

The life history and control of *Pityophthorus juglandis*
Blackman on *Juglans nigra* L. in eastern Tennessee

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DEDICATION

To

Jeremy Wayne Nix

my husband

thank you for all your support during this process, I could not have done it without you

and

Fredrick McClure Avery

and

Carol Barron Avery

my parents

who have always believed in me and gave me the confidence in myself to overcome all
obstacles

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ABSTRACT

In the last decade, western states have experienced an increasing mortality rate in *Juglans nigra* L., black walnut, as a result of the fungal species *Geosmithia morbida* Kolařík et al. that results in numerous cankers that girdle the branches, resulting in dieback and tree mortality. The only known vector of *G. morbida* is the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman. This newly recognized disease/insect complex has been named Thousand Cankers Disease (TCD) due to the quantity of cankers produced by *G. morbida*.

Recently, TCD was discovered in the eastern U.S. To limit the spread of TCD in eastern Tennessee, a better understanding of the life histories of *G. morbida* and *P. juglandis* is imperative.

The primary objective of this study was to determine the life history of WTB in eastern Tennessee using field-infested black walnuts bolts. From this data, it was determined that WTB has at least three larval instars. Gallery structures of the different WTB life stages were determined. Secondly, a survey was initiated to identify native predators and parasitoids for use as potential biological control agents of WTB. The consumption rates of WTB by the collected the predators were recorded and a potential listing of native predators was developed. From this survey, three clerid species were observed to feed on WTB. Nine additional coleopteran species and two parasitoid species were found in association with WTB infested logs. Concentration levels and translocation of two systemic insecticides (imidacloprid and dinotefuran) were studied in mature *J. nigra* and tissue types affected were identified. Concentrations of all the

chemicals were determined using high pressure liquid chromatography (HPLC). Imidacloprid concentrations were detected in all tissue types tested including nutmeat. Dinotefuran was only detected in trace amounts in the first sampling period.

TABLE OF CONTENTS

CHAPTER I.....	1
Introduction and Literature Review.....	1
<i>Juglans nigra</i>	1
Pests of <i>Juglans nigra</i>	4
Thousand Cankers Disease.....	5
<i>Pityophthorus juglandis</i>	7
Morphological Characteristics.....	8
<i>Pityophthorus laetus</i>	8
<i>Geosmithia morbida</i>	9
Impact of Thousand Cankers Disease on <i>Juglans nigra</i>	11
Chemical Control of Bark Beetles.....	13
Imidacloprid and Dinotefuran.....	13
Research Objectives.....	15
CHAPTER II.....	16
The Behavior and Development of the Walnut Twig Beetle in eastern Tennessee.....	16
Abstract.....	16
Introduction.....	17
Materials and Methods.....	20
Results and Discussion.....	24
Conclusions.....	41
CHAPTER III.....	44

Survey of Natural Enemies of Walnut Twig Beetle in Eastern Tennessee.....	44
Abstract.....	44
Introduction.....	44
Materials and Methods.....	46
Results and Discussion.....	49
Conclusions.....	61
CHAPTER IV.....	62
Concentration Levels of Imidacloprid and Dinotefuran in <i>Juglans nigra</i>	62
Abstract.....	62
Introduction.....	62
Materials and Methods.....	65
Results and Discussion.....	70
Conclusions.....	79
Overall Conclusions.....	80
List of References.....	83
Vita.....	93

LIST OF TABLES

Table 1. Population density of walnut twig beetles in infested black walnut.....	27
Table 2. Average length, width and range of walnut twig beetle pupae and adult Galleries	40
Table 3. Potential predators and parasitoids of walnut twig beetle collected in Knox County, TN	50
Table 4. Imidacloprid concentrations (ppb) $\pm$ SE, determined using HPLC/MS/MS for tree tissue of <i>Juglans nigra</i> L.....	71
Table 5. Dinotefuran concentrations (ppb) $\pm$ SE, determined using HPLC/MS/MS for tree tissue from <i>Juglans nigra</i> L	74

LIST OF FIGURES

Figure 1. Native range of <i>Juglans nigra</i> in the eastern U.S. (Williams 1990)	2
Figure 2 (a-c). (a) <i>Geosmithia morbida</i> conidia and conidiphores; (b) <i>Geosmithia morbida</i> conidial chains; (c) <i>Geosmithia morbida</i> cultures	10
Figure 3. Walnut twig beetle emergence numbers from black walnut samples held in the laboratory from September 2010-May 2011.....	26
Figure 4. Mean $\pm$ SE (n=6) of walnut twig beetle larvae determined monthly over two years	29
Figure 5. Mean $\pm$ SE (n=6) of walnut twig beetle pupae determined monthly over two years	30
Figure 6. Mean $\pm$ SE (n=6) of walnut twig beetle adults determined monthly over two years	31
Figure 7. Clerid larva present in walnut twig beetle larval galleries	32
Figure 8 (a-d). (a) Parasitoid pupae present in walnut twig beetle larval galleries; (b) Lateral view of parasitoid pupa; (c) Ventral view of parasitoid pupa	32
Figure 9. Walnut twig beetle larvae body dimensions.....	34
Figure 10 (a-b). (a) Female gallery with mycelia of <i>Geosmithia morbida</i> ; (b) Close up of the same female gallery	35
Figure 11 (a-d). (a) Entrance/Exit hole of WTB; (b) Female walnut twig beetle gallery with frass caps present; (c) Newly laid egg in female gallery covered with frass cap and WTB female to right; (d) Female gallery with mycelia, including conidia, and larval galleries perpendicular to the main female tunnel	35

Figure 12 (a-d). (a) Close up of walnut twig beetle egg; (b) Walnut twig beetle larva; (c) Walnut twig beetle pupae with mycelia present in chamber; (d) Walnut twig beetle teneral adult with mycelia of <i>Geosmithia morbida</i> present in chamber.....	36
Figure 13. Frass caps in female walnut twig beetle gallery	38
Figure 14. Walnut twig beetle larvae gallery length and width.....	40
Figure 15. Emergence numbers of walnut twig beetle in traps from late September 2012 to mid-March 2013	42
Figure 16 (a-d). (a) Dorsal view of <i>Enoclerus ichneumoneus</i> ; (b) Dorsal view of <i>Madoniella dislocatus</i> ; (c) Dorsal view of <i>Pyticeroidea laticornis</i> ; (d) Dorsal view of <i>Enoclerus nigripes</i>	51
Figure 17 (a-c). (a) Dorsal view of <i>Leptophloeus angustulus</i> ; (b) Dorsal view of <i>Narthecius grandiceps</i> ; (c) Dorsal view of <i>Dysmerus basalis</i>	53
Figure 18 (a-d). (a) Dorsal view of <i>Aeletes floridiae</i> ; (b) Dorsal view of <i>Tenebroides marginatus</i> ; (c) Dorsal view of <i>Bitoma quadriguttata</i> ; (d) Dorsal view of <i>Colydium lineola</i>	53
Figure 19 (a-c). <i>Theocolax</i> species found emerging from <i>Juglans nigra</i> bolts: (a-b) Lateral view of <i>Theocolax</i> sp.; (c) Ventral view of <i>Theocolax</i> sp	54
Figure 20. Mean $\pm$ SE live and dead specimens consumed by <i>Madoniella dislocatus</i> (MD) and <i>Pyticeroidea laticornis</i> (PL).....	56
Figure 21. (a) Mean $\pm$ SE time between attacks (seconds) by <i>Madoniella dislocatus</i> (MD) and <i>Pyticeroidea laticornis</i> (PL); (b) Mean $\pm$ SE predator response time by <i>Madoniella dislocatus</i> (MD) and <i>Pyticeroidea laticornis</i> (PL); (c) Mean $\pm$ SE total feeding time (seconds) by <i>Madoniella dislocatus</i> (MD) and <i>Pyticeroidea</i>	

<i>laticornis</i> (PL); (d) Mean $\pm$ SE total preening time (seconds) by <i>Madoniella</i> <i>dislocatus</i> (MD) and <i>Pyticeroidea laticornis</i> (PL).....	57
Figure 22. (a) Mean $\pm$ SE <i>Pityophthorus juglandis</i> (WTB) and <i>Tribolium confusum</i> (TC) specimens attack by <i>Madoniella dislocatus</i> (MD); (b) Mean $\pm$ SE <i>Pityophthorus</i> <i>juglandis</i> (WTB) and <i>Xylosandrus crassiusculus</i> (XC) specimens by <i>Madoniella</i> <i>dislocatus</i> (MD); (c) Mean $\pm$ SE <i>Pityophthorus juglandis</i> (WTB) and <i>Tribolium</i> <i>confusum</i> (TC) specimens attack by <i>Pyticeroidea laticornis</i> (PL); (d) Mean $\pm$ SE <i>Pityophthorus juglandis</i> (WTB) and <i>Xylosandrus crassiusculus</i> (XC) specimens attack by <i>Pyticeroidea laticornis</i> (PL)	59
Figure 23(a-c). (a) CoreTect (imidacloprid) soil pellets; (b) Hole dug around the base of a black walnut tree in preparation for one soil pellet; (c) CoreTect pellet being placed into a hole at the base of a black walnut tree.....	67
Figure 24 (a-b). (a) Measuring the DBH of a black walnut tree; (b) Spraying Safari (dinotefuran) on trunks of black walnuts with a hand sprayer.....	67
Figure 25 (a-c). (a) Cutting black walnut leaf tissue with scalpel; (b) Placing nut tissue into 10-dram glass vial; (c) 10-dram glass vials ready to be processed through HPLC/MS/MS machine	69
Figure 26. Mean $\pm$ SE imidacloprid concentration (ppb) for walnuts and walnut husk maggot in each tree stratum	73
Figure 27. Mean $\pm$ SE olefin concentration (ppb) for each stratum and tissue type.....	77
Figure 28. Mean $\pm$ SE walnut husk maggot abundance (mean number of individuals/walnut within walnut husk for each stratum	78

CHAPTER I INTRODUCTION AND LITERATURE REVIEW

Juglans nigra

Black walnut, *Juglans nigra* L., (Family Juglandaceae) is also known as American walnut and eastern black walnut (Williams 1990). Black walnut is native to the eastern part of North America, where it occurs in forest coves and well-drained river bottoms (Williams 1990, Tisserat et al. 2009). In the U.S., its distribution ranges from New England to the Florida panhandle and from the east coast west to the far side of the Great Plains (Williams 1990, Tisserat et al. 2009) (Figure 1). The seven *Juglans* species native to the U.S. are: *J. cinerea* L., *J. hindsii* (Jeps), *J. californica* S. Watson, *J. microcarpa* Berl., and *J. major* Torr., and *J. nigra*. Of these, only *J. nigra* and *J. cinerea* are valuable from a lumber standpoint (Hardin et al. 2001).

Black walnuts are deciduous trees with pinnately compound, alternate leaves which are yellow-green with a lanceolate shape and 30-61cm long. From 9-23 are usually leaflets attached to the rachis composing a leaf. The leaves turn yellow in the fall and quickly drop (Hardin et al. 2001). Their growing period only lasts around 115-135 days (Brinkman 1965). Twigs have chambered piths and the bark is dark brown sometimes described as chocolate colored. Black walnuts have rough bark with deep furrows (Hardin et al. 2001). Black walnuts are monoecious and unlikely to self-pollinate due a difference in timing of production of female and male flowers (Beineke 1974). In the northern most part of the range for black walnut, flowers appear in June whereas, in the southern part of their range flowers appear in mid-April (Williams 1990). Nuts usually form in clusters of 2 to 3, are 4 to 8 cm diameter and have a thick yellow-green husk (Hardin et al. 2001). Nuts are dispersed primarily by squirrels and other rodents as a consequence of their use as food (Hardin et al. 2001).



Figure 1. Native range of *Juglans nigra* in the eastern U.S. (Williams 1990).

Black walnuts are frequently found as individual trees scattered throughout the forest (Eyre 1980). Black walnuts are intolerant of shade (Baker 1948, Hardin et al. 2001). In the forest, black walnut trees form a tall, straight bole with branches near the top of the tree (Hardin et al. 2001). A taproot is present early in the life of a black walnut, but the roots spread laterally with time (Hardin et al. 2001). Tree species likely to be associated with black walnut include, but are not limited to: yellow-poplar, *Liriodendron tulipifera* L., white ash, *Fraxinus americana* L., black cherry, *Prunus serotina* Ehrh., basswood, *Tilia Americana* L., beech, *Fagus grandifolia* Ehrh., sugar maple, *Acer saccharum* Marshall, oaks, *Quercus* spp., and hickories, *Carya* spp. (Brinkman 1965, Hardin et al. 2001). In addition, *J. nigra* and *J. cinerea* can be found in the same stands, but do not cross with each other (Williams 1990, Hardin et al. 2001). Black walnuts produce juglone, an allelopathic compound, in their leaves, bark, nut husks, and roots (MacDaniels and Pinnow 1976, Rietveld 1979). Juglone is toxic to many plants, including white pine, *Pinus strobus* L., apple trees, *Malus* spp., and tomatoes, *Solanum* spp. (MacDaniels and Pinnow 1976, Rietveld 1979), and will prevent them from growing around black walnuts. Black walnuts trees grow best on neutral soils such as alfisols and entisols and do well in sandy loam, loam, or silt loam textured soils due to water retention in the soils so that the trees can use retained moisture during periods of drought (Losche 1973). Mature trees can be 31 to 37 m in tall and 76 to 102 cm diameter at breast height (DBH) (Williams 1990, Landt and Phares 1973). These trees are resilient and will re-sprout from the stump after being destroyed by fire or cut down (Williams 1990).

Due to black walnut's straight-grained, and dark colored wood, lumber of black walnut is valued gunstocks, furniture, cabinetry, and other finished wood products (Williams 1990, Hardin

et al. 2001). The estimated value of standing black walnut timber is more than half a trillion dollars (Hardin et al. 2001, Newton et al. 2009).

Veneer is the primary use of black walnut and logs suitable for veneer are highly prized (Das et al. 2001, Nicodemus et al. 2008). Nuts from mature trees are often sold for use in baked goods or ice cream, and are a food source for both humans and wildlife (Williams 1990, Hardin et al. 2001). Squirrels feed on both ripe and non-ripe nuts (Weber et al. 1992). In addition, ground nut shells are used as jet engine cleaner, filler in dynamite, nonslip agent in automobile tires, and as a flour-like carrying agent in various insecticides (Williams 1990).

On average, 10,287 metric tons of hulled nuts and 907.2 metric tons of black walnut kernels are purchased annually, with average yearly demand increasing annually (Warmund et al. 2008). Nut crops vary in quantity with large nut crops every 2 to 3 years (Hardin et al. 2001). The nuts from producing trees ripen in the fall between September and October. Black walnut is widely planted outside of its native range in the western U.S. for timber and as an ornamental (Tisserat et al. 2009).

Pests of *Juglans nigra*

Approximately 300 insect species are associated with black walnut (Weber et al. 1992) most of which do not significantly damage the host tree and are not considered to be pests. Pest species include defoliators, such as the walnut caterpillar, *Datana integerrima* Grote and Robinson, and the fall webworm, *Hyphantria cunea* (Drury); boring insects such ambrosia beetle, *Xylosandrus germanus* (Blandford), flatheaded apple tree borer, *Chrysobothris femorata* (Olivier), larvae which feed in the phloem, walnut curculio, *Conotrachelus retentus* (Say), which harms young nuts, and walnut shoot moth, *Acrobasis demotella* Grote (Weber et al. 1992). The walnut husk maggot, *Rhagoletis suavis* Cresson, is considered a minor pest of the black walnut

in the eastern U.S. (Brooks 1921). Both *R. suavis* and the walnut husk fly, *Rhagoletis completa* Cresson, are found throughout the central U.S. The larvae feed and burrow into the walnut husk of almost mature nuts (Weber et al. 1992). The larvae feeding cause the husks to become slimy and stick to the shell (Weber et al. 1992).

In addition, fungal diseases and pathogens can occur on limbs, trunks, leaves, and roots of black walnut (Weber et al. 1992). All of these pests are considered to be of minor economic importance whereas the newly described disease/insect complex, Thousand Cankers Disease (TCD), can destroy entire stands of black walnut trees (Tisserat et al. 2009). TCD is similar to the relationship between *X. germanus* and *Fusarium* spp. *X. germanus* introduces conidia of *Fusarium* spp. into the trees as the female tunnels into the trees. Elongated cankers form on the main stem that appears as a dark stain beneath the bark. Entrance/exit holes from *X. germanus* are often visible and have a canker surrounding the area. Infections by *Fusarium* spp. produce cankers which cause symptoms such as dieback and resprouting (Weber et al. 1992). Affected trees should be pruned or removed with the debris burned (Weber et al. 1992). Although infections by *Fusarium* spp. have some similarities to infections by *Geosmithia morbida* Kolařík et al., it differs in that it usually affects younger trees, whereas infections by *G. morbida* are most often found in mature black walnuts.

Thousand Cankers Disease

In the last decade, the western states have experienced increasing mortality in *J. nigra* (Leslie et al. 2010, Seybold et al. 2010b). This massive decline was first noticed in 2001-2003 by arborists and foresters in Boulder and Colorado Counties, Colorado (Tisserat et al. 2009). By fall of 2008, 700 black walnuts had died and were removed from the Boulder area alone, representing the majority of the black walnuts in that area (Tisserat et al. 2009). Tree deaths

were first attributed to drought stress; however, research later revealed that an unknown and undescribed fungal species of *Geosmithia* was the causative agent (Leslie et al. 2010, Seybold et al. 2010b, Tisserat et al. 2009). Previously, the genus *Geosmithia* was not known to contain plant pathogens (Seybold et al. 2010b, Tisserat et al. 2009). However, *Geosmithia morbida* (Ascomycota: Hypocreales: Bionectriaceae) was found to be associated with WTB, isolated from both beetle galleries and adult beetles from the tree host *J. nigra* (Kolařík et al. 2011).

Symptoms of TCD include yellowing and thinning of foliage in the upper crown, followed by branch dieback, and tree mortality (Seybold et al. 2010b, Tisserat et al. 2009). Foliage may wilt in advanced cases of TCD (Seybold et al. 2010b). Cankers originate around beetle entrance holes where *G. morbida* enters the cambial tissue (beneath the bark) (Leslie et al. 2010, Seybold et al. 2010b). The fungus does not infect trees systemically and cankers resulted from infections are limited to areas near feeding sites and tunnels of WTBs. However, if trees are attacked by high numbers of WTBs, the number of cankers in a single tree can be numbered in the thousands (Tisserat et al. 2009). The surface of the bark may show only dark stains or cracking above cankers (Seybold et al. 2010b, Tisserat et al. 2009). Initial cankers are small, oblong, and develop in WTB galleries. These small cankers can coalesce to obtain lengths up to two meters, encompassing half the circumference of the trunk, yet remaining invisible from the outside (Tisserat et al. 2009). The only known vector of *G. morbida* is the walnut twig beetle (WTB), (Coleoptera: Curculionidae: Scolytinae), *Pityophthorus juglandis* Blackman (Leslie et al. 2010, Newton et al. 2009, Seybold et al. 2010b, Tisserat et al. 2009). The beetle enters the tree by creating a pin-sized shot hole through the bark (Tisserat et al. 2009). To detect the tunnels of WTB, the top layer of bark must be carefully removed to observe the tunnels which are formed throughout the bark and phloem of the tree. WTB transports *G. morbida* into the tree

as it feeds in the phloem tissue of the walnut and creates its tunnels (Tisserat et al. 2009). This newly recognized disease/insect complex gets its name, Thousand Cankers Disease (TCD), from numerous cankers produced by *G. morbida* (Seybold et al. 2010b).

Pityophthorus juglandis

The genus *Pityophthorus* was first described in 1864 by Eichhoff and revised by Blackman in 1928. This genus is widespread with more than 200 species found throughout North and Central America and more than 100 species found throughout the U.S. (Bright 1981, Arnett et al. 2002). *P. juglandis* Blackman was first described by Blackman in 1928 from specimens collected from *Juglans nigra* L. in Lone Mountain, New Mexico and Paradise, Arizona (Blackman 1928, Cranshaw 2011).

WTB was collected in New Mexico by 1896 and its known range included Arizona, southern California, and Chihuahua, Mexico by 1992 (Bright 1981, Wood and Bright 1992). Its known western range now includes northern New Mexico, Colorado, Utah, Idaho, Washington, Oregon, and the beetles are often found in dying *J. nigra* (Cranshaw 2011). The known tree hosts of WTB are *J. nigra*, *J. californica*, *J. hindsii*, and *J. major*, but other walnuts may serve as a host (Newton et al. 2009, Wood and Bright 1992).

In Bright's (1981) description of the *Pityophthorus* genus, 26 of 220 species in both North and Central America are described as attacking deciduous trees. The majority of this genus attacks coniferous trees, such as *Abies*, *Picea*, *Larix*, and *Pseudotsuga* (Bright 1981). *Pityophthorus juglandis* is in the Juglandis group with 12 other species. From this group, the majority do not feed on coniferous plant material (Bright 1981) and *Juglans* spp. are the only known hosts of *P. juglandis* (Tisserat et al. 2009).

The WTB has historically been a minor pest of walnut twigs, but never reportedly caused tree mortality (Bright 1981, Graves et al. 2009, Leslie et al. 2010, Seybold et al. 2010b). No synonyms are present in the literature for this species. In 2004 and 2005, WTB was collected from dying black walnut trees, but was considered a secondary pest since it had never been reported to cause tree death (Tisserat et al. 2009).

