Biological Control of Emerald Ash Borer in the Southern U.S.: Seasonality, Phenological Synchrony, and Implications for Management

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Dedication

*To **my parents** – Thank you for always loving and supporting me, through all the late nights and long days. You always pushed me to be more than I was and I could have never gotten to where I am today without you.*

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Abstract

The emerald ash borer (EAB), *Agrilus planipennis* Fairmare (Coleoptera: Buprestidae), is an invasive wood-boring beetle from Asia. Believed to have been introduced on wooden packaging materials in the early 1990s it was first discovered in the United States in 2002. Since this initial discovery it has spread to 32 states as well as two Canadian provinces. The primary host of EAB is ash trees (*Fraxinus* spp.), in which EAB can cause mortality in as little as four years. To mitigate the spread and impact of this invasive pest, management plans incorporating the use of conventional pesticides and biological control agents were developed. Of these biological control agents, three larval parasitoids native to the same area as EAB were chosen: *Spathius agrili* Yang (Hymenoptera: Braconidae), *Spathius galinae* Belokobylskij and Strazanac (Hymenoptera: Braconidae), and *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae). Along with these introduced parasitoids, several native parasitoids have been evaluated as potential biological control agents of EAB in the United States.

A two-year research project to enhance our knowledge of natural enemies of EAB and to assess introduced parasitoids of EAB in the southern United States was initiated. The primary research goals of this project were to: 1) assess establishment of introduced parasitoids and incidence of potential native parasitoids of EAB, 2) determine seasonality and phenological synchrony of EAB and its introduced parasitoids in a southern climate, and 3) assess native parasitoids of EAB and their potential for rearing and release.

The results of this research will provide essential knowledge on the current status of introduced parasitoids of EAB in the south as well as insights into the native

parasitoids of EAB. This information will inform executive decisions on the management of EAB as a whole and will help to guide future biological control efforts.

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CHAPTER I

INTRODUCTION/LITERATURE REVIEW

The planet Earth is an ever changing and evolving system of interacting forces, one of these forces is the interaction between living organisms. These interactions between organisms, as well as their interactions with their surroundings, is a facet of the field of study known as Ecology. One of the most important concepts of Ecology is the phenomenon of invasive species. An invasive species is a plant, fungus, or animal species that is not native to a specific location and which has the tendency to spread to a degree believed to cause damage to the environment, human economy, or human health (Non-native Species Secretariat 2017). The U.S. Department of Agriculture (USDA) definition of an invasive species is any species that is: 1) non-native (or alien) to the ecosystem under consideration and 2) whose introduction causes or is likely to cause economic or environmental harm or harm to human health (USDA National Agricultural Library, 2016)

The movement of these species from their native habitats to a new one is often facilitated through human activities. With the continuous growth of the human population and by extent the demand for international trade and travel, the unintentional spread of invasive species is also being accelerated (Pimentel et al. 2000). These new transported species have the potential to cause extensive damage to both the natural world and human society. The primary way in which they cause damage to the natural environment is through their competitive pressure on the environment. When an invasive species enters a new environment they have virtually no abiotic and biotic limitations, which allow them to rapidly expand and maintain their populations. This population expansion allows them to outcompete native species. Of the 958 species which are classified as “Endangered” under the Endangered Species Act, 400 of them are estimated to be at

risk due to competition from invasive species (Pimentel et al. 2000). In terms of damages to humans it is estimated that invasive species cause an economic loss of up to $120 billion per year (Pimentel et al. 2000). These damages accrue through everything from agricultural losses to public safety, to the costs of trying to control and manage the impact of these invasive pests. One such pest that is responsible for both economic and natural damages is the emerald ash borer (EAB) (*Agrilus planipennis* Fairmare) (Coleoptera: Buprestidae), an invasive wood-boring beetle native to eastern Asia whose main habitat and food source is ash trees (*Fraxinus* spp.).

Ash Tree

Members of the *Fraxinus* genus (Oleaceae) are some of the most widely spread species of trees in the North American continent, with ash trees comprising some 7.5% of the volume of hardwood saw timber in the Eastern United States alone, with an undiscounted stumpage value estimated to be at least $25.1 billion (Poland and McCollough 2006). More than 40 species of ash occur in the United States, where green (*Fraxinus pennsylvanica)* and white ash (*Fraxinus americana*) have the widest distributions (Global Biological Information Facility 2017) (Figs. 1.1 and 1.2, respectively).

Members of the *Fraxinus* genus are valued both ecologically and economically. Ecologically, the two most widely spread species (green and white ash) fill various roles in their respective forest systems. Green ash provides both habitat and food for a plethora of organisms, such as game and non-game birds, deer, bison, and other small mammals. Green ash is also utilized in the reclamation of coal mining sites because it has the ability to quickly establish (Gucker 2005). White ash fills many of the same roles as green ash providing shelter and food for many organisms, as well as being prone to forming cavities making them a prime candidate

for woodpecker habitation. Once again similar to green ash, white ash is also used in the reclamation of disturbed sites such as previous coal mining sites (Griffith 1991).

