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Assessment of *Pityophthorus juglandis* colonization characteristics and implications for further spread of thousand cankers disease

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Assessment of *Pityophthorus juglandis* colonization characteristics and implications for further spread of thousand cankers disease

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ABSTRACT

Nonnative, invasive forest insect pests are a significant threat to the health of global forest resources. Thousand cankers disease, a recently described disease threatening walnuts, is the result of an invasive insect-pathogen complex in which the walnut twig beetle (*Pityophthorus juglandis* Blackman) is a vector to the associated fungal pathogen, *Geosmithia morbida*. Both are native to the southwestern U.S. into northern Mexico, however, have been found well beyond their historic range, and now threaten black walnut (*Juglans nigra*) in the eastern U.S. Beetles have likely been spread via the transport of infested walnut logs.

The goal of this project was to identify potential pathways by assessing *P. juglandis* colonization of *J. nigra* logs and seedlings, and to identify treatments to prevent colonization in logs. Beetle colonization of logs and lumber treated by phytosanitation measures was tested. When exposed to high colonization pressures and baited with a pheromone lure, beetles attacked steam heated, methyl bromide fumigated, and kiln-dried samples. Colonization in a reduced pressure exposure scenario was inconclusive.

Azadirachtin, borate, and permethrin were tested as a means of preventing *P. juglandis* colonization of black walnut logs. Beetle survival rates over a 120 hour exposure period, and colonization activity was compared. Permethrin was the only insecticide to kill all beetles and to prevent any attacks. A 30% concentration of borate showed some control, reducing survival rate compared to control levels, however, beetles successfully attacked the material, and its use as a control method is questionable. Azadirachtin was not effective in the doses tested.

Finally, *P. juglandis* colonization of *J. nigra* nursery stock seedlings was tested. Seedlings were exposed to beetles in no-choice and choice assays. For no-choice assays, beetles were caged directly onto the stems. In choice assays, seedlings were placed near infested logs, presenting the seedlings to beetles emerging from the infested material. Beetles attacked stems in all diameter size classes tested (0.5 – 2.0 cm), however, showed a preference for larger diameter trees. No attacks were observed in the choice assays and may have been confounded by the method used to introduce beetles to seedlings.

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Chapter 1 Introduction, Background and Literature Review

INVASIVE FOREST INSECT PESTS

Nonnative, invasive species pose a significant threat to native biodiversity in ecosystems across the globe (Liebhold et al. 1995, Wilcove et al. 1998, Ludsin and Wolfe 2001, Sakai et al. 2001, Ricciardi 2007, Aukema et al. 2010). Species invasions cause an estimated $1.4 trillion in global economic losses annually and pose a threat to native biodiversity in the United States from exotic invasive species ranks second only to that of habitat loss (Wilcove et al. 1998). A nonnative invasive species is generally defined as any species that has been introduced (i.e. not historically known from) and established within a new ecosystem, and causes ecological or economic harm (USDA Forest Service 2014, NOAA 2014, EPA 2014). Introductions of these invaders are increasing on a global scale as a byproduct of increased international trade (Ricciardi 2007, Westphal et al. 2008, Aukema et al. 2010).

Invasive arthropods rank at the top of the list of invaders. This is often attributed to their small, inconspicuous size and their ability to inhabit—or occupy temporarily—commonly used shipping materials such as crates and commonly traded goods (Haack and Petrice 2009, Aukema et al. 2010). This is especially true of subcortical beetles which encompass species belonging to the families Buprestidae, Cerambycidae, and Curculionidae. Species from each of these three families develop and feed in the xylem or phloem of woody species (Triplehorn and Johnson 2005). Species of Cerambycids and Curculionids have been recovered from steam heated logs and from small patches ($100 – 1,000$ cm^2) of bark left on boards that had also been steam heated in accordance with accepted phytosanitation regulations (Haack and Petrice 2009). This observed behavior and the continued increase global trade are likely contributing factors in the rise in phloem and wood feeding insect pest introductions. Aukema et al. (2010) report phloem and wood boring insects represented 56% of all insect introductions from 1980 to 2006. Several species of bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) are known to be significant tree killing pests (Raffa and Berryman 1982, Wood 1982) and the continued introduction of species from this family poses a significant threat to forests in North America and globally (Haack 2001, Brockerhoff et al. 2006, Haack 2006).

Another life history trait common to Curculionids is an association with a variety of fungi (Paine et al. 1997). Bark and ambrosia beetles carry the spores of several symbiotic fungi externally on their elytra, or in specialized organs called mycangia (Triplehorn and Johnson 2005). Increasingly, these fungal symbionts associated with nonnative beetles—often non-pathogenic in their native ranges—are reported to be pathogenic to naïve tree species in introduced ranges (Hulcr and Dunn 2011). These

symbioses play a significant role in the tree killing efficacy of Curculionids. Tree mortality is often the result of the confluence of beetle feeding and fungal decomposition of the tissues (Raffa and Berryman 1982). Such is the case in the recently discovered insect-pathogen complex thousand cankers disease, that is causing widespread mortality of walnut species in North America (Tisserat et al. 2009). The fungal pathogen *Geosmithia morbida* was not previously known as a pathogenic agent, and represents the first observed species within the genus *Geosmithia* displaying pathogenic capabilities(Kolařík et al. 2011). Nonnative, invasive, phytophagous coleopterans and their fungal symbionts represent a significant threat to the ecological and economic wellbeing of North America's forest resources.

THOUSAND CANKERS DISEASE

Thousand cankers disease is the result of an invasive insect-pathogen disease complex causing mortality of species in the genus *Juglans*. The complex includes the walnut twig beetle (*Pityophthorus juglandis* Blackman) which vectors the fungal pathogen *Geosmithia morbida* (Tisserat et al. 2009, Kolařík et al. 2011). *Pityophthorus juglandis* is native to the southwestern U.S., historically known from Arizona, New Mexico, and northern Mexico, associated with the range of Arizona walnut (*Juglans major*) (Torrey) A. Heller (Seybold et al. 2010, Cranshaw 2011). The beetle and pathogen are believed to have co-evolved with *J. major,* as neither appear to be a major pest to the host, and *Juglans major* appears to be the least susceptible species in the *Juglans* genus (Cranshaw 2011, Utley et al. 2013). Conversely, black walnut (*J. nigra* L.) and butternut (*J. cinerea* L.) are particularly susceptible to the pathogen. In an inoculation study, the largest cankers (measured as the total surface area of necrotic tissue) were observed on *J. nigra* and *J. cinera* (Utley et al. 2013).

Thousand cankers disease has been confirmed throughout the western U.S. (Cranshaw 2011, Tisserat et al. 2011, Seybold et al. 2012a) and in several states in the eastern U.S. within the native range of black walnut (Cranshaw 2011, Grant et al. 2011). The disease was first described from dying *J. nigra* in Boulder, CO in 2008 (Tisserat et al. 2009). It was soon confirmed on hundreds of out-planted black walnuts (Cranshaw 2011), from native western walnut species (*J. hindsii* and *J. californica*), and from the commercially important *J. regia* (native to Asia) across the western U.S. (Seybold et al. 2012a). Thousand cankers disease was thought to only threaten black walnuts planted well beyond the native range; however, in 2010, the disease was discovered within the native range of *J. nigra,* recovered from infested walnuts in Knoxville, TN (Grant et al. 2011). The disease was soon confirmed in Virginia, Pennsylvania (Seybold et al. 2012a), North Carolina (Hadziabdic et al. 2013), Ohio (Fisher et al. 2013),

and Maryland (MDA 2013). In the fall of 2013, the beetle and pathogen were confirmed in Italy, the first recorded case of thousand cankers disease outside of the United States (Montecchio et al. 2014). In 2014, *G. morbida* was recovered in Indiana, however, to date, no captures of *P. juglandis* have been reported (Juzwik et al. 2015). The exact method of introduction is not known, however, it appears likely that the beetle is transported in walnut wood or plant material as a result of commerce and/or firewood movement (Newton and Fowler 2009).

Thousand cankers disease is characterized by a progressive out-in or top-down mortality, beginning in the crown and moving down the main stem, often killing the tree (Tisserat et al. 2009). Damage to the phloem tissue results from *P. juglandis* feeding and from tissue necrosis caused by *G. morbida*. Cankers occur beneath the bark and are closely associated with beetle galleries, only extending several centimeters beyond the margins of the gallery (Tisserat et al. 2009). Black staining and/or bark cracking may be visible at the surface, especially in the smooth-barked *J. regia* (Tisserat et al. 2009, Graves et al. 2009).

Confluence of feeding galleries and cankers, resulting from numerous attacks, severs the nutrient flow within the tree, causing die off of portions of the tree (Graves et al. 2009, Seybold et al. 2012b). Symptoms of thousand cankers disease begin with premature yellowing and wilting of leaves in the crown. As the disease progresses, thinning foliage, flagging, branch dieback, crown dieback, and epicormic shoots become evident (Graves et al. 2009, Tisserat et al. 2009). The length of time between initial attack and the onset of symptoms is not known, however, mortality can occur within three years once symptoms manifest (Seybold et al. 2013b).

WALNUT TWIG BEETLE

The walnut twig beetle (*Pityophthorus juglandis*) Blackman (Coleoptera: Curculionidae: Scolytinae) belongs to a group of bark beetles commonly known as twig beetles. Members of the genus include beetle species that feed and reproduce within the phloem or pith of a host species (Blackman 1928, Bright 1981). Most species of *Pityophthorus* attack damaged or dying conifers, particularly *Pinus* spp., however, some species are known to attack hardwoods, woody vines, and shrubs (Bright 1981).

Pityophthorus juglandis was first described by Blackman in 1928, collected from black walnut (*J. nigra*) in Lone Mountain, New Mexico and from *Juglans* spp. from Paradise, Arizona (Blackman 1928, Bright

1981). The range reported for WTB included New Mexico, west to southern California and south into northern Mexico (Bright 1981, Wood and Bright 1992). Although originally described from black walnut material, the historic range coincides with the range of Arizona walnut (*J. major*) and with that of California walnut (*J. californica*) in southern California (Bright and Stark 1973, Wood and Bright 1992). It appears likely that the beetle evolved in the region with both Arizona and California walnut as original host species (Seybold et al. 2010, Cranshaw 2011).

In recent decades, *P. juglandis* has greatly expanded its range and is now found across the American West (Cranshaw 2011) and in several eastern states (Grant et al. 2011, Seybold et al. 2012a). All species of walnut found in North America are known hosts of the beetle including the two species native in the East, black walnut (*J. nigra*) and butternut (*J. cinerea*), the native Western species Arizona walnut (*J. major*), California walnut (*J. californica*), and Northern California walnut (*J. hindsii*), and the nonnative but widely cultivated English (or Persian) walnut (*J. regia*) (Tisserat et al. 2009, Seybold et al. 2010, Seybold et al. 2013a). *P. juglandis* has also been reported from the closely related wignut (*Pterocarya* sp.) in California (Seybold et al. 2013a).

P. juglandis is a small yellowish to dark reddish-brown bark beetle (Bright 1981, Seybold et al. 2013b). The beetle is 1.5 – 2.0 mm long and approximately 3 times as long as it is wide (Blackman 1928, Bright 1981). Distinction of *P. juglandis* from other *Pityophthorus* can be difficult as most species share similar morphology, however, *P. jugalndis* possesses 4 – 6 concentric rows of asperities on the anterior of the pronotum (Bright 1981, Seybold et al. 2013b). WTB also has a shallow, often shiny, declivity (anterior portion of the elytra) (Seybold et al. 2013b).

Although male and female *P. jugalndis* superficially look similar, the species exhibits sexual dimorphism (Bright et al. 1981). Females possess a distinct tuft of yellow to golden setae on the frons (area of the head between the eyes). Conversely, males have a sparsely pubescent frons and may include a narrow brush of setae just above the mandibles (Seybold et al. 2013a). Males also have granules along the first and third interstrial spaces whereas the female declivity is smooth.

As is typical with many phloeophagus scolytines, males enter the bark first and create a nuptial chamber, followed by one of more females (Bright 1981). Upon entering the phloem, males produce an aggregation pheromone attracting females and other males (Seybold 2012, Seybold et al. 2013a). As

females follow a male into the nuptial chamber, mating ensues and the females excavate egg galleries, generally in the tangential direction. Females chew out notches along the tunnel walls, insert an egg, and cover the egg with a frass cap (Nix 2013). Though female fecundity was not empirically tested, Nix (2013) reported an average of 16.6 eggs per female. WTB have three larval instars (Dallara et al. 2012, Nix 2013) and were reported to develop in 4-5 weeks in a laboratory setting from samples in Tennessee (Nix 2013) and in 7 weeks in Colorado (Tisserat et al. 2009).

P. jugalndis adults begin to emerge in early March and remain active through much of November in Tennessee and California (Seybold et al. 2012b, Nix 2013). Essentially, beetles are active when the average air temperature is at or above 18-19°C (Seybold et al. 2012b, Chen and Seybold 2014). The beetles exhibit bimodal flight patterns with most flight activity occurring at dusk and dawn (Seybold et al. 2012b, Chen and Seybold 2014). Flight ceases during mid-day and at night. The seasonality of trap catches indicate *P. juglandis* activity is driven by temperature and beetle flight drops off significantly as daily mean temperatures drop below 17°C (Chen and Seybold 2014). When ambient air temperatures remain below this threshold, generally corresponding to winter months in the Northern hemisphere flight activity ceases (Nix 2013, Chen and Seybold 2014).

P. jugalndis overwinters under the bark, typically as an adult or larvae, however, pupae and teneral adults were also observed in eastern Tennessee during winter (Nix 2013). The beetle was shown to be intolerant of below freezing temperatures in experiments to determine the lethal lower temperature and super cooling point (SCP). Lower lethal temperatures that achieve 50% mortality (LT_{50}) were -16.7°C and -16.9°C for adults and larvae respectively, and SCPs were -16°C and -18.1°C for adults and larvae respectively (Peachey 2012). Such intolerance to direct exposure to winter temperatures helps explain the beetle's observed overwintering behavior and observed seasonal flight activity.

Beyond out-planted black walnut, *P. juglandis* and thousand cankers disease is currently causing mortality within the native walnuts of California and in plantations of English walnut (Seybold et al. 2010, Tisserat et al. 2011, Seybold et al. 2012a), and in black walnut and butternut in their native range in the eastern U.S. (Grant et al. 2011, Randolph et al. 2013). Beetles have been observed attacking apparently healthy trees, stressed trees, and cut logs (Tisserat et al. 2009, Mayfield 2014). It has also been shown that *P. juglandis* can persist within cut logs for at least 18 months, with evidence of beetle colonization of the cut material during that period (Peachey 2012).

Effective control methods for *P. juglandis* in live trees have not yet been determined (Moltzan 2011). Peachey et al. (2011) found only partial control (i.e. reduced numbers) of beetles in cut infested *J. nigra* logs when heat treated via solar radiation using clear plastic tarps, or through topical applications of bifenthrin, permetherin, and biodiesel. Steam heating of cut logs and fumigation with methyl bromide have been shown to be effective at removing WTB from cut logs. Mayfield et al. (2014) found steam heating logs to a core temperature of at least 52°C for 40 min to be effective in eliminating the beetle. Fumigation with methyl bromide at 120 mg/L at 4.5°C for 48 hrs is also an effective means of eliminating the beetle from logs (Myers unpublished). These treatments may be useful for movement of walnut logs across quarantine boundaries, or to meet the phytosanitary requirements of importing countries receiving walnut logs from the United States.

Means of preventing attacks of and mitigating damage to walnut trees are also being researched. Tissue accumulations of two systemic insecticides, imidacloprid and dinotefuran, were examined in *J. nigra* in eastern Tennessee. Nix et al. (2013) found that imidacloprid levels exceeded acceptable levels in walnut nut meat, but found only trace amounts of dinotefuran. Efficacy of the systemic insecticides on *P. juglandis* mortality was not tested, however, given the concentrations of imidacloprid found in the nut meat, the use of this insecticide may be limited; however, dinotefuran may provide a viable alternative (Nix et al. 2013). Several native predators have also been discovered. Two species of clerid beetles (Coleoptera: Cleridae), *Madoniella dislocatus* and *Pyticeroides laticornis,* were observed feeding on *P. juglandis* in a laboratory setting (Nix 2013). A previously unidentified parasitoid wasp (Hymenoptera: Pteromalidae) *Theocolax* sp. was also identified within *P. juglandis* galleries and observed parasitizing larvae. Such naturally occurring predators may provide potential biological controls for this invasive bark beetle (Nix 2013).

GEOSMITHIA MORBIDA

The pathogen in the thousand cankers disease complex is *Geosmithia morbida* M. Kolařík, E. Freeland, C. Utley,and N. Tisserat (Ascomycota: Hypocreales) (Tisserat et al. 2009, Kolařík et al. 2011). When the disease was first described, the associated *Geosmithia* fungus found was previously unknown. The discovery of what is now named *Geosmithia morbida* represents the first known case of phytopathogenicty in a species of *Geosmithia* (Kolařík et al. 2011). *G. morbida* is described as having yellowish conidia en masse with distinctly verrucose conidiophores with a stipe measuring 20-200 x 2.5-3 µm and a base with curved and atypically branched cells (Kolařík et al. 2011). Conidia inside beetle

galleries appear whitish and have been observed lining the tunnels of both adults and larvae (Tisserat et al. 2009, Nix 2013). Spores have also been observed and recovered from the bodies of *P. juglandis* (Tisserat et al. 2009, Kolařík et al. 2011).

P. juglandis is the only known vector of *G. morbida* (Lesile et al. 2010), however, it has been posited that other bark and ambrosia beetles could vector the pathogen (Newton and Fowler 2009, Reed et al. 2013). *Geosmithia* are commonly associated with numerous species of Scolytinae and globally distributed (Kolařík et al. 2004, Kolařík et al. 2007, Kolařík and Kirkendall 2010). Several other species of sub-cortical beetles have been reported from declining black walnut, including nonnative ambrosia beetles such as *Xyleborinus saxesenii* and *Xylosandrus crassiusculus* (Reed et al. 2013, Seybold et al. 2013b). Incidence of thousand cankers disease in the seven eastern states overlap with *Pityophthorus lautus,* a native bark beetle associated with walnut and several other hardwood species (Bright 1981, Lesile et al. 2010, Reed et al. 2013). It has been speculated that these or similar species could become a vector of the pathogen. In fact, *G. morbida* was recovered from the small bark dwelling weevil *Stenomimus pallidus* from a walnut plantation in Yellowwood State Forest in Brown CO., Indiana in June of 2014 (Juzwik et al. 2015). This represents a new state record for thousand cankers disease and the first instance in which the pathogen has been recovered from a species other than *P. juglandis*. To date, *P. juglandis* has not been recovered in Indiana (INDNR 2014). Further testing is required to see if other sub-cortical coleopterans can vector the *G. morbida* pathogen*.*

Geosmithia morbida has not been observed to move systemically within the host, meaning the fungus does not spread within the host or from host to host directly through grafting, but rather appears to produce annual cankers within the phloem (Tisserat et al. 2009, Hadziabdic et al. 2013). As a result, the pathogen must be repeatedly introduced into a host by numerous beetle attacks in order to propagate throughout a host. This occurs each time a *P. juglandis* chews its way into the phloem and the spores come into contact with viable phloem material (Tisserat et al. 2009, Kolařík et al. 2011). The phenomenon of what appeared to be hundreds of brown to black cankers on dying walnuts in Colorado resulted in the naming of the complex thousand cankers disease (Tisserat et al. 2009).

As with the walnut twig beetle, effective management strategies preventing infestation within hosts have not been determined (Moltzan 2011). Effective phytosanitation methods of removing the pathogen from cut wood have been found. The same steam heat treatment reported for the beetle—

52°C for at least 40 min—also prevented recovery of the pathogen (Mayfield et al. 2014). Methyl bromide fumigation that eliminates the beetle has produced mixed results for eradication of *G. morbida* as the fungus was recovered at low rates in tests in 2013 and 2014 (Myers unpublished data). To date, no data from testing fungicides or other chemical management options in preventing *G. morbida* inoculation in walnuts have been published.

THREAT TO *JUGLANS NIGRA*

Juglans nigra L. is a native deciduous hardwood, widely distributed across the Midwest and eastern deciduous forests from Florida to eastern Texas, north to eastern South Dakota and east across southern Quebec, CA and as far east as Massachusetts (Williams 1990). *J. nigra* is a tree reaching 30 m. in height with alternate, pinnately compound leaves reaching 0.3-0.6 m. long with 9-21 leaflets (Kirkman et al. 2007). Twigs are stout and brown with a dark brown chambered pith. Bark is dark gray to brown with deep furrows and distinctive, interconnecting ridges. Black walnut fruits are nuts within an irregularly ridged shell inside a distinctive spherical green husk that turns black as it ripens (Krikman et al. 2007). *J. nigra* looks similar to its co-occurring relative butternut (*J. cinerea*), however, butternut's fruits are ovoid in shape and the bark lacks the interlaced ridges found on black walnut (Kirkman et al. 2007).

J. nigra is found from open fields to forests as scattered individuals or in small clusters from moist, well-drained sites (Williams 1990). Occurrence of black walnut can be used as an indicator of moist, rich, well protected soils as the species is highly sensitive to soil characteristics, possessing a narrow ecological amplitude (Barnes et al. 1998). Although considered shade intolerant (Williams 1990), it is considered a gap-phase species as it can persist in the understory and grows quickly in response to disturbance events that open gaps in the canopy (Barnes et al. 1998).

