

THESIS

STUDIES ON THE WALNUT TWIG BEETLE (WTB), *PITYOPHTHORUS JUGLANDIS*, IN
RELATION TO ITS ASSOCIATION WITH *GEOSMITHIA MORBIDA*, ITS SURVIVAL IN
FELLED LOGS, AND ITS SENSITIVITY TO TEMPERATURE EXTREMES

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ABSTRACT

STUDIES ON THE WALNUT TWIG BEETLE (WTB), *PITYOPHTHORUS JUGLANDIS*, IN RELATION TO ITS ASSOCIATION WITH *GEOSMITHIA MORBIDA*, ITS SURVIVAL IN FELLED LOGS, AND ITS SENSITIVITY TO TEMPERATURE EXTREMES

The walnut twig beetle (WTB, *Pityophthorus juglandis*) is the vector of *Geosmithia morbida* and a causal agent of thousand cankers disease (TCD) of black walnut (*Juglans nigra*). In addition to aggressive feeding by WTB, damage to walnut occurs from canker development around beetle galleries due to *G. morbida*. Cankers coalesce and girdle the branches and trunk eventually causing mortality. To better understand WTB, studies were initiated to: 1) determine if WTB is attracted to *G. morbida*, or other bark fungi, when given a choice; 2) determine whether clear plastic tarping or applications of bifenthrin, permethrin, and biodiesel to felled logs affected the persistence of the walnut twig beetle or altered the suitability of logs for future breeding by the insect; and 3) establish upper and lower lethal temperature (LT) limits of the beetle as well as determine supercooling point (SCP) and seasonal variation of SCP of walnut twig beetle.

For the first study, in order to determine if WTB was attracted to *G. morbida* or other bark fungi, bioassays for both adult and larval WTBs were conducted. No differences in adult WTB numbers were detected between tubes that were filled with either potato dextrose agar (PDA) or PDA colonized by *G. morbida*. However, *Xyleborinus saxeseni*, an ambrosia beetle commonly associated with TCD during the later stages of decline was collected in higher numbers from tubes containing PDA compared to PDA colonized by *G. morbida*. Larvae preferentially migrated towards agar plugs colonized by *G. morbida* when tested against PDA. Similarly,

larvae also migrated toward plugs colonized by *Fusarium solani* or *Penicillium solitum* when tested against PDA. However, when given a choice between two fungi, no preference in larval movement was observed. These results suggest that larvae are attracted to the bark fungi tested in general, but not specifically to *G. morbida*.

In the second study (testing cultural and chemical controls to felled black walnut logs), beetle emergence from all logs was variable six months after trees were felled; no beetle emergence was recorded in a portion of both treated and untreated logs and overall detection of WTBs was poor. Nevertheless, only logs treated with bifenthrin were consistently devoid of beetles. Walnut twig beetles emerged from a small proportion of the treated and untreated logs for up to 21 months after sampling and even from some logs in which the beetles previously had not been detected suggesting colonization of felled logs. *Geosmithia morbida* was isolated from the beetle at each sampling date. Neither plastic sheeting nor the chemical applications of biodiesel or permethrin were an effective means of disinfesting black walnut logs of WTB.

For the third study, based on logistic regression models, the lower median lethal temperature (LT_{50}) and lower lethal temperature required to kill 99% of the population (LT_{99}) for WTB adults was $-16.7\text{ }^{\circ}\text{C}$ and $-22.97\text{ }^{\circ}\text{C}$ respectively and for larvae was $-16.9\text{ }^{\circ}\text{C}$ and $-25.19\text{ }^{\circ}\text{C}$ respectively. The upper LT_{50} and upper LT_{99} for WTB adults was $48\text{ }^{\circ}\text{C}$ and $53\text{ }^{\circ}\text{C}$ respectively and for larvae was $47\text{ }^{\circ}\text{C}$ and $48\text{ }^{\circ}\text{C}$ respectively. Mean monthly SCPs for WTB larvae and adults from January to June 2012, were between $-16\text{ }^{\circ}\text{C}$ to $-18.5\text{ }^{\circ}\text{C}$. Several differences among monthly means were detected but those differences were no larger than $2\text{ }^{\circ}\text{C}$.

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CHAPTER I. Attraction of Walnut Twig Beetle (Coleoptera: Curculionidae) to the fungus *Geosmithia morbida*

INTRODUCTION

As early as the mid 1990s, black walnut (*Juglans nigra*) trees were experiencing decline and mortality in the western United States (Cranshaw 2011; Pscheidt and O'Camb 2010). In 2001, this decline was first associated with the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman, in the Espanola Valley of New Mexico (USDA Forest Service 2002). Walnut twig beetle was again associated with crown dieback and tree mortality in 2005 from several areas along the Front Range in Colorado (USDA Forest Service 2005). It was first assumed that this decline was caused by the drought conditions experienced in 2002-2003 but even in areas receiving supplemental irrigation and after normal precipitation returned in 2004, black walnuts continued to die (Tisserat et al. 2009). Symptoms included yellowing leaves on flagging branches, thinning of the upper canopy, branch die back beginning with smaller diameter branches and progressing to larger diameter branches, extensive canker development just beneath the bark, and tree mortality two to four years after the first noticeable symptoms (Tisserat et al. 2009). In 2009 an undescribed *Geosmithia* sp. was found to be associated with canker formation surrounding WTB galleries, and the walnut decline was named "thousand cankers disease" (TCD; Tisserat et al. 2009, 2011). The fungus has since been described as *Geosmithia morbida* (Kolarik et al. 2011). TCD is now recognized as the disease responsible for killing thousands of black walnuts in several western states (AZ, CA, CO, ID, NV, OR, UT, and WA) where it is planted for ornamental value, and has recently been found in the native stands of black walnuts in the East (TN, PA, and VA) (Cranshaw 2011; Kolarik et al. 2011; Tisserat et al.

2009; Seybold et al. 2010). TCD is a disease complex involving the minute WTB which vectors the newly described fungus.

The WTB was originally described from Arizona walnut (*J. major*) in New Mexico (Blackman 1928), a host that has a native range that includes New Mexico, Arizona, and extends south into Chihuahua, Mexico (Wood & Bright 1992). The beetle is native to Arizona walnut, and likely southern California walnut (*J. californica*). In native stands of Arizona walnut it behaves like a typical twig beetle, attacking small or diseased and weakened branches (Cranshaw 2011, Seybold et al. 2010). However, much more serious injuries result from infestation of non-native hosts, notably black walnut. On black walnut the WTB does not discriminate against branch size and can heavily attack and breed in all areas of the tree including the main stem (Tisserat et al. 2009).

The WTB is a reddish brown, minute twig beetle that measures about two mm in length and can be differentiated from other closely related species by the asperities on the pronotum which form regular, concentric rows (Blackman 1928). Walnut twig beetle is believed to have overlapping generations in Colorado and can complete its life cycle from egg to adult in four to six weeks (Tisserat et al. 2009). Male WTB are the first to colonize branches and are followed by females after the release of a pheromone (Graves et al. 2010). Larvae are nondescript, white, C-shaped, and tunnel heavily in the phloem. Larval galleries run with the grain while egg galleries are constructed against the grain (Seybold et al. 2010)

The genus *Geosmithia* is a little studied group of fungi that is consistently associated with phloeophagous bark beetles (Kolarik et al. 2007, 2008; Kubatova et al. 2004). The relationships between the beetles and the fungi are uncertain but it is thought that *Geosmithia* spp. are vector-specific fungi despite lack of entomochory-related characteristics (i.e., absence of sticky spores)

often associated with insect-vectored fungi (Kolarik et al. 2008). *Geosmithia morbida* is the only species in the genus *Geosmithia* or in the family Bionectriaceae that is described as a plant pathogen (Tisserat et al. 2009) although *G. langdonii* and *G. pallida* reduced stem growth of garden cress seedlings (Cizkova et al. 2005).

Geosmithia morbida is often seen growing within WTB galleries coating the pupae and lining the pupal chambers. It is consistently isolated from WTB adults and necrotic tissues surrounding the beetle galleries and *G. morbida* spores are spread to new hosts incidentally during tunneling by WTB. However, little detail is known about the association of the two causal organisms involved in TCD.

A wide range of relationships between insects and fungi are known and well developed insect-fungi relationships are widespread among bark beetles (Kruess 2002). Many instances occur where the relationship is mutualistic providing benefit to both the insect and the fungus. For example, fungi may be transported by the insect acting as a vector and the insect in turn receives either a nutritional benefit from the fungi (Batra 1963; Kukor et al. 1988; Six and Paine 1998, Six 2003) or a reproductive benefit (Honda et al. 1988; Six and Paine 1998). A classic example of this mutualism is seen with the mountain pine beetle, *Dendroctonus ponderosae* (MPB) and one of its associated blue stain fungi, *Ophiostoma clavigerum* (Robinson-Jeffery & David) Harrington. In this relationship, MPB is unable to reproduce without the fungus (Six and Paine 1998) and it acts as a nutritional source for adults which feed on the thick layer of fungus that lines the pupal chamber (Six and Paine 1998, Six 2003). In return, the fungus needs MPB for movement among hosts and for the ability to reach desired tissues. Like *O. clavigerum*, *G. morbida* densely lines the pupal chambers in WTB galleries. Examples of antagonistic

relationships are less common; fungi may be entomopathogenic (Castrillo et al. 2011) or their secondary metabolites may be toxic to the insect (Rohlf's et al. 2007).

Insect attraction to fungal volatiles has been demonstrated by several groups (Honda et al. 1988; Faldt et al. 1999; and El-Hamalawi 2008) and this can be important in their interaction. No information is available regarding the relationship between *G. morbida* and its vector WTB. To better understand this, a series of trials were established to determine if *G. morbida* is attractive to the walnut twig beetle.

MATERIALS AND METHODS

Experimental Overview

Walnut twig beetle adults and larvae were tested for their attraction to fungi that colonize necrotic bark surrounding galleries in TCD-affected trees. For the adult choice test, a log infested with WTBs was placed in a chamber and emerging adults were given a choice between gathering in a tube containing ¼- strength potato dextrose agar modified with 100mg/liter streptomycin sulfate and 100mg/liter chloramphenicol (PDA) or a tube containing PDA colonized with *G. morbida*. For the larval choice bioassay, a larva was placed in the center of a Petri dish and given the choice of migrating towards an agar plug of PDA, or a plug of PDA colonized by either *G. morbida*, *Penicillium solitum*, or *Fusarium solani* (in various combinations).

Sample Material

Insects

Black walnut trees showing symptoms of TCD were felled in December 2010 in Boulder, CO. The logs were cut into manageable pieces and left uncovered outside at a central location in

Boulder, until the experiments began. For the adult choice test, logs 30-cm-diameter by 20-cm-long were transported to the Colorado State University (CSU) campus in Fort Collins, CO. This experiment was conducted inside of the laboratory between the months of April to October 2011. Before the experiment began, a small (6 cm x 4 cm) area of bark from the log was removed and dissected to confirm the presence of living WTBs. For the larval choice test experiment, second and third instar larvae were extracted from bark and placed individually into 5 mL tubes then starved for 48 hours in the dark at 4°C.

Fungi

Single-spore isolates of *G. morbida* isolate 1217, *F. solani* isolate 1179, and *P. solitum* isolate 1281 were obtained from the Tisserat Culture Collection of Fungi at CSU and grown for two weeks on PDA in Petri plates or 50 mL tubes. The *G. morbida* isolate chosen was the first to be isolated and shown to be pathogenic to black walnut (Tisserat et al. 2009). *Fusarium solani* is a fungus commonly isolated from necrotic bark of black walnut during the final stages of TCD (Tisserat et al. 2009) and is also pathogenic to walnut. *Penicillium solitum* was also consistently isolated from decaying walnut bark but is not considered pathogenic.

Experimental set-up

Adult Choice Test

For the adult choice test study, an insect emergence chamber was constructed using a 19-liter bucket modified to include a 50 mL tube on either side, so the bottom of the tubes were flush with the base of the bucket (Figure 1.1). The tubes were attached to the chamber with 45° polyvinyl chloride (PVC) elbows oriented in a downward position. The elbows were sanded along the inner rim, with a rotary sander tool, providing a ramp for the adults into the collection tube and a textured surface for them to walk. Preliminary observations showed that WTB adults

easily succumbed to static electricity on the smooth surface of the plastic bucket so 100-grit sandpaper was placed in the bottom to provide traction for walking. The bucket had a lid containing a 10-cm-diameter hole covered with a fine mesh fabric to provide ventilation. The 50 mL collection tubes were filled with either PDA or PDA colonized by *G. morbida*.



Figure 1.1 *Adult choice test.* Walnut twig beetle infested log was placed inside of bucket and beetle emergence into the two tubs was recorded. Tubes were filled with either $\frac{1}{4}$ -strength potato dextrose agar modified with 100mg/liter streptomycin sulfate and 100mg/liter chloramphenicol (PDA) or PDA colonized by *G. morbida*.

Five infested logs were placed inside five buckets and left undisturbed for ten days. Collection tubes were switched to account for differences in light and temperature, and buckets were left ten more days at which time the tubes were harvested. Contents of the tubes were extracted and insects were identified, sexed, and counted. The experiment was conducted a total of 5 times using the same logs.

Larval Choice Test

For the larval choice test bioassay, a 100- mm-diameter x 15-mm-deep Petri dish was filled with water agar. At each side of the dish, 5-mm-diameter plugs of water agar were removed. The holes were filled with PDA or agar colonized by one of the test fungi. Walnut twig beetle larvae were tested immediately after insertion of the fungal plugs by placing a larva in the center of the dish (Figure 1.2A). The dishes were incubated at 25°C in the dark for 48 hours at which time larvae were removed. The movement of the larva was visualized 24 to 48 hours later by observing the small amount of fungal/bacterial growth associated with the larval movement (Figure 1.2B). Trails were traced using a permanent marker (Figure 1.2C). The movement of the larvae relative to the position of agar plugs containing *G. morbida* was tested against larval movement in relation to plugs of PDA (n=55), agar colonized by *F. solani* (n=50), or agar colonized by *P. solitum* (n=50). Larval movement relative to the position of PDA or plugs colonized by *F. solani* was also tested (n=35). Larval movement was scored as either movement towards one agar plug only, movement towards both agar plugs, or movement toward neither plug. Occasionally a larva would pupate or die and was omitted from the study.

Statistics

Adult Choice Test

A chi-square goodness of fit test was used to compare the beetle counts related to the two treatments: *Geosmithia morbida* verses PDA (CHITEST, Excel 2007). No post hoc comparisons were made.

Larval Choice Test

Comparisons of counts of larval preferences using a chi-square goodness of fit test were made to determine whether there was a preference between the four categories (i.e. one of the two active treatments, both treatments or neither treatment). Distribution of WTB larvae and adults among the treatments was tested against the distribution of the expected homogeneous values (CHITEST, Excel 2007). Post hoc comparisons looking at the two active treatments were then made using a Z-test of single proportions.

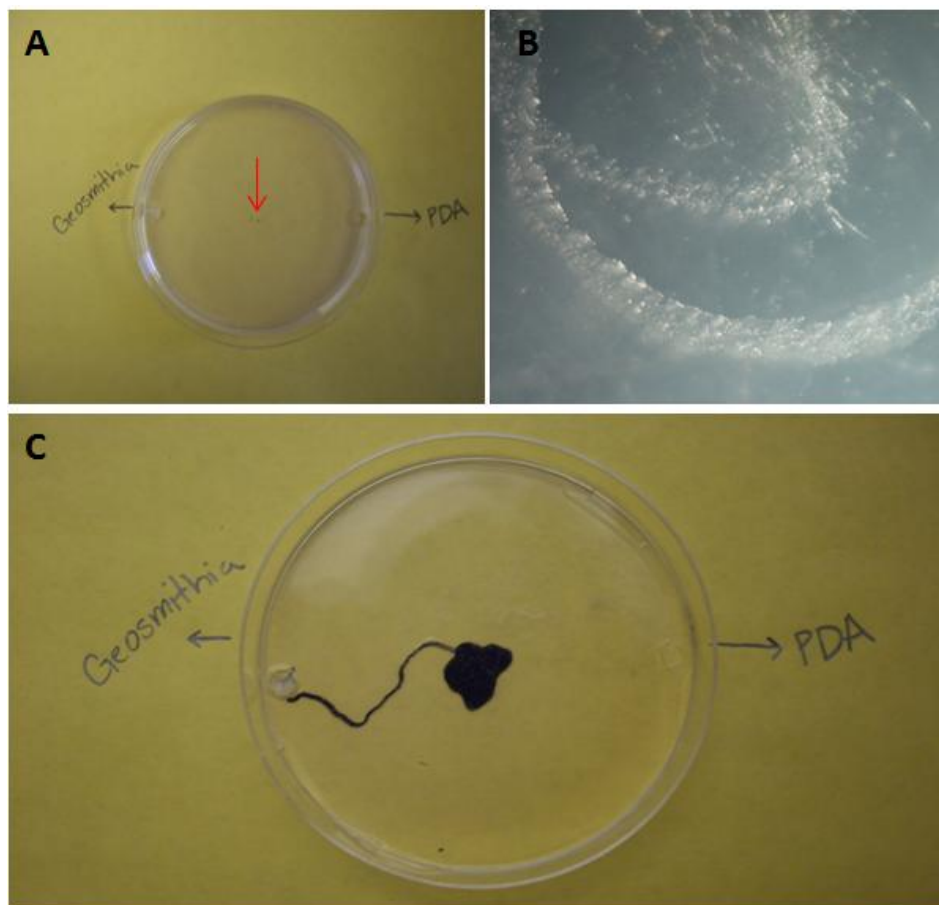


Figure 1.2 A-C. Larval choice test bioassay. (A) A walnut twig beetle larva (indicated by red arrow) was placed on a Petri plate filled with water agar. At each side of the dish, plugs of water agar were removed. The holes were filled with either PDA or PDA colonized by one of the test fungi (*Geosmithia morbida*, *Penicillium solitum*, or *Fusarium solani*). Larva was removed after 48 hours. (B) Bacterial/fungal growth under magnification outlining the trail of walnut twig beetle larvae. (C) Example of larval trail that was traced with permanent marker.

