

**Incidence, Dispersal, and Risk Assessment of Walnut Twig Beetle,
Pityophthorus juglandis, on Black Walnut in Appalachian Forests**

**A Thesis Presented for the
Master of Science
Degree
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Dedication

To

Nicole Elizabeth Hensley

my wife

Thank you for your constant encouragement and patience through this process

and

Douglas Wayne Hensley

and

Rita Elaine Hensley

my parents

For their unwavering confidence in my abilities and their support of myself and my family
from beginning to end

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Abstract

The native range of walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman, includes Arizona, California, New Mexico, and parts of Mexico. In 2010, WTB was found in the eastern United States, the native range of black walnut, *Juglans nigra* L. Although WTB is not believed to kill black walnut, it carries a fungal pathogen, *Geosmithia morbida* M. Kolařík et al., which was identified as the causal agent of thousand cankers disease (TCD). This disease complex has killed millions of trees in the United States. Studies have documented the movement of WTB in urban settings; however, its movement in forested systems is not well understood. The goal of this research is to understand the role of forests in TCD epidemiology and the risk of WTB to black walnut resources. This project will emphasize three research objectives, which are to: 1) document incidence and distribution of WTB on black walnut in Appalachian forests, 2) assess dispersal of WTB in forests, and 3) determine the dispersion pattern of WTB in black walnut orchards.

Pheromone-baited funnel traps ($n = 33$) were deployed and monitored from April through November at 14 locations in eastern Tennessee and western North Carolina. Trap collection numbers suggest a low incidence of WTB in forests. To measure the dispersal of WTB in forests, a study was initiated in Morgan County, TN, using equally spaced traps along three transects from a central release point. No significant trends in dispersal were identified, as beetles were recovered at a range of distances and directions. Dispersion patterns of WTB were assessed in two black walnut orchards, in Anderson and Knox Counties. Analysis of trap collections revealed a clumped distribution of WTB at sites in both counties. Findings from this research can be used to inform risk assessments of WTB in forests, and to enhance current knowledge of TCD epidemiology in the native range of black walnut.

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CHAPTER I

Introduction

Black Walnut

Black walnut, *Juglans nigra* L. (Juglandaceae) is a deciduous, nut-bearing tree, and is one of six native walnut species identified in the continental United States (Randolph et al. 2013). The native range of black walnut spans from the central to eastern United States (Fig. 1.1). Because black walnut is considered to be an excellent shade tree due to its dense canopy, it is regularly planted along neighborhood streets, city parks, and other ornamental settings (Williams 1990). This growth characteristic also has enhanced its popularity outside its native range, where it has been planted extensively in urban landscapes throughout the western states and southern Canada (Seybold et al. 2010). Black walnut was also introduced in Europe by the early 1600s (Nicolescu 1998).

Black walnut trees tend to grow slowly, especially if grown with other black walnut trees. They rarely occur in large stands in a forest setting, but rather in small patches or clusters, typically containing only a few mature trees. Some specimens have been recorded to live as long as 200 years. The average height of mature black walnut is 25 m, but some can be over 30 m tall (Williams 1990). Leaves of the black walnut tree are arranged in an alternating pattern along the branch, and range in length from 30-60 cm. Leaves are compound, generally consisting of 9-23 lanceolate-shaped leaflets. Each leaflet can range in length from 6-10 cm and can be found with finely toothed edges and slight pubescence along the leaf surface (Mielke and Ostry 2004). These foliage features are reasonably distinct and are often used as a key component in species identification (Fig. 1.2).

Black walnut is an important tree – aesthetically, commercially, and culturally. As described above, the shade provided by the large canopy provides comfort but, aesthetically, the large canopy also provides a pleasing view in the landscape. Because of their large canopy, black walnuts also have been used in agroforestry systems, grown alongside row crops like corn, soybeans, and winter wheat, and are commonly employed as a wind break along the perimeter of farmland (Williams 1990).

Commercially, black walnut is considered a valuable lumber commodity (Mielke and Ostry 2004). In 2010, the commercial value of urban and forested black walnut trees in Tennessee was estimated at \$2.84 billion worth of standing timber (Tennessee Department of Agriculture 2010). Within the native range of black walnut, there are an estimated 306 million living trees, estimated at over \$500 billion worth of growing stock (Newton et al. 2009), with Missouri, Ohio, and Kentucky containing nearly 35% of the living black walnut trees in the United States (Randolph et al. 2013). Black walnut is prized for the rich, dark color found in the wood grain. Black walnut wood has a reputation for durability, withstanding the rigors of kiln treatments while maintaining its shape and strength (Williams 1990). Throughout American history the wood was used to create valuable ornaments and trinkets, and yet could also be found in everything from fence railing to cabins constructed entirely of black walnut lumber (Quigley and Lindmark 1967). Kitchen cabinets, tables, hardwood flooring, and gun stocks are just a few examples of the most common items made from black walnut wood today (Newton et al. 2009).

In addition to its value as a lumber commodity, black walnut is also valued as a food source and as a product that can be processed into many forms. The raw nut produced by the tree presents a valuable browsing resource for turkeys, bears, racoons, and other foraging animals (Brooks 1951). The nutmeat is often added to cakes, cookies, and other baked goods,

offering more protein per ounce than any other tree nut (Câmara and Schlegel 2016). Hammons Products Company® in Stockton, MO is one of the largest distributors of raw black walnut products and estimates the 2018 value of black walnut kernels to be roughly \$0.75 cents per 28 grams. Brian Hammons, president of Hammons Products Company, stated that his company purchases approximately 10 million kg of raw walnuts per year, and he expected that number to increase in the future (Gounley 2016). Nuts can be pressed into concentrated cooking oil, offering a distinctive, rich flavor that is unique to walnuts. Dried walnut husks are often used in abrasive products, such as sand blasting media, and are effective as filtration media in water filters and smokestack scrubbers. Flesh from the walnut fruit contains tannins that are processed into expensive dyes and veneer wood stains (Newton et al. 2009). Additionally, the exterior flesh of the nut contains a chemical compound known as juglone, 5-hydroxy-1,4-naphthoquinone (Chao et al. 2001). Fabrics are often dyed with solutions containing juglone that has been extracted from black walnut husks. These fabrics exhibit antibacterial properties against gram-positive *Staphylococcus* species and gram-negative *Escherichia* species (Mirjalili and Karimi 2013).

Culturally, black walnut is an important food, economic resource, and wood for some families in the United States, especially in rural areas. These families often collect enormous amounts of nuts to sell as extra income or to use as ingredients in various cooked goods (Jones et al. 1998). Nuts are primarily dispersed by squirrels and other rodents, which are hunted as a source of food for those living in rural areas (Peattie 1950). The tree can also be cut into wood for personal use or sold for extra income.

One of the well-known characteristics of black walnut is its allelopathic properties. Allelopathy is a chemical interaction between two or more organisms that has the ability to

change the behavior, growth, and overall survival of those organisms (Coder 1999). The effects can be either positive or negative depending on the relationship (Brooks 1951). Stem growth, root growth, seed germination, nutrient uptake, and mycorrhizal relationships within the soil can be affected by varying concentrations of allelopathic chemicals (Chao et al. 2001). Many different plant species employ this defensive process as a means to control their surroundings (Coder 1999). The main allelopathic chemical produced by black walnut is juglone, which has been shown to exhibit allelopathic properties toward a wide variety of solanaceous plants. Juglone is released from the leaves, roots and nuts, and commonly acts as a respiration inhibitor (Dana and Lerner 2001). Juglone is able to disrupt the preinitiation step of transcription, ultimately preventing RNA transcription, resulting in genetic flaws within susceptible organisms (Chao et al. 2001). These flaws are manifested in various on susceptible plant species when exposed to juglone. Symptoms can include low germination rates, wilting foliage, stunted growth, yellowing foliage, and plant death (Brooks 1951). In a natural setting, black walnut can effect species composition with the use of this allelopathic defense.

In addition to producing allelopathic compounds, black walnut releases volatile organic compounds (VOC's) into the air (Blood et al. 2018). Humans have harnessed VOC's for their own purposes throughout history, as flavoring for food products, antiseptics for illness or injury, or as pleasing perfumes. In nature, these compounds serve a similar purpose for the plant. Produced in various parts of the plant, VOC's attract animals to pollinate flowers and disperse seeds. These compounds can also attract predators and parasitoids to protect the plant from attacks by unwelcome pests and offer defense against some plant pathogens (Rosenkranz and Schnitzler 2016).

Even with the protection of chemical compounds black walnut is not immune to all diseases or damaging pests. Crown gall, caused by *Agrobacterium tumefaciens*, is one of the leading soil-borne diseases of black walnut. Fungal species of *Phytophthora* and *Armillaria* infect through the root system and lead to crown and root rot. A common foliage disease of black walnut, walnut blight, is caused by the fungus *Xanthomonas aboricola* pv. *juglandis*. Infection by plant pathogens weakens the tree, leaving it susceptible to attack by damaging insect pests. One of these pests, fall webworm, *Hyphantria cunea* (Drury), forms silken webs at the tips of limbs, killing the leaves trapped inside, but is considered to pose little threat to overall tree health (Yang and Zhang 2007). Larvae of *Rhagoletis suavis* (Loew), the walnut husk maggot, cause premature nut drop as they develop inside the flesh of the husk, leading to a decrease in viable nuts produced (Nix et al. 2014).

Walnut Twig Beetle

The walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae), was first described in 1928, from specimens collected in New Mexico and Arizona. The native range of *P. juglandis* extends through Mexico and southern California (Blackman 1928, Cranshaw 2011). WTB is a small (1.5 - 3.0 mm long), reddish-brown, wood-boring beetle that exhibits multiple generations per year (Cranshaw and Tisserat 2008). In the western United States WTB generations typically occur during the warmer months of the year, as adult WTB have been collected in traps from mid-April to late October (Seybold et al. 2013). Laboratory flight mill tests revealed that WTB fly an average distance of 372 m but are capable of flying over 1 km during a single flight period (Hefty et al. 2016).

Male and female WTB appear to be nearly identical, but upon closer inspection, a cluster (also described as a halo or crown) of yellow setae can be seen on the frons of the female beetle

(Fig. 1.3) (Seybold et al. 2012). WTB males release aggregation pheromones as they create feeding galleries, eventually colonizing phloem tissue of a black walnut tree (Seybold et al. 2016). The main purpose of aggregation pheromones is to attract a female for reproduction, yet copious amounts of WTB males have been recorded responding to these compounds, presumably to outcompete other males (Hefty et al. 2016). Once mating is completed, the female feeds along phloem tissue, ovipositing in the horizontal galleries made as she feeds. Once the eggs hatch, the larvae feed outward, vertically, from the original adult gallery (Nix 2013). Though the life cycle of WTB is not completely understood in all regions where it has been found, it is believed that WTB adults will overwinter inside the bark of the scaffold limbs and trunk of black walnut (Luna et al. 2014).

WTB quietly coexisted with its native host, Arizona walnut, *J. major* (Torr.) A. Heller, in Arizona, New Mexico and northern Mexico (Tisserat et al. 2009). Only recently (2010), WTB was discovered on black walnut in Tennessee (Grant et al. 2011), and has been detected in Maryland, North Carolina, Ohio, Pennsylvania, and Virginia (Hansen et al. 2011, Rhodes et al. 2012, Fisher et al. 2013, Hadziabdic et al. 2014). The spread of WTB has benefited from the quick and extensive movement of infested black walnut wood into regions that it had not previously inhabited. Wood mills, veneer plants, nursery suppliers and even campgrounds have all been shown to contribute to the unintentional spread of WTB throughout the United States (Nix 2013). New techniques, such as kiln-treating wood products, are being studied to aid in slowing the spread of WTB into new areas (Mayfield et al. 2014). Natural predators and parasitoids may also contribute as a means of biological control of WTB. Three clerid beetles species, *Enoclerus nigripes* (Say), *Pyticeroides laticornis* (Say), and *Madoniella dislocatus* (Say), were found to occur within WTB galleries and feed on WTB. Parasitoid wasps

(*Neocalosoter* sp. and *Theocolax* sp. (Cerocephalinae)) were also recovered from bolts of black walnut and seen actively feeding on WTB larvae in the galleries (Lambdin et al. 2015). Though not host specific, these predators appear to be promising biological control agents of WTB as they are already present within the native range of black walnut and have demonstrated that they can feed on WTB. Further studies to investigate their potential in suppressing TCD are needed. Overall, WTB has been monitored in more than 120 counties across the United States, with many of the trapping locations found in western states and focused in disturbed urban areas (Seybold et al. 2016). However, little is known about the distribution and dispersal ability of WTB within a natural forest system. A mixed forest setting may act as a natural buffer for susceptible black walnut trees (Wiggins et al. 2014), and likely contains a more diverse array of biological control agents acting on WTB, in turn protecting black walnut from new infestations of WTB.

***Geosmithia morbida* and Thousand Cankers Disease**

In the late 1990s, researchers in the western United States began to observe that black walnut trees were exhibiting an increase in symptoms, such as yellowing foliage, flagging limbs, and crown dieback (Tisserat et al. 2009, Flint et al. 2010). In 2008, it was discovered that the rise in tree mortality was caused by a previously unnamed fungus, *Geosmithia morbida* M. Kolařík et al. (Ascomycota: Hypocreales: Bionectriaceae) (Kolarik et al. 2011). The *Geosmithia* genus of fungi is typically associated with saprotrophic beetle species that rely on decaying organic material for their nutritional needs, but these fungi had never been recorded as plant pathogens (Kolařík et al. 2007). Currently, only two known *Geosmithia* species, *G. morbida* and *G. pallida* (G. Smith) M. Kolařík, Kubátová & Pažoutová, have been characterized as plant pathogens (Lynch et al. 2014). *G. morbida* forms several small annual cankers within the bark

of *Juglans* species, spreading throughout the cambium tissue, and effectively reducing the flow of nutrients throughout the canopy of the infected host (Utley et al. 2009). Although *G. morbida* has demonstrated that it can infect all *Juglans* species, *J. nigra* was confirmed to be the most susceptible host among those species endemic to the United States (Utley et al. 2013).

The substantial number of *G. morbida* cankers found on declining and dead black walnut trees are due to the feeding behavior of a primary insect vector, identified as WTB (Tisserat et al. 2009). As the beetle explores the tree for a suitable entry point into the vascular tissue, it has the potential to deposit numerous conidia of *G. morbida* at each probing site. Conidia are generally carried by setae on the head and elytra of adult beetles (Fig. 1.4). The association between WTB and *G. morbida* was discovered when beetle galleries and adult beetles collected from host trees were found to contain *G. morbida* conidia (Kolařík et al. 2011). The formation of thousands of small cankers on the tree prompted scientists to name this insect/pathogen complex ‘thousand cankers disease’ (TCD) (Tisserat et al. 2009). It is believed that both WTB and *G. morbida* coevolved over time, using *J. major*, *J. hindsii* (Jepson) R.E. Smith, and *J. californica* S. Wats as their primary hosts (Seybold et al. 2012). WTB will respond to the presence of volatiles associated with *G. morbida* growth, supporting the idea of coevolution (Blood et al. 2018).

In 2010, the TCD complex was detected in Knoxville, TN. This incident marked the first time that TCD had been detected east of Colorado and marked the first occurrence of the pathogen within the native range of *J. nigra* (Grant et al. 2011). In 2013, a town in northeast Italy reported symptomatic trees ranging in age from 17-year-old timber stock to 80-year-old trees in a homeowner’s garden. The noted symptoms were discolored foliage, wilting, and crown dieback. It was later confirmed that these walnut trees were infected with *G. morbida* and infested with WTB; hence, TCD on these trees was confirmed (Montecchio and Faccoli 2014).

By 2015, seven states within the native range of eastern black walnut confirmed the presence of diseased trees, WTB, or *G. morbida* (Fig. 1.5).

While most notably associated with several *Juglans* species, TCD has also been documented to affect *Pterocarya* species, such as wingnut, *Pterocarya fraxinifolia* (Lam.) Spach (Hishinuma et al. 2016). If left unchecked, TCD could potentially cause billions of dollars in economic losses across several states, threatening the genetic resources of Juglandaceae and other potential hosts in the United States (Daniels et al. 2016).