Morphological Characteristics

The original collection of WTB was taken from small branches of *J. nigra* and the original description is based on the holotype and 73 other specimens (Blackman 1928). WTB is a small, yellowish to reddish brown, wood-boring beetle, size ranging from 1.5 to 2.0 mm long and 2.8 to 3.1 times long as it is wide (Seybold et al. 2010b, Blackman 1928). Teneral adults are light yellow and turn a reddish-brown as they harden (Blackman 1928).

Males appear similar to females except for a tuft of fine short blonde setae on the front of the head and more coarsely punctured elytra. The male also has coarser punctures on the elytra. In addition, the male has distinct granules on both the suture and lateral convexities on the elytra (Blackman 1928). The beetle is difficult to distinguish from other members of *Pityophthorus* due to similar morphological characteristics. However, WTB has a steep declivity in the elytra and 4 to 6 concentric rows of asperities on the pronotum that are usually broken and overlap at the median line (Tisserat et al. 2009, Blackman 1928, Bright 1981). In addition, the punctures on the elytra are in rows (Blackman 1928).

Pityophthorus lautus

Pityophthorus lautus Eichhoff has similar characteristics and is difficult to distinguish from *P. juglandis*. *P. lautus* is also in the genus *Pityophthorus* (Bright 1981). *P. lautus* is approximately 1.5mm long, 2.5 times as long as wide. It is also reddish brown: the same color as

WTB. LaBonte and Rabaglia (2012) developed a pictorial key to emphasize key characteristics separating *P. juglandis* from other *Pityophthorus* species. According to this key, WTB is less than 3 mm long, it has distinct concentric rows on its pronotum, the apex of its elytra is rounded, striae 1 and 2 are impressed on the elytra, and the non-impressed declivity is dull and roughened. Lastly, the anterior edge of the pronotum has 12 asperities (LaBonte and Rabaglia 2012). To differentiate *P. lautus* from *P. juglandis*, *P. lautus* lacks fused asperities on the first two lines on the pronotum as well as a steep declivity on the elytra (Blackman 1928).

This distinction is only an issue in eastern North America, where *P. lautus* is native and *J. nigra* serves as one of its hosts (Bright 1976, 1981). *P. lautus* and *P. juglandis* are both associated with hardwoods and are the only species in the *Pityophthorus* genus found to infest the *Juglans* genus (Bright 1976, 1981, Graves et al. 2009, Tisserat et al. 2009). In surveys conducted in August-September of 2010 in Tennessee, both *P. juglandis* and *P. lautus* were collected from *J. nigra* in eastern Tennessee (Grant et al. 2011). Since *P. lautus* and *P. juglandis* have been collected from *J. nigra*, it is important to know the characteristics that distinguish them as separate species.

Geosmithia morbida

Geosmithia morbida was found to be associated with WTB, isolated from both beetle galleries and adult beetles from the tree host *J. nigra* (Kolařík et al. 2011). In addition, cankers caused by *Fusarium solani* (Mart.) Sacc. were also isolated from trees infected with TCD (Leslie et al. 2010). However, it is unclear if *F. solani* plays a role in TCD or with *G. morbida* (Leslie et al. 2010). The three main characteristics that differentiate *G. morbida* from other *Geosmithia* are: its inability to grow on Czapek-Dox agar (CDA), its thermotolerance, and the monilioid or atypically branched base of the conidiophores (Figure 2) (Kolařík et al. 2011). To date, *G.*



Figure 2 (a-c): (a) *Geosmithia morbida* conidia and conidiophores; (b) *Geosmithia morbida* conidial chains; (c) *Geosmithia morbida* cultures; photos (a-c) Dr. Alan Windham, University of Tennessee.

morbida is the only phytopathogenic species in this genus or in the family Bionectriaceae (Kolařík et al. 2011). To date, WTB is the only known vector for *G. morbida* (Leslie et al. 2010, Kolařík et al. 2011). Although it is possible that other wood boring beetles or other insects associated with *Juglans* may act as vectors for this *G. morbida* (Leslie et al. 2010, Kolařík et al. 2011).

Geosmithia morbida conidia are suspected to be carried on the elytra of WTB and eventually come into contact with phloem tissue that way (Newton et al. 2009). It is theorized that in the western U.S., WTB and *G. morbida* have been present for a long time and are not a recent introduction (Kolařík et al. 2011). The genus *Geosmithia* has a diversity of species associated with bark beetles and has a worldwide distribution (Kolařík et al. 2004, Tylová et al. 2011). In general, the *Geosmithia* genus is associated with a diversity of wood-boring beetles in the Scolytinae tribe (Kolařík et al. 2007, Kolařík and Kirkendall 2010). However, other beetles, such as members of Bostrichidae, have also been found to disperse this genus (Kolařík et al. 2007). Little is known about the ecological role this genus plays or impact it has on its hosts (Kolařík et al. 2005). In addition, the genetic diversity of *G. morbida* is poorly understood (Hadziabdic 2011). However, research done by Hadziabdic et al. (2011) identified 16 microsatellite markers for loci of *G. morbida*. These microsatellites markers can be used in population diversity studies and be used to develop techniques for molecular diagnosis of this disease.

Impact of Thousand Cankers Disease on *Juglans nigra*

Since the discovery of TCD, the disease has been identified as a major cause of mortality of black walnut (and other walnut species) in the western states of Colorado, Idaho, Oregon,

Utah, Washington, Arizona, New Mexico, Nevada, and California (Cranshaw 2011, Newton et al. 2009, Seybold et al. 2010b, Tisserat et al. 2009). It has recently been detected in four eastern states including Tennessee, Virginia, Pennsylvania, and North Carolina (Tennessee DAC 2012, Virginia DAC 2012, Pennsylvania DAC 2012, North Carolina DAC 2013). Grant et al. (2011) confirmed both WTB and *G. morbida* in Anderson, Blount, Knox and Union counties in Tennessee. Many uncertainties associated with this disease remain unanswered, i.e., what is the origin of *G. morbida* and why has disease/insect complex become such a recent threat since the beetle and fungus seem to be from similar regions?

Juglans nigra is very susceptible when compared to the susceptibility of other *Juglans* species to TCD (Newton et al. 2009). Other *Juglans* species such as *J. hindsii* and *J. californica*, have also experienced symptoms of TCD and WTB has been recovered from these species, although mortality has been low compared with *J. nigra* (Newton et al. 2009). *Juglans regia* L. and *J. major* both appear to have some natural resistance to TCD (Newton et al. 2009). The range of TCD is predicted to spread throughout the western U.S. due to extensive ornamental planting of black walnut and English walnut (Graves et al. 2009). If TCD becomes widespread in the eastern U.S., it has the potential to decimate native populations of *J. nigra*.

On August 5, 2010, the Tennessee Department of Agriculture announced the presence of TCD in East Tennessee (Oliver 2010). Anderson, Blount, Jefferson, Knox, Loudon, Sevier, and Union counties are currently quarantined for TCD (Tennessee DAC 2012). In addition, Virginia, Pennsylvania, and North Carolina have confirmed outbreaks of TCD and have quarantined several of their counties (Pennsylvania DAC 2012, Virginia DAC 2012, North Carolina DAC 2013). Since TCD may spread through transportation of firewood, no hardwood firewood can be moved outside of quarantined counties (Leslie et al. 2010, Oliver

2010). In addition, the Tennessee Department of Agriculture has designated buffer counties around the quarantined counties which include Campbell, Claiborne, Cocke, Grainger, Hamblen, McMinn, Monroe, Morgan, Rhea, Roane and Scott counties (Tennessee DAC 2012). Walnut tree products and hardwood firewood can be moved within buffer counties, but not outside (Oliver 2010). Furthermore, walnut products can be moved into a quarantine county but not removed (Oliver 2010). Many state departments of agriculture are prohibiting entry of certain walnut products from the states with TCD infestations. Once limited to tree hosts in the western states, TCD could have a catastrophic impact on the commercial industry and ecological roles associated with *J. nigra*, comparable to damage resulting from the invasive chestnut blight or Dutch elm disease. In Tennessee, an estimated 26 million black walnut trees exist on public and private timberland with a value estimated at around \$1.47 billion (Tennessee Government 2012).

Chemical Control for Bark Beetles

Imidacloprid and Dinotefuran

Imidacloprid and dinotefuran are systemic insecticides and part of the neonicotinoid class of insecticides (Elbert et al. 1998, Kodaka et al. 1998, Cowles 2010). Neonicotinoids target the insect's nicotinic acetylcholine receptor which affects the mechanics of the central nervous system (Tomizawa and Yamamoto 1993). They are efficient at killing insects while at the same time posing little risk to humans and other mammals due to their inability to bind well to mammalian nerve cells (Cowles 2010). In addition, imidacloprid has a short half-life (1.4 d), breaks down quickly in sunlight, and will bind to organic matter which reduces run off and its impact on non-target aquatic invertebrates (Mullins and Christie 1995, Cowles 2010).

Neonicotinoids are currently used in agriculture, pest control, landscape pest management, and arboriculture (Cowles 2010). Products of imidacloprid (CoreTect[®], Merit[®] and Xytect[®]) and dinotefuran (Safari[®] and Transtect[®]) are applied as soil injections and drenches, foliar sprays, trunk sprays and trunk injections (Cowles 2010). The roots absorb the soil application where it eventually ends up in the xylem and spreads to areas of transpiration such as the leaves. Imidacloprid metabolizes into olefin which is 16 times more toxic to some insects than imidacloprid (Nauen et al. 1998).

Imidacloprid and dinotefuran are used to treat a variety of forest pests. Imidacloprid is used to treat *Tomicus piniperda* L., a bark beetle that attacks the shoots of young scotch pine, *Pissus sylvestris* L. (McCullough and Smitley 1995) and is the primary insecticide used to control the phloem-feeding larvae of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Poland et al. 2006, Russell et al. 2010, Ugine et al. 2012). In addition, bronze birch borer, *Agilus anxius* Gory, larvae feed within the phloem and phloem-xylem regions of the tree (Loerch and Cameron 1983) and are treated with systemic insecticides, including imidacloprid, emamectin benzoate, and dinotefuran (Herms et al. 2009, Smitley et al. 2010). Topical applications of imidacloprid are used in nut-producing trees due to the low mammalian toxicity and reduced systemic movement within plants to combat such pest (Leicht 1996); however, systemic applications have not been approved for black walnut since it is considered to be in group 14-tree nut (OFR 2010). Not all wood-boring insects are sensitive to imidacloprid and dinotefuran. For example, Grosman and Upton (2006) determined that dinotefuran and imidacloprid were ineffective at treating loblolly pine, *Pinus taeda* L., infested with *Ips* spp. However, emamectin benzoate and fipronil have been found to significantly reduce *Ips* spp. and southern pine beetle, *Dendroctonus frontalis* Zimmermann, in *P. taeda* (Grosman and Upton

2006, Grosman et al. 2009). Emamectin benzoate and fipronil may be worth evaluating for their effectiveness against WTB, if imidacloprid and dinotefuran are ineffective.

Research Objectives

Currently, the primary means of managing TCD is rapid detection and removal of infected trees (Oliver 2010). Homeowners in eastern Tennessee should watch for symptoms such as yellowing of leaves, branch dieback, sap staining, or pin-sized WTB entrance holes for detection of TCD (Graves et al. 2009, Oliver 2010, Seybold et al. 2010b).

To further limit the spread of TCD in eastern Tennessee, a better understanding of *G. morbida* and *P. juglandis* life history is imperative. In addition, a rapid, effective method of managing pest organisms in live trees needs to be established to help provide initial management of TCD. By having a rapid tactic available, such as a pesticide, initial control can be established, while experts research more ecologically stable tactics (i.e., biological control).

Therefore this research will focus on the life history of *P. juglandis* its potential native predators in eastern Tennessee; thus, allowing for a more ecologically sound control tactic of TCD. In addition, this project will determine the translocation and within-tree concentration of two insecticides. Concentrations of the insecticides will be determined using an HPLC machine. Particularly, the nutmeat will be analyzed for presence of insecticides since this is the part that is consumed by humans and wildlife.

The objectives for this study were to:

1. Determine the life history for the immature and adult stages of the WTB in eastern Tennessee.
2. Identify potential predators and parasitoids for use as biological control agents of WTB.
3. Determine the translocation of two systemic pesticides with-in mature *Juglans nigra*.

CHAPTER II

THE BEHAVIOR AND DEVELOPMENT OF THE WALNUT TWIG BEETLE IN EASTERN TENNESSEE

Abstract

The establishment of Thousand Cankers Disease in the eastern U.S. could threaten black walnut, *Juglans nigra*, in its native range. Currently, the primary means of managing Thousand Cankers Disease is rapid detection and removal of infected trees. The disease is characterized by a series of cankers caused by the fungus, *Geosmithia morbida*, vectored by the walnut twig beetle (WTB), *Pityophthorus juglandis*. The life history of *P. juglandis*, WTB, was investigated in eastern Tennessee on *J. nigra*. Eleven galleries were found with both male and females in the same tunnel (excluding teneral adults). Of these, five galleries had one male and one female, while six galleries had one male with two females. Eggs were individual females produced a mean of 16.6 (7.0-25.0) eggs per gallery was recorded from 17 tunnels with individual females present. Egg and immature development occurs within galleries excavated by the females at the interface of the bark and cambial tissue consisting of entrance tunnels and brood chambers. Three larval instars were recovered from WTB tunnels. Larvae produce tunnels perpendicular to the adult female's gallery. The last larval instar expands the end of the gallery prior to pupation. This species overwinters as larvae and adults within the galleries of black walnut. Flight activity was recorded from the end of September until the beginning of December (traps were only present from September 2012-March 2013). In addition, native enemies were identified in the galleries with WTB, which may serve as potential biological control agents against this invasive pest.

Introduction

Pityophthorus juglandis Blackman (Coleoptera: Curculionidae: Scolytinae), the walnut twig beetle (WTB), was originally described from specimens collected in Lone Mountain, New Mexico and Arizona on *Juglans nigra* L. by Blackman (1928). The beetle is native to Chihuahua (Mexico), Arizona, California, and New Mexico (Bright 1981, Newton et al. 2009, Tisserat et al. 2009, Wood and Bright 1992). WTB was documented to be responsible for the transmission of Thousand Cankers Disease (TCD) (Tisserat et al. 2009), a complex of the WTB and a fungal pathogen (*Geosmithia morbida*), that threatens the destruction of black walnut throughout the U.S.

Since 1992, the known range of WTB expanded to include Colorado, Idaho, Nevada, Washington, Oregon, Utah, and Wyoming (Graves et al. 2010, Cranshaw 2011) where beetles are found in dying *J. nigra*. In 2010, populations of WTB and TCD were discovered in Knox Co. in east Tennessee representing the first documentation of these pests east of the Mississippi River (Grant et al. 2011). The beetle and pathogen have since been found in Virginia and Pennsylvania (Thousand Cankers Disease 2011, 2012, Hansen et al. 2011). Haywood County, North Carolina confirmed the presence of TCD in the fall of 2012 (North Carolina DAC 2012). Butler County Ohio found WTB in December of 2012 though the pathogen *G. morbida* has not been detected (Ohio DNR 2012).

The walnut twig beetle is a small, yellowish to reddish brown, wood-boring beetle, ranging in size from 1.5 to 2.0 mm long, with a length 2.8 to 3.1 times greater than its width (Seybold et al. 2010b, Blackman 1928). WTB is only known to feed on species within the genus *Juglans*, and has been confirmed to feed on more than ten species or hybrids (Seybold et al. 2010a).

WTB is believed to prefer branches 1.5 cm or greater in diameter, although it will colonize the trunk of the tree, making the “twig” in its name somewhat misleading (Seybold et al. 2010a).

WTB has historically been considered a minor pest of *Juglans* sp. in the southwestern U.S. and Mexico, but was never reported to cause walnut mortality (Bright 1981, Graves et al. 2009, Leslie et al. 2010). However, in 2004 and 2005 in Colorado, WTB was collected from dying black walnut trees where it was initially considered a secondary pest (Tisserat et al. 2009). Tree deaths were initially attributed to drought stress; however, additional research revealed that a previously undescribed fungal species of *Geosmithia* (*G. morbida*) was responsible (Leslie et al. 2010, Seybold et al. 2010b, Tisserat et al. 2009). Previously, species within the genus *Geosmithia* had not been considered a plant pathogen (Seybold et al. 2010b, Tisserat et al. 2009).

The only known vector of *G. morbida* is the WTB (Leslie et al. 2010, Newton et al. 2009, Tisserat et al. 2009). Before 2000, *G. morbida* was not known to be associated with WTB (Tisserat et al. 2009). It is hypothesized that the beetle co-evolved with *J. californica* and *J. hindsii* in the western U.S.; although the potential evolution of WTB with *G. morbida* remains unproven (Seybold et al. 2010a). WTB is an effective vector of *G. morbida* since even short feeding galleries result in cankers attributed to *G. morbida* (Seybold et al. 2010a). The beetle enters the tree by creating a pin-sized shot hole through the bark (Tisserat et al. 2009). To detect the tunnels of WTB, the top layer of bark must be carefully removed to observe the tunnels which are formed at the interface of the inner bark and cambial tissue. WTB carries *G. morbida* into the tree as it feeds on the phloem tissue of the walnut while creating tunnels (Tisserat et al. 2009, Seybold et al. 2010b). This newly recognized disease/insect complex derives its name, TCD, from the numerous cankers produced by *G. morbida* (Tisserat et al. 2009, Seybold et al. 2010b).

Several similar studies on the biology of WTB have been completed in California. Using aggregation pheromone-baited traps, Seybold et al. (2012a), found that WTB fly primarily at dusk during late June and early July in CA. Later that year in tests conducted in August and early November, they discovered the majority of WTB still fly at dusk although time of flight was more varied. They concluded that WTB flies little or not at all during the night. In addition, Seybold et al. (2012a) associated temperature with flight activity and discovered that WTB flight activity peaks at temperatures between 23-24°C (73.4-75.2°F) and cease flying in temperatures below 17-18°C (62.6-64.4°F). In California, the only month WTB flight did not occur was in December (Seybold et al. 2012b).

An aggregation pheromone has been found to which both sexes of WTB respond, although more females than males are caught in traps using this pheromone (Seybold et al. 2010a, 2012a). This pheromone bait is now commercially available and being used as a survey tool for WTB. Traps have not been used to assess population levels, only to detect the presence of WTB (Seybold et al. 2012b). Suggested survey trapping periods are June-August although it has been suggested that trapping in the south might begin in April or May (Seybold et al. 2012b). Additional trapping revealed that WTB flies in Tennessee and Virginia into late November and in January (Seybold et al. 2012b). Three instars of WTB larvae have been identified in California (Dallara et al. 2012). These instars were determined by measuring and analyzing larval head capsule widths (Dallara et al. 2012). Kishi (1971) proposed that counting the larval exuviae in galleries was more reliable than measuring head capsule widths; however, Dallara et al. (2012) argue that finding a complete collection of exuviae in galleries is difficult due to their delicate nature and by the possible breakdown of exuviae by detritivores. These studies help lay the groundwork to better understand the development and behavior of WTB which is important

knowledge to have when searching for control tactics.

Currently, the primary means of managing TCD is rapid detection and removal of infected trees (Oliver 2010). No biological or chemical controls are currently available to stop the spread or rehabilitate infested trees. To further stop the spread of TCD in eastern Tennessee, a better understanding of the life histories of *G. morbida* and *P. juglandis* is imperative (Oliver 2010). Therefore, research was initiated to understand the behavior and development of WTB in eastern Tennessee.

Materials and Methods

Population Density and Laboratory Emergence

Experiments were conducted to estimate the beetle's population density in infested black walnut logs. Six branch samples were taken from infested black walnut trees felled in August 2010. Three of the samples came from Fountain City (36°2'13.03"N; 83°54'46.72"W) and three samples came from Lakeshore in Knox County (35°55'23.59"N; 83°59'29.47"W). Each sample consisted of two or three limb sections (bolts) each between 24 to 34 cm long and 7 to 12 cm diameter placed in separate plexi-glass containers (30.5 cm x 40.0 cm) for evaluation and observation. Air circulation was provided by cutting one circle (14 cm) on either side of the boxes and covering with mesh netting. The front of each box had a circular hole cut-out with a cloth covering the hole. The cloth provided a way to aspirate emerging beetles without the danger of the minute beetles escaping. The cloth was tied in a knot when not in use to prevent beetle escape. Boxes were observed daily for newly-emerged adult WTB. When beetles were discovered, they were removed from the boxes by an aspirator, and the number and date of emerged beetles recorded. The beetles were placed in 95% ethanol and labeled.

In late September 2010, six additional samples each between 25 to 30 cm long and 8 to

10 cm diameter were collected from the Lakeshore site. These samples were placed in the laboratory in smaller clear plastic containers (38.0 cm x 25.5 cm) located near the original six samples. For air circulation, the top of each container was removed and replaced with the same mesh netting used in the first containers. Beetle emergence was recorded daily. From the number of WTB collected from walnut logs, a population density was calculated by dividing the total number of WTB by the total bark surface area (35856.90 cm²) of black walnut logs.