RESULTS

Adult Choice Test

There was no difference ($P > 0.10$) between the number of emerged WTB adults that were collected in tubes containing agar colonized by *G. morbida* and to those in tubes containing agar only (Figure 1.3A). The number of male and female WTBs collected in tubes with *G. morbida* was similar; however, more males than females ($P < .05$) were collected in tubes containing agar only (Figure 1.3A). More *Xyleborinus saxeseni*, an ambrosia beetle commonly found in black walnut wood in the later stages of TCD, were collected in tubes containing agar only compared to *G. morbida* ($P < .001$)(Figure 1.3B).

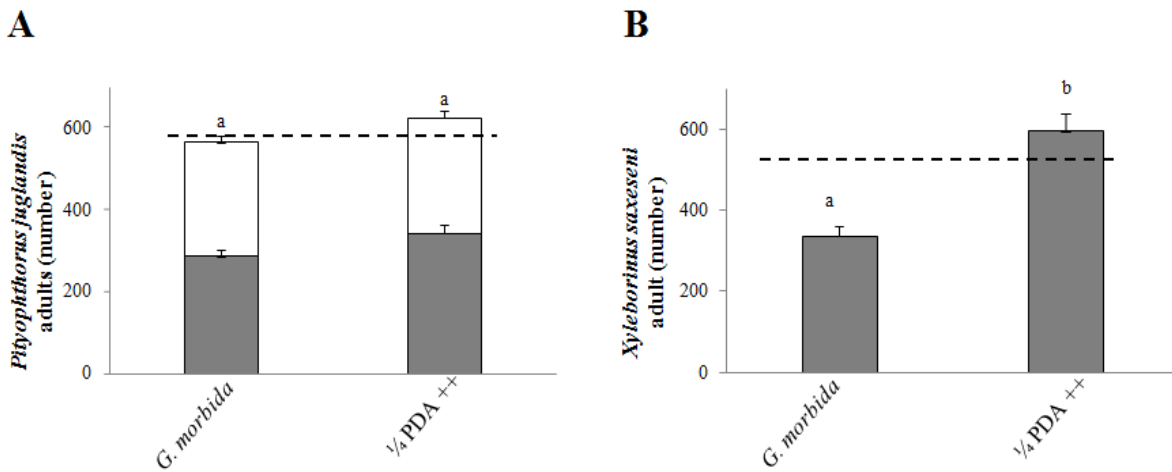


Figure 1.3 A,B Adult Choice Test. Newly emerged adult walnut twig beetles (WTB) were given two options: a collection chamber filled with PDA or; PDA colonized by *Geosmithia morbida*. (A) Number of emerged *Pityophthorus juglandis* adults (\pm SD) per treatment. Columns are divided into proportions of male and female *P. juglandis*. White portions are female, grey are male. (B) Number of emerged *Xyleborinus saxeseni* (\pm SD) per treatment ($p < .001$). Results were analyzed using a chi-square goodness of fit test. Dashed lines represent expected values for the chi square goodness of fit analysis. Bars with different letters are statistically significant at $p < 0.01$

Larval Choice Test

Walnut twig beetle larvae migrated more frequently towards an agar plug colonized by *G. morbida* (Figure 1.4A) ($P < 0.0001$, $\chi^2 = 20.2$, $df = 3$) or *Fusarium solani* (Figure 1.4D) ($P < 0.0001$, $\chi^2 = 18.78$, $df = 3$) compared to an un-colonized agar plug. No larval preference was observed when plugs colonized by *F. solani* and *G. morbida* ($P = 0.32$, $\chi^2 = 0.31$, $df = 3$) were placed in the same plate (Figure 1.4B). Similarly no differences in larval preference were observed when plugs colonized by *G. morbida* and *P. solitum* ($P = 0.75$, $\chi^2 = 2.84$, $df = 3$) were placed in the same plate (Figure 1.4C).

In experiments comparing larval movement towards a fungus (*G. morbida*, *F. solani*, or *P. solitum*) the larvae usually traveled in a more or less direct route from the center of the Petri dish to the colonized plug (Figure 1.2 C). In many of the bioassays, larvae would stay on the fungal plug or burrow inside of it and remain there for the duration of the experiment (Figures 1.5 A and B). In experiments where the larvae were given the choice between two different fungi, the larval trails were more or less sporadic and seemed to cover more area of the water agar Petri plate.

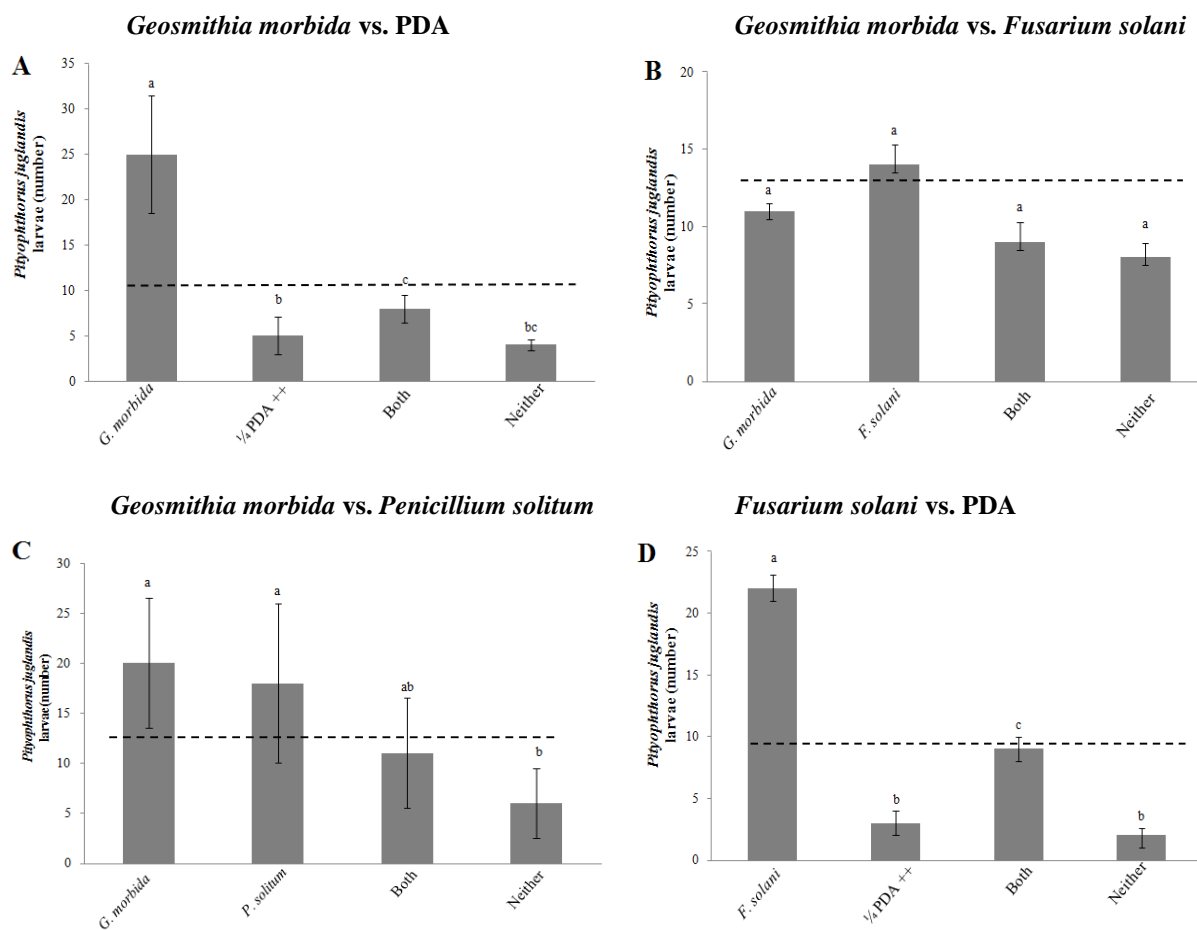


Figure 1.4 A-D Larval choice test. A Petri dish filled with water agar was used to conduct a larval bioassay. On either side of the dish a plug of water agar was removed and filled with either (A) *Geosmithia morbida* vs. PDA, (B) *G. morbida* vs. *Fusarium solani*, (C) *G. morbida* vs. *Penicillium solitum* or (D) *F. solani* vs. PDA. A larva was placed in the center of the dish and left for 48 hours. Its behavior was scored in one of the following ways: 1) larva picked one or the other active treatment (i.e. test fungi); 2) larva chose both active treatments; 3) larva went to neither treatment or 4) larva went to both treatments. Results were analyzed using a chi-square goodness of fit test. Dashed lines represent expected values for the chi square goodness of fit analysis. Post-hoc comparisons were made using a Z-test of single proportions. Bars with different letters are statistically significant at $p < 0.01$.

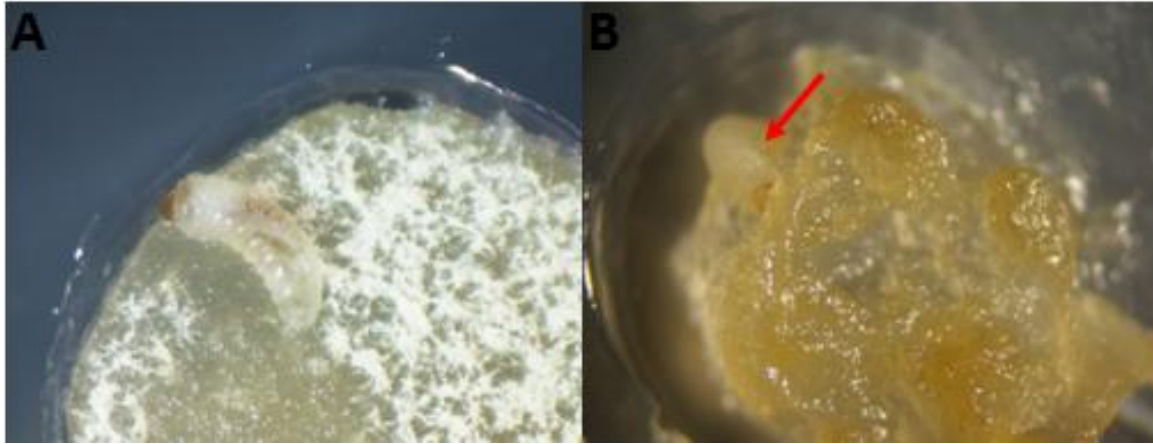


Figure 1.5 A,B (A) Walnut twig beetle (WTB) larva residing on an agar plug colonized by *Geosmithia morbida*. (B) Plug of agar colonized by *G. morbida* that has been burrowed through by WTB larva. Larva is indicated by the red arrow.

DISCUSSION

Insect attraction to fungal volatiles is documented in several orders of insects, most notably Coleoptera, Diptera, Lepidoptera, and Hymenoptera. Fungal volatiles as oviposition attractants were shown by Honda et al. (1988), who found the yellow peach moth, *Conogethes punctiferalis*, was more attracted to fruit inoculated with fungi rather than healthy fruit. Additionally, moths were attracted to moldy rice cakes and fungi grown on an artificial medium. Insect attraction to fungal volatiles was also demonstrated by Faldt et al. (1999). Volatiles were collected in situ from several bracket fungi and analyzed by gas chromatography–mass spectrometry. In this study, a general fungal volatile, oct-1-en-3-ol (Pyysalo 1976; Kaminski et al. 1980), attracted several species of wood inhabiting beetle generalists.

Our results indicate an elicited response by WTB larvae for several fungi associated with the bark of black walnut. Larvae were attracted to the agar plugs colonized by *G. morbida*, *P. solitum*, and *F. solani* but not to PDA alone. The motivation for this attraction is unknown but may be caused by chemical or carbon dioxide emissions from the fungi, or other unknown factors such as humidity or temperature differences between treatments.

There was no preferential movement of emerging adult WTBs to tubes containing *G. morbida*. This suggests that volatile chemicals produced by the fungus are not attractive to the WTB. However, the newly emerged adults may not have been sensitive to the treatments. Alternatively, it is possible the experimental design may have precluded finding the influence of any fungal volatiles because of the length of time the tubes were left on the boxes could have diluted the volatile concentration gradient within the emergence chambers.

Adult *X. saxeseni* chose containers filled with PDA over containers colonized by *G. morbida*. Our findings show that clearly no attraction exists between *G. morbida* and the wood inhabiting ambrosia beetle, and if any relationship exists at all, it seems to be antagonistic i.e. the fungus acts as a deterrent to the beetle. One hypothesis on antagonistic relationships with fungi is the chemical shield hypothesis; where secondary metabolites act as a resistance mechanism to the fungi in order to ward off or harm predators and competitors (Chinnici and Bettinger, 1984; Rohlfs and Obmann, 2009). It has long been assumed that an important role of fungal secondary metabolites is to act as protection against fungivory (Demain and Fang 2000; Scheu and Simmerling 2004). This theory was demonstrated experimentally by Rohlfs et al. (2007) with *Aspergillus* mutants that lacked the *laeA* gene that regulates secondary metabolites. The springtail, *Folsomia candida*, preferred the mutants over the wild type and demonstrated higher reproductive success when fed a diet of the mutated fungus.

Insect attraction to volatiles of pathogens they vector are seen in several insect families including sap beetles (Coleoptera: Nitidulidae), fruit flies (Diptera: Drosophilidae) and shore flies (Diptera: Ephydriidae). One example is seen with the oak wilt pathogen, *Ceratocystis fagacearum*. Here the fungus produces fungal mats underneath the bark which produce sticky spores that are disseminated by nitidulids and drosophilids (Dorsey and Leach 1956; Collins and

Kalnins 1965). Profiles of headspace volatiles from *C. fagacearum* reveal many components commonly associated with fruit odors are attractive to nitidulids suggesting that this fungus mimics food odors in order to attract nitidulids and other insect vectors such as *Drosophila* (Griswold 1958; Collins and Kalnins 1965; Lin and Phelan 1992). Nitidulids also vector the fungus *Fusarium verticillioides*, the causal agent of an important ear-rot disease of corn (Windels et al. 1976). In a study by Bartelt and Wicklow (1999), attraction to volatiles of *F. verticillioides* were observed and correlated with the alcohol, acetaldehyde and ethyl acetate components of the headspace profile versus the phenolic component. Another insect-vector attraction example is seen in shore flies. El-Hamalawi (2008) observed adult shore flies (known aerial vectors of many plant pathogens) were attracted to sporulating cultures or plant tissues infected with *Verticillium dahlia*, *Fusarium oxysporum* f.sp. *basilica* and *Thielaviopsis basicola*.

Chemical cues whether volatile or non-volatile undoubtedly are a factor in the biological/physiological processes of their associated insects. Certainly WTBs utilize fungi in some unknown manner and the discovery of this utilization could uncover mechanisms to control this insect and stop the spread of TCD.

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CHAPTER II. Persistence of walnut twig beetle as affected by chemical and cultural treatments

INTRODUCTION

Black walnut is native to the central and eastern United States and serves as an important food source for wildlife (Harlow & Harrar 1969). In the lumber industry, black walnut wood is used for gunstocks, cabinetry, and furniture and is one of the most highly desired timber species in North America (Dirr 1998). The estimated value of black walnut growing stock in the East alone is over 500 billion dollars (Newton et al. 2009). Horticulturally, the nuts are grown for human consumption. The tree also has ornamental value in the western states where is widely planted in landscapes.