Members of the *Fraxinus* genus are also valued economically, used widely as crafting materials and landscape trees. For example, ash trees provided roughly 14.4% of the total leaf cover in an urban setting in Chicago (Cappaert et al. 2005). Because they are so numerous, the potential cost of removing urban ash trees in the United States is estimated at between $20-60 billion dollars, and this estimate does not include the cost of replacing them (Cappaert et al. 2005). Ash has also long been considered a "cash crop' due to the strength, hardness, heavy weight, and elasticity of its wood. Thus, it is often used in furniture, architecture, and sports equipment, such as the famous Louisville Slugger (Poland and McCollough 2006). Ash is also used as pulp for the manufacturing of paper and cardboard (Cappaert et al. 2005).

Agrilus planipennis

The EAB is an invasive bark-boring beetle native to eastern Asia whose primary host is ash trees. The species was first discovered to be the cause of ash mortality in southeast Michigan and areas of Canada (Gould et al. 2017). It is theorized that the beetle was transported to the United States in the early 1990s in some form of infested wooden packaging material carried on cargo ships or planes originating from Asia (Gould et al. 2017). Since its initial discovery in 2002, EAB has been found in 32 states, as well as in Ontario and Quebec in Canada (USDA FS 2017) (Fig. 1.3). EAB was first discovered in Tennessee in Knox County in 2010. After an initial assessment conducted by USDA APHIS (Animal and Plant Health Inspection Service) PPQ (Plant Protection and Quarantine) and the Tennessee Department of Agriculture, EAB was found to be present in two counties (Knox and Loudon) (Powell et al. 2012). Since then EAB has spread, resulting in the quarantining of 59 counties in Tennessee (as of September 2017) (Fig.

1.4). This quarantine includes the regulation of firewood of all hardwood (non-coniferous) species; nursery stock, green lumber, and other material living, dead, cut, or fallen, including logs, stumps, roots, branches, and composted and uncomposted chips of the genus *Fraxinus.* These regulated articles can only be moved outside of a quarantine zone through the use of special permits and after they are checked and cleared to be moved by an inspector (7 C.F.R § 301.53-5).

EAB is a relatively small beetle, 8.5-13.5 mm long and 3.1-3.4 mm wide in its adult stage. Adults are predominantly metallic green, which accounts for their name; however, they do have other color morphs ranging from sapphire blues to rust colors (Buck 2015). EAB emerge as adults in spring and reach peak oviposition in late June to early July, with each female laying between 60 to 90 eggs in her lifetime (Buck 2015). These eggs then hatch within 2 to 3 weeks and the larva enter directly into the bark where they tunnel until reaching the cambium layer. Once larvae have reached the cambium layer, they feed on the phloem tissues and outer sapwood. This feeding continues through four larval instars until late summer and fall when the larvae excavate a pupation chamber in the xylem where they overwinter. Pupation generally takes place in early spring and adults emerge beginning this process anew (Bauer et al. 2003). It has been observed that EAB in the northern United States exhibits what is known as a 1½-year life cycle, where not every member of each generation is able to reach the pupation stage in a given year (Bauer et al. 2003, Herms and McCollough 2014). It is yet unknown whether this life cycle differs in the southern United States.

EAB causes damage to its host tree through feeding on the phloem layer of the tree during the larval stages. This feeding creates distinctive “S” shaped galleries, which are then filled with frass (Fig. 1.5). Normally this type of feeding would not be harmful to the host tree,

however, EAB occurs in such great numbers that their combined galleries have a girdling effect on the host over time. EAB infestations are subtle and often difficult to detect until the host tree begins to show significant canopy dieback. EAB can cause mortality in the host tree within 2 to 3 years, and after its initial discovery in 2002, EAB has caused the deaths of millions of ash trees in North America (Cappaert et al. 2005, USDA FS 2017).