Field Emergence

To detect the field emergence and calculate the number of degree-days accumulated prior to WTB emergence in the spring, six walnut bolts (5-7 cm diameter, 25 cm length) were collected using a pole saw from an infested tree at the Lakeshore site in November 2010. Six additional samples were taken monthly from December 2010- February 2011 to increase the likelihood of obtaining infested samples. These samples were placed in the Helping Hands Kitchen Garden located in the University of Tennessee (UT) Gardens (35°56'38.82"N; 83°56'13.61"W). The containers used to house these samples were glass, 3.8 liter-sized jars. Each infested sample was individually placed in a glass jar. The lids were replaced with the same mesh netting as used previously and described for air circulation. To reduce the amount of light entering the glass jars and prevent over-heating, the jars were placed underneath a bench for shade. This experiment was replicated in December 2011- February 2012 to compare WTB adult emergence dates with those observed in 2010-2011 and calculate the number of degree-days accumulated until WTB emergence. Degree-days were calculated using weather information available through McGhee Tyson Airport, TN, U.S. Celsius-based heating degree-days (dd) were calculated using a base temperature of 10°C and standards given in Herms (2004). The dd were calculated from January 1st until emergence for both years.

Seasonal Development

Because WTB develops underneath the bark, seasonal development was assessed by collecting and dissecting the bolts over time to observe and document developmental stages. Three branch samples were collected once every two weeks from infested *J. nigra* from September 2010-October 2012. Infested trees from three locations (Lakeshore [35°55'23.59"N; 83°59'29.47"W], Emory Road [36°5'5.33"N; 83°55'54.98"W], and Fountain City [36°2'13.03"N; 83°54'46.72"W]) were used to collect WTB infested samples. The bark was shaved off each set of samples with a draw knife, revealing the WTB galleries, any developmental stage of WTB present, and other organisms within the branch. Photographs of the galleries and life stages of WTB were taken to better represent their life history and behavior. In addition, the quantity and stages of development of collected walnut twig beetles were recorded.

WTB Development in Bolts Baited with Pheromone Lures

Three mature trees of *J. nigra* were selected from three different locations in Knoxville including Choto (35°49'17.79"N; 84°8'46.65"W), Kensington (35°53'12.9"N; 84°2'18.06"W), and Burkhart Road (36°4'46.61"N; 83°51'28.43"W) in August 2012. Each tree selected was confirmed to be heavily infested with WTB through branch sampling or previous trapping. Fifteen fresh bolts from uninfested *J. nigra* limbs were collected weekly from the Plant Science Farm in Knoxville, TN (35°35'41.79"N; 83°57'18.96"W). The size of bolts ranged from 5-7 cm in diameter and each was approximately 25 cm long. At each of the three sites, five uninfested bolts of *J. nigra* were tied together and hung on the outer edges of limbs in each of the three trees. One WTB lure (Contech Enterprises Inc.) was stapled to one of the bolts to attract flying WTB to the tree. After one week, the bolts were removed and replaced with fresh, uninfested

bolts. One of the five bolts was randomly selected from each site and cut with a draw knife to confirm that infestation had or had not occurred. Bolts were then placed individually in 3.8 liter-sized glass jars used and held in the laboratory at 21°C. Bolts were changed and the experiment continued for six weeks. The first bolts were removed and maintained in the laboratory for six weeks, the second set for five weeks, and so on. When the bolts had set for the appropriate time between one-six weeks, bolts were dissected with a draw knife, and life stages of WTB observed and quantified. Head capsules of the larval samples were measured (length and width) with an ocular micrometer (Blackman 1915, Dallara et al. 2012) and recorded to determine the number of instars. Adult WTB were sexed and gallery structures were recorded. Gallery structure, length, width, shape, presence or absence of visible mycelia, and life stages and sexes of WTB present in the gallery were recorded. Only galleries with a clear beginning and end were measured for this study. In addition, only galleries with an associated WTB were measured to ensure that every gallery could be positively identified as having been made by a specific life stage.

Overwintering flight behavior of WTB

Four traps were hung at the Lakeshore site, eight at the Burkhart site, and four traps on the Agricultural campus site (35°56'55.39"N; 83°56'30.13"W) of the University of Tennessee. Traps were placed and maintained in these field sites from September 2012-April 2013. From this experiment, the flight behavior of WTB during the winter months and their emergence of the spring population were evaluated. Pheromone-baited traps were hung in both the upper and lower strata of the test trees. Traps were constructed using 2-liter soda bottles coupled with Nalgene® 250 mL wide-mouth bottles screwed into the opening of the soda bottle lids. The bottoms of the Nalgene® bottles were removed and replaced with mesh netting fastened with hot

glue to allow rain water to pass through without drowning the specimens. Two circular holes (10-12 cm diameter) were cut on either side of the soda bottles to allow insects to enter the trap. A WTB pheromone lure was attached with a zip tie to the middle of each trap. Traps were placed on the outer edges of limbs and checked every two weeks for WTB; the pheromone bait was changed every two months. Collected WTB were placed into 118 mL plastic specimen cups and taken to the laboratory. The collected WTB were sexed and, if still alive, were either fed to clerid predators or placed on fresh black walnut bolts in clear plastic boxes (8.4 x 7 cm area and 4.6 cm deep) at 22°C to rear laboratory populations of WTB. These containers had a square hole (14 cm x 11 cm) cut out of the top of each container which was covered with a fine mesh glued over the hole to allow for air circulation and prevent WTB from escaping.

Data Analysis: Shapiro-Wilks W test of normality and Levene's test of homogeneity of variances were used to verify that the data (gallery width, gallery length, larvae head capsule and size differences, as well as the amount of larvae, pupae, and adults collected over two years) conformed to the assumptions of analysis of variance (ANOVA). Differences in larval gallery width and length as well as differences in larval head capsules and numbers of WTB collected over two years were analyzed using PROC Mixed ANOVA (SAS 2005). ANOVA and Least Significant Differences (LSD) procedures were run on data ($P < 0.05$) to determine differences between larval gallery widths, lengths, as well as head capsule widths, and numbers of WTB collected over two years.

Results and Discussion

Population Density and Laboratory Emergence

From the logs held in the laboratory at 21°C from August 2010-May 2011, emergence began in September 2010 and ceased after February 2011 with a total emergence of 4,839 WTB

(Figure 3). Overall emergence peaked in November and December 2010. Therefore, the population density was 0.135 WTB per cm² of black walnut material (Table 1). The population density will fluctuate depending on the intensity of the infestation. This wood was similar in size to firewood and was not waxed or had any water added to it once it was cut. The wood had been drying for several months before the population peaked in November and December.

This experiment demonstrates that WTB can still emerge from logs six months after being cut when held at a constant temperature. Frass appeared on the bottoms of containers as WTB recolonized the already infested, dry wood after first emergence. In addition, WTB were observed re-entering logs. Re-colonization was probably due to the inability of WTB to access fresh wood since they were held in containers. To minimize WTB re-colonization, WTB were removed on a daily basis. WTB will continue to emerge from cut logs up to six months. However, it is likely that WTB were emerging from these trees before logs were cut, thus, it is likely that WTB might have emerged for seven or eight months. This prolonged emergence is important to keep in mind, when considering the transportation of firewood. Raw wood acts as a key pathway for the movement of forest pests including bark and ambrosia beetles (Newton et al. 2009). With the decline of black walnut from Thousand Cankers Disease, infested trees will continue to be cut and transported to various areas around the country, thus, enhancing the spread of this disease.

Outside Emergence

From the bolts held in the glass jars outside in the UT Gardens in November 2010-April 2011 and November 2011-April 2012. One WTB emerged on April 11, 2011. Two WTB emerged on March 20, 2012. For year one, 404.5 degree-days (dd) were accumulated and 312.5 dd for year two. Due to low numbers of observations, beetles, no conclusions could be drawn

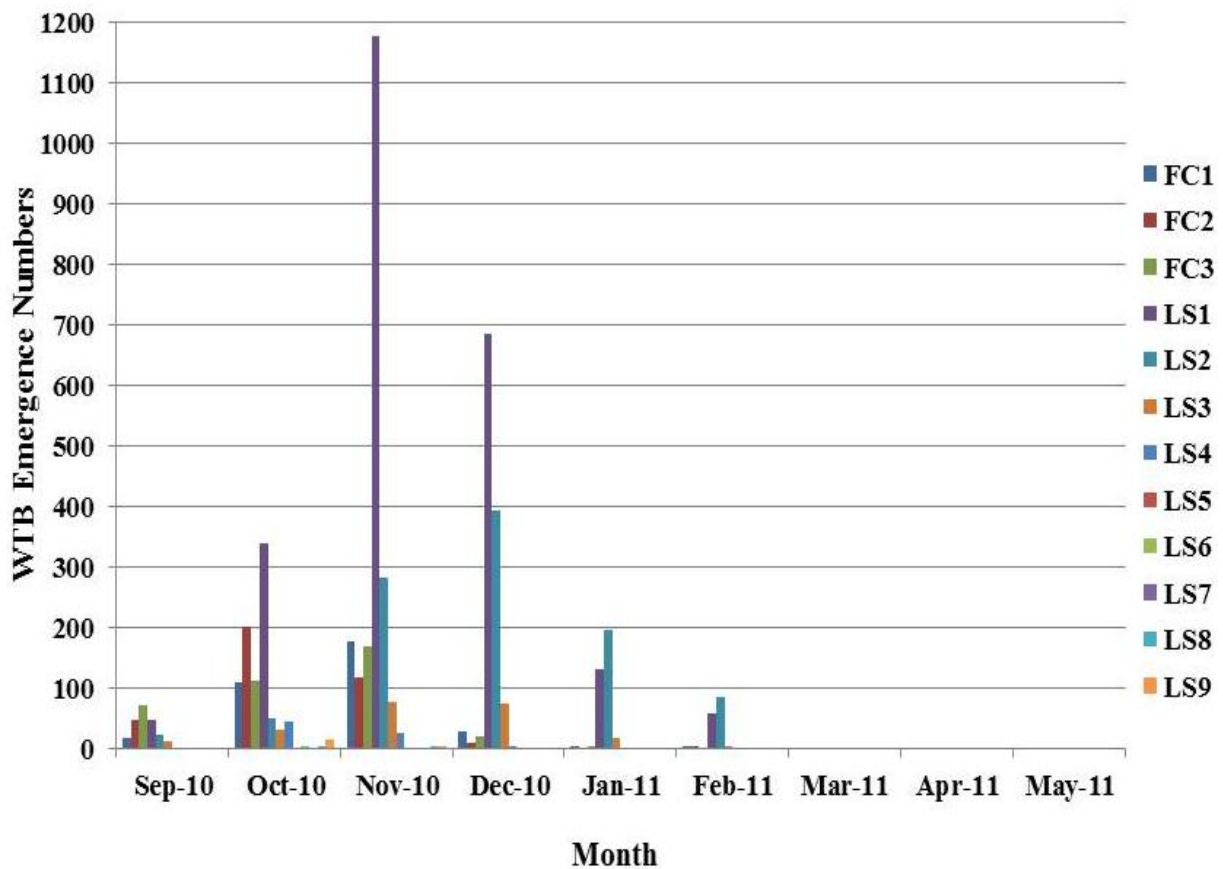


Figure 3. Walnut twig beetle emergence numbers from black walnut samples held in the laboratory from September 2010-May 2011. Log labels are in the legend. LS- Lakeshore and FC- Fountain City.

Table 1. Population density of walnut twig beetles in infested black walnut			
Containers	Total WTB emerged	Surface area of bark (cm²)	WTB/cm²
FC 1	322	13224.900	0.024
FC 2	376	2306.600	0.163
FC 3	375	1881.800	0.199
LS 1	2436	2364.300	1.030
LS 2	1029	3460.100	0.297
LS 3	210	3093.300	0.068
LS 4	71	2811.700	0.025
LS 5	0	1407.400	0.000
LS 6	1	1715.300	0.001
LS 7	0	1473.400	0.000
LS 8	1	1017.900	0.001
LS 9	18	1100.200	0.016
Totals	4839	35856.900	0.135

from these experiments. More data is needed to determine a dd range. The reason for low emergence numbers is that the infestation density in that tree was low or that the tree was sampled in the wrong location for optimum population densities. Perhaps a better approach would be to place traps next to infested trees through the winter and record spring emergence.

Dissected Monthly Logs

Over the course of two years, larval presence was detected in every month, although not in every month in each year (Figure 4). Larvae were present during the winter months in both years. The number of generations of WTB per year remains unclear (Figures 4-6). WTB pupae were present during all months except April, May, and June (Figure 5). Adult WTB were recovered in all months, but not in every month from both years (Figure 6). These data suggest that larval, pupal, and adult stages of WTB are present during the winter in eastern TN. Pupae are present in a small number during the winter with larval and adults being the most common stages to overwinter. However, egg location and gallery structures were not determined until the final months of this project, meaning eggs were not recovered from any of these samples and could have been present during the winter.

From the logs dissected, some natural enemies of the WTB were found in WTB galleries. These include clerid larvae (Figure 7), scolytid parasitoids in the genus *Theoclast* (both adults and pupae), *Aeletes floridae* (Marseul), *Leptophloeus angustulus* (LeConte), and *Bitoma quadriguttata* (Say). *Theoclast* in particular was found several times as pupae in WTB larval galleries with larvae in adjacent galleries, but not in the gallery with the pupae (Figure 8). This is possibly because the larva that was present in that gallery was eaten by the parasitoid.

Number of WTB Instars

By measuring head widths with an ocular micrometer and graphing the results to see peak

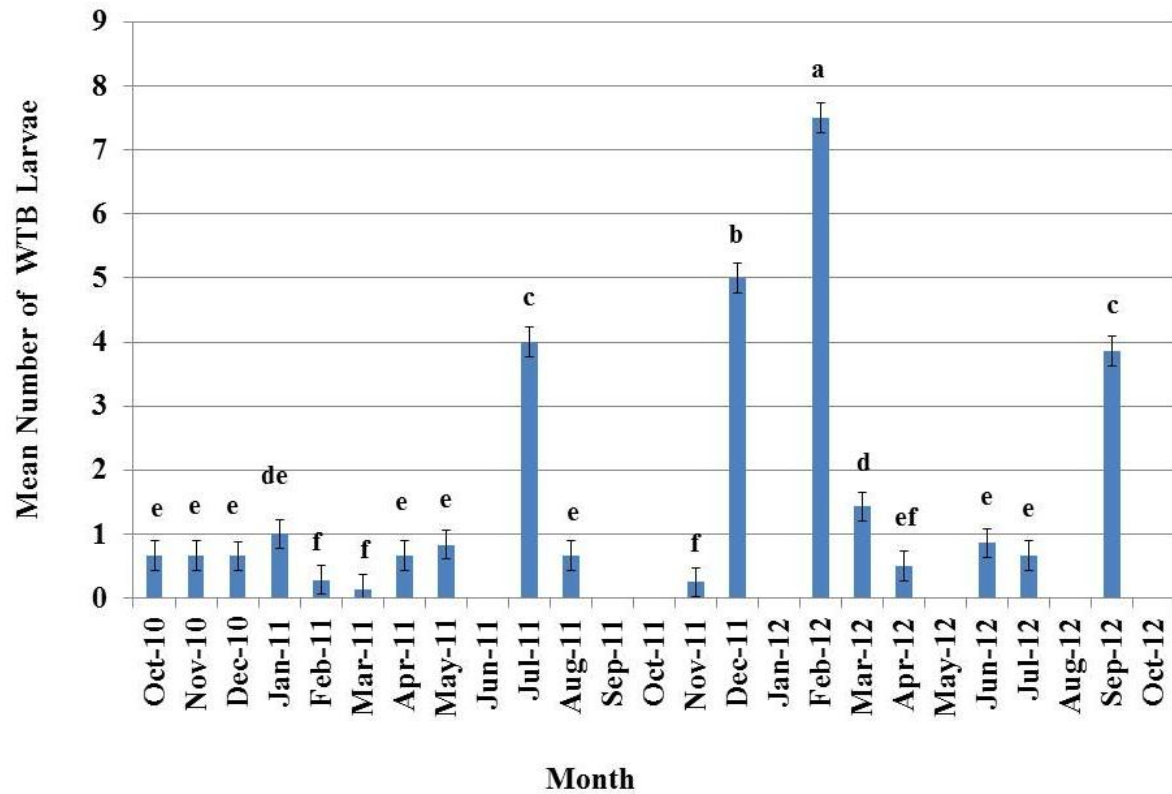


Figure 4. Mean $\pm$ SE (n=6) of walnut twig beetle larvae determined monthly over two years. Means followed by different lowercase letters are significantly different (LSD test; $P < 0.05$).

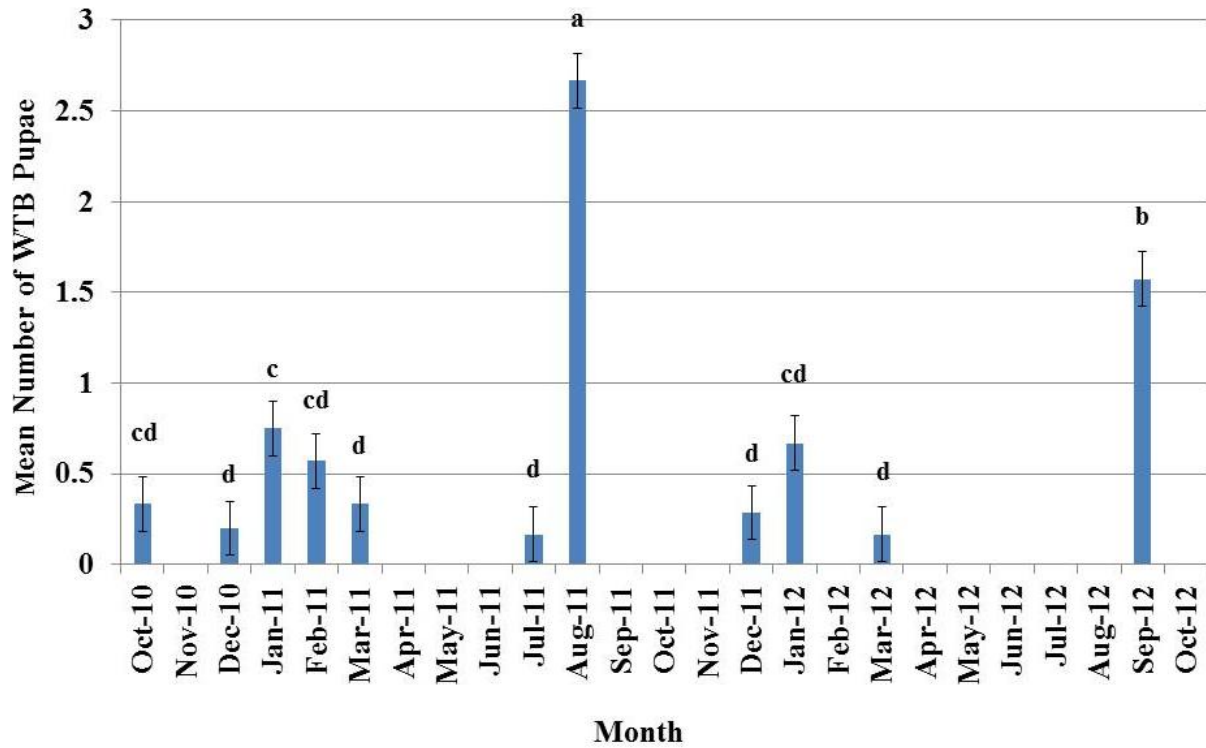


Figure 5. Mean $\pm$ SE (n=6) of walnut twig beetle pupae determined monthly over two years. Means followed by different lowercase letters are significantly different (LSD test; $P < 0.05$).

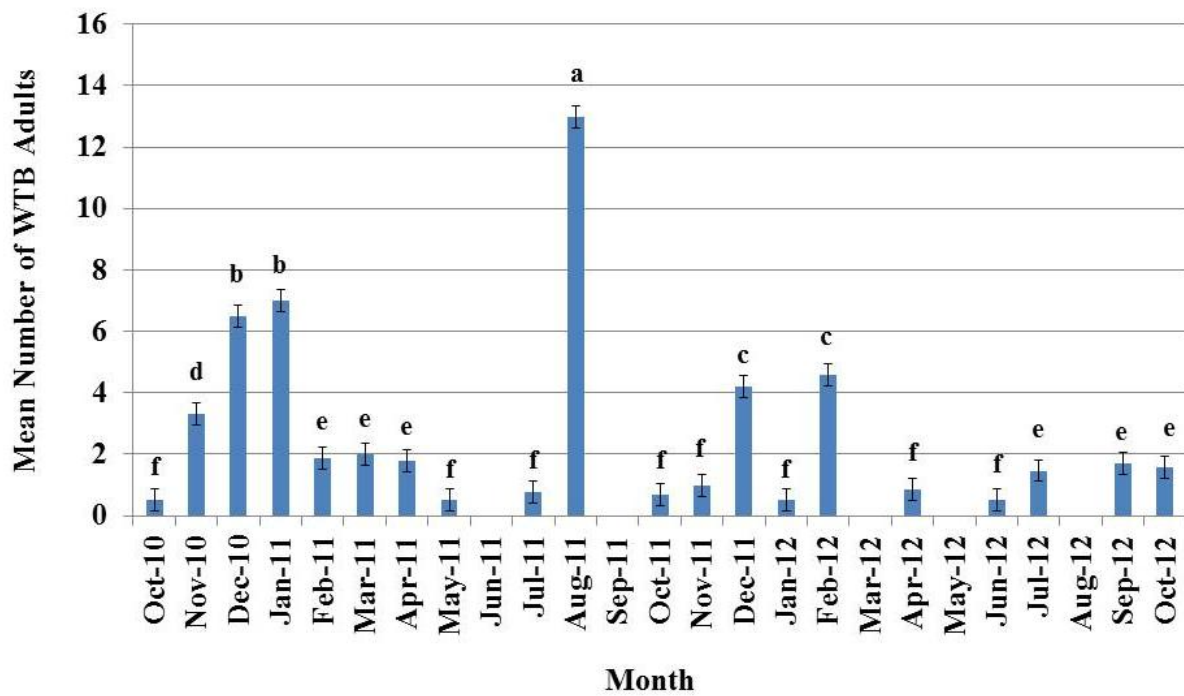


Figure 6. Mean $\pm$ SE (n=6) of walnut twig beetle adults determined monthly over two years. Means followed by different lowercase letters are significantly different (LSD test; $P < 0.05$).



