The chemical ecology of bark and ambrosia beetles that colonize deciduous trees

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SUMMARY

The health and productivity of U.S. forests are increasingly threatened by wood-boring insects (Solomon 1995, Haack 2006). These threats are predicted to increase as climate change decreases host resistance and international trade facilitates the introduction of exotic species (Kuhnholz et al. 2003, Brockerhoff et al. 2006). Compromised host resistance predisposes trees to increased colonization by wood-borers and associated pathogens (Raffa and Berryman 1983). Exotic forest pests, most of which are bark and ambrosia beetles (Coleoptera: Scolytinae), pose a greater risk than native species due to a lack of co-evolved defenses in tree hosts (Brockerhoff et al. 2006, Haack 2006, Hulcr and Dunn 2011). To locate susceptible hosts, many bark and ambrosia beetles rely on behaviorally active compounds, known as semiochemicals. Many attractive and repellent semiochemicals have been exploited within integrated pest management (IPM) programs (Otvos 2004, Pedigo and Rice 2014). While diverse semiochemicals of conifer-infesting bark and ambrosia beetles have been described, compounds that attract or repel scolytines which colonize deciduous trees remain relatively unknown. The identification of additional semiochemicals of bark and ambrosia beetles of deciduous trees will increase our capacity to sustainably manage some of the most destructive biological threats to valuable tree species.

As few semiochemicals of deciduous tree pests have been identified, the capacity to monitor and manage wood-boring pests in managed and natural hardwood forests is limited. The *overall goal of my research* is to identify specific compounds or combinations that bark and ambrosia beetle pests of deciduous trees utilize to locate host trees and mates. I will identify specific attractive and repellent compounds that can be used in IPM programs to protect valuable trees. My *working hypothesis* is that bark and ambrosia beetles that colonize deciduous trees utilize semiochemicals for host and mate discovery, but, due to differences in reproductive strategies, ambrosia beetles rely solely on host-associated volatiles. In addition to identifying novel semiochemicals, I also aim to increase trapping efficiency by measuring the attractive range of

pheromone and host volatile lures. My proposed research focuses on some of the most injurious bark and ambrosia beetles affecting native hardwood tree species; these include the peach bark beetle (PBB, *Phloeotribus liminaris* Harris), the walnut twig beetle (WTB, *Pityophthorus juglandis* Blackman), and several species of exotic ambrosia beetles (AMB; *Xylosandrus germanus* Blandford, *Xylosandrus crassiusculus* Motschulsky, and *Xyleborinus saxeseni* Ratzeburg). To achieve my research goals I have the following objectives:

1. **Describe the aggregation pheromone of the peach bark beetle and test its operational capacity in mass trapping;**
2. **Determine the extent to which host-associated and fungal volatiles attract the walnut twig beetle;**
3. **Identify host-associated compounds which attract or repel the exotic ambrosia beetles *Xylosandrus germanus*, *Xylosandrus crassiusculus*, and *Xyleborinus saxeseni*; and**
4. **Establish the active space of walnut twig beetle pheromone and ambrosia beetle ethanol lures.**

The identification of species-specific behavioral chemicals will increase the efficacy and availability of semiochemical-based IPM tactics. Also, compounds that attract a broad range of species could improve exotic species monitoring, while species-specific compounds may increase the efficiency of mass trapping for pest management. For those species with fungal associates, volatiles from the symbiont may increase attraction to known host-associated (i.e., ethanol) and pheromone attractants. Moreover, by measuring the attractive range of pheromone and host volatile lures, I will better understand the chemically-mediated dispersal of adult beetles to inform trapping protocols. Expanding the use of semiochemical-based tactics within IPM programs will increase the sustainability of pest management efforts, thereby reducing reliance on insecticides to manage bark and ambrosia beetle pests of deciduous trees. In addition, an understanding of the signaling strategies of scolytine beetles with disparate life-history traits and reproductive strategieswill inform future efforts to exploit the chemically mediated host-colonization to manage scolytine pests of hardwood trees.

GENERAL INFORMATION

Bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) are small, wood-boring weevils that share similar life histories and ecological niches, yet differ in nutrient sources and reproductive strategies. Adult bark beetles tunnel and feed in phloem and cambium tissue of host trees, where, after mating, eggs are deposited in niches carved into gallery walls (Wood 1982a). Larvae develop as they consume cambium and phloem tissue (Raffa et al. 2015). In most conifer bark beetle species, symbiotic fungi are inoculated into gallery walls that act to decrease the efficacy of host defenses and concentrate nutrients (Six 2012). Many bark beetles rely on mass attack by conspecifics in order to overcome host defenses and create conditions conducive to larval survival. Due to nutritional requirements, bark beetles are usually restricted to specific species or families of trees (Raffa et al. 2015). In contrast, ambrosia beetles create nuptial galleries into the xylem, where they are further protected from predators and adverse environmental conditions (Wood 1982a). Adult ambrosia beetles carry symbiotic fungi in mycangia, specialized sacs associated with the mandibles, during migration and overwintering. Cultivating the fungus after boring into susceptible hosts provides spores for larval consumption. Fungivory has created nutritional independence from hosts and, as a result, ambrosia beetles utilize a much broader assemblage of host species (Wood 1982a, Kunholtz et al. 2001).

Bark and ambrosia beetle colonization results in economic loss due to decreased tree growth, destruction of harvested logs, tree mortality, and degradation of lumber affected by fungal infestations (Wood 1982a). Destruction of host phloem inhibits the transfer of photosynthate and defensive compounds to tree tissues. Symbiotic fungi often disrupt water conduction, plug vascular tissue with resin, or produce toxins, all of which can lead to diminished growth or host mortality (Paine et al. 1997). Ambrosia beetles often cause less mechanical damage to host tissues, but inoculated fungi can inhibit water and nutrient transfer, often resulting in tree mortality from wilt diseases (e.g., Fraedrich 2008). Ambrosia beetles also bore deep into sapwood tissue, providing entry points for other injurious insects, fungi, and bacteria.