Numerous studies have examined the incidence of WTB in the United States and/or the impact associated with high concentrations of WTB on black walnut (Newton et al. 2009, Flint et al. 2010, Cranshaw 2011, Grant et al. 2011, Seybold et al. 2012, Randolph et al. 2013, Daniels et al. 2016). Yet, those previous studies were primarily focused on WTB occurrence and black walnut mortality in developed areas, such as city parks and residential areas. Many of these studies also were completed outside of the native range of black walnut, where thousands of black walnut trees had been transplanted. The discovery of WTB and the TCD complex within the native range of black walnut prompted the need for research focused on naturally-occurring trees found in mixed forests. Of further concern was the need for a risk assessment that may prove useful to commercial black walnut growers in the eastern and midwestern states. This information will provide insight on how WTB may progress through an existing stand of black walnut, enabling growers to develop better management strategies for future WTB infestations.

Research Objectives

To enhance the knowledge of TCD epidemiology within the native range of black walnut, and to better understand the role that forests may play in the spread of WTB, the focus of this research was to:

- 1) Document incidence and distribution of WTB on black walnut in Appalachian forests,
- 2) Assess dispersal of WTB in forests, and
- 3) Determine the dispersion pattern of WTB in black walnut orchards.

CHAPTER II

Incidence and Distribution of Walnut Twig Beetle Populations in Eastern Tennessee and Western North Carolina

Introduction

Black walnut, *Juglans nigra* L. (Juglandaceae), has experienced an increase in mortality since the early 1990s. Overwhelmingly, the largest number of dead and dying black walnut was seen throughout the western United States, outside its native range (Tisserat et al. 2009, Flint et al. 2010). In 2008, this increased mortality was determined to be caused by an insect-pathogen complex, later named thousand cankers disease (TCD), in reference to the amount of necrotic tissue, or number of cankers, that form as the pathogen grows within the bark (Seybold et al. 2010). The two primary organisms identified in this complex are the walnut twig beetle (WTB), *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae), and the phytopathogenic fungus *Geosmithia morbida* M. Kolařík et al. (Ascomycota: Hypocreales: Bionectriaceae). These organisms may have coevolved for centuries in the southwestern United States, with WTB transporting conidia of *G. morbida* on the outside of their bodies and leaving those conidia behind as they feed outside and inside the bark (Tisserat et al. 2009, Seybold et al. 2013).

WTB has been known to occur on other members of the *Juglans* genus, such as Arizona walnut, *Juglans major* (Torr.) Heller, and was not known to cause harm to species within its native range. Once black walnut trees were transplanted within the native range of WTB, it shifted to the new host and became problematic. WTB has shown a preference for feeding on and reproducing in black walnut over all other *Juglans* species, and black walnut exhibits the most severe symptoms to WTB and to the introduction of *G. morbida* (Utley et al. 2009, Utley et

al. 2013). As the number of dead trees increased among transplanted black walnut, so did the concern that a pathway may exist that would allow this devastating complex to be introduced to the native range of black walnut in the eastern United States. In 2009, eastern states were considered at “low risk” levels for the introduction of TCD (Newton et al. 2009). In 2010, those concerns about the spread of TCD were realized as the first occurrence of TCD east of the Mississippi River was reported in Knoxville, TN (Grant et al. 2011). As a result, several surrounding states have implemented county and state-wide quarantines of black walnut material in an attempt to prevent the potential introduction of TCD. Black walnut materials, such as logs, nursery stock, packaging materials, and roots or stumps, were regulated and prohibited from movement outside of designated areas. This study was designed to answer questions about the presence of WTB in Appalachian forests: where do they occur and are previously reported populations of WTB spreading throughout the native range of black walnut?

Materials and Methods

Site Selection and Trap Deployment

In 2016, 33 traps were deployed at several locations in eastern Tennessee and western North Carolina (Table 2.1, Fig. 2.1). These locations were selected within counties where TCD had been previously documented, and within surrounding quarantine buffer counties (Anderson, Blount, Knox, Monroe, Morgan, and Union Counties in Tennessee, Haywood County in North Carolina). At each location, conditions such as the presence of symptomatic trees, the number of neighboring trees, tree height, etc., were used to determine whether two to four Lindgren four-funnel stack traps (Synergy Semiochemical Corporation®) baited with WTB lure (Contech Enterprises®) were installed (Fig. 2.2). Three funnel traps were placed within the mixed forests at Chuck Swan Wildlife Management Area. Three traps were also placed at Loyston Point

Recreational Area, managed by the Tennessee Valley Authority (TVA). Two traps were placed among black walnuts on a privately-owned cattle farm at the Povo Road site (Monroe County, TN), which had not previously been monitored for WTB or TCD. Two funnel traps were placed in a wood lot in southern Knox County, TN, one near the edge of the forest and the other approximately 50 m away from the forest edge. The South Cumberland site was located at the University of Tennessee Forest Resources AgResearch and Education Center in Morgan County, TN, where two traps were placed near the forest edge in the only two black walnut trees detected in the area. The site in Blount County, TN was located on the Maryville College campus, within a large forested area, commonly used for recreational activities. Three funnel traps were placed at this site which had been used in previous WTB studies. Collaborators at Oak Ridge National Laboratory (ORNL) (Anderson County, TN) deployed and monitored four traps within the ORNL “Reservation” forested area. The final site in Tennessee was at Doyle Farm, which was located in northwest Knox County, TN. Two traps were installed within this 5.6 ha woodlot, which was primarily composed of declining green ash, *Fraxinus pennsylvanica*, and black walnut, both of which had been planted by the land owner more than 30 years earlier. Twelve traps were deployed across four sites within Haywood County, NC, with each site receiving three funnel traps suspended in or near black walnut (Table 2.1).

For the 2017 trapping season, the number of traps deployed for this study was reduced from 33 to 16 (Table 2.2, Fig. 2.3). Due to the high number of WTB recovered ($n=134$) at the Doyle Farm site in 2016, a new dispersion pattern study was initiated to assess the existing WTB infestation found there. As a result, 17 Lindgren traps were repurposed to support this new study. The remaining 16 traps were monitored as described below.

During both years at each site, limbs on symptomatic black walnut, ca. 6 to 12 m from the ground, were selected for trap placement in or near the tree canopy. In some cases, traps were placed on a neighboring tree, but was located no further than 6 m from a black walnut. Traps were filled with approximately 200 mL of an equal parts mixture of propylene glycol and water in the respective 500 mL collection cups. Each trap received a *P. juglandis*-specific 10 cm bubble cap pheromone lure (Contech Enterprises®) that was attached to the inner frame via zip ties, as specified by published trap construction guidelines (Seybold et al. 2013). To ensure efficacy, each lure was replaced every 30 d throughout the trapping season, April to November.

Sample Collection

Every two weeks, collection cups were removed from the corresponding traps, the contents were poured into 190-micron mesh paint filters (TCP Global®), and the traps were recharged with the propylene glycol mixture (Fig. 2.4). After samples were collected, the filters were placed into resealable plastic bags (3.8-liter), and the collection date, location name, and trap name/number were recorded on the outside of the bag. Samples were then taken to the Integrated Pest Management and Biological Control Laboratory at the University of Tennessee. Samples were stored in a Fisher Scientific Isotemp Incubator Model 304R at 1.5°C, until they were processed and analyzed for the presence of WTB adults. *P. juglandis* identification was achieved with the use of reference materials (such as Seybold et al. 2013), and with the assistance of Dr. Greg Wiggins and Dr. Jerome Grant, The University of Tennessee in Knoxville, TN.

Results and Discussion

2016 Collections

In 2016, 140 WTB adults were collected from five traps in three forested areas (Anderson, Blount, and Knox Counties) in TN (Table 2.1). WTB was documented for the first time at two sites, Doyle Farm and a forested location at Oak Ridge National Laboratory (ORNL). These sites represented the first documentation of WTB in forests in Knox and Anderson County, respectively. The greatest number of WTB ($n=134$) was collected at the Doyle Farm site, which prompted further investigation. While walnut trees at this site are not symptomatic, trees may begin to exhibit symptoms of TCD in the near future. One trap located at the ORNL location in Anderson County, TN recovered one WTB during the trapping season, but the remaining three traps failed to collect any WTB. Collections of WTB at the Maryville College trapping site were much lower ($n=5$) in 2016, than collections during previous years of trapping ($n=143$ in 2014, $n=338$ in 2015).

Conversely, WTB was not collected from 83% of traps ($n=25$) at eight locations. No WTB adults were collected from Chuck Swan Wildlife Management Area (three traps), Loyston Point Recreational Area (three traps), Povo Road (two traps), southern Knox County (two traps), or South Cumberland (two traps). None of the traps placed in Haywood County, NC recovered WTB.

2017 Collections

In 2017, the number of WTB recovered remained low (Table 2.2). Of the 16 funnel traps deployed across eastern Tennessee and western North Carolina, 82% ($n=13$) failed to collect adult WTB. The three traps located at the Maryville College site collected 10 WTB. Trap 1 recovered one WTB, with trap 2 and trap 3 recovering seven and two WTB, respectively. WTB

adults were not recovered from the ORNL forested site after its initial detection there in 2016. No WTB were collected from April to November from the remaining traps at all other 2017 trapping sites used in this study.

Summary

Though WTB has been found in traps throughout eastern Tennessee and western North Carolina since it was first detected within the native range of black walnut in 2010, the overall WTB population appears to be declining in these areas as evidenced by monitoring data collected over the past two years. Trapping at some of these sites, in the past, collected hundreds of WTB in a one-month period; traps at these sites now consistently reflect populations totaling less than 10 WTB collected throughout an entire trapping season of six or seven months at those same sites, two or three years later. While peaks in WTB emergence within mixed forests continue to mirror the trends seen in previous years, with population peaks in June and September, the overall numbers of emerging adults are low by comparison. While clumps of infestation have been detected, they have become increasingly difficult to find, suggesting a low incidence of WTB within forested systems.

This information suggests that WTB poses minimal risk to forested black walnut within its native range. This minimal risk is attributed to several factors. Variations in climate, such as warmer winters and early spring freezes, may affect the physiology of WTB within these regions (Hefty et al. 2016), which lie outside its native range. Native predators and pathogens may be impacting populations of WTB, inhibiting their ability to establish and spread in forests (Nix 2013). Also, an increase in annual precipitation may reduce the stress placed on black walnut, leaving the trees better equipped to handle an infestation of WTB, avoiding the release of stress pheromones which may attract more WTB (Blood et al. 2018). Finally, the ability of WTB to

move throughout urban areas and along forest edges has been documented in previous studies, but WTB may simply be unable to easily navigate through a mixed stand of undisturbed forest.

Further research on the ability of WTB to disperse throughout a mixed forest system will offer insight into whether WTB can effectively target specific pheromones, navigate through foreign semiochemicals, and ultimately colonize a new host. It is interesting to note that many of the original trees that tested positive for the TCD complex, both in urban and forested areas, now seem to have recovered from their initial infestation and infection. Some TCD-positive black walnuts in areas experiencing increased precipitation increased their percentage of live crown from 40% to 90% within three years (Griffin 2015). Perhaps a host tree struggles with a new infestation of WTB initially, but adapts over time, or the infestation/infection rate slows enough to allow the tree to manage the disease. Whether this recovery is permanent remains to be seen, but evidence suggests that the risk to survival of black walnut within its native range is minimal given appropriate environmental conditions.

CHAPTER III

Assessment of Dispersal of Walnut Twig Beetle Within a Forest System

Introduction

Thousand cankers disease (TCD) has been responsible for the death of thousands of black walnut trees since the disease was first identified and named in 2008 (Tisserat et al. 2009). Across the western United States transplanted black walnuts experienced an increase in mortality throughout the early 1990s. Initially, a small (1.5 to 2 mm) wood-boring beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae) (walnut twig beetle [WTB]), was believed to be the organism responsible for this increase in mortality (Cranshaw and Tisserat 2008). However, it was later discovered that a pathogenic fungus, *Geosmithia morbida* M. Kolařík et al. (Ascomycota: Hypocreales: Bionectriaceae), was a contributing factor in TCD. This fungus, which had been isolated from the tissue of dead and dying walnut trees, grew and spread throughout the phloem of walnut trees, cutting off nutrient supplies and leading to crown dieback and potential death (Utley et al. 2013).

WTB and *G. morbida* share a unique insect/fungal relationship in that the beetle transports the fungal conidia on the outside of its body and, as it tunnels under the bark, deposits conidia at several different sites. Once *G. morbida* has found a suitable entry point into the phloem, it begins to clog the vascular tissue of the tree as it grows. In response to this stress, the tree will release volatile compounds which attract more WTB transporting even more conidia of *G. morbida*. As male beetles colonize the host tree, they release an aggregation pheromone that attracts females as well as other competing males. Each newly arrived WTB will feed outside

and within the bark with the potential to deposit any conidia adhering to its body. A canker may form at each infection site.

In 2010, TCD was first reported within the native range of black walnut in a suburban area of Knoxville, TN (Grant et al. 2011). Surrounding counties and states implemented quarantines, just as areas in the western United States had done previously, to prevent and reduce the spread of TCD into new areas. However, most monitoring of TCD movement had been done in urban or disturbed habitats. WTB has been collected in traps placed in or near forested black walnut (Wiggins et al. 2014), and concern was high that the disease complex would place millions of trees at risk of infestation and infection. This study was designed to help characterize how WTB disperse in a mixed forest system, and to investigate potential dispersal patterns of WTB that may add to current TCD understanding and WTB control practices.

Materials and Methods

Site Selection and Trap Deployment

A three-year assessment of the dispersal ability of WTB within a forest was initiated at The University of Tennessee Forest Resources AgResearch and Education Center (Morgan County, TN) on 6 June 2016 (Fig. 3.1). Morgan County was previously designated a TCD quarantine county, which made it an ideal county for the release and recovery of WTB during this experiment. Before installation of the trapping area commenced, approximately 20 ha of forest area were scouted for the presence of mature black walnut (Fig. 3.2). No black walnut trees were located within the search area. Furthermore, no WTB had been previously collected at this site.

Four-funnel Lindgren stack traps ($n=78$) were baited with the 10 cm bubble cap lure (Fig. 2.2) containing a proprietary formulation (Contech Enterprises®) which mimicked the

aggregation pheromone released by male WTB. Attached to each trap was a 500 mL collection cup filled with approximately 200 mL of an equal parts mixture of propylene glycol and water. Traps were arranged in three transects, with each transect containing 24 traps (Fig. 3.3, Fig. 3.4). Each transect was positioned to follow naturally-occurring topographic features (i.e., ravines and stream beds), offering a type of natural barrier on either side of the row of traps. These topographical barriers are believed to aid in restricting released WTB adults to their respective release transects. The 24 funnel traps were deployed along each transect, suspended from 3 m long pieces of 1.27 cm diameter, thin-walled galvanized steel conduit, using heavy-gauge wire. Each piece of conduit was slid over a 1.2 m long section of 1.3 cm thick rebar, which had been driven 30 cm into the ground for stability. Sliding the conduit piping on and off the rebar allowed easy access to the collection cups. The traps were placed every 8 m (8 to 96 m) uphill and downhill from a central release site (Fig. 3.3). Transects were located approximately 100 m apart from one another, with the closest transect located approximately 560 m from the forest edge (Fig. 3.4). Additionally, each transect had one trap placed 50 m to the east and 50 m to the west of the center release point to monitor for movement among the transects.

Collection and Release of Walnut Twig Beetle

To collect sufficient numbers of WTB adults for each release, black walnut materials (limbs, trunk, and bark fragments) were collected from areas known to have populations of WTB from previous trapping efforts. Materials were collected from 17 April through 13 September 2016, with new material collected every six to eight days. For the 2017 study, black walnut materials were collected from 1 May through 5 September 2017, with new material collected every seven to ten days. This study is currently being repeated for a third year, and harvesting of walnut materials began on 16 May 2018, and is ongoing, with collection of new materials

occurring every seven to ten days. Collected black walnut materials were stored in 60 L transparent plastic storage containers (67.3 cm x 40.6 cm x 31.1 cm) with vented, mesh-lined lids (Fig. 3.5), labelled with the name of the collection site and date collected. A log sheet was provided to record the number of live WTB removed from each container. Limbs were collected at five separate locations across east Tennessee at Maryville College in Blount County (2016 and 2017), Choto Road in Knox County (2016 and 2017), Doyle Farm in Knox County (2016, 2017, and 2018), Oak Ridge National Laboratories in Anderson County (2017), and Daus Community Center in Sequatchie County (2018). The limb pieces ranged in size from 2.5 to 16 cm in diameter. Trunk and bark samples, ranging from 25 to 39 cm diameter, were collected from a black walnut (25 m) that had fallen during a severe weather event at the Maryville College campus in June 2017.