As previously stated, the primary host of EAB is ash trees. As ash declined rapidly in North America due to EAB infestation, concerns arose as to whether or not EAB could potentially infest other members of the Oleaceae family, such as olives and lilacs. Studies have been conducted to investigate how well EAB survived on alternative hosts, such as black walnut (*Juglans nigra*), olive (*Oleaceae europaea*), or lilacs (*Oleaceae synringa)*. The first of these studies examined EAB oviposition and larval development on green, white, black (*Fraxinus nigra*) and blue (*Fraxinus quadrangulata*) ash, as well as six potential alternative hosts including privet (*Oleaceae ligustrum*), Japanese tree lilac (*Syringa reticulata*), American elm (*Ulumus americana*), black walnut, hickory (*Carya tomentosa*), and hackberry (*Celtis occidentalis*) (Anulewicz et al. 2006). In this no-choice study, EAB laid eggs on all test species with larvae reaching the second instar on all species of ash and privet before the braches desiccated. Larvae attempted to feed on black walnut, Japanese lilac, American elm, and hackberry but died as first instars. No feeding was observed on hickory branches. A similar study examined EAB landing rates, oviposition, and larval development on North American ash species and congeners of its reported hosts in Asia (Anulewicz et al. 2008). Like the previous study, this study observed EAB on the non-ash species: American elm, hackberry, black walnut, shagbark hickory, and Japanese tree lilac. Similar to the earlier findings, this study reported that EAB laid eggs on all test species, though few were usually laid on non-ash species. Larval

feeding and development were observed normally on ash species, with no EAB larvae able to survive, grow, or develop on non-ash species.

In a more recent study, EAB was found to survive and complete multiple generations on white fringetree (*Chionanthus virginicus* L.) (Cipollini 2015). This study reported that 4 of 20 mature trees that were examined in Dayton, OH, exhibited symptoms of EAB damage including: canopy dieback, bark splitting, and adult exit holes. The removal of bark from these trees showed evidence of at least three generations of larval damage, as well as actively feeding larvae (Cipollini 2015).

Another study focused on biotic factors affecting mortality of immature EAB on ash in the Russian Far East and found low rates of EAB larval mortality caused by undetermined biotic factors on introduced North American green ash (3-27%). This study also observed higher EAB larval mortality caused by putative plant resistance in Oriental ash species, indicating that ash species from the native range of EAB are naturally resistant to EAB (Duan et al. 2012a) From these studies, it is evident that while EAB poses a great threat to the ash populations, as well as species related to ash, it poses little threat to other tree species in the United States.

Biological Control of Emerald Ash Borer

Initial insecticidal treatments for EAB generated mixed results, with some treatments providing adequate protection from EAB one year and then failing to do so the following year. Since then, several advancements, in particular the use of systemic insecticides, has given rise to new more effective treatments, such as soil injection or drenches and insecticides injected into the base of the trunk (Herms and McCullough 2014). While these advances have dramatically increased the likelihood that ash trees will be protected from EAB, these insecticides require specific timing to work properly against EAB. Because it is difficult to detect early EAB

infestations, treatments may be applied too late to provide adequate control. Treatment costs to control EAB on a large urban scale or in a forest also prohibit their use across a landscape scale. As such, foresters and land managers developed a management plan for EAB, which included the use of natural enemies as a key tactic in managing EAB populations (Buck 2015).

During the early phase of the invasion of EAB into North America, surveys of EAB-infested wood were conducted to determine whether potential native parasitoids were present that could be used against EAB. A survey conducted in western Pennsylvania in 2009 yielded a variety of hymenopteran parasitoid candidates including *Balcha indica* (Eupelmidae) (Mani & Kaul), *Eupelmus pini* (Eupelmidae), *Dolichomitus vitticrus* Townes (Ichneumonidae), as well as two additional unidentified ichneumonids, *Orthizema* sp. Townes and *Cubocephalus* sp. Townes. Collectively, these parasitoids provided approximately 3.6% parasitism of EAB in the field (Duan et al. 2009). The most abundant of these parasitoids was *B. indica*, which comprised ca. 82% of all the parasitoids recovered during this survey. It was concluded from this survey that these parasitoids may do well when combined with classical biological control agents (i.e. introduced parasitoids) in North America (Duan et al. 2009).

Three exotic larval parasitoids, native to the natural range of EAB, have been investigated and cleared for release in the United States. These three parasitoid species are *Spathius agrili* (Yang) (Hymenoptera: Braconidae), *Spathius galinae* (Belokobylskij and Strazanac) (Hymenoptera: Braconidae), and *Tetrastichus plannipennisi* (Yang) (Hymenoptera: Eulophidae) (Gould et al. 2017).

Spathius agrili is a gregarious idiobiont ectoparasitoid which was first described in China in 2004 and was the first parasitoid to be reared from EAB. *S. agrili* parasitizes the larvae of EAB beneath the bark, and the number of parasitoid eggs laid per host were positively correlated

with larval size as measured by larval weight, pronotum width, body length, and body width (Wang et al. 2008). One study found that the parasitism rate for *S. agrili* in the field ranged from 30-90% with 35 eggs being found in a single host. On average 8.4 adults can be reared from a single host with a sex ratio of 3:1 (female to male) (Yang et al. 2005). Using 18 wood-boring insect species either in the genus *Agrilus* or associated with ash forests, researchers demonstrated that *S. agrili* can parasitize the larvae of other *Agrilus* species but that attacks were significantly lower on these other species when compared with attacks on its natural host (EAB) (Yang et al. 2008). This strong relationship specifically with EAB is what made *S. agrili* one of the prime candidates for release in the United States. *S. agrili* typically exhibits 3 to 4 generations per year and overwinters as a prepupa in a cocoon inside the host gallery. On average females live 29.1 days while males live 23.6 days. *S. agrili* develop through five larval instars, and the larvae feed gregariously on the host's hemolymph. The generation time from egg to adult wasp is approximately 27-28 days at 22-26 C (Yang et al. 2010).