Host tree resistance to colonization by wood-boring pests is predicted to decrease in response to warming temperatures, drought, flooding, increased urbanization, and soil nutrient deficiencies

(Bentz et al. 2010). Rapid increases in the introduction of exotic bark and ambrosia beetles also expose hosts to large numbers of novel pests (Brockerhoff et al. 2006). Introduced ambrosia beetles are especially problematic, as they can go undetected in wood products and packing materials (Kunholtz et al. 2003). Unique reproductive strategies, such as consanguineous polygyny (sib-mating), also promote establishment by exotic ambrosia beetles, as only one fertilized female is necessary for colonization and egg production in a susceptible host (Wood 1982a, Jordal et al. 2001). In many cases, native host trees lack resistance to exotic beetles and their symbiotic fungi, resulting in greater mortality when subject to invasive-species colonization (Kuhnholz et al. 2001, Hulcr and Dunn 2011). For example, the redbay ambrosia beetle (*Xyleborus glabrutus* Eichhoff) is an exotic insect that vectors a symbiotic fungus (*Raffaela lauricola* T.C. Harr., Fraedrich & Aghayeva), the cause of laurel wilt (Fraedrich 2008). This combination of exotic vector and fungus has caused high mortality in several deciduous tree species, including redbay (*Persea borbonia* L.), avocado (*Persea americana* Mill.), and other trees in the Lauraceae (Hanula et al. 2008).

Due to the cryptic life cycle of scolytine beetles, managing these pests with sprayed pesticides is often ineffective. For most of their life cycle, bark and ambrosia beetles exist as larvae, protected below bark and woody tissue (Raffa et al. 2015). Once adults emerge from their protective hosts, little time is spent on bark where contact with insecticides may occur (Gitau et al. 2013). Many sprayed insecticides that are effective against some bark beetles have dramatic negative consequences for beneficial insects and other wildlife, making this method of control unsustainable (Wood 1982a). Systemic compounds, such as emamectin benzoate and fipronil, may be effective for protection of individual trees, but applications are impractical for forest pest management and fungal inoculation can occur before beetle death (Grosman et al. 2009). Cultural controls can also be used to decrease the impact of bark and ambrosia beetles, but even increased tree vigor may not be sufficient to defend against invasive insect pests (Brockerhoff et al. 2006). The long-term protection of hardwood trees at risk to bark and ambrosia beetles require the integration of multiple IPM tactics, and there is a critical need for more effective methods for managing these important pests. Targeting chemically mediated behaviors, such as host and mate location, is central to developing effective IPM tactics.

Research since the 1960s has demonstrated that many wood-boring beetles utilize semiochemicals to mediate host and mate location (Borden 1989, Gitau et al. 2013). Bark and ambrosia beetles are able to detect a broad array of compounds that signal the physiological state of nearby host trees or the presence of conspecifics (Kuhnholtz et al. 2003). These semiochemicals can be characterized as host-associated volatiles, conspecific pheromones, or kairomones produced by associated species, and mediate a variety of behaviors, including host detection, competition avoidance, and mate location (Borden 1989). Many of these compounds are utilized by multiple species, which then use additional strategies, such as differences in flight period and pheromone component ratios, to maintain reproductive isolation.

Many deciduous-infesting species are attracted to ethanol, a volatile generally associated with stressed trees (Graham 1968, Miller and Rabaglia 2009, Ranger et al. 2016). Another host-associated volatile, conophthorin, is attractive to several exotic ambrosia beetles (VanDeerLan and Ginzel 2013, Ranger et al. 2014). Verbenone, a compound first identified in conifer-infesting bark beetles, is a minor repellent to two deciduous-infesting ambrosia beetles (VanDerLaan and Ginzel 2013). Nevertheless, the operational capacity of these compounds to manage pest populations is relatively unknown. To date, there are only a handful of examples where semiochemicals have been used to manipulate populations of pest beetles in hardwood systems. Several pest species of exotic ambrosia beetles are managed using ethanol-baited traps, and several native bark beetles, such as the walnut twig beetle, are controlled using pheromone-baited traps (Seybold et al. 2015, Ranger et al. 2016). The lack of knowledge regarding semiochemicals of deciduous tree pests exposes economically important forest and nursery resources to increasing injury and damage. Sustainable monitoring and management can be improved by increasing our understanding of the chemical ecology of deciduous-infesting bark and ambrosia beetles.

To efficiently utilize semiochemicals in trapping programs, it is paramount to determine the behaviorally active range of the lure. By understanding the lure active space, the three-dimensional space in which receivers respond to a signal, traps can be placed at intervals to maximize lure coverage and minimize cost (Bossert and Wilson 1963, Byers 2008). Our understanding of volatile odor plumes and effective attraction radius has increased over the last

several decades, but quantifying pheromone dispersal remains relatively difficult (Murlis 1992, Schlyter 1992, Byers 2008). Abiotic factors, such as wind direction, temperature, and relative humidity, can make estimating active space difficult, but approximate ranges have been determined by placing lured traps at different distances from insect sources and using capture rates to inform the active space area and relative efficiencies at each distance. For example, the optimal distance between monitoring traps for the coffee leaf borer (*Leucoptera coffeela*) was determined by placing baited traps at various distances (Bacca et al. 2006). Determining the attractive range of commercially available lures will aid in developing more efficacious and cost-effective IPM programs for bark and ambrosia beetle pests.