The walnut twig beetle (WTB), *Pityophthorus juglandis*, along with the pathogenic fungus *Geosmithia morbida*, cause thousand cankers disease (TCD) of black walnut (*Juglans nigra*) that has resulted in mortality in many western states (AZ, CA, CO, ID, NV, OR, UT, and WA) and several states in the East (TN, PN, and VA) (Cranshaw 2011; Kolarik et al. 2011; Tisserat et al. 2009; Seybold et al. 2010). Because of the recent discovery of TCD, control methods have not been developed. With the rapidly increasing spread of the disease front, and with the recent disease conformations in the native habitat of black walnut, mechanisms to control WTB and stop the spread of TCD are urgently needed.

Effective bark beetle control via insecticides has been demonstrated at both the individual tree level and at the forest level. McCambridge (1982) protected individual ponderosa pines from mountain pine beetle (*Dendroctonus ponderosae*; MPB) by topical applications of carbaryl-water suspensions or by application of lindane emulsion. Systemic translocation of neem-based insecticide was effective in reducing *Ips pini* populations in lodgepole pine in Canada (Duthie-Holt et al. 1999).

Diesel fuel oil (no. 2) has commonly been used as a solvent for insecticides such as ethylene dibromide, orthodichlorobenzene, dichlorodiphenyltrichloroethane (DDT), and dichlorvos (Massey and Wygant 1954; Schmid, 1972; Cibulsky and Hyche, 1974). The use of diesel fuel oil alone has also been used as an effective means of control for several species of bark beetles (Cibulsky and Hyche, 1974; Schmid, 1972; Mata et al. 2002). Cibulsky and Hyche (1974) noted increased *Ips* mortality on loblolly pine (*Pinus taeda*) when bolts were treated with the fuel alone and similarly, Schmid (1972) saw an average of 90% mortality of the spruce beetle, *Dendroctonus rufipennis* (Kirby) when diesel fuel oil was applied to cull logs. Likewise, diesel fuel oil applied to logs also decreased MPB survival (Mata et al. 2002).

Effective control against some bark beetle species has also been demonstrated by the use of permethrin and bifenthrin (Grosman & Upton 2006). No chemical insecticides are currently labeled specifically for control of WTB on black walnut. In addition to lack of data on efficacy, black walnut's production of nuts fit for human consumption entails restrictions on the pesticides available for use on the tree. The use of any insecticide management program has limitations such as cost effectiveness, disruption of natural insect fauna, the potential risks involved with application of chemicals, and the possibility for chemical drift (Billings 1980).

Temperature is a limiting factor in the development, distribution and often, percent mortality (Grahm 1924) of subcortical insects. The use of solar radiation to provide control to subcortical insects has long been utilized (Craighead 1920; Buffam and Lucht, 1968; Mitchel & Schmid 1974; Holsten and Werner 1993; Negron et al. 2001). Craighead (1920) found that several types of wood directly exposed to sunlight, and turned weekly during the summer months controlled wood inhabiting insects in Arizona, Mississippi, and Virginia. In Arizona, Buffam and Lucht (1968) found that 4-mil clear polyethylene sheeting, used as a tarp over slash piles of

ponderosa, raised pile temperatures to nearly 56° C and successfully treated *Ips* beetles. Mitchel & Schmid (1972) solar treated logs in Wyoming and found up to 90% mortality in spruce beetle (*Dendroctonus rufipennis*) infested logs on the top surfaces but not to the sides. Greater spruce beetle mortality (on average 94%) was observed if logs were turned between July and August. However, in Alaska, the use of solar heat combined with polyethylene sheeting did not increase subcortical temperatures high enough to affect spruce beetle brood mortality (Holsten and Werner, 1993). Negron et al. (2001) noted the use of 6-mil plastic sheeting, or solar radiation without plastic sheeting, was an effective method for reducing MPB numbers in lodgepole and ponderosa pine logs.

Because no chemical or cultural controls for WTB have been established, we elected to measure the effectiveness of several chemical applications and the use of solar heat as a means to control WTB to felled black walnut logs. Two trials testing permethrin, biodiesel, and solar heat were conducted between 2010-2011 and one trial testing the above mentioned treatments plus bifenthrin was conducted in 2010 to evaluate the efficacy of these chemical and cultural control methods.

MATERIALS AND METHODS

Experimental Overview

Trees showing symptoms of TCD were felled and used for experiments conducted in two different locations along the Front Range in Colorado (Boulder and Wheat Ridge). Logs were treated with chemicals or covered with 4-mil clear plastic sheeting. At periodic intervals, a section of each log was removed, brought to the campus at Colorado State University (CSU), tested for the presence of *G. morbida*, and monitored for WTB emergence.

Experiment 1

Trees showing symptoms of TCD in Boulder, CO were felled between November and December 2009, and transported to the Boulder City Forestry lot. In March 2010, individual logs approximately 1.5-2-meters-long were divided into small (15.2- 21.6-cm), medium (22.9-29.2 cm), large (30.5- 45.7 cm) and extra-large (>45.7 cm) diameter classes. Each log was sealed at each end with a paraffin-based wax paint to prevent desiccation (Anchorseal, U.C. Coatings Corporation, Buffalo, NY).

A single log selected from the medium diameter class was sprayed to runoff (10.65 L/log) with permethrin (0.5% emulsion of Astro), biodiesel (0.5% emulsion of Blue Sun 80) or water on 30 March, 2010. Biodiesel was chosen as an alternative to petroleum diesel because of pesticide regulations in Boulder (Secrist, 2002).

A log from each of the four diameter classes was left untreated (control) or covered with a 4-mil clear plastic tarp for 6 months as a means to solar heat the logs and potentially kill WTBs.

Logs in all treatments were placed in a randomized block design with five replicates (Figure 2.1). Five samples in total were taken from each log over the duration of the experiment (one pre-treatment and four post-treatment). Samples were obtained by removing a 7.6 cm section from the end of the log with a chain saw. Wood moisture readings were obtained from each log at each sampling date using a hand held moisture meter (Pin Moisture Meter mini-Ligno E/D, Lignomat USA Ltd., Portland, OR). Sampling dates were 17 March, 20 May, and 10 December 2010, and 2 June and 19 December 2011. Samples were transported to the campus at CSU where they were stored at 4°C until they were evaluated for the presence of *G. morbida*; the

cut ends of each sample were then painted with paraffin wax, and placed into insect emergence containers where they were monitored for WTB adult emergence.

Row 1	Permethrin 23	Control 62 78 29 35	Plastic 100 30 54 20	Biodiesel 60	Water Control 33
Row 2	Biodiesel 45	Control 18 59 26 109	Plastic 111 64 129 128	Water Control 32	Permethrin 106
Row 3	Control 131 107 41 114	Permethrin 108	Plastic 40 25 21 135	Water Control 39	Biodiesel 34
Row 4	Water Control 110	Biodiesel 101	Control 133 12 119 52	Plastic 132 125 63 2	Permethrin 56
Row 5	Plastic 137 112 103 126	Biodiesel 123	Permethrin 22	Water Control 113	Control 138 27 104 36

Figure 2.1 *Experiment 1*. Schematic of experimental design in Boulder, CO. Numbers in each treatment and row represent individual black walnut logs that were sampled during the experiment. In the case of the untreated control and the plastic tarp treatment, four logs representing different diameter classes were included.

Experiment 2

Experiment 2 was conducted at Prospect Park in Wheat Ridge, CO. Thousand cankers disease-affected trees were felled between September and November 2009, and stored in the park until the experiment began. Logs were evaluated as described for experiment one and arranged in a randomized complete block design with five replications on 14 May 2010 (Figure 2.2). In addition to the five treatments listed in experiment one, an insecticide treatment application of bifenthrin, sprayed to runoff (10.65 L/log, 0.25% emulsion of Onyx) was also used. Diameter

classes were determined as described in experiment one except the extra-large diameter class was omitted from this study. Treatments were applied to the logs on 7 June 2010. The plastic sheeting remained over the logs for a period of 4 months. A total of four samples (one pre-treatment and three post-treatment samples) were taken from each log on 14 May, 1 August, and 16 December 2010 and 7 June 2011. Logs and the samples from the logs were processed as described in experiment one.

Row 1	Water Check	Plastic	Permethrin	Biodiesel	Control	Bifenthrin
	233	271	213	244	246	206
	216	298	251	218	268	270
Row 2	265	299	276	254	255	237
	Bifenthrin	Water Check	Permethrin	Control	Biodiesel	Plastic
	234	286	261	229	269	217
Row 3	280	247	221	248	294	205
	215	263	291	225	220	250
	Permethrin	Bifenthrin	Water Check	Control	Biodiesel	Plastic
Row 4	292	282	278	283	219	274
	235	230	210	232	203	208
	222	260	277	287	238	290
Row 5	Bifenthrin	Plastic	Permethrin	Control	Water Check	Biodiesel
	281	297	240	212	243	207
	227	264	209	267	257	252
Row 6	272	214	226	285	204	275
	Plastic	Permethrin	Biodiesel	Control	Water Check	Bifenthrin
	262	200	201	266	258	224
Row 7	289	256	241	211	293	245
	249	242	259	239	253	228

Figure 2.2 *Experiment 2*. Schematic of experimental design in Wheat Ridge, CO. Numbers in each treatment and row represent individual black walnut logs that were sampled during the experiment. In all treatment scenarios, three logs were used representing different diameter classes

Experiment 3

Black walnut trees in Boulder, CO were felled between September and November 2010, and were moved to the same location as listed in experiment one. Logs were arranged into a randomized complete block design with 24 replications on 22 December 2010 (Figure 2.3). In contrast to experiments one and two, for this experiment, only one log, ranging in diameter from 17.8 cm to 35.6 cm, was used in each treatment replication. Treatments were as described in experiment one and were applied to the logs on 5 May 2011. Three samples in total (one pre-treatment and two post-treatment samples) were obtained from each log on 22 December 2010, and 2 June and 19 December 2011.

Isolation of *Geosmithia morbida* from Log Samples

Small bark chips (approximately 1-5 mm) were aseptically removed with a scalpel from the margin of necrotic phloem near the cambium of each sample tested. Five to ten chips were placed in a Petri dish filled with ¼- strength potato dextrose agar modified with 100mg/liter streptomycin sulfate and 100mg/liter chloramphenicol (PDA) and incubated at 23° C with 12 h of light for up to two weeks. For the pre-treatment sampling dates in each experiment, isolation of *G. morbida* was attempted on each sample and was discontinued when either a positive identification of the fungus was confirmed or after several unsuccessful attempts. For each subsequent sampling date, twenty to thirty log samples per experiment were randomly chosen to attempt to isolate *G. morbida*. Growth of *G. morbida* from any of the chips was considered a positive presence of the fungus in the log; if no growth was detected the fungus then was recorded as absent from the log. Data was presented as the proportion of samples testing positive for the fungus over the total number of samples tested.

R1	Control 301 1	Permethrin 302 1	Water Control 303 1	Biodiesel 304 1	Plastic 305 1	Permethrin 306 2	Control 307 2	Water Control 308 2	Biodiesel 309 2	Plastic 310 2	Biodiesel 311 3	Control 312 3	Permethrin 313 3	Water Control 314 3	Plastic 315 3	Permethrin 316 4	Water Control 317 4	Control 318 4	Plastic 319 4	Biodiesel 320 4
R2	Permethrin 321 5	Plastic 322 5	Biodiesel 323 5	Water Control 324 5	Control 325 5	Water Control 326 6	Control 327 6	Biodiesel 328 6	Plastic 329 6	Permethrin 330 6	Permethrin 331 7	Water Control 332 7	Plastic 333 7	Biodiesel 334 7	Control 335 7	Plastic 336 8	Water Control 337 8	Biodiesel 338 8	Permethrin 339 8	Control 340 8
R3	Water Control 341 9	Biodiesel 342 9	Permethrin 343 9	Plastic 344 9	Control 345 9	Water Control 346 10	Plastic 347 10	Permethrin 348 10	Biodiesel 349 10	Control 350 10	Permethrin 351 11	Biodiesel 352 11	Water Control 353 11	Control 354 11	Plastic 355 11	Permethrin 356 12	Control 357 12	Water Control 358 12	Biodiesel 359 12	Plastic 360 12
R4	Water Control 361 13	Biodiesel 362 13	Plastic 363 13	Permethrin 364 13	Control 365 13	Permethrin 366 14	Control 367 14	Water 368 14	Biodiesel 369 14	Plastic 370 14	Water Control 371 15	Plastic 372 15	Control 373 15	Biodiesel 374 15	Permethrin 375 15	Control 376 16	Plastic 377 16	Water Control 379 16	Permethrin 379 16	Biodiesel 380 16
R5	Plastic 381 17	Water Control 382 17	Control 383 17	Biodiesel 384 17	Permethrin 385 17	Plastic 386 18	Permethrin 387 18	Water Control 388 18	Biodiesel 389 18	Control 390 18	Plastic 391 19	Water Control 392 19	Control 393 19	Biodiesel 394 19	Permethrin 395 19	Water Control 396 20	Permethrin 397 20	Biodiesel 398 20	Control 399 20	Plastic 400 20
R6	Control 401 21	Plastic 402 21	Water Control 403 21	Permethrin 404 21	Biodiesel 405 21	Water Control 406 22	Permethrin 407 22	Plastic 408 22	Control 409 22	Biodiesel 410 22	Biodiesel 411 23	Water Control 412 23	Permethrin 413 23	Control 414 23	Plastic 415 23	Water Control 416 24	Biodiesel 417 24	Permethrin 418 24	Plastic 419 24	Control 420 24

Figure 2.3 *Experiment 3*. Schematic of experimental design in Boulder, CO and walnut twig beetle (WTB) emergence by sampling date. Numbers in each treatment and row (R1-R6) represent an individual black walnut log that was sampled during the experiment. Logs highlighted in yellow, represent WTB emergence from pre-treatment samples collected. Logs with blue and orange borders represent WTB emergence from samples collected 6 and 12 months post-treatment respectively.

Insect Emergence Containers

An emergence container for each sample was constructed using a 45.7 x 30.5 x 7.6 cm cardboard box modified to include a 50 mL clear plastic tube attached to the center of the 30.5 cm side of the box where the side meets the base (Figure 2.4). Tubes were attached to the boxes with a 45° polyvinyl chloride (PVC) elbow oriented in a downward position that connected to the tube. Elbows were sanded along the inner rim with a rotary sander tool to provide a ramp for the adults into the collection tube and a textured surface for the emerged insects to walk on. To prevent escape and because WTBs are attracted to light (personal observation) care was taken to seal the boxes at each end with duct tape. When beetles (and other insects) emerged from the sample, they were attracted to the light and would proceed into the clear collection tube. Emergence chambers were monitored weekly for WTBs, and other insects, for up to eight months or until 12 consecutive weeks passed with no insect emergence.



Figure 2.4 Emergence containers constructed from cardboard boxes and adapted with 50 mL collection tubes.

Temperature Monitoring

In the third experiment, temperature data loggers (Watchdog 200 Series Data Loggers and Watchdog B-Series Button Loggers, Spectrum Technologies, Inc. Plainfield, IL) were used to record temperatures at 30-minute-intervals. For the solar heated treatment, temperature loggers were placed in the inner bark at the cambium interface on both the top and the bottom of the log, as well as in the airspace under the plastic. A data logger was also placed at the cambium interface on the top of a control log. Ambient air temperatures were obtained from the National Oceanic and Atmospheric Administration; Earth System Research Laboratory (NOAA/ESRL) located less than 5 miles from experiment.

Statistics

Efficacy of treatments for all experiments was determined by comparing the presence or absence of WTBs from treatment groups at each sampling date. Because beetle emergence varied considerably, WTB survival was documented as the total percent emergence from logs belonging to each treatment group at each sampling date. Data were analyzed by PROC GLIMMIX (SAS Institute 2010) to determine significant differences among treatments.

RESULTS

Experiment 1

Walnut twig beetle emergence numbers varied considerably during the experiment, ranging from 0 to 199 beetles for a specific log and sampling period (Figure 2.5). Walnut twig beetles emerged from 22 % of all bark samples collected from pre-treated logs whereas 38% of all logs (both treated and untreated) had beetle emergence at one or more sampling dates during the study (Figure 2.6). Thus, WTBs were detected in 9 logs that did not initially have beetle emergence in the pre-treatment sampling.