Figure 7. Clerid larva present in walnut twig beetle larval galleries.

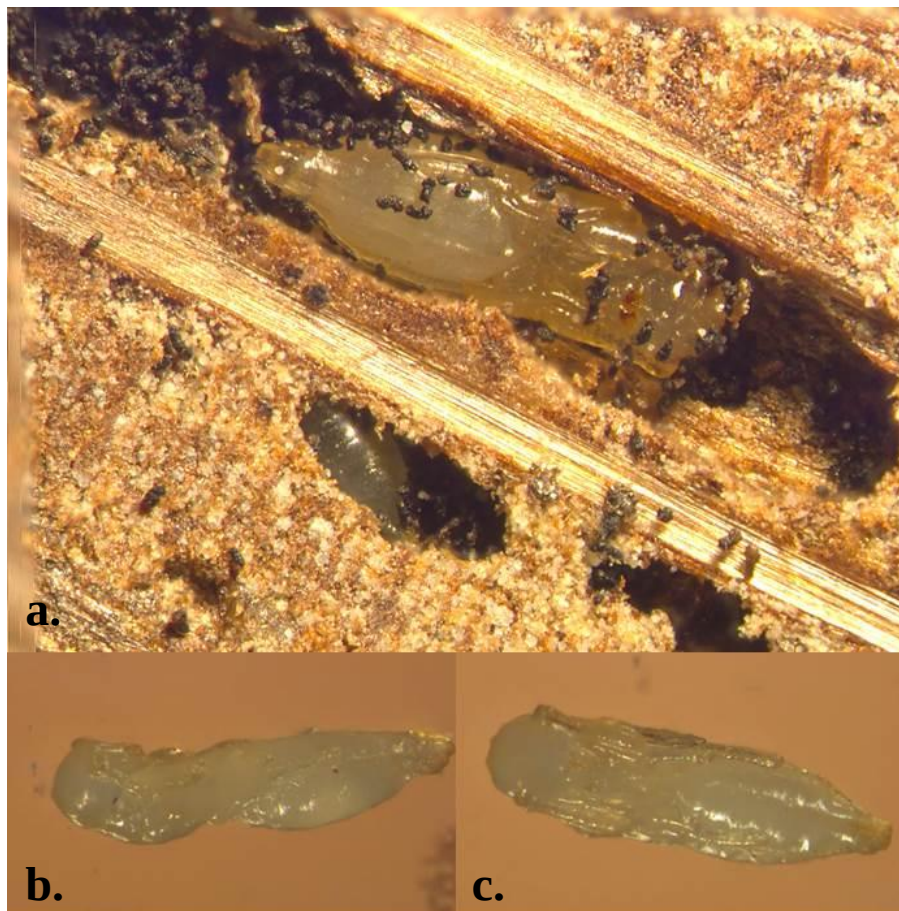


Figure 8(a-d). (a) Parasitoid pupae present in walnut twig beetle larval galleries; (b) Lateral view of parasitoid pupa; (c) Ventral view of parasitoid pupa.

points, WTB in eastern Tennessee were determined to have at least three larval instars (Figure 9). Peaks were observed in head capsule width (mm) in weeks two, three, and four; suggesting three larval instars. This result agrees with research conducted in California (Dallara et al. 2012) where specimens of WTB were reported to have three instars under both laboratory and field conditions. In addition, *P. micrographus* L. (Lekander 1968), *P. orarius* Swaine (Hedlin and Ruth 1970), and *Hylesinus californicus* (Swaine) (Langor and Hergert 1993) were all reported to have three larval instars.

Head capsule width and length and body width and length all peak at week 4, then decline in weeks 5 and 6 (Figure 9). This decline is probably due to progeny hatching that were laid at a later date than the first group of eggs. In regard to larval development time, bolts held in the laboratory after just one week and then cut revealed no larval tunnels, though eggs were present. Larvae were recovered during all subsequent weeks (2-6). One pupa and one teneral adult were recovered after three weeks in the laboratory. However, more pupae were obtained in week 4 than week 3, as well as teneral adults. By counting the one week that the bolts remained in the field to become infested and the three to four weeks bolts were maintained in the laboratory, it takes WTB between four and five weeks after infestation to reproduce from egg to adult. Eggs hatched between two to three weeks after oviposition in laboratory conditions.

WTB Gallery Structure

Fungal mycelia were visible macroscopically within the female egg-laying galleries, pupal, and teneral adult chambers (Figure 10a-b, Figure 11d, Figure 12c-d) and confirmed to be *G. morbida*. *G. morbida* was isolated from female egg-laying chambers and teneral adults collected from dissected bolts. Mycelia were not visible in the larval galleries, or in galleries with only WTB males. Mycelia were only present in galleries in bolts held under laboratory

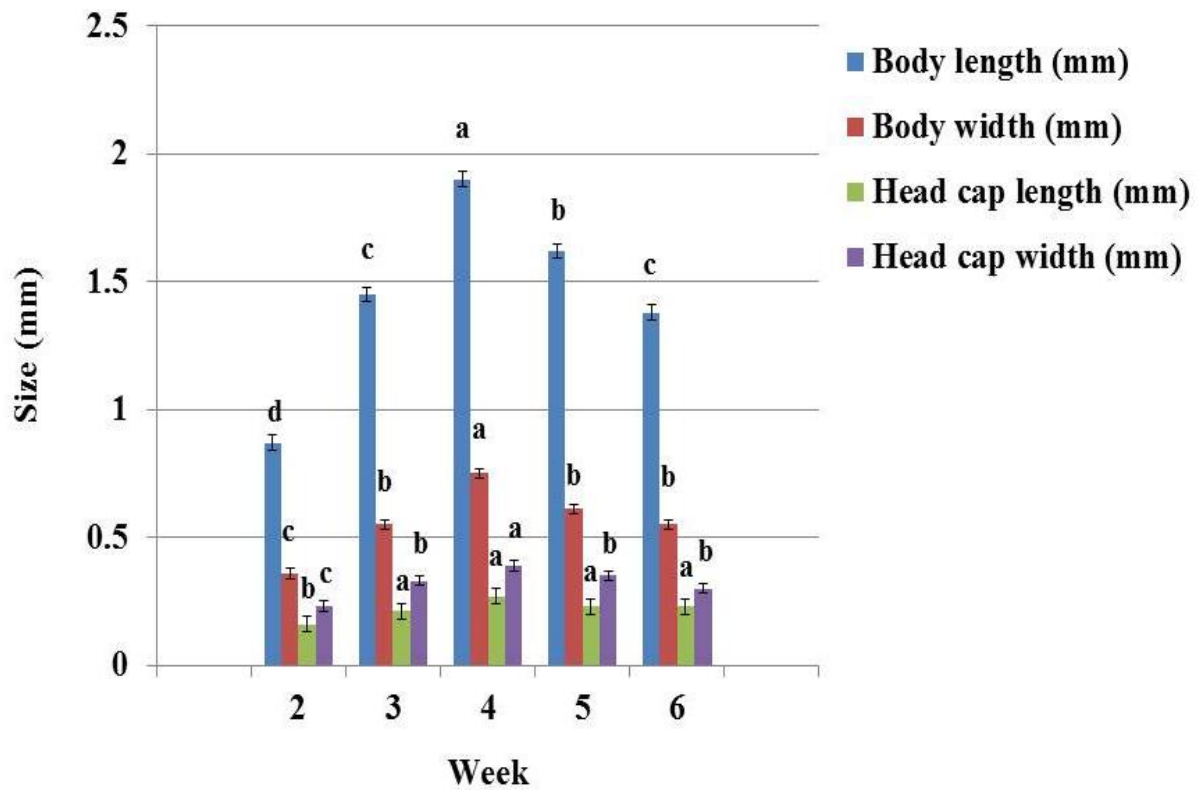


Figure 9. Walnut twig beetle larvae body dimensions. Means (n=38) larvae within the same morphological category (indicated by color) followed by the same lowercase letter(s) are not significantly different ($P > 0.05$; LSD test).

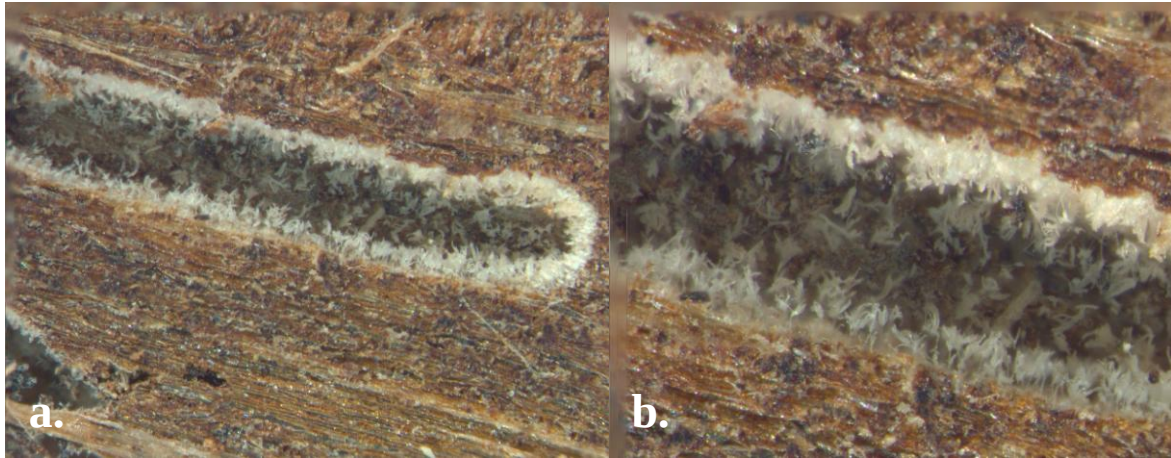


Figure 10(a-b). (a) Female gallery with mycelia of *Geosmithia morbida*; (b) Close up of the same female gallery. Note conidiophores and chains of conidia.

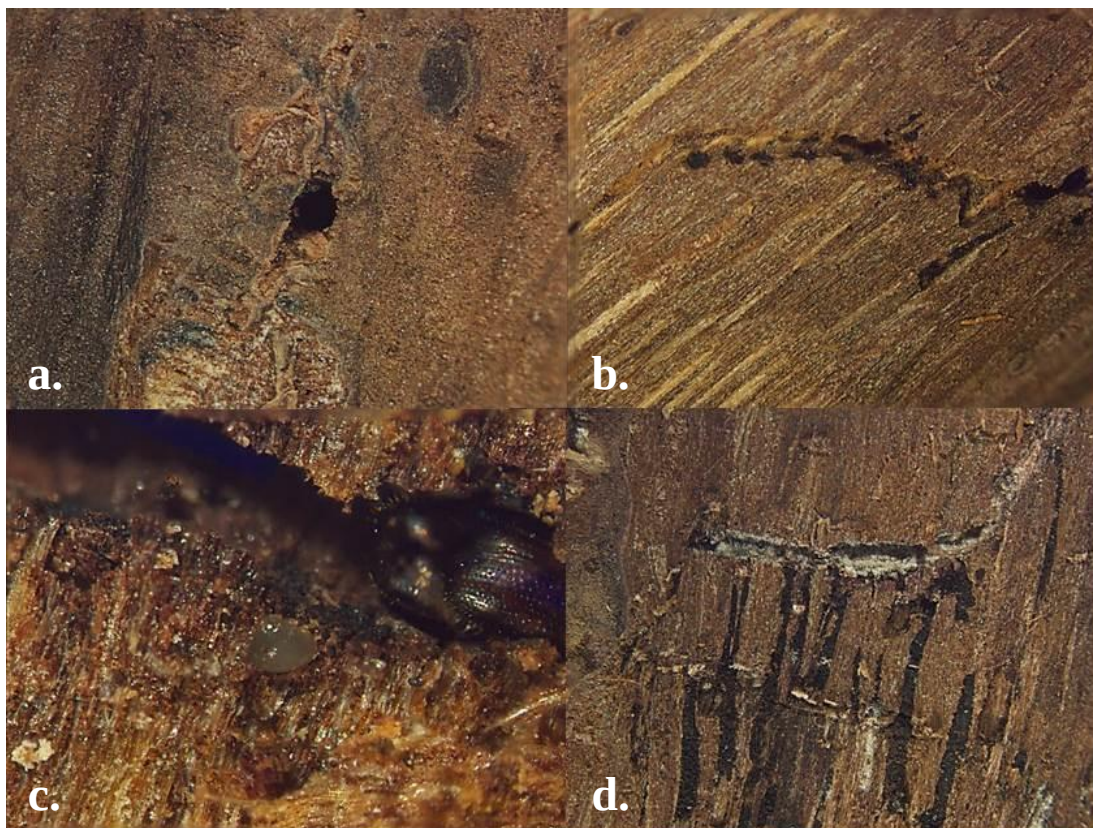


Figure 11(a-d). (a) Entrance/Exit hole of WTB; (b) Female walnut twig beetle gallery with frass caps present; (c) Newly laid egg in female gallery covered with frass cap and walnut twig beetle female to right; (d) Female gallery with mycelia, including conidia, and larval galleries perpendicular to the main female tunnel.

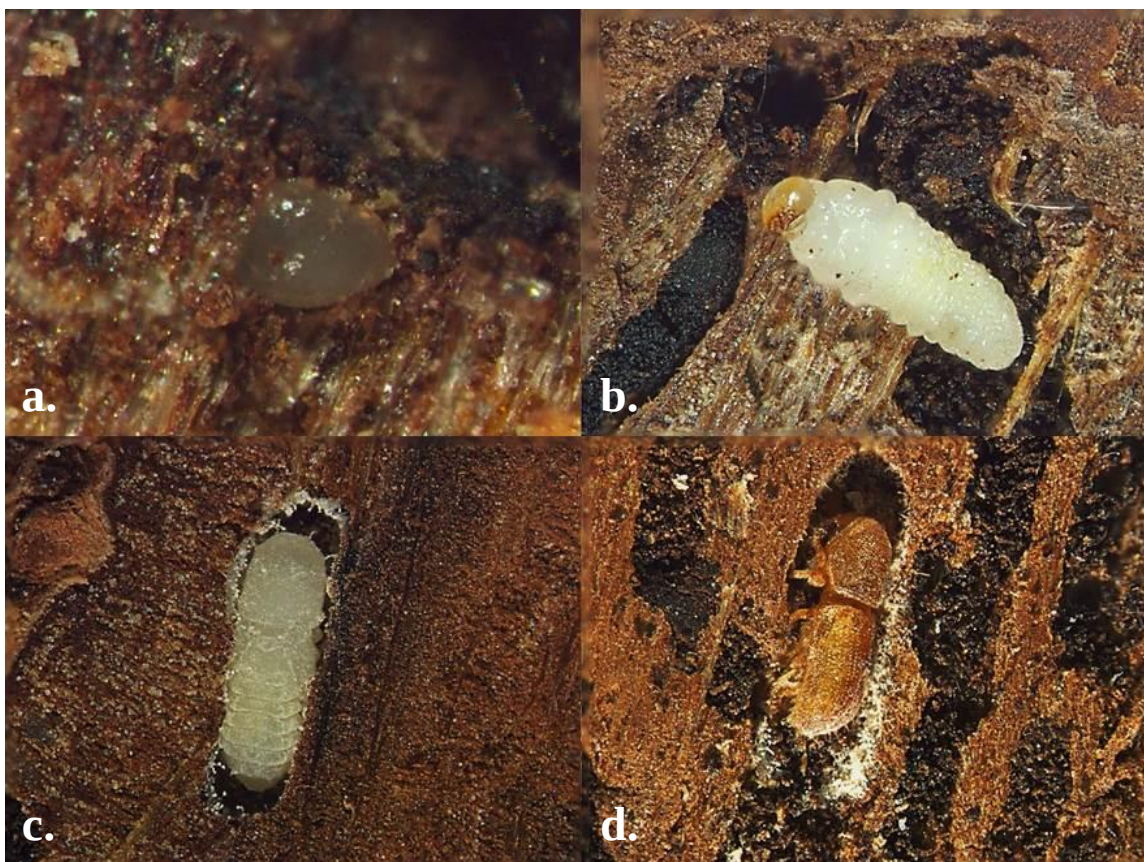


Figure 12(a-d). (a) Close up of walnut twig beetle egg; (b) Walnut twig beetle larva; (c) WTB pupae with mycelia present in chamber; (d) Walnut twig beetle teneral adult with mycelia of *Geosmithia morbida* present in chamber.

conditions for three weeks. Mycelia were not visible in bolts cut between weeks 1 and 2. Frass was not present in female egg-laying chambers or in male chambers, but was present only in larval, pupal, and teneral adult galleries.

Egg and Female Galleries. The female's egg galleries were constructed against the grain of the wood in a straight line or angled on the wood underneath the bark usually in the phloem region (Figure 11b-d). The female chewed a series of small U-shaped indentions along either side of the gallery wall where she deposited a single egg into each of the indentions and then covered them with the frass cap. The frass cap may be useful in protecting the eggs from enemies and keep them from desiccating (Figure 13; Figure 11b-c; Figure 12a). This frass cap is not easily broken or shifted like other frass particles, but is solid and glued together with the aid of the salivary material mixed with the excrement to form the cap (Kirkendall 1983). Some springtail eggs were found scattered throughout an abandoned WTB gallery. While WTB eggs are found in the walls of the female gallery, they were never observed in open tunnels. The absolute fecundity of the female is unknown at this time. However, the average number of egg indentions found in one tunnel with only one female present was 16.6 (7-25) eggs from 17 tunnels

(Table 2). The egg-laying galleries and number of egg indentions are similar to those of other scolytid species, including *P. lautus* (Kirkendall 1983). Kirkendall (1983) suggests that scolytid females construct more than one egg-laying gallery before dying. It is plausible that females will only construct one egg-laying gallery in material that cannot support multiple broods. Females may even re-emerge and find additional plant material to lay their eggs in and to regain their energy (Kirkendall 1983). Re-emerging WTB poses a management problem with because contaminated beetles may possibly be moving multiple times between walnut trees and/or new areas on an already infested walnut tree, thus re-infesting the same tree or infesting new material.

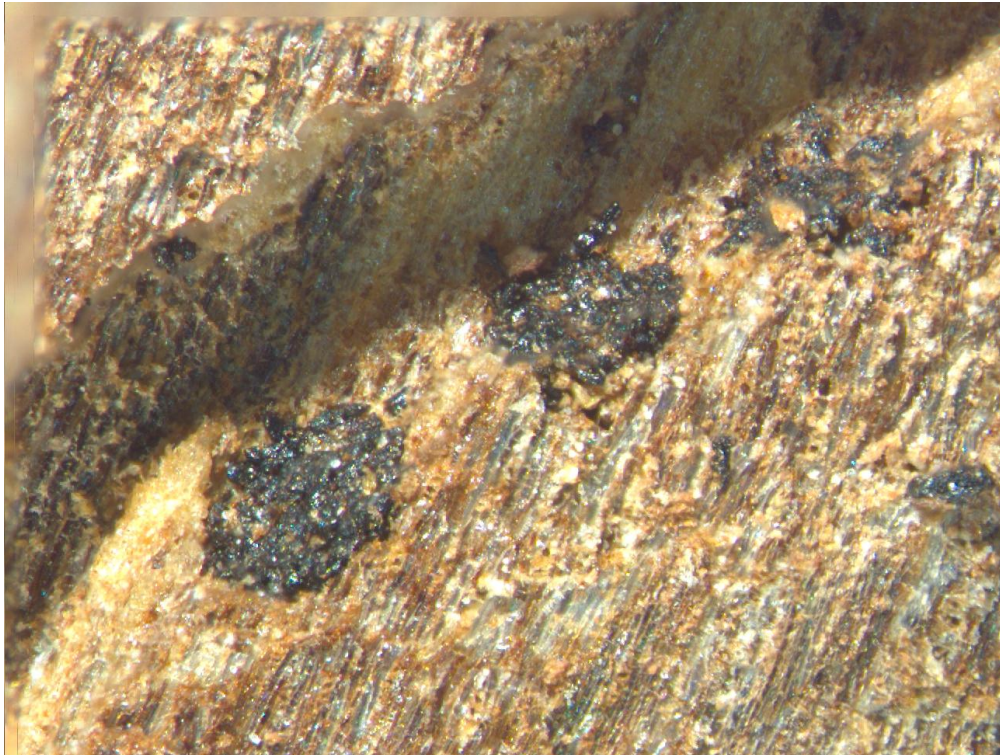


Figure 13. Frass caps in female walnut twig beetle gallery.

