies in the laboratory do have inherent limitations. Tethered flight studies limit the influence of many factors that affect flight during natural dispersal, such as ambient air temperature, light intensity, and barometric pressure that have been shown previously to be associated with *P. juglandis* flight (Chen and Seybold 2014). Wind speed, for example, negatively impacts the flight of *P. juglandis*, with the most active flight happening at wind speeds below 4 km/h (Chen and Seybold 2014). Wind-aided dispersal can profoundly impact small-

bodied insects such as bark beetles (Furniss and Furniss 1972, Nilssen 1984, Crossley and Hogg 2015). *Ips typographus*, for example, can fly distances of >8 km and reach 19-55 km when aided by wind (Botterweg 1982, Forsse and Solbreck 1985). Similarly, *D. ponderosae* has been observed to enter atmospheric wind currents and travel hundreds of kilometers in a single day (Furniss and Furniss 1972, Jackson et al. 2008, de la Giroday et al. 2011).

Another environmental factor that influences flight is relative humidity (RH), especially for small-bodied insects (Zhang et al. 2008, Chen et al. in review). Fahrner et al. (2015), for example, found that the flight speed of *A. planipennis* declined with an increase in humidity during a 24 h trial on the same flight mills used herein. In another laboratory, flight frequency of white pine cone beetle, *Conophthorus coniperda* (Schwarz), increased with exposure to dry air (Henson 1962). The ambient humidity levels for our study were relatively low, with daily means between 11-33% RH (complete data not shown). The mortality of all but nine beetles by the end of the 24 h flight trial may have been due to desiccation caused by low humidity; however, we did not find an effect of humidity on distance flown ($F_{1, 291} = 0.14$, $P = 0.71$). In the field, increased humidity is associated with decreased flight of *P. juglandis.* This pattern may be due to a higher, more costly wing-beat frequency instigated by higher humidities (Chen et al. in review).

We use discretion when relating laboratory-based flight potential to natural dispersal, although some studies of insects in tethered flight have been found to correlate well with field observations of dispersal (Forsse and Solbreck 1985, Jactel and Gaillard 1991, Evenden et al. 2014). Given the size of *P. juglandis*, we expect that natural flight capacity of *P. juglandis* is limited, and likely not more than 3 or 4 km at the extremes. This limited dispersal poses serious challenges for sampling established, but unknown populations on the landscape (Venette et al. 2002). As further information regarding the natural dispersal of *P. juglandis* becomes available, this basic knowledge of flight capability will aid in informing management strategies to inhibit the spread of this invasive pest.

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7.8 Figures

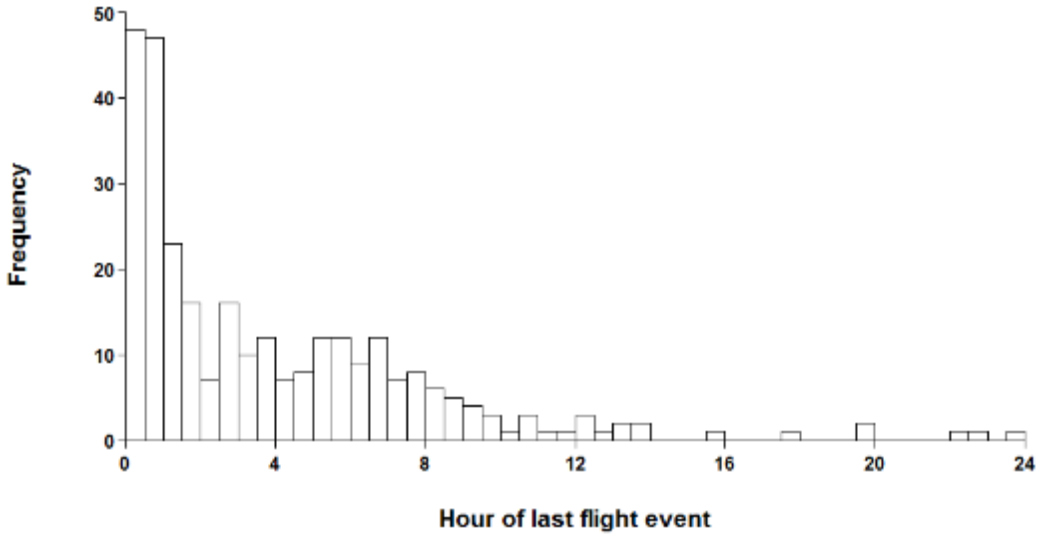

Figure 7.1 Hour of last flight event of male and female walnut twig beetles, *Pityophthorus juglandis*, recorded on a computer-monitored flight mill over a 24-hour (*n* = 654 beetles).