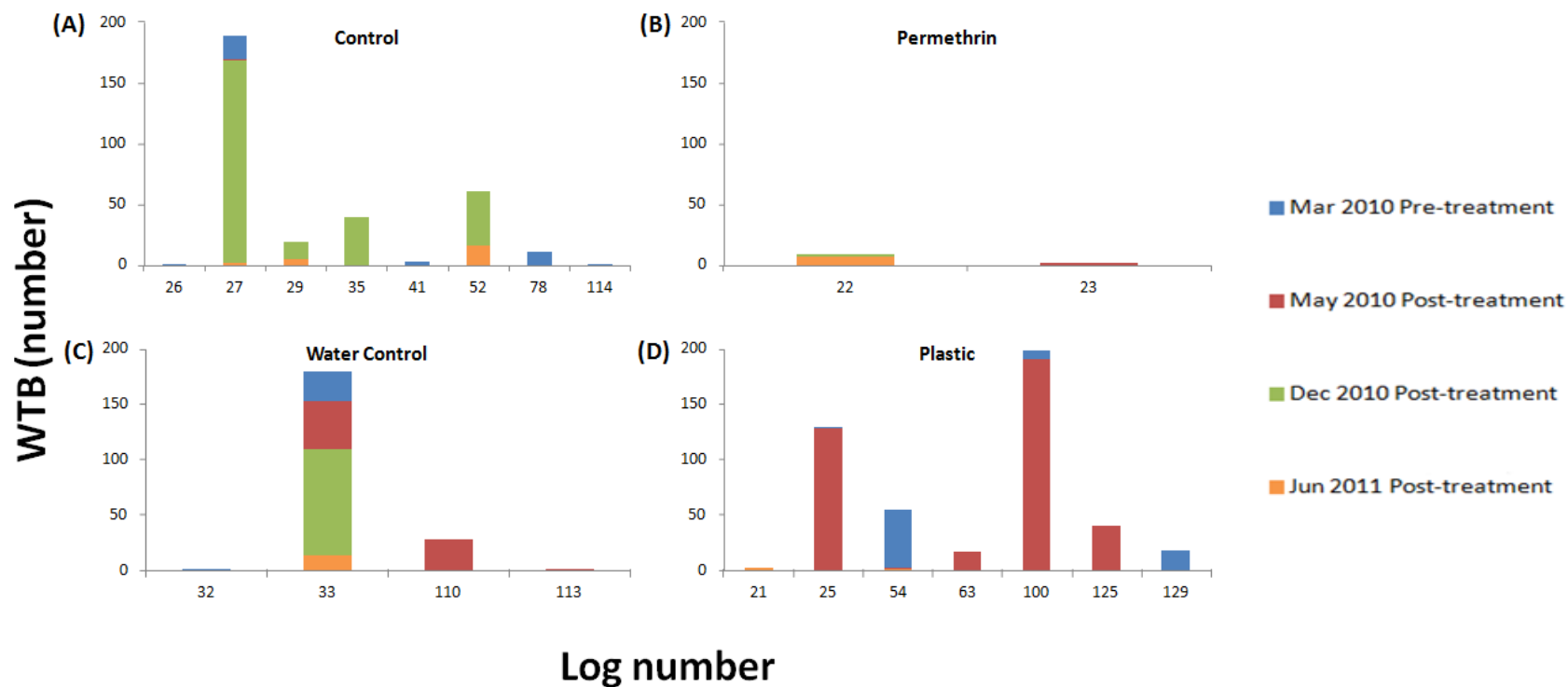


Figure 2.5 *Experiment 1*. Walnut twig beetle (WTB) emergence by date from samples collected from A) untreated black walnut logs, B) logs treated with permethrin, C) logs sprayed with water only, or D) logs covered with 4-mil plastic sheeting. Log samples with no WTB emergence during the experiment, including all logs treated with biodiesel, were omitted from the figure. Blue bars indicate WTB emergence from pre-treated samples taken in March 2010. Red, green and orange bars represent WTB emergence from post-treated samples taken in May and December 2010 and June 2011 respectively.

Row 1	Permethrin 23	Control 62 78 29 35	Plastic 100 30 54 20	Biodiesel 60	Water Control 33
Row 2	Biodiesel 45	Control 18 59 26 109	Plastic 111 64 129 128	Water Control 32	Permethrin 106
Row 3	Control 131 107 41 114	Permethrin 108	Plastic 40 25 21 135	Water Control 39	Biodiesel 34
Row 4	Water Control 110	Biodiesel 101	Control 133 12 119 52	Plastic 132 125 63 2	Permethrin 56
Row 5	Plastic 137 112 103 126	Biodiesel 123	Permethrin 22	Water Control 113	Control 138 27 104 36

Figure 2.6 *Experiment 1*. Schematic of experimental design in Boulder, CO and walnut twig beetle (WTB) emergence by sampling date. Numbers in each treatment and row represent individual black walnut logs that were sampled during the experiment. In the case of the untreated control and the plastic tarp treatment, four logs representing different diameter classes were included. Logs highlighted in yellow represent WTB emergence from pre-treatment samples collected from each log. Logs with blue, orange and green borders represent WTB emergence from samples collected 2, 9 and 15 months post-treatment respectively.

Samples taken from all treatments at 2, 9, 15, 21 months post-treatment had WTB emergence percentages of 15, 13, 13 and 0 respectively (Table 2.1). Total WTB emergence percentages for the study in the extra-large, large, medium, and small logs were 10%, 40%, 44%, and 50% respectively.

Table 2.1 *Experiment 1.* Percentage of black walnut log samples with walnut twig beetle emergence between March 2010 and December 2011 in Boulder, CO.

Treatment	<i>n</i>	Infested log samples (%)				
		Pre-treatment	2 months post-treatment	9 months post-treatment	15 months post-treatment	21 months post-treatment
Control	20	25	5	15	15	0
Permethrin	5	0	2	2	2	0
Biodiesel	5	0	0	0	0	0
Water Check	5	40	60	20	20	0
Plastic tarp	20	25	15	10	10	0
Total		22	15	13	13	0
Emergence						

To better understand the emergence of WTBs in logs over time, three logs in the study with the longest recorded emergence were evaluated. One log had been treated with water (log 33), one with permethrin (log 22), and one was not treated (log 52). Walnut twig beetle emergence was observed for 16 to 21 months, depending on the individual log (Figure 2.7). In the control log, beetle emergence was not detected until the second post-treatment sampling date (9 months) and was also observed in the subsequent sampling date at (15 months). Beetle emergence from this sample was recorded for 6 months after collection, for a total of 21 months after the experiment began. *Geosmithia morbida* was isolated from 86% of pre-treated log samples and samples continued to test positive for the fungus, although at a decreased percentage, for up to 15 months after treatment at which time attempts to culture *G. morbida* were unsuccessful (Figure 2.8).

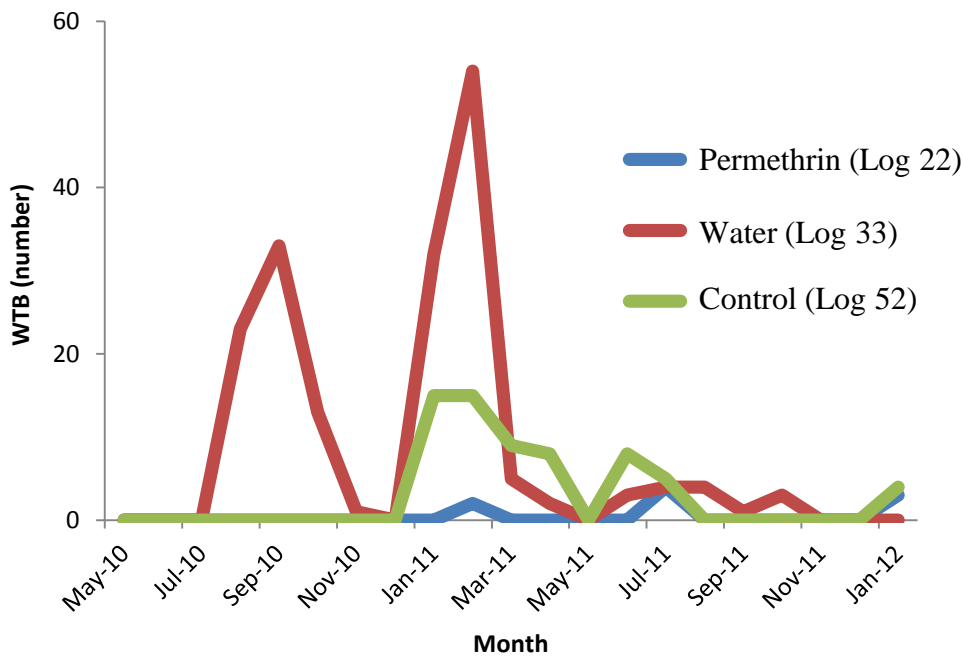


Figure 2.7 *Experiment 1.* Emergence of walnut twig beetles from the three black walnut logs with the longest duration of emergence.

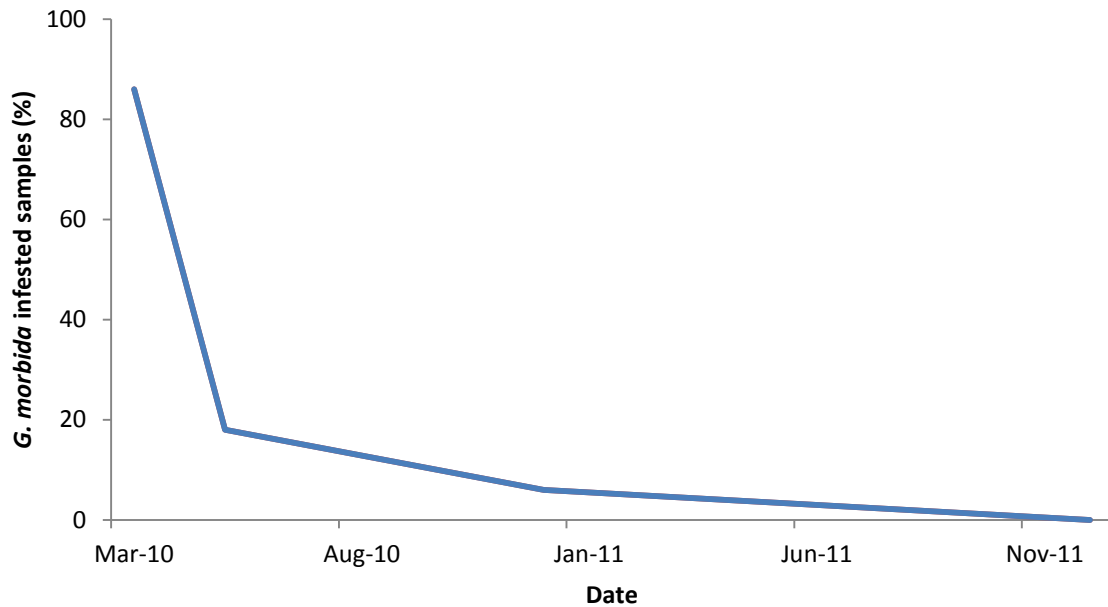


Figure 2.8 *Experiment 1*. Proportion of black walnut sample/subsample that tested positive for *G. morbida* in Boulder, CO over a period of 21 months.

Because of the low percentages of beetle emergence, and the variability in beetle numbers, statistical differences among treatments were not conducted. However, based on emergence data, plastic tarping and permethrin sprays to logs did not completely eliminate WTB at all sampling dates. Although no WTB emergence was observed in logs treated with biodiesel, they were also not detected in these logs prior to treatment (Figure 2.6).

Experiment 2

As with experiment one, WTB emergence was variable, and ranged from 0 to 378 beetles for a specific log and sampling period (Figure 2.9). Walnut twig beetles emerged from 51% of all bark samples collected from pre-treated logs and from 62 % of all logs during the study (Figure 2.10). Thus, WTBs were detected in only 3 logs that did not initially have beetle emergence in the pre-treatment sampling. Emergence percentages of WTB for all samples taken at 3, 7, and 13 months were 6, 3 and 0 respectively, and much lower than observed at the

Boulder site (Table 2.2). Total WTB emergence did not differ ($P>0.05$) among small (37%), medium (71%), and large (51%) diameter log classes. *Geosmithia morbida* was isolated at a much lower frequency from the pretreated (8%) and post-treatment samples than logs from the Boulder site (Figure 2.11). No differences were observed among treatments ($P>.05$), although differences in percentages of infested logs differed ($P = 0.001$) among the three sampling dates (Table 2.2).

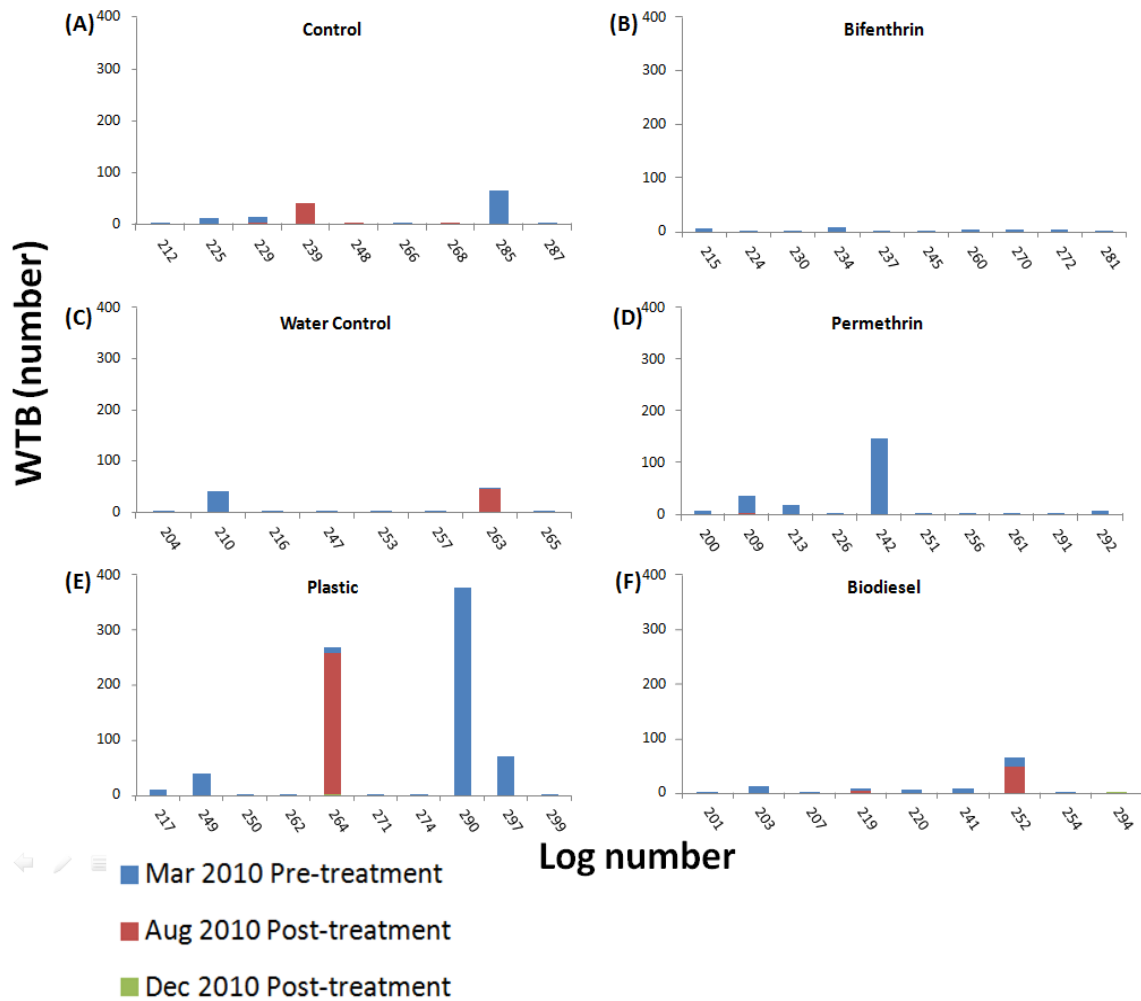


Figure 2.9 Experiment 2. Walnut twig beetle (WTB) emergence by date from samples collected from A) untreated black walnut logs, B) logs treated with bifenthrin, C) logs sprayed with water only, D) logs treated with permethrin, E) logs covered with 4-mil plastic sheeting or F) logs treated with biodiesel. Log samples with no WTB emergence during the experiment were omitted from the figure. Blue bars indicate WTB emergence from pre-treated samples taken in March 2011. Red and green bars represent WTB emergence from post-treated samples taken in August and December 2010 respectively.