Larval and Pupal Galleries. Final larval galleries are constructed perpendicular to the female gallery in a straight line going with the grain of the wood (Figure 11d). Frass and exuviae are often found in larval galleries as they tunnel away from the female gallery. At the end of the larval tunnel, the larvae pupate and develop into teneral adults. In heavily-infested bolts larval chambers overlapped and it was impossible to tell when one began and one ended. Larval galleries were not detected during the first week of observations. However, larval gallery lengths increased every week from week 2 to 5 (Figure 14). Larvae are vermiform in shape and are white with a reddish brown head capsule (Figure 12b). Pupae are white, exarate with their body parts distinguishable (Figure 12c). Pupae and teneral adults resided in the largest average gallery lengths of all WTB life stages (Table 2). Teneral adults are yellowish-brown and soft before they darken to a reddish brown and their elytra harden (Figure 13d) (Blackman 1928). After pupation is completed and teneral adults have hardened, they chew an exit hole through the wood and seek a mate (Figure 11a). Teneral adults likely transport *G. morbida* although their effectiveness is unknown.

Male Galleries. Adult males were found either present in female galleries with one or two females or by themselves in a globular (irregular) shaped gallery. Often the globular male gallery was attached to one or two female egg-laying galleries. This globular gallery may be used for mating since scolytids are known to mate under the bark, and it is the largest chamber (width wise) found from any life stage, with the average width being 2.21 mm which is large enough for two WTB to fit side by side (Table 2). In addition, this entry way chamber or “nuptial chamber” has been documented in other scolytid species where the male remains with the female during the ovipositional process (Kirkendall 1983). Eleven galleries were recorded with both male and females present (excluding teneral adults), five galleries had one male and

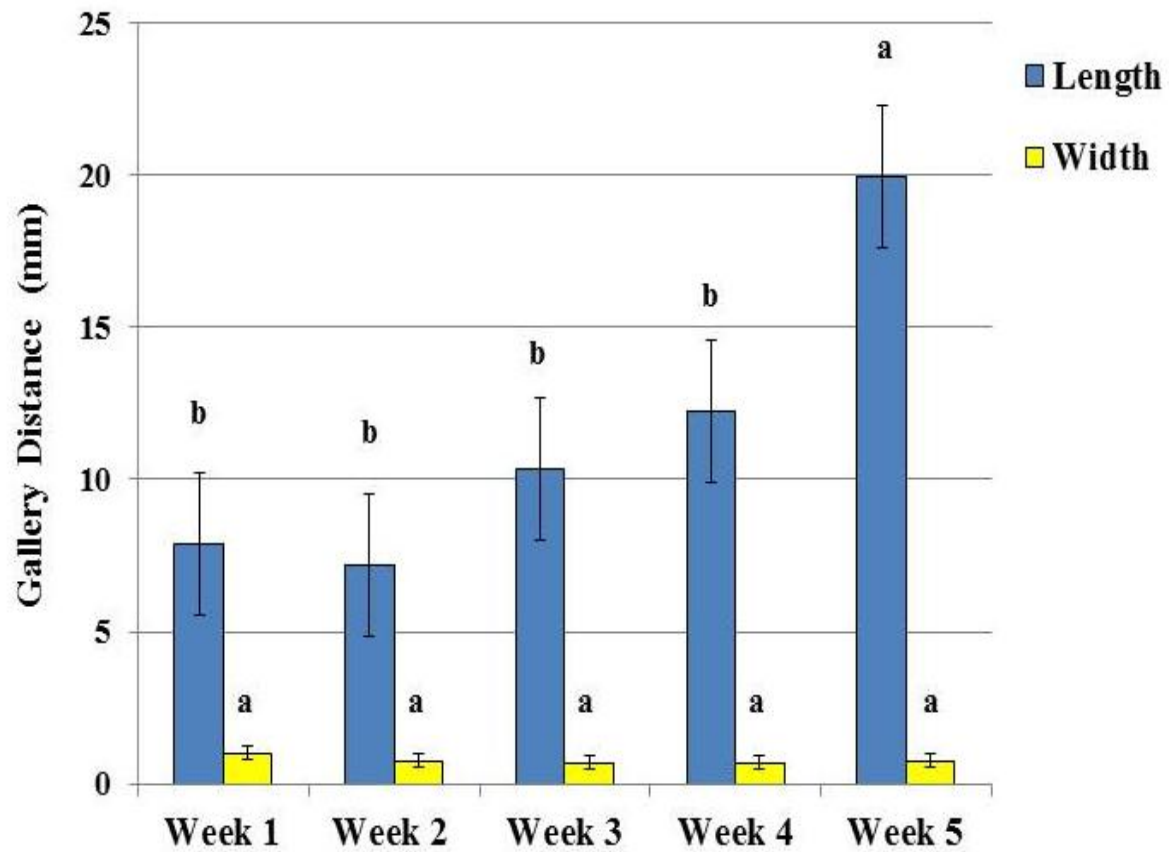


Figure 14. Walnut twig beetle larvae gallery length and width. Means (n=6) within columns of the same distance groups (colors) followed by the same lowercase letter(s) are not significantly different (LSD test; $P > 0.05$).

Table 2. Average length, width and range of walnut twig pupae and adult galleries.

Life Stage	Number of galleries recorded	Gallery Length (Average)	Gallery Width (Average)	Gallery Length (Range)	Gallery Width (Range)
Pupa	18	23.09mm	1.00mm	11.00-31.00mm	1.00mm
Teneral Adult	12	19.70mm	1.28mm	15.00-30.00mm	1.25-1.75mm
Female Adult	33	15.94mm	1.05mm	1.50-30.00mm	0.50-1.50mm
Male Adult	8	8.29mm	2.21mm	4.00-14.00mm	1.00-4.00mm

one female, while six galleries had one male with two females. This suggests that WTB males may mate with multiple females. This type of mating behavior is common in scolytids and has evolved independently at least five times in this family according to Kirkendall (1983). Copulation was not observed during this study. It has been noted in several other scolytid species that the male stays with the female during the ovipositional process, perhaps to prevent other males from copulating with her (Kirkendall 1983, 1984). Another theory is that males block the entrance, thus, protecting the female and eggs from predators and parasitoids (Kirkendall et al. 1997). Lastly, Kirkendall et al. (1997) theorized that the male may block the tunnel entrance to keep the humidity higher for the eggs, thus, increasing the probability of the eggs hatching. Neither conidia nor frass was observed within male galleries.

Winter Trapping

More females (n=83) than males (n=26) were captured in WTB traps (Figure 15). Flight activity was recorded from the end of September till the beginning of December. No WTB were captured in January or February 2012. One male WTB was captured on 15 March 2013. Traps continue to be checked every two weeks for emergence to detect flight activity later into spring.

Conclusions

WTB overwinter primarily as adults and larvae. Although pupae are occasionally present in overwintering populations, they are few in numbers. Eggs were not detected during the winter although it is possible they were present in the galleries formed by adult females. Females lay between 7-25 eggs in the walls of their galleries. The female covers her eggs with frass caps possibly for protection against predators. Absolute fecundity was not determined. Females create a gallery against the grain of the wood in a straight line. Frass was only present in larval, pupal, and teneral adult galleries. Mycelia of *G. morbida* were found only in female, pupal, and

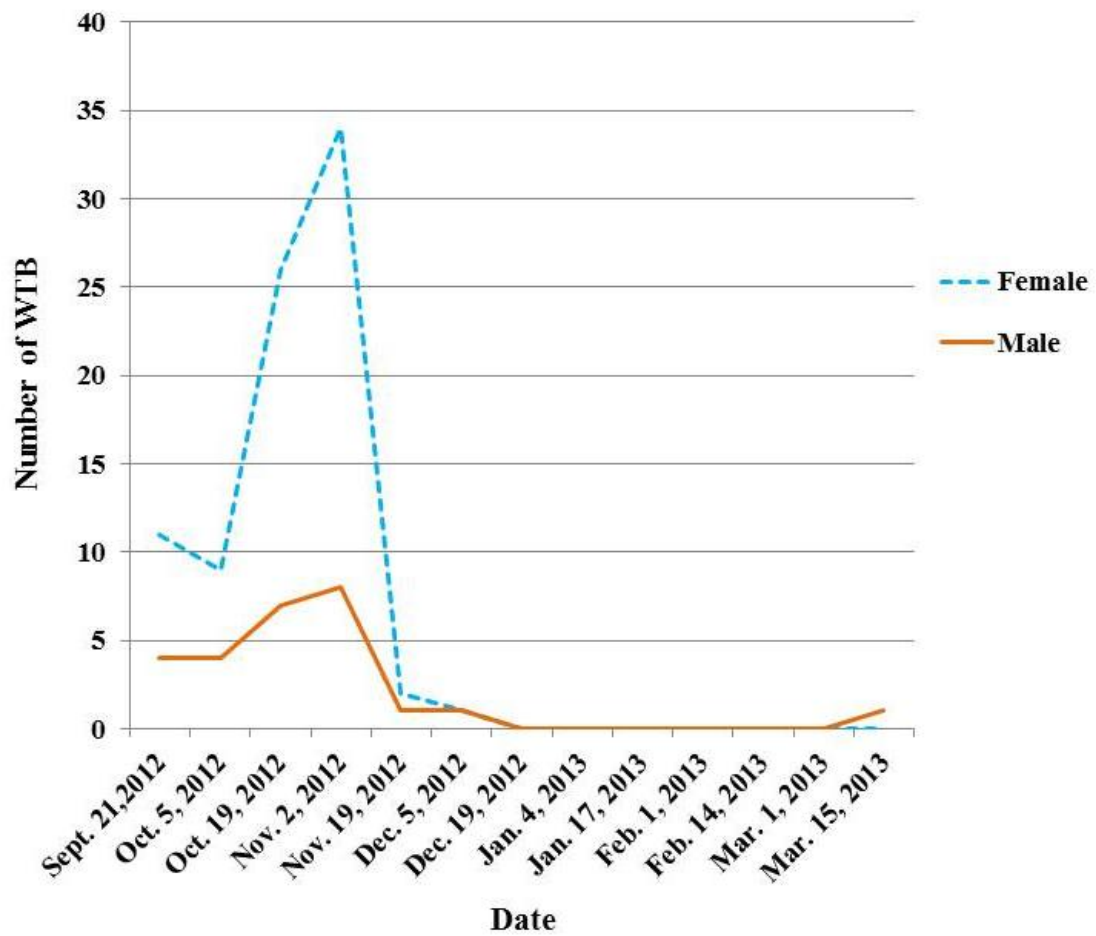


Figure 15. Emergence numbers of walnut twig beetles in traps from late September 2012 to mid-March 2013.

teneral adult galleries. Larval galleries are perpendicular to the female egg-laying gallery and in a straight line parallel with the grain of the wood. Males create a globular gallery where they are suspected to mate with the female. This gallery is often attached to one or more female galleries. It is suspected that male WTB mate with 1 to 2 females.

Immature WTB takes 4 to 5 weeks to develop into adults in laboratory conditions. Three larval instars were recovered from WTB tunnels though more are possible. WTB flight ceases (or slows down) during the winter months of late December-early March in eastern Tennessee. One WTB emerged from jars in April 2011 and two WTB in March 2012. WTB emerged for six months from cut logs that were held in laboratory conditions with no added moisture. Natural enemies are present in the galleries of WTB and may possibly be used as biological control agents. Hopefully, this information can be used to better understand the insect/disease complex of Thousand Cankers Disease and be used to detect and manage this disease.

CHAPTER III

SURVEY OF NATURAL ENEMIES OF THE WALNUT TWIG BEETLE IN EASTERN TENNESSEE

Abstract

Twelve coleopteran species representing five families were collected and identified as potential predators of the WTB in eastern Tennessee. Of the five clerid species collected, three (*Enoclerus nigripes* (Say), *Madoniella dislocatus* (Say), and *Pyticeroidea laticornis* (Say)), were observed to feed on WTB. Clerid larvae were observed within the galleries of the WTB. Consumption rates for three (*E. nigripes*, *M. dislocatus*, and *P. laticornis*) of the five clerid species were recorded. Behavioral comparisons for *M. dislocatus* and *P. laticornis* consisted of predator recognition time, time between attacks, total feeding time, and preening behavior. In addition to the 12 coleopteran species, two parasitoids (*Neocalosoter* sp. and *Theocolax* sp. (Cerocephalinae)) emerged from WTB infested *J. nigra*. The identification of key natural enemies associated with WTB has important implications for the implementation of biological control of WTB in newly infested areas.

Introduction

Black walnut is native to eastern North America where it occurs in forest coves and well-drained river bottoms (Williams 1990, Tisserat et al. 2009). Within the last decade, western states have experienced increasing mortality of *J. nigra* (Leslie et al. 2010, Seybold et al. 2010b). Tree deaths are attributed to the fungal species, *Geosmithia morbida* Kolařík et al. (Leslie et al. 2010, Seybold et al. 2010b, Tisserat et al. 2009). The only known vector of *G. morbida* is the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Newton et al. 2009, Tisserat et.

al. 2009). This newly recognized disease/insect complex was named Thousand Cankers Disease (TCD) due to the number of coalescing cankers produced by *G. morbida* (Seybold et al. 2010b).

Like several other bark beetles, WTB bores into the phloem-cambial region of twigs, branches, or trunks of walnuts. Minute emergence holes in the bark are a good indication of infestation by WTB. In 2010, TCD was discovered in Knox Co., TN representing the first documentation of these pests east of the Mississippi River (Grant et al. 2011). Infestations of WTB have since been documented in VA and PA (Virginia DAC 2012, Pennsylvania DAC 2012) and NC (North Carolina DAC 2013). Various management methods are currently being investigated to prevent further spread of this disease including chemical studies to evaluate various insecticide products and application techniques for controlling WTB. While the status and usefulness of insecticides for control of WTB remain unclear, natural enemies of this pest may provide the most feasible and sustainable method of suppressing pest populations. Although little is known about the life history or the occurrence and impact of natural enemies on this invasive pest, generalist predators have been documented to reduce pest populations of bark beetles (Cronin et al. 2000).

Some generalist predaceous beetles in the families Laemophloeidae, Monotomidae, and Trogossitidae were found to be associated with WTB on walnut in California, as well as two Hymenoptera species in the families Bethyridae and Pteromalidae (Seybold 2010a). In 2010, surveys were initiated in eastern Tennessee to search for potential biological control agents for use against WTB or that may play a role in regulating WTB populations.

Materials and Methods

Study Sites and Experimental Design

Three field sites were surveyed for predators and parasitoids of WTB from September 2010 to November 2012. These field sites were selected based on preliminary observations of WTB-infested walnut trees. Walnut bolts (30-35 cm long and 10-14 cm diameter) were cut from infested *J. nigra* obtained from two sites (Lakeshore (35°55'23.59"N; 83°59'29.47"W) and Emory Road (36°5'5.33"N; 83°55'54.98"W) in Knox Co., TN). Samples were taken to the laboratory and maintained in plexi-glass containers at 22°C. Containers were observed daily for emergence of native predators of WTB from August 2010-August 2011.

At the third site located at Strong Stock Farm (36° 3'7.1526"N ; 83° 47'23.3802"W) in Knox Co., TN, a survey for potential native predators was initiated using two soda bottle (2-liter) traps hung in the upper and lower canopies of six mature black walnut trees from May 2012-October 2012. Trees selected for evaluation were located either on the edge of forested areas or in clumps in open fields. Twelve soda bottle traps were each baited with a black walnut bolt (~12 cm long and ~4 cm diameter), each with a 1.27 cm hole drilled to a depth of 11.5 cm into the center of the bolt and filled with 95% ethanol. Traps were constructed using 2-liter soda bottles coupled with Nalgene® 250 mL wide mouth bottles screwed into the opening of the soda bottle lid. The bottoms of the Nalgene® bottles were removed and replaced with mesh netting fastened with hot glued to allow rain water to pass through without drowning the specimens. Two holes (10-12 cm diameter) were cut on either side of the soda bottles to allow insects to enter the trap. Traps were placed on the outer edge of limbs and checked every other day for potential native predators. Collected predators were placed into 118 ml plastic specimen cups and taken to the laboratory for observation. Each predator was identified and individually placed

in a Petri dishes (9 cm) provided with a sheet of filter paper, a wet cotton ball and live specimens (2-5) of WTB to sustain the predators. Specimens of WTB were reared in the laboratory by placing fresh black walnut bolts into acrylic glass boxes (size 40.5cm x 30.5cm) containing WTB infested bolts and maintained at 22°C. These acrylic glass containers had a square hole (14 cm x 11 cm) on the top of each container and covered with a fine mesh glued over the hole to allow for air circulation and prevent WTB from escaping.

Of the predators collected, two species, *Madoniella dislocatus* (Say) and *Pyticeroides laticornis* (Say), were selected to assess their feeding behavior. To determine if adults from the two clerid species exhibited a preference for live or dead WTB and if they were generalist feeders, experiments were conducted under laboratory conditions. Experiments were conducted as choice tests replicated seven times and consisted of exposing three live and three dead prey of each specific species to predators for 2 weeks. Prior to each test, predators (n=14) selected for the behavior tests were starved for 24 hours prior to evaluation. Data catalogued from these tests included: predator recognition time, time between attacks, total feeding time, preening behavior, and consumption rates. Each test was terminated after 15 minutes if the specimen failed to feed (Frazier et al. 1981). Observations continued until the specimen declined to feed on prey (waited 15 minutes after last feeding). Prey escape behavior was noted in addition to predator feeding and preening behavior (Frazier et al. 1981). Tests were timed using a stop watch and specific behavior activity was recorded in seconds. Consumption rates were recorded for *Enoclerus nigripes* (Say), *M. dislocatus*, and *P. laticornis*. In addition, a choice/preference test was run with three *P. laticornis* and two *M. dislocatus* in which the predators were each fed seven WTB and seven *Xylosandrus crassiusculus* (Motschulsky) for 10 days. Lastly, a choice test performed

for 10 days using seven *Tribolium confusum* Jacquelin du Val and seven WTB as potential prey for three *P. laticornis* and two *M. dislocatus*.

Data Analysis

Shapiro-Wilks W test of normality and Levene's test of homogeneity of variances were used to verify that the behavioral data (species, number of specimens, prey recognition time, time between attacks, total feeding time, consumption rates, total preening time, and feeding preference) collected conformed to the assumptions of the student's *t*-test (SAS 2005).

Results and Discussion

Twelve predator species and two parasitoid species were associated with WTB on *J. nigra* in Tennessee (Table 3). From these, two clerid species consisting of seven *M. dislocatus* and seven *P. laticornis* (Figure 16b, c) were captured in soda bottle traps and later observed to feed on WTB. Two specimens of *E. nigripes* (Figure 16d) were collected from WTB-infested bolts confined in acrylic glass containers in the laboratory. From these two specimens, only one lived and consumed 198 WTB in 89 days for an average consumption rate of 2.23 live WTB per day. *E. nigripes* is a native species distributed throughout the eastern and central U.S. (Leavengood 2008). Its range extends from Maine to Minnesota and from South Carolina to Oklahoma. This predator species has been recorded to feed on adults, pupae, and larvae of wood-boring beetles on hardwoods (Arnett et al. 2002). Two additional species of clerids, *Enoclerus ichneumoneus* (Fab.) and *Placopterus thoracicus* (Olivier), were captured in soda bottle traps although their consumption of WTB is unknown (Figure 16a). Larval and adult specimens of the clerids were observed on the bark of WTB-infested logs, while only clerid larvae were observed within the galleries with WTB in black walnut bolts maintained in the

laboratory. In one instance, a clerid larva consumed one WTB larva by lifting it up in the air and pinching its side with its mandibles.

Table 3. Potential predators and parasitoids of walnut twig beetle collected in Knox County, TN.