My proposed research focuses on some of the most injurious bark and ambrosia beetles affecting native hardwood species. The peach bark beetle (PBB, *Phloeotribus liminaris* Harris) causes gummosis in black cherry (*Prunus serotina* Ehrh.), which degrades the timber quality of this high-value hardwood species (Rexrode 1981). Managing this pest before host colonization is critical, as induced host defenses cause aesthetic wood defects which severely decrease the value of harvested wood. The invasive ambrosia beetles *Xylosandrus germanus* Blandford, *Xylosandrus crassiusculus* Motschulsky, and *Xyleborinus saxeseni* Ratzeburg, are all pests of ornamental and hardwood nurseries in the eastern United States. Each of these insect species is attracted to ethanol, but species-specific attractants are needed to increase the efficacy of management strategies, and the attractive range of ethanol lures, which are currently employed in mass trapping, is unknown. The walnut twig beetle (WTB, *Pityophthorus juglandis* Blackman) vectors the fungal pathogen *Geosmithia morbida* Kolarik, which is responsible for Thousand Cankers Disease (TCD) in high-value black walnut (Tisserat et al. 2009). The pheromone used by WTB to locate mates has been identified and is used for monitoring and managing pest populations (Seybold 2015), but trapping results are highly variable and the attractive range of the lure appears to be limited.

APPROACH

Objective 1: Identify the aggregation pheromone of the peach bark beetle and test its operational capacity in mass trapping.

Background

The peach bark beetle (PBB, *Phloeotribus liminaris* Harris) is a small, dark-colored insect native to eastern North America (Wood 1982a). Adult PBB colonize susceptible *Prunus* trees for overwintering and reproduction, and their primary hosts are black cherry (*Prunus serotina* Ehrh.). Stressed and wounded hosts are colonized more often than healthy trees, although both can be infested at high PBB densities (Rexrode 1981). In response to colonization, cherry trees produce and exude a defensive polysaccharide gum out through the bark, creating gum spots throughout the wood. These gum spots make subsequently harvested wood unsuitable for veneer, thereby decreasing its value by 50–90 percent (Rexrode and Baumgras 1984, Cassens 2004). Current management of PBB relies on cultural methods, including reducing cherry stumpage and post-harvest sanitation, but these remain insufficient to manage high PBB densities present in many areas of the Central Hardwood Forest. To increase black cherry veneer production in the Central hardwoods additional management tactics are imperative.

Rationale

Management of many bark and ambrosia beetle pests relies on using semiochemicals to manipulate their behavior. The pioneer sex of many bark beetle species use volatile compounds associated with hosts to locate susceptible trees (Wood 1982b, Reddy and Guerrero 2004). Following initial colonization, pheromones, volatile compounds produced by conspecifics, mediate mass aggregation of additional beetles to overcome host defenses and promote encounters with mates (Borden 1989). Although most described bark beetle pheromones remain limited to conifer-infesting species, preliminary results suggest that adult PBB aggregate in response to female-produced pheromones (Ginzel et al., unpublished data). The identification and development of semiochemical lures for PBB will increase the ability to manage PBB populations, thereby increasing black cherry veneer production, and possibly expand the geographic area where harvestable cherry is produced.

Preliminary data

When adult PBBs were exposed to both conspecific-infested and wounded black cherry bolts in a straight-tube olfactometer under laboratory conditions, female-infested bolts elicited greater walking response in males (Fig. 1). In field assays using traps baited with PBB-infested bolts, more adult PBB of both sexes were attracted to female-infested bolts than male-infested or control cherry bolts (Fig. 2). These results provide evidence for the presence of a female-produced pheromone responsible for the aggregation of adult PBB to colonized hosts.

Figure 1: Attraction of adult PBB to different odor sources in straight-tube olfactometer assay. Five adult PBB of each sex were placed in one end of the olfactometer while volatiles from four odor sources were pulled over the beetles. The location of beetles at the end of 10 min. was recorded and an attraction index was calculated for each sex by odor source combination. Differences in means were tested with ANOVA and compared to the control by a Dunnett's test. Female-infested cherry was significantly more attractive to male PBB when compared with all other host-PBB combinations ($F_{7,194}$=6.35, p<0.0001, Dunnett's test p<0.0001)