On 10 June 2016, a total of 150 WTB adults, which had been collected from the containers within the previous 48 hours, were taken to the Morgan County site and released across the three arranged transects (25 females and 25 males per transect). The beetles were transported to the release areas inside six Petri dishes (50 x 9 mm) containing a damp filter (47 mm membrane). Three Petri dishes contained 25 male WTB each, with the remaining three Petri dishes containing 25 female WTB. At the central point within each transect, the lid and filter membrane of each Petri dish were removed and placed next to one another, and the beetles allowed to disperse. Once the beetles had been released, the traps were monitored every seven to ten days, for a period of five weeks. Contents of the collection cups were emptied into 190-micron mesh paint filters (TCP Global®), which were then placed into resealable plastic bags (3.8-liter). The collection date and trap name/number were recorded on the outside of the bag and traps were recharged with the propylene glycol mixture. Samples were then taken to the

Integrated Pest Management and Biological Control Laboratory at the University of Tennessee and examined under a microscope for the presence of WTB. Suspect beetles were stored in 7.3 ml vials containing 190-proof ethanol solution (75% ethanol, 25% water, until positive WTB identification was confirmed by use of the University of California identification publication (Seybold et al. 2013), and the assistance of Dr. Gregory Wiggins and Dr. Jerome Grant of the Entomology and Plant Pathology Department at The University of Tennessee in Knoxville.

To decrease the risk of overlapping collection data, all 78 traps were deactivated (i.e., lures removed) for five weeks between the Summer and Fall releases. Collection cups were left empty and lures were not replaced until two days before the second release. On 31 August 2016, a second release of 150 adult WTB occurred, utilizing the same procedures described for the first release. This release was followed by five additional weeks of monitoring every seven to ten days. Following all releases, the number of WTB recovered, date of recovery, trap location, and replication number were recorded on each collecting date.

Data Analysis

Linear regression analysis was used to assess both Summer and Fall releases to identify trends in WTB movement throughout a mixed forest using SPSS (IBM Corp. 2017). Summer and Fall releases for each year were analyzed separately using the total number of WTB collected from all three transects after each release. Comparisons were conducted by associating the absolute distance of the trap from the central release point to the number of recovered WTB at each distance. Criterion alpha was set at $\alpha = 0.05$, and r-square was calculated.

Results and Discussion

Dispersal Study- 2016

WTB were recovered in traps at a range of distances (8 to 96 m) and in all cardinal directions following both Summer and Fall releases. No apparent trend in WTB dispersal within a mixed forest was detected from collections following both releases. Of the initial 300 adult beetles released in 2016, only 7.3% ($n=22$) were recovered. Recovery of WTB was higher during the Summer release ($n=14$) than during the Fall release ($n=8$) (Fig. 3.6). The recovery of only 22 WTB (7.3%) from the original 300 that were released suggests WTB may not navigate effectively to the lures and traps when used in a mixed forest system.

Linear regression analysis following Summer ($R^2= 0.030$, $p= 0.571$) (Fig. 3.7A) and Fall ($R^2= 0.008$, $p= 0.771$) (Fig. 3.7B) found no significant trends. The sex ratio of the recovered beetles following the Summer release was 6:1 (twelve males and two females). For the Fall release, eight males and no females were recovered. This result mirrors the phenomenon of male *P. juglandis* responding to aggregation pheromones in greater numbers than females in previous studies (Hefty et al. 2016).

The expectation prior to these releases was that most of the WTB would fly to the nearby traps within the first weeks and be collected; however, beetles were recovered each week of the five-week trapping cycle, with 18 traps recovering WTB, and 11 of those traps located 50 m or farther from the central release point. The recovery distances demonstrate the ability of WTB to disperse within a mixed forest in search of a suitable host. However, dispersal may have been enhanced by the close proximity of traps to one another, creating a ‘chemical corridor’ to assist dispersal to traps farther away. The presence of foreign compounds or chemicals produced by

various plant and animal species within the forest system may also have added to any confusion or stress experienced by the WTB upon release.

Dispersal Study- 2017

All traps were reset with fresh propylene glycol mixture and new WTB lures on 2 June 2017. The Summer 2017 release and recovery was scheduled to begin 5 June 2017, but low emergence of WTB adults from collected black walnut material failed to produce sufficient numbers at any one time to be able to replicate the study. Collections were made during the original five-week study period even though no WTB had been released. As expected, no WTB were recovered within the study area, suggesting that the 22 WTB collected during the 2016 releases were, in fact, the same beetles that had been artificially introduced for the purposes of this study. A lack of WTB collections at this site in 2017 also supports the Chapter 1 conclusion that incidence of WTB in a mixed forest system is low. The transects were recharged on 28 August 2017, in anticipation of the Fall release, scheduled to begin 30 August 2017. However, low adult emergence from the containers of walnut material forced the study to be abandoned once again.

Dispersal Study- 2018

Traps used for the 2018 releases were recharged with new WTB lures and fresh propylene glycol mixture on 31 May 2018 in preparation for the Summer 2018 release cycle. However, black walnut material stored in emergence containers continued to yield low numbers of adult WTB. Efforts are ongoing and new material will be collected and stored to monitor for appropriate WTB emergence that will allow for this study to be replicated in 2018.

Summary

In 2016, WTB were collected in traps at a range of distances and directions from both Summer and Fall releases. No apparent trend in dispersal was observed. Out of 78 traps deployed across three transects, 18 traps recovered WTB throughout the entire 2016 releases. WTB did demonstrate small-scale dispersal ability within the forest system. Dispersal to traps located farther away from the central release point may have been enhanced by the proximity of traps to one another, creating a ‘chemical corridor’ capable of guiding WTB to other traps.

Further research is needed to determine the effects that trap spacing may have on dispersal. Although no black walnut were identified within the study area, volatiles produced by other organisms existing within a mixed forest setting may have attracted or repelled the released WTB, causing beetles to navigate ineffectively. While care was taken to avoid premature death of released adults, it is possible that some WTB were weakened by the stress of transport between the laboratory facilities and the study site and, as a result, never have taken flight. Previous studies had suggested that age of the beetle may affect flight duration, with older beetles having depleted lipid content and being less likely to fly as far as younger beetles (Hefty et al. 2016). With this in mind, beetles used in releases had been collected within 48 hours of emergence to offer the best chance at dispersing from the release point. Furthermore, predatory animals, insects, and bacteria that inhabit forested systems may have reduced the number of beetles available for recovery in baited traps during the 5-week period. Assessment of the procedures utilized in the collection and subsequent release of adult WTB could yield more effective methods of rearing and recovery.

CHAPTER IV

Determining Dispersion Patterns of Walnut Twig Beetle in Black Walnut Orchards

Introduction

In 2010, a devastating disease complex named thousand cankers disease (TCD) was discovered within the native range of black walnut, *Juglans nigra* L. (Juglandaceae) (Grant et al. 2011), which extends from the Atlantic Coast of the United States and ends just west of the Mississippi River, and from the Gulf Coast states into southern Canada (Fig 1.5). TCD was identified as the condition responsible for thousands of black walnut deaths since the late 1980s. The complex consists of a small curculionid beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae), the walnut twig beetle (WTB), and a newly described pathogenic fungus, *Geosmithia morbida* M. Kolařík et al. (Ascomycota: Hypocreales: Bionectriaceae) (Tisserat et al. 2009, Kolařík et al. 2011). The fungus relies on WTB reaching new host trees to colonize. The beetle excavates galleries within the tree bark, where they produce future progeny. As they feed outside and under the bark, beetles deposit microscopic conidia of *G. morbida*. These conidia grow and coalesce within the phloem of the tree, blocking crucial nutrient pathways and causing limbs to die (Tisserat et al. 2009, Utley et al. 2009).

Though Tennessee comprises a smaller component of native black walnut, other states, such as Missouri, Ohio, and Kentucky, are home to nearly 35% of the live black walnut trees found throughout the eastern United States (Randolph et al. 2013). Many of these trees occur in orchard systems and carry a tremendous amount of economic value for nut, nursery, and timber producers across the native range. Previous research (Wiggins et al. 2014) detected the limited

presence of WTB within forested locations of black walnut, suggesting that trees located within the interior areas of a forest may be at lower risk to WTB infestation due to a buffer of other hardwood species. Further study of the dispersal and dispersion patterns displayed by WTB was necessary.

Dispersion is a pattern of spatial arrangement found within a population, and can be classified into three categories: random, clumped, and uniform (Armstrong 1977). Random dispersion patterns occur when individuals within a population are found independent of one another. To observe this pattern, attractants and repellents, such as pheromones, would have no effect on the behavior of an individual beetle. Because WTB have been shown to be dependent on aggregation signaling (Hefty et al. 2016, Blood et al. 2018), clustering or clumped aggregation is likely when populations move into a new area. Thus, clumped dispersion may be expected with WTB. A clumped dispersion is generally influenced by resource limitations or behavior and would be indicated by groupings or clusters of individuals. If individuals within the study area are found to display uniform dispersion, they will be evenly distributed, often a result of territorial behavior (Walker 2011).

A study was designed to 1) focus on the movement of WTB within planted stands of black walnut over the course of a growing season, and 2) assess the risk of WTB infestation to neighboring trees by determining whether WTB exhibited a random, clumped, or uniform dispersion pattern. Two sites were selected, and the extent of WTB infestation was documented throughout the year and the dispersion of WTB throughout the summer season assessed using spatial analysis.

Materials and Methods

In the spring of 2017, two sites were selected, Doyle Farm in Knox County, and an orchard at Oak Ridge National Laboratory (ORNL) in Anderson County (Fig 4.1). Pheromone-baited traps were used to assess presence of WTB at both sites in 2017 and 2018.

Doyle Farm

This study site was an existing stand of black walnut at Doyle Farm in Knox County, TN (Fig. 4.2). The designated research area was ca. 5.6 ha; according to the landowner, this area contained over 1,300 black walnut trees, planted as seeds in the mid-1990s, and grown alongside approximately 5,000 declining green ash (*Fraxinus pennsylvanica* Marshall). The original intent of the landowner was to harvest the trees for timber several years later. All black walnut trees at least 5 cm in diameter ($n = 276$) were georeferenced using a Garmin® Montana 650t global positioning unit and tagged with 15 cm aluminum tags (Loma Industries®) to record the reference number assigned to each tree. The largest recorded tree diameter at Doyle Farm was 64 cm. The mean diameter across the site was 27 cm.

Walnut trees in this orchard were not regularly distributed or planted at consistent intervals because 1) the use of seeds instead of saplings resulted in inconsistent germination, and 2) volunteer trees originating from more mature trees were not removed. Therefore, using a grid to randomize the trap trees throughout the site would not account for the localized density and distribution of trees. Thus, the site was divided into clusters of adjacent trees using the “Grouping Analysis” tool in ArcMap 10 (ESRI 2011) to better represent tree density and dispersion. To determine the number of traps needed to adequately sample the site (and thus the number of groups needed) a power analysis was conducted by a statistician at The University of Tennessee, Dr. Arnold Saxton, and determined that a sample of 25 trees would provide a

sufficient representative sample of the georeferenced tree population. Grouping analysis was set to 25 groups (Fig. 4.3A) and one tree from each resulting group was selected as a spatial representative for that group of trees (Fig. 4.3B). This method allowed for a more representative distribution of trap trees throughout the site. An additional five “check” traps were positioned in randomly selected black walnut trees and were used to test the accuracy of the dispersion model that would be generated at the end of the collection season.

The 30 Lindgren funnel traps (25 monitoring traps, 5 check traps) were baited with WTB lure (Contech Enterprises®) and deployed in the selected walnut canopies, 7 to 10 m from the ground depending upon limb arrangement, with one trap per tree. Traps were suspended by 15 to 24 m lengths of paracord rope (3 mm) which were draped over limbs and allowed to operate in a pulley-like fashion, raising and lowering the attached funnel trap when needed. Traps were monitored every 10 to 14 days, with samples collected in 190-micron mesh paint filters (TCP Global®), placed into sealable plastic bags (1.75 ml), with the collection date, trap number, and site name written on the bag. The number of WTB captured in each trap on each collection date was recorded as samples were processed. Parameters, such as diameter at breast height (dbh) and tree density, were considered to account for variations in beetle distribution.

Oak Ridge National Laboratory

A second black walnut orchard (ca. 6 ha), managed by Oak Ridge National Laboratory (ORNL) in Anderson County, TN, was selected to replicate of the study (Fig. 4.1). Unlike Doyle Farm, this site consisted solely of black walnut trees, arranged in rows, and fairly evenly spaced (12 to 15 m apart). Some mortality was observed but appeared to occur randomly to individual trees rather than in large patches consisting of several trees. Of the 339 black walnuts identified within the study area, 14 (4.3%) were identified as dead, producing no new growth in the spring.

These dead trees were excluded from the overall count and were not candidates to receive traps, leaving 325 viable trees available for the study. The remaining 325 black walnuts were georeferenced using a Garmin® Montana 650t global positioning unit and each tree tagged with a 15 cm aluminum tag displaying a unique number for field identification.

Black walnut at the ORNL site had been planted in three discrete, noncontinuous sections. One section (ca. 1.6 ha), containing 155 black walnuts trees (Fig. 4.4A), was selected as the study area to be modelled. Because the trees were fairly uniform in spacing, age, and density, trap tree selection at ORNL was much easier to perform than at Doyle Farm. While walking back and forth along the rows of black walnut, 15 trees were randomly selected to receive traps (Fig. 4.4B). Trap deployment commenced on 9 June 2017 in identical fashion and with the same materials as described for the Doyle Farm study. Whenever possible, traps were hung 7 to 10 m from the ground, depending on limb arrangement of the selected tree. Collections of trap catches began on 21 June and continued through 30 October 2017. Samples were collected every 10 to 14 days and taken to the University of Tennessee for processing. Trap number, date collected, and number of beetles collected were recorded for each corresponding sample. As with Doyle Farm, diameter and tree density were noted for comparison among trap collections.

Data Analysis

Cumulative trap catch data were analyzed using linear regression in SPSS (IBM Corp. 2017) to explain the effects of tree density within 10 m of trap trees and dbh on the number of WTB collected. Criterion alpha was set at $\alpha = 0.05$, and r-square was calculated.

Spatial analysis was conducted to estimate infestation of WTB in trees neighboring trap trees based on trap collections, as well as illustrate variations in the dispersion pattern of WTB

throughout the trapping season. Weekly and cumulative trap catch data were used to model infestation of adjacent trees using the interpolation method inverse distance weighting (IDW) in ArcMap 10 (ESRI 2011). This method estimates values near a sample point by linearly weighting neighboring points as a function of their distance from the sample point. For the analysis, the weighting power was set at two, and the search radius used to conduct the interpolation was set to the nearest 12 trees to each trap tree. The surface maps generated from IDW analysis predicted WTB numbers in trees adjacent to the trap tree. The maps from each sampling date can be compared to estimate dispersion patterns of WTB within the site over the season.

To determine if dispersion within the site was significantly clumped, autocorrelation analysis was conducted using the Incremental Spatial Autocorrelation tool in ArcMap. This tool measures spatial autocorrelation for a series of distances using the Global Moran's I index. This index measures spatial autocorrelation using both location and feature values. Based on the fit of observed compared to expected values, a coefficient and associated p-value that reflect the intensity of spatial clustering at distances among sampled points were calculated, resulting in estimates of the significant distances at which spatial clustering occurs. Cumulative trap catches for the entire season were the feature values, and the number of distance bands was set at 10, with distances calculated using the Euclidean method. Distances at which significant aggregation occur were then used as a threshold to calculate the Getis-Ord G_i^* statistic. This statistic estimates where areas of significant clustering occur within the site and is calculated in ArcMap using the Hot Spot Analysis tool. A coefficient and associated p-value were generated, identifying where features with either high or low values cluster spatially and where non-random

clustering is most pronounced. This study was repeated in 2018, and data from the 2018 study is still being collected and was not available for inclusion.

Results and Discussion

Doyle Farm

During 2017, 299 WTB were collected. Throughout the trapping season, WTB were found to occur during all but one collection period (6 September 2017). The lack of WTB adults collected on this sampling date may be due to a possible decrease in the number of WTB adults taking flight, as populations were between generations and the second seasonal peak in emergence occurred a few weeks later. Emergence peaks were seen in June and September, mirroring previous years of collection data for WTB emergence in east Tennessee .

Sequential examination of the images generated from IDW interpolation illustrated seasonal dispersion of WTB at Doyle Farm. Dispersion of WTB within the site varied from week to week, with WTB collected at the eastern and western ends of the site, and little to no occurrence in traps in the middle of the study area (Fig. 4.5). When all 2017 collections are combined, and interpolation conducted, certain areas of the site exhibit higher concentrations of WTB. These areas occurred on the southwest and northeast edges and corners of the site. In contrast, the traps in the middle of the site had few to no WTB collected throughout the entire trapping season.