Family	Genus	species	Author	Collection Method	Habits	Distribution
Cleridae	<i>Enoclerus</i>	<i>nigripes</i>	(Say)	Emerged from WTB infested walnut bolts in laboratory.	Feeds on all life stages of bark beetles. (Leavengood 2008) Len. 5-7mm	DE, District of Columbia, FL, GA, IL, IN, KS, ME, ML, MD, MI, MN, MO, NH, NY, NC, OH, OK, PA, SC, TN, VA, WI, and Manitoba
Cleridae	<i>Enoclerus</i>	<i>ichneumoneus</i>	(Fab.)	Soda bottle trap hung in <i>J. nigra</i> .	Predator of bark beetles (Leavengood 2008) Len. 8-11mm	ON, NY, PA, MD, OH, IN, IL, WI, DC, VA, AL, SC, GA, FL, AR, TX, KS, IA (Downie and Arnett 1996)
Cleridae	<i>Madoniella</i>	<i>dislocatus</i>	(Say)	Soda bottle trap hung in <i>J. nigra</i> .	Generalist predator, feeds on bark beetles (Leavengood 2008) Len. 3.5-6mm	PQ, ON, NY, NJ, PA, OH, IN, IL, WI, GA, SC, WV, TX, KS, CO
Cleridae	<i>Pyticeroidea</i>	<i>laticornis</i>	(Say)	Soda bottle trap hung in <i>J. nigra</i> .	Generalist predator, feeds on bark beetles (Leavengood 2008) Len. 4-7mm	PQ, ON, NY, CT, NJ, MD, PA, OH, IL, IN, VA, WV, NC, SC, GA, FL (Downie and Arnett 1996)
Cleridae	<i>Placopterus</i>	<i>thoracicus</i>	(Olivier)	Soda bottle trap hung in <i>J. nigra</i> .	Predator of bark beetles (Leavengood 2008) Len. 5-8mm	IL, IN, OH, PA, PQ, NY, NJ, MD, VA, SC, GA, FL, SD (Downie and Arnett 1996)
Histeridae	<i>Aeletes</i>	<i>floridae</i>	(Marseul)	Emerged from WTB infested walnut bolts in laboratory. Found in WTB tunnels.	Predator (possibly of eggs).	Eastern US MD to OH south FL to TX.
Laemophloeidae	<i>Dysmerus</i>	<i>basalis</i>	Casey	Emerged from WTB infested walnut bolts in laboratory.	Found in Scolytine burrows in oaks Len. 1.7mm	D.C., NJ, AL, FL
Laemophloeidae	<i>Leptophloeus</i>	<i>angustulus</i>	(LeConte)	Emerged from WTB infested walnut bolts in laboratory. Found in WTB tunnels.	Scolytine predator especially in oaks Len. 1.6-2.0mm	IN, MD, OH, FL, OK, MO
Laemophloeidae	<i>Narthecius</i>	<i>grandiceps</i>	LeConte	Emerged from WTB infested walnut bolts in laboratory.	Scolytine predator L. 3.0mm	PA, FL, OK OK, FL, PA, and MI.
Pteromalidae	<i>Neocalosoter</i>	sp.		Emerged from WTB infested walnut bolts in laboratory.	Scolytine parasitoid Associated with <i>Pityophthorus consimilis</i> , <i>Styphlosoma granulatatum</i> , <i>Thysanoes fimbicornis</i> (Natural History Museum 2012)	NC, FL, Costa Rica, Nearctic (Natural History Museum 2012)
Pteromalidae	<i>Theocolax</i>	unidentified		Emerged from WTB infested walnut bolts in laboratory. Pupae found in WTB larval tunnels.	Found in WTB tunnels. Parasitoids of bark beetles (Xiao and Huang 2001)	China and eastern U.S.
Trogossitidae	<i>Tenebroides</i>	<i>marginatus</i>	P.Beauv.	Emerged from WTB infested walnut bolts in laboratory.	Larvae are predacious and hunt in galleries of wood boring insects. Adults are fungivores and feed on dead trees. (Downie and Arnett 1996) Len. 4.3-7mm	NY, PA, OH, IL, WV, NC, SC, GA, AL, FL, KY, MS, LA, TX, KS, IA. (Downie and Arnett 1996)
Zopheridae	<i>Bitoma</i>	<i>quadriguttata</i>	(Say)	Emerged from WTB infested walnut bolts in laboratory. Found in WTB tunnels.	Found in under the bark of dead hardwoods and pines. (Lord et al 2011)	CT, DE, IN, MD, NJ, NH, NY, OH, AL, FL, GA, MI, NC, SC, TN, WV, VA, OK, TX, USA; Ontario, Canada (Lord et al. 2011)
Zopheridae	<i>Colydium</i>	<i>lineola</i>	Say	Emerged from WTB infested walnut bolts in laboratory.	Predator on ambrosia beetles and other wood boring insects (Miller 1984)	Washington D.C., DE, IL, IN, MD, NJ, NY, PA, AL, AR, FL, GA, LA, MI, SC, TN, AZ, CA, OK, TX, OR, WA, MO, USA; British Columbia, Ontario, Canada (Lord et al. 2011)

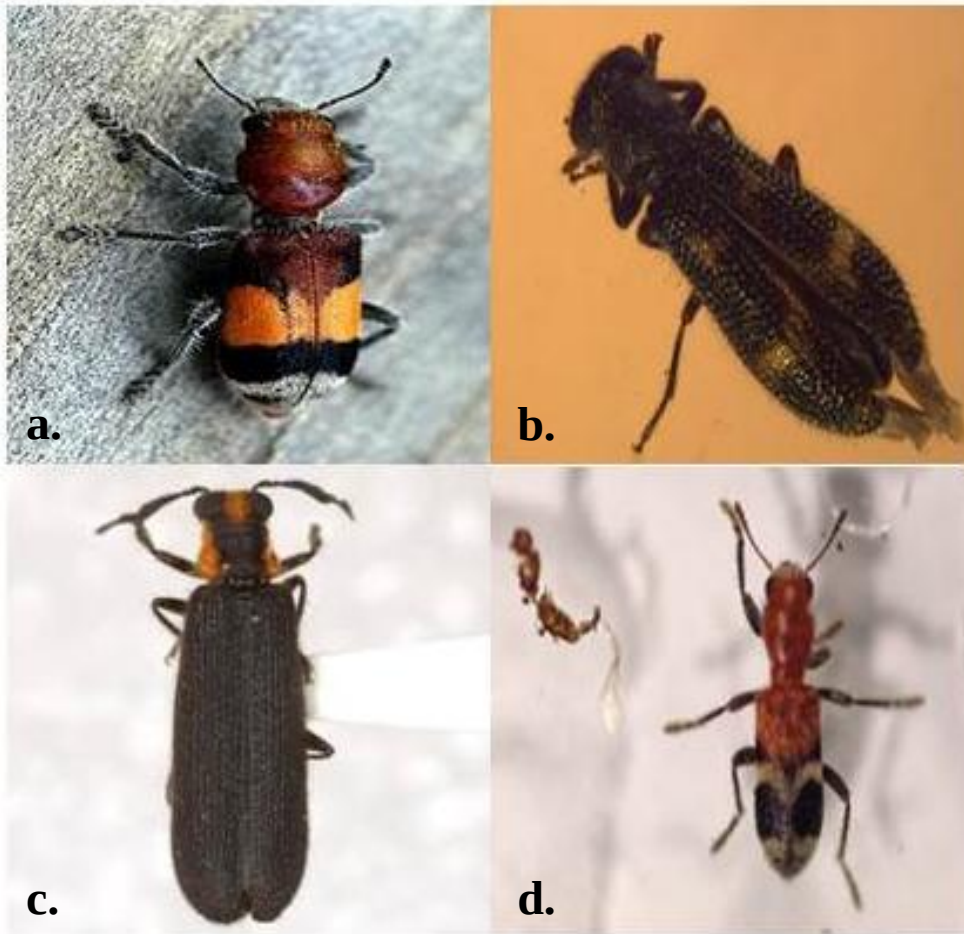


Figure 16 (a-d): (a) Dorsal view of *Enoclerus ichneumoneus*; (b) Dorsal view of *Madoniella dislocatus*; (c) Dorsal view of *Pyticeroides laticornis*; (d) Dorsal view of *Enoclerus nigripes* (photos a,c, Mike Quinn, TexasEnto.net).

Seven additional coleopteran species that are potential predators of WTB emerged from infested bolts held in the acrylic glass containers. Some of these included *Leptophloeus angustulus* (LeConte) (Laemophloeidae) (Figure 17a), a native predator in the eastern U.S. and Canada with a range extending from Maryland to Mississippi and from Florida to Canada (Arnett et al. 2002; Majka and Chandler 2009). *L. angustulus* is often found in scolytine galleries on hardwoods. Adult *Narthecius grandiceps* LeConte (Laemophloeidae) (Figure 17b) are predaceous and are native to the southern and eastern U.S. ranging from Oklahoma to Florida and north to Pennsylvania and Michigan (Arnett et al. 2002, Thomas 2007). The predator *Tenebroides marginatus* P. Beauv. (Trogossitidae) (Figure 18b) is native to the U.S. (Arnett et al. 2002). *Bitoma quadriguttata* (Say) (Zopheridae) (Figure 18c) is native to the eastern U.S. ranging from Florida, New Hampshire, and Texas (Arnett et al. 2002). *B. quadriguttata* is reported as a saproxylic beetle from oak by Ulyshen and Hanula (2009) and perceived to feed on conidia within galleries (Lawrence 1977). *Aeletes floridae* (Marseul) (Histeridae) (Figure 18a) is widespread throughout the eastern U.S. ranging from Ohio south to Florida and Texas. This species appears to develop underneath the bark of dead or dying trees (Arnett et al. 2002). All of these coleopteran species have been confirmed to emerge from *J. nigra* and are known to feed on prey in the subfamily Scolytinae.

In addition, two species of parasitoids (Cerocephalinae) emerged from WTB-infested bolts of *J. nigra* (Table 3). An undetermined species in the genus *Theocolax* pupated in the larval galleries of WTB; thus, it may possibly be an ectoparasitoid on WTB larvae. Several adult *Theocolax* were also found searching female WTB galleries possibly for a suitable place to lay their eggs (Figure 19a-c). This particular species emerged in high numbers in infested walnut bolts held in containers in the laboratory implying a possible parasitoid relationship with WTB in

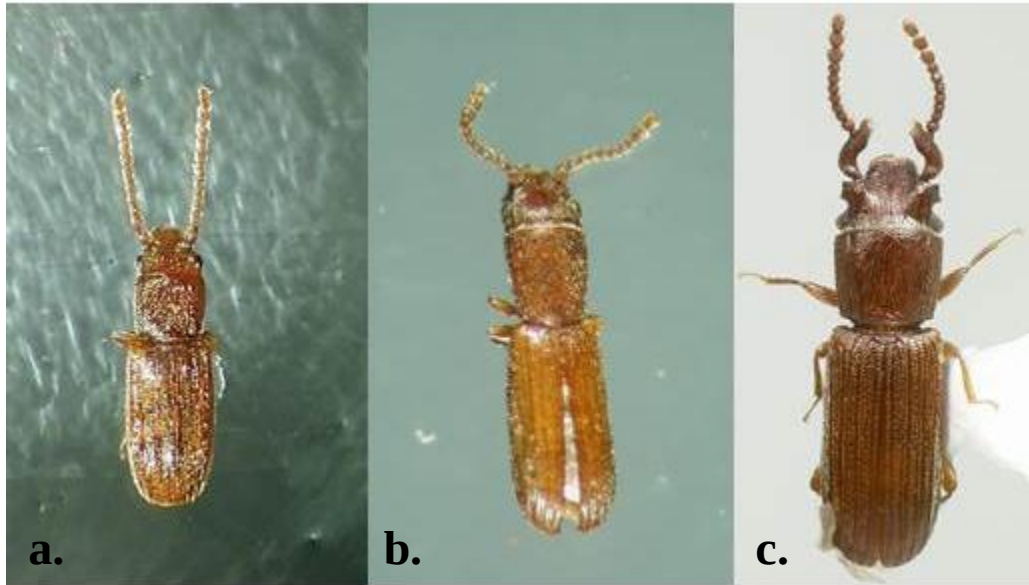


Figure 17 (a-c): (a) Dorsal view of *Leptophloeus angustulus*; (b) Dorsal view of *Narthecius grandiceps*; (c) Dorsal view of *Dysmerus basalis* (photo c, Michael C. Thomas, 2009).



Figure 18 (a-d): (a) Dorsal view of *Aeletes floridiae*; (b) Dorsal view of *Tenebroides marginatus*; (c) Dorsal view of *Bitoma quadriguttata*; (d) Dorsal view of *Colydium lineola* (photo a, Jeffrey P. Gruber, 2002 Madison, WI; photo d, Ashley Bradford, 2012 Alexandria, Fairfax County, VA).



Figure 19(a-c): *Theocolax* species found emerging from *Juglans nigra* bolts: (a-b) Lateral view of *Theocolax* sp.; (c) Ventral view of *Theocolax* sp.

black walnut. The other parasitoid species collected was *Neocalosoter* sp.; however, only one specimen was recovered during the collecting period.

In the behavioral tests conducted on *M. dislocatus* and *P. laticornis*, both similarities and differences in feeding and preening behaviors were found between the two species. For example, both *M. dislocatus* and *P. laticornis* strongly (t test; $t=1.45$; $df=2$; $P < 0.05$) prefer live rather than dead WTB prey (Figure 20). This infers both species are unlikely to be scavengers on WTB should live WTB be present. In addition, WTB exhibited thanatosis, death feigning, as a defense mechanism against predator attacks. This behavior was occasionally observed when a predator attacked WTB. WTB would tuck its legs and antennae close to its body and lay very still. The predator would struggle to pick up its prey and after a few seconds cease its attack and wander away from its prey. In addition to WTB, both *M. dislocatus* and *P. laticornis* were observed to death feign when being removed from their Petri dish in order to clean out the debris from a feeding. Both clerid species would tuck their legs and antennae close to their body and lay on their dorsal side.

Pyticeroideis laticornis was observed to feed on dead prey and is more likely to exhibit a scavenger behavior than *M. dislocatus*, although its scavenger behavior in the laboratory may be attributed to its higher consumption rate, meaning it may not have been provided sufficient live prey to satiate its hunger. *P. laticornis* consumed a significantly (t -test; $t=1.45$; $df = 2$; $P < 0.05$) greater number (27% more) of WTB in 2 weeks than *M. dislocatus* (Figure 20). This is probably due to *P. laticornis* being larger than *M. dislocatus* and requiring more food to sustain life functions. In addition, *M. dislocatus* was significantly (t -test; $t = 1.42$; $df = 2$; $P < 0.05$) faster in its recognition time, time between attacks, and total feeding time than *P. laticornis* (Figure 21a-c). *M. dislocatus*; however, did spend significantly (t -test; $t = 1.66$; $df = 2$; $P < 0.05$) more time

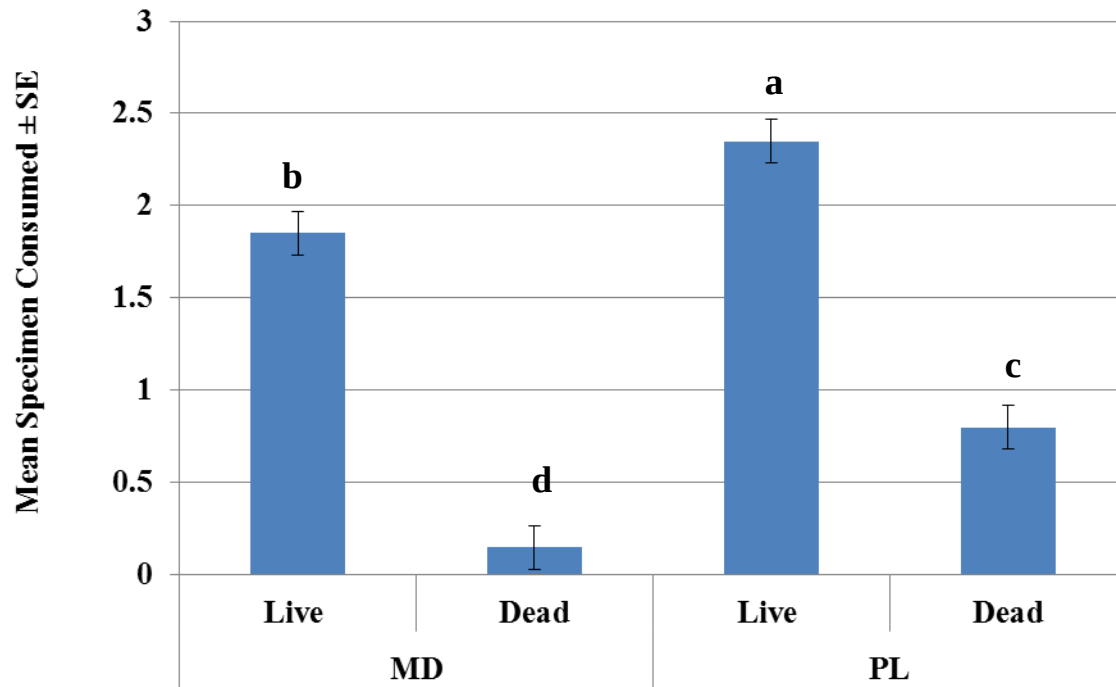


Figure 20. Mean $\pm$ SE live and dead specimens consumed by *Madoniella dislocatus* (MD) and *Pyticeroides laticornis* (PL). Means followed by different lowercase letter(s) are significantly different (*t*-test; $P < 0.05$).

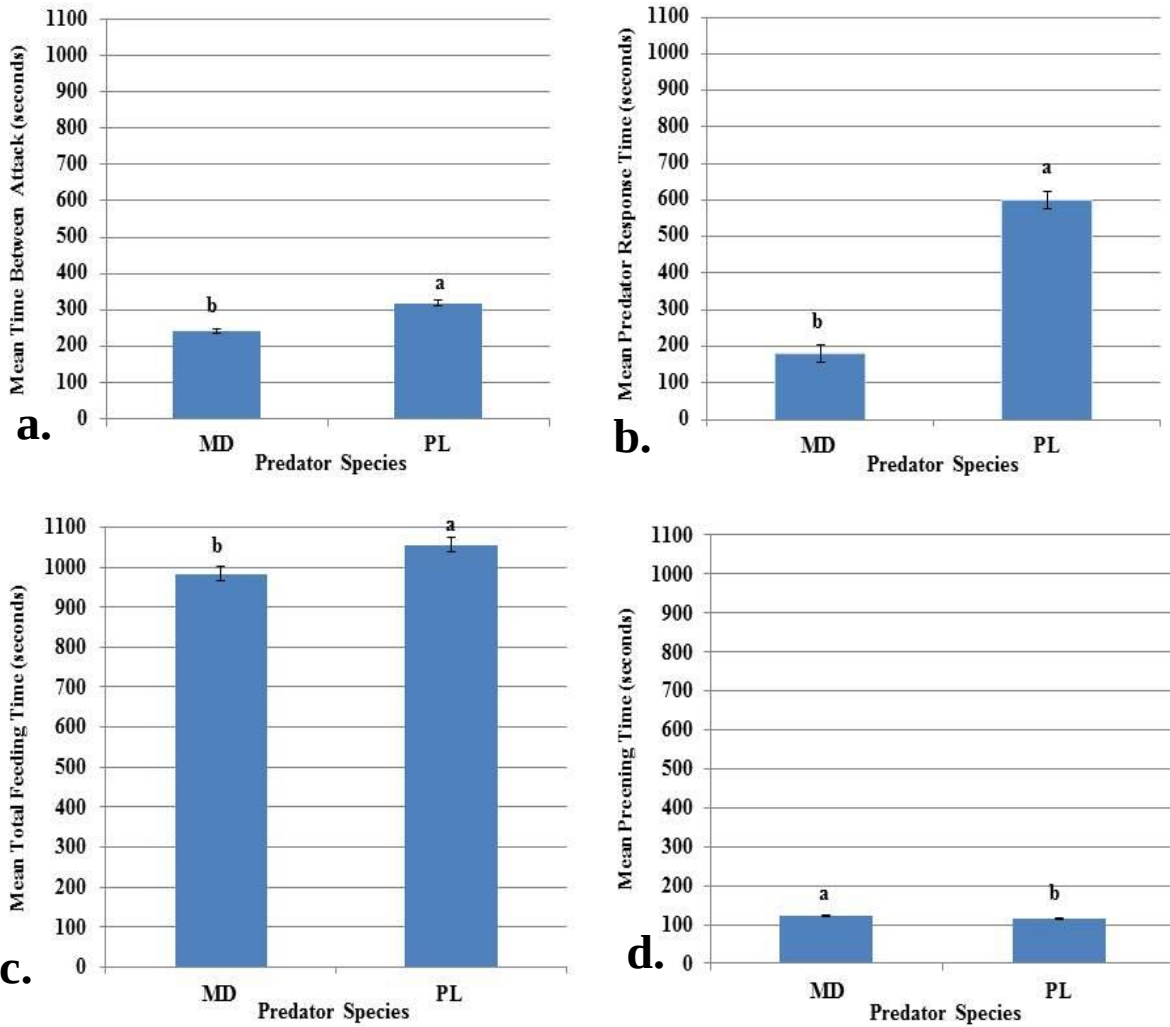


Figure 21. (a) Mean $\pm$ SE time between attacks (seconds) *Madoniella dislocatus* (MD) and *Pyticeroides laticornis* (PL); (b) Mean $\pm$ SE predator response time, by *Madoniella dislocatus* (MD) and *Pyticeroides laticornis* (PL); (c) Mean $\pm$ SE total feeding time (seconds) by *Madoniella dislocatus* (MD) and *Pyticeroides laticornis* (PL); (d) Mean $\pm$ SE total preening time (seconds) by *Madoniella dislocatus* (MD) and *Pyticeroides laticornis* (PL). Means followed by different lowercase letter(s) are significantly different (t -test; $P < 0.05$).

preening than *P. laticornis* (Figure 21d). Neither species consumed specimens of *T. confusum* in the choice test between WTB and *T. confusum* (Figure 22a, c). The lack of consumption of *T. confusum* may have been due to the size difference between *T. confusum* and both clerid species tested. *T. confusum* is similar in size (3.2 mm) to either species (Table 3), which may have made it unsuitable as a prey species. In the choice test between WTB and *X. crassiusculus*, *P. laticornis* significantly (*t*-test; $t = 1.85$; $df = 2$; $P < 0.05$) preferred WTB (1.5-1.9 mm) over *X. crassiusculus* (2.1-2.9 mm) (Figure 22d) (Blackman 1928). These findings support earlier conclusions by researchers that prey size can influence prey preference (McKemey et al. 2003, Troost et al. 2008). However, *P. laticornis* did feed on *X. crassiusculus*, while *M. dislocatus* did not feed on *X. crassiusculus* (Figure 22b, d). *X. crassiusculus* is present in black walnut trees and was collected in the same soda bottle traps used to collect the clerid species. From collection data, we found specimens of *X. crassiusculus* to be present within the same trees as *P. laticornis*.