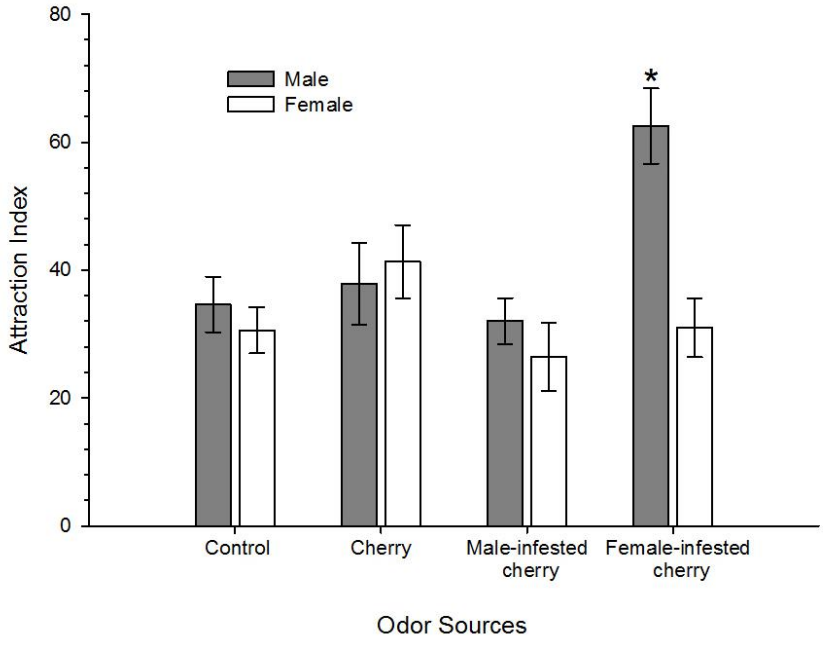
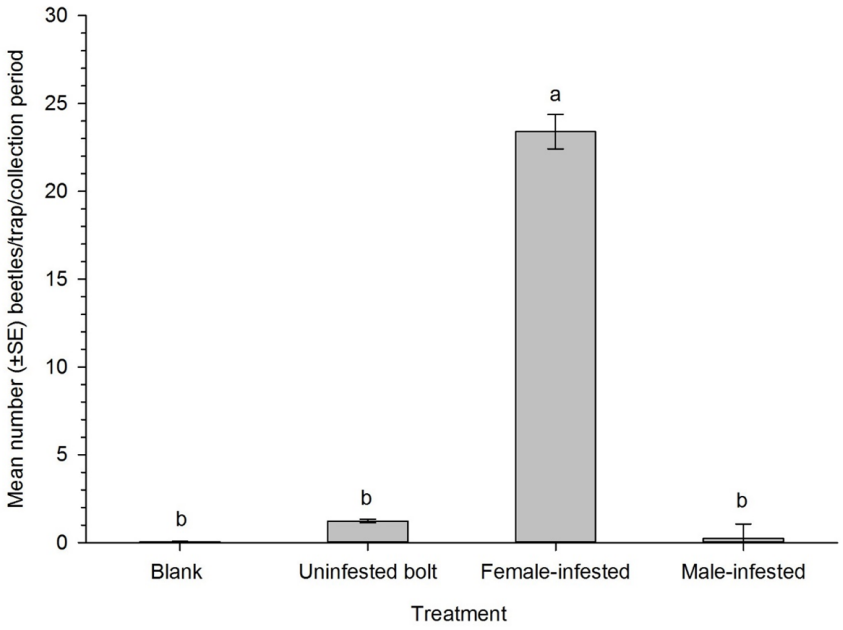


Figure 2: Adult PBB (male and female) captured in field assay using infested-bolt lures. Small bolts of black cherry (infested or not infested with 40 adult PBB of one sex) were placed in mesh bags and hung on 4-unit multi-funnel traps over seven weeks in 2015. Captured beetles were collected daily, counted, and sexed. Differences in means were tested using a non-parametric Friedman's test. Letters over the bars indicate significant differences at p = 0.05 (Friedman's $Q_{4,60}$=15.5, p<0.01).



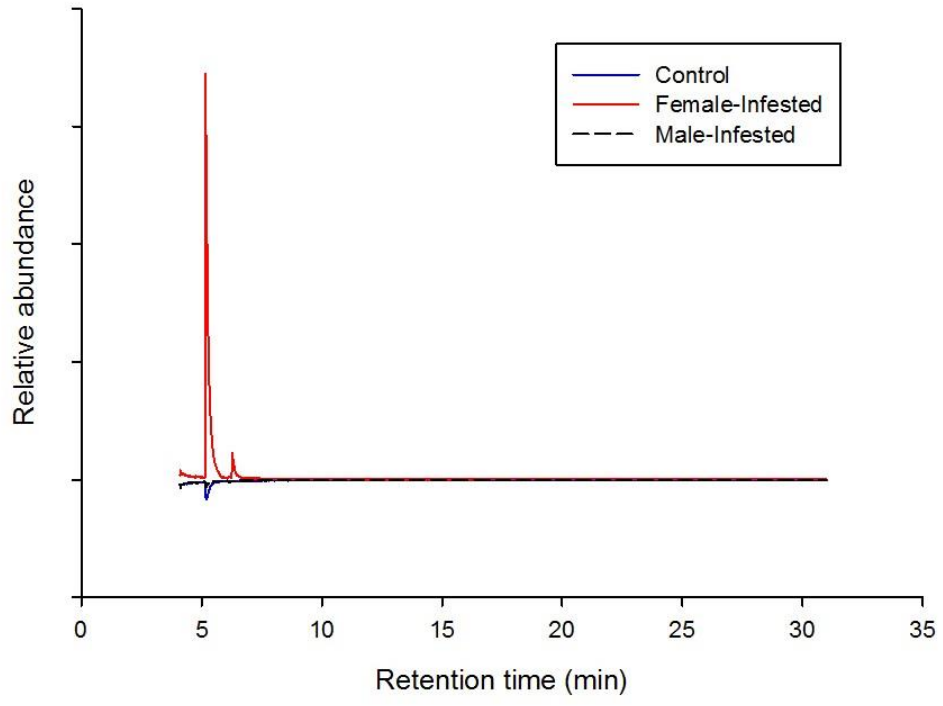
Identification of volatile attractant

To identify the volatile compounds associated with female-infested bolts, I infested small bolts (10 cm length) of black cherry with (1) no PBB (control), (2) 40 adult female PBB, or (3) 40 adult male PBB. To infest the bolts, 40 small holes (2 x 2 mm) were drilled into the bark and an adult PBB was placed into each hole; holes were drilled in the control bolt, but no beetles were introduced. Bolts were placed in large glass tubes attached by Tygon® tubing to a laboratory vacuum system pulling at ~1 L/min. An absorbent filter containing 100 mg of 80/100 mesh HayeSep Q (Ohio Valley Specialty Company, Marietta, OH) was placed between the glass tube and vacuum system. I collected headspace volatiles by leaving filters in place for 12 h and subsequently eluting each sample with three 0.5-mL aliquots of $MeCl_2$. Extracts were analyzed using a Hewlett-Packard 6890N gas chromatograph, in splitless mode, coupled to a Hewlett-Packard 5973 mass spectrometer using electron-impact ionization (70 eV) (as in Hughes et al.