Autocorrelation analysis indicated that significant ($p = 0.0087$) clustering of WTB occurred at distances ≤ 108 m. Using this threshold, Hot Spot Analysis indicated significant (p -values range from 0.0032 to 0.0461) non-random clustering of WTB in the southwest corner of Doyle Farm (Fig. 4.6). These are areas where WTB is expected to be encountered. “Cool spots,” or areas where WTB is not expected to be encountered, were found to be significant (p -

values ranged from 0.0036 to 0.0416) in the center of Doyle Farm. The dispersion pattern of WTB at Doyle Farm is more spatially clustered along the southwestern edge of the site than would be expected by random chance, likely due to the density of black walnut surrounding those trap trees.

Linear regression analysis of density of walnut trees within 10 m of trap trees did not significantly influence the total number of WTB collected throughout the season ($R^2 = 0.09$, $p = 0.149$) (Fig. 4.7). When all trap trees were analyzed, the dbh of trap trees did not influence the total number of WTB collected throughout the ($R^2 = 0.006$, $p = 0.719$) (Fig. 4.8A). However, a large diameter (60 cm) tree was identified among the 25 trap trees and the other 24 trap trees had a diameter range of 15 to 35 m. If the 60 cm ‘outlier’ is removed, dbh of the remaining 24 trap trees does significantly influence WTB collections ($R^2 = 0.194$, $p = 0.0310$) (Fig. 4.8B).

As previously discussed, the walnut orchard at Doyle Farm was not a conventional or intensively managed orchard. Black walnut was found to occur sporadically across the site, often intermingled with the remains of what once were living green ash trees. The emerald ash borer, *Agrilus planipennis* Fairmaire, infested the Doyle Farm site many years ago, and few living ash trees remain. The dead and declining ash trees may play a role in the dispersal of WTB and other species throughout this site. With more light and moisture reaching the ground below, a more diverse community of herbaceous plant species is becoming established. In the future, black walnut will be the dominant tree found in the canopy zone at Doyle Farm. This increase in herbaceous plant biodiversity may in turn bring new predators, diseases, and other stress factors into the area, affecting both black walnut and WTB in beneficial or negative ways. Revisiting the Doyle Farm site over the next few years will provide more definitive information on how the changing landscape impacts WTB populations.

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The trend of low numbers of WTB adults across east Tennessee was also seen at the ORNL site in 2017. Low numbers of WTB were not entirely unexpected, as only one WTB had previously been collected near this location in the past, indicating the presence of a smaller population here than existed at Doyle Farm. In total, six male WTB were recovered at ORNL from 9 June to 30 October 2017. Male WTB are considered the pioneering sex that seeks out a viable host before attracting a female and are more likely to be collected as they explore their surroundings. Two beetles were collected on 21 June, one collected on 11 July, two more collected on 1 August, and the last beetle was collected on 29 August (Fig. 4.9). From a stand of 155 live black walnuts, all six WTB were collected from just two trees located roughly 33 m apart. No other trees or topographical barriers existed between the two trap trees. The larger than average gap of more than 33 m effectively placed these trees at the ends of long rows of black walnut with no overlapping limbs or canopy. Perhaps this arrangement created an edge effect, which WTB have shown a preference for at other trapping sites.

Interpolation (IDW) indicated apparent clumped distribution among WTB at ORNL (Fig. 4.10). However, autocorrelation analysis detected no significant associations (lowest p-value was 0.139 at 52.8 m). Linear regression analysis did not reveal a significant association between the number of WTB collected to tree dbh ($R^2 = 0.096$, $p = 0.182$) (Fig. 4.11A) or tree density at 10 m ($R^2 = 0.008$, $p = 0.693$) (Fig. 4.11B). The low number of WTB collected at this site came from only two trees, suggesting a relatively new infestation. As a result of the low catch numbers during the 2017 season no further analysis was conducted. This location was reset on 11 June 2018, all traps were recharged fresh WTB lure and collection cups filled with new propylene glycol solution. Collections are ongoing and will continue through October 2018.

Summary

WTB were primarily collected from trees located in the northeastern and southwestern corners of the Doyle Farm site. Beetle catches varied between collection periods most likely due to periodic peaks in emergence and beetle movement across the site. WTB expressed a clumped distribution within both study sites. Given ample supply of host material and less natural barriers to dispersal, as would be found in a mixed forest situation, WTB seemed able to locate other black walnut trees as hosts. Though the ORNL site produced too few beetles to perform meaningful analysis, the pattern seen at Doyle Farm was also observed there. WTB collected at ORNL revealed a similar preference to clump within only a few hosts, rather than disperse randomly across the entire site. Tree stress, presence of predators or disease, and the length of overall infestation may have an effect on WTB populations at these sites. Traps located near the ORNL site had only recently detected the presence of WTB in the past year, whereas WTB at Doyle Farm, whose densities were much higher, had likely been present at that location for some time. Continued monitoring at each site would provide additional insight on how length of infestation influences dispersion patterns. Diameter of the trap tree, as well as neighboring trees, was not found to have a significant impact on WTB density within the site. The results of this study will be useful to black walnut producers in several states as it will document how WTB may progress through orchards and will inform risk assessments of existing black walnut stands.

Future studies of WTB dispersion patterns within black walnut orchards could enhance development of prediction models. For example, increasing the number of traps within each site may provide a better representation of the clustering events leading to more accurate predictive models. Analyzing more variables, such as temperature, relative humidity, topography, and soil moisture, and including those data in other interpolation methods both within each site as well as

between these sites may tease out additional information which may generate new models of beetle distribution, answering how and why WTB seem to colonize one particular host over another. Knowing which areas of a black walnut stand are infested and how WTB may progress throughout the stand will enable land owners to update their current management strategies to reduce the risk of future WTB infestations and avoid economic, ecological, and environmental losses.

CHAPTER V

Conclusions

The health and survival of black walnut within its native range have been threatened for nearly a decade. With thousands of trees succumbing to thousand cankers disease (TCD) in the western United States, the prognosis appeared bleak for the future of black walnut in the eastern United States. TCD is an insect-pathogen complex. A small (1.5 to 2 mm) wood-boring beetle, *Pityophthorus juglandis* Blackman (Coleoptera: Curculionidae), the walnut twig beetle (WTB), has been identified as the primary vector for *Geosmithia morbida* M. Kolařík et al. (Ascomycota: Hypocreales: Bionectriaceae), a phytopathogenic fungus (Tisserat et al. 2009, Kolařík et al. 2011). The barrel-shaped conidia of *G. morbida* are transported by WTB to new host trees and are deposited at multiple locations as the beetles probe for feeding sites both outside and inside the bark. The conidia grow to a determinant size within the phloem of the tree, obstructing the flow of nutrients, which leads to the formation of cankers. If enough cankers coalesce the tree will begin to show symptoms of stress (yellow foliage, flagging limbs, crown dieback), and may potentially die (Tisserat et al. 2009, Utley et al. 2009).

Past trapping for WTB in eastern Tennessee collected hundreds of adult beetles at some locations (Wiggins et al. 2014). WTB populations in those same areas, however, appear to be declining. Areas that once collected hundreds of WTB in a single funnel trap now recover, at the most, double digit numbers. In most areas, the occurrence of WTB can still be detected, but the overall number of beetles present has declined. Reports of WTB in new counties and states has slowed and finding new infestations of WTB has become increasingly difficult. These findings suggest that WTB poses low risk to forested black walnut. The discovery of new infestations are most likely due to infested black walnut materials being moved anthropogenically. The rapid

implementation of state-enacted quarantines undoubtedly helped slow the spread of infested/infected materials.

Incidence and movement of WTB within a mixed forest are limited. When WTB adults were released into a forested system in the absence of black walnut hosts, they were unable to properly identify pheromone baited traps, as only 22 of an initial 300 released beetles were recovered in lure-baited traps. More WTB males ($n=20$) were recovered than females ($n=2$), which was generally the case in natural trapping scenarios, as male WTB are considered the pioneering sex. The recovered WTB displayed no trend in dispersal patterns, as they were collected at a range of distances (8 to 96 m) and directions (North, South, East, and West) from a central release point. Dispersal of WTB into and across forested areas is likely impaired by interference from a mixture of volatiles produced by other plants or insects, acting as either attractants or repellents of WTB (Blood et al. 2018). Perhaps, in an urban setting, the pheromone lures are sufficient to attract WTB, but in the interior of a mixed forest those signals are obscured or altogether lost.

In an orchard setting, where multiple hosts were present with limited interference, WTB colonized clusters of trees, in close proximity to one another. Beetles were collected in nearly every one of the 30 lure-baited traps positioned throughout the site, but the most active locations were seen along the perimeter of the orchard. Significant nonrandom clustering was detected in the southwest corner of the Doyle Farm site, demonstrating a clumped distribution. At the ORNL orchard site, only six WTB were collected throughout 2017, but those beetles also displayed a clumped distribution, as they were collected from two adjacent black walnut trees along the perimeter of the site. In contrast, the middle area of both sites was rarely shown to collect any WTB, suggesting that WTB prefer the outer edges and corners of the sites where the

beetle densities were highest. WTB seem to prefer the edges of wooded areas rather than the interiors of stands. This information could prove useful to orchard and forest managers alike by informing risk assessment decisions to focus control efforts (tree removal, chemical pesticides, pheromone trapping, or biological control) along the perimeter of a site instead of spending time and money on black walnut located deep within a site.

The variations in the activities of WTB documented in these studies could be due to changes in climate, such as warmer winter or cooler spring months, as well as periods of drought versus periods of high rainfall. The sites themselves could be undergoing some sort of transition in species composition or other disturbances. Perhaps predators or pathogens were able to reduce the number of WTB (eggs, larvae, pupae, and adults) within these sites and have contributed to reducing or slowing the spread of WTB into new areas. Public outreach and education programs combined with the implementation of county and state quarantine guidelines have also assisted in the lower occurrence of WTB within the native range of black walnut.

The future of black walnut within its native range is still in question. Will WTB populations and reports of new beetle infestations increase in the future? Will *G. morbida* successfully infect black walnut with the aid of new insect vectors? How will emerging technologies enhance the ability to detect TCD? Continued monitoring of WTB is necessary to provide accurate information about population densities or movement into new areas and educating the public about the latest information, such as quarantine restrictions and infestation/infection pathways, through publications and public outreach. Effective and efficient coordination between state and federal government is essential to minimize wasted resources, such as time, money, and black walnut trees.

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APPENDIX

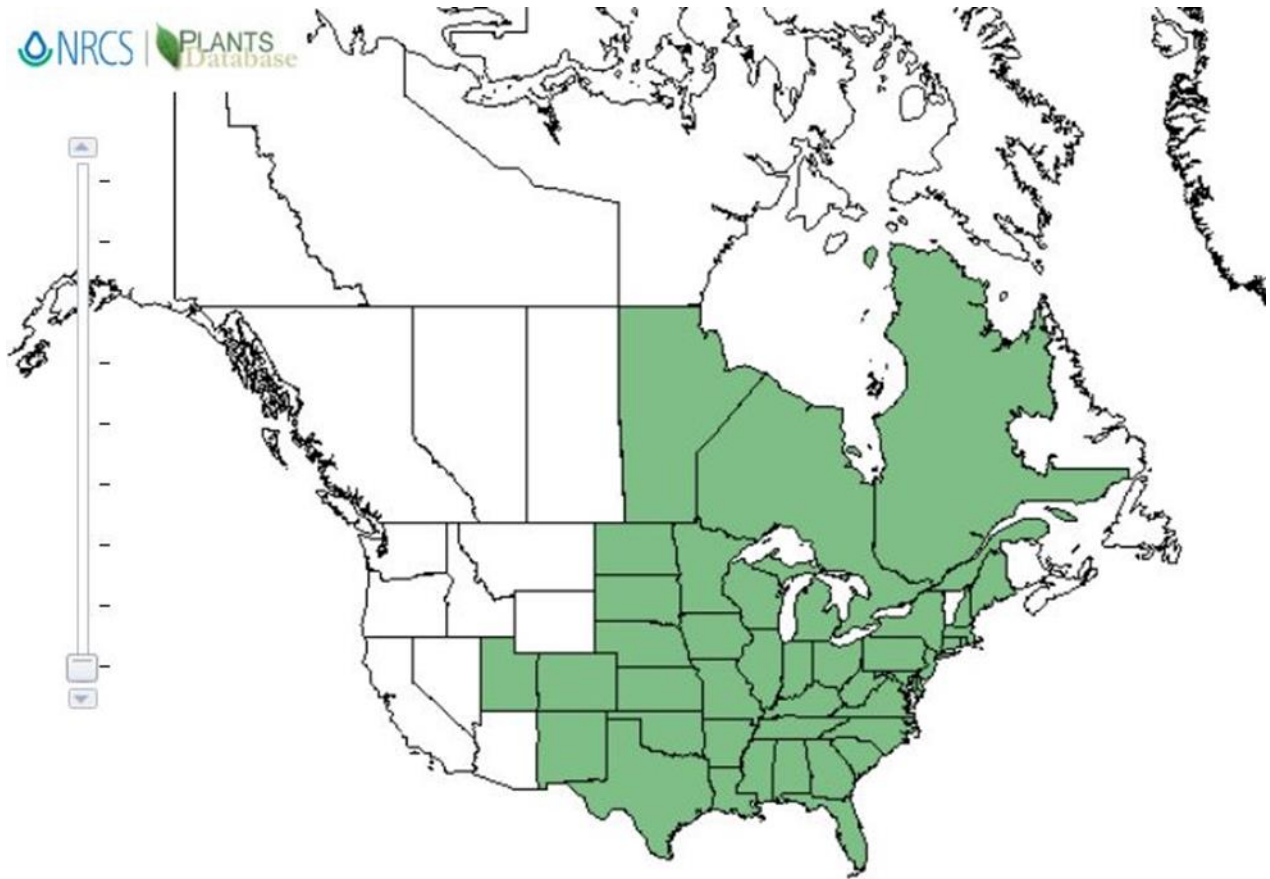




Figure 1.2 Identifying characteristics of black walnut, *Juglans nigra* L., include compound leaves with lanceolate shaped leaflets and round green-fleshed fruit (Brockman 1968).



Figure 1.3 Female (A) and male (B) walnut twig beetle, *Pityophthorus juglandis*. Note the additional pubescence on the frons of the female (courtesy of Bugwood.org).

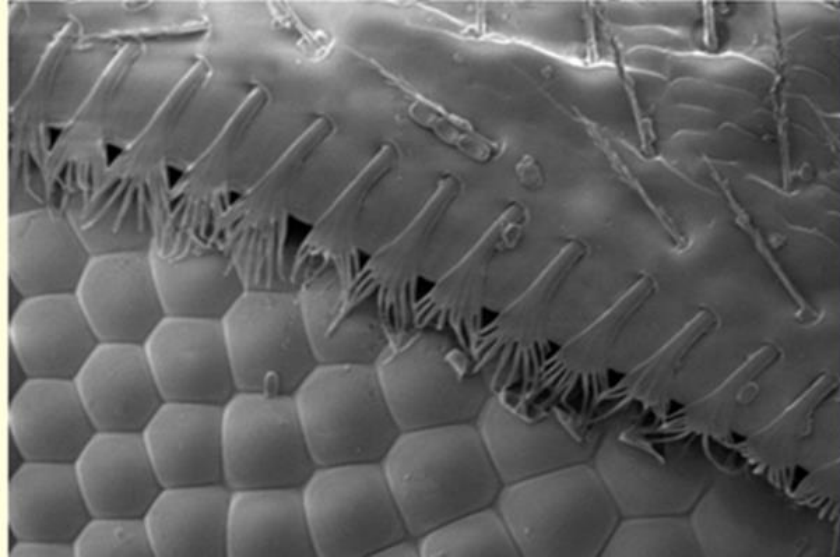


Figure 1.4 Conidia of *G. morbida* on the head of walnut twig beetle, *Pityophthorus juglandis*, (2.21K magnification) (courtesy of Paris Lambdin).