Row 1	Water Check 233 216 265	Plastic 271 298 299	Permethrin 213 251 276	Biodiesel 244 218 254	Control 246 268 255	Bifenthrin 206 270 237
Row 2	Bifenthrin 234 280 215	Water Check 286 247 263	Permethrin 261 221 291	Control 229 248 225	Biodiesel 269 294 220	Plastic 217 205 250
Row 3	Permethrin 292 235 222	Bifenthrin 282 230 260	Water Check 278 210 277	Control 283 232 287	Biodiesel 219 203 238	Plastic 274 208 290
Row 4	Bifenthrin 281 227 272	Plastic 297 264 214	Permethrin 240 209 226	Control 212 267 285	Water Check 243 257 204	Biodiesel 207 252 275
Row 5	Plastic 262 289 249	Permethrin 200 256 242	Biodiesel 201 241 259	Control 266 211 239	Water Check 258 293 253	Bifenthrin 224 245 228

Figure 2.10 *Experiment 2*. Schematic of experimental design in Wheat Ridge, CO and walnut twig beetle (WTB) emergence by sampling date. Numbers in each treatment and row represent individual black walnut logs that were sampled during the experiment. In all treatment scenarios, three logs were used representing different diameter classes. Logs highlighted in yellow represent WTB emergence from pre-treatment samples collected from each log. Logs with blue and orange borders represent WTB emergence from 3 and 7 months post-treatment respectively.

Table 2.2 *Experiment 2.* Percentage of black walnut samples with walnut twig beetle emergence between May 2010 and June 2011 in Wheat Ridge, CO.

Treatment	<i>n</i>	Infested Log Samples (%) ^{ab}			
		Pre-treatment	3 months post-treatment	7 months post-treatment	13 months post-treatment
Control	15	40	27	0	0
Permethrin	15	67	7	7	0
Biodiesel	15	53	13	7	0
Water Check	15	53	7	0	0
Plastic tarp	15	67	7	7	0
Bifenthrin	15	67	0	0	0
Total Emergence		51	6	3	0

^a No significant difference among treatments (SAS PROC GLIMMIX) $P > .05$

^b Differences detected between sampling dates and WTB emergence $P = .001$

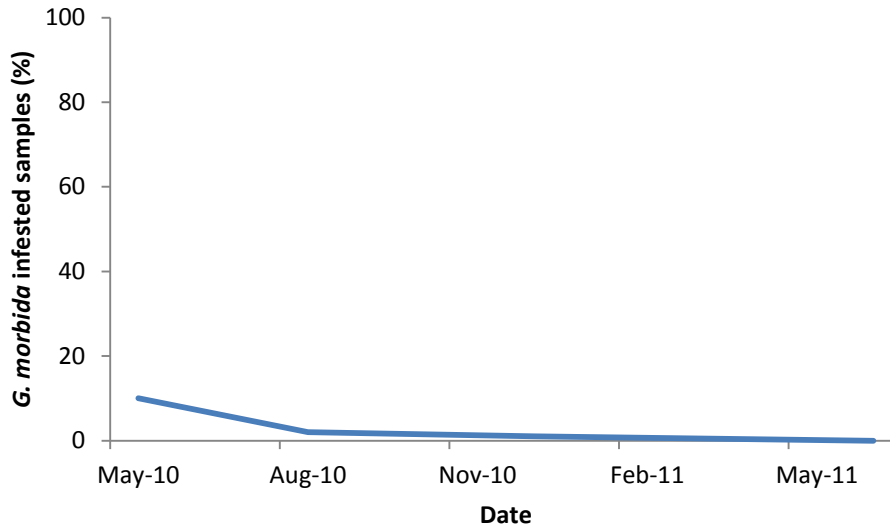


Figure 2.11 Experiment 2. Percentage of black walnut log samples testing positive for *G. morbida* in Wheat Ridge, CO.

Experiment 3

Walnut twig beetle emergence ranged from 0 beetles to 115 beetles for a specific log and sampling period (Figure 2.12). Walnut twig beetles emerged from 57% of all bark samples collected from pre-treated logs and from 59 % of all logs at least one sampling date during the study (Figure 2.13). Thus, WTBs were detected in 8 logs that did not initially have beetle emergence in the pre-treatment sampling. There were no treatment effects on WTB emergence from samples on any sampling date ($P>.10$) (Table 2.3), but there was a slight effect of log placement ($P=.08$) where more beetles emerged from logs in the inner blocks (Figure 2.13). There were also WTB emergence differences ($P<.0001$) among sampling dates, with higher numbers associated with the pretreatment date (Figure 2.12, Table 2.3). Nearly 80% of pre-treated log samples were positive for *Geosmithia morbida* and percentages gradually declined as time progressed (Figure 2.14).

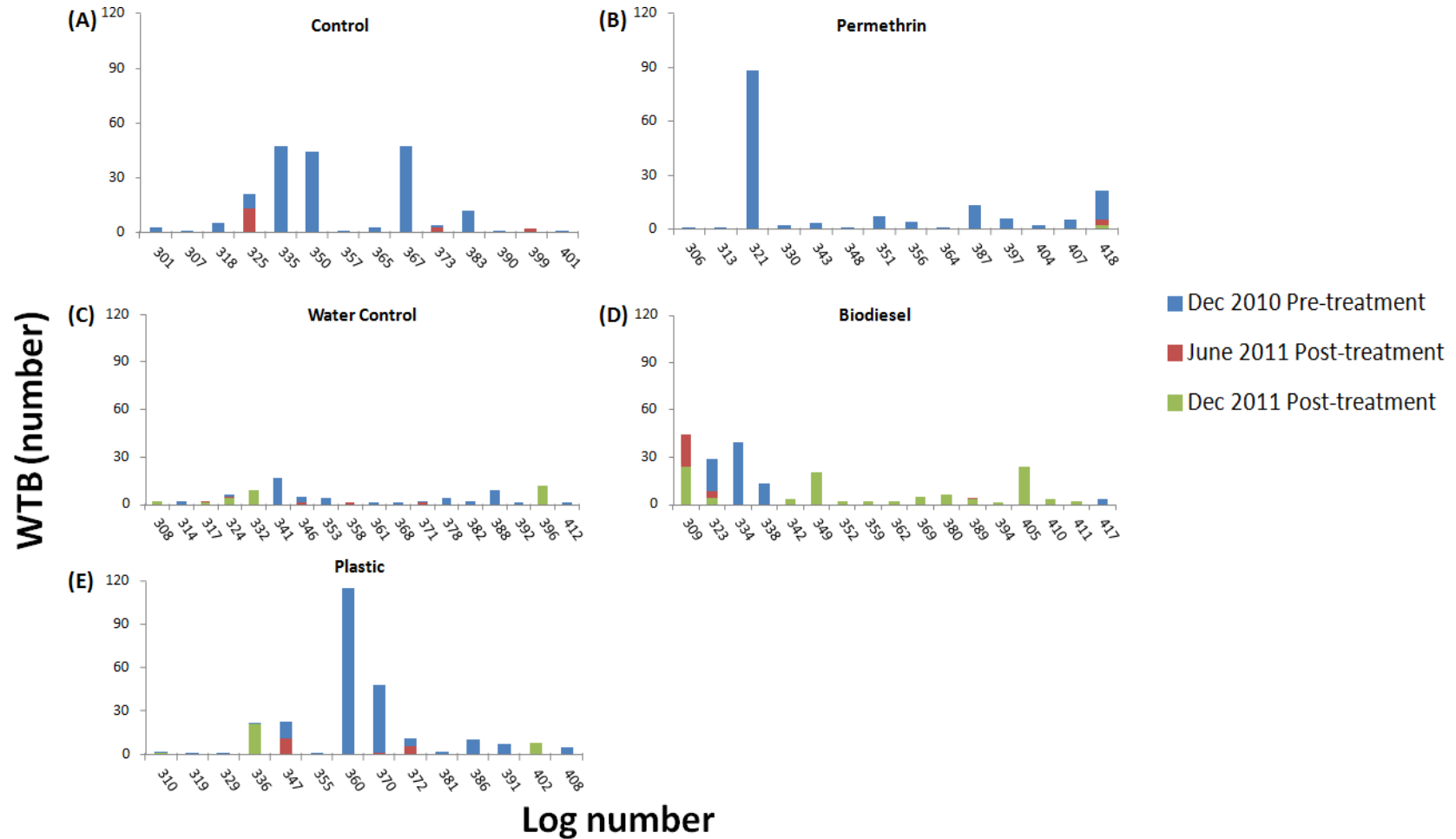


Figure 2.12 *Experiment 3.* Walnut twig beetle (WTB) emergence by date from samples collected from A) untreated black walnut logs, B) logs treated with permethrin, C) logs sprayed with water only, D) logs treated with biodiesel, or E) logs covered with 4-mil plastic sheeting. No bifenthrin treatment was used in Boulder, CO. Blue bars indicated WTB emergence from pre-treated samples taken in December 2010. Red, and green bars represent WTB emergence from post-treated samples taken June and December 2011 respectively.

R1	Control 301	Permethrin 302	Water Control 303	Biodiesel 304	Plastic 305	Permethrin 306	Control 307	Water Control 308	Biodiesel 309	Plastic 310	Biodiesel 311	Control 312	Permethrin 313	Water Control 314	Plastic 315	Permethrin 316	Water Control 317	Control 318	Plastic 319	Biodiesel 320
R2	Permethrin 321	Plastic 322	Biodiesel 323	Water Control 324	Control 325	Water Control 326	Control 327	Biodiesel 328	Plastic 329	Permethrin 330	Permethrin 331	Water Control 332	Plastic 333	Biodiesel 334	Control 335	Plastic 336	Water Control 337	Biodiesel 338	Permethrin 339	Control 340
R3	Water Control 341	Biodiesel 342	Permethrin 343	Plastic 344	Control 345	Water Control 346	Plastic 347	Permethrin 348	Biodiesel 349	Control 350	Permethrin 351	Biodiesel 352	Water Control 353	Control 354	Plastic 355	Permethrin 356	Control 357	Water Control 358	Biodiesel 359	Plastic 360
R4	Water Control 361	Biodiesel 362	Plastic 363	Permethrin 364	Control 365	Permethrin 366	Control 367	Water 368	Biodiesel 369	Plastic 370	Water Control 371	Plastic 372	Control 373	Biodiesel 374	Permethrin 375	Control 376	Plastic 377	Water Control 378	Permethrin 379	Biodiesel 380
R5	Plastic 381	Water Control 382	Control 383	Biodiesel 384	Permethrin 385	Plastic 386	Permethrin 387	Water Control 388	Biodiesel 389	Control 390	Plastic 391	Water Control 392	Control 393	Biodiesel 394	Permethrin 395	Water Control 396	Permethrin 397	Biodiesel 398	Control 399	Plastic 400
R6	Control 401	Plastic 402	Water Control 403	Permethrin 404	Biodiesel 405	Water Control 406	Permethrin 407	Plastic 408	Control 409	Biodiesel 410	Biodiesel 411	Water Control 412	Permethrin 413	Control 414	Plastic 415	Water Control 416	Biodiesel 417	Permethrin 418	Plastic 419	Control 420

Figure 2.13 *Experiment 3*. Schematic of experimental design in Boulder, CO and walnut twig beetle (WTB) emergence by sampling date. Numbers in each treatment and row (R1-R6) represent an individual black walnut log that was sampled during the experiment. Logs highlighted in yellow, represent WTB emergence from pre-treatment samples collected. Logs with blue and orange borders represent WTB emergence from samples collected 6 and 12 months post-treatment respectively.

Table 2.3 *Experiment 3*. Percentage of black walnut samples with walnut twig beetle emergence between December 2010 and December 2011

Treatment	<i>n</i>	Infested Log Samples(%) ^{ab}		
		Pre-treatment	6 months post-treatment	12 months post-treatment
Control	24	58	12.5	0
Permethrin	24	58	4	8
Biodiesel	24	58	12.5	12.5
Water Check	24	54	21	21
Plastic tarp	24	54	12.5	12.5
Total Emergence		57	12.5	11

^a No significant difference among treatments (SAS PROC GLIMMIX) $P > .05$

^b Differences detected between sampling dates and WTB emergence $P = .001$

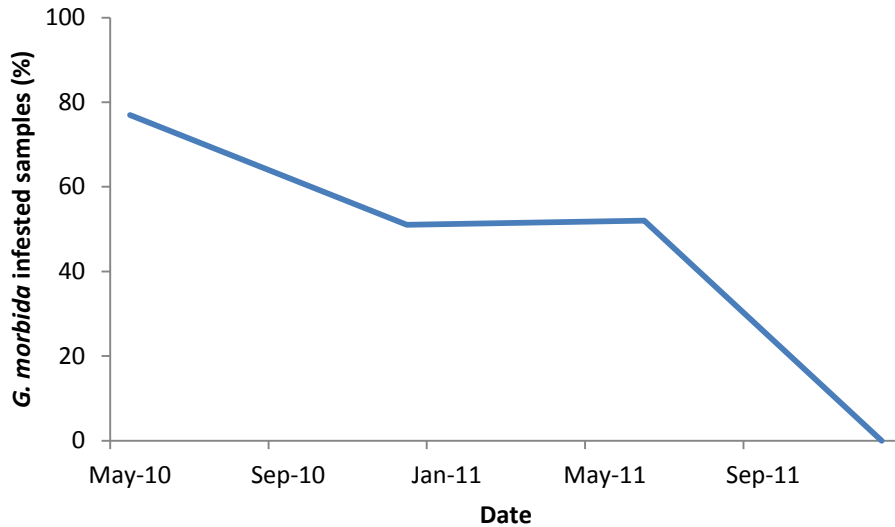


Figure 2.14 *Experiment 3.* Percentage of black walnut log samples testing positive for *G. morbida* in Boulder, CO.

The plastic tarp treatment failed to reduce WTB emergence numbers on any sampling date relative to untreated logs ($P > 0.10$). Inner bark temperatures at the top of the log reached between 60°C to 63°C thirteen days in July 2011 and registered in the upper 50°C 's for many days in July and August (Figure 2.15). Inner bark temperatures at the bottom of the log reached 41°C for three days but were consistently in the 30°C 's and similar to the ambient air temperature for much of the summer (Figure 2.15). Temperatures of the airspace underneath the plastic were usually between inner bark temperatures at the bottom and the top of the logs (Figure 2.15). Inner bark temperatures recorded on the top of plastic-covered and uncovered logs were similar (Figure 2.16)

Wood moisture in black walnut logs decreased with time ($P = .034$) but it was not affected by treatment ($P = .12$) (Figure 2.17).

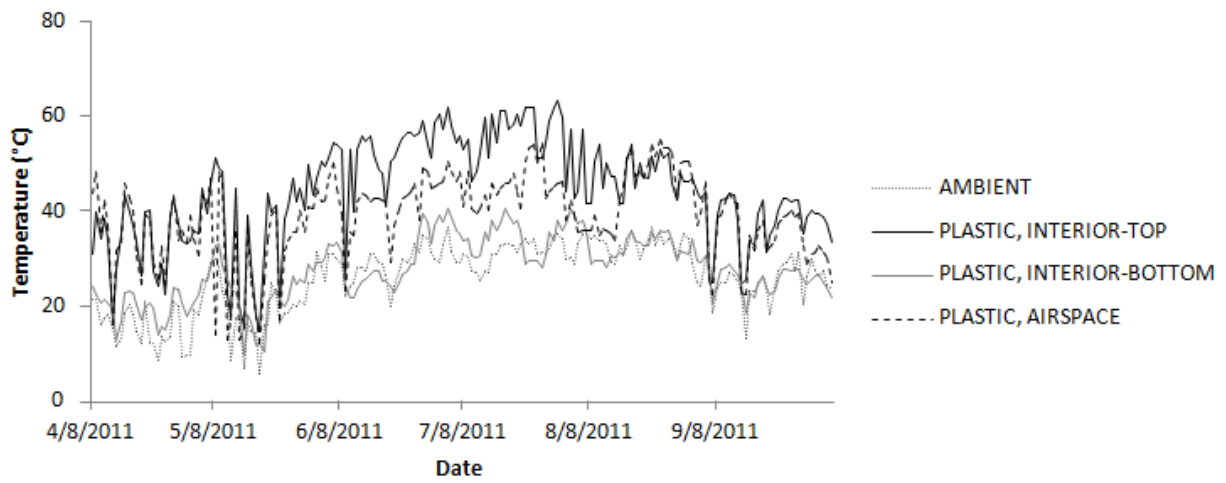


Figure 2.15 *Experiment 3.* Airspace temperatures and inner bark temperatures at the top and bottom of a plastic covered black walnut log compared with ambient temperature.