J. nigra is one of the most economically valuable North American hardwood species (Shifley 2004, Newton and Fowler 2009, Moltzan 2011). Total value of standing black walnut timber for lumber and veneer production in the U.S. is estimated at more than $500 billion (Newton and Fowler 2009). *J. nigra* wood is commonly used for gun stocks, cabinetry, and several other finished wood products and is prized for its strong, durable, wood and distinctive dark brown heartwood (Williams 1990, Kirkman et al. 2007, Moltzan 2011). Veneer quality logs are exceptionally valuable (Moltzan 2011).

J. nigra nuts are edible and are sold commercially, although the nut from English walnut (*J. regia*) is more commonly available (Kirkman et al. 2007, Newton and Fowler 2009, Moltzan 2011). They are also used in several industrial applications, due to the abrasive properties of the shells (Michler et al. 2006). *J. nigra* is a commonly planted landscape tree, and a valuable species for wildlife, making it an important species in the ecology of the eastern hardwood forest (Shifley 2004, Michler et al. 2006, Newton and Fowler 2009).

Initial response to thousand cankers disease has been to adopt state quarantines restricting the movement of walnut wood and products out of infested counties (Newton and Fowler 2009, Moltzan 2011). Although the exact pathway of *P. juglandis* spread across the West and into the East is not known, beetles were likely carried in infested wood as logs, flitches, firewood, or burls (Newton and Fowler 2009). The infestation in Pennsylvania was traced to flitches of *J. hindsii* shipped to a mill from a western source (Turcotte et al. 2013). Nursery stock has also been implicated as a potential pathway (Newton and Fowler 2009, Moltzan 2011).

Items restricted for movement from any county and state infested with thousand cankers disease are as follows: "the walnut twig beetle; *Geosmithia morbida*; all plants and parts of plants—excluding nuts—in the genus *Juglans* including nursery stock, budwood, scionwood, green lumber, and other material living, dead, cut, or fallen, including logs, stumps, roots, branches, mulch and composted and un-composted chips; and any hardwood firewood" (TDA 2014). In order to move any of the restricted materials out of a quarantined area, the material must receive a phytosanitation certification or a compliance agreement (TDA 2014). Effective steam heat treatment, 52˚C for > 40 min, has been reported as a means of eliminating the vector and pathogen from wood and wood products (Mayfield et al. 2014). Other approved means of phytosanitation include fumigation and squared edged kiln dried lumber (TDA 2014).

RESEARCH OBJECTIVES

In order to mitigate damages from the invasive walnut twig beetle and thousand cankers disease, it is imperative that pathways of spread be identified and managed. Ideally, effective management of thousand cankers disease will include management of the disease where it occurs, mitigation of the impacts, and prevention of new anthropogenic introductions, while maintaining economic viability of industries associated with walnut, including lumber, nut, and nursery industries (Moltzan 2011).

Invasive species follow a general invasion process that can broadly be broken into four steps: (1) Introduction; (2) Establishment; (3) Spread; and (4) Impact (Sakai et al. 2001, Hulme 2006). Prevailing invasive species management theory suggests that prevention of a species introduction offers the most effective means of management (Simberloff et al. 2005, Hulme 2006). A crucial component to preventing nonnative invasive species introductions is the effective management of the pathways of introduction. Successful thousand cankers disease management relies upon comprehensive knowledge of how *Pityophthorus juglandis* can be spread so that strategies can devised to prevent further introduction.

The objectives of the following studies were to identify potential pathways of introduction or spread by studying *P. juglandis* colonization behavior in commercially transported goods, and to identify potential tools for preventing colonization of cut logs. Given the potential for the beetle to colonize aged logs (Mayfield unpublished data, Peachey 2012) and possibly chemically and culturally treated logs (Peachey 2012), the beetle's ability to colonize steam heated and methyl bromide fumigated *J. nigra* logs were tested. Although evidence suggests *P. juglandis* can attack small diameter branches (Seybold et al. 2010), no studies have been reported on the beetle's colonization behavior in young, small diameter trees, common for nursery stock. Thus, experiments were conducted to test *P. juglandis* colonization in *J. nigra* nursery seedlings. Finally, laboratory scale assays were conducted testing potential chemical treatments for preventing beetle colonization of *J. nigra* logs. Results of these studies help to better elucidate the risk of anthropogenic spread of thousand cankers disease and to better inform management and quarantine restrictions.

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Chapter 2 Effect of phytosanitation methods on post-treatment colonization of *Juglans nigra* logs by *Pityophthorus juglandis*

ABSTRACT

North American walnuts (*Juglans* spp.) are currently under threat from thousand cankers disease caused by an insect-pathogen complex: the walnut twig beetle (*Pityophthorus juglandis* Blackman) and the associated fungal pathogen *Geosmithia morbida*. Of particular concern is the potential damage to eastern black walnut (*Juglans nigra* L.), one of North America's most valuable hardwood lumber species. The purpose of this study was to determine potential pathways of spread of the beetle in forest products. We tested currently accepted phytosanitation treatments for any residual effect on beetle colonization. Steam heated, methyl bromide fumigated, and kiln-dried samples of *J. nigra* were exposed to *P. jugalndis* colonization pressure in two exposure scenarios. In a high-exposure scenario, beetles readily colonized steam heated and methyl bromide fumigated logs. Beetles were also recovered from kiln-dried lumber with bark left on. Under a more realistic exposure scenario, beetles were again recovered from steam heated logs, but were not recovered from kiln-dried lumber with bark left intact. Results from the second trial may have been confounded by overall low beetle recovery. These data suggest logs and lumber treated with phytosanitation methods should not be exposed to subsequent *P. jugalndis* colonization pressure. Further protection may be required to ensure walnut wood transported out of quarantine areas does not pose a risk of further introduction or spread of *P. jugalndis* and thousand cankers disease.

BACKGROUND

Eastern black walnut (*Juglans nigra* L.) is currently threatened by thousand cankers disease, caused by the insect-pathogen complex comprising the walnut twig beetle (*Pityophthorus juglandis* Blackman, Coleoptera, Curculionidae, Scolytinae) and the fungal pathogen *Geosmithia morbida* (Tisserat et al. 2009, Kolařík et al. 2011). Adult *P. juglandis* introduce the pathogen as they enter the phloem of the trunk and branches of a host walnut tree. *Geosmithia morbida* is not a systemic pathogen, and instead requires repeated introductions to cause new cankers (Tisserat et al. 2009). *Pityophthorus juglandis* is native to the American southwest, historically known from Arizona, New Mexico, and northern Mexico, associated with the range of Arizona walnut *Juglans major* (Torr.) A. Heller (Cranshaw 2011, Seybold 2012a). *Juglans major* appears to be the least susceptible species in the *Juglans* genus (Moltzan 2011, Utley et al. 2013). Conversely, *J. nigra* and butternut (*J. cinerea* L.) have been shown to be particularly susceptible to the pathogen, developing the largest cankers when inoculated with the fungus (Utley et al. 2013).

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In recent decades, the beetle's range has greatly expanded. Recoveries have been reported from dead and dying walnuts—particularly out-planted *J. nigra*—throughout the western U.S. (Cranshaw 2011, Tisserat et al. 2011, Seybold et al. 2012a). More recently, *P. juglandis* has been found in several states in the eastern U.S. within the native range of *J. nigra* (Cranshaw 2011) Including Tennessee (Grant et al. 2011), Virginia, Pennsylvania (Seybold et al. 2012a), North Carolina (Hadziabdic et al. 2013), Maryland (MDA 2013), and Ohio (Fisher et al. 2013). The pathogen has been recovered from trees in a single county in Indiana; however, no *P. jugalndis* been detected yet (Juzwik et al. 2015). Beetle introductions beyond its native range are hypothesized to have occurred through the transport of infested walnut wood material through commerce or personal firewood movement (Newton and Fowler 2009).

In response to the range expansion of *P. juglandis* and threat from thousand cankers disease, states have established quarantines restricting the movement of walnut (Newton and Fowler 2009). Items restricted from movement out of any county infested with thousand cankers disease include: all plants and parts of plants—except nuts—in the genus *Juglans* including nursery stock, budwood, scionwood, green lumber, logs, stumps, roots, branches, mulch, and any hardwood firewood (TDA 2014). In order to move any of the restricted materials out of a quarantine area, the material must receive a phytosanitation certification or a compliance agreement from the receiving state (Newton and Fowler 2009, TDA 2014).

Effective phytosanitation methods to eradicate thousand cankers disease from infested walnut wood have been reported. Mayfield et al. (2014) reported that heating the outer sapwood (at a depth of 1 cm) of a log to at least 52°C for 40 minutes killed all life stages of *P. juglandis* and prevented any recovery of *G. morbida*. Because walnut is sometimes co-mingled with ash in loads of firewood, Mayfield et al. (2014) recommend sanitizing walnut firewood with the same 60°C (core temperature) for 60 min standard currently implemented for sanitizing ash logs infested with *Agrilus planipennis* Fairmaire (Myers et al. 2009). Because walnut and ash may be under quarantine in the same area (e.g. much of east Tennessee), utilization of the same standard provides a consistent heat treatment option.

Fumigation with methyl bromide or several alternative compounds is commonly used as a phytosanitary treatment on logs for export in international trade (Haack and Brockerhoff 2011). Work to develop a methyl bromide treatment schedule for thousand cankers disease has found that treatments of 120 mg/L at 4.5°C for 48 hours are effective in eliminating *P. juglandis* from infested *J. nigra* logs. However,

G morbida is capable of surviving this treatment and will require substantially higher rates to achieve quarantine level control (Myers unpublished)

Although effective phytosanitary measures to eradicate thousand cankers disease have been determined, no work has evaluated the risk of re-infestation of walnut material post treatment (Mayfield et al. 2014). There is evidence to suggest some species will colonize and persist within these treated materials. Haack and Petrice (2009) assessed the ability of bark and wood boring insects (Curcculionidae and Cerambicidae) to infest heat treated lumber with bark left on, and found that certain species from both families of wood attacking insects readily colonized the treated materials.

J. nigra is one of the most economically valuable North American hardwood species, prized for its dark heartwood (Shifley 2004, Newton and Fowler 2009, Moltzan 2011). Total standing U.S. *J. nigra* timber stock for lumber and veneer production is estimated to be more than $500 billion (Newton and Fowler 2009). Continued spread of thousand cankers disease is likely to have significant impacts on lumber, veneer, and other related wood products industries as a result of restrictions on the transport of *J. nigra* and other North American *Juglans* species (Newton and Fowler 2009).

Given the economic, ecological, and cultural importance of *J. nigra* (Newton and Fowler 2009, Moltzan 2011), it is imperative to determine all anthropogenic pathways available to *P. juglandis* and *G. morbida*. Comprehensive assessment of phytosanitation measures can be considered in two parts: First, assessing the efficacy of eliminating a pest or pathogen from infested material, thus rendering material pest free; Second, assessment of treatment efficacy in preventing new attack or colonization of the sanitized material. The goal of this study was to assess the latter aspect with respect to the thousand cankers disease system.

In order to assess the risk of colonization of previously treated *J. nigra* wood by *P. juglandis*, two field based experiments in which treated samples of walnut were exposed to beetle colonization pressure were implemented. The first experiment was intended to expose treated samples to extreme beetle pressure as a "worst case scenario." Samples were baited with a *P. juglandis* aggregation pheromone lure (Seybold et al. 2013, Mayfield et al. 2014) and placed in the crowns of actively infested *J. nigra* trees (Mayfield et al. 2014). We hypothesized that *P. juglandis* would colonize the steam heated and methyl bromide fumigated samples, but that the two kiln-dried lumber samples would not be colonized by the

beetles. We expected little to no residual effect of the phytosanitation treatments that would prevent colonization, concurrent with the findings of Haack and Petrice (2009), but did expect the dehydrated nature of the lumber treatments would deter beetle colonization.

In a second experiment, we attempted to create a more realistic, lumberyard exposure scenario in which sanitized logs and lumber were placed on the ground within close proximity to infested logs. We also sought to elucidate the effect of the pheromone lure had on observed colonization. All treated *J. nigra* samples were divided into two blocks. In the first block, all samples were baited with the lure, and in the second block, no samples were baited. We predicted *P. juglandis* would colonize the steam heated logs (fumigation was not included) but not the two kiln-dried lumber samples following the same logic as above. We also expected that the baited samples would be attacked more than non-baited samples across treatments, because the lure simulates a beetle-produced signal indicating a viable host has been found (Seybold et al. 2013).

MATERIALS AND METHODS

EXTREME EXPOSURE STUDY

The first experiment, hereafter referred to as the extreme exposure study, involved the exposure of treated samples of *Juglans nigra* wood (N=150) to *Pityophthorus juglandis* colonization pressure in a field-based, full factorial experiment. Round-wood logs (bolts) (n=90) measuring roughly 61 cm in length and 7-16 cm in diameter and boards of sawn lumber (n=60) measuring 61 cm long by 5 cm wide and 15 cm deep, were treated to phytosanitation specifications outlined in the quarantine regulations (TDA 2014). Bolts were cut from felled *J. nigra* obtained from an orchard in Nebo, NC, a county in which no evidence of *P. juglandis* or thousand cankers disease has been reported, on 23 July 2013 and brought back to Knoxville, TN for processing and treatment at the University of Tennessee. Ends of each bolt were sealed by dipping the end approximately 2 cm deep into melted Parrafin wax, resulting in a barrier that retarded moisture loss (Mayfield et al. 2014). Once sealed, bolts were distributed amongst three groups of roughly equal diameter distributions (visually estimated). Once distributed, samples were tagged and the diameter measured using a caliper and recorded. Groups were then assigned to one of three treatments: steam heat (n=30), methyl bromide fumigation (n=30), or no treatment (control; n=30).

For the steam heat treatment group, a 4 mm diameter hole was drilled through the bark at the log midpoint to a depth of ½ of the bolt's diameter. Bolts were placed on wooden racks inside a walk-in kiln (SII Dry Kilns, Lexington, NC) at the University of Tennessee and a type K, 22-guage thermocouple was inserted into each hole to measure the temperature at the core of each log (Taylor and Lloyd 2009). Thermocouple wires were wedged into the hole with round-wood toothpicks, creating a seal reducing ambient heat measurements. Temperature data were recorded at 1 minute intervals using a Keithly Model 2700 multimeter-data acquisition system (Keithley Instruments, Inc., Cleveland, OH) interfaced with a laptop computer (Taylor and Lloyd 2009, Mayfield et al. 2014). The core of each bolt was brought to 60°C and maintained for a duration of 60 minutes (Myers et al. 2009, Mayfield et al. 2014) with the kiln vents closed keeping relative humidity inside the kiln near 100% (Mayfield et al. 2014).

Samples to be fumigated were placed into two 245 L, gas-tight sealable steel chambers housed inside a standard 6.1 m refrigerated shipping container to maintain temperature at 4.5°C. An initial dose of 120 mg/L methyl bromide (Meth-O-Gas Q, Great Lakes Chemical Inc., West Layfayette, IN) over 48 hr exposure period was used based on prior work that had shown this treatment to provide complete control of *P. juglandis*. (Myers unpublished). Methyl bromide headspace concentrations were monitored during the fumigation using an Agilent 490 micro gas chromatograph (Agilent Technologies Inc.) with a 10 m poraplot Q column and thermos conductivity detector. Samples were drawn at 2 hr intervals through 2 m lengths of 0.762 mm ID polyether etherketone (PEEK) tubing via a 6-channel stream selector valve (Valco Instrument Co. Inc.). Concentration x time products were calculated using mid-point reimann sums for each of the two fumigations. Upon completion of the treatment, the lids to the containers were removed and the bolts allowed to off-gas for 48 hrs. All treated bolts were housed in the lab for 3-5 days at approximately 20°C until deployment in the field.

Samples for the two lumber treatments were cut from a *J. nigra* log purchased from, and sawn into boards roughly 2.4 m × 30 cm × 5 cm at a mill in Oak Ridge, TN. Boards were taken back to the University of Tennessee and cut into 60 pieces measuring roughly 61 cm × 15 cm × 5 cm. Bark was removed from 30 boards ("Kiln-dried lumber no bark" treatment) and left intact on the remaining 30 ("Kiln-dried lumber with bark"). Boards were placed in the kiln at the University of Tennessee and dried to approximately 8% moisture content following the standard *J. nigra* drying schedule (Boone et al. 1988). Samples were removed from the kiln and housed in the lab until deployment in the field.

Field sites were located around the greater Knoxville, TN area and were selected based on severity of visual symptoms in walnut trees, accessibility, and in some cases based on previous trapping or study success (Mayfield et al. 2014). Thirty trees at 11 sites were selected in which to hang five bolts per tree—one each of the five treatments. Nylon ropes (1.27 cm diameter) were hung in the infested walnut trees using a Big Shot™ slingshot (Jameson LLC, Clover, SC) to launch a weighted line over branches in the canopy. Ropes were pulled over each branch using the line and tied on the trunk of the tree on one end as described by Mayfield et al. (2014). One *P. juglandis* pheromone lure (product #300000736, Contech Enterprises Inc., Delta, BC) was stapled to each sample midway along the length of each sample. On the opposite side, a white 7.6 x 17.5 cm sticky card with a 1 cm^2 grid pattern (AlphaScents Inc. West Linn, OR). Sticky cards were used to confirm the presence of beetles in flight at each site. An eye-bolt was screwed into the tops of the wood samples and the free end of rope was tied onto each eye-bolt and hoisted into position in each of the 30 trees. Samples were spaced ≥ 2 m apart.

Samples were deployed for 30 days from 5 August to 5 September 2013. At the conclusion of 30 days all wood samples were brought back to the University of Tennessee for processing. Each sample was cut in half and either the top half or bottom half was randomly assigned to an emergence chamber. All samples were brushed vigorously with a handheld broom to remove any beetles residing on the surface prior to placement within the emergence chamber.

Emergence chambers were constructed from 18.2 L paint mixing buckets and a hook was attached to the inside of the lid. Bucket bottoms were cut off and replaced with a funnel leading to a Nalgene collection cup (Reed et al. 2013, Mayfield et al. 2014). Collection cups contained propylene glycol at a depth of approximately 2 cm to kill and temporarily preserve emerged beetles (Mayfield et al. 2014). Samples were collected every 4 weeks for a duration of 5 months. Collections were made using paint strainers to filter insects from the propylene glycol and kept refrigerated. Emerged insects were observed using a dissecting stereoscope and all *P. juglandis* were identified using a screening aid (Seybold et al. 2013) and tallied.

The remaining half of each sample was assessed for colonization of and reproduction within treated material by measuring total *P. juglandis* gallery lengths (cm) and counting life stages. A sub-sample of the middle 1/3 of bark (10 cm) on each steam heated, fumigated, and control bolt was removed by scoring the bark with a band-saw and peeling back to the cambial layer using a chisel (Figure 2.1).

Neither lumber treatment was included in this analysis because the source of the log purchased was not known and may have come from an infested area in TN, potentially confounding results. Galleries of adult *P. juglandis* were measured using a Scalex MapWheel© (Scalex Corp. Carlsbad, CA). Counts of *P. juglandis*—adults, pupae, and larvae—in each bark sample were recorded. Only galleries and life stages found within the innermost portion of the phloem were measured as neither the phloem nor the bark was further dissected.

Total emergence of *P. juglandis* was divided by the surface area of bark for each sample (beetles/cm^2) of bark. This controlled for differences in bolt diameters among bolts and lumber treatments. Mean emergence was Log10(y+1) transformed to ensure homogeneity of variance based on Levene's test (α=0.05) and analysis of variance (ANOVA) was used to test the null hypothesis of no difference in mean emergence between treatments (PROC ANOVA; α=0.05). Mean gallery lengths were also Log10(y+1) transformed to satisfy homogeneity of variance. Transformed mean gallery lengths per treatment and mean life stage count (pooled adult, pupae, and larvae) per treatment were compared using ANOVA as well. Post hoc Tukey's HSD tests were performed to identify and differences found between means with ANOVA. *P* values < 0.05 were considered significant for all analyses. All statistics presented represent analysis on transformed data, however, tables and figures represent non-transformed data. All analyses were performed using SAS V.9.3 with Enterprise Guide software (SAS Institute 2013).

LUMBERYARD EXPOSURE STUDY

The second experiment, hereafter referred to as the lumberyard exposure study, consisted of treated *J. nigra* samples (N=80) subsequently exposed to *P. juglandis* colonization pressure. Treatments included steam heated bolts, non-treated (control) bolts, kiln-dried lumber with bark on, and kiln-dried lumber with bark removed. Methyl bromide fumigation was not included in this experiment based on an observed similar performance to steam heated material from the first study. Twenty replicates of each treatment were split between two blocks, block A in which all samples received the pheromone lure and block B in which no samples received the lure. Bolts were again cut from felled *J. nigra* in Nebo, NC. Samples were cut to roughly 30.5 cm long and ranged between 6 and 17 cm in diameter. Cut ends were sealed in the field using AnchorSeal (UC Coatings Corp., Buffalo, NY) and brought to Knoxville, TN for treatment at the University of Tennessee. Bolts were randomly assigned to steam heat or control groups. Treated samples were heated until the core temperature was raised to 60°C for 60 min as previously described. Control samples were housed in the lab at approximately 20°C until deployment.