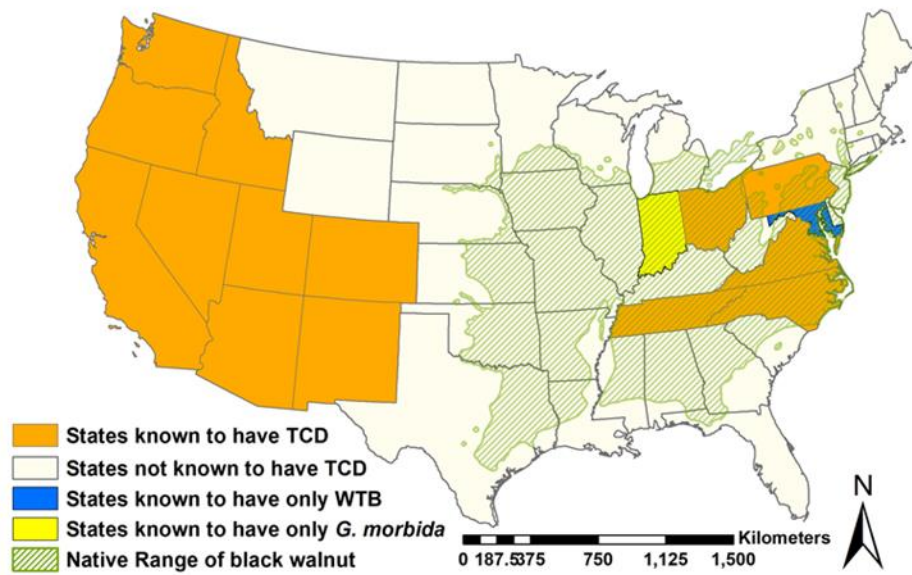


Figure 1.5 Map of states confirmed to have thousand cankers disease (TCD), and active quarantine zones (courtesy of www.thousandcankersdisease.com, 2018).

Chapter II

Table 2.1 Trap locations and total number of walnut twig beetles collected from forested black walnuts in Tennessee and North Carolina, 2016.

Trap Name	Latitude	Longitude	Distance (m) ¹	County	State	WTB ²
Chuck Swan 1	36.33787	-83.93489	105	Union	TN	0
Chuck Swan 2	36.35059	-83.90076	220	Union	TN	0
Chuck Swan 3	36.37043	-83.89767	250	Union	TN	0
Doyle Farm 1	36.05921	-84.05929	75	Knox	TN	131
Doyle Farm 2	36.05815	-84.06011	75	Knox	TN	3
Maryville College 1	35.74875	-83.96189	1	Blount	TN	1
Maryville College 2	35.74788	-83.96299	92	Blount	TN	0
Maryville College 3	35.74783	-83.96229	70	Blount	TN	4
ORNLO5	35.96133	-84.23975	90	Anderson	TN	1
ORNLO2	35.96262	-84.23445	312	Anderson	TN	0
ORNLO3	35.93862	-84.24973	108	Anderson	TN	0
ORNLO4	35.93283	-84.24505	0	Anderson	TN	0
Loyston Point 1	36.27603	-83.95559	53	Union	TN	0
Loyston Point 2	36.27748	-83.95460	125	Union	TN	0
Loyston Point 3	36.27727	-83.95812	105	Union	TN	0
Povo 1	35.51352	-84.31053	35	Monroe	TN	0
Povo 2	35.51365	-84.31013	55	Monroe	TN	0
South Cumberland 1	36.04555	-84.43437	0	Morgan	TN	0
South Cumberland 2	36.04499	-84.43444	3	Morgan	TN	0
South Knox 1	35.91436	-83.84081	0	Knox	TN	0
South Knox 2	35.91401	-83.84042	50	Knox	TN	0
UTNC 01.01	35.76213	-83.10484	0	Haywood	NC	0
UTNC 01.02	35.76213	-83.10484	0	Haywood	NC	0
UTNC 01.03	35.76213	-83.10484	0	Haywood	NC	0
UTNC 02.01	35.76221	-82.98059	1	Haywood	NC	0
UTNC 02.02	35.76221	-82.98059	1	Haywood	NC	0
UTNC 02.03	35.76221	-82.98059	1	Haywood	NC	0
UTNC 03.01	35.60957	-83.01167	0	Haywood	NC	0
UTNC 03.02	35.60957	-83.01167	0	Haywood	NC	0
UTNC 03.03	35.60957	-83.01167	0	Haywood	NC	0
UTNC 04.01	35.61873	-83.03313	1	Haywood	NC	0
UTNC 04.02	35.61873	-83.03313	1	Haywood	NC	0
UTNC 04.03	35.61873	-83.03313	1	Haywood	NC	0

¹ Approximate distance in meters from the forest edge. ² Number of walnut twig beetle collected from trap.

Table 2.2 Trap locations and total number of walnut twig beetles collected from forested black walnuts in Tennessee and North Carolina, 2017.

Trap Name	Latitude	Longitude	Distance (m) ¹	County	State	WTB ²
Chuck Swan 1	36.33787	-83.93489	105	Union	TN	0
Chuck Swan 2	36.35059	-83.90076	220	Union	TN	0
Chuck Swan 3	36.37043	-83.89767	250	Union	TN	0
Maryville College 1	35.74819	-83.96204	32	Blount	TN	1
Maryville College 2	35.74788	-83.96299	92	Blount	TN	7
Maryville College 3	35.74783	-83.96229	70	Blount	TN	2
ORNLO5	35.96133	-84.23975	90	Anderson	TN	0
ORNLO2	35.96262	-84.23445	312	Anderson	TN	0
ORNLO3	35.93862	-84.24973	108	Anderson	TN	0
ORNLO4	35.93283	-84.24505	0	Anderson	TN	0
Loyston Point 1	36.27603	-83.95559	53	Union	TN	0
South Cumberland 1	36.04555	-84.43437	0	Morgan	TN	0
South Cumberland 2	36.04499	-84.43444	3	Morgan	TN	0
UTNC 01.01	35.76213	-83.10484	0	Haywood	NC	0
UTNC 02.01	35.76221	-82.98059	1	Haywood	NC	0
UTNC 02.02	35.76221	-82.98059	1	Haywood	NC	0

¹ Approximate distance in meters from the forest edge. ² Number of walnut twig beetle collected from trap.

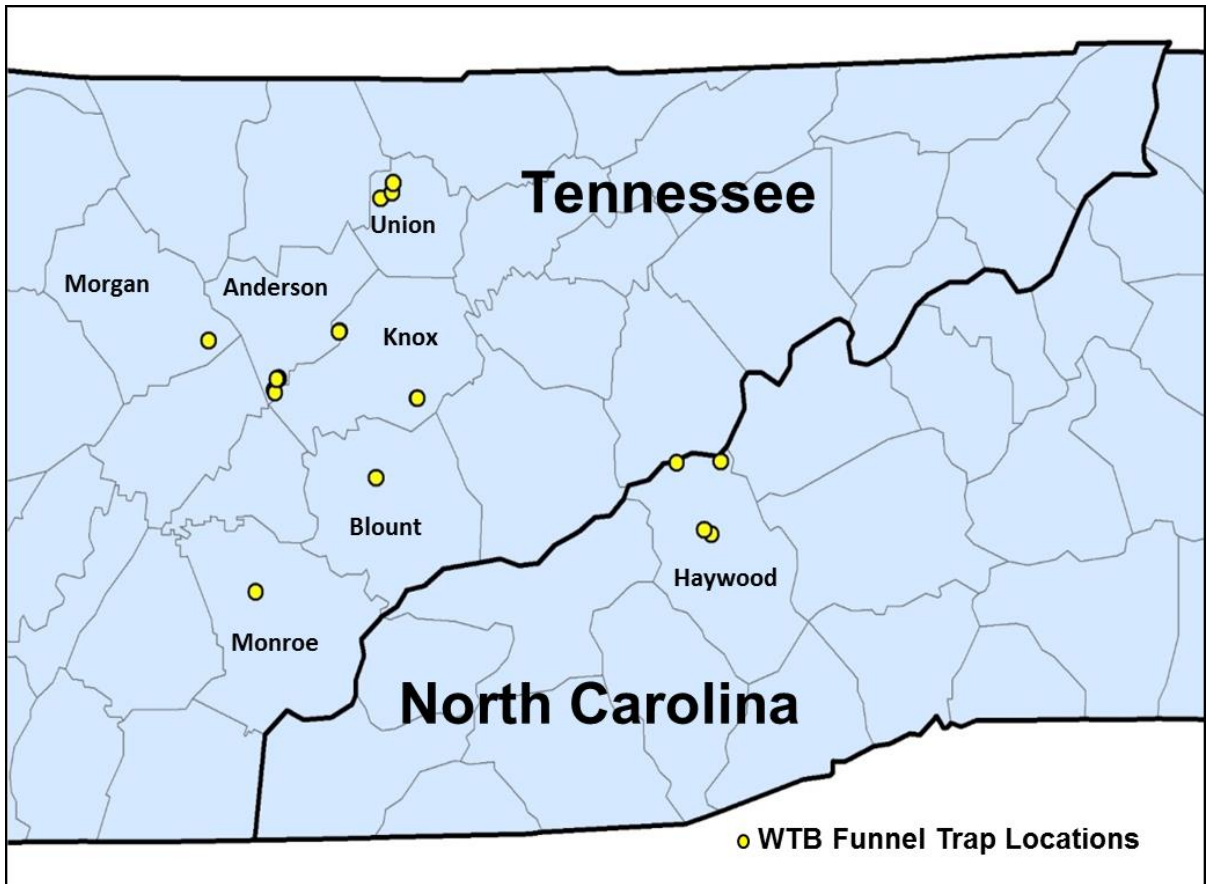


Figure 2.1 Locations of Lindgren funnel traps ($n=33$) used to collect walnut twig beetle (WTB) in forested areas in eastern Tennessee and western North Carolina counties, 2016.



**WTB lures
(Contech Enterprises)**

Figure 2.2 Lindgren four-funnel stack trap and Contech Enterprises® walnut twig beetle lure (Seybold et al. 2013) used to collect walnut twig beetles, 2016 and 2017.

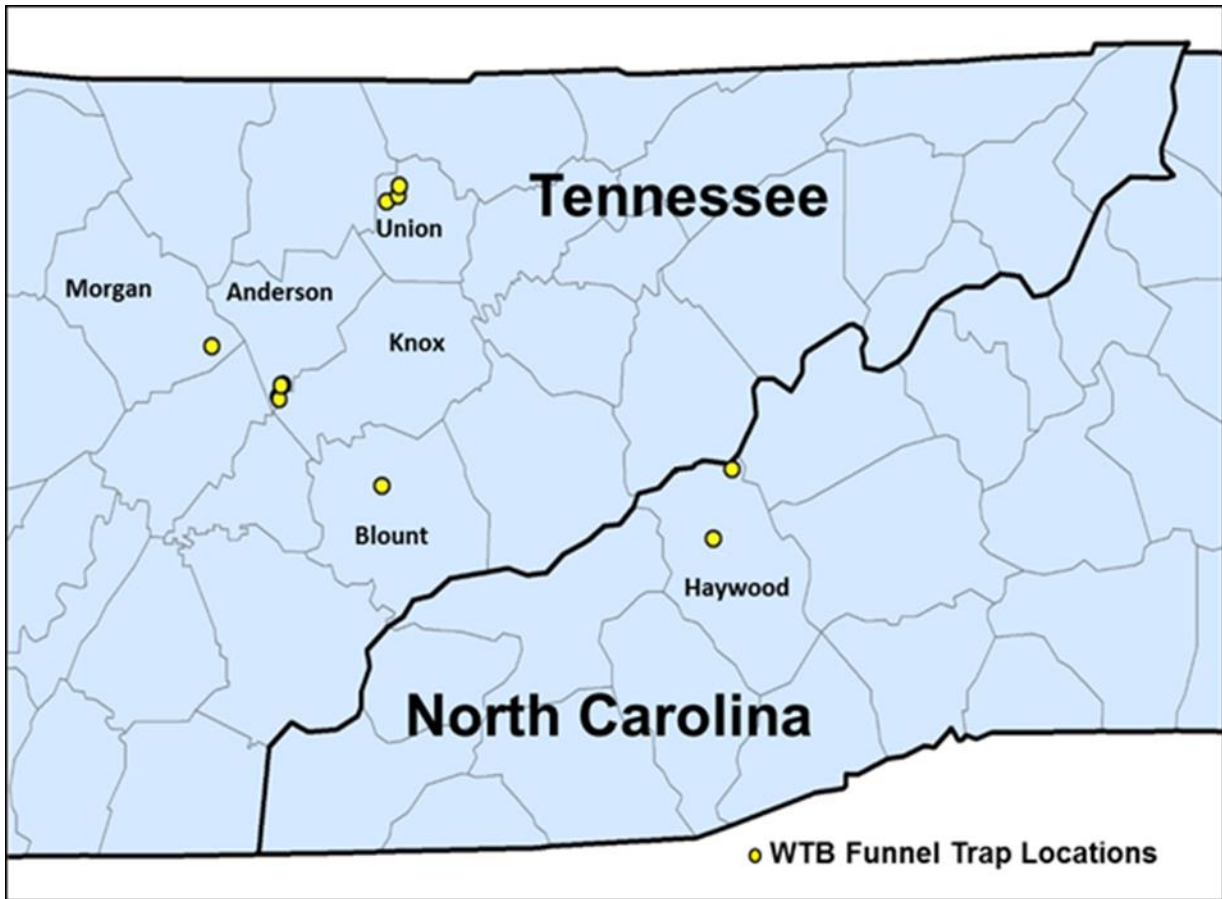


Figure 2.3 Locations of Lindgren funnel traps ($n=15$) used to collect walnut twig beetle (WTB) in forested areas in eastern Tennessee and western North Carolina counties, 2017.



Figure 2.4 Collection of funnel trap samples, 2016 and 2017.

Chapter III

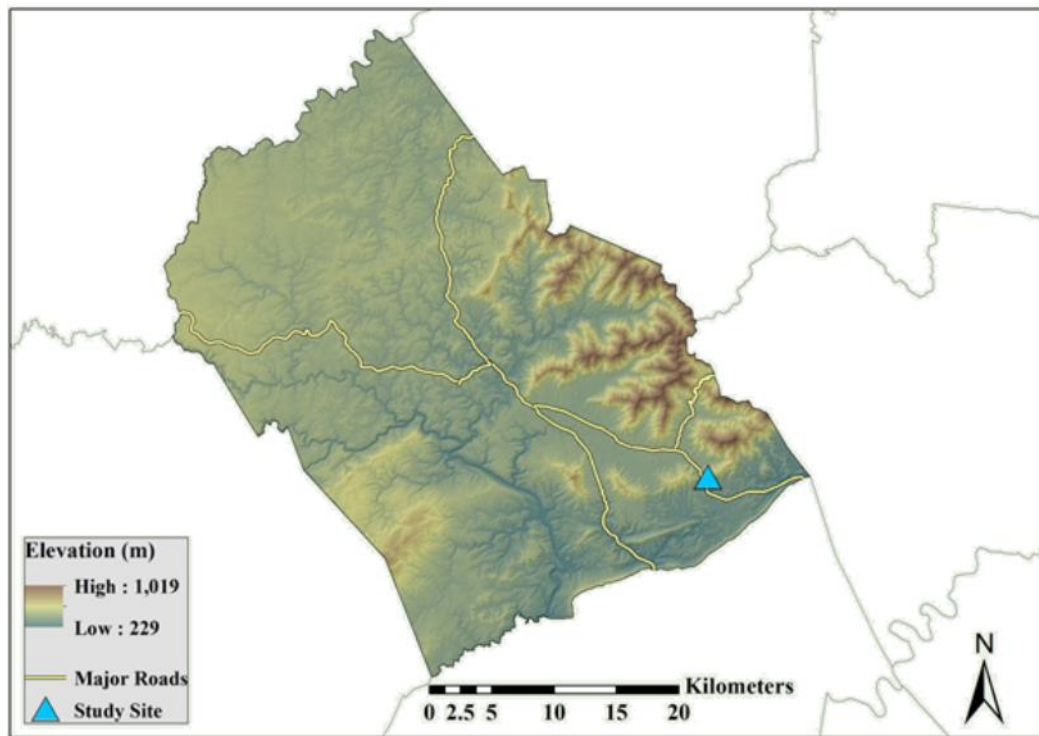


Figure 3.1 Location of WTB dispersal study site in southeast Morgan County, TN, 2016 and 2017.