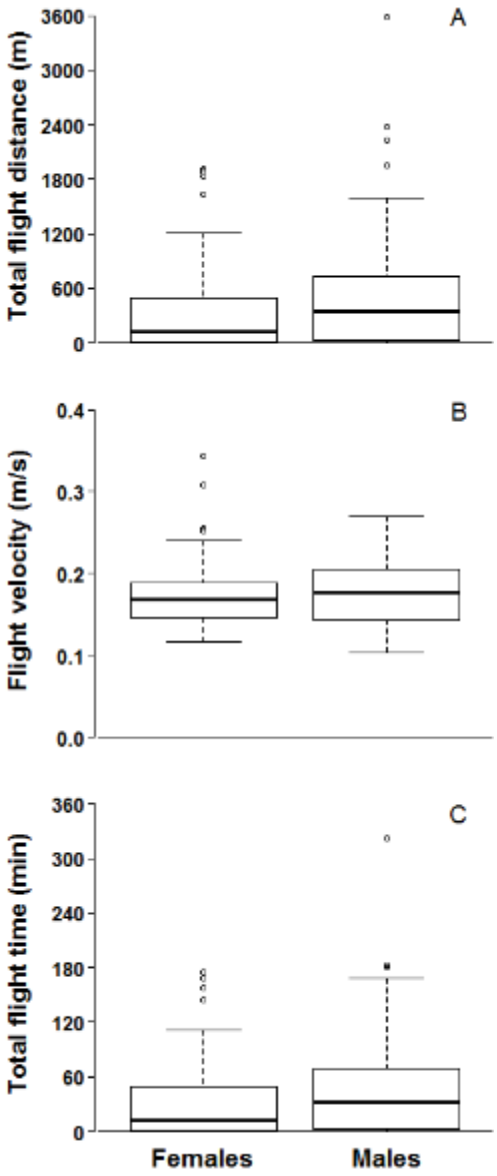

Figure 7.2 Comparison of flight metrics of female and male walnut twig beetles, *Pityophthorus juglandis,* on a computer-monitored flight mill over a 24-hour period (*n* = 95 females and 83 males). A) Total flight distance. B) Flight velocity. C) Total flight time. Boxplots represent quartiles and median; whiskers represent the smaller of maximum value or 1.5 times the interquartile range of the data; and dots represent values beyond the whisker range.

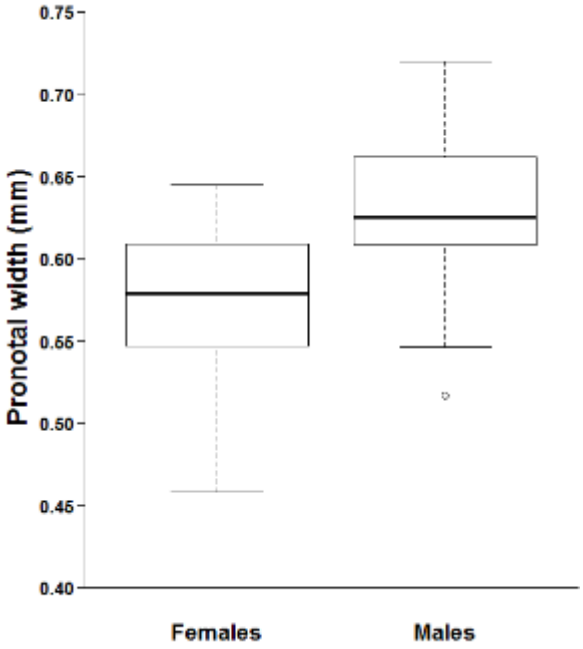

Figure 7.3 Comparison of pronotal widths of female and male walnut twig beetles, *Pityophthorus juglandis* (*n* = 30 females and 30 males).

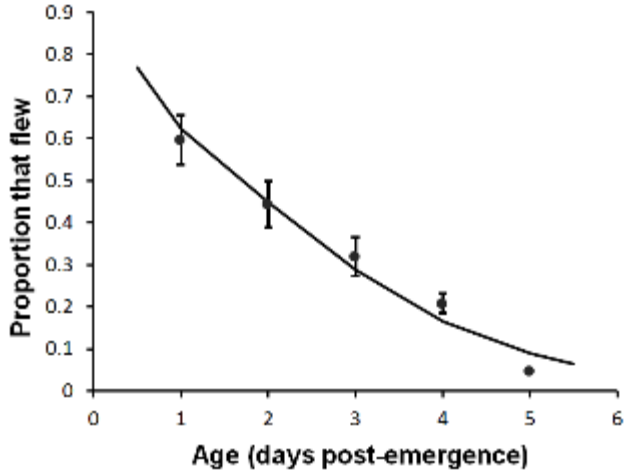

Figure 7.4 Effect of age on the proportion of walnut twig beetles, *Pityophthorus juglandis*, which initiated flight 1 to 5 days post-emergence on a computer-monitored flight mill (±SE). Probability of flight equals expy/(1+expy) where y = 1.20689 – 0.70562(age in days); *n* = 67, 72, 72, 77, 84 insects from days one to five.