Lumber was once again procured from a log purchased and cut at a lumber mill in Oak Ridge, TN. Boards were sawn from the log as described above and the bark completely removed from half of the samples. Boards were cut to 30.5 cm in length and placed onto racks within the walk-in kiln and dried to 8% moisture content following the same schedule used in the first experiment (Boone et al. 1988).

To create a "lumberyard exposure" scenario, 10 circular plots 6 m in diameter were arranged along two 38 m long transects (5 ea). Each plot was spaced 2 m apart (from edge to edge) and the two transects were arranged so that the perimeters of plots were 10 m apart (Figure 2.2). Transects ran East to West within the field. Plots were established on an open field on the Holston River Farm Research Unit located east of downtown Knoxville, TN along the Tennessee River (35.959055° N, -83.855090° W). The study site was located on the shoulder of a hill sloping towards the river. The field was located south of an equipment barn for the farm. A row of large box elder (*Acer negundo* L.) bordered the field on the east and a row of small eastern red buds (*Cercis canadensis* L.) bordered the field to the north along a gravel access road behind the barn. There were no *J. nigra* located within 200 m of study area.

Each circular plot was divided in half along an east-west gradient so that 4 spaces were evenly distributed along the perimeter of the northern half and 4 spaces distributed along the southern half (Figure 2.2). Treated samples were randomly assigned to a space in a plot by first assigning two samples of each treatment to a plot (numbered 1-10) by generating a random numbers table and assigning corresponding sample tag numbers a plot beginning with plot 1 and moving chronologically. Next each pair of samples was randomly assigned to either the northern half or the southern half. Finally, a random numbers table (1-4) was generated where 1=control, 2=steam heated, 3=kiln-dried lumber with bark, and 4=kiln-dried lumber without bark. Placement began at the easternmost position on the northern half of each plot and moved in a counter clockwise fashion (east to west on the northern half and west to east on the southern) until all samples were assigned to a position.

An infested source bolt was placed in the center of each plot as the source material for *P. juglandis* colonization pressure. To infest source bolts, 40-70 cm long bolts were cut from a single tree at Nebo, NC and from three trees at the University of Tennessee Arboretum in Oak Ridge, TN. Each bolt was baited with pheromone lure and hung in known infested trees as described in the previous experiment. Each bolt was hung for 30 days prior to deployment in the field. After 30 days, a 10 cm long sub-sample was cut off of the top of each and placed into a rearing chamber as a means of estimating the total

beetle emergence. Beetle emergence for each sub-sample was monitored for eight weeks and extrapolated to estimate the total emergence per source log. A source log was randomly assigned to each of the 10 exposure plots and placed on-end at the center.

Samples were exposed to emerging beetles at the field plots for 57 days from 20 August to 16 October 2014. Exposure time was originally planned for 30 days but was extended based on low beetle emergence from the infested source bolt sub-samples. Supplemental beetles (10M, 10F beetles at each plot) were released on two occasions in an attempt to increase the number of potential colonizers. Supplementary release beetles were reared from infested bolts in the lab, transferred to plastic petri dishes for transport to the field site, and placed onto a small wooden platform set atop the source bolt. Supplementary beetles were released between 5:30 and 6:30 pm EST based on reported peak flight activity (Seybold et al. 2012b, Chen and Seybold 2014). After exposure concluded, all samples were placed into emergence chambers for five months.

Parametric ANOVA could not be used because multiple treatments had zero or near zero values, thus nonparametric ANOVA procedures were used. The Kruskal-Wallis test was used to test the null hypothesis that there was not difference in mean beetle emergence (# beetles/100 cm^2 of bark) among treatments within each block, and the Dwass, Steel, Critchlow-Fligner (DSCF) multiple comparisons procedure was used to identify means that differed between groups in pairwise tests (Hollander and Wolfe 1999). The Wilcoxon two sample Z-test was used to test the null hypothesis that mean beetle emergence was not different between each treatment across blocks (with lure and without lure). All *P* values < 0.05 were considered significant. Nonparametric ANOVA was performed using PROC NPAR1WAY in SAS V.9.3 with SAS Enterprise Guide software package (SAS Institute 2013).
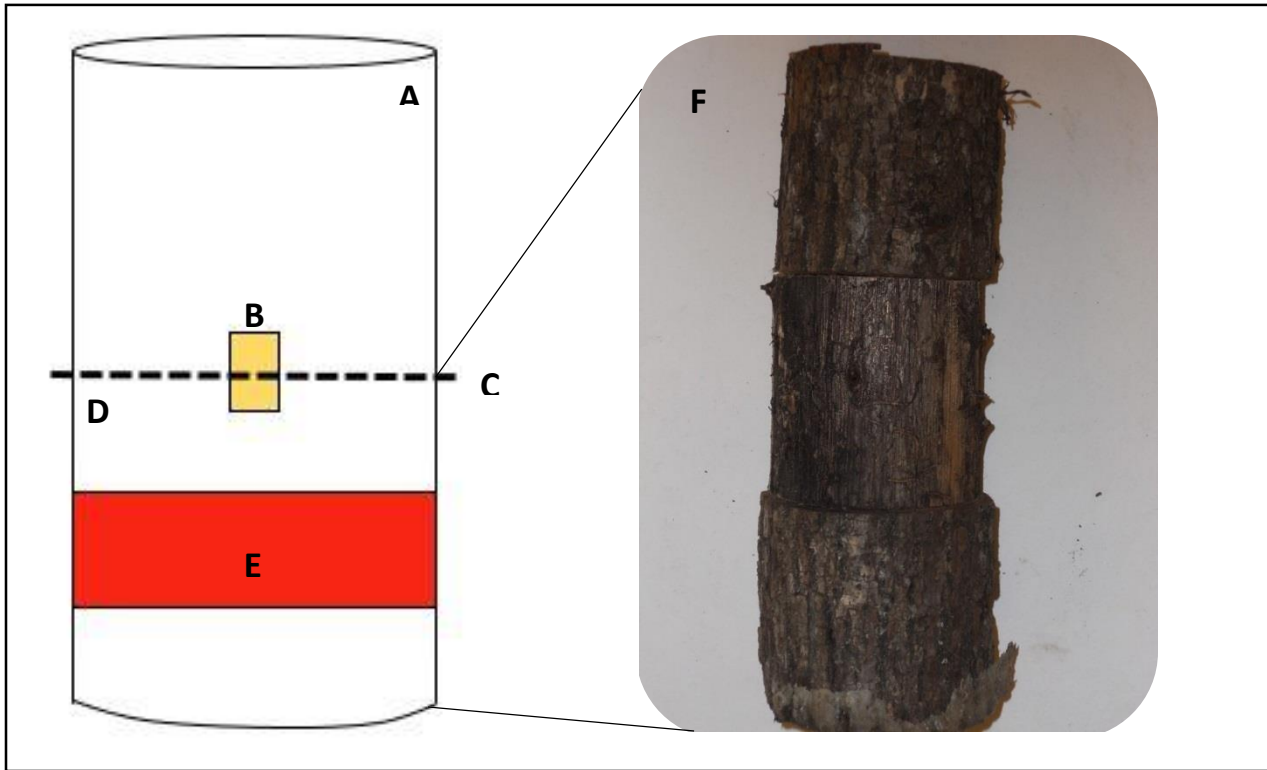
Figure 2.1. Diagram of a sample log. Where (A) is the top half of the log, randomly assigned to an emergence chamber, (B) the yellow box indicates where the pheromone lure is attached, (C) indicates the cut line dividing the whole log in half, (D) represents the bottom half of the log, assigned to further sub-sampling, (E) the red rectangle indicates the middle third of the lower half where the sub-sample of bark was taken, and (F) depicts an example of a sample log half with the bark removed for analysis.
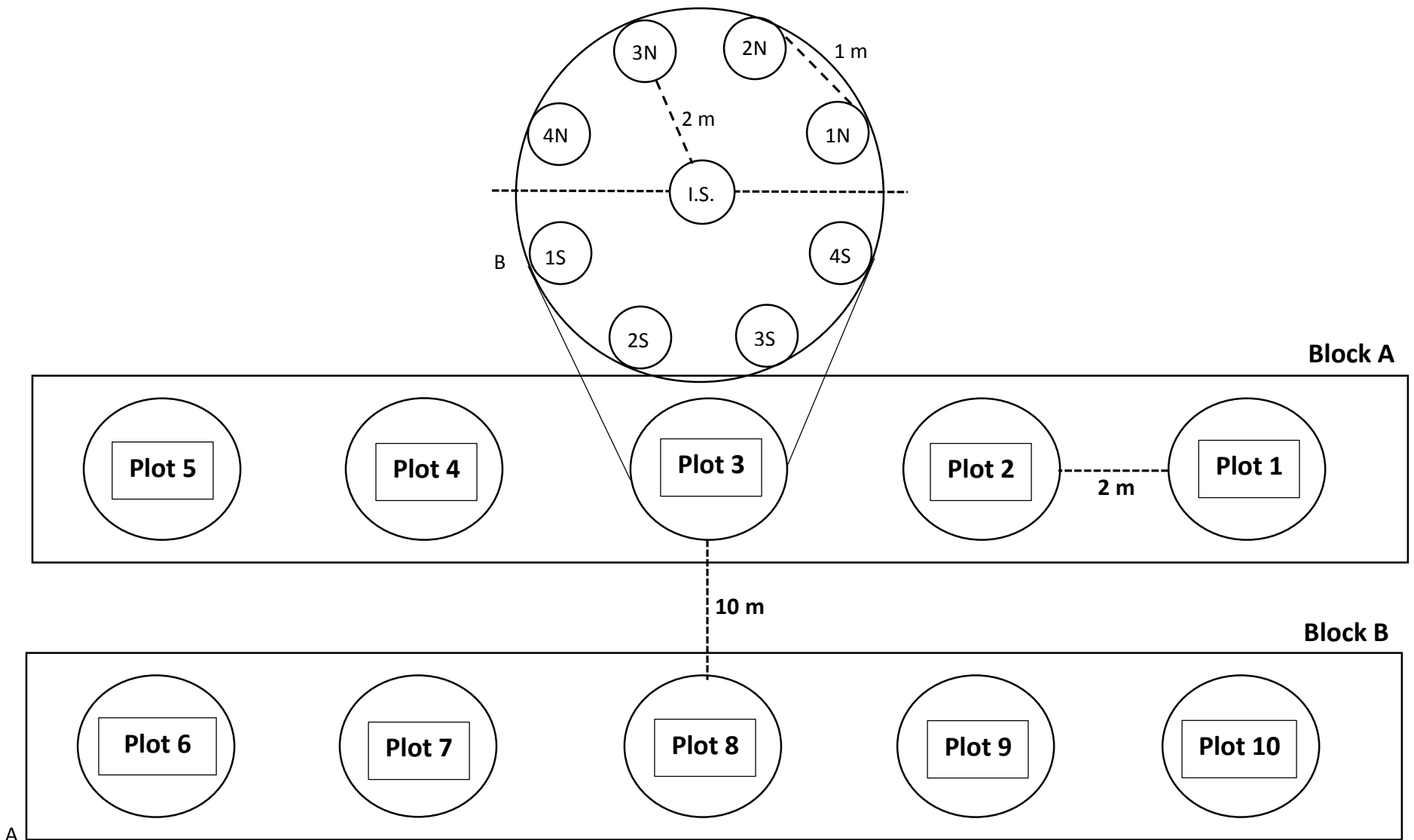
Figure 2.2. Diagram of the circular plots arranged on two transects Block A and Block B, each with 5 plots (A). Example showing the detailed setup of a single plot (B). Detailed plot shows the division between the 4 positions on the northern half of the plot and the 4 positions on the southern half of the plot. I.S. indicates the position for the Infested Source material.

RESULTS

EXTREME EXPOSURE STUDY

A total of 43,455 *P. juglandis* were collected from 105 samples (Table 2.1). The majority, 64%, of beetles emerged from the control samples. Four beetles were recovered in the collection cup of 2 kiln-dried lumber with no bark samples, however, a *t*-Test indicated there was no evidence to suggest the true mean was greater than 0 (t_{29} = 1.28; P = 0.21). Therefore, kiln-dried lumber with no bark samples were excluded from ANOVA. The number of emerging beetles differed significantly among the samples tested ($F_{3, 116}$ = 60.23; P < 0.01). Emergence was greatest from the control samples, then followed by steam heated and fumigated bots, and the kiln-dried bark on samples had the lowest emergence (Figure 2.3).

P. juglandis gallery lengths differed significantly among control and treated samples ($F_{2, 87}$ = 44.70; P < 0.01). Although Tukey's HSD test did not indicate a difference in mean beetle emergence between steam heated and fumigated bolts, however, mean gallery length was approximately 4 times shorter in the fumigated samples (Figure 2.4).

Mean pooled *P. jugalndis* life stages also varied among samples ($F_{2, 87}$ = 6.30; P < 0.01). Control and fumigated samples were found to be different, while steam heated and fumigated samples were not different (Figure 2.5).

Mean emergence was plotted over the monitoring period to detect temporal patterns among the treatments (Figure 2.6). The control, steam heated, and fumigated bolts all had a similar pattern. Peak emergence occurred within eight weeks and was followed by a steady decline over the remaining 12 weeks. Conversely, emergence from the kiln-dried bark on samples followed a different pattern. Eighty-seven percent of all emergence was captured within the first 4 weeks of monitoring (Figure 2.6).

LUMBERYARD EXPOSURE STUDY

Total *P. juglandis* colonization pressure per cluster was estimated by calculating the number of beetles that emerged per unit surface area of bark as previously described from each of the 10 cm long source bolt sub-samples (Table 2.2). The sub-sample emergence was then extrapolated to the source bolt by multiplying the total number of beetles per sub-sample by the total surface area of bark for each source log. The two supplemental releases totals (20/cluster/release) were added to the extrapolated beetle emergence to get the total per plot colonization pressure (Table 2.2).

Despite an average estimated number of potential colonizers of 281 beetles per plot, at total of only 288 *P. juglandis* were collected from the samples across the four treatments. The majority of beetles, approximately 80%, emerged from control bolts and the remaining 20% emerged from steam heated bolts. Of the beetles emerged from control bolts, 80% were collected from bolts in Block A. Of the 57 beetles collected from steam heated samples, 46% were collected from samples in Block A and 54% were collected from Block B. All beetles were collected from only eight bolts (10%), five control and three steam heated samples.

The Kruskal-Wallis test indicated there was evidence of a variation across the treatments in Block A (X^2 = 9.52; P = 0.02), however, the DSCF multiple comparisons did not indicate any pairwise differences (Table 2.3). There was no evidence to indicate a difference among treatments in Block B (X^2 = 2.05; P = 0.56). Similarly, the Wilcoxon two sample Z-test indicated there was no evidence of variation in mean beetle emergence between any of the treatments across the two blocks (Table 2.4).

DISCUSSION

EXTREME EXPOSURE STUDY

Pityophthorus juglandis readily colonized the phloem of steam heated and methyl bromide fumigated *J. nigra* logs that were baited with a pheromone lure. Although treatments reduced the number of beetles that emerged compared with the untreated samples (Figure 2.3), residual effects from the steam heat and methyl bromide fumigation sanitation treatments did not impede beetles from attacking and reproducing within the material. Both mean beetle emergence and mean gallery length of the inner phloem layer are well above zero (Figures 2.3 & 2.4). Thus the steam heat and fumigation sanitation treatments reduce beetle preference of the material but do not preclude subsequent colonization and reproduction.

Recovery of all three life stages from sub-samples of both treatments and the timing of peak emergence (Figure 2.6) supports the conclusion that steam heat and methyl bromide fumigation did not prohibit *P. juglandis* reproduction in treated logs. Emergence trends for the two sanitation treatments appear very similar to the emergence trend of the control logs, which are known to produce subsequent generations (Peachey 2012, Nix 2013, Mayfield et al. 2014). Emergence in all three treatments peaked within eight weeks of the beginning of emergence monitoring and is consistent with the previously reported generation time of seven weeks (Tisserat et al. 2009).

Although the control samples had three times as many beetles emerge as the steam heated samples (Table 2.1), the mean pooled life stages between the controls and steam heated bolts were not different (Figure 2.5). Another surprising trend observed was the mean gallery length from the steam heated samples was more than 4 times longer than the mean gallery length from the fumigated samples (Figure 2.4). Given the similar number of beetle emergence (Figure 2.3) we expected to see less pronounced of a difference. These findings may be the result of an uneven distribution of *P. juglandis* colonization throughout a log, which would help explain why our sub-sample of bark did not reflect the same trends observed from the emergence monitoring. Instead, beetles may congregate in spots along the profile of a log, perhaps in response to the aggregation pheromone produced by males (Seybold et al. 2013).

Sixty-three beetles were recovered from 14 samples of kiln-dried lumber with bark on (Table 2.2) indicating that *P. juglandis* can enter bark dried to 8% moisture content when baited with a pheromone lure. Given the dehydrated state of kiln dried bark and phloem, this was a surprising find. Although there is evidence that beetles can enter and persist for some time within the dried bark, there is no evidence of successful reproduction within the material. Unlike the heated, fumigated, and control bolts, peak emergence occurred within the first four weeks of emergence and rapidly decreased to zero (Figure 2.6). Despite not being viable material for reproduction, kiln-dried bark may provide a temporary refuge allowing for accidental transport of adult *P. juglandis*.

On the other hand, the kiln-dried lumber with bark removed samples were not successfully attacked by the beetles, even when baited with pheromone lure. Four beetles were recovered from 2 samples during a single collection (Table 2.1), however, these were likely cross contamination and not successful attacks. It is possible beetles managed to escape from adjacent emergence chambers and either found a way into the two chambers, or into the two collection cups while cups were removed, prior to straining the sample. Another possible explanation is that the beetles resided in the crevices created from splits within the boards. Both samples did have sizeable ($\geq$ 5 cm long) splits, and beetles may have remained wedged in the crevices, eventually falling into the respective collection cups. Presence of the beetles could provide indication of the extreme colonization pressure the samples were subjected to. However, it seems unlikely any beetle would remain in such in a confined area without any food resources for

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Table 2.1. Total number of *Pityophthorus juglandis* emerged from each of the five treatments and the number of samples colonized per treatment tested in the extreme exposure study.

Treatment	Total *P. juglandis*	Mean (±SE) SA Bark*	Beetles/cm^2 bark	Num. samples**
Steam Heated	9509	1160.6 ± 42.0	788	29
Fumigated	6022	1230.5 ± 34.7	468	30
KDLB†	63	79.7 ± 0.8	81	14
KDLNB‡	4	0 ± 0	*NA*	2
Control	27857	1094.5 ± 33.2	2578	30
Total	43455		3914	105

Note: all numbers were rounded to the nearest whole beetle.

NA: Not applicable as there was no bark on these samples.

*Surface area of bark in cm^2.

**Number of samples with $\geq$ 1 beetle emergence.

†Kiln-dried lumber with bark intact.

‡Kiln-dried lumber with no bark.

Table 2.2. Estimated number of potential *Pityophthorus juglandis* colonizers for each circular plot from the lumberyard replication study.

Circular Plot	Block	Est. # from Source Bolt*	Supp. Releases	Est. Potential Col.†
1	A	82	40	122
2	A	46	40	86
3	A	48	40	88
4	A	1686	40	1726
5	A	56	40	96
6	B	7	40	47
7	B	137	40	177
8	B	112	40	152
9	B	26	40	66
10	B	210	40	250
			Total	2810

*Estimated numbers of potential colonizers were estimated by extrapolating the number of beetles per total surface area of bark emerged from a 10 cm long sub-sample of each bolt, multiplied by the total surface area of the source bolt. All beetle estimates are rounded to the nearest whole beetle.