2013). Compounds were identified by comparing mass spectra to that of known compounds in the National Institute of Standards and Technology (NIST) database (http://chemdata.nist.gov).

Three major volatile components were identified from all bolts, but one was more abundant in extracts from female-infested bolts. During the first two weeks following infestation, α-longipinene and cyclosativene were abundant in the headspace of all bolts. These compounds may be associated with wounding of black cherry. Starting at day 14 benzaldehyde was also present in all extracts, but the relative abundance in female-infested bolts was approximately 10 times as much as that present in male-infested and control bolts (Fig. 3). These finding provide evidence that benzaldehyde is used as a volatile pheromone for PBB. These results are surprising, as this represents a novel use of host defensive compounds as a pheromone previously undescribed in bark and ambrosia beetle literature.

Figure 3: Chromatogram from dynamic headspace collection of control, male-, and female-infested black cherry bolts. The large peak has been identified as benzaldehyde.



Objective 1a: Determine the extent to which compounds present in infested bolts attract adult PBB in the field.

A field assay will be used to test the extent to which PBB are attracted to each of the major volatile components identified from the experiment described above. The major components were identified as benzaldehyde, α-longipinene, and cyclosativene. The three compounds will be used to bait transects of four-unit Lindgren funnel traps placed at three sites of high cherry density: one managed plantation (ArborAmerica) and two managed forests (Purdue Martell Forest and Purdue Wildlife Area). Each transect will consist of ten traps, each attached to the top of a 2-m aluminum pole and baited with a semi-permeable polyethylene sachet(s) containing 1 mL of one of the following compounds or combinations: (1) no lure (control), (2) benzaldehyde (1:4 benzaldehyde:ethanol), (3) α-longipinene (1:20 α-longipinene:methanol), (4) cyclosativene, (5) benzaldehyde + α-longipinene, (6) benzaldehyde + cyclosativene, (7) α-longipinene + cyclosativene, (8) benzaldehyde + α-longipinene + cyclosativene, (9) ethanol, or (10) methanol. Ethanol and methanol are included as solvents for benzaldehyde and α-longipinene, respectively, and are therefore tested for attraction in each transect. Transects will be established during periods of high PBB flight activity, usually occurring from April–June and August–September. The traps will be maintained over six weeks for each replicate, with two replicates per year at each site. Captured beetles will be collected weekly and identified to species and sex. The number of PBB captured will be used to determine the extent of attraction to each compound, while the number of each sex collected will be used to determine if differences exist in attraction between the sexes. Depending on results, compound concentrations and lure combinations may be modified.

Objective 1b: Test the operational capacity of benzaldehyde to manage PBB

To determine the efficacy of using benzaldehyde lures to manage PBB, I will test the capacity of baited Lindgren multi-funnel traps placed at different densities to reduce beetle populations and host tree colonization. Four stands of approximately the same area, located in managed forests (Purdue Martell Forest, Purdue Wildlife Area), with high cherry density will be used for the two year experiment. In the first year a series of Lindgren 4-unit, multi-funnel traps will be placed in each stand at one of the following densities: (1) 25 m, (2) 50 m, (3) 100 m, or (4) 200 m. Traps will be placed on metal conduit (~3 m tall) and baited with benzaldehyde lures, which will

consist of 1 mL benzaldehyde (1:4 ethanol) within a semi-permeable polyethylene sachet. Lures will be replaced weekly and captured beetles will be typed and sexed. Traps will be maintained for eight weeks during periods of high PBB flight activity, during both April–June and August–September. In the second year, one Lindgren 4-unit, multi-funnel trap will be placed at the center of each stand and baited with a benzaldehyde lure. Lure replacement and beetle collection will be the same as the first year. The mean number of captured beetles per trap collection will be used to compare PBB densities between years in each stand. The density-dependent change in beetle capture (e.g. the change in beetle capture in relation to trap density) will be used to establish recommended densities of baited traps for PBB management. In addition to trap collection, trees within each stand will be rated for PBB colonization by counting gum spots found on the bark of the black cherry trees. Gum accumulates on tree bark in response to pest colonization, most often to PBB attacks (Rexrode 1981). Several focus trees within each stand will be randomly selected (number based on cherry density within the stand) and external gum spots will be counted each year during high PBB activity. Gum spots will be counted on the main stem, from the ground up to 2 m. After each gum spot is counted, it will be scraped off so that new gum spots can be easily identified. The mean number of gum spots will also be used to compare differences in colonization between years for each trap density.

Objective 2: Determine the extent to which host-associated and fungal volatiles attract the walnut twig beetle.

Background

The walnut twig beetle (WTB, *Pityophthorus juglandis*) is a small, dark-colored insect native to the southwestern United States (Wood 1982a) and vector a pathogenic fungus (*Geosmithia morbida*) responsible for Thosand Cankers Disease (TCD), which has caused the widespread death of black walnut (*Juglans nigra*) in many western states, and has recently been detected in the East (Tisserat et al. 2009, Grant et al. 2011).. The fungus causes cankers to form in the phloem tissue of susceptible walnut (*Juglans*) hosts (Kolarik et al. 2011) and at high densities, cankers coalesce and cause limb dieback or tree mortality. This disease threatens black walnut trees, whose wood is among the most valued in the U.S., especially for its use as veneer.