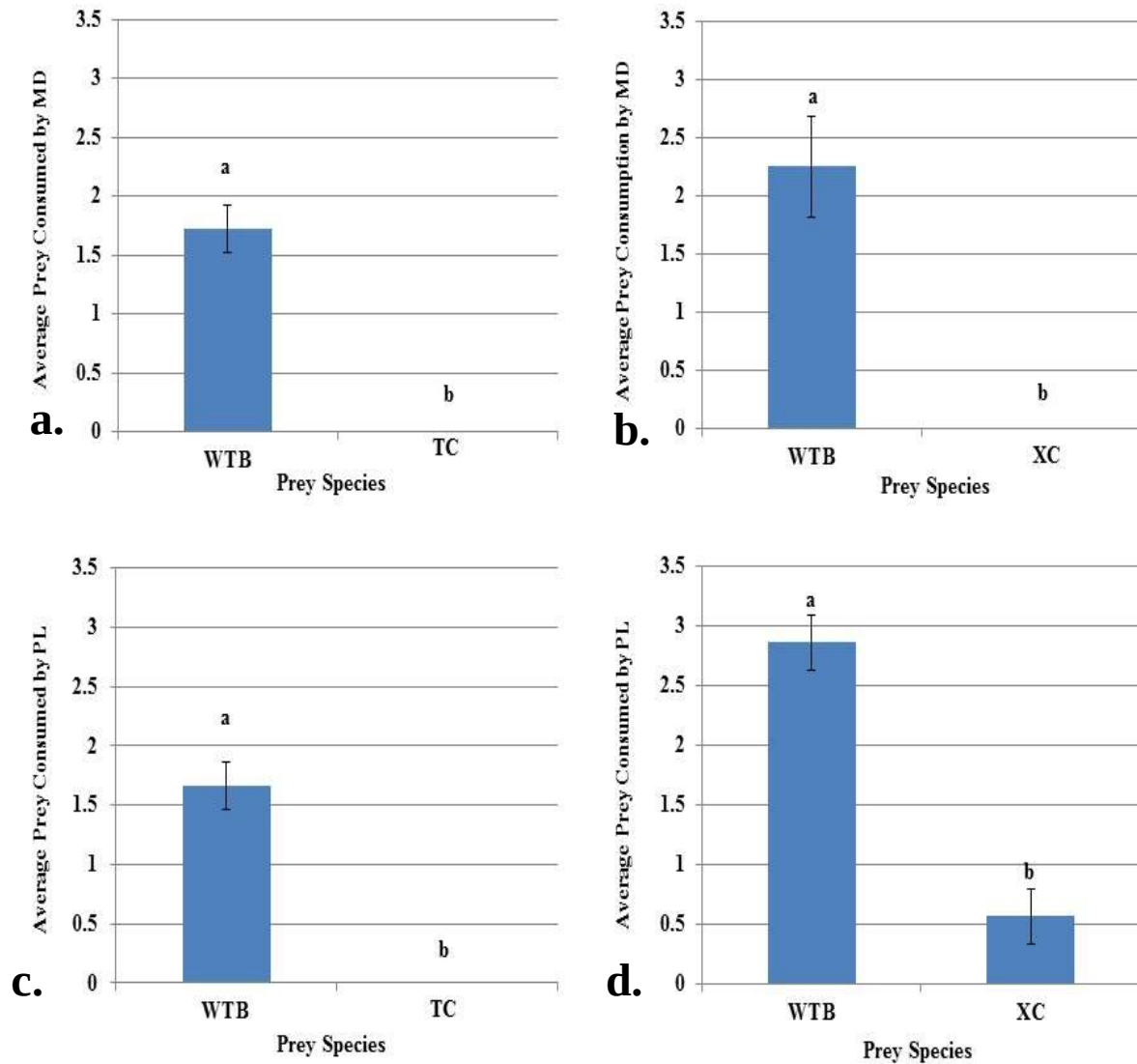


Figure 22. (a) Mean $\pm$ SE *Pityophthorus juglandis* (WTB) and *Tribolium confusum* (TC) specimens attack by *Madoniella dislocatus* (MD); (b) Mean $\pm$ SE *Pityophthorus juglandis* (WTB) and *Xylosandrus crassiusculus* (XC) specimens attack by *Madoniella dislocatus* (MD); (c) Mean $\pm$ SE *Pityophthorus juglandis* (WTB) and *Tribolium confusum* (TC) specimens attack by *Pyticeroides laticornis* (PL); (d) Mean $\pm$ SE *Pityophthorus juglandis* (WTB) and *Xylosandrus crassiusculus* (XC) specimens attack by *Pyticeroides laticornis* (PL). Means followed by different lowercase letter(s) are significantly different (*t*-test; $P < 0.05$).

and *M. dislocatus* and are a food source for *P. laticornis*, but not for *M. dislocatus*. Again, size appears to play a significant behavioral role in the clerid's feeding dynamic as *M. dislocatus* is too small to eat *X. crassiusculus*, while *P. laticornis* can manage to feed on *X. crassiusculus*, when necessary. *P. laticornis*'s consumption rate for *X. crassiusculus* was low compared to WTB; however, *P. laticornis* may not need to feed on as many *X. crassiusculus* to reach satiation due to the larger size of *X. crassiusculus*.

Both *M. dislocatus* and *P. laticornis* exhibited feeding and hunting behaviors similar to *Thanasimus dubius* (Fab.) (Frazier et al. 1981). Upon contact with a potential prey source, both predators moved their antennae in an up-and-down motion while walking rapidly in search of prey. Based on their rapid recognition of the prey source and their actions, it appears these predators respond to bark beetle chemical and visual cues. Once the predator recognized the prey source and attacked, it seized the prey, positioned the prey on its back with the underside of the prey's head aligned with the predator's mandibles, and began feeding. The predators used their pro and mesothoracic legs to manipulate the prey and their metathoracic legs to stabilize and balance their body during feeding. Both *P. laticornis* and *M. dislocatus* fed on soft tissue - usually in the cervix region of the prey. Both predator species frequently decapitated the prey before proceeding to feed on the internal tissue. The predator often left the head capsule, elytra and wings uneaten. Grooming or preening behaviors occurred either between attacks or at the end of the feeding period. Preening behavior consisted of the predator grooming their prothoracic legs with their mandibles and/or pulling down their antennae with their prothoracic legs one at a time and grooming them with their mandibles. *P. laticornis* and *M. dislocatus* expressed different temperaments in the laboratory. *M. dislocatus* specimens appeared calmer, less active and easier to care for because they were not constantly active and trying to escape. *P.*

laticornis specimens would often try to fly away when food was dropped into their Petri dish, which sometimes resulted in damage to the specimens.

Conclusions

Native predators have been confirmed to feed on WTB from cut bolts and in laboratory consumption tests, although *M. dislocatus* and *P. laticornis* have significant behavioral differences when feeding on WTB. Due to size differences, it appears *M. dislocatus* may be a more effective predator because it focuses on smaller prey close to the size range of WTB. In addition, *M. dislocatus* is easier to maintain in the laboratory due to its calmer disposition compared with *P. laticornis*. The discovery of these predators and parasitoids provides the means to develop potential biological control agents for suppressing WTB populations in the future. Native predators and parasitoids are currently interacting with WTB and decreasing its population. However, the overall effect these predators have on the WTB population has not been investigated. In addition, two parasitoids were observed to be infesting WTB adults within the galleries. These predators and parasitoids may have evolved to feed on *P. lautus*, which is native to this area and found on *J. nigra*. Because these indigenous predators are widespread throughout the eastern U.S. covering the range of black walnut and possess multifarious traits, they should be further investigated as potential biological control agents against the WTB to suppress TCD. A comprehensive study of predator and parasitoid populations in forested areas is critical to understanding the risk that WTB will have on native black walnut populations. If predator and parasitoid populations are already in place in forested areas, then the spread of TCD could be lessened and the forests better protected against this new insect/disease complex.

CHAPTER IV

CONCENTRATION LEVELS OF IMIDACLOPRID AND DINOTEFURAN IN *JUGLANS NIGRA*

Abstract

Black walnut, a valuable economic and environmentally important species, is threatened by Thousand Cankers Disease. Systemic applications of imidacloprid and dinotefuran were made to mature black walnut trees to evaluate their translocation and concentration in various tissue types. The metabolism of imidacloprid in plants produces a toxicologically important metabolite, olefin, which has been documented to have high levels of toxicity in other systems. The objective of this study was to evaluate the movement of systemically applied dinotefuran, imidacloprid and the metabolite olefin in mature black walnut leaf, twig, core, nutmeat, nut husk, and into a known herbivore, the walnut husk maggot. CoreTect imidacloprid soil pellets and a trunk spray of Safari dinotefuran were applied to mature black walnuts in spring 2011. With the use of liquid chromatography coupled with tandem mass spectrometry, imidacloprid and olefin concentrations were detected in both the lower and upper strata in all tissue types tested. Dinotefuran was only detected in the first sampling period and was found in low concentration levels in leaf and twig tissue types. Dinotefuran was not detected in the nutmeat, nut husk, or walnut husk maggot tissue. Populations of walnut husk maggot were significantly reduced in trees treated with imidacloprid compared to control trees indicating a negative impact on this pest species.

Introduction

Black walnut, *Juglans nigra* L., is native to eastern North America, where it grows in forest coves and well-drained river bottoms (Williams 1990, Harlow and Harrar 1969). In the U.S., its distribution extends from New England to the Florida and from the east coast to the

western delineation of the Great Plains (Harlow and Harrar 1969). These native trees are prized for their economic, ornamental, and ecological importance in the eastern U.S. (Smith and Follmer 1972).

The establishment of Thousand Cankers Disease in the eastern US, once limited to tree hosts in the western states, threatens the economic industries and ecological roles associated with black walnut in the region. In Tennessee, an estimated 26 million black walnut trees exist on public and private timberland with a value estimated at around \$1.47 billion (Tennessee Government 2012). Due to black walnut's fine, straight-grained wood, it is considered premium lumber for timber, gunstock, furniture, cabinetry, and other finished wood products (Williams 1990, Harlow and Harrar 1969). Also, some homeowners and landowners harvest this valuable nut crop for personal use or to sell for income, and this type of economic use certainly has growth potential (Newton et al. 2009). Industrial use of black walnuts include ground walnut shells used as jet engine cleaner, filler in dynamite, nonslip agent in automobile tires, and as a flour-like carrying agent in various insecticides (Williams 1990). On average, 10,287 metric tons of hulled nuts and 907.2 metric tons of black walnut kernels are purchased each year, with average demand increasing annually (Reid et al. 2004).

Environmentally, it is difficult to assess what the loss of these trees would have on wildlife within the various habitats as well as the negative impact on the aesthetic quality of the areas they inhabit. More than over 300 insect species have been documented on this valuable tree (Weber et al. 1992). Of these, few are considered pests (Williams 1990). Despite the small number of major pests associated with black walnut, these trees face some severe health risks. Thousand Cankers Disease (TCD) is a serious insect/disease complex that poses a significant threat to black walnut within its native range. Tree deaths from (TCD) are attributed to the

fungus species, *Geosmithia morbida* Kolařík et al. (Leslie et al. 2010, Seybold et al. 2010b, Tisserat et al. 2009). The only known vector of *G. morbida* is the walnut twig beetle *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae: Scolytinae) (WTB) (Newton et al. 2009, Tisserat et al. 2009). Like several other bark beetles, WTB bores beneath the bark into the phloem region of twigs, branches, or trunks of black walnuts. Currently, the primary means of managing TCD is rapid detection and removal of infected trees (Oliver 2010). Various control methods, including biological controls and systemic chemicals, are currently under investigation to prevent further spread of this TCD.

Two chemicals used to manage bark beetle populations are imidacloprid and dinotefuran. Imidacloprid is used against a variety of forest pests including *Tomicus piniperda* L., a bark beetle that attacks the shoots of young scotch pine, *Pinus sylvestris* L. (McCullough and Smitley 1995), and is the primary insecticide used to control the phloem-feeding larvae of the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky) (Poland et al. 2006, Russell et al. 2010, Ugine et al. 2012). In addition, bronze birch borer, *Agrilus anxius* Gory, larvae feed within the phloem and phloem-xylem regions of the tree (Loerch and Cameron 1983) and are treated with systemic insecticides including imidacloprid, emamectin benzoate, and dinotefuran (Herms et al. 2009, Smitley et al. 2010). Topical applications of imidacloprid are often used in nut-producing trees due to the low mammalian toxicity and reduced systemic movement within plants to combat such pests (Leicht 1996). However, systemic applications have not been approved for black walnuts since it is considered a group 14 nut tree (OFR 2010).

Little is known about the di-trophic movement of these two systemic applied pesticides, imidacloprid and dinotefuran, within the tree tissue, nor have the effects on non-target species in mature black walnut trees been effectively evaluated. Additionally, little is known about the

imidacloprid metabolite, olefin, which is more toxic than imidacloprid (Coots 2012), and it is unknown at what levels and for how long olefin can remain within deciduous plant material including nutmeat. Although research has been conducted on the spatial and temporal movement of imidacloprid in eastern hemlocks (Dilling et al. 2010), it is important to understand the movement of derivative chemicals, i.e., olefin, through black walnuts if they are going to be considered for control of TCD and other wood-boring insects.

By studying the movement of olefin along with other chemicals, the effectiveness of these compounds on insect populations can be better understood and potential negative effects on non-target species can be recognized. Therefore, the objective of this project is to document the translocation and within-tree concentrations of imidacloprid, olefin, and dinotefuran within various types of black walnut tissue, including leaves, twigs, core, nut husk, and nutmeat, as well as non-target walnut husk maggot larvae, *Rhagoletis suavis* Cresson, dwelling within the nut husk of mature black walnuts.

Materials and Methods

Study Sites

Mature black walnuts (n=21) were selected from Strong Stock Farm (36° 3'7.1526"N; 83° 47'23.3802"W), Knoxville, TN in April 2011. Trees selected were located on either the edge of the forest, individually in open fields or clumped in open fields. Tree heights averaged 17.8 m (8.5 - 27.4 m) with a diameter at breast height (DBH) of 51.6 cm (27.4-116.1 cm). Trees were tagged with yellow flagging tape to mark their location. Selected trees were arranged in a randomized complete split block design, consisting of seven blocks of three trees per block. Samples were taken from two locations (lower and upper strata) on each tree with the designated lower stratum consisting of the region of trunk below 5.18 m, while the upper stratum was

considered to be above 5.18 m. In addition to sampling, tree characteristics (tree height, transparency, density, crown class, DBH, foliage color, overall appearance, and crown conditions) were documented in June 2011 and again in June 2012. Treatments consisted of CoreTect (imidacloprid), a trunk spray of Safari (dinotefuran), and no treatment (control). Chemicals were applied in the spring on 20 April 2011. CoreTect, a systemic soil pellet, was applied to the soil at 1 pellet per 2.5 cm of tree DBH. Pellets were placed 30.5 cm away from the trunk and buried 5.1-12.7 cm deep in the soil encircling the tree (Figure 23a-c). Multi-stem tree rates were based on cumulative centimeters of DBH of all stems (Figure 24a). A trunk spray of Safari (dinotefuran) and was applied at 1.13 AI per 3.8 liters of water and sprayed on the tree trunk from base to a height of 2.5 m until saturation using a 18.9 liter hand held sprayer (Figure 24b). Multi-stem tree rates were based on cumulative centimeters of DBH of all stems.

Sampling

Samples were taken one, three, five, eight, and 12 months posttreatment. Samples were taken from the lower and upper tree strata to monitor the translocation of imidacloprid, olefin, and dinotefuran. Samples consisted of two leaf, two twig, two core, and two nut (when present) samples from each tree stratum for a total of 16 samples per tree. Two 24-cm branch and two nut (when present) samples (Dilling et al. 2010) were collected from each tree stratum using a 2.5 m pole pruner. Two-5 cm trunk core samples were collected at breast height from each tree using a 5 mm increment borer. All samples were immediately sealed in plastic bags, packed in ice and taken to the laboratory to be stored in a freezer at -18°C until extraction could take place. To detect the concentration levels of dinotefuran, imidacloprid and its metabolite, olefin, within the nutmeat, nut samples were separated into nut husk and nutmeat. To track the movement of



Figure 23 (a-c): (a) CoreTect (imidacloprid) soil pellets; (b) Hole dug around the base of a black walnut tree in preparation for one soil pellet; (c) CoreTect pellet being placed into a hole at the base of a black walnut tree.



Figure 24 (a-b): (a) Measuring the DBH of a black walnut tree; (b) Spraying Safari (dinotefuran) on trunks of black walnuts with a hand sprayer.

dinotefuran, imidacloprid and olefin into non-target pests, the walnut husk maggots present in the nut husks were counted, dried, ground, weighed, and placed into 1-g units for chemical analysis. All tissue types were divided into 1-g units, cut, crushed using a scalpel, and added to 10.0 ml of histological grade acetone in 10-dram glass vials (Figure 25a-c).

HPLC/MS/MS Method:

Extraction, Clean-Up, and Quantification of Chemicals

To determine imidacloprid concentrations in tree matrices (foliage, twig, core, nutmeat, and husk tissue from black walnut trees) the chemical extractions, sample clean-up, and HPLC/MS/MS quantification protocol established by Schöning and Schmuck (2003) were used. To determine dinotefuran concentration in tree matrices, the protocol established by Kamel (2010) was used.

HPLC/MS/MS was conducted using an Hewlett Packard HP 1100 high pressure liquid chromatograph coupled with a tandem triple quadrupole Applied Biosystems API 3000 mass spectrometer fitted with a Phenomenex Luna C18 reversed phase column (15 cm length x 4.6 mm i.d.). Parameters for the HPLC/MS/MS were as follows: injection volume: 50 µl, oven temperature: 40°C, mobile phase A: water + 0.1 ml acetic acid per liter, mobile phase B: acetonitrile + 0.1 ml acetic acid per liter, gradient; 0-10 minutes 20% mobile phase B, 11-15 minutes 90% mobile phase B, 16-19 minutes 20% mobile phase B, stop time: 19 minutes, flow (column/MS): 1.0 / 0.15 ml / minute, retention time for imidacloprid: approximately 9.1 minutes, retention time for dinotefuran: 3.3 minutes, interface: electrospray, turbo-ion spray potential: + 5000 V, temperature: 300°C, scan type: multiple reaction monitoring (MRM), polarity: positive, and collision gas: nitrogen 5.0 (99.999% purity), 0.87 l/minute. Chemicals were obtained from



Figure 25 (a-c): (a) Cutting black walnut leaf tissue with scalpel; (b) Placing nut tissue into 10-dram glass vial; (c) 10-dram glass vials ready to be processed through HPLC/MS/MS machine.

Sigma-Aldrich (St. Louis, MO) and consisted of HPLC grade water (99.9%) and acetonitrile (99.9%). Standards of imidacloprid (96.9%) and dinotefuran (99.7%) were obtained from Sigma Aldrich (St. Louis, MO), and standards of olefin (97.5%) were obtained from Crescent Chemical Company (Islandia, NY).

Calculations and Data Analysis

Residuals in parts per billion (ppb) of imidacloprid, olefin, and dinotefuran were calculated using the average of peak areas of imidacloprid and dinotefuran conversions for each analyte using the formula described by Schöning and Schmuck (2003). Shapiro-Wilks W test for normality and Levene's test of homogeneity of variances were used to verify that chemical concentration data conformed to the assumptions of analysis of variance (ANOVA). Imidacloprid, olefin, and dinotefuran concentration data were converted from ng g^{-1} of each analyte to ppb and placed into an Excel[®] file and analyzed using PROC Mixed ANOVA in SAS (SAS 2005). ANOVA and Least Significant Differences (LSD) procedures were run on chemical concentration data ($P < 0.05$) to determine concentration differences across tree tissue types and time.

Results and Discussion

Concentration Levels of Imidacloprid in Walnut Tissue Types

Mean imidacloprid concentration levels differed significantly (LSD test; $P < 0.05$) by stratum, tissue type, and time (Table 4). Imidacloprid concentrations were highest in foliage tissue taken from the lower stratum for all sampling periods and lowest in core tissue for all sampling periods, except in September samples. In September, the lowest imidacloprid concentrations were in the walnut husk maggot tissue taken from walnuts collected from the upper stratum. Imidacloprid concentrations were significantly higher (LSD test; $P < 0.05$) in

Table 4. Imidacloprid concentrations (ppb)* $\pm$ SE, determined using HPLC/MS/MS from tree tissue of <i>Juglans nigra</i> L.					
Sampling Time					
	May-11	Jul-11	Sep-11	Dec-11	Apr-12
Treatment Group					
CONTROL					
Upper Twig	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Upper Foliage	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Upper Nut Meat	—	—	0.0 $\pm$ 0.0aA	—	—
Upper Nut Husk	—	—	0.0 $\pm$ 0.0aA	—	—
Upper Maggots	—	—	0.0 $\pm$ 0.0aA	—	—
Lower Twig	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Foliage	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Nut Meat	—	—	0.0 $\pm$ 0.0aA	—	—
Lower Nut Husk	—	—	0.0 $\pm$ 0.0aA	—	—
Core	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Maggots	—	—	0.0 $\pm$ 0.0aA	—	—
IMIDACLOPRID					
Upper Twig	11.98 $\pm$ 1.74dE	63.62 $\pm$ 2.55dD	87.30 $\pm$ 1.34dC	101.23 $\pm$ 2.34dB	123.11 $\pm$ 2.10dA
Upper Foliage	12.89 $\pm$ 1.25cE	68.85 $\pm$ 3.25cD	97.13 $\pm$ 1.78cC	123.09 $\pm$ 2.14.0cB	134.22 $\pm$ 1.09cA
Upper Nut Meat	—	—	72.19 $\pm$ 1.99eA	—	—
Upper Nut Husk	—	—	11.79 $\pm$ 1.13hA	—	—
Lower Twig	24.79 $\pm$ 1.66 bE	96.79 $\pm$ 2.06bD	112.97 $\pm$ 1.24bC	134.45 $\pm$ 2.67bB	157.43 $\pm$ 3.42bA
Lower Foliage	31.46 $\pm$ 1.89aE	99.45 $\pm$ 2.69aD	129.26 $\pm$ 1.44aC	157.32 $\pm$ 2.34aB	171.29 $\pm$ 1.31aA
Lower Nut Meat	—	—	84.02 $\pm$ 2.56eA	—	—
Lower Nut Husk	—	—	56.4 $\pm$ 2.09fA	—	—
Core	1.25 $\pm$ 0.54eC	34.56 $\pm$ 2.13eB	45.05 $\pm$ 6.45gA	47.13 $\pm$ 5.39eA	49.56 $\pm$ 4.97eA
Lower Maggots	—	—	11.72 $\pm$ 0.22a	—	—
Upper Maggots	—	—	9.25 $\pm$ 0.14b	—	—

*ppb= parts per billion

*Means (n=14) within columns of the same treatment group followed by the same lowercase letter(s) and means (n=14) within the same row followed by the same uppercase letter(s) are not significantly different (LSD test; $P > 0.05$).