†Total estimated number of potential colonizers
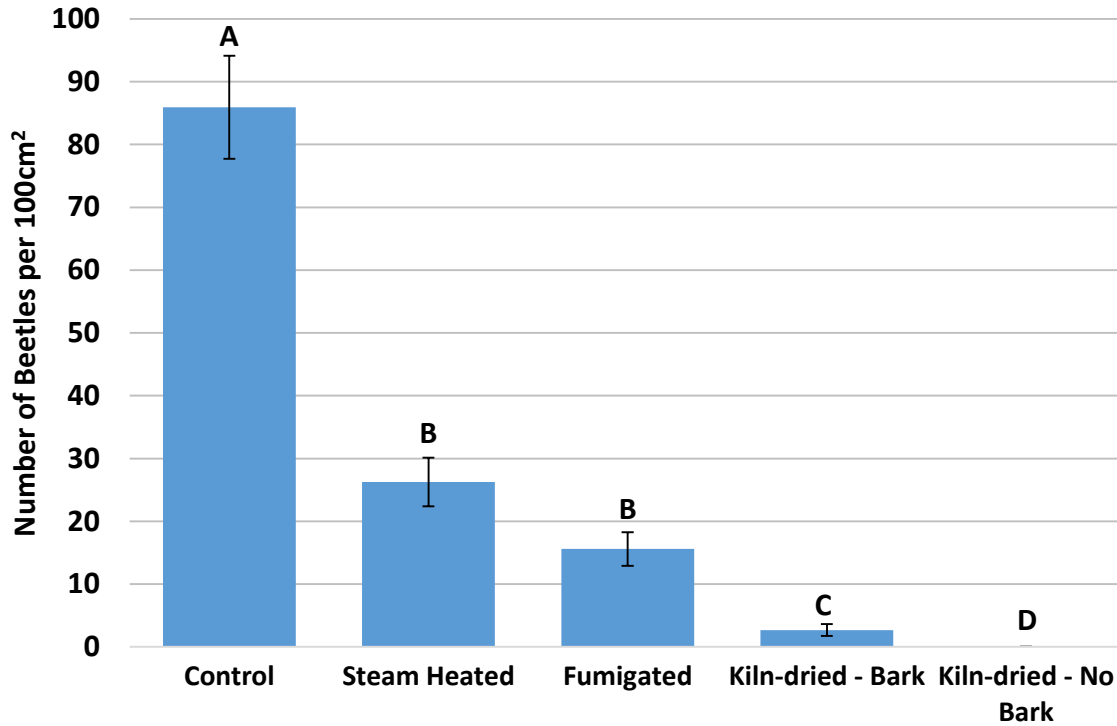
Figure 2.3. Mean number of beetles/100cm^2 of bark (± SE) by treatment emerged from treated samples of *Juglans nigra* baited with pheromone lure and exposed to *Pityophthorus juglandis* during the extreme exposure scenario trial. Different letters indicate different means based on Tukey's HSD test (α = 0.05).
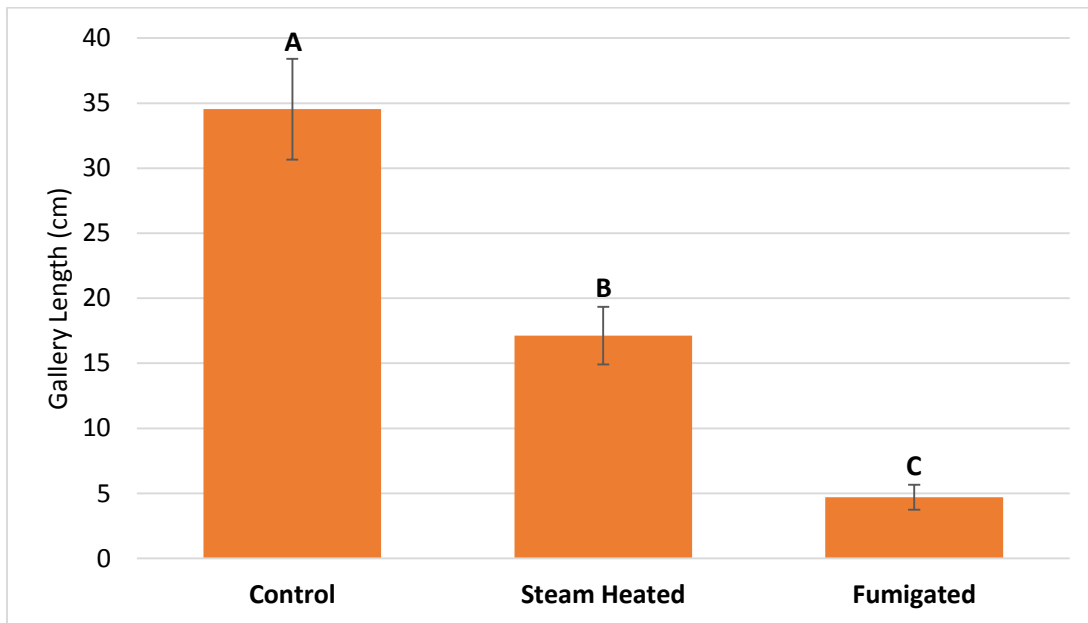
Figure 2.4. Mean gallery lengths (± SE) measured from sub-sample of bark for heated, fumigated, and control samples. Different letters indicate different means based on Tukey's HSD test (α = 0.05).
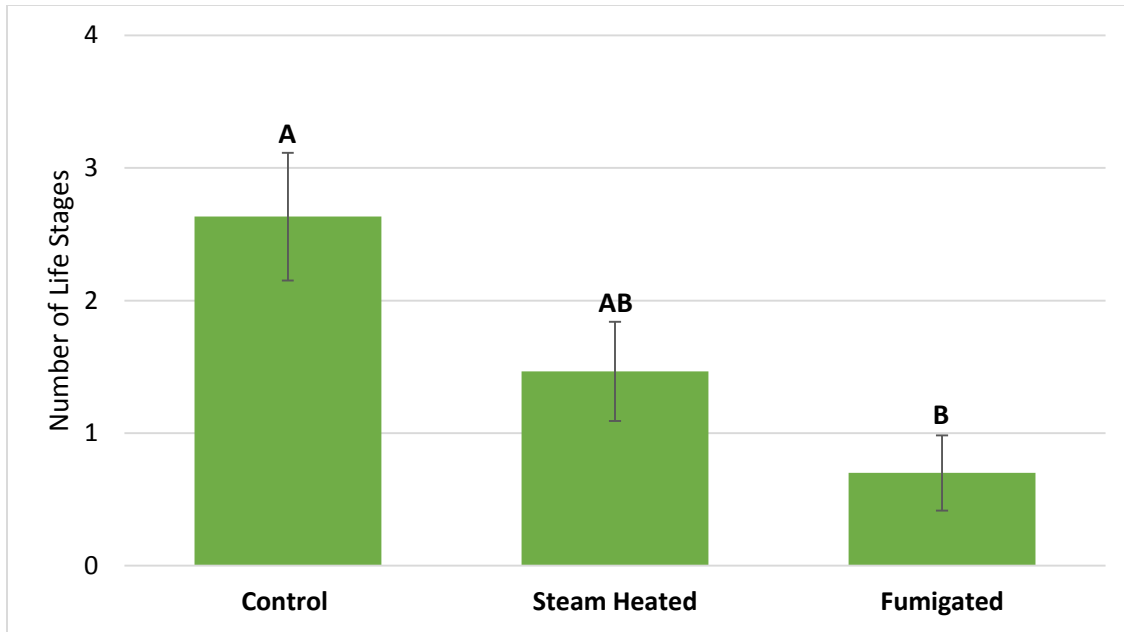
Figure 2.5. Mean number of *P. juglandis* life stages (± SE) (larvae, pupae, & adult) pooled per treatment. Different letters indicate different means based on Tukey's HSD test.
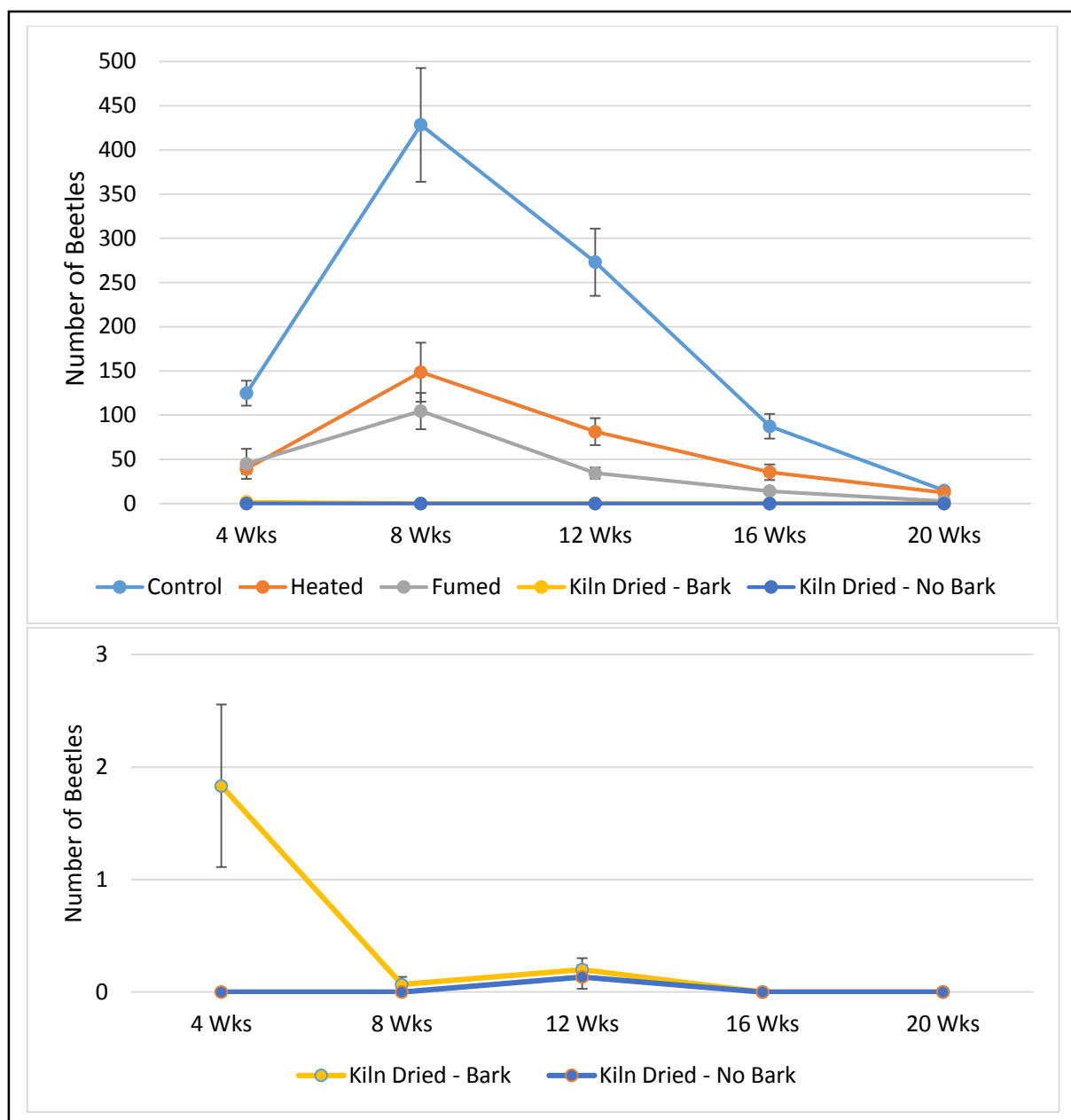
Figure 2.6. Mean *P. juglandis* emergence per treatment per collection time (± SE) plotted over the 20 weeks of emergence. Top plot shows all five treatments, bottom plot separates the two kiln-dried lumber samples and was plotted on a smaller scale to improve visibility.

Table 2.3. Pairwise Two-Sided Multiple Comparison Analysis of mean beetle emergence/100 cm² of bark using the Dwass, Steel, Critchlow-Fligner Method comparing treatments within Block A and within Block B.

	Block A		
Treatment	Wilcoxon Z	DSCF Value	*P* - Value
Control vs. Steam	1.49	2.11	0.44
Control vs. KDB*	2.16	3.06	0.13
Control vs. KDNB†	2.16	3.06	0.13
Steam vs. KDB*	1	1.41	0.75
Steam vs. KDNB†	1	1.41	0.75
KDB* vs. KDNB†	0	0	1
	Block B		
Treatment	Wilcoxon Z	DSCF Value	*P* - Value
Control vs. Steam	-0.49	0.69	0.96
Control vs. KDB*	1	1.41	0.75
Control vs. KDNB†	1	1.41	0.75
Steam vs. KDB*	1.45	2.05	0.47
Steam vs. KDNB†	1.45	2.05	0.47
KDB* vs. KDNB†	0	0	1

Bold *P* values are significant at $\alpha = 0.05$.
*Kiln-dried Lumber with Bark.
†Kiln-dried Lumber with No-Bark.

Table 2.4. Wilcoxon two-sample tests comparing the number of *Pityophthorus juglandis* emerged per 100 cm² of bark between treatments across the two blocks from the lumberyard exposure study.

Treatment	Z score	*P*-Value
Control	-1.34	0.18
Steamed	0.55	0.58
KDB*	0.00	1.00
KDNB†	0.00	1.00

*Kiln-dried Lumber with Bark
†Kiln-dried Lumber with No Bark

long, and all four beetles were collected after 12 weeks (Figure 2.5). In this case, it appears likely the beetles represent cross contamination as a result of inconsistencies with the emergence chambers. Inclusion of de-barked lumber was, in part, to act as a negative control and to serve as an indicator of issues with the experiment design.

LUMBERYARD EXPOSURE STUDY

A clear majority of the beetles that were captured during emergence monitoring were recovered from untreated control bolts; however, analyses indicated no differences in mean beetle emergence existed, even between the controls and the kiln-dried lumber samples (which had no emergence) in either block (Table 2.3). A majority of samples in both the control and steam heated groups, and all of the kiln-dried lumber samples were devoid of *P. juglandis* emergence, likely confounding true differences that may exist among the treatments tested. The large number of zero values likely also explains why no difference was detected within treatments across the two blocks (with lure A and without lure B).

Although we estimated an average of more than 200 potential colonizers per plot, these estimations may have been artificially inflated. The method used to extrapolate the number of beetles emerging from a sub-sample assumes that the beetle density is evenly distributed across the log. Evidence from the extreme exposure study may contradict that assumption and our results may have been confounded by a relatively low beetle population emerging from the source bolts. Several researchers observed a later start to the *P. juglandis* flight season and lower than expected numbers of *P. juglandis* trap catch across east Tennessee for 2014 compared with 2013 field observations (Klingeman personal communication; Grant personal communication; Lambdin personal communication). We suspect that the source material used in the lumberyard exposure study was not heavily infested with beetles due to relatively low *P. juglandis* populations in the area in 2014.

An alternative explanation could be that the source bolts were actually acting as a beetle sink. Emerging *P. juglandis* may have been more attracted to the source bolt in a neighboring cluster or perhaps emerging beetles were not compelled to leave the source material at all. Little is known about either the dispersal characteristics of the beetle or what factors promote dispersal of individual beetles from a host resource. It is possible that a significant proportion of the emerging population may enter the same log or branch after a short period of crawling along the bark surface.

CONCLUSIONS

Understanding potential pathways of introduction of a quarantined pest or pathogen is a crucial aspect of effective management. Effective phytosanitation treatments for eliminating *Pityophthorus juglandis*—the vectoring agent of thousand cankers disease—from *J. nigra* logs have been determined (Mayfield et al. 2014, Myers unpublished data). These data provide evidence that *P. juglandis* can colonize steam heated and methyl bromide fumigated bolts when baited with a pheromone lure and exposed to extreme beetle colonization pressure. There does not appear to be a residual treatment effect strong enough to prevent successful colonization or reproduction within these treated materials. We also demonstrated that *P. juglandis* can enter and persist within kiln-dried bark for a short time, though reproduction likely did not occur. These results are supported by the findings of Haack and Petrice (2009) who demonstrated that numerous species of bark and ambrosia beetles can colonize previously steam heat treated logs and wood packing materials.

The ability of the pathogen *G. morbida* to colonize the treated wood material was not addressed in this study. Presence of the pathogen is a key component to the presence of thousand cankers disease. If the fungus cannot develop in the phloem of steam heated, fumigated logs, or kiln-dried lumber with bark intact, the threat of spreading thousand cankers disease may be reduced even if *P. jugalndis* can still colonize the material. Colonization of treated walnut wood should be tested through inoculation studies. However, if beetles can still emerge from the material with evidence of *G. morbida* spores, it will likely not matter from a regulatory perspective. Thus, fungal recovery from beetles that have emerged from the treated material should also be examined.

Much of the biology and ecology of *P. juglandis* has yet to be determined. It is still unclear how the beetle finds and selects a suitable host tree. Bark beetles are known to use host plant volatile cues (Wood 1982), visual cues (Mayfield and Brownie 2013), and even landing and "tasting" (Raffa and Berryman 1982) potential hosts prior to selection. The combination of cues utilized by *P. juglandis* needs to be investigated to provide researchers and managers with a better understanding of how host selection is made. Such knowledge would enable better protection of living walnut trees and commercially transported walnut wood.

These findings indicate that logs and lumber (bark on) treated by approved phytosanitary measures, should not be exposed to *P. juglandis* post-treatment. However, results suggest that the risk of *P.*

juglandis colonizing kiln dried bark is significantly less than steam heated or fumigated bark, and this material may be a low risk pathway. Further testing is required to determine definitively whether or not *P. juglandis* can colonize un-baited kiln-dried lumber with bark left on. Future tests could expose kiln-dried lumber samples to beetles from actively infested trees without the pheromone lure. Samples could be placed on the ground or on an elevated platform 10-20 meters from the base of the infested trees. This exposure would represent an exposure scenario less extreme than the "worst case scenario" and perhaps more reliable than the lumberyard exposure scenario presented in this study.

Should subsequent exposure of treated logs to *P. juglandis* occur, walnut wood could remain an anthropogenic pathway for further spread of the beetle and thousand cankers disease. Veneer logs and firewood (Newton and Fowler 2009) are two examples of walnut wood products in which phytosanitized walnut may still provide a pathway of accidental introduction. Preventing further anthropogenic spread of *P. juglandis* is crucial to protecting *J. nigra* from further damage by thousand cankers disease.

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Chapter 3 Efficacy of superficially applied insecticides in preventing *Pityophthorus juglandis* colonization of *Juglans nigra* logs.

ABSTRACT

The health and commercial viability of one of North America's most valuable hardwood lumber species, eastern black walnut (*Juglans nigra* L.), is currently under threat from thousand cankers disease. This emergent and often fatal disease is the result of an invasive bark beetle species, *Pityophthorus juglandis* Blackman, and an associated fungal pathogen, *Geosmithia morbida*. Range expansion of the beetle and pathogen has likely been facilitated by transport of infested walnut logs and wood products. Preventing colonization of walnut logs and lumber is crucial to preventing further thousand cankers disease spread, and to the continued viability of the walnut lumber industry. Insecticide treatments may provide a tool to accomplish that goal. This study evaluated the efficacy of three insecticides in reducing *P. juglandis* survival rates and colonization activity on *J. nigra* bolts, 3 – 5 cm in diameter, following dip treatment applications. Treatments included 0.003% azadirachtin, 15% disodium octaborate tetrahydrate [DOT], 0.5% permethrin, and water in Trial 1; and 0.013% azadirachtin, 30% DOT, 0.5% permethrin, and water in Trial 2. Two pairs (2M:2F) of *P. juglandis* were placed into containers with a treated sample and observed for 120 hours. Permethrin was the most effective treatment in both trials in reducing beetle survival rates, and was the only treatment to effectively prevent beetle colonization. The 30% DOT treatment reduced beetle survival rate compared to the control; however, it did not significantly reduce the number of attacks or gallery length. Azadirachtin was not effective at either concentration tested. Results f suggest the use of insecticide dip treatments coud be helpful in preventing *P. juglandis* from colonizing cut *J. nigra* logs. Treatments could be used in conjunction with phytosanitation to help prevent further spread of thousand cankers disease while allowing for the continued transport of walnut logs and wood products.

BACKGROUND

Eastern black walnut (*Juglans nigra* L.) and several other *Juglans* species in North America are currently under threat from an emergent, often fatal disease known as thousand cankers disease (Tisserat et al. 2009, Seybold et al. 2010). The disease is caused by the phloem-feeding bark beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae), and an associated fungal pathogen *Geosmithia morbida* (Tisserat et al. 2009, Kolařík et al. 2011). Adult *P. juglandis* introduce the pathogen upon entering the phloem of branches and trunk of a host tree. *G. morbida* is not a systemic pathogen and numerous beetle attacks are required to induce mortality (Tisserat et al. 2009). Mortality generally occurs in the branches of the canopy first and progresses down the main stem until the host tree dies.

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Dieback is the result of cankers and beetle gallery formation coalescing in the phloem, girdling branches and ultimately the main stem (Tisserat et al. 2009).

Neither the beetle nor the pathogen are native to the eastern United States. Rather, *P. juglandis* is historically known from the American southwest, originally associated with Arizona walnut (*Juglans major*) (Torr) A. Heller (Cranshaw 2011, Seybold et al. 2012). *J. major* is hypothesized to be the primary host of the beetle and pathogen in its native, historic range (Moltzan 2011, Utley et al. 2013), and significant mortality as the result of thousand cankers disease on *J. major* has not been reported. Over the past two decades the range of *P. juglandis* has greatly expanded. The beetle has been collected primarily from declining *J. nigra* across the western states where it is not native, but has been extensively out-planted (Cranshaw 2011, Tisserat et al. 2011, Seybold et al. 2012). In 2010, *P. juglandis* was first discovered within *J. nigra*'s native range on infested trees in Knoxville, TN (Grant et al. 2011). Since that time, the beetle and pathogen have been recovered in Pennsylvania, Virginia (Seybold et al. 2012), North Carolina (Hadziabdic et al. 2013, Wiggins et al. 2014), and Ohio (Fisher et al. 2013). The pathogen has been recovered from symptomatic trees in Indiana (Juzwik et al. 2015), however, to date no captures of *P. juglandis* have been reported. Both the beetle and pathogen were recovered from a walnut grove in Italy (Montecchio et al. 2014), constituting the first international occurrence of thousand cankers disease. Given the tremendous distance and disjoint nature of these introductions, anthropogenic influence is likely involved in the transport of the beetle. Introductions may be the result of the commercial transport of infested walnut material in which the bark was left intact e.g. logs, un-edged lumber, or firewood (Newton and Fowler 2009, Turcotte et al. 2013).

Continued spread threatens one of North America's most economically valuable hardwood species in *J. nigra* (Shifley 2004, Newton and Fowler 2009, Moltzan 2011). Total value of standing *J. nigra* timber for lumber and veneer production is estimated at more than $500 billion (Newton and Fowler 2009). Black walnut wood is commonly used for gun stocks, cabinetry, and several other finished wood products and is prized for its strong and durable wood, especially the distinctive, dark-brown heartwood (Williams 1990, Kirkman et al. 2007, Moltzan 2011). The highest quality logs are sold as veneer logs and veneer-quality *J. nigra* is exceptionally valuable (Moltzan 2011).