Currently *S. agrili* has been unable to establish in the northern United States, therefore, the EAB Biological Control Program limits the release of *S. agrili* to states below the 40th parallel north (Bauer et al. 2015). However, releases in these more southern states, such as Kentucky, Missouri, Tennessee, Virginia, and West Virginia are still too recent for establishment confirmation (Gould et al. 2017). In another study, *S. agrili* was recovered one year after releases in eastern Tennessee, showing that the parasitoid had the potential to overwinter in the south; however, as previously stated, the standard amount of time to begin to assess for establishment after release is three years making these recoveries too early to be indications of establishment (Hooie et al. 2015).

Spathius galinae is the newest biological control agent to be introduced into the United States as part of the efforts to control EAB. *S. galinae* is a gregarious idiobiont larval ectoparasitoid that was relatively recently described from the Russian Far East (Duan et al. 2014, Watt and Duan 2014). Initial studies of *S. galinae* found that it takes approximately 1 month (29 days) to complete a single generation under normal rearing conditions (25 ± 1°C, 65 ± 10% RH, and a photoperiod of 16:8 L:D). Larvae of *S. galinae* go through five instars with the fifth instar spinning cocoons for pupation. Adult female wasps had an average life span of 7 weeks with peak fecundity at 3 weeks after emergence when reared in groups, and after 2 weeks when reared in pairs. It was also found that throughout its life span a single female *S. galinae* produces an average of 31 progeny when reared in groups, and an average of 47 progeny when reared in pairs (Duan et al. 2014).

In a no-choice study when *S. galinae* was presented with 16 species or groups of North American wood-boring insects, including *Agrilus planipennis, A. anxius, A. masculinus, A. sulcicollis, A. bilineatus, A. auroguttatus, Chrysobothris* spp., *Anoplophora glabripennis, Elaphidion mucronatum, Neoclytus acuminatus, Urographus fasciatus, Isorhipis oblique,* unknown Cerambycidae, *Jylesinus fraxini, Podosesia* spp., and *Janus abbreviatus*, that *S. galinae* attacked only one of these species (the gold spotted oak borer [GSOB], *A. auroguttatus,* an invasive species of oak in North America) (Flint et al. 2013). Parasitism was significantly lower on GSOB than on EAB (Duan et al. 2015). The native distribution of this species, being from the Russian Far East and South Korea, indicate it may be more adapted to cold regions than that of *S. agrili* making is a good candidate for North American releases (Belokobylskijj et al. 2012). The life cycle of *S. galinae* as well as this host specificity made it a prime candidate as a biological control agent against EAB in the United States and it was cleared for release in 2015

(Bauer et al. 2015). Due to *S. galinae* being a relatively new biological control agent its establishment in release areas is currently unknown. Releases of *S. galinae* are ongoing in the northern and southern United States.

Tetrastichus plannipennisi is a gregarious larval koinobiont endoparasitoid from China that is currently deployed in the United States as a biological control agent of EAB. In a laboratory study, it was determined that a single generation takes approximately 4 weeks to complete under normal rearing conditions (25 ± 2°C, 65 ± 10% RH, and L:D 16:8 hr photoperiod). It was also determined that male wasps live for an average of 5 weeks, while females live significantly longer with a median survival time of 6 weeks and a maximum survival time of 9 weeks (Duan et al. 2011). The average number of progeny per reproductive female remained constant through the first 6 weeks of this study, with each female producing between 23 and 26 progeny each week. Lifetime realized fecundity was determined to be an average of 57 progeny per reproductively active female, with a female to male sex ratio of 3:1 (Duan et al. 2011). In another study, both male and female *T. planipennisi* were exposed to actively feeding larvae of eight buprestids (*Agrilus anxius, A. bilineatus, A. ruficollis, A. subcinctus, A.* sp., *Chrysobothris femorata, C. floricola,* and *C. sexsignata*), five cerambycids (*Neoclytus acuminatus, Megacyllene robiniae, Astylopsis sexguttata, Monochamus scutellatus*, and an unknown sp. in maple), and a sawfly (*Janus abbreviatus*). All species were implanted in small branches of their respective host plants, and actively feeding larvae of EAB were implanted in ash branches. This study resulted in *T. planipennisi* rejecting all species except the actively feeding larvae of EAB (Liu and Bauer 2006). This host specificity, as well as its long lifespan and gregarious nature, led to the selection of this parasitoid species as one of the primary candidates for biological control of EAB in the United States.