Rationale

Monitoring and manipulation of WTB populations currently rely on traps baited with pheromone lures (Seybold et al. 2015). This lure has increased WTB detection, but trapping results where WTB occurs can be highly variable and the lure appears to only operate at short distances (~20 m). To increase the efficacy of monitoring and management of WTB additional semiochemicals are needed to enhance attraction to the pheromone-baited traps. Many bark beetles are attracted to compounds associated with susceptible hosts (Byers 1989, Reddy and Guerrero 2004). Additionally, volatile compounds associated with symbiotic fungi attract many bark and ambrosia beetle species (Byers 1989, Hulcr et al. 2011). I will conduct experiments to identify host- and fungal-associated compounds which are: (1) attractive to WTB, and/or (2) enhance attraction to WTB pheromone lures.

Objective 2a. Identify host-associated compounds which are attractive or enhance attraction to WTB pheromone lure

Two field experiments will be conducted to determine the extent to which compounds associated with walnut hosts attract WTB, or enhance attraction to the pheromone lure. Volatiles collected from the leaves of various genotypes of black walnut contained a number of compounds that will be used in the assays (Ginzel et al., unpublished). To test WTB attraction to these compounds, one transect of eleven, 4-unit Lindgren funnel traps will be placed in a large block of WTB-infested black walnut near Walla Walla, WA. Each trap will be hung at 3 m on metal conduit and baited with one of the following compounds: (1) no lure (control), (2) WTB pheromone (positive control), (3) limonene, (4) camphene, (5) cymene, (6) sabinene, (7) β-pinene, (8) α-pinene, (9) decane, (10) β-caryophyllene, or (11) a host volatile blend. The host volatile blend will consist of a solution containing the host compounds (compounds 3-10) present in ratios similar to that described from walnut volatile collections (Ginzel et al., unpublished).

Each lure will consist of 350 μL of pure compound placed in a capped, 400 μL plastic vial, which allows for a slow release of the enclosed compound. The trap transect will be installed during periods of WTB active flight. Captured beetles will be collected weekly and shipped to the Purdue Forest Entomology Lab, where they will be counted and sexed. The number of captured beetles will be used to determine if compounds are attractive. Sub-samples from

captured beetles will be sexed to determine if there are sex-specific differences among compounds with respect to attraction.

In a separate experiment, I will determine the extent to which host-associated volatiles enhance attraction of WTB to pheromone-baited traps. Specifically, individual traps along a transect will be baited with one of the following: (1) no lure (negative control), (2) WTB pheromone (positive control), (3) limonene + WTB lure, (4) camphene + WTB lure, (5) cymene + WTB lure, (6) sabinene + WTB lure, (7) β-pinene + WTB lure, (8) α-pinene + WTB lure, (9) decane + WTB lure, (10) β-caryophyllene + WTB lure, or (11) host volatile blend + WTB lure. Captured beetles will be treated as described above. Treatments will be compared to the WTB pheromone treatment using a REGWQ test (SAS) to determine if any of the host-associated compounds have increased attraction to the lure.

Objective 2b: Identify fungal volatiles which attract or enhance attraction to pheromone lure, of adult WTB.

I will also conduct two experiments to determine the extent to which symbiotic and associated fungal volatiles attract WTB, or enhance attraction their pheromone lure. Previous research has identified isoamyl alcohol and isobutyl alcohol as major components of volatiles associated with growing *G. morbida* (Ginzel et al., unpublished). In addition to these compounds, several others (listed below in treatments) are associated with fungi vectored by other bark and ambrosia beetles known to infest walnut (C. Ranger, pers. comm.). One transect of ten, 4-unit Lindgren funnel traps will be placed in a block of WTB-infested black walnut near Walla Walla, WA. Each trap will be hung at 3 m on metal conduit and baited with one of the following compounds: (1) no lure (control), (2) WTB pheromone (positive control), (3) isoamyl alcohol, (4) isobutyl alcohol, (5) benzyl alcohol, (6) phenethyl alcohol, (7) methyl phenylacetate, (8) 2-ethyl-1-hexanol, (9) methyl benzoate, or (10) a fungal volatile blend. The fungal volatile blend will consist of a solution containing each of the compounds (3-9) in a 1:1 ratio. Each lure will consist of 1 mL of pure compound in a semi-permeable polyethylene sachet. Transects will be installed during periods of WTB active flight. Captured beetles will be collected weekly and shipped to the Purdue Forest Entomology Lab, where they will be counted and sexed. The number of captured beetles will be used to determine if compounds are attractive. Sub-samples from

captured beetles will be sexed to determine if there are sex-specific differences among compounds with respect to attraction.

To determine if the fungal volatiles enhance attraction to the WTB pheromone lure, another transect will be placed with similar lures, although each will also have an attached WTB pheromone lure. Thus, each trap in the second transect will be baited with one of the following: (1) no lure (control), (2) WTB pheromone (positive control), (3) isoamyl alcohol + WTB lure, (4) isobutyl alcohol + WTB lure, (5) benzyl alcohol + WTB lure, (6) phenethyl alcohol + WTB lure, (7) methyl phenylacetate + WTB lure, (8) 2-ethyl-1-hexanol + WTB lure, (9) methyl benzoate + WTB lure, or fungal volatile blend + WTB lure. Captured beetles will be treated as described above. Treatments will be compared to the WTB pheromone treatment to determine if any of the fungal compounds have increased attraction to the lure.

Objective 3: Find host-associated compounds which are attractive or repellent to the exotic ambrosia beetles *Xylosandrus germanus*, *Xylosandrus crassiusculus*, and *Xyleborinus saxeseni*.