In response to the spread of thousand cankers disease, states have established quarantines to restrict the movement of walnut logs and wood products (Newton and Fowler 2009, Moltzan 2011). In

Tennessee, regulated articles include: "the walnut twig beetle; *Geosmithia morbida*; all plants and parts of plants in the genus *Juglans* including nursery stock, budwood, scionwood, green lumber, and other material living, dead, cut, or fallen, including logs, stumps, roots, branches, mulch and composted and un-composted chips; and any hardwood firewood" (TDA 2014). Other states that have enacted quarantines have similar lists of restricted materials. In order to remove any of these items from counties in which thousand cankers disease have been confirmed, a phytosanitation certification or a compliance agreement must first be obtained (Newton and Fowler 2009, TDA 2014).

Although effective phytosanitation methods, such as steam heat (Mayfield et al. 2014), have been reported for *P. juglandis* and *G. morbida*, recent evidence suggests the beetle can colonize the bark of treated logs (Audley, unpublished data, CH 2 above). Haack and Petrice (2009) also reported colonization of heat treated logs and boards of lumber with bark left intact by species of bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) and of long horned beetles (Coleoptera: Cerambycidae). The study by Haack and Petrice (2009) provided significant evidence to support a revision in the ISPM 15 guidelines requiring complete debarking prior to phytosanitation for wood packing materials(Haack and Brockerhoff 2011). The requirement for debarking logs or lumber is burdensome to the walnut wood industry. Producing lumber that is completely bark-free reduces the volume of top quality boards as a result of over-edging (NHLA 2003). Veneer log buyers require the bark remain intact on the log to protect the wood from drying and fungal discoloration prior to processing at the mill.

Further precautions may be required to ensure no *P. juglandis* are transported on phytosanitarily treated wood. One approach could be the use of insecticides applied to the surface of the log. Insecticides are a commonly used management technique for protecting individual trees from bark beetle attack (Fettig et al. 2006) and have been shown to be effective against attack on cut logs and lumber (Strom and Roton 2009, Fettig et al. 2011).

The aim of this study was to assess the efficacy of readily-available topical insecticides labeled for logs and wood products on the colonization of phytosanitized *J. nigra* logs by *P. juglandis*. Three chemicals were tested, azadirachtin, borate (disodium octaborate tetrahyrate [DOT]), and permethrin. The first chemical of interest was the botanical insecticide azadirachtin, a terpenoid extract from the neem tree (*Azadirachta indica*) (Schmutterer 1990, Nauman et al. 1994, Newberry et al. 2013). Azadirachtin's

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primary mode of action is as a growth regulator and its structure is similar to that of ecdysone, an insect hormone that regulates metamorphosis. Azadirachtin acts as an ecdysone blocker and prevents successful development, primarily in the larval stages (Newberry et al. 2013, Schmutterer 1990). Studies of the chemical also provide evidence of feeding deterrence, mating disruption, adult sterilization, and repellency of all life stages of several insect pests (Schmutterer 1990, Nauman et al. 1994, Schmutterer 1995, Xie et al. 1995, Newberry et al. 2013). Azadirachtin is a broad spectrum insecticide with low mammalian toxicity.

Tests of azadirachtin's efficacy against bark and wood boring beetles have yielded mixed results. In a study testing systemic injections of azadirachtin on *Agrilus plannipenis* Fairmaire in ash (*Fraxinus* spp.) trees, 100% of larvae failed to complete development at doses $\geq$ 13.6 mg/cm diameter breast height; however, all doses tested were not effective in controlling adults (McKenzie et al. 2010). *Anoplophora glabripennis* Motschulsky larval mortality reached 60% when fed a diet with an azadirachtin concentration of 50 ppm (Poland et al. 2006). Systemic treatments were also effective against larvae of *Dendroctonus ponderosae* Hopkins in treated lodgepole pine, *Pinus contorta* var. *latifolia* Engelmann (Nauman et al. 1994, Naumann and Rankin 1999). However, Duthie-Holt and Borden (1999) found no reduction in the attack rate of adult *D. ponderosae* Hopkins on lodgepole pine treated with a topical azadirachtin emulsion.

Borates are commonly used wood preservatives (Taylor and Lloyd 2009) and have been shown to have broad spectrum insecticidal and fungicidal efficacy across agricultural and domestic applications with low mammalian toxicity (Lloyd 1998). Slahor et al. (2005) demonstrated cost-effective borate retention coupled with heat treatment in lumber used for pallets, thus lending support to the idea of practical application in an industry setting for *J. nirgra* lumber and veneer logs as well. Tests of dip and spray treatments of DOT indicated only a marginal effect as a sanitizing agent (post colonization) when tested on the wood boring beetle *A. planipennis*, however, only low doses (1.22 – 6.6%) were tested (Nzokou et al. 2006). In an assay of the effect of DOT on the structure infesting beetle *Hemicoelus gibbicollis* LeConte, larvae were prevented from entering Douglas fir timbers with > 95% efficacy (Suomi and Akre 1992).

Permethrin is a synthetic pyrethroid similar to the pyrethrin extracts of pyrethrum flowers (Casida et al. 1983). Pyrethroids are characterized by an acute toxicity to a wide range of insects with low toxicity in

mammals. Permethrin has been effective in reducing attacks of several species of Curculionids including *D. brevivomis* Le Conte (Shea et al. 1984), *D. frontalis* Zimmermann (Strom and Roton 2009), and *Xyleborus glabratus* Eichhoff (Carrillo et al. 2013). A spray treatment of *J. nigra* bolts that were hung in field exposure tests were also shown to be 100% effective in preventing *P. juglandis* attack (Mayfield and Juzwik unpublished).

Objectives of this study were to assess the efficacy of the aforementioned insecticides in (1) inducing adult *P. juglandis* mortality and (2) reducing colonization success in treated *J. nigra* samples. The three insecticides were tested in laboratory scale, small-bolt dip-treatment bioassays.

MATERIALS & METHODS

Ten branch segments, one m long and between two and five cm in diameter, were cut from three felled *J. nigra* from the University of Tennessee Arboretum (35.998891° N, -84.218106° W) in Oak Ridge, TN in July 2014. The ends of each segment were coated with Anchorseal (UC Coatings Corp., Buffalo, NY) to reduce moisture loss, and the samples were returned to the University of Tennessee in Knoxville, TN where they were kept in refrigerated (4.5°C) storage. In August, branches were removed from storage and placed onto wooden racks in a walk-in kiln (SII Dry Kilns, Lexington, NC) and steam heated for 81 min (21 min to raise the core temperature to 60°C) to achieve a core temperature of 60°C for 60 minutes (Mayfield et al. 2014). After steam heating, branches were removed and cut into approximately 4.5 cm long bolts with a radial arm saw.

The diameter of each bolt was measured and bolts were divided into eight groups of 10 bolts (to be further divided into two sets of four groups; four per trial) of roughly equal diameters (visually estimated). Ends were again coated with Anchorseal and samples were housed in the lab, maintained at approximately 20°C. All bolts were visually inspected for any bark and wood boring beetle activity (entrance/exit holes) prior to testing. Any holes were marked so as to not confound *P. juglandis* colonization activity measurements.

INSECTICIDE TREATMENTS

The eight groups were divided in half between Trial 1 (N = 40) and Trial 2 (N = 40) so that mean diameters were not different among the four groups in each respective Trial. Mean diameters were compared by analysis of variance (ANOVA) and the assumption of equal variance checked using Levene's

test ($P > 0.05$). Mean bolt diameters were not different in Trial 1 ($F_{3,36} = 0.16$, $P = 0.93$). ANOVA comparisons of the Trial 2 bolts indicated a difference in the mean diameters ($F_{3,36} = 3.30$, $P = 0.03$), however, post-hoc Tukey's HSD test did not indicate a variance among the means. Each group was randomly assigned to one of the following treatments: DOT (Tim-bor® AI 98%; Nisus Corp., Rockford, TN); azadirachtin (AzaSol® AI 6%; Arborjet Inc., Woburn, MA); permethrin (Astro® AI 36.8%; FMC Corp., Philadelphia, PA); and water (control).

Insecticide products were dissolved in water to create a solution bath into which bolts were submerged. Concentrations (as % g/ml of water) in Trial 1 were as follows: 0.003% azadirachtin (Arborjet Inc. 2014), 15% DOT (Nisus Corp. 2014), and 0.5% permethrin (FMC Corp. 2014). Concentrations were determined based on each products label. All solutes were dissolved in 1892.7 mL (measured using a graduated cylinder) of water at ambient room temperature (approx. 20° C). For the control group, 1892.7 mL of non-heated, tap water was used. Each bolt was submerged into its respective solution and held submerged with tongs for 120 sec to ensure complete coverage of the bark surface. All bolts were allowed to dry overnight (approx. 16 hr) prior to beetle introductions.

Trial 2 was a complete replication of the first, however, treatment concentrations for DOT and azadirachtin were both increased based on the results of Trial 1. The azadirachtin concentration was increased to 0.013% by dissolving 3.97 g in 1892.7 mL of water at room temperature (Arborjet Inc 2014). A 30% solution of DOT was made by dissolving 566.98 g in 1892.7 mL of water at 50° C, water was heated to increase the solubility in order to attain the 30% concentration (Nisus Corp. 2014). Permethrin and control treatments were attained by following the same protocol as previously outlined. Again all bolts were submerged for 120 sec, and then allowed to dry overnight.

BEETLE REARING FOR ASSAYS

All *P. juglandis* were reared from *J. nigra* logs that were infested in the field. Beetles were captured by hanging logs with a *P. juglandis* pheromone lure (Seybold et al. 2013) in infested trees across Knoxville, TN (Mayfield et al. 2014). Beetles were collected in dry collection cups out of emergence chambers. Beetles were kept on filter paper inside of petri dishes in a refrigerator (4.5° C) until they were used in the experiments. Only beetles kept for ≤ 3 days were used. All beetles were sexed using a dissecting stereo microscope and arranged into male and female pairs prior to use in the bioassays.

OBSERVATIONS OF BEETLES EXPOSED TO TREATED SAMPLES

A laboratory scale bioassay, similar to the design used by Strom and Roton (2009), was used to evaluate the treatments. Arenas consisted of a 125 mL Nalgene® cup (6 cm in diameter x 6 cm tall) with a 19.6 cm^2 mesh screen lid to allow gas circulation within the arena. Branch samples were cut, dip treated, and placed into the arena on top of a sheet filter paper. On the morning of deployment for each trial, two pairs (2M:2F) of *P. juglandis* were placed onto the filter paper next to the bolt. Beetles were handled and transferred on the bristles of a fine tipped paintbrush to avoid injury. Lids to each arena were immediately fastened and housed inside a fume hood in the lab (the fume hood fan was not used). Lights to the fume hood were turned on each morning at 8:00 am and turned off each night between 6:00 and 7:00 pm to simulate daily photoperiod cycles.

A total of eight observations per sample were made over a 120 hour period for each trial (Fettig et al. 2011). Two observations per 24 hour period were made during the first 72 hours after exposure (HAE), followed by a single observation per 24 hour period for the remaining 48 hours. During each observation, all live and dead beetles and attack holes were counted. Beetles were confirmed dead by gently probing with the bristles of a fine tipped paintbrush under the stereo microscope. Mortality was recorded as a proportion of beetles per sample and the time of observation (expressed as the number of hours post-exposure) was used to estimate the time to mortality for each individual.

After the final observation (120 hours), a thorough visual inspection for all *P. juglandis* attack holes was conducted. Bark was then removed using a wood chisel and any adults found within the bark were recorded as alive or dead. Gallery lengths were measured using a Scalex MapWheel™ (Scalex Corp. Carlsbad, CA) as an indication of successful colonization (Strom and Roton 2009). Trial 1 was conducted from 3-8 Sept. 2014 and Trial 2 was conducted from 16-21 Sept. 2014 (both ranges include the day of insecticide treatment followed by 5 days of beetle exposure).

STATISTICAL ANALYSIS

P. jugladis survival rates were estimated for each treatment using Kaplan-Meyer product-limit survival analysis (Lee and Wang 2003), a similar method to the Life-table method used by Fetting et al. (2011). Survival curves for each of the four treatments were compared using multiple pairwise comparisons with the nonparametric Log-Rank test and the Bonferroni adjustment (SAS Institute Inc. 2013). The Log-Rank test was used instead of the Wilcoxon test due to the relatively large number of censored values

(101 out of 320), which indicate individuals that did not die during the testing period (Lee and Wang 2003). Survival curves were estimated using the SAS Lifetest procedure in the SAS Enterprise Guide software package (SAS Institute 2013). All analyses were considered significant at α = 0.05.

Analysis of variance (ANOVA) was used to test the null hypothesis that mean number of attack holes and mean gallery lengths did not differ by insecticide treatment. Post-hoc means comparisons were made using Tukey's HSD test (α = 0.05). Mean total attack holes per treatment were Log10(y+1) transformed in Trial 2 so the data adhered more closely to a normal distribution to satisfy the assumptions of ANOVA. Mean gallery lengths for Trial 2 were also Log10(y+1) transformed to satisfy the equality of variance assumption. Data from Trial 1 did not require any adjustments for normality or homogeneity of variance. All reported statistics are based on the analysis of transformed data (where applicable), however, means and standard errors reported in all tables and figures reflect non-transformed data. Analysis was performed using the JMP Pro 11.1.1 statistical software package by SAS (JMP Pro 2013).

RESULTS

In Trial 1, *P. juglandis* survival on the permethrin 0.5% (% A.I. in g/ml of solvent) treatment was significantly less than on the control treatment (X^2 = 54.82, *P* < 0.01). Similar differences existed between permethrin and azadirachtin and permethrin and DOT as well (Table 3.1). There were no differences in beetle survival rate among azadirachtin, DOT, and control treatments (Table 3.1). Permethrin was the only treatment on which 100% beetle mortality was achieved within 120 HAE, all individuals died by 72 HAE (Table 3.2). Survival on the other two insecticide treatments and on the controls only decreased to approximately 50%.

Beetle survival on the bolts treated with permethrin was similar in Trial 2. Again, the survival curve from the permethrin treatment was the only one to reach 0% survival. In fact, beetle mortality appears to have occurred at an even faster rate. All *P. juglandis* died within 36 HAE (Table 3.2). Unlike in Trial 1, however, the increased concentration of DOT decreased beetle survival rate to a level significantly below that of both the control (X^2 = 10.90 *P* = 0.01) and azadirachtin (X^2 = 22.889, *P* < 0.01) treatments. Increasing the concentration of azadirachtin in Trial 2 did not produce a similar result, and there was no evidence of a difference in beetle survival between azadirachtin and the control treatments (Table 3.1). In fact, beetles appeared to have survived slightly better on bolts dip treated with azadirachtin than on bolts only treated with water (Table 3.2).

Investigation of the bark surface for attack holes at 120 HAE revealed no *P. jugalndis* had tunneled into the bark of bolts treated with permethrin in either Trial 1 or Trial 2 (Table 3.2). Therefore, the permethrin samples were not included in ANOVA analysis of mean attack holes or mean total gallery length so as to not violate the assumption of homogeneity of variance.

In Trial 1, there was no significant difference in the mean number of attack holes among azadirachtin, DOT, and water treatments (Figure 3.1A). Similarly, no difference was found among the three treatments in the mean gallery lengths found (Figure 3.2A). Such a result was not surprising given similar beetle survival rates observed for each of the treatments during the first trial.

In Trial 2, the mean number of attack holes differed by treatment ($F_{2, 27}$ = 3.96; *P* = 0.03). The increased concentration of DOT in this trial reduced the number of attacks compared with the azadirachtin treatment (Figure 3.1B). Despite what appears to be an improved performance, Tukey's HSD test indicated DOT did not perform significantly better than water. The same trend was mirrored in the ANOVA tests of mean total gallery length (Figure 3.2B) as well.

DISCUSSION

The permethrin treatment of 0.5% was most effective in reducing *P. juglandis* survival in both trials (Table 3.1). Permethrin at a 0.5% concentration was the only treatment to successfully prevent *P. juglandis* colonization and achieve 100% mortality within 120 HAE of beetles to dip treated, small bolts of *J. nigra* in either trial (Figures 3.2 - 3.4). A key aspect of the permethrin treatment was the complete lack of evidence of colonization in either trial. No attack holes were observed (Table 3.2, Figure 3.1) and thus there were no galleries to be measured (Table 3.2). The same level of control (no colonization) was found in a field exposure study in which larger *J. nigra* bolts were exposed to *P. juglandis* for 30 days (Juzwik and Mayfield unpublished data). As has been shown in previous bioassays with other bark and ambrosia beetle species (Pajares and Lanier 1989, Fettig et al. 2006), permethrin appears to have acute toxic effects as a contact insecticide on *P. juglandis*. Permethrin is a potentially attractive treatment for

Table 3.1. Multiple pairwise comparisons of *Pityophthorus juglandis* survival analysis using the Bonferroni corrected nonparametric Log-Rank test among four topically applied insecticide treatments.

Trial 1				
Treatment Comparison			*P*-Values	
Topical Insecticide	Topical Insecticide	Chi-Square	Raw	Bonferroni
Azadirachtin 0.003%	DOT 15%	0.34	0.56	1
Azadirachtin 0.003%	Permethrin 0.5%	56.26	<0.01	**<0.01**
Azadirachtin 0.003%	Water	0.02	0.90	1
DOT 15%	Permethrin 0.5%	46.89	<0.01	**<0.01**
DOT 15%	Water	0.21	0.64	1
Permethrin 0.5%	Water	54.82	<0.01	**<0.01**
Trial 2				
Treatment Comparison			*P*-Values	
Topical Insecticide	Topical Insecticide	Chi-Square	Raw	Bonferroni
Azadirachtin 0.013%	DOT 30%	22.89	<0.01	**<0.01**
Azadirachtin 0.013%	Permethrin 0.5%	83.13	<0.01	**<0.01**
Azadirachtin 0.013%	Water	1.82	0.18	1
DOT 30%	Permethrin 0.5%	11.57	<0.01	**<0.01**
DOT 30%	Water	10.90	<0.01	**0.01**
Permethrin 0.5%	Water	52.73	<0.01	**<0.01**

P-Values in bold indicate significant differences at α = 0.05

Table 3.2. *Pityophthorus juglandis* mean cumulative mortality % (± SE) at each observation period (hours after exposure), mean (± SE) attack holes, and mean gallery length (± SE) for each of the four insecticides tested in the laboratory scale assay for each trial.

Trial 1										
	Mortality % at HAE* (±SE)								Activity after 120hr	
Treatment	8hr	24hr	32hr	48hr	56hr	72hr	96hr	120hr	AH[†] (±SE)	GL[‡] (±SE)
Azadirachtin 0.003%	0 ± 0	0 ± 0	8 ± 4	13 ± 4	15 ± 4	25 ± 8	43 ± 11	50 ± 10	2.0 ± 0.3	6.5 ± 1.2
DOT 15%	0 ± 0	8 ± 4	15 ± 6	33 ± 8	33 ± 8	43 ± 5	48 ± 6	55 ± 7	2.0 ± 1.1	8.3 ± 4.5
Permethrin 0.5%	75 ± 5	83 ± 7	83 ± 7	95 ± 3	98 ± 3	100 ± 0.0	100 ± 0	100 ± 0	0.0 ± 0.0	0.0 ± 0.0
Water	5 ± 3	8 ± 5	10 ± 6	20 ± 7	28 ± 9	38 ± 9	43 ± 9	50 ± 9	2.0 ± 1.1	7.5 ± 2.9

Trial 2										
	Mortality % at HAE* (±SE)								Activity after 120hr	
Treatment	12hr	22hr	36hr	44hr	58hr	72hr	96hr	120hr	AH[†] (±SE)	GL[‡] (±SE)
Azadirachtin 0.013%	5 ± 3	8 ± 5	18 ± 7	23 ± 7	28 ± 9	30 ± 8	38 ± 9	38 ± 11	3.0 ± 0.8	13.5 ± 7.6
DOT 30%	13 ± 6	20 ± 7	50 ± 8	63 ± 9	80 ± 10	83 ± 8	88 ± 6	93 ± 4	2.0 ± 0.8	4.05 ± 2.1
Permethrin 0.5%	60 ± 7	78 ± 6	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	100 ± 0	0.0 ± 0.0	0.0 ± 0.0
Water	0 ± 0	5 ± 3	20 ± 6	28 ± 6	38 ± 7	43 ± 8	50 ± 9	60 ± 9	3.0 ± 0.8	8.4 ± 5.6

All averaged mortality percentages were rounded to the nearest whole number. Average number of attack holes were also rounded to the nearest whole number.

*HAE = Hours after exposure.

[†]AH = Attack Holes.