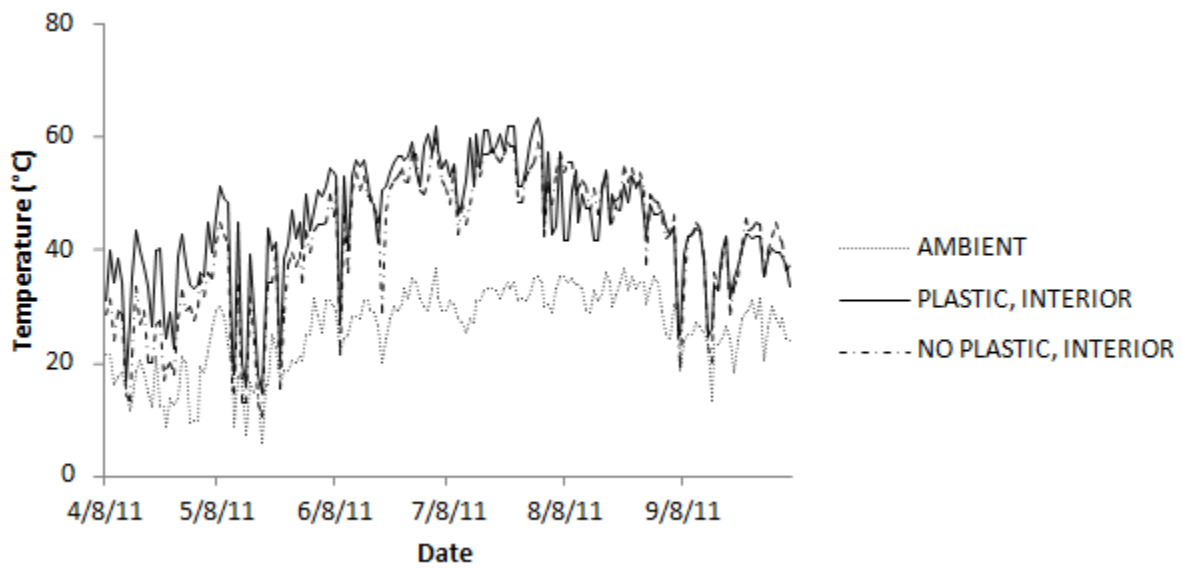


Figure 2.16 *Experiment 3.* Ambient temperature compared with the inner bark temperature at the top of a plastic covered black walnut log and the inner bark temperature at the top of a control log.

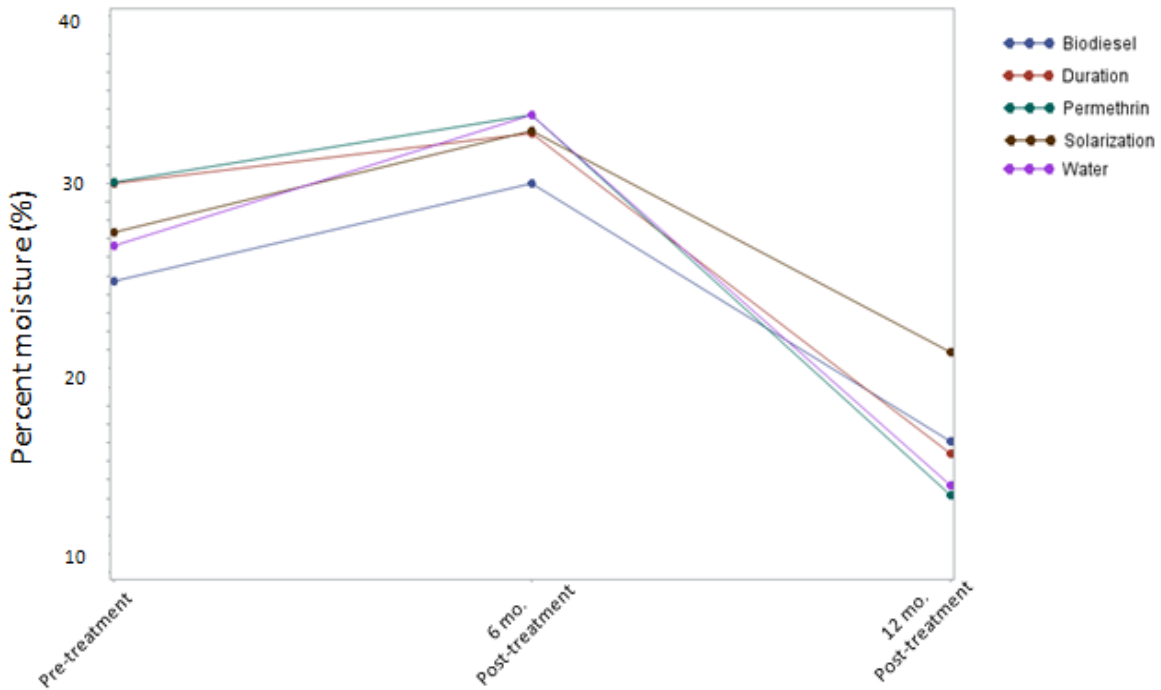


Figure 2.17 Experiment 3. Average percentage wood moisture of black walnut logs over time.

DISCUSSION

There was large variation (22-55%) in WTB emergence from pre-treatment samples in the three experiments, and in all cases beetle detection was relatively poor. This made treatment comparisons difficult. The problem was partially overcome in the third experiment by adding more replicates to each treatment.

There may be several reasons for the low WTB presence/emergence from pretreated logs. The WTB likely are not uniformly distributed in the trunk, and it is possible that the relatively small-sized bark pieces collected from the logs may not have sampled a beetle-infested area. In fact, WTB galleries tend to be aggregated in black walnut, with more on the bottom side of branches, and in some cases, only on certain areas of the trunks (Ned Tisserat, Colorado State University, personal communication, 2012). Other bark beetles show a similar aggregation in the bark (Choi et al. 2008). The spatial attack pattern of the ambrosia beetle, *Platypus koryoensis*

varies from uniform to non uniform depending on the attack density; as density increases, attack patterns become more uniformly distributed (Choi et al. 2008). In addition to WTB attack density rate, areas of the bark could be infested with other insects resulting in a depletion of resources. I observed frequent tunneling by the flatheaded borer, *Chrysobothris femorata* (Olivier). When areas of the bark were inhabited by this beetle, WTB galleries were less frequent. This aggregation problem could be overcome by visually inspecting each log to ensure it is infested with WTBs. However, WTB entrance/exit holes are very small (< 1 mm diameter) and often found in bark crevices making visual inspection difficult. An alternative approach would be to sample and destructively remove a portion of bark to look for galleries in each log. However this approach is time-consuming and wouldn't necessarily address the aggregation problem. Any further studies involving log/wood treatments will need to incorporate large numbers of replicates to overcome this sampling problem.

In some cases, logs that initially had no WTB emergence, continued to have no detectable infestation for the experiment duration, regardless of treatment. This suggests that these logs did not serve as a breeding ground for the WTB after they were felled. However, in all experiments, some logs which had no WTB emergence in pre-treatment sampling had beetle emergence at later sampling dates. This may have been a result of the aggregated nature of the galleries as previously described, such that galleries were missed in the initial sampling process but later sampled. Alternatively, it could indicate that logs initially free of WTB were later infested or reinfested and served as a breeding ground over an extended period. This is especially true in logs where WTB emergence was detected 16 to 21 months after pre-treatment sampling. Previous studies have shown that WTB adults emerge from logs, then re-enter the same log to

lay eggs, potentially resulting in enormous populations (Whitney Cranshaw, Colorado State University, personal communication, 2012).

In either case, these studies show that felled black walnut logs may harbor WTB for at least 21 months. This is longer than the period of time that elm logs remain a viable source for *Scolytus* spp populations. Here, logs dry out and cannot sustain a beetles after a year (Cranshaw 2009). However, sanitation efforts similar to those utilized for Dutch elm disease (i.e. chipping, burning, bark removal or removal of logs to landfills) could be effective in management of TCD (Anonymous, 1948; Haugen, 1998; Cranshaw, 2009).

None of the treatments tested were effective at consistently eliminating WTB from infested logs. Chemical applications of permethrin and biodiesel to kill existing WTB in the bark and prevent reinfestation were repeatedly unsuccessful. Walnut twig beetle emergence was detected in at least one permethrin-treated log 3 months after application in each of the experiments. Similarly, WTB was detected in biodiesel-treated logs after treatment. Biodiesel, although similar to petroleum diesel in molecule size, differs considerably in chemical structure. Biodiesel is almost entirely made up of fatty acid methyl esters (FAME) which are unsaturated “olefin” compounds, while petroleum diesel is mostly made of saturated hydrocarbons (Ciolkosz 2009). Additionally, biodiesel contains virtually no sulfur while petroleum diesel contains 15 ppm of sulfur (Anonymous, 2000). It is possible that petroleum diesel could provide better control for WTB in felled walnut logs and its effectiveness should be explored in subsequent studies.

In Wheat Ridge, no emergence was recorded in post-treatment samples of bifenthrin, but because of the initial low emergence percentage, and the low number of beetle emergence for the sample, no differences for this treatment were detected. This treatment should be tested again.

Plastic tarping has been used to solarize logs and disinfest them of bark beetles (Buffam and Lucht, 1968). However, this method typically reduces beetle numbers rather than eliminates them (Mitchel and Schmid, 1972; Negron et al. 2001). Regardless, this technique was ineffective for WTB; emergence was observed in post-treatment samples in all experiments. In fact, in two of the experiments there was an increased beetle emergence in post-treatment samples relative to pre-treatment samples. It is possible that an increased level of wood moisture (Figure 2.14) contributed to the logs ability to sustain WTB populations, but this statement is cautioned because of the limited accuracy of the moisture reader. Although, the average moisture readings of log samples for each treatment and sampling date were within the accuracy range of the moisture meter (6-45%).

Inner bark temperatures of at least 51° C for 30 minutes are needed for control of WTB (Peachey et al. 2012). While inner bark temperatures reached lethal boundaries for WTB in the top of the logs (63° C), inner bark temperatures exceeding 51° C were not attained on the bottom of the logs. Temperature differences between the top and bottom of logs are consistent with data obtained by Negron et al. (2001) and Mitchell and Schmid (1972). The weekly turning of logs during summer months could be an effective control of WTB and should be explored at a later time however, this method is quite labor intensive and may not be practical.

In summary, storage of felled black walnut logs with the bark intact could be a source of inoculum for healthy black walnuts and should be avoided. Neither plastic sheeting nor the chemical applications of biodiesel or permethrin were an effective means of disinfesting black walnut logs of WTBs. Solar treatments used in conjunction with log turning and chemical applications of petroleum diesel or bifenthrin deserve further study. Perhaps like Dutch elm disease, a combination of management strategies combining sanitation, control of insect vectors,

preventative fungicide applications and the use of resistant cultivars will be necessary for effective management of thousand cankers disease (Haugen, 1998; Cranshaw, 2009).

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CHAPTER III. The effect of temperature on survival of *Pityophthorus juglandis* Coleoptera: Curculionidae (Scolytinae)

INTRODUCTION

The walnut twig beetle (WTB), *Pityophthorus juglandis*, vectors *Geosmithia morbida*, the casual agent of thousand cankers disease (TDC) of black walnut (*Juglans nigra*) (Tisserat et al. 2009). Arizona walnut (*J. major*), is a native host of WTB, and is distributed in the southwestern United States where winter temperatures are higher than those found in much of the native range of black walnut (Figure 3.1 A-C)(Blackman 1928; Seybold et al. 2010). On its native hosts, WTB behaves like a typical twig beetle causing limited damage however; much more serious injuries result from infestations of non-native hosts, particularly black walnut. On black walnut, WTB is found feeding on larger diameter branches, and even the stem of the tree (Tisserat et al. 2009). The lifecycle of WTB is not fully understood but in Colorado, there are thought be two or more overlapping generations per year and adults are observed flying from April to October (Tisserat et al. 2009).

Damage to black walnut occurs first from repeated feeding by WTB followed by extensive canker formation at each beetle entry/exit hole. Initial symptoms of TCD-affected trees are yellow flagging in the upper canopy, followed by branch dieback and overall thinning of the crown, eventually leading to tree mortality (Kolarik et al. 2011; Tisserat et al. 2009). After noticeable symptoms appear, trees are usually killed within two years (Tisserat et al. 2009).

Temperature is a key factor in the lifecycle of subcortical insects, and its effects are noticed both behaviorally and physiologically (Graham 1924; Lombardero et al. 2000). Temperature extremes, both high and low, influence the population dynamics of many insects and can offer safe and effective approaches to implement into many pest management programs (Hallman and Denlinger 1998).

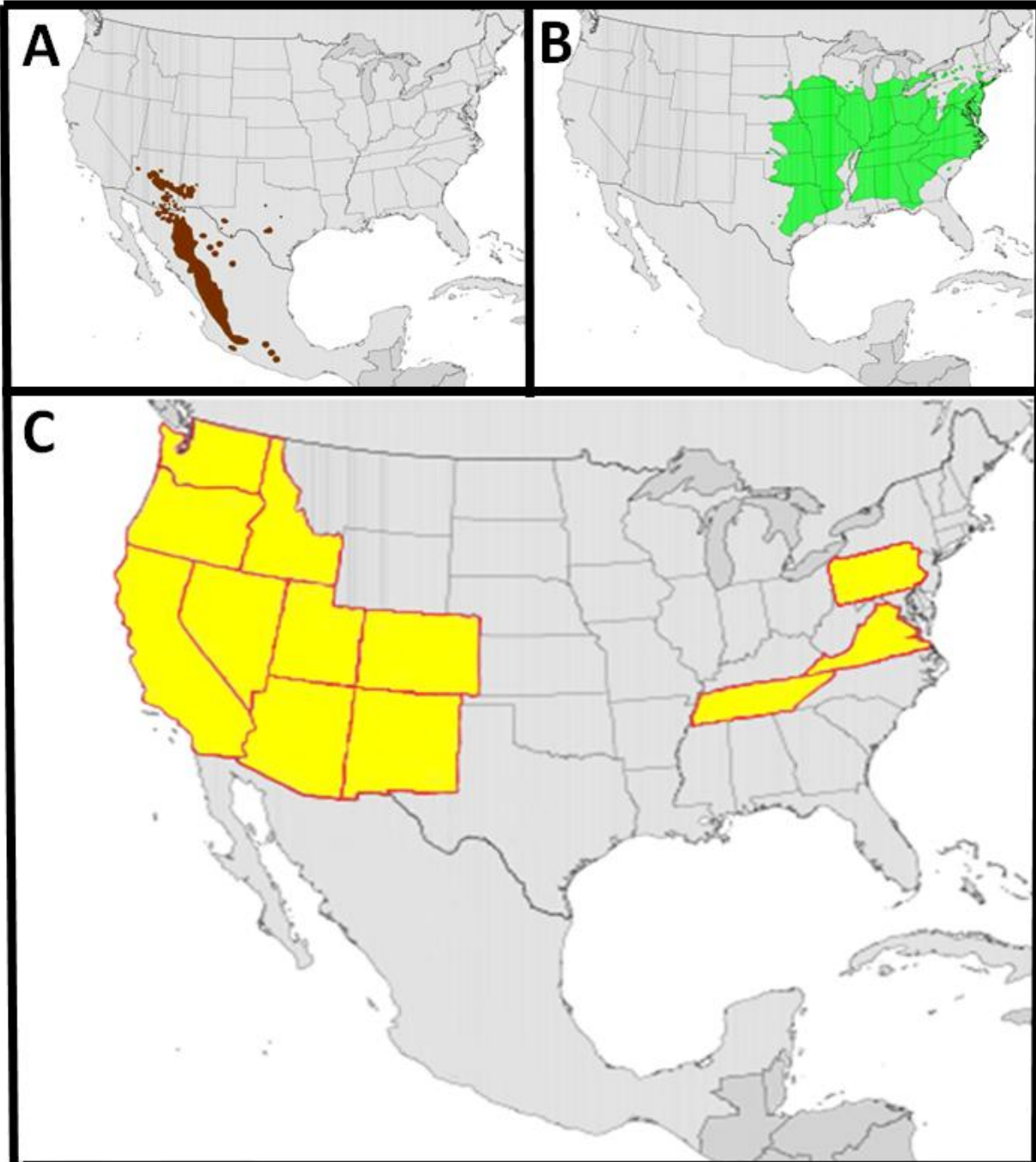


Figure 3.1. *Distribution maps.* (A) Native distribution of Arizona walnut. (B) Native distribution of black walnut. (C) States with confirmed cases of thousand cankers disease.