Background

The ambrosia beetles *Xylosandrus germanus* Blandford, *Xylosandrus crassiusculus* Motschulsky, and *Xyleborinus saxeseni* Ratzeburg share similar life histories and nutritional requirements. All three are exotic species with native ranges throughout Asia (Ranger et al. 2016). Each colonizes a wide variety of hardwood tree species and vectors a symbiotic fungus species, on which its progeny will feed. These species are considered pests of ornamental nursery stock and can be locally abundant throughout areas of the eastern U.S., where there are usually two generations per year (Oliver and Mannion 2001). Management of these pests is necessary to protect valuable nursery trees, especially during the first several years of growth when trees have little phloem and growth is usually promoted above defense. These beetles are difficult to manage due to their cryptic lifestyle deep in the xylem. Because sprayed pesticides are ineffective and systemic pesticides are usually impractical on a large scale, semiochemicals are used for management of ambrosia beetle species.

Rationale

Ambrosia beetles, like bark beetles, rely on semiochemicals to find new hosts for reproduction (Gitau et al. 2013, Ranger et al. 2016). However, due to reproductive strategies that diminish the need to search for mates (i.e. sib-mating), ambrosia beetles are most likely attracted to volatiles associated with susceptible hosts rather than compounds produced by conspecifics or other associated insects. Although the ambrosia beetles are attracted to the tree stress volatile ethanol (Miller and Rabaglia 2009), in some studies colonization of surrounding trees has occurred even in the presence of ethanol-baited traps, suggesting other compounds play a role in chemically-mediated host finding (Coyle et al. 2005, Gandhi et al. 2010, Ranger et al. 2011). Several other tree volatiles (i.e. alpha-pinene, conophthorin) have caused variable increases in attraction, but the response appears to depend on species and habitat (Ranger et al. 2011, VanDerLaan and Ginzel 2013, Ranger et al. 2014, Miller et al. 2015). Although "healthy" hosts can attract ambrosia beetles (Kuhnholz et al. 2001), biotic and abiotic stress can increase the production of host-associated volatiles in trees, often increasing attraction of bark and ambrosia beetles (Miller and Rabaglia 2009, Ranger et al. 2015). Thus, I aim to collect volatiles from both healthy and stressed trees and test the extent to which volatile compounds attract the target species.

Objective 3a: Identify volatiles associated with healthy and stressed chestnut trees.

To identify compounds associated with regularly colonized hosts, I will describe the compounds associated with healthy, as well as artificially stressed, American chestnut (*Castanea dentata* Borkh.) trees. Blocks of chestnut trees have been observed to suffer regular attack from ambrosia beetles in Tennessee and in several Purdue managed forests, yet volatiles associated with these hosts have not been described (Oliver and Mannion 2001). For healthy trees, volatiles will be gathered from two sources: (1) individual three-year old potted plants maintained in a greenhouse setting, and (2) four-year old plants present in a small block of chestnut at the Purdue Martell Forest. For stressed trees, volatiles will also be gathered from two sources: (1) individual three-year old potted plants in a greenhouse setting, with roots submerged in standing water (simulating water stress; see Ranger et al. 2013) during volatile collection, and (2) four-year old plants from the same block as above (Martell Forest), with the main stem girdled (simulating stress from mechanical damage or insect colonization) previous to volatile collection. Volatiles will be collected from leaves, branches, and bark of each tree during bud flush, as well as after

leaves have fully expanded. Many ambrosia beetles colonize hosts as leaves are expanding, therefore, there may be unique semiochemicals present before full leaf flush. Volatiles will be gathered from four trees of each source, for a total of 16 trees.

To collect volatiles, a turkey-baking bag will be secured around the tissue of interest and connected by Tygon tubing to a small vacuum pump, which will blow air into the bag and remove it at ~1 L/min. An absorbent filter containing 100 mg of 80/100 mesh HayeSep Q will be in the tube connecting the bag to the vacuum pump. The vacuum will pull air through the filter for 8 h for each sample. After volatile collection, each HayeSep Q filter will be eluted with three 0.5-mL aliquots of $MeCl_2$. Extracts will be analyzed using a Hewlett-Packard 6890N gas chromatograph, in splitless mode, coupled to a Hewlett-Packard 5973 mass spectrometer using electron impacy ionization (70 eV). Compounds will be identified by comparing mass spectra to that of known compounds in the NIST database. Actual abundance and ratios of detected compounds will be calculated by comparing peak areas to those of known amounts of 1-nonanol.

Objective 3b: Determine the biological activity of identified compounds in the laboratory.
I will test the behavioral response of adult beetles to major compounds identified from the previously described chestnut collections. To test responses, a combination of olfactometer assays and gas chromatography-electroantennographic detection (GC-EAD) will be used. For both assays adult beetles will be reared from infested material from nearby forest or nurseries. GC-EAD is a method that records voltage change in insect antennae in respose to exposure to volatile compounds, which are simultaneously identified by flame ionization detection (Leal et al. 1995). Beetle antennae will be prepared by pinning the antennal club against the side of the head and piercing it with a small pipette filled with AgCl solution, which pipette is then attached to a recording electrode. The antennae will be exposed to elutions from the previous experiment (3a) and the electrophysiological response of the antennae to particular compounds within the sample will be recorded. The response to ethanol will be used as a positive control. The response of three adult beetles of each sex for each species will be tested. Responses within GC-EAD assays do not always correlate with attraction or repellence; thus the need for olfactometer assays.