[‡]GL = Gallery length in mm.
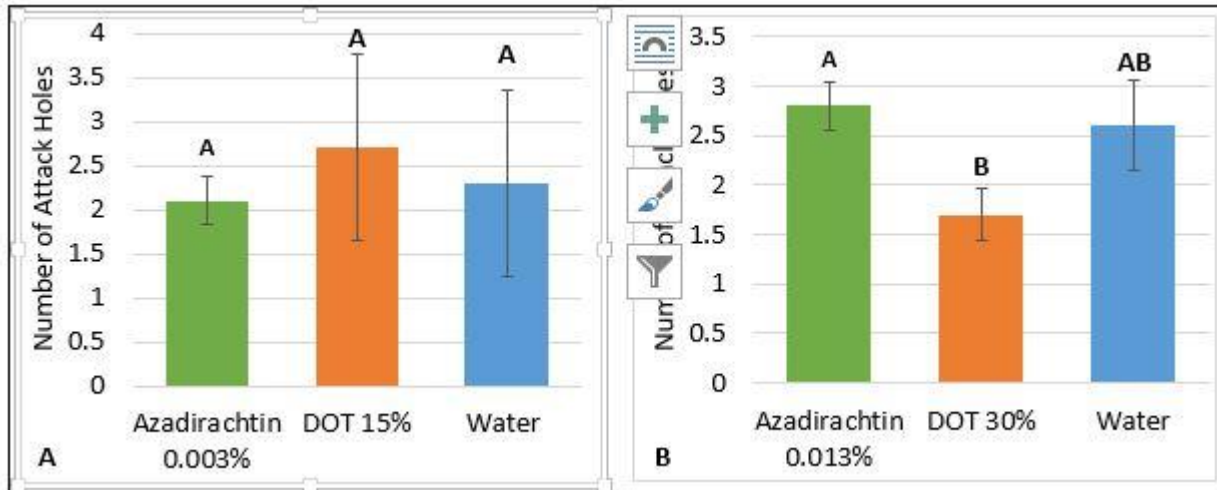
Figure 3.1. ANOVA comparisons of mean number of attack holes (± SE) per treatment for Trial 1 (A) and Trial 2 (B). No difference was found across treatments in Trial 1 ($F_{2,27}$ = 0.930; P = 0.407). A difference in the mean number of attack holes did exist in Trial 2 ($F_{2,27}$ = 3.960; P = 0.031). Different letters indicate different means based on a post hoc Tukey's HSD test (α = 0.05). Permethrin was excluded from analysis in both trials as there were no attack holes observed.
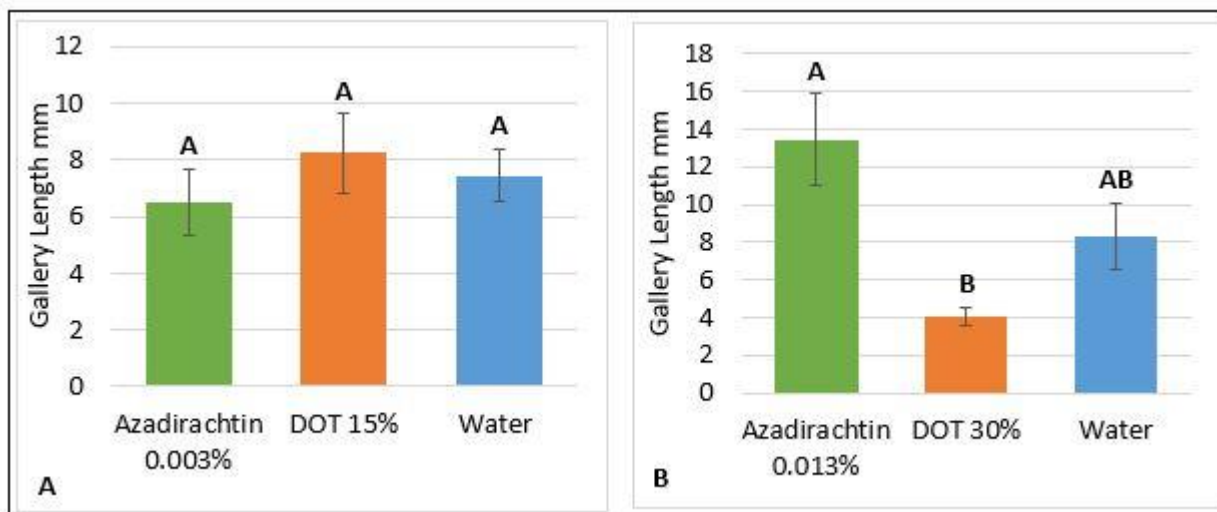
Figure 3.2. ANOVA comparisons of the mean total gallery lengths (± SE) per treatment for Trial 1 (A) and Trial 2 (B). No difference was found across treatments in Trial 1 ($F_{2,27}$ = 0.541; P = 0.589). A difference in the mean number of attack holes did exist in Trial 2 ($F_{2,27}$ = 8.766; P = 0.001). Different letters indicate different means based on a post hoc Tukey's HSD test (a = 0.05). Permethrin was excluded from analysis in both trials as there were no attack holes observed, and thus no gallery lengths to measure.
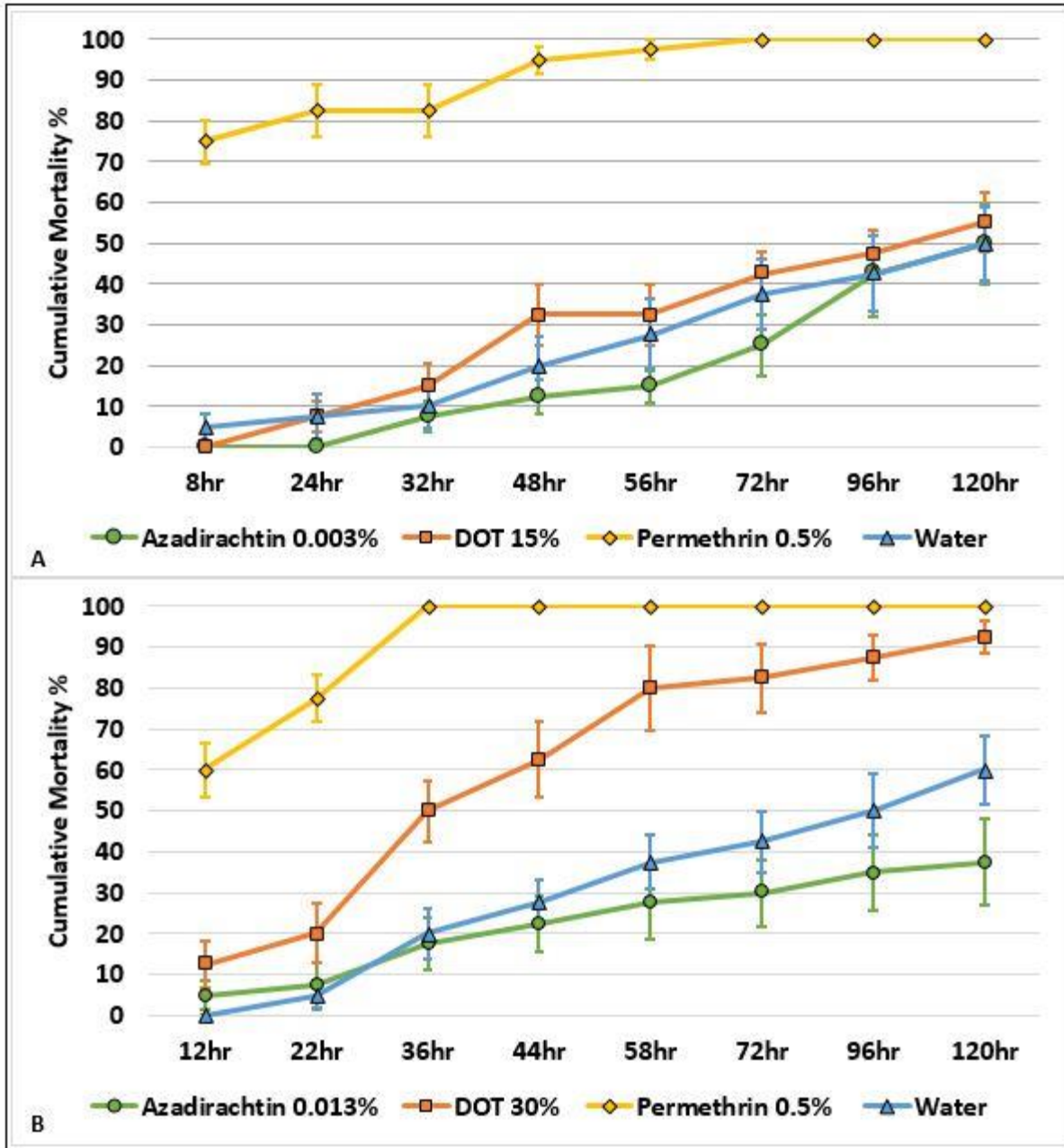
Figure 3.3. Mean cumulative *Pityophthorus juglandis* mortality % with standard error bars for each observation period per insecticide treatment for Trial 1 (A) and Trial 2 (B).

preventing accidental anthropogenic spread of *P. jugalndis* post-phytosanitation as beetles are unlikely to be transported unless they are capable of residing under the surface of the bark.

Although permethrin has a relatively low mammalian toxicity (Schmutterer 1995, Newberry et al. 2013), the US Environmental Protection Agency has noted the chemical's potential as a human carcinogen, and also cites risks to aquatic ecosystems with improper application or disposal of the insecticide (EPA Permethrin Facts 2006). Per the Astro® label, treated surfaces should not be touched with exposed skin until the surface is completely dry (FMC 2014). This may present logistical issues for industry applications via bottlenecks with treatment times (waiting for logs to dry) or via increased costs through investment in protective equipment. Although risk of toxic effects may be low, future studies could consider the lowest dose requirement for effective beetle management to reduce exposure to industry workers. Unlike with permethrin, DOT is not a skin irritant, and is of little concern should solution come into contact with the skin (Nisus 2014).

The 15% DOT treatment in Trial 1 did not perform any better than azadirachtin or the water control (Table 3.1, Figure 3.3). Increasing the DOT concentration to 30% in Trial 2 improved performance, and the observed beetle survival rate was significantly reduced compared to the control and azadirachtin (Table 3.1). However, even the 30% DOT dip treatment did not reduce beetle attacks or gallery length. This may be explained by the mode of action of DOT as an insecticide. The poison must be ingested to be effective, and thus some level of adult feeding must occur (Suomi and Akre 1992, Strong et al. 1993). Therefore, DOT man not provide an effective tool in preventing the spread of beetles. Although the liquid Tim-Bor® solution applied to samples in this study did not prevent colonization activity, DOT can be applied as a thicker emulsion in concentrations up to 50% in the commercial product CelluTreat 50® (Nisus 2014). The resulting surface barrier may better deter *P. juglandis* attacks on treated logs; however, applying the more viscous emulsion may not be practical within an industry setting.

Azadirachtin performed the worst of the three insecticides tested. No reductions in the survival rate, in the number of attack holes, or in the gallery lengths compared with the water controls were observed for either trial. The lack of efficacy on adult *P. juglandis* in this study is consistent with the findings of McKenzie et al. (2010) who reported no effect on adult *Agrilus planniplanipenis*, and with Duthie-Holt and Borden (1999) who observed no reduction in *Dendroctonous ponderosae* attacks. Although azadirachtin can induce mortality and reduce adult feeding in some species of herbivorous beetles (Xie

et al. 1995), the effects are less conclusive in bark and wood boring species. This is consistent with the poor performance of azadirachtin in this study.

Another possible explanation for the ineffective performance observed may simply be that the concentrations tested in this trial were too low. Products with a greater % of A.I. exist, such as NeemAzal (42.3% A.I.) (McKenzie et al. 2010). A greater concentration may be more effective in reducing adult *P. juglandis* survival rates on treated bolts. However, even in greater concentrations, azadirachtin may not have the acute toxicity required to effectively prevent short term colonization of bark by bark beetles from a regulatory perspective. In systemic application bioassays using cerambycide larvae, Poland et al. (2006) found a prolonged feeding period was required before the insecticide produced significant mortality. Given the apparent reduced effectiveness on adult beetles, azadirachtin is likely not an effective management tool for preventing *P. juglandis* transport in treated *J. nigra* logs.

CONCLUSION

The results of this study suggest permethrin when applied as a dip-treatment can be an effective tool in preventing adult *P. juglandis* colonization on *J. nigra* logs. The efficacy of preventing *P. juglandis* colonization of other insecticides labeled for use on logs, such as imidacloprid (Nzokou et al. 2006, Poland et al. 2006), with the same dip-treatment method should be investigated in future studies. Submerging logs into insecticide solutions may provide an effective and efficient means of protecting walnut logs from subsequent exposures to *P. juglandis* with limited regulatory interference of commercial operations.

In order to effectively manage the threat from thousand cankers disease, anthropogenic pathways of spread must be understood and managed (Newton and Fowler 2009, Moltzan 2011). Although it is imperative that the spread of thousand cankers disease be mitigated, management must not unnecessarily restrict the walnut lumber industry (Moltzan 2011). As such, regulations that can help minimize future introductions of *P. juglandis* and allow for the continued transport of saw logs and wood products so as to not compromise the quality of a valuable timber resource should be of high priority.

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Chapter 4 Assessment of *Pityophthorus juglandis* colonization of *Juglans nigra* nursery stock

ABSTRACT

Thousand cankers disease currently threatens the health of eastern black walnut (*Juglans nigra* L.) across United States. The disease, caused by the invasive bark beetle *Pityophthorus juglandis* Blackman and an associated fungal pathogen *Geosmithia morbida*, is characterized by a top-down dieback of infested trees, eventually leading to mortality. The beetle and pathogen have likely been introduced outside of their native range through the transport of infested walnut wood. Another possible anthropogenic pathway of introduction is via walnut nursery stock. *G. morbida* is reported to develop in seedlings when inoculated, however, the ability of *P. juglandis* to colonize young, small diameter trees has not been investigated. The goal of this study was to elucidate the risk of spreading thousand cankers disease via the nursery industry by assessing the beetle's colonization behavior on *J. nigra* nursery stock. Beetles were caged directly onto the stems of seedlings from various sources and of various basal diameter sizes in a no-choice assay. In a second experiment, potted seedlings were exposed to *P. juglandis* in a limited choice assay. Beetles attacked nursery seedlings as small as 0.5 cm in diameter when caged directly onto stems, although a greater response to larger diameter stems was observed. Although seedlings across various sources and across various diameters were attacked, there was no evidence of successful progeny development within the young trees. No beetle attacks were found on seedlings exposed to beetle colonization in the choice assays. This may be indicative of a lack in preference for or an inability to recognize the seedlings as a viable host, a low number of *P. jugalndis* colonizers, or issues with the exposure methods used. Further investigation is required to better elucidate the risk nursery stock poses as a pathway for thousand cankers disease causal organisms.

BACKGROUND

Continued range expansion of the walnut twig beetle (*Pityophthorus juglandis* Blackman) and an associated fungal pathogen *Geosmithia morbida* currently threatens the health of a valuable hardwood lumber species, eastern black walnut (*Juglans nigra* L.). This insect-pathogen complex causes an emergent and often fatal disease known as thousand cankers disease (Tisserat et al. 2009, Seybold et al. 2013). Thousand cankers disease can occur in all North American walnuts, however, *Juglans nigra* is particularly susceptible, and is of significant management concern given its economic value (Shifley 2004, Newton and Fowler 2009, Moltzan 2011). The disease is the result of the gallery formation and feeding of the phloeophagus bark beetle, *Pityophthorus juglandis* (Blackman, Coleoptera: Curculionidae: Scolytinae), and the canker formation of *Geosmithia morbida* radiating into surrounding phloem tissue from the galleries (Tisserat et al. 2009, Kolařík et al. 2011). Adult *P. juglandis* introduce the pathogen

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upon entering the phloem of a host tree. *G. morbida* is not a systemic pathogen and numerous attacks are required to induce mortality in the host (Tisserat et al. 2009). Currently, *P. juglandis* is the only known vector of the pathogen (Tisserat et al. 2009), however, *G. morbida* has been recovered from a species of weevil (*Stenomimus pallidus* Boheman) in Indiana (Juzwik et al. 2015). Mortality generally follows a top-down, out-in pattern, and dieback is the result of cankers and beetle gallery formation coalescing in the phloem, girdling branches and ultimately the main stem (Tisserat et al. 2009).

The beetle and pathogen are historically known from the American southwest (Cranshaw 2011, Seybold et al. 2012a); however, in recent decades both have been recovered significantly beyond the historic range. Their current distribution encompasses most of the western states (Cranshaw 2011, Tisserat et al. 2011, Seybold et al. 2012a), and several eastern states within the native range of *J. nigra* including: Tennessee (Grant et al. 2011); Pennsylvania; Virginia (Seybold et al. 2012a); North Carolina (Hadziabdic et al. 2013); Ohio (Fisher et al. 2013); and Maryland (MDA 2013). *G. morbida* was recently recovered from declining *J. nigra* in Indiana (Juzwik et al. 2015), but to date, presence *P. juglandis* has not been confirmed. Both the beetle and pathogen have also been recovered from a walnut grove in Italy (Montecchio et al. 2014). Given the tremendous distance and disjoint nature of these introductions, anthropogenic influence must be involved in the transport of the beetle. Introductions are likely the result of the commercial transport of infested walnut material (Newton and Fowler 2009, Turcotte et al. 2013).

One possible pathway is via the movement of walnut nursery stock (Newton and Fowler 2009). Live plant material represents a significant pathway of global translocations of arthropod pests including Curculionids (Haack 2006, McCullough et al. 2006, Hulme et al. 2008). Ports of entry and commercial nurseries are often points of introduction and establishment for several non-native bark and ambrosia beetles (Haack 2006, McCullough et al. 2006, Gandhi and Herms 2010). Such species can cause significant damage to nursery crops, and are often of management concern (Oliver and Mannion 2001, Adkins et al. 2010).

Currently, nursery stock is included in state issued thousand cankers disease quarantines (Newton and Fowler 2009, Moltzan 2011). Once a county has been confirmed positive for the disease, transport of walnut nursery stock out of the area is restricted. Introduction of *P. juglandis* and thousand cankers disease into areas with major nursery growing operations is likely to have economic ramifications.

(Cranshaw and Tisserat 2011). *Geosmithia morbida* has previously been shown to develop in nursery seedlings of several walnut species including *J. nigra* (Utley et al. 2013), however, no studies have addressed the beetle's colonization behavior in young walnut trees. Current knowledge of *P. juglandis* biology suggests that the beetle has a preference for branches $\geq$ 1.5 cm in diameter but it has been observed from branch segments of smaller diameters (Seybold et al. 2010). This would suggest the beetles could occupy nursery trees.

The objective of this study was to determine if *P. juglandis* will attack and colonize young, small diameter, *J. nigra* nursery stock. Two experiments were designed. A no-choice assay in which beetles were caged onto stems in a controlled, greenhouse setting, and a choice assay in which beetles were released in close proximity to seedlings in an open field. We hypothesized that the beetles would attack and colonize the young trees and reproduce within the host material under both experimental settings. We also hypothesized the beetles would attack larger diameter ($\geq$ 1.5 cm) seedlings in greater frequency and produce more progeny than in seedlings < 1.5 cm in diameter.

MATERIALS AND METHODS

A total of 172 seedlings were procured from five nurseries from five states within the native range of *J. nigra* (Table 4.1). Basal diameters were measured using calipers by averaging measurements of two points perpendicular to one another, at a height of one cm above the root collar of each stem.

Nursery liner stock was obtained as bare-root transplants from the Willis Orchards (GA) and North Carolina Division of Forestry (NC) nurseries. Both stocks were kept in walk-in coolers with the roots wrapped in plastic after being dug from the field growing sites. Upon receipt in Tennessee, trees were potted into 23 L plastic molded containers (Classic 2800, Nursery Supplies Inc.® Kissimmee, FL) using a custom blend of 50% aged pine bark, 20% peat, 20% sand, 5% ash, and 5% perlite mix (Salifu et al. 2006). Stems from GA were dug in December 2013 and shipped to the University of Tennessee (UT) in Knoxville, TN on 31 December 2013. Liners were potted on 28-29 January 2014 and kept in a plastic lined high tunnel until 6 March, when the stems were placed in a greenhouse. The NC stock was picked up from Asheville, NC on 22 May, potted on 23 May, and placed into the greenhouse on 30 May 2014.

Seedlings from the remaining three nursery sources (TN, MO, and OH) were in containers at the time of procurement. All stems were placed into the greenhouse at UT by the end of May 2014. The greenhouse

bay was maintained between 24° and 30°C under a natural light cycle. Relative humidity was maintained between 60 and 80% for the duration of the study. All trees were kept in the greenhouse until deployment in the respective experiment to prevent exposure to any ambient *P. juglandis.* Stems were watered twice a week (between 960 and 1600 ml per watering event, based on the diameter of the stem, with the largest stems receiving the most water) throughout the duration of the study.

Fertilizer was not added in during the study. Seedlings were inspected weekly for the presence of greenhouse pests. Biological control agents were deployed on two occasions to deal with outbreaks of spider mites and aphids during the late summer and fall of 2014. Two species of predatory mites, *Neoseiulus californicus* (August) and *Amblyseius californicus* (October) were released to control spider mites and a midge, *Aphidoletes aphidimyza*, was released once in August to control aphids.