A survey of populations of hymenopteran parasitoids associated with larval stages of EAB was conducted in 2009 and 2010 in recently invaded areas in the north central United States, namely Michigan (Duan et al. 2012b). Results of these surveys indicated that several hymenopteran species had become associated with EAB in Michigan; and that among these parasitoids *T. planipennisi* was the most abundant. *T. planipennisi* accounted for 93% of the parasitoid individuals recovered in 2009 and for 58% in 2010, with low levels of parasitism (1 to 5%) being consistently detected at the survey sites across both years (Duan et al. 2012b). Another study (Duan et al. 2013) examined specifically the establishment of *T. planipennisi* in three counties (Ingham, Gratiot, and Shawassee) in southern Michigan where 3,311 – 4,597 female and 1,500 male *T. planipennisi* had been released into six forested sites per county between 2007 - 2010. This study found that in both parasitoid release and control plots by fall of 2012 the portion of sampled trees with one or more broods of *T. planipennisi* had increased from 33% and 4% in the first year after release to 92 and 83%, respectively. It was also observed that the mean number of broods found in sampled trees increased from less than one per tree in the first year after release to 2.46 and 3.08 in control and release plots, respectively. The level of EAB larval parasitism in the release and control plots also increased, rising from 1.2 and 0.2% to 21.2 and 12.8% respectively (Duan et al. 2013). These studies indicate that *T. planipennisi* has been able to adequately establish in the northern United States and that it will likely play a pivotal role in the future biological control of EAB.

Research Objectives

While much research has been conducted on these introduced parasitoids, as well as their native counterparts in the northern United States, relatively little research has been conducted in the southern United States. The phenology of EAB and both its introduced and native parasitoids

in the south is still largely unknown, as is the variety of native parasitoids present in the south. This information could provide vital insights into the current condition of the parasitoids being deployed in the south and whether or not they have been as effective as in the northern United States. Therefore, research was initiated to investigate aspects of the biology of EAB and its parasitoids in a southern climate.

To achieve a better understanding of the current situation of biological control of EAB in the south the focus of this research is threefold:

1) Assess the establishment of the introduced parasitoids of EAB in the south,

2) Determine the seasonality and phenological synchrony of EAB and its introduced parasitoids in a southern climate, and

3) Assess the native parasitoids of EAB in the south and their potential for rearing and release.

CHAPTER II

ASSESSMENT OF INCIDENCE OF NATIVE PARASITOIDS AND ESTABLISHMENT OF INTRODUCED PARASITOIDS OF EMERALD ASH BORER

Introduction

The emerald ash borer (EAB) was first discovered in the United States in 2002 in Detroit, MI, and has since spread to 32 states and two Canadian provinces. An integrated pest management (IPM) program was immediately established and deployed by the U. S. Department of Agriculture Animal and Plant Health Inspection Service (USDA APHIS) to attempt to minimize the damage caused by EAB. One of the foundational components of this IPM program was the discovery and evaluation of parasitoids that were native to the same area (e.g., Asia) as EAB and specifically targeted EAB as their hosts. Research on several parasitoid species, including both egg and larval parasitoids, was conducted. After this research was completed, one EAB egg parasitoid (*Oobius agrili* Zhang and Huang) (Hymenoptera: Encyrtidae) and three EAB larval parasitoid species were selected as promising biological control agents for release in the United States, though the egg parasitoid was not part of the focus of this study. The three larval parasitoids were members of two genera: two species of *Spathius* (*Spathius agrili* Yang and *Spathius galinae* Belokobylskil & Strazanac) (Hymenoptera: Braconidae) and one species of *Tetrastichus* (*Tetrastichus plannipennisi* Yang) (Hymenoptera: Eulophidae). These parasitoids have displayed the ability to survive in the northern United States, but it is imperative to study their behavior in the southern United States as well (Duan et al. 2011, Duan et al. 2013).

An important characteristic to study when evaluating a biological control agent for release is whether or not that agent is able to establish within the area where it is released. In other words: Is that species able to live where it will have the opportunity to impact the pest that it is released against? Also, understanding how these biological control agents will interact with each other (e.g., their potential to be released together or their encounters with each other after release) is imperative to a successful biological control program (Ulyshen et al. 2010).

Therefore, a study was designed to assess the establishment, seasonality, and phenology of two introduced larval parasitoid species (*S. agrili* and *T. plannipennisi*), as well as incidence of native parasioids of EAB larvae, in Tennessee. The questions to be answered in this study included: Are the introduced parasitoids of EAB able to establish in the southern United States, and during what period in the year are these parasitoids active? This study also addresses the question: What native parasitoids occur and when do they occur?