Olfactometer assays are used to quantify behavioral response, using a walking response, of insects to different odor sources (Knolhoff and Heckel 2014). Pure solutions of major antennally-active compounds identified from exp. 3b will be procured from Sigma-Aldrich, St. Louis, USA. Five adult beetles of one sex of each species will be placed in a Y-tube olfactometer and exposed to different odor sources in each arm. Each of the tested compounds will be tested against: (1) ambient air passed through an activated charcoal filter, and (2) ethanol (positive control). The residence time of each beetle within segments of the olfactometer will be recorded and used to determine attraction to each odor source, with the arm location of the beetle recorded each minute for 10 min. A difference in residence times in each branch will indicate that one odor source is more attractive than the other. For each species, males and females will be tested separately and the experiment will be repeated six times for each compound.

Objective 3c: Determine attraction of biologically active compounds in the field.
Those compounds to which AMB show a response in GC-EAD and olfactometer assays will be tested for attraction in field assays. Transects of 4-unit Lindgren funnel traps will be placed in each of two field sites. Each trap will be attached to the top of a 2-m aluminum pole and treated with the following: no lure (negative control), ethanol (positive control), or one of the compounds to be tested. The number of traps in each transect will depend on the number of compounds to be tested. Transects will be installed during periods of high AMB flight, normally occurring from April–June and August–September. Each transect will be repeated at least once for a total of four replicates (two sites x two flights). The number of beetles captured will be used to determine the extent to which the tested compounds attract AMB.

Objective 4: Measure the active space of walnut twig beetle pheromone and ambrosia beetle ethanol lures.

Background

The efficiency of lured traps to monitor and manage insect pests is influenced by the spatial density of traps. The active range of semiochemical lures varies depending on characteristics of the compound and receiving insect (Byers 2008). Distances between lured traps may thus influence the ability to use attractive or repellent compounds to monitor and manage insect pests.

The distance at which a semiochemical can affect the behavior of a receiver is known as the effective radius, and is relatively unknown for most of the lures currently used for management of wood-boring beetles (Byers 1989, 2008). The optimal space between lured traps in monitoring and management programs can be improved by determining the active space of semiochemical lures.

Rationale

To better inform trapping protocols for WTB and ambrosia beetles, I will determine the active space of lures by placing semiochemical-baited traps at various distances from the discrete edge of beetle-infested hardwood stands. The number of beetles captured at each distance will allow for estimates of the relative efficiency of lures at different distances from beetle sources, as well as the maximum attractive distance at which each lure attracts beetles. This knowledge can be used to better plan for coverage of lure compounds in monitoring and mass trapping efforts.

Objective 4a: Describe the active space of WTB attraction to commercially available pheromone lures

Established and incipient populations of WTB are currently managed using Lindgren multi-funnel traps baited with a commercially available pheromone lure (Seybold et al. 2015). The active range of WTB pheromone lures remains unknown. To measure the distance at which WTB lures attract adult beetles, traps will be placed at different distances from a discrete block (approximately 1800 m^2) of black walnut trees in a WTB infested plantation near Walla Walla, WA. Although the active range of lures may be affected by forest density and composition, the majority of protectable walnut reside in managed plantations; thus, this experiment would mirror similar conditions.

Walnut twig beetle pheromone lures (The Scotts Miracle Gro Company, Marysville, OH) will be placed on traps during the active flight of WTB, usually occurring from late spring to early fall. Pairs of 4-unit, Lindgren multi-funnel traps will be placed at 2, 5, 10, 25, 50, and 100 m from edge of the block of trees. In each pair, one trap will be baited with a commercially available WTB lure and the other trap will have no lure (a control). Distances from the stand edge for each pair of traps will be random, not sequential (e.g., 2, 5, 10, etc.). Each trap in a pair will be at least

5 m from the other, while pairs of traps will be at least 10 m from each other. All traps will be at least 200 m from other walnut trees to avoid confounding results. To visualize an example of the design (e.g., various distances), a diagram of pheromone trap placement from a previous study is shown in Fig. 5. Captured beetles will be collected weekly by collaborators and shipped to Purdue University for identification and counting. The experiment will be repeated four times over a two year period. The rate of WTB capture (mean number of beetles captured per trap per collection period) will be used to determine the effective range of the pheromone lure. The distance at which traps capture at least one beetle will be used to determine the maximum range of the lure.

Figure 4: Trap placement to determine the attractive range of a pheromone lure for cerambycid beetles (from Dunn et al. 2016)

Objective 4b: Describe the active space of ambrosia beetle ethanol lures.

The bulk of exotic and native ambrosia beetles are currently monitored and managed using Lindgren multi-funnel traps baited with ethanol lures. To determine the attractive distance of

ethanol lures, traps will be placed at different distances from small blocks of American chestnut trees routinely attacked by ambrosia beetles. Trees are located at the Purdue Richard D. Lugar Forestry Farm as well as the Purdue Martell Forest. Trapping will occur during active flight of adult ambrosia beetles, either April–June or August–October. Spatial placement of traps will be similar to above experiment, although a greater number of distances will be used to account for possible species-specific differences in attractive range. Pairs of 4-unit, Lindgren funnel traps will be placed at 2, 5, 10, 25, 50, 100, 150, and 200 m from blocks of trees. Trap distances will be randomized from one end of the experimental space to another and each trap will be at least 10 m from other traps. At each distance, two traps will be placed: one blank (control) trap and one baited with an ultra-high release (UHR) ethanol lure (Synergy Semiochemicals, Burnaby, BC). All traps will be at least 200 m from other deciduous trees to avoid confounding beetle captures. Traps will be in place for six weeks and captured beetles will be collected weekly, identified, and counted. Trapping will occur during two flights at two sites, for a total of four replicates. The rate of AMB capture (mean beetle/collection period) for each species will be used to determine the effective range of the lure. The maximum active range will be determined using traps that captured at least one beetle. The ranges will be determined for all AMB species captured.

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