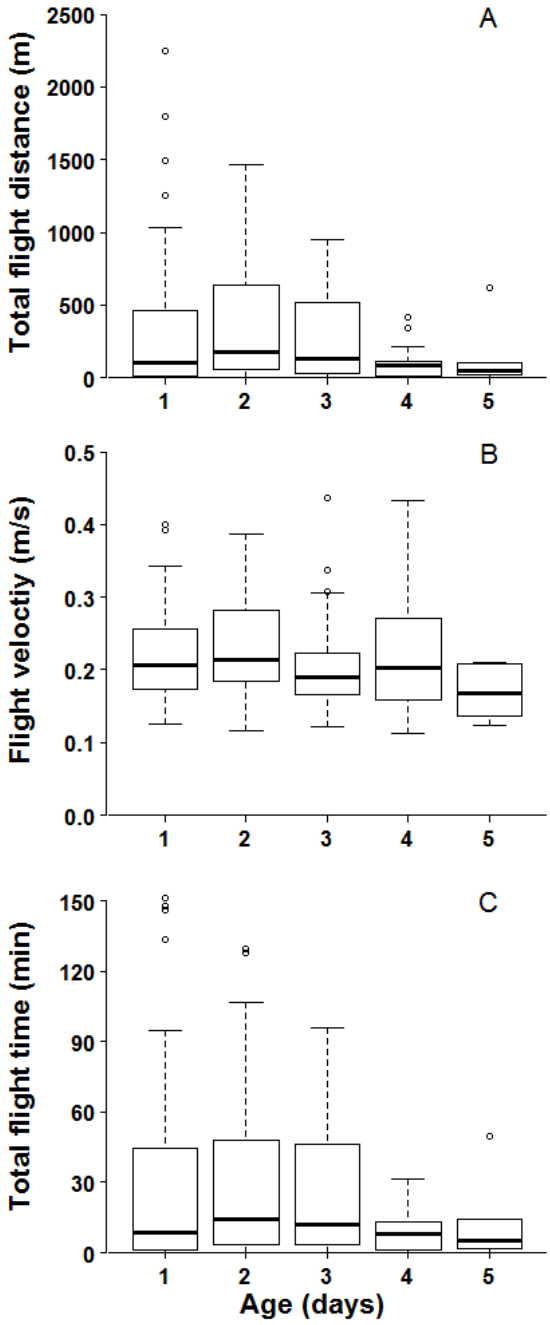

Figure 7.5 Comparison of flight metrics of walnut twig beetles, *Pityophthorus juglandis* 1 to 5 days post-emergence on a computer-monitored flight mill. *n* = 40, 32, 23, 14, 6 fliers for days one to five. A) Total flight distance. B) Flight velocity. C) Total flight time.

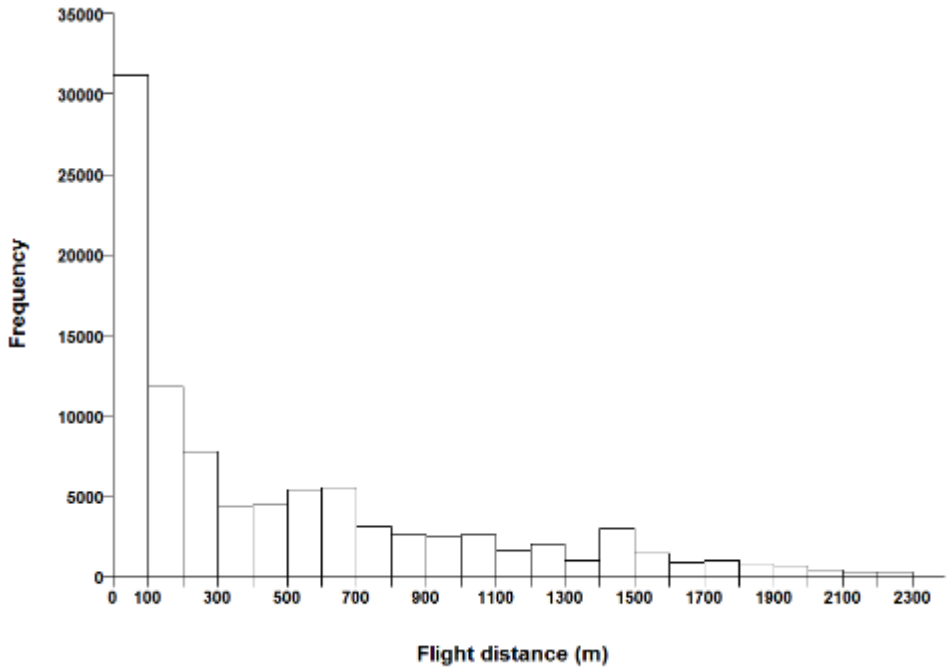

Figure 7.6 Monte Carlo method simulation of continuous flight over 5 days post-emergence integrating empirical results from experiments reflecting propensity to fly and age post-emergence. Histogram reflects a hypothetical distribution of 100,000 insects.