NO-CHOICE ASSAY

Two no-choice assays were conducted (Trial 1 and Trial 2) in a greenhouse setting under natural light conditions. Temperature was maintained between 24° and 30°C based on the temperatures associated with observed maximum *P. juglandis* flight activity (Seybold et al. 2012b). Ten beetles were caged onto the stems of each replicate in a similar fashion to Mayfield et al. (2008) to determine if the beetles would utilize the young trees given no other choice. A sex ratio of 5F:5M was utilized whenever possible; however, the number of beetles were limited in Trial 1, and several cages received skewed sex ratios based on the beetle availability. *P. juglandis* were reared from *J. nigra* bolts that were hung in trees symptomatic of thousand cankers disease in Knoxville and Maryville, TN and were donated to our study from a trapping study conducted by Klingeman et al. (unpublished data). All beetles were visually grouped by gender using a stereoscope (Seybold et al. 2013a), and placed into a petri dish assigned to each replicate until each dish had 10 beetles. Dishes were kept in a refrigerator at 4.5°C for $\leq$ 3 days prior to deployment. Initially, the number of reared beetles was low and inconsistent, and resulted in a range of staggered start dates in Trial 1. Beetle emergence significantly improved by the start of Trial 2, thus all exposure times began the same day. An increased number of available beetles also allowed for a one-time, second release of beetles on several trees in Trial 2 to replace any observed dead individuals 3 days after the initial introduction.

Beetles were caged onto eight replicates from each of the five sources (except for the TN source in which 4 trees were excluded for poor health [N=36]) in Trial 1 and onto 12 replicates (N=36) of three of

the sources (NC, MO, and OH) in Trial 2 (Table 4.1). Cages were constructed using 15 cm long Mylar tubes 4 cm in diameter. Two windows were cut into the tubes and covered with a fine mesh cloth to allow for gas exchange into the cage. Each tube was cut down the length of one side and wrapped around the stem and sealed with packaging tape. Finally, both ends were plugged with Memory foam plugs (Figure 4.1 A). Foam plugs were cut from a mattress pad that was 4 cm thick and were cut to roughly 6.5 cm in diameter to better fill the space around the stems. A middle section was taken out of each plug with a cork borer, using three different diameters, 0.3, 0.5, and 1 cm, corresponding to small, medium, and large diameter stems.

Beetles were placed into a randomly assigned cage and monitored for 30 seconds after initial release to ensure beetles were ambulatory inside the cage environment. The number of trees with $\geq$ 1 beetle crawling on the stem within the first 30 seconds was recorded. Cages were then observed daily for 15 days. During each observation, the total number of dead and alive *P. juglandis* were noted, and all attack holes were counted (Figure 4.1 B). As previously mentioned, dead beetles in each cage were replaced on the third day in Trial 2 in order to extend the amount of exposure time for each stem to 10 live potential colonizers. Beetles were replaced in equal sex ratios—regardless of the sex of dead beetles, which was not confirmed until the end of the experiment to prevent loss of live beetles—and in the case of an odd number of dead beetles, we alternated between male and female skewed ratios. Dead beetles were not replaced during Trial 1 as a result of a lack of available beetles at the time.

After 15 days of exposure, each stem was harvested by cutting roughly 3 cm above and below each plug. Attack holes were observed outside of the caged area on a few stems (within 1-2 cm of the end of the foam plugs), thus, the total area analyzed for each stem was increased to reflect this observation. Approximately 27 cm (total length) of each stem was analyzed. Plugs were carefully removed over a sheet of white paper to prevent the loss of any beetles, and all dead beetles were counted and sexed. The bark surface beneath the foam plugs and just outside of the cage area were also investigated for attack holes as several beetles were observed wedging themselves between the foam and both the tube and the stem. At the time of harvest, the phloem width (mm) for each segment was determined by measuring from the outer edge of the xylem to the inner edge of the bark for both the top and bottom portions of the stem, and then averaging the two measurements. Phloem widths ranged from 0.3 to 3.0 mm and differed among the five sources (ANOVA; $F_{4, 67}$ = 16.14, P = < 0.01). Mean phloem widths per source are found in Table 4.2.

Each stem was placed into a zip lock bag for transport and placed into an emergence chamber the same day of harvest. Chambers were fashioned from 18.9 L paint buckets with the bucket bottoms removed and replaced with a plastic funnel terminating into a collection cup (Reed et al. 2013, Mayfield et al. 2014).

Collection cups were filled with a small amount of propylene glycol to kill and preserve the emerged beetles. Samples were collected once every 4 weeks for 20 weeks. All beetles collected were sexed as previously described and recorded. Progeny (F_1 generation) were determined by subtracting the recovered adults from the initial exposure numbers. Each stem was removed from the chamber and examined a final time. Bark was carefully scraped away around all attack holes with a scalpel blade to reveal beetle galleries. Total gallery lengths were measured using a Scalex MapWheel™ (Scalex Corp. Carlsbad, CA), and any beetles found were recorded by life stage.

In Trial 1, beetle exposure was conducted from 12 July to 15 August 2014 (staggered start dates) and emergence was monitored from 27 July 2014 through 20 January 2015. In Trial 2, beetle exposure ran from 8-23 September 2014 and emergence was monitored from 23 September 2014 through 23 February 2015. Phloem diameter, source, and basal diameter were tested for correlation (PROC CORR) with number of attack holes, gallery lengths (mm), and for the presence (Y/N) of attack. Results from the correlation analysis were used to inform grouping of the data for further analysis (i.e. by source or BD classes). Basal diameter was correlated most strongly with number of attack holes, gallery length, and presence of attack in both trials (Table 4.3). Correlation analysis was used to inform further analysis and data were organized in to basal diameter classes of $\leq$ 0.80 cm (A), 0.81-1.10 cm (B), 1.11-1.60 cm (C), and $\geq$ 1.61 cm (D). All four basal diameter classes were represented in both trials despite the fact that only three sources (NC, MO, OH), of the original five, were represented in Trial 2.

Total gallery length was divided by the number of attack holes to standardize the measurements for comparisons among the basal diameter classes. Analysis of variance (ANOVA) was used to test the null hypothesis that there was no difference in the mean number of attack holes, gallery lengths per attack hole, emerged progeny, and the presence of attack among the four basal diameter classes. Significantly different means were identified using a post-hoc Tukey's honest significant difference (HSD) test (PROC ANOVA).

Table 4.1. Nursery source, state of origin, and number of potted *J. nigra* seedlings used in each trial of the No-Choice and Choice experiments.

Source	No-Choice		Choice		
State	Trial 1	Trial 2	Trial 1	Trial 2	Total Seedlings
GA	8	NA	10	10	28
OH	8	12	10	10	40
TN	4*	NA	10	NA	14
MO	8	12	10	10	40
NC	8	12	10	10	40
				TOTAL	162

*Only 4 replicates were included from the TN source as a result of poor health.
NA indicates no replicates from a particular source were included during the corresponding trial.

Table 4.2. Mean phloem width (± SE) per source.

Source	Phloem Width (mm)	
NC	0.6 ± 0.1	A
GA	1.1 ± 0.1	AB
TN	1.4 ± 0.3	BC
MO	1.4 ± 0.1	BC
OH	1.6 ± 0.1	C

Different letters indicate different means based
on Tukey's HSD test (α = 0.05).

Table 4.3. Pearson's Correlation Coefficients for source, phloem width, basal diameter, and total number of beetle introduced correlated with the total number of attack holes, gallery lengths, and presence of attack on the seedlings tested in the No-Choice assays, Trial 1 and Trial 2.

Explanatory Variables		Trial 1			Trial 2		
		Attack Holes	Gallery Lengths	Presence of Attack	Attack Holes	Gallery Lengths	Presence of Attack
Source	Pear. Coef.	**0.36**	0.24	**0.43**	0.28	0.09	**0.41**
	P - Value	**0.03**	0.17	**0.01**	0.10	0.61	**0.01**
Phloem Width	Pear. Coef.	0.11	0.12	0.00	0.18	0.05	**0.40**
	P - Value	0.53	0.50	1.00	0.33	0.77	**0.02**
Basal Diameter	Pear. Coef.	**0.38**	0.22	**0.45**	**0.36**	0.14	**0.43**
	P - Value	**0.02**	0.20	**0.01**	**0.03**	0.43	**0.01**
Total Beetles	Pear. Coef.	0.00	0.00	0.00	**-0.37**	-0.27	**-0.41**
	P - Value	0.00	0.00	0.00	**0.03**	0.12	**0.01**

Pear. Coef. = Pearson's correlation coefficient. All bold coefficients and *P*-values are significant at α = 0.05.

Figure 4.1. A. Example of the cage design implemented on a *J. nigra* seedling. **B.** Example of an attack hole (inside orange circle) as seen through the cage during the no-choice assays.

Logistic regression (PROC LOGISTIC) was used to estimate the probability of attack based on basal diameter, phloem diameter, source, and diameter class. Stepwise selection was used to determine which parameters were included in the model with α = 0.15 to enter the model and α = 0.05 to stay in the model. The R^2 value and the receiver operator curve (ROC) were used as an indication of accuracy in predicting the probability of attack (Hood and Bentz 2007). An ROC of 0.5 indicates the model is no better than random chance and a value of 1.0 indicates a perfect fit. A value between 0.7 and 0.8 is considered acceptable, between 0.8 and 0.9 is excellent, and above 0.9 is outstanding (Hosmer and Lemeshow 2000). All analyses were performed using SAS V 9.3 Enterprise Guide statistical analysis software (SAS Institute 2013) and all *P*-values < 0.05 were considered significant.

CHOICE ASSAY

To further elucidate the risk of *P. juglandis* attack on *J. nigra* nursery stock, two choice assays (Trial 1 and Trial 2) were designed in which potted trees were placed in close proximity to infested source material in an open-field setting. Ten seedlings from each of the five sources (Table 4.1; N=50) were randomly assigned to a position in a circular plot (Figure 4.2B) so that each replicate was equally spaced around the infested source bolts (2-3 per plot) which were placed in the center. Only four of the five sources were included in the second trial (Table 4.1; N=40), as not enough healthy seedlings from the TN source were available. In Trial 2, seedlings from the four sources were randomly assigned to a spot within the circular plot skipping the 0° position to account for the missing source replicate (Figure 4.2B)

The assays were conducted in an open grass field on the Holston River Farm Research Unit located east of downtown Knoxville, TN along the Tennessee River (35.959055° N, -83.855090° W). The field was located on the shoulder of a hill sloping towards the river. A row of large box elder (*Acer negundo* L.) bordered the field on the east and a row of small eastern red buds (*Cercis canadensis* L.) bordered the field to the north along a gravel access road. No other *J. nigra* trees were located within 200 m of study area. A total of ten plots were arranged along two transects (5 per transect) that ran from East to West. Both transects were spaced so that the southern edge of plots in transect 1 were 10 m apart from the northern edge of plots in transect 2 (Figure 4.2A).

Circular plots were 4 m in diameter and plots were spaced 2 m apart (edge to edge) along each transect (Figure 4.2A). Five spots around the circumference of the plot were established at 0°, 72°, 144°, 216°,

and 288°. A hole was dug at each spot roughly 30 cm in diameter and 30 cm deep using an auger and posthole diggers. Pots were placed into the holes so that each stem sat nearly flush with the ground.

To obtain infested source material, 40 cm long bolts were cut from three felled *J. nigra* trees at the University of Tennessee Arboretum in Oak Ridge, TN (35.999000° N, -84.219065° W). To prevent moisture loss, cut ends from each bolt were coated with Anchorseal (UC Coatings Corp., Buffalo, NY). Each bolt was baited with a *P. juglandis* pheromone lure (product #300000736, Contech Enterprises Inc., Delta, BC) and hung in known infested trees across the greater Knoxville area. Each bolt hung for 30 days prior to deployment in the choice assay plots. A 10 cm section was cut off of the top of each bolt and placed into a rearing chamber just before deployment in the assays. Emergence from the sub-section was monitored for eight weeks. Total number of emerged *P. juglandis* per cm^2 of bark for each sub-sample was then multiplied by the total surface area of bark for each bolt to estimate the total number of potential colonizers for the whole source bolt. Sub-sample emergence monitoring occurred simultaneously with the source bolt deployment in the field so as to not lose beetle pressure on the seedlings.

Within eight weeks it was apparent from the sub-sample emergence monitoring that several infested source bolts were producing little to no beetle emergence. Therefore, additional beetles were released in each circular plot in order to supplement the number of potential colonizers per plot. Additional beetles were reared from bolts in the laboratory, transferred into plastic petri dishes with a fine bristled paint brush, and taken to the study site. Three releases of 10 beetles each were made during Trial 1 (n=30 per plot) and two releases of 20 beetles each were made during Trial 2 (n=40 per plot). Releases were made after 6 p.m. based on the optimal flight patterns observed by Seybold et al. (2012b) and Chen and Seybold (2014). During each release, beetles were transferred from petri dishes onto a small wooden platform (5 cm x 5 cm) placed on top of the source bolt. Total estimated beetle pressure per plot can be found in Table 4.4.

To monitor the proportion of potential *P. juglandis* colonizers leaving the study area, four Lindgren funnel traps (4-unit) were hung at four positions around the perimeter of the study area. Traps were hung 10 m from the edge of the nearest circular plot based on the monitoring guidelines established by Seybold et al. (2013), along the perimeter of the study area. Each trap was baited with a *P. juglandis*

B

Spot 1
0°

2 m

Spot 5
288°

Source
bolts

Spot 2
72°

2 m

Spot 4
216°

Spot 3
144°

C

Transect 1 ←

Plot 5 Plot 4 Plot 3 Plot 2 Plot 1

2 m

10 m

Transect 2 ←

Plot 10 Plot 9 Plot 8 Plot 7 Plot 6

A
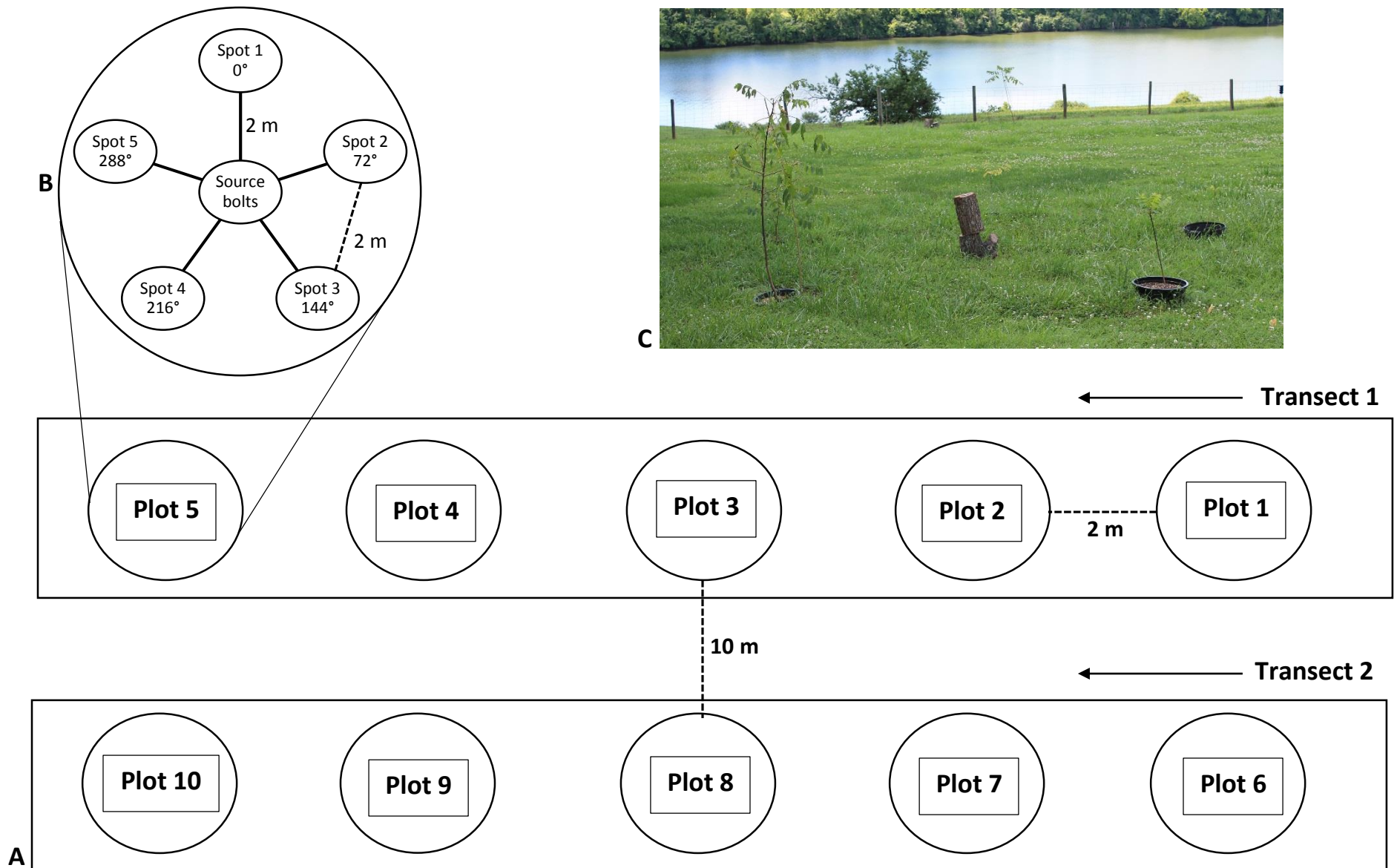
Figure 4.2. Diagram depicting the two 28 m long transects (A) with 5 circular plots arranged along each, a breakdown of the seedling placement in each plot (B), and a picture of seedlings surrounding the infested source material in Plot 1 at the Holston River Farms site (C). In Trial 2, Spot 1 was left empty since only four sources were included.

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Table 4.4. Estimated number of potential *Pityophthorus juglandis* colonizers per plot for the choice assay Trials 1 and 2.

	Trial 1				Trial 2		
Plot #	Est. from Source Bolts	Sup. Release*	Est. Total	Plot #	Est. from Source Bolts	Sup. Release*	Est. Total
1	3	30	33	1	146	40	186
2	73	30	103	2	75	40	115
3	59	30	89	3	5	40	45
4	14	30	44	4	294	40	334
5	56	30	86	5	103	40	143
6	32	30	62	6	68	40	108
7	37	30	67	7	26	40	66
8	82	30	112	8	45	40	85
9	30	30	60	9	109	40	149
10	413	30	443	10	51	40	91
Totals	799	300	1099	55	922	400	1322

The number of potential colonizers was estimated by extrapolating the emergence collected from a 10 cm sub-sample taken from the end of each source bolt to the whole log based on the number of beetles emerged per cm^2 of bark. Estimated beetle pressures were rounded to the nearest whole beetle.
*Supplemental release beetles.

pheromone lure. Collection cups were filled approximately half-way with propylene glycol and were checked once every four weeks throughout the duration of the study.

The seedlings were deployed from 4 June through 19 August 2014 in Trial 1, and from 20 August through 17 October 2014 in Trial 2, for a total exposure time of roughly 12 and 9 weeks respectively. Trial 1 was extended as beetles were not available for supplemental releases until late July, and thus more time was needed to allot for the additional beetle colonization time. At the conclusion of each trial, all foliage from each tree was removed and stems were cut into roughly 30 cm long sections—including all limbs—and placed into zip-lock bags for transport back to the lab. Once back at the lab, each segment was thoroughly inspected for attack holes. ANOVA was used to test the null hypothesis that there was no difference in the mean number of attacks among the nursery sources tested.

RESULTS

NO-CHOICE EXPERIMENTS

In Trial 1, 92% of trees (33/36) and in Trial 2, 61% of trees (22/36) had $\geq$ 1 *P. juglandis* crawling along the stem within 30 seconds of introduction. In both trials all replicates that were attacked had at least one attack hole within the first five days except for two trees in Trial 2. New attack holes were only observed in six trees (17%) beyond day five and in four trees (11%) beyond day 10 in Trial 1. In Trial 2, new attack holes were observed on nine trees (25%) beyond day five and on only two trees (6%) beyond day 10. Increasing the total number of beetles released per cage in Trial 2 did not yield an increase in attack rates. Rather, higher beetle populations were negatively correlated with the number of attacks or the presence of attack on the trees (Table 4.3).

In Trial 1, there were no differences in either mean number of attack holes (ANOVA; $F_{3, 32} = 1.17$, $P = 0.34$) or mean gallery lengths/hole (ANOVA; $F_{3, 32} = 2.13$, $P = 0.12$) among the four basal diameter classes (Table 4.5). Similarly, there were no difference in the mean gallery lengths/hole (ANOVA; $F_{3,32} = 0.37$, $P = 0.77$) in Trial 2 (Table 4.5). ANOVA indicated there was evidence of a difference in the mean number of attack holes in Trial 2 (ANOVA; $F_{3,32} = 3.05$, $P = 0.04$); however, Tukey's HSD test indicated no pairwise differences in means existed (Table 4.5).

In Trial 1, the mean percent of seedlings attacked differed significantly among the basal diameter classes (ANOVA; $F_{3,32}$ = 3.74, P = 0.02). Tukey's HSD test indicated more trees in the largest diameter class (D) were attacked than in the smallest class (Table 4.5). Data from Trial 2 failed the ANOVA assumption of equal variance at a 0.05 significance level (Levene's test; $F_{3,32}$ = 1.90, P < 0.01) and thus a non-parametric procedure was used (PROC NPAIR1WAY). Results of a Kurskal-Wallis test indicated a difference in the mean proportion of trees attacked (X^2 = 8.76, P = 0.03). The Dwass, Steel, Critchlow-Flinger method (Hollander and Wolfe 1999) was used to calculate pairwise, two-sided multiple comparisons, and indicated the only difference in mean proportions to exist between basal diameter classes A and D (DSCF value = 3.96, P = 0.03). In both trials all trees (100%) in the largest diameter class were attacked. Class A was the least attacked in both trials with 33% and 30% of the trees attacked in Trials 1 and 2 respectively (Table 4.5).