(LSD test; $P < 0.05$) concentrations of imidacloprid followed by twig, core, nutmeat, husk, and samples from the lower stratum within each tissue type (foliage, twig, core, husk, nutmeat, and walnut husk maggot) (Table 4). Within each stratum category, foliage had significantly higher walnut husk maggot.

Concentration levels within nutmeat samples exceeded the established nut crop acceptable tolerance level (50 ppb) for imidacloprid (OFR 2010) (Figure 26). Therefore, using a spring treatment of the CoreTect formula for treating black walnut trees will most likely cause imidacloprid concentrations to rise above the acceptable level in nut crops. It is possible that a fall treatment result in concentrations below the acceptable levels for imidacloprid. However, it is unknown how long the imidacloprid would persist in the black walnut nut crops in the following years. Concentration levels of imidacloprid were highest in the last sampling period one year after treatment (April 2012). Over the time period tested, imidacloprid concentrations progressively increased, while dinotefuran concentrations diminished (Tables 4-5).

The same trend of higher concentration levels of imidacloprid in lower tree strata was observed in eastern hemlock, *Tsuga canadensis* L., although the tissue types differ. In a two-year study tracking the translocation and concentrations of imidacloprid in eastern hemlock, significantly higher concentrations of imidacloprid were found in the lower stratum compared to the upper (Dilling et al. 2010). In addition, 293 insect species were documented to be associated with eastern hemlock and at least 33 species were impacted by systemic imidacloprid treatments (Dilling et al. 2009). Furthermore, two non-target predators species that feed on hemlock woolly adelgids that had consumed imidacloprid exhibited both lethal and sublethal effects from the chemical (Eisenback et al. 2010). The distribution of various nutrients, including nitrogen and potassium, has been documented in both evergreen and deciduous trees. Deciduous trees had

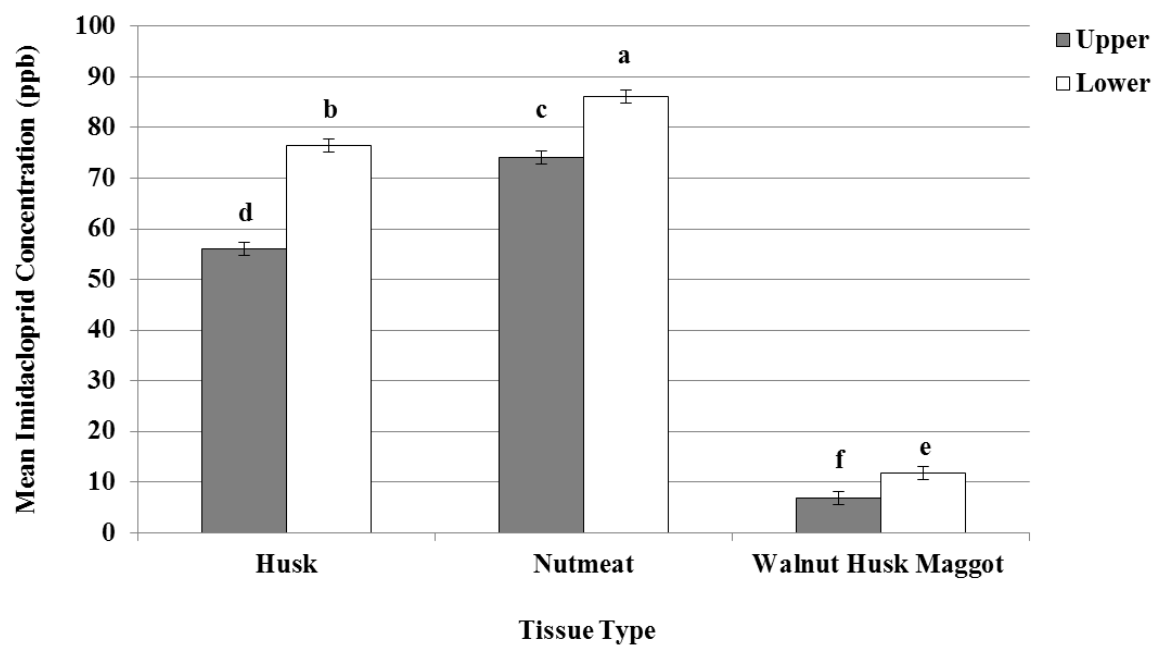


Figure 26. Mean $\pm$ SE imidacloprid concentration (ppb) for walnuts and walnut husk maggot in each tree stratum. Means ($n = 84$) in a column followed by same letter do not differ significantly (LSD test; $P < 0.05$).

Table 5. Dinotefuran concentrations (ppb)* $\pm$ SE, determined using HPLC/MS/MS from tree tissue of <i>Juglans nigra</i> L.					
Sampling Time					
	May-11	Jul-11	Sep-11	Dec-11	Apr-12
Treatment Group					
CONTROL					
Upper Twig	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Upper Foliage	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Upper Nut Meat	—	—	0.0 $\pm$ 0.0aA	—	—
Upper Nut Husk	—	—	0.0 $\pm$ 0.0aA	—	—
Upper Maggots	—	—	0.0 $\pm$ 0.0aA	—	—
Lower Twig	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Foliage	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Nut Meat	—	—	0.0 $\pm$ 0.0aA	—	—
Lower Nut Husk	—	—	0.0 $\pm$ 0.0aA	—	—
Core	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA	0.0 $\pm$ 0.0aA
Lower Maggots	—	—	0.0 $\pm$ 0.0aA	—	—
DINOTEFURAN					
Upper Twig	1.13 $\pm$ 0.34aA	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB
Upper Foliage	1.56 $\pm$ 0.68aA	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB
Upper Nut Meat	—	—	0.0 $\pm$ 0.0aB	—	—
Upper Nut Husk	—	—	0.0 $\pm$ 0.0aB	—	—
Upper Maggots	—	—	0.0 $\pm$ 0.0aB	—	—
Lower Twig	1.26 $\pm$ 0.56aA	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB
Lower Foliage	1.41 $\pm$ 0.73aA	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB
Lower Nut Meat	—	—	0.0 $\pm$ 0.0aB	—	—
Lower Nut Husk	—	—	0.0 $\pm$ 0.0aB	—	—
Core	0.0 $\pm$ 0.0bA	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB	0.0 $\pm$ 0.0aB
Lower Maggots	—	—	0.0 $\pm$ 0.0aB	—	—

*ppb= parts per billion

Means (n=14) within columns of the same treatment group followed by the same lowercase letter(s) and means (n=14) within the same row followed by the same uppercase letter(s) are not significantly different (LSD test; $P > 0.05$).

higher nutrient translocation rates compared to evergreen trees (Luxmoore et al. 1981). As such, not only is the translocation of imidacloprid and olefin dependent on their chemical composition and how they react to organic material, but the movement of the chemicals may be dependent on the type of tree or plant material to which they are applied.

Concentration Levels of Dinotefuran in Walnut Tissue Types

Concentrations of dinotefuran were only detected in the first sampling period one month post-treatment (Table 5). Concentration levels were highest in the upper foliage followed by upper twig, lower foliage, and the lower twig. However, there were no significant differences in dinotefuran concentrations between these tissue types as only trace amounts were detected. The only tissue type that was significantly different during the first sampling period was the core sample which did not have any dinotefuran present in the samples compared to the trace amounts found in the other tissue types. Dinotefuran was not detected in the nutmeat, nut husk, or in the walnut husk maggot. These data imply that with a spring treatment of dinotefuran, this chemical will have degraded before walnuts are ready to harvest in the fall. Dinotefuran concentrations were not detected beyond the first month of sampling. These results are probably due to dinotefuran moving quickly through the plant and its chemical behavior of not being able to bind well to organic material. Since black walnut is labeled as a group 14 crop, chemical concentration levels in the nutmeat are carefully controlled. Other members of this group include almonds, other *Juglans* species, pecans, etc. However, concentration levels of dinotefuran for group 14 have not yet been established by federal government (OFR 2005).

Concentration Levels of Olefin in Walnut Husk, Walnut Nutmeat, Walnut Husk Maggot

Olefin, a metabolite of imidacloprid, was only tested in nutmeat, husk, and the walnut husk maggot in the fifth month post-treatment. Olefin concentrations differed significantly (LSD

test; $P < 0.05$) by stratum and tissue type (Figure 27). Olefin concentrations were highest in nutmeat tissue from the upper stratum and lowest in walnut husk maggot tissue and husk tissue taken from walnuts collected from the lower stratum. Olefin concentrations were significantly greater (LSD test; $P < 0.05$) in the upper stratum within each tissue type (husk, nutmeat, and walnut husk maggot). Within each stratum category, nutmeat had the highest (LSD test; $P < 0.05$) concentrations of olefin followed by husk and walnut husk maggot. Olefin concentrations were significantly lower (LSD test; $P < 0.05$) than imidacloprid concentrations within each respective stratum and tissue type during the time period of this study. For example, imidacloprid concentrations ranged from 8.0 to 134.0 ppb and olefin concentrations ranged from 2.0 to 4.5 ppb. However, olefin concentrations have been documented to progressively increase as imidacloprid concentrations decreased over time. The olefin metabolite, one of 12 known metabolites of imidacloprid, has been reported to be at least ten times more active than its parent compound against the green peach aphid, *Aphis gossypii* Glover (Nauen et al. 1998), and 13 times more active against hemlock woolly adelgid in eastern hemlocks (Coots 2012). Another metabolite, i.e., 5-hydroxy, has been documented to be slightly less active than the parent imidacloprid; however, 5-hydroxy still retains relatively high toxicological properties. These data infer longer-term residual effects that could result in longer control of pest insects due to the toxicity of imidacloprid's metabolites.

Abundance of Walnut Husk Maggot in Treated and Untreated Trees

The mean number of walnut husk maggots in black walnuts was significantly lower (LSD test; $P < 0.05$) in trees treated with imidacloprid in both the upper and lower strata compared to untreated controls (Figure 28). The lower stratum of imidacloprid-treated trees had the lowest mean number of walnut husk maggots.

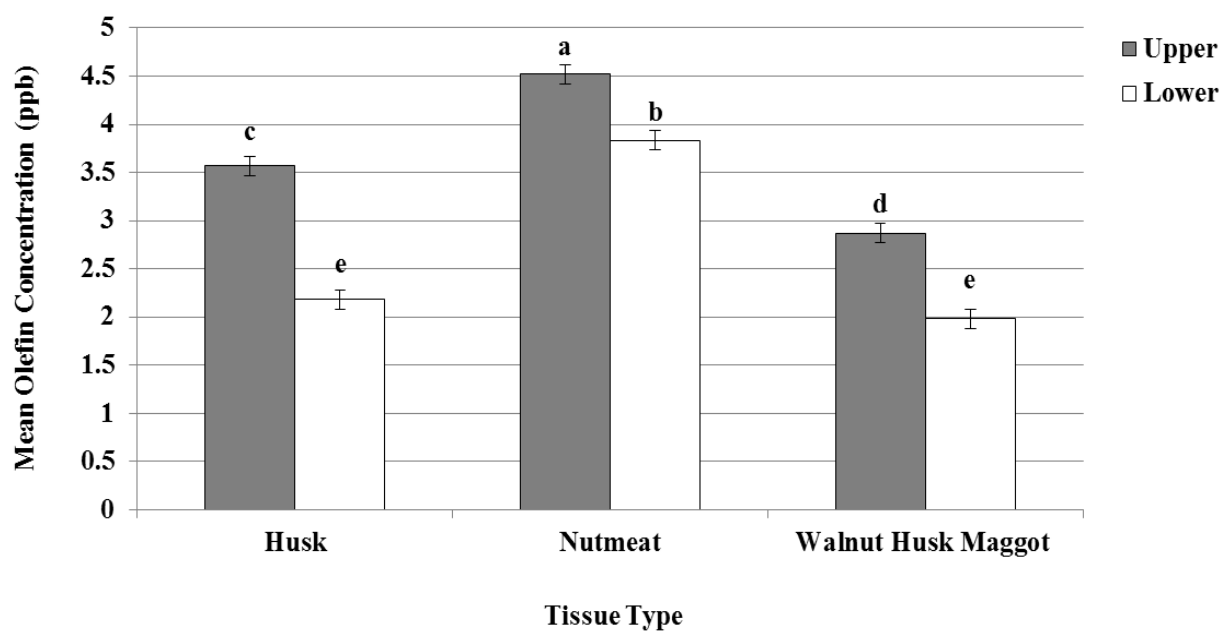


Figure 27. Mean $\pm$ SE olefin concentration (ppb) for each stratum and tissue type. Means (n=84) with different letters are significantly different (LSD test; $P < 0.05$).

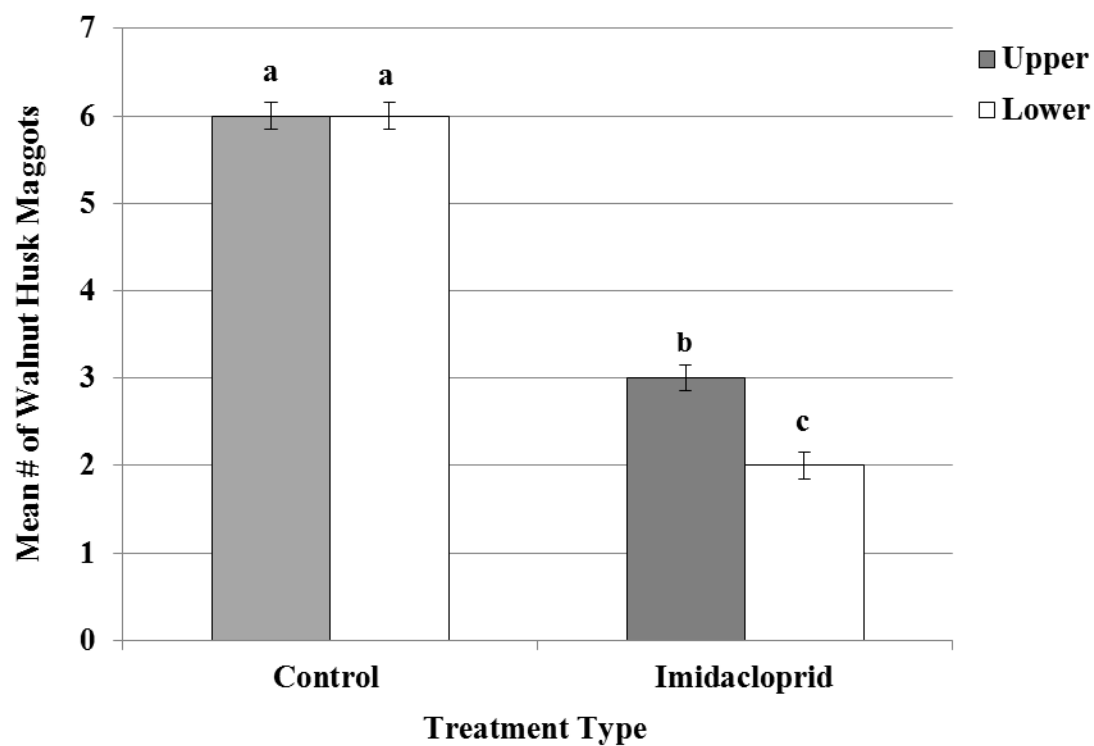


Figure 28. Mean walnut husk maggot abundance (mean number of individuals/walnut $\pm$ SE) within walnut husk for each stratum. Means with different letters are significantly different (LSD test; $P < 0.05$).

Conclusions

Imidacloprid concentrations were significantly greater in the lower stratum in all tissue types tested, and foliage in the lower stratum had the highest concentrations of imidacloprid. When testing for olefin, higher concentrations were found in the upper stratum with nutmeat having the highest concentrations in the tissue types tested (nutmeat, nut husk, and walnut husk maggot). The walnut husk maggot, a non-target species of this treatment, had detectable concentrations of both imidacloprid and olefin in both strata. In addition, walnut husk maggot numbers were significantly lower in treated trees compared to control trees implying that systemic chemical treatment of imidacloprid decreased their numbers. In addition, this research implies that black walnuts which are treated in the spring with the soil pellet CoreTect (imidacloprid) nutmeat concentration levels (ppb) are above the current established acceptable concentration levels for imidacloprid in nutmeat established by the OFR (2010). Thus, this formula for imidacloprid may not be an acceptable method to use for controlling pests on black walnut trees due to contaminating nut crops. However, dinotefuran moved quickly through the system and was not detectable in any of the nut tissue or any tissue after the first sampling period.

CHAPTER V

OVERALL CONCLUSION

WTB overwinter primarily as adults and larvae. Although pupae are occasionally present in overwintering populations, they are few in numbers. Eggs were not detected during the winter although it is possible they were present in the galleries formed by adult females. Females lay between 7-25 eggs in the walls of their galleries. The female covers her eggs with frass caps possibly for protection against predators. Absolute fecundity was not determined. Females create a gallery against the grain of the wood in a straight line. Frass was only present in larval, pupal, and teneral adult galleries. Mycelia of *G. morbida* were found only in female, pupal, and teneral adult galleries. Larval galleries are perpendicular to the female egg-laying gallery and in a straight line parallel with the grain of the wood. Males create a globular gallery where they are suspected to mate with the female. This gallery is often attached to one or more female galleries. It is suspected that male WTB mate with 1 to 2 females.

Immature WTB takes 4 to 5 weeks to develop into adults in laboratory conditions. Three larval instars were recovered from WTB tunnels though more are possible. WTB flight ceases (or slows down) during the winter months of late December-early March in east Tennessee. One WTB emerged from jars in April 2011 and two WTB in March 2012. WTB emerged for six months from cut logs that were held in laboratory conditions with no added moisture. Natural enemies are present in the galleries of WTB and may possibly be used as biological control agents.

Three native predators have been confirmed to feed on WTB from cut bolts and in laboratory consumption tests, although *Madoniella dislocatus* and *Pyticeroidea laticornis* have significant behavioral differences when feeding on WTB. Due to size differences, it appears *M. dislocatus* may be a more effective predator because it focuses on smaller prey close to the size

range of WTB. However, *M. dislocatus* is easier to maintain in the laboratory due to its calmer disposition compared with *P. laticornis*. Nine additional predators and two parasitoids have been found in association with WTB in infested black walnut logs. The discovery of these predators and parasitoids provides the means to develop potential biological control agents for suppressing WTB populations in the future. Native predators and parasitoids are currently interacting with WTB and decreasing its population. However, the overall effect these predators have on the WTB population has not been investigated. In addition, two parasitoids were observed to be infesting WTB adults within the galleries. Because these indigenous predators are widespread throughout the eastern U.S. covering the range of black walnut and possess multifarious traits, they should be further investigated as potential biological control agents against the WTB to suppress TCD. A comprehensive study of predator and parasitoid populations in forested areas is critical to understanding the risk that WTB will have on native black walnut populations. If predator and parasitoid populations are already in place in forested areas, then the spread of TCD could be lessened and the forests better protected against this new insect/disease complex.

Through liquid chromatography coupled with tandem mass spectrometry, concentration levels of dinotefuran, imidacloprid, and olefin (metabolite of imidacloprid) were detected in treated mature black walnuts. Trace amounts of dinotefuran were found in the first sampling period one month posttreatment. No amounts of dinotefuran were detected in nut husks or nutmeat. Imidacloprid and olefin concentrations were detected in black walnut husks and nutmeat as well as in walnut husk maggots, in both the lower and upper strata. Imidacloprid concentrations were significantly greater in the lower strata in all tissue types tested including foliage, twig, core, nut husk, nutmeat, and walnut husk maggot. Olefin was only tested on

nutmeat, nut husk, and walnut husk maggot. When testing for olefin, higher concentrations were found in the upper strata with nutmeat having the highest concentrations in the tissue types. The walnut husk maggot, a non-target species of this treatment, had detectable concentrations of both imidacloprid and olefin in both strata. In addition, walnut husk maggot numbers were significantly lower in treated trees compared to control trees implying that systemic chemical treatment decreased their numbers.

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