Materials and Methods

Parasitoid Releases

In 2012, releases of two introduced larval parasitoids of EAB, *S. agrili* and *T. plannipennisi* were initiated at eight locations in eastern Tennessee (Fig. 2.1). Two locations each in Blount and Knox Counties and one location each in Anderson, Hamblen, Monroe, and Morgan Counties were selected for parasitoid releases. In a peripheral study, the egg parasitoid *O. agrili* was also released at these same locations. All released parasitoids were obtained from USDA APHIS PPQ (Plant Protection and Quarantine) Biological Control Production Facility in Brighton, MI.

Parasitoid releases were made from 2012-2015, and the number of each species released each year are listed in Table 2.1. Although releases were made during 2012-2015, only five sites

(Cowan Park, Haven Hill, Lorino Park, Ramsey Historic House, and Miser Station) where releases had been made three years before assessments began were used in this study. The typical time to assess establishment is three years after the last release.

In Blount County, two release locations were selected to monitor at Haven Hill Road and Miser Station Road (Table 2.1). Releases of *S. agrili* and *T. plannipennisi* began in 2012 at Haven Hill and Miser Station Road, followed by releases of *O. agrili* beginning at both sites in 2013. These releases continued until 2014 with a total of 4,808 *O. agrili,* 5,412 *S. agrili,* and 13,914 *T. plannipennisi* released from 2012-2014 at Haven Hill and 4,515 *O. agrili*, 4,757 *S. agrili*, and 13,927 *T. plannipennisi* released from 2012-2014 at Miser Station.

In Knox County, two release locations were selected to monitor at Cowan Park and the Ramsey Historical House, where releases of *S. agrili and T. plannipennisi* began in 2012 and 2013, respectively, and releases of *O. agrili* began in 2013 and 2014 at Cowan Park and Ramsey Historic House, respectively (Table 2.1). These releases continued until 2014 with a total of 4,941 *O. agrili,* 4,736 *S. agrili,* and 13,894 *T. plannipennisi* released at Cowan Park; and 5,679 *O. agrili,* 3,949 *S. agrili,* and 21,447 *T. plannipennisi* released at Ramsey Historic House. In Hamblen County, one location was selected at Lorino Park, where releases began in 2013 for all species and continued through 2014, with a total of 5,629 *O. agrili,* 4,918 *S. agrili,* and 19,181 *T. plannipennisi* released (Table 2.1).

Pan Trap Sampling of Parasitoids

Beginning in 2015, three years after the initial releases of parasitoids, sampling was begun to monitor three sites (Cowan Park, Haven Hill, and Miser Station); and in 2016 for the two remaining sites (Lorino Park and Ramsey Historic House) for parasitoid recovery and to assess establishment. The method chosen for monitoring for establishment of introduced EAB

parasitoids was yellow pan traps, first invented by Volker Moericke (Gould 2017). A pan trap consisted of yellow plastic bowls (355 ml) attached to 15.24 cm right-angled metal brackets, which were then attached to a tree within the release site (Fig. 2.2). Traps were placed roughly at chest height and were positioned on the tree to protect them from outside interference, often being placed on the backside of the trees. These traps were then numbered 1 - 10 using a permanent marker after placement on the tree.

Each trap consisted of two plastic bowls. The base (or bottom) bowl had two pairs of holes, four holes total, drilled into the bottom of it, each pair was located on the opposite side of the bowl relative to each other. This base bowl was then attached to the metal bracket via zip ties by threading the ties through the pairs of holes in the bottom of the bowl and then threading the front tie through the small hole at the tip of the metal bracket and then cinching it tight while the back tie is simple cinched around the bracket. The other bowl was left completely intact and placed inside the base bowl; it was then secured by clipping two binder clips to the edge of the two bowls. Ten pan traps were placed on 10 trees (one trap/tree) which were distributed throughout the release area. Traps were filled roughly one third full with propylene glycol diluted 50% with water, which acted as both a killing agent and as a preservative. Traps were monitored, changed and recharged every week during the spring and summer months and every other week during the fall and winter months to allow sufficient time for recovery of established parasitoids.

Recovery and Identification of Parasitoids

Specimens from pan traps were collected by pouring the contents of the inside bowl through a TCP Global® paint filters (TCP Global Corporation) with 190 micron filter tips. The location, date of collection, and trap number were written on the filter. This protocol allowed the

antifreeze remaining in the bowl to pour through the filter. The filter which contained parasitoid specimens was placed in a resealable bag (3.8 liter) and transported to the laboratory. Paint filters were chosen based on Cooperative Agricultural Pest Survey (CAPS) protocols for bark beetle trapping.