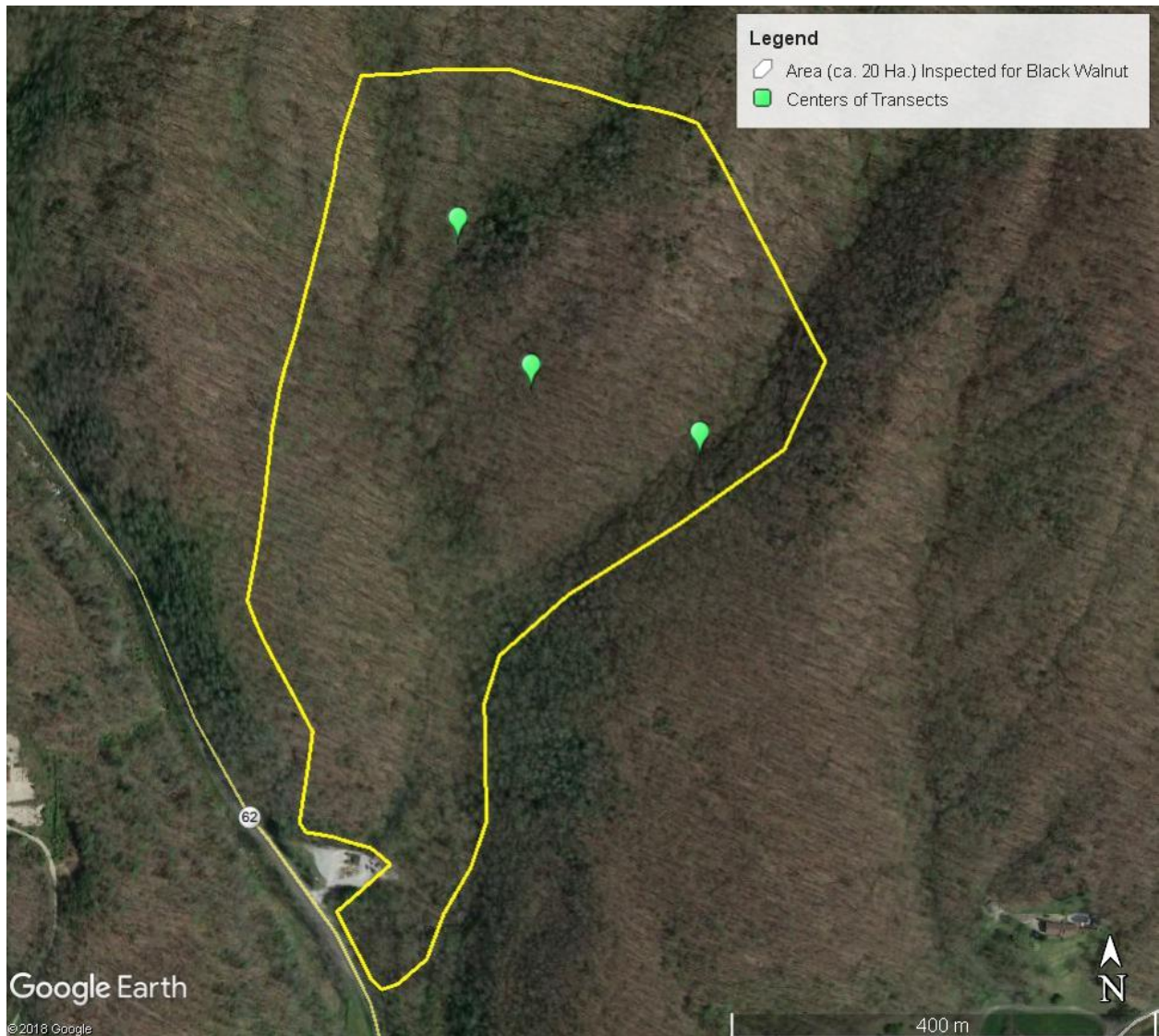


Figure 3.2 Area of research site (Morgan Co.) that was surveyed for the presence of mature black walnut trees, 2016. Markers indicate approximate locations of central release points within each of the three transects.

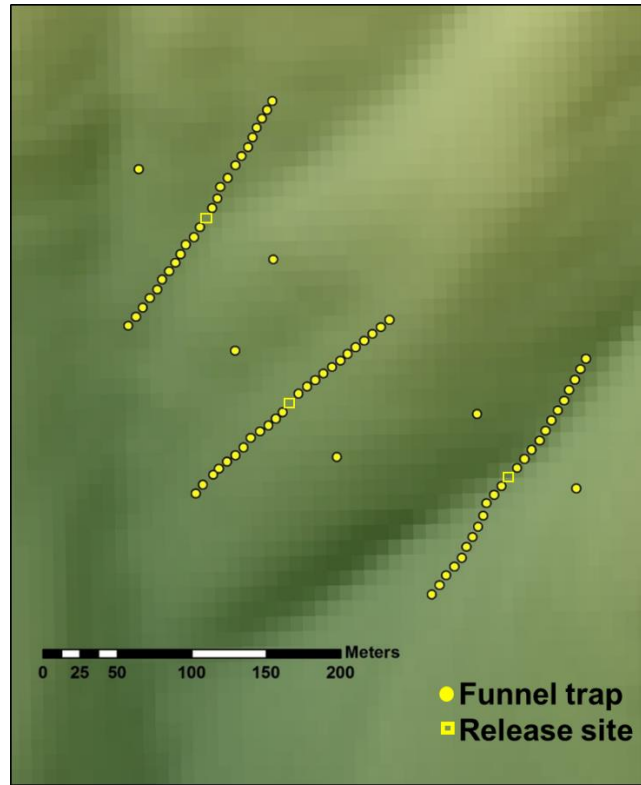


Figure 3.3 Arrangement of Lindgren four-funnel traps ($n=78$) deployed in three transects in Morgan County, 2016 and 2017.



Figure 3.4 Uphill view of Lindgren trap arrangement (yellow boxes) within a transect at dispersal study site in Morgan County.



Figure 3.5 Examples of transparent containers used to collect walnut twig beetle adults emerging from field-collected walnut.

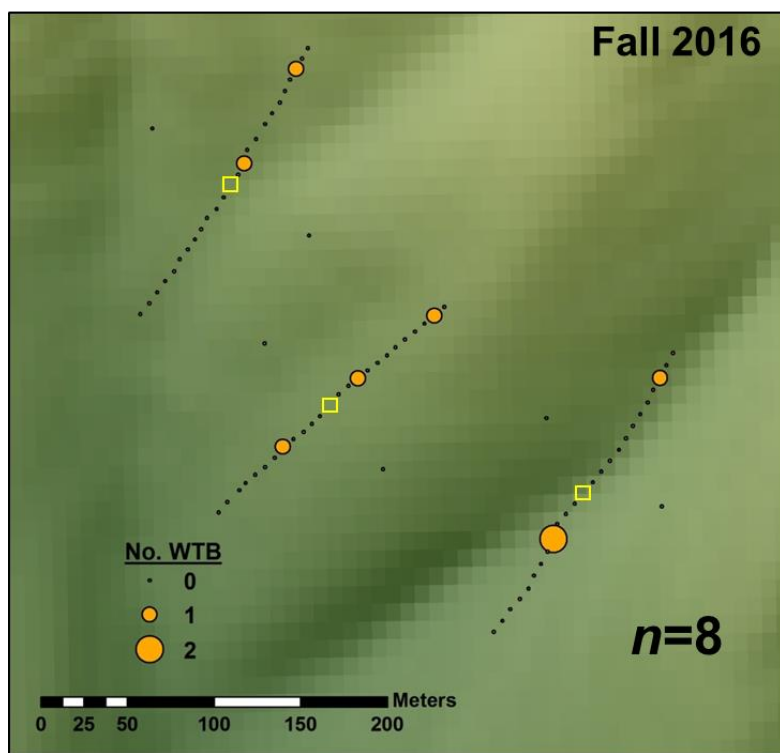
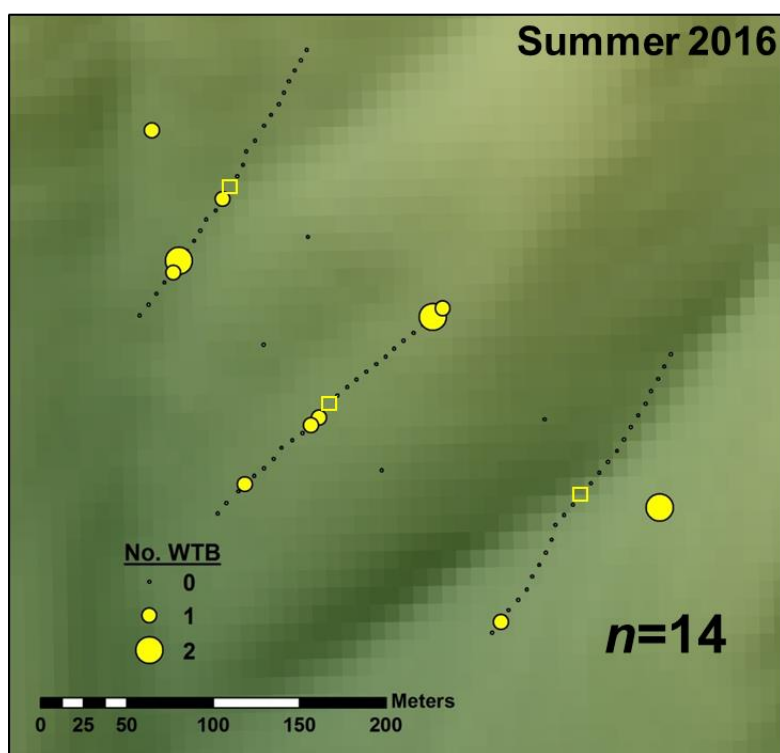


Figure 3.6 Recovery of released walnut twig beetle (WTB), *Pityophthorus juglandis*, during Summer ($n=14$) and Fall ($n=8$) of 2016. Large yellow circles identify traps that collected two or more WTB, smaller yellow circles identify one WTB collected.

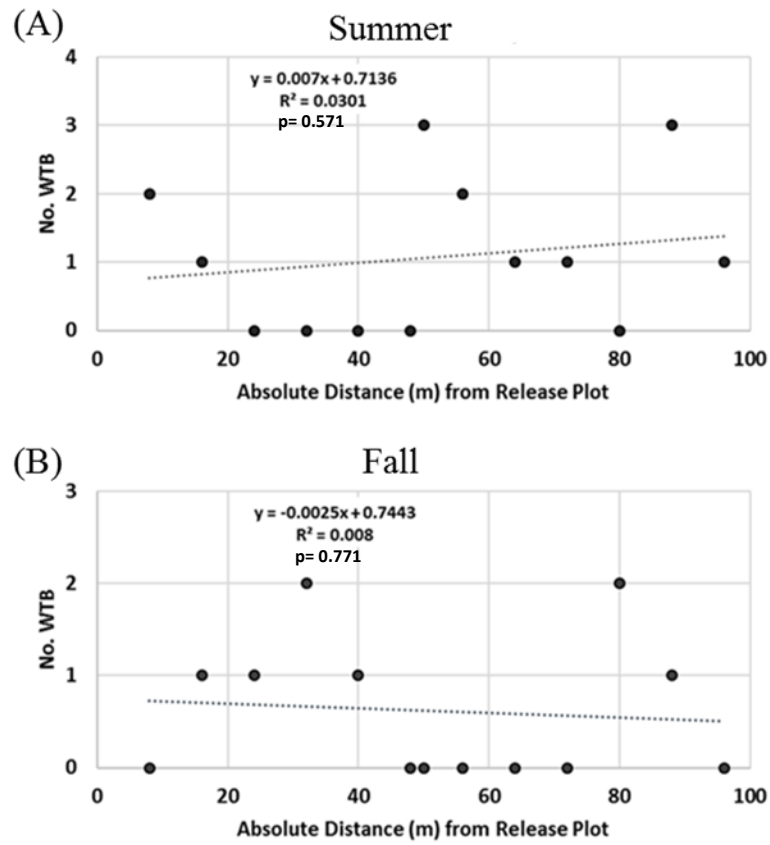


Figure 3.7 Linear regression analysis of number of walnut twig beetle (WTB), *Pityophthorus juglandis*, collected at each distance for (A) Summer and (B) Fall release and recovery, 2016.

Chapter IV

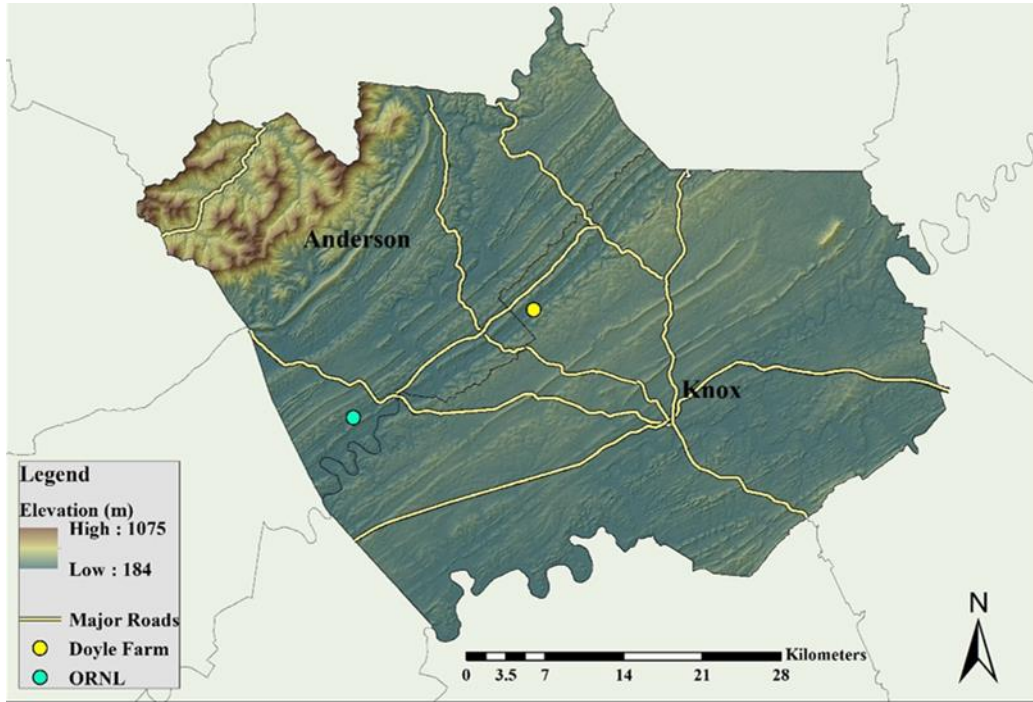


Figure 4.1 Study sites in Knox County, TN (Doyle Farm, yellow) and Anderson County, TN (Oak Ridge National Laboratory [ORNL], blue), 2017.

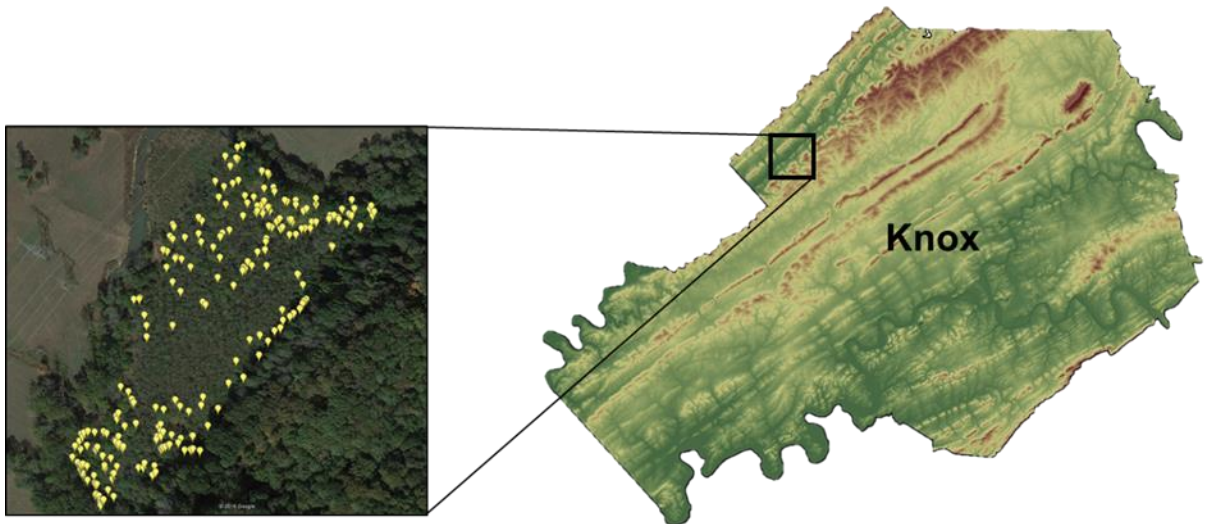


Figure 4.2 Georeferenced black walnut ($n=276$) at Doyle Farm (ca. 5.6 ha), Knox County, TN.