Emergence in Trial 1 and Trial 2 did not exceed the number of adults introduced (F_0 generation) for any replicate. Had the F_0 generation successfully reproduced within the material, we would expect to see more emerged adults than the number of adults exposed to the stem (after accounting for the dead adults that were recovered). This measure may be confounded in that many beetles were unaccounted for at the conclusion of the experiments. A total of 231 of 360 (64%) beetles were not accounted for in Trial 1 and 123 of 539 (23%) beetles were not accounted for in Trial 2. Beetles likely escaped by wedging themselves between the foam plug and the bark or between the foam plug and the cage wall. Several individuals were observed escaping via the latter method.

Logistic regression analysis revealed that basal diameter was the most important parameter tested in predicting attack in both trials (Trial 1: X^2 = 7.31, P = 0.01; Trial 2: X^2 = 6.73, P = 0.01) and was the only parameter included in the model. The maximum rescaled R^2 was relatively low (0.31 and 0.25) in both trials, indicating that only 31% and 25% of the variation in the data were explained by the model in Trials 1 and 2 respectively. However, in both cases the ROC levels were within the acceptable range of model accuracy outlined by Hosmer and Lemeshow (2000) with values of 0.79 and 0.74, respectively, indicating both models predicted the probability of attack better than random chance. The regression curves produced for both trials indicate the probability of attack approaches 100% when the stem basal diameter is around 2 cm (Figure 4.3).

Table 4.5. Mean number of total attacks (± SE), mean gallery length per attack hole (± SE), and mean percent of seedlings attacked (± SE) by basal diameter class for each of the two trials.

Trial	Basal Diameter Class*		n†	Attack Holes	Gallery Length/Hole‡	% of Trees Attacked
Trial 1	A	≤ 0.80	9	1.0 ± 0.6 A	1.8 ± 0.9 A	33 ± 17 A
	B	0.81-1.10	9	3.0 ± 0.8 A	6.1 ± 1.6 A	78 ± 15 AB
	C	1.11-1.60	10	3.0 ± 1.0 A	5.8 ± 1.6 A	70 ± 15 AB
	D	≥ 1.61	8	4.0 ± 1.2 A	3.6 ± 1.4 A	100 ± 0 B
Trial 2	A	≤ 0.80	10	1.0 ± 0.5 a	3.4 ± 0.1 a	30 ± 15 a
	B	0.81-1.10	13	1.0 ± 0.3 a	5.5 ± 0.1 a	46 ± 14 ab
	C	1.11-1.60	6	1.0 ± 0.3 a	3.9 ± 0.0 a	67 ± 21 ab
	D	≥ 1.61	7	4.0 ± 1.5 a	6.2 ± 0.1 a	100 ± 0 b

Different letters—not including the diameter class distinctions—indicate significantly different means based on Tukey's HSD test (α = 0.05). Letters indicating different means of the percent of trees attacked in Trial 2 are based on Dwass, Steel, Critchlow-Flinger pairwise comparisons (α = 0.05). Mean attack holes were rounded to the nearest whole number.

*Basal Diameter Classes in cm.

†Number of observations per diameter class.

‡Gallery Lengths in mm.

CHOICE EXPERIMENTS

Estimated mean number of potential *P. juglandis* colonizers was similar for Trial 1 and Trial 2 (ANOVA; $F_{1,18}$ = 0.24, *P* = 0.63). The mean number of potential colonizers per plot in Trial 1 was 110 ± 38, and ranged from 33 to 443 beetles. Total trap catch in the four Lindgren funnel traps located on the perimeter of the study plot was low during Trial 1. Only 23 beetles were caught between the four traps over the 2.5 month period. The mean trap catch was 1.4 ± 0.5 beetles, and total catch among the four traps ranged from 2 to 11 beetles. The mean estimated number of potential colonizers per plot in Trial 2 was 132 ± 26 beetles and ranged from 45 to 334. More beetles were captured in the funnel traps during the second trial with a total catch of 41 over 2 months. The mean trap catch was 3.4 ± 1.9 beetles, and catch totals for the four traps ranged from 1 to 29 beetles. Despite an indication that there should have been *P. juglandis* colonizers present during both trials, no evidence of attack holes could be found on any of the seedlings tested in either trial.

DISCUSSION

NO CHOICE EXPERIMENT

Pityophthorus juglandis attacked young, small diameter *J. nigra* nursery stock, when caged directly onto stems in a no-choice exposure assay. At least one replicate from each of the four diameter classes and from each of the nursery sources tested was attacked in both trials. In part because mean stem diameters differed across the nursery sources (ANOVA $F_{4,67}$ = 138.65, *P* = <0.01), effects of seedling origin on *P. juglandis* attack responses cannot be discerned in this test. Beetle attack response did increase on the larger diameter stems, as evident by an increased probability of attack as basal diameter increases (Figure 4.3). This observation coincides with other reports of an increased rate of *P. juglandis* attack on branch diameters greater than 1.5 (Seybold et al. 2010) and 2 cm (Tisserat et al. 2009). Both of our logistic regression models predicted that the probability of attack increases to nearly 100% as stem basal diameter approaches and exceeds 2 cm.

Despite replacing dead beetles with new live beetles during Trial 2, thus effectively increasing the total number of *P. jugalndis* each stem was exposed to, the smaller diameter stems (basal diameter class A) were less likely to be attacked. This observation provides further evidence that *P. juglandis* attack response is greater on larger diameter stems.

Contrary to our expectations, neither mean number of attacks nor mean gallery lengths differed among nursery liner basal diameter classes tested in both trials. A large amount of variation in the number of observed attacks on individual stems may help explain the apparent lack in response to diameter class. For instance, all of the replicates in the largest basal diameter class (D) were positive for attack, the number of attack holes ranged from 1 to 10 and 1 to 12 holes in Trial 1 and Trial 2 respectively. Some of this variation could be explained by differences in beetle age. Once yellowish to light brown teneral adults darken (Blackman 1928, Nix 2013), there is no means to discern approximate age of darker reddish-brown adults (Blackman 1928, Seybold 2013a). Because all of the source beetles were taken from populations that had emerged from *J. nigra* logs, there was no way to distinguish between adults that had just matured from adults that had re-emerged after having just reproduced. Re-emerged adults may have been less likely to attack or less successful in attacking a new stem.

Similarly, there was no significant differences in the mean gallery lengths per attack hole among the basal diameter classes tested. We expected to see longer mean lengths in the larger diameter stems, coinciding with the observed increased rate of attack response on the larger stems. Instead, it appears as though once a given beetle responds to some stimulus triggering colonization behaviors, the total gallery length remains consistent, irrespective of stem diameter. An interesting observation from both trials, was that several of the attack holes on the largest stems were very short, apparently abandoned galleries, ranging from < 1 mm to 3 mm long. Such spots may constitute evidence of "tasting" or gustation (Raffa and Berryman 1982, Wood 1982, Graves 2008) by *P. juglandis* as a means of assessing host suitability. It is unclear why so many of these holes occurred on the largest diameter stems but did not appear on the smaller stems.

There was little evidence of successful reproduction in any of the replicates tested. Gallery excavations yielded a total of 15 larvae from only three trees, two from the largest diameter class and one from the second to smallest class (B). Twelve larvae (80%) were recovered from a single tree from the largest diameter class, the same tree that had the most attacks (12 holes) and had the longest gallery length. All larvae found appeared to be dead (Figure 4.4), and no pupae were recovered. Several dead adults from the F_0 generation (initial colonizers) were also found dead inside their galleries. During the experiment, outer bark was observed splitting behind excavating adults (Figure 4.5) from several of the smaller diameter seedlings with thin outer bark. The observed bark splitting likely contributed to phloem desiccation, thus may have contributed to prematurely death of adults, developing larvae, and eggs. A

study by Wagner et al. (1979) revealed that larvae of *Dendroctonus frontalis* Zimmerman were susceptible to delayed development and mortality when phloem moisture content decreased during larval development. This observation is consistent with a previous study (see Chapter 2) in which *P. juglandis* attacked samples of kiln dried bark but did not produce any offspring in the material (Audley, unpublished data). Harvesting the small stems likely also contributed to the rapid desiccation of phloem as stems and limbs of smaller diameters lose moisture faster than larger diameter material (Hayes et al. 2009, Nicholls and Brackley 2009). Offspring development may have been more successful in the seedlings with outer bark thick enough to prevent splitting had the stem not been harvested.

CHOICE EXPERIMENT

No attacks were observed on any of the seedlings in either of the choice assays. This may have been the result of several factors. Young, small diameter trees may not have provided a sufficient visual cue for the beetles to recognize as a host (Kogan 1994, Mayfield and Brownie 2013). Young trees may also lack the necessary volatiles required by beetles to enable host detection (Wood 1982, Kogan 1994, Bruce et al. 2005). Volatile organic compounds that function in host recognition by some bark beetles (Byers 1996, Bruce et al. 2005), can change in composition and profile depending on tree age (Adams and Hagerman 1976, Nunes and Pio 2001, Pallardy 2010). It is entirely possible that *P. juglandis* may not register *J. nigra* seedlings as a potential host within the landscape, despite our observation of attacks on seedlings in a no-choice setting. Forcing beetles onto the stem may have circumvented any host detection stimuli that the young trees may lack.

Another possible explanation for the lack of observed attacks may be that the source logs presented the most attractive host material, and acted as a beetle sinks rather than sources. *P. juglandis* have been observed re-entering the same host log in a laboratory setting (Nix 2013) and can attack cut *J. nigra* logs for several months after harvest (Alley, unpublished data). Populations within the source logs could have been actively producing aggregation pheromone, thus drawing newly emerged adults into the same log or into a nearby log. Bark beetle dispersal presents a high risk of mortality for adult beetles (Byers 1995). Although it has not been determined for *P. juglandis*, other species of scolytids, such as the southern pine beetle (*D. frontalis*), produce a density dependent, anti-aggregation pheromone verbenone (Rudinsky et al. 1974). *P. juglandis* could have a similar currently unknown density dependent trigger for dispersal. If population densities were not high enough within the source bolts to trigger a density dependent response the source bolts may have been the most attractive material available to emerging

beetles, and thus drawing potential colonizers away from the nursery liners. This could also help explain why the perimeter funnel traps did not capture many beetles compared to the estimated number of potential colonizers.

CONCLUSIONS

Our results support the hypothesis that *P. jugalndis* can attack and persist within young, small diameter *J. nigra*, thus supporting the restrictions on transporting nursery stock, as these trees may provide an anthropogenic pathway for further beetle and pathogen spread (Newton and Fowler 2009). Beetle attack was best explained by basal diameter, with the larger diameter stems more likely to be attacked than smaller diameter stems. Thus, it follows that the risk of *P. juglandis* attack on *J. nigra* nursery stock increases in older, larger trees.

Although beetles did not successfully reproduce in the nursery stock, it appears the method for monitoring emergence may have influenced that success, at least in the larger diameter seedlings. Beetle reproduction in nursery stock should be further tested with the trees kept intact throughout the duration of the experiment to better control for phloem desiccation. Knowledge of *P. jugalndis* fecundity within nursery hosts could better inform management options for thousand cankers disease in a nursery setting.

The lack of attacks on the seedlings in the choice assays raises more questions about the true risk of *P. juglandis* and thousand cankers disease for the nursery industry. Future tests of the beetle's ability to detect young *J. nigra* on the landscape should attempt avoid the confounding factors we encountered. Exposing potted nursery stock to a more consistent source of potential colonizers by placing the stems within 10 – 20 m of the base of actively infested *J. nigra* trees. Another possible solution could be to release beetles into a large, caged arena where beetles are presented a limited-choice of various nursery seedlings. Future studies could also investigate beetle response to different regional sources by testing trees of similar age and diameters across different sources. Further investigation should be pursued to better elucidate these risks in order to inform industry practices, protect valuable commercial material, and manage further anthropogenic spread of a lethal insect-disease complex.
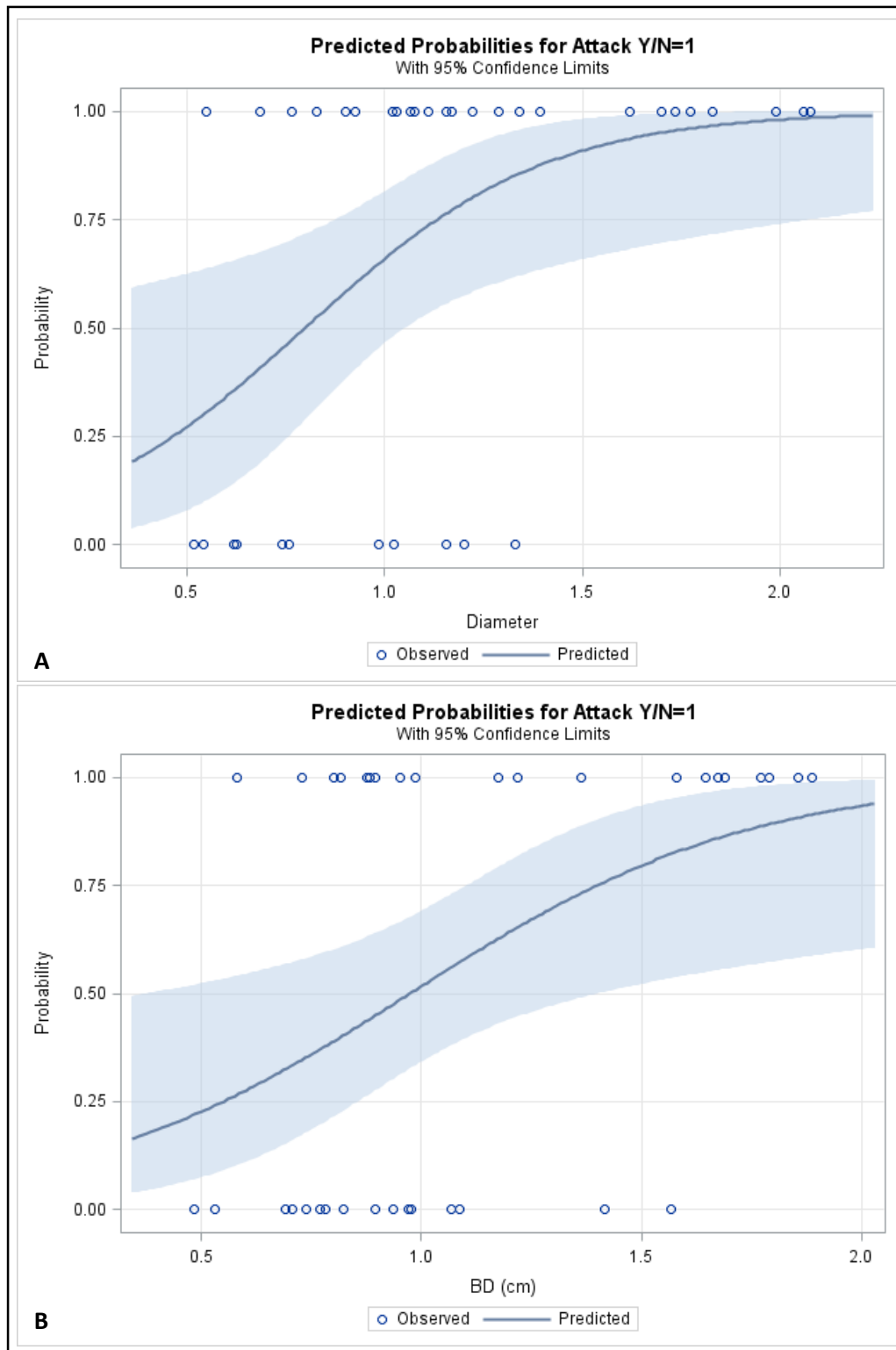
Figure 4.3. Predicted probabilities of attack based on logistical regression analysis of observed attacks and basal diameters for _J. nigra_ nursery seedlings exposed to _P. juglandis_ colonization pressure in No-Choice assay Trial 1 (A) and Trial 2 (B).

Figure 4.4. Desiccated *P. juglandis* larvae found via gallery excavation of a *J. nigra* seedling from the No-Choice assay. Bark was carefully peeled back using a scalpel blade.

Figure 4.5. Outer bark of a *J. nigra* seedling observed splitting behind two tunneling adult *P. jugalndis* (dead). Photograph was taken prior to gallery excavation with a scalpel.

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Chapter 5 Conclusions

Pityophthorus juglandis readily colonizes steam heated and methyl bromide fumigated *J. nigra* logs when baited with a pheromone lure and hung in actively infested trees. Beetle emergence from heated and fumigated material was reduced compared to the untreated control group; however, these treatments likely did not achieve sufficient colonization reduction to meet desired regulatory standards. There was also evidence of successful brood production within the treated materials. The inability to prevent beetle colonization and the ability for beetles to sustain and propagate within the logs, indicates wood treated by steam heating or methyl bromide fumigation may still provide a potential pathway should subsequent, post-treatment exposure to *P. juglandis* occur.

Beetles were also recovered from several kiln-dried lumber samples with bark left intact that were exposed to high colonization pressure and baited with a lure. Unlike the heated and fumigated treatments, however, there was no evidence of successful reproduction. It appears beetles may be able to persist for relatively short periods of time in the dried bark. Even though reproduction is unlikely, dried lumber with bark intact may provide refuge for beetles to be transported.

Results were not conclusive when treated samples were exposed to *P. juglandis* colonization pressure in a more realistic, lumberyard replication scenario. Although no beetles were recovered from kiln-dried samples (baited or un-baited), a relatively low number of beetles were recovered overall, even from untreated controls. This suggests the results may have been confounded by a lower than estimated number of potential colonizers, or perhaps by the methods of exposure employed. Further investigation is required to better elucidate the risks of colonization associated with treated wood.

This study did not address the ability of the pathogen, *Geosmithia morbida,* to colonize logs and lumber upon exposure post-treatment by phytosanitary methods. Although the pathogen requires *P. juglandis* for introductions into host material, thousand cankers disease results from the combination of beetle and pathogen activity within a host. Thus, the ability of *G. morbida* to persist within treated logs and/or wood products should also be investigated.

Topical insecticides samples may provide an effective means of repelling *P. juglandis* colonization in cut wood. Permethrin (0.5% g/ml water) reduced beetle survival rates to 0 and prevented any attacks in both trials. DOT produced mixed results. At a 15% concentration, beetle survival rates were not reduced compared to water treated controls. When the concentration was increased to 30% in the second trial,

survival rate was reduced but at a lower rate than from permethrin samples. In neither case were beetles repelled from attacking the material however. No difference in the number of attacks or the total gallery length excavated was found compared to the controls. Azadirachtin performed the worst of the three chemicals. Neither concentration tested (0.003 and 0.013%) reduced *P. juglandis* survival rates nor reduced the colonization activity of the treated samples.

P. juglandis can colonize young, small diameter *J. nigra* seedlings when beetles are caged directly onto the stem. Beetles successfully attacked trees from each of the four basal diameter classes ranging from 0.5 to 2.0 cm in diameter. Although beetles attacked even the smallest stems presented, they exhibited a clear preference for larger diameter stems and the proportion of trees attacked increased as basal diameter increased. Despite successful attacks, there was no evidence of successful brood development. This may be an artifact of stem moisture loss as a result of harvesting the stems to place samples into rearing chambers. The beetle's fecundity in young walnuts should be investigated further.

No attacks were observed on any of the size classes of seedlings tested in the choice experiments, despite estimating beetle colonization pressure to be roughly 500 beetles per test plot. Although this result may represent an inability of the beetles to recognize the young trees as a potential host, the success of the beetles in the no-choice assays suggests the experiment was likely impacted by confounding variables. It may be that the source material was actually acting as a sink and actually presented the most attractive host material to the emerging beetles. Given such uncertainty, no conclusions about the true risk of attack and colonization of *J. nigra* nursery stock by *P. jugalndis* can yet be made.

In conclusion, *P. juglandis* appears able to colonize both treated wood and young, small diameter seedlings. These findings further support the continued restrictions of these items in order to mitigate the damage associated with thousand cankers disease.

Vita

Jackson Paul Audley was born May 18, 1988 in Washington D.C. Jackson lived in Vail, CO for several years as a young child, but was raised primarily in Roswell, GA where he graduated from Roswell High School in May 2006. He proceeded to attend the University of Georgia where he obtained a dual Bachelor of Science degree in Wildlife and Natural Resource Recreation & Tourism in December 2009. Following completion of his undergraduate degree, he worked as a research technician in the Forest Entomology Lab at the University of Georgia under the supervision of Dr. Kamal Gandhi. In this capacity, he fostered a passion for investigating invasive forest insect pests. Prior to beginning his Master's degree, he spent a year as an AmeriCorps volunteer collecting land health data across the state of Nevada. Jackson began his Masters of Science program in the Department of Forestry, Wildlife, and Fisheries at the University of Tennessee in August 2013 under the guidance of Dr. Adam Taylor. During his program, Jackson presented numerous posters and oral presentations while attending several local, regional, and national meetings. He co-moderated the graduate student session of Southern Forest Insect Workshop Conference in July 2014. Jackson is an active member of the Entomological Society of America.