In the laboratory, most specimens were sorted on the same day of collection. However, if same-day sorting was not possible, the resealable bags containing filters and specimens were stored in a Fisher Scientific Isotemp Incubator Model 304R set to 1.5°C to ensure that samples remained fresh enough for species identification. Assessment of the contents collected from the traps was done by first examining any loose foliage that may have been collected from the trap, such as leaves, under a microscope to check for specimens that may adhere to them. After this foliage was examined, the paint filter was split from the side and the remaining contents were transferred to a 100x15 mm petri dish with a shallow layer (just enough to separate and disperse the contents of the sample) of 70% alcohol. This petri dish was then placed under a microscope and forceps were used to sift through the materials. All suspected EAB parasitoids (based on a key provided by Juli Gould, USDA APHIS) were removed and placed into 17x60 8-ml vials containing 5 ml of 95% alcohol, labeled with the date of collection and location, and mailed to Juli Gould for confirmation. This process was repeated for every pan trap sample collected.

After receiving confirmation of the species collected, a table cataloging the occurrence of these introduced parasitoids throughout the year, as well as that of any native parasitoids collected, was developed. This information was used to determine the seasonality and phenology of these parasitoids in a southern climate. This information will provide insight into the status of the released parasitoids of EAB at each collection site and throughout the year.

Results and Discussion

Parasitoid Recovery in 2015

Recoveries of introduced parasitoids in 2015 were low, with only 1 *S. agrili* (Haven Hill) and no *T. plannipennisi* collected (Table 2.2). Collections at Cowan Park, Miser Station, and Haven Hill began three years after the introduced parasitoids were released into them, meaning that the low recovery of them in this initial recovery period indicates that the parasitoids have not been able to effectively establish. However, the recovery of the single *S. agrili* does indicate that the parasitoids are present in some capacity.

Collections of native parasitoids were greater across all sites from May into October, with *S. floridanus* collected in particularly high numbers (Table 2.2). These data can also be used to derive the potential time period during the year in which these native parasitoids are active (June to October). This information can be compared to phenological data of EAB itself to determine if the parasitoids are phenologically synchronized or not (see Chapter IV). The comparatively high number of native parasitoids, in particular *S. floridanus,* that were recovered indicated that these parasitoids might be potential biological control agents that could be explored for use against EAB.

Parasitoid Recovery in 2016

Beginning in 2016 monitoring for the establishment of introduced parasitoids at Lorino Park and Ramsey Historic House was initiated, while sampling at 2015 sites continued. Similar to initial parasitoid recoveries of 2015, low numbers of introduced parasitoids were recovered in 2016, with only one specimen of *T. plannipennisi* and one specimen of *S. agrili* collected, both of which were collected from Carl Cowan Park (Table 2.3). These data further support that the introduced parasitoids have not been able to establish in their southern environments. These data

also support that the active period for these parasitoids is as early as April for *T. plannipennisi* and that, similar to the previous year, this period is a little later in the year for *S. agrili*. However, in contrast to observations the previous year, the number of native parasitoids recovered during this year of collections was much lower than during the previous year (Table 2.3). The low number of parasitoids could be related to environmental conditions (such as the drought during 2016) that differed from the previous year, but could also imply that the EAB infestation at these sites may have reached its final stages and as such the EAB have already begun to move from these sites resulting in lower numbers of native parasitoids.

Parasitoid Recovery in 2017

Samples collected during 2017 have not yet been fully processed. Therefore, no results on parasitoid recovery from 2017 are included in this thesis.

It should also be mentioned that even though *O. agrili* was released in great numbers at numerous sites in the south, no *O. agrili* were recovered from any release sites during the course of this study. Further research on EAB eggs and parasitoid incidence throughout the year is needed.

Summary

Recoveries of introduced parasitoids in the southern United States have been extremely low. These data support that the parasitoids have been unable to effectively establish in the south. This inability to establish could indicate that the parasitoids are not able to survive in the southern United States either through the inability to adapt to climate or through phenological asynchrony with EAB in the south. Several native parasitoids were also collected; however, the number of native parasitoids collected has declined in recent years. This decline is possibly due to adverse environmental conditions present from year to year, such as warming periods during

the winter months, or could possibly indicate that the EAB infestation in the study sites has progressed to a point where the EAB have moved to other areas, reducing availability of host resources for these parasitoids. Further study of the phenology of the introduced parasitoids in the south as well as how their phenology synchronizes with that of EAB in the south is necessary to determine if they should still be deployed in the south or if other, more adapted, biological control agents should be pursued.