Insects exposed to low temperatures are threatened by ice formation (Denlinger and Lee 1998). A commonly accepted hypothesis on freezing separates insects into two categories: freeze-tolerant species or freeze-susceptible species (Salt 1961; Lee and Denlinger 1991; Bale

2002). Freeze-tolerant insects have adaptations that protect their cells from damage during the freezing process. On the contrary, freeze-susceptible insects can avoid freezing by overwintering in protected habitats, using cryoprotectant substances (such as glycerol) to lower the point of ice nucleation (Lee and Denlinger 1991) and clearing their gut content of food materials which could act as ice nucleators (Bale 2002). In freeze-susceptible insects, the supercooling point (SCP) is defined as the temperature at which spontaneous freezing occurs (Regniere and Bentz 2007). SCP is usually the absolute lower lethal temperature (LT) threshold (Lee 1991).

Insects exposed to high temperatures become susceptible to high rates of water loss resulting in mortality (Denlinger and Lee 1998). The use of high temperature treatments as a pest management technique has long been used as a safe alternative to pesticides and is an effective alternative in programs where pesticide resistance is an issue. Heat treatments to felled logs and lumber are used as an effective means of disinfestation and are mandated by several agencies for sanitation of many pest species. For example, the United States Department of Agriculture Animal (USDA), Animal and Plant Health Inspection Service (APHIS) requires that a specific heat treatment (in a heat treatment facility approved by APHIS) is applied to firewood or logs of ash (*Fraxinus* spp.) moving out of quarantined areas to stop the spread of the emerald ash borer (USDA, 2011)

Lower lethal limit temperatures for WTB are unknown but potentially lethal low temperatures may occur in native regions of black walnut and could affect the population dynamics of the beetle. Recent research to determine a heat treatment schedule for the sanitation of black walnut logs found that no WTBs survived in logs when core log temperatures reached 50.1° C for thirty minutes, but a core temperature 56 ° C for thirty minutes was recommended as the minimum temperature required to rid black walnut logs of WTB (Costanzo

2012). To better understand temperature effects on survival of WTB, we initiated studies to determine: (1) seasonal variations in cold tolerance, as measured by the supercooling point (SCP), and (2) upper and lower lethal temperatures.

MATERIALS AND METHODS

Experimental Overview

Walnut twig beetles were exposed to decreasingly cooler temperatures and SCPs were measured, or specimens were removed at predetermined subzero temperatures and checked for survival to establish lower lethal limits. In the lower limit study, *Xyleborinus saxeseni* was also tested for comparison. Walnut twig beetles were also exposed to increasingly higher temperatures, removed at specific temperatures, and checked for survival after 24 hours to establish upper lethal limits.

Supercooling Point Determination

Black walnut trees showing symptoms of TCD were felled in December 2011. Logs infested with WTB adults and larvae were stored outdoors in a shaded screened facility at Colorado State University. Adult and larval (Figure 3.2, A) WTBs were extracted from bark and stored individually in 0.2 mL plastic tubes at 25° C, for no longer than 24 hours before each monthly experiment began .

Beetles were attached between a 36-gauge copper-constantan thermocouple and an aluminum rod, using a thin layer of high vacuum grease (Dow Corning, Midland, MI), and then placed into an aluminum block (designed for temperature stabilization) (Figure 3.2, B & C). Beetles were subsequently placed into an environmental test chamber programmable freezer (Tenny Jr. Programmable Freezer, Tenny, Inc., South Brunswick, N.J.) at an initial temperature of 25° C. The chamber temperature was then lowered at a rate of -0.2° C per minute. Using a

multi-channel data logger (DaqPro-5300, Portable Handheld Data Logger, Omega Engineering, Inc., Stamford, CT.) attached to the thermocouples, temperatures were recorded every second. Data were graphed and SCP was determined by visualizing the lowest temperature reached before a sudden temperature increase greater than 0.5°C indicating the onset of freezing (Figure 3.3). The number of WTB adult and larvae tested in each month are presented in Tables 3.1 and 3.2, respectively.

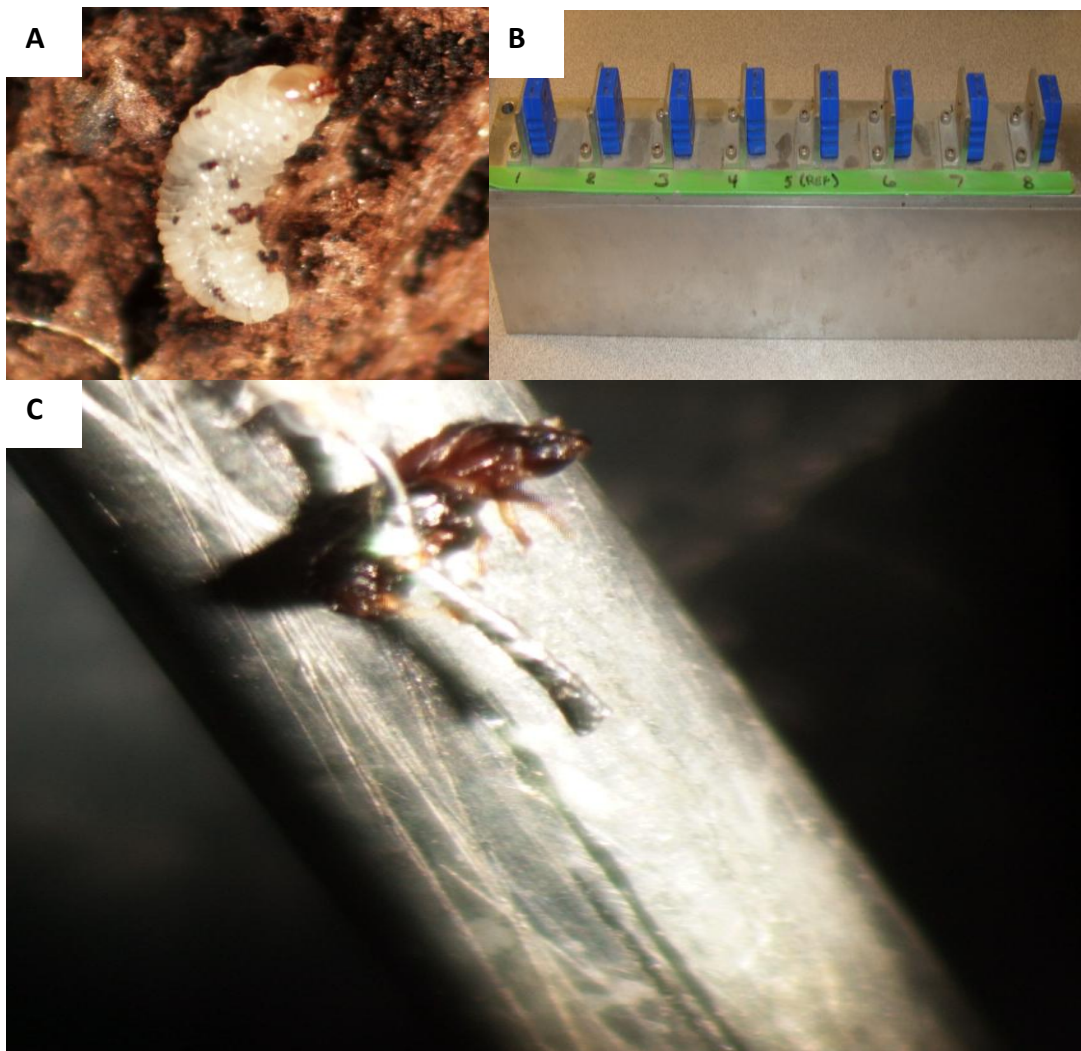


Figure 3.2. *Supercooling point (SCP) study.* (A) Walnut twig beetle (WTB) larva in a piece of bark. (B) Aluminum block used to stabilize temperatures in SCP study. (C) Adult WTB placed between a 36-gauged copper-constantan thermocouple and aluminum rod.

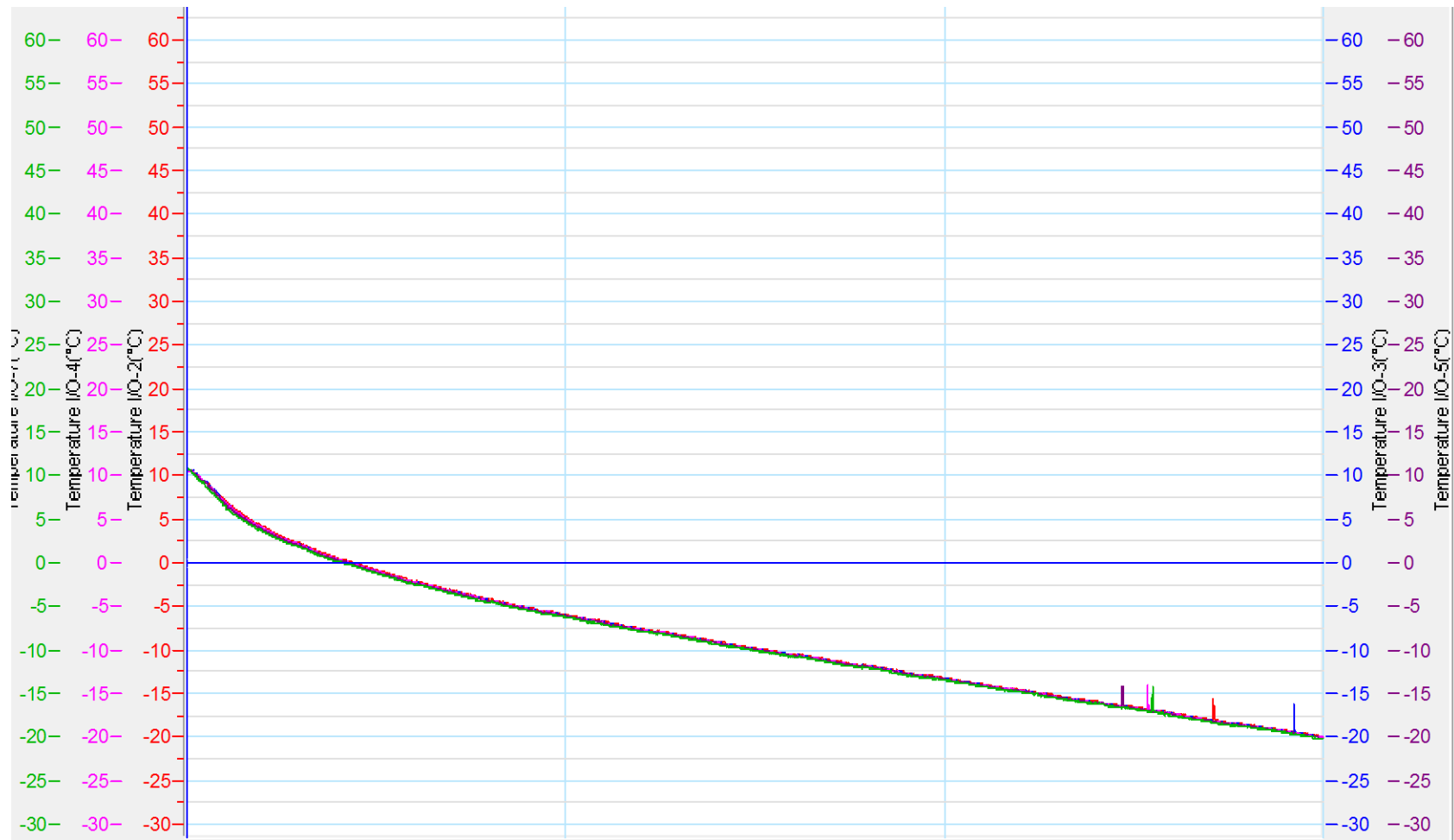


Figure 3.3. *Supercooling point (SCP) study.* Example of the sudden release of heat ($>0.5^{\circ}\text{C}$) as walnut twig beetles adults freeze. The supercooling point is defined as the lowest temperature reached before this temperature increase. Each line in the graphic represents a single walnut twig beetle adult.

Table 3.1. *Supercooling point (SCP) study.* Means (\pm SE) and ranges of SCP for walnut twig beetle adults each month.

Month	n^a	Mean SCP ($^{\circ}$ C) \pm SE	Range ($^{\circ}$ C)
January	9	-16.94 \pm 0.32	-15.6, -18.7
February	18	-18.1 \pm 0.3	-16.6, -21.4
March	20	-17.06 \pm 0.28	-14.9, -19.6
April	17	-16.01 \pm 0.1	-15.4, -16.8
May	15	-17.75 \pm 0.42	-15.5, -22.3
June	16	-17.95 \pm 0.6	-13, -21.8

^aNumber of adult beetles tested in each month.

Table 3.2. *Supercooling point (SCP) study.* Mean (\pm SE) and range of SCP for walnut twig beetle larvae in each month.

2012 Month	n^a	Mean SCP ($^{\circ}$ C) \pm SE	Range ($^{\circ}$ C)
January	11	-16.81 \pm 0.26	-15.1, -17.6
February	15	-17.84 \pm 0.2	-16.6, -19.5
March	13	-17.08 \pm 0.34	-15.6, -20.5
April	N/A	N/A	N/A
May	16	-17.16 \pm 0.35	-15.5, -21.4
June	12	-16.49 \pm 0.28	-14.9, -18.2

^aNumber of larvae tested in each month.

Lower Lethal Temperature Experiment

Black walnut trees infested with WTB were felled between September and November 2010 and stored outdoors. In April 2011, adult and larval WTBs were collected as described for the SCP study, and held for no more than 48 hours at 4 $^{\circ}$ C until an experiment began. In addition to WTBs, *X. saxeseni* adults were tested as a comparison species. Emerged *X. saxeseni* adults were collected and stored as described for WTBs. All insects were kept individually in 0.2 mL plastic tubes for the duration of the experiment. The insects were checked for survival, placed into the programmable freezer and held at 0 $^{\circ}$ C for 12 hours. The temperature in the chamber was then lowered at a rate of - 4 $^{\circ}$ C per hour to -25 $^{\circ}$ C.

Seventeen to 36 tubes (depending how many specimens were available at the time of the experiment) were removed from the freezer at the following temperatures as measured by the freezer: -5, -10, -13, -15, -16, -19, -20, and -23° C. After removal, each insect was left at 25° C for 30 minutes and checked for survival based on movement after provoking with a fine bristled paintbrush. The percentage mortality at each temperature was recorded. The experiment was repeated four times.

Upper Lethal Temperature Experiment

In January 2012, WTB adults and larvae were collected using the same techniques described for SCP study. Eight 0.2 mL tubes (each containing 4 beetles) were placed in each row of a thermal cycler (Biorad, My Cycler, Hercules, CA). Four tubes contained adult beetles, and 4 contained larvae; for a total of 16 beetles per row. A temperature gradient option was selected on the thermal cycler. Beetles exposed to temperatures ranging from 37° C to 51° C increasing at a rate of 0.2° C per minute. Beetles were removed and 30 minutes and then transferred to 25° C for 24 hours and checked for survival based on movement as described for lower temperature study.

Statistics

For SCP study, mean monthly SCP differences were detected using the wilcoxon method to make multiple pairwise comparisons and account for non-normality and unequal variances (SAS, JMP). For upper and lower LT determination studies, data were fitted using a logistical regression (PROC LOGISTIC; SAS Institute 2010) and max-rescaled R-square values were used. In the upper LT determination study, an offset was used (C=0.2112) for the logistic regression of the WTB larvae to account for consistent background mortality.

RESULTS

Supercooling Point Determination

Monthly mean SCPs of adult WTBs ranged between $-16.1\text{ }^{\circ}\text{C}$ in April to $-18.1\text{ }^{\circ}\text{C}$ in February (Figure 3.4; Table 3.1). Larval monthly mean SCPs ranged between $-16.5\text{ }^{\circ}\text{C}$ in June to $-17.8\text{ }^{\circ}\text{C}$ in January (Figure 3.5, Table 3.2).