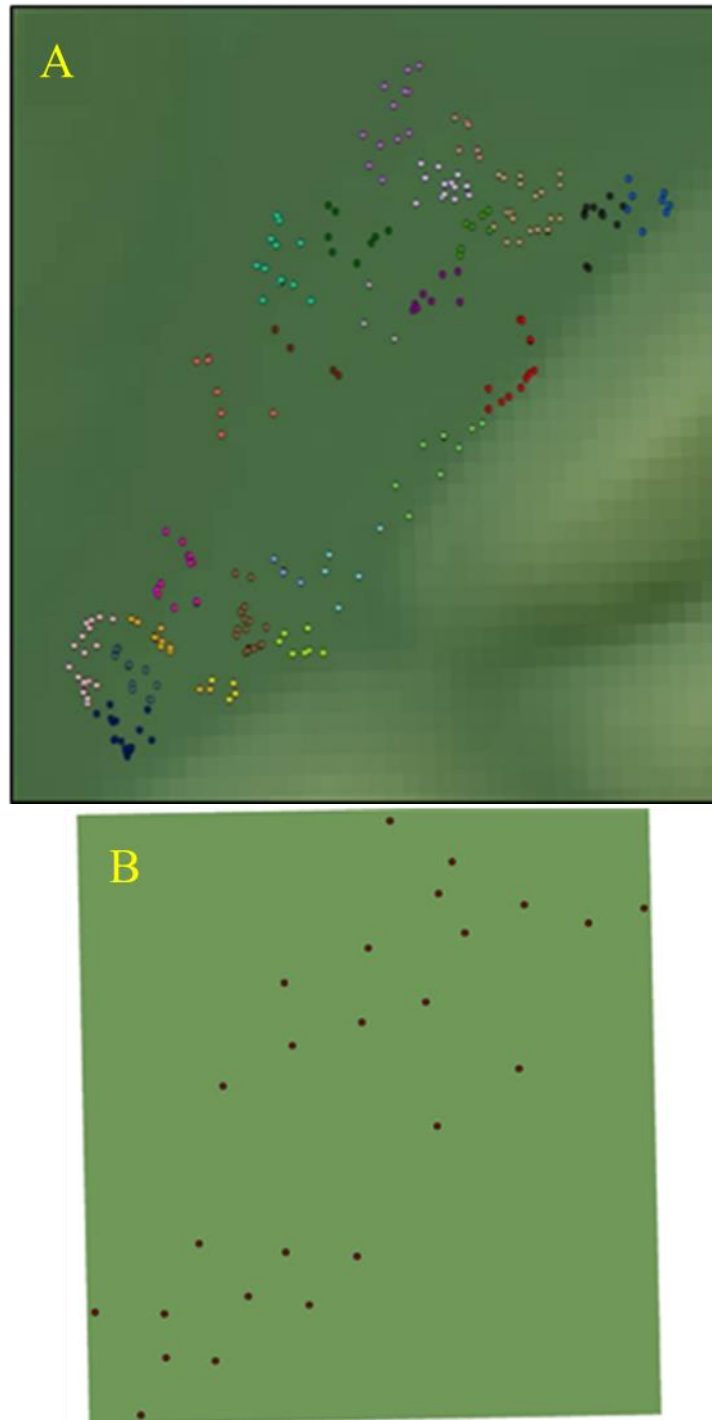


Figure 4.3 Doyle Farm site divided into (A) 25 color-coded clusters with ArcMap “grouping analysis” tool, with (B) one trap tree selected from each cluster.

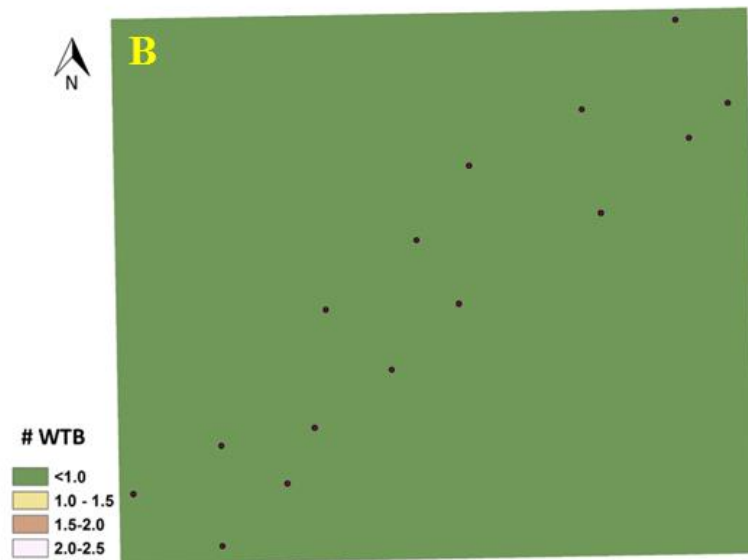


Figure 4.4 (A) Georeferenced black walnut ($n= 155$) at Oak Ridge National Laboratory (ORNL) site, ca. 6 ha, Anderson County, TN, (B) Location of trap trees ($n=15$) designated by black dots.

Figure 4.5 Inverse distance weighting (IDW) model showing seasonal distribution (19 June to 18 October 2017) of walnut twig beetle, *Pityophthorus juglandis*, at Doyle Farm.

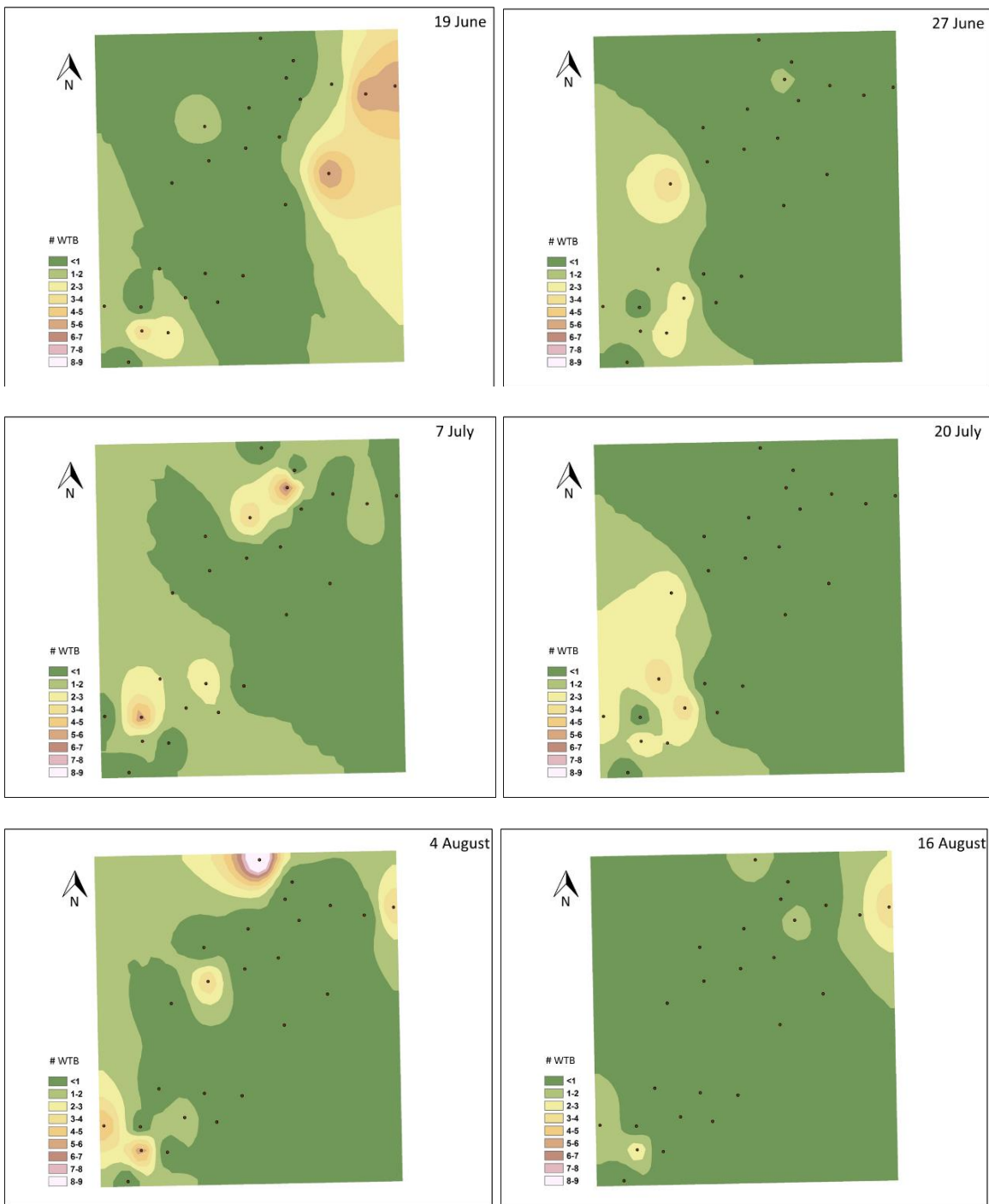


Figure 4.5 continued

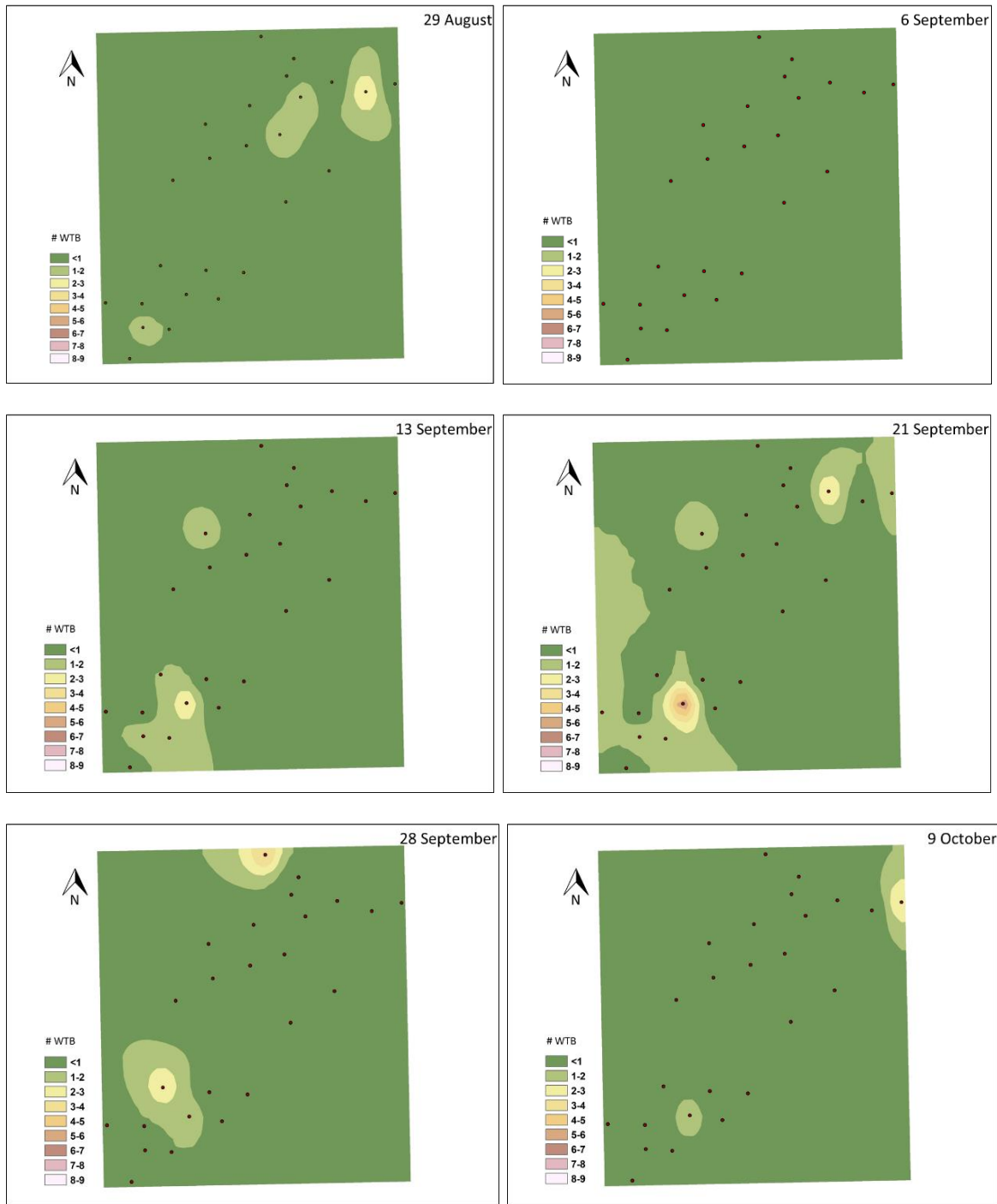


Figure 4.5 continued

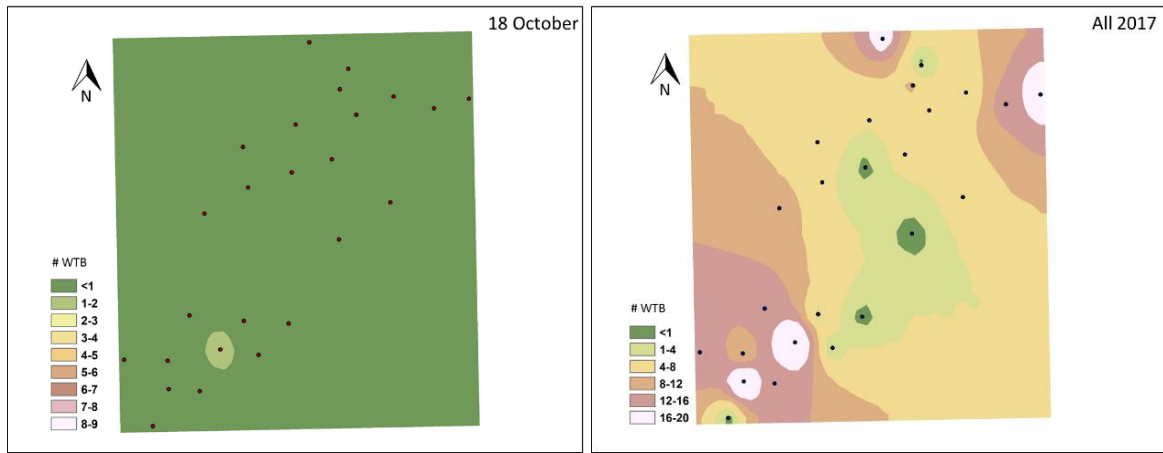


Figure 4.5 continued

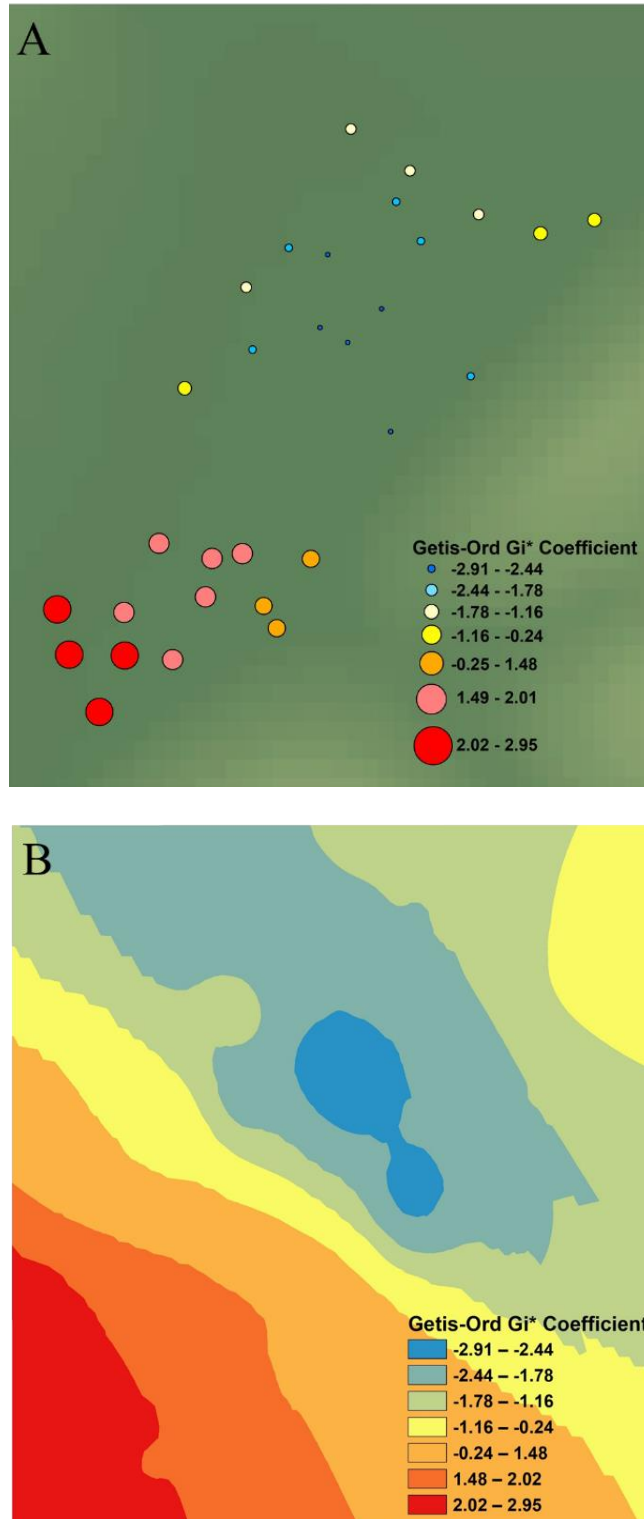


Figure 4.6 Clustering of walnut twig beetle (WTB), *Pityophthorus juglandis*, A) in individual trap trees and B) across the site, Doyle Farm, 2017. Red area denotes high levels of non-random clustering of WTB. Blue area denotes low levels of non-random clustering of WTB.

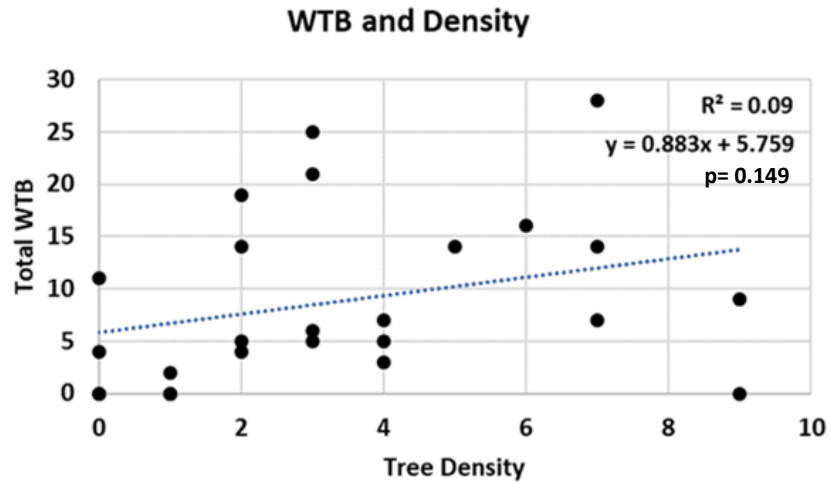


Figure 4.7 Linear regression model of black walnut density around each trap to the number of walnut twig beetles, *Pityophthorus juglandis*, collected in each trap, Doyle Farm, 2017.

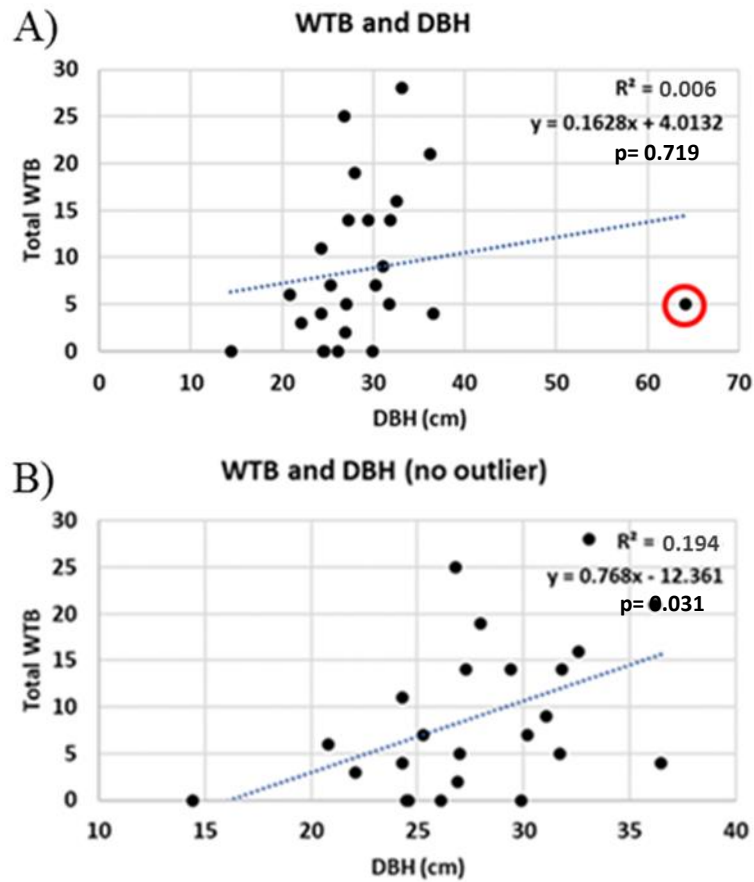


Figure 4.8 (A) Linear regression of diameter at breast height (dbh) and the number of walnut twig beetles, *Pityophthorus juglandis*, collected, and 65 cm outlier (red circle). (B) Regression with outlier removed.

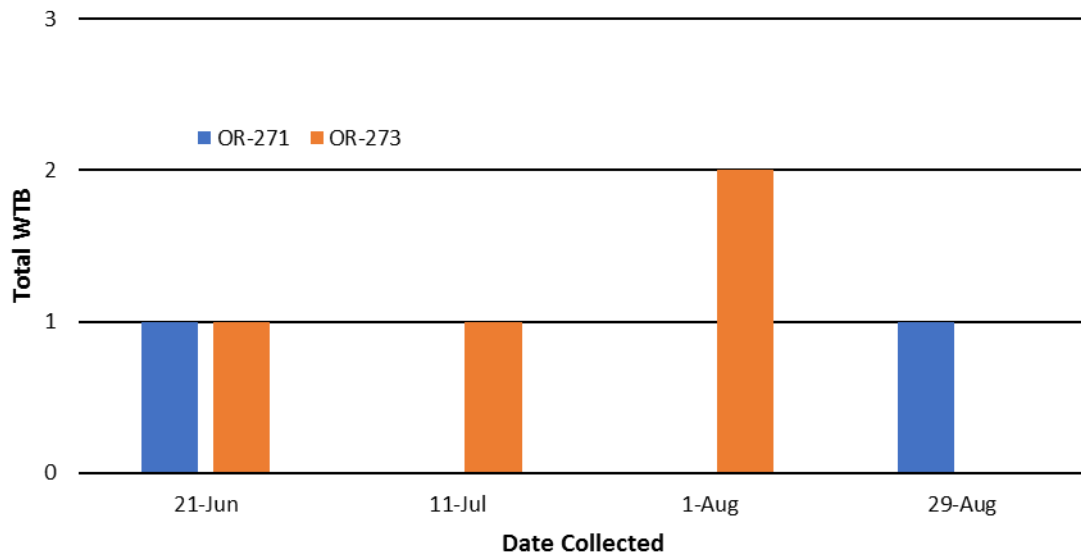


Figure 4.9 Number of walnut twig beetles, *Pityophthorus juglandis*, collected in traps (OR-271, OR-273) at Oak Ridge National Laboratory from 21 June to 30 October 2017.

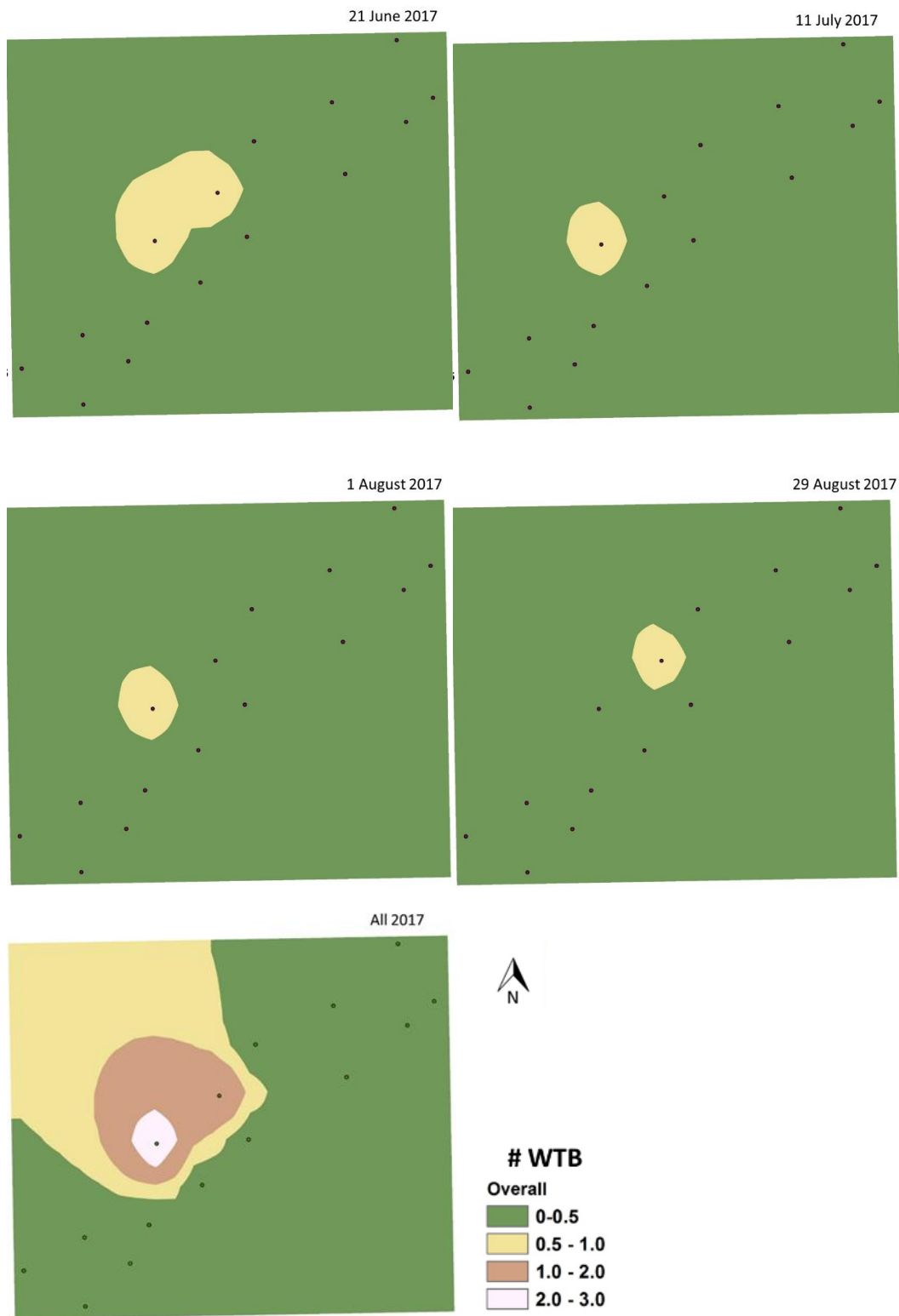


Figure 4.10 Inverse distance weighting (IDW) model of walnut twig beetle, *Pityophthorus juglandis*, seasonal distribution (9 June to 30 October 2017) at Oak Ridge National Laboratory.

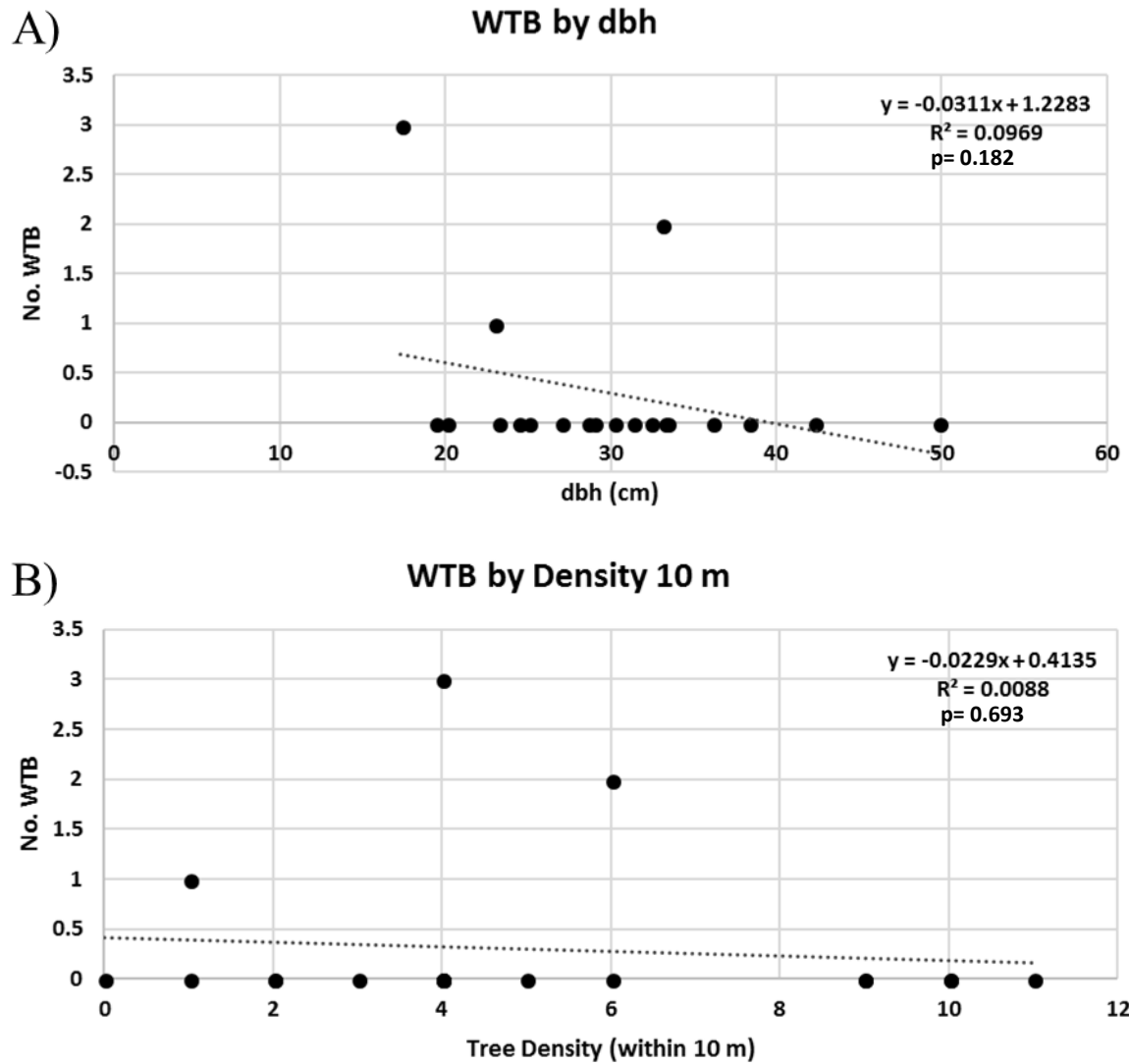


Figure 4.11 Linear regression analysis of number of walnut twig beetle (WTB), *Pityophthorus juglandis*, collected at Oak Ridge National Laboratory (A) by dbh and (B) by tree density at 10 m, 2017.

VITA

Philip Gerald Hensley was born on May 26, 1980, in Rock Hill, South Carolina, to his parents Douglas and Rita Hensley. As a child, he enjoyed exploring the forest that surrounded his home in northern Louisiana. In 1989, Philip and his family moved to the east Tennessee area and eventually settled in Sevierville, TN. The proximity of the Great Smoky Mountains National Park to his home in Sevierville allowed Philip countless opportunities to explore the wilderness and feed his curiosity.

In May of 1998, he graduated with honors from Sevier County High School in Sevierville, TN. After high school, he attended Pellissippi State Community College, majoring in Hospitality Management, and graduated with his Associate of Science degree in the spring of 2002. Over the next ten years, Philip would manage several hotel and restaurant locations before deciding that he wanted to continue his education.

Philip began attending classes at The University of Tennessee in Knoxville, TN in May of 2012, majoring in Plant Sciences, with a Bioenergy concentration, and minoring in Entomology and Plant Pathology. He obtained his Bachelor of Science degree in May of 2016. In the fall of 2016, Philip began a Master of Science program in the Department of Entomology and Plant Pathology at the University of Tennessee and graduated with his degree in August of 2018. Philip is a member of the Tennessee Entomological Society and the Entomological Society of America.