CHAPTER III

DETERMINE SEASONALITY AND PHENOLOGICAL SYNCHRONY OF EMERALD ASH BORER AND ITS INTRODUCED PARASITOIDS IN A SOUTHERN CLIMATE

(Part of a cooperative geographical assessment of emerald ash borer parasitoids in New York and Tennessee)

Introduction

As was discussed in Chapter I, the invasive wood-boring emerald ash borer (EAB) (*Agrilus planipennis* Fairmare) (Coleoptera: Buprestidae) was introduced into the United States in 2002 and has since then spread across 32 states, as well as two Canadian provinces. This distribution places EAB in a number of different environments, from the warmer south to the cooler north, meaning that the introduced parasitoids being released against it will also be exposed to these varying environments. When using a biological control agent, ensuring that it can survive in the environment into which it is released is important; however, it is not only important to know that it can survive, but to also understand how it survives.

Phenology is the study of recurring plant and animal life cycle stages, especially their timing and relationships with weather and climate (Schwartz 2013). Essentially it describes the temporal aspects of the life cycle of an organism in its respective habitat, for example, when an insect begins to emerge in the summer or when it begins to overwinter. Understanding the phenology of a biological control agent and its host is vital to the success of a biological control program, and this concept is true for EAB and its introduced parasitoids. Insights on phenology will allow researchers to determine when and where to release these parasitoids of EAB so that

they will have the most impact on EAB by utilizing them in the best circumstances to facilitate parasitism. Releasing these parasitoids at the wrong time, or into an environment to which they are not properly acclimated, could result in what is known as “phenological asynchrony”. If two organisms are phenologically asynchronous, their life cycles are not properly synched and they may not interact. For example, if parasitoids begin to emerge too early in a new environment due to differences in temperature from that of their original habitat, they could emerge before their host organism is at a proper life stage for them to parasitize.

Therefore, a study was designed to determine the seasonality and phenological synchrony of EAB and its introduced parasitoids in a southern climate. Questions to be answered in this study include: what is the phenology of EAB and its introduced parasitoids in the southern United States, and are these phenologies synchronous in the southern United States?

Materials and Methods

Assessment of Parasitoid Phenology: Growth Chamber Study

To assess the phenology of introduced parasitoids of EAB, a study was designed to rear and evaluate these parasitoids in growth chambers. This method was chosen to provide a controlled environment for assessment.

Before this study was initiated, a series of five HOBO data loggers (HOBO U23-001 Pro V2) with external temperature probes were placed on ash trees on Brian Ostby’s property in Knox County. The probes of each HOBO unit were placed underneath the bark of ash trees to record hourly temperature readings. Temperatures were recorded from June 2015 to August 2017. These temperature readings were then downloaded roughly every two weeks and formatted into programs which were uploaded to two Percival E-36-L growth chambers with expanded program capabilities, so that the chambers could run hourly temperatures to simulate

temperatures recorded in the field (i.e., temperatures common to a southern climate). The temperatures programmed to run on these growth chambers used data recorded from the previous calendar year. After these growth chambers were programmed for field temperatures, parasitoids (*Spathius agrili*, *S. galinea*, and *Tetrastichus plannipennisi*) were requested from the USDA APHIS PPQ Biological Control Production Facility in Brighton, MI. These parasitoids were either received in rearing bolts, small bolts of ash wood infested with EAB larvae by the Facility to be parasitized by parasitoids, or as adults. Parasitoids received in rearing bolts were placed into round plastic containers (1.9 liter), which were then placed into the growth chambers until parasitoids emerged.

Once adult parasitoids emerged, or arrived as adults, they were counted and sexed, and were allowed to mingle in plastic containers (2 liter) with a supply of honey and water for one week to improve chances of mating. Parasitoids were then separated into groups consisting of two females and one male (when possible). To prepare EAB larvae as hosts for native parasitoids, fresh wood was obtained from a grove of young ash (roughly 10-13 years old) trees at the Ramsey Historical House in Knox County. This wood was cut into small bolts between 10.16 and 11.43 cm in height with a diameter of roughly 5.08 cm and then artificially infested with EAB. These artificial infestations were done by forming artificial galleries in fresh wood.

Ash wood was artificially infested by forming galleries using an X-Acto® knife to form two parallel cuts over the area where the gallery would be placed, and then a smaller cut connecting these two parallel cuts essentially forming three sides. A chisel was used to peel and raise the resulting bark flap, starting from the cut, and then to carefully peel along the length of the parallel cuts (Fig. 3.1A). Care was taken not to damage the peeled bark area where the gallery was to be placed as that area needed to be resealed after larval placement in the gallery.

After peeling and raising the bark, one hand was used to keep the bark held away from the wood while the other hand used a Dremel® tool to form the artificial gallery (Fig. 3.1B). The gallery was made by using the Dremel® tool to form a small trench in which one EAB larva was placed.

The larvae (second and third instar, when possible) used in these artificial infestations were collected from the field. In the unlikely event that sufficient numbers of these larval instars were not available, younger or older larvae were used. Care was used to place EAB larvae inside the new gallery correctly as it needed to be placed with its dorsal side facing away from the wood and its head pointing to the top of the wood. After placing the larva into the artificial gallery, the bark