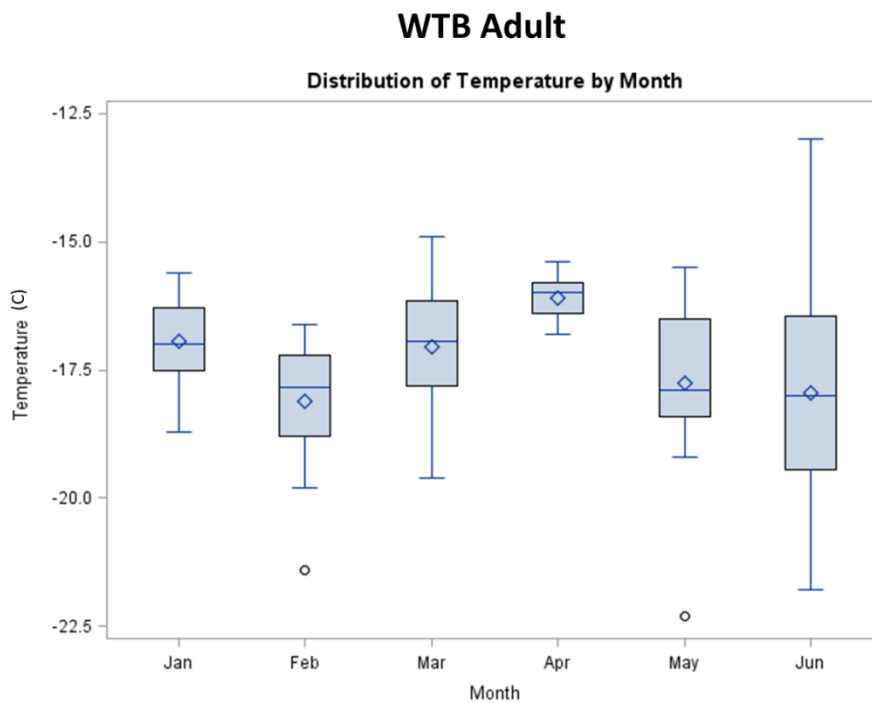


Figure 3.4. *Supercooling point (SCP) study.* Seasonal variation of SCP of walnut twig beetle (WTB) adults from January through June 2012. The central horizontal line in each box corresponds to the sample median and the diamond corresponds to the mean. Minimum and maximum observations are indicated by horizontal line at either end of the whiskers, the length of the box represents the interquartile range (between 25% and 75%). Circles represent outliers.

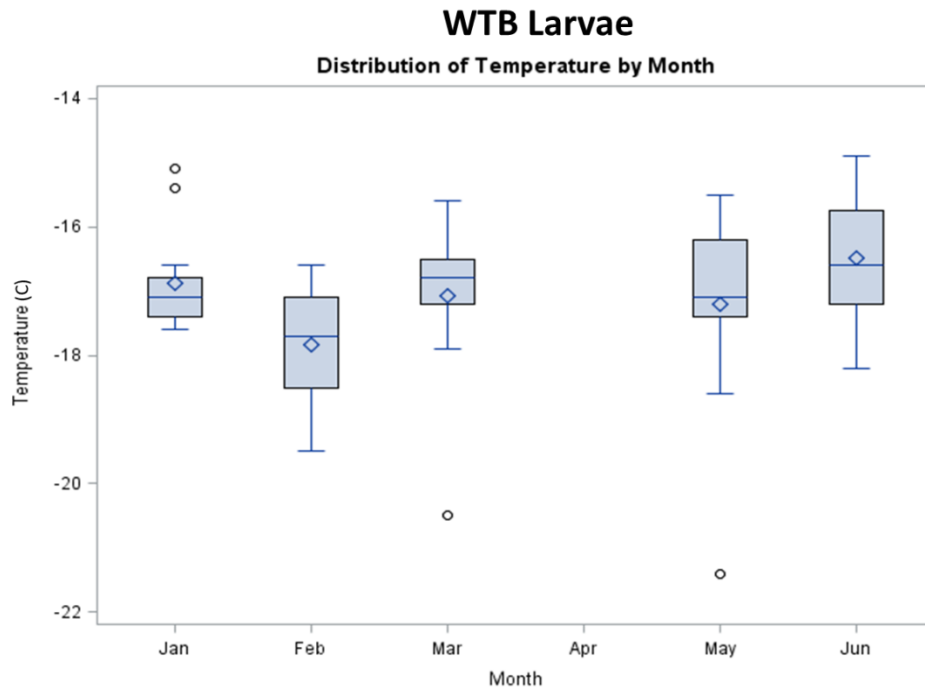


Figure 3.5. *Supercooling point (SCP) study.* Seasonal variation in SCP points of walnut twig beetle (WTB) larvae from January through June 2012. The central horizontal line in each box corresponds to the sample median and the diamond corresponds to the mean. Minimum and maximum observations are indicated by horizontal line at either end of the whiskers, the length of the box represents the interquartile range (between 25% and 75%). Circles represent outliers.

Several differences in monthly mean SCPs were observed. For adults, the monthly mean SCP in April differed from each month tested, and the mean SCP in February was different from the mean SCP in January and March. Mean SCP increased ≈ 2 °C between February and April ($P < 0.0001$), and increased ≈ 1 °C between March and April ($P = .0063$). Adult monthly mean SCP differences detected in February were marginally lower at a difference of ≈ 1 °C ($P = 0.0219$) and 1.16 °C ($P = 0.0165$) than January and March respectively.

For larvae, monthly mean SCPs differed in February from all other months tested. Here mean SCPs increased ≈ 0.7 °C ($P = 0.0195$) in May to 1.35 °C ($P = 0.0014$) in June. No other differences in monthly mean SCPs were detected for larvae. Larval specimens were not available for the month of April therefore; no data is shown for that month.

Lower Lethal Temperature Experiment

Mortality at -15°C and -23°C was 19 and 100% respectively for WTB adults and 33 and 100% respectively for WTB larvae. *Xyleborinus saxeseni* was more cold tolerant, with 13 and 58% mortality at the respective temperatures (Figure 3.6). Lower median lethal temperature (LT₅₀) for WTB adults and larvae was -16.7 °C and -16.9 °C respectively; while LT₉₉ for adults and larvae was -22.97°C and -25.19 °C respectively (Figure 3.6;Table 3.3). For *X. saxeseni*, LT₅₀ was -24.7 °C, and LT₉₉ was not estimated because 100% mortality was not achieved during the study (Figure 3.6; Table 3.3). Logistic regression for adult WTBs produced the following equation with a max $R^2=0.9174$ (n=172):

$$y = \text{EXP}(-12.2617+0.7339(X)) / (1+\text{EXP}(-12.2617+0.7339(X)))$$

Logistic regression for larval WTBs is as follows with a max $R^2=0.9158$ (n=380):

$$y = \text{EXP}(-9.3944+0.5554(X)) / (1+\text{EXP}(-9.3944+0.5554(X)))$$

and logistic regression for *X. saxeseni* with max $R^2= 0.6856$ (n=172):

$$y = \text{EXP}(-5.56558+ -0.2294(X)) / (1+\text{EXP}(-5.6558+-0.2294(X))).$$

Upper Lethal Temperature Experiment

Mortality at 46.2°C and 50.2°C was observed at 5 and 100% respectively for WTB adults and observed at 26 and 100% respectively for WTB larvae (Figure 3.7). Upper LT₅₀ for WTB adults and larvae was 48 °C and 47°C; while LT₉₉ for WTB adults and larvae was 53 °C and 48 °C respectively (Figure 3.7;Table 3.3) Logistic regression for adult WTBs produced the following equation with a max $R^2=0.8277$ (n=432):

$$y = \text{EXP}(-45.8012+0.9557(X)) / (1+\text{EXP}(-45.8012+0.9557(X)))$$

To account for background mortality at low temperatures in the larval WTB logistic regression an offset was used (C=.2112). Logistic regression is as follows with a max $R^2=0.58268$

(n=426):

$$y = C + ((1 - C) * \text{EXP}(-290.973 + 6.1476(X)) / (1 + \text{EXP}(-290.973 + 6.1476(X))))$$

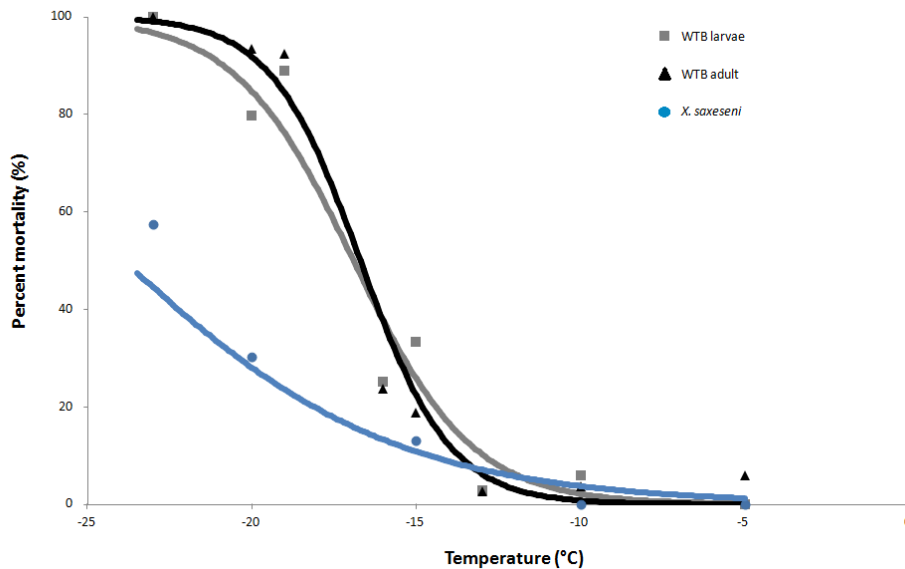


Figure 3.6 Lower lethal temperature (LT) determination. Percent mortality of walnut twig beetle (WTB) adults (▲), larvae (■), and *X. saxeseni* (●) when exposed to decreasingly lower temperatures. Logistic regressions for WTB adults ($y = \text{EXP}(-12.2617 + 0.7339(X)) / (1 + \text{EXP}(-12.2617 + 0.7339(X)))$; —; $R^2=0.9174$) and larvae ($y = \text{EXP}(-9.3944 + 0.5554(X)) / (1 + \text{EXP}(-9.3944 + 0.5554(X)))$; —; $R^2=0.9158$) and adult *X. saxeseni* ($y = \text{EXP}(-5.56558 + 0.2294(X)) / (1 + \text{EXP}(-5.56558 + 0.2294(X)))$; —; $R^2=.6856$) are displayed.

Table 3.3. Upper and lower lethal temperature (LT) estimates for walnut twig beetle adults and larvae, and lower LT estimates for *X. saxeseni* adults.

Species/Stage	Upper and Lower Mortality Temperature Estimates			
	Upper LT ₅₀	Upper LT ₉₉	Lower LT ₅₀	Lower LT ₉₉
	°C	°C	°C	°C
WTB adults	47.93	52.73	-16.71	-22.97
WTB larvae	47.33	48.08	-16.91	-25.19
<i>X. saxeseni</i> adults	N/A	N/A	-24.65	N/A

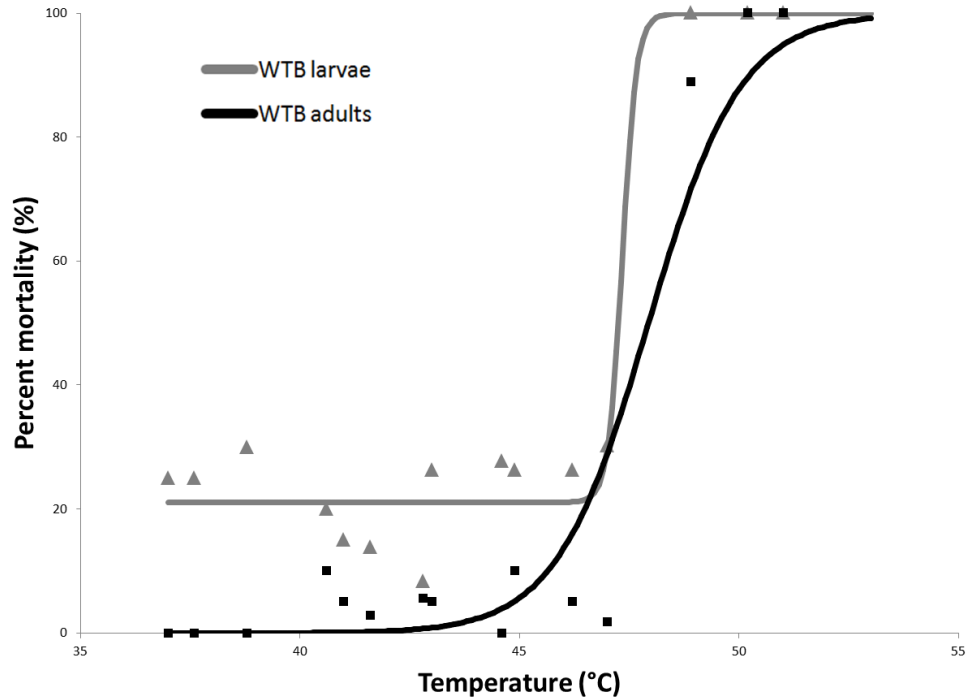


Figure 3.7 Upper lethal temperature (*LT*) determination. Percent mortality of walnut twig beetle (WTB) adults (■) and larvae (▲) when exposed to increasingly higher temperatures and held at final high temperature for 30 minutes. Logistic regressions for WTB adults ($y = \text{EXP}(-45.8012+0.9557(X)) / (1+\text{EXP}(-45.8012+0.9557(X)))$); —; $R^2=0.8277$) and larvae ($y = C+((1-C)*\text{EXP}(-290.973+6.1476(X)) / (1+\text{EXP}(-290.973+6.1476(X))))$); —; $R^2=0.58268$) are displayed. For larvae, an offset (SAS: PROC PROBIT with OPTC option) was used ($C=.2112$) to account for background mortality at lower tempera

DISCUSSION

Based on my results, it appears that the lower *LT* is consistent with the SCPs for WTB adults or larvae. This suggests that the SCP is a reliable measure of cold tolerance for this insect. Adult and larval monthly mean SCPs were between -16°C and -18.1°C while the lower LT_{50} was -16.7°C and -16.9°C for adults and larvae. Variation could be due to differences in methods between the two studies or beetles could have acclimated in the lower *LT* experiment, insects were held at 0°C for 12 hours before exposure to subzero temperatures while in the SCP the temperature decreased at a rate of -4°C per hour consistently from 25°C temperature to -25°C . On another note, the plastic vials used in the lower lethal determination study could have

provided the beetles with protection. This experiment should be repeated using the same methods as described in the SCP study.

After SCPs were reached, complete mortality of WTBs was observed suggesting that WTBs are freeze-intolerant. This is consistent with other bark beetles in the subfamily Scolytinae (Lombardero et al 2000; Bentz and Mullins 1999). Further research is needed gathering SCP data during winter months in order to determine whether or not WTBs can adjust their SCPs in response to seasonal changes. Although moderate changes in SCPs in response to seasonal changes were detected in this study, the magnitude of these differences was not as extreme as noted in other beetle species (Lombardero et al 2000). In future experiments, survival at temperatures just above the SCP should be tested in order to better understand WTB survival at subzero temperatures. Variation of SCPs within a given month for WTBs could be a result of gut content. Studies by Worland & Convey (2001) found patterns SCPs in Antarctic microarthropods were divided into two groups, high temperature groups (in the summer) and low temperature groups (in the winter). This seasonal dichotomy of SCPs was attributed to food particles acting as ice nucleators which are present in the gut during summer months but not in winter months when the insect is in the overwintering “starved” state. Likewise, cold tolerance has been modeled based on three states in which the individual beetles pass through; one of them being an intermediate state where ice-nucleating agents have been cleared from their gut (Regniere and Bentz, 2007). Here SCP variation is also observed. Future studies testing cold tolerance of WTB should starve the beetles to insure food material is not occupying the gut and acting as ice nucleators. Additionally, in some species, sexual dimorphism could result in SCP differences (Renault et al. 2002). Further studies should investigate whether differences exist between SCPs in male and female WTBs.

Seasonal variations of SCP for WTB adults and larvae, from January 2012 to May 2012, were between -16° C and -18° C. The lower lethal temperature in April 2011 was between -18° C and -19° C. Walnut twig beetle survival may be limited by extreme cold events in the northern native range of black walnut, where temperatures of -18° C are not uncommon. However, along the Front Range in Colorado, winter temperatures in 2010 reached -25° C and WTBs were still found living in the bark of the trunk in the spring of 2011 suggesting the bark plays an important role in providing protection to the insects.

Developing an effective heat treatment program for WTB logs requires 100% mortality of WTB. In this study, critical temperatures for WTB adult and larval mortality were established. Complete mortality was observed when WTBs were exposed to temperatures at or exceeding 50.2°C and survival estimations given by the logistic regression yielded LT₉₉ temperature estimates of 53°C and 48°C for adults and larvae respectively.

Undoubtedly, black walnut bark adds some protection from the elements to developing WTBs. The temperature estimates given based on the logistic regressions do not account for this protection however, the survival data is consistent with data gathered by Costanzo (2012) where no survivors were seen when sections of logs were exposed to 50.1°C. This study is in agreement with the work done by Costanzo, that logs should be heated to a minimum of 56°C to achieve complete sanitation of the log with the bark attached. Additional studies should look at rates of heating as they can have an impact on insect mortality (Kells et al. 2011). Further studies should also look at whether or not reinfestation can occur after logs have been heat treated.

In closing, understanding the effects of temperature on the survival of WTB can be a key factor in understanding its population dynamics and can also help to develop effective pest

management techniques. Our results provide a baseline of upper and lower lethal temperatures as well as begin to describe the overwintering biology of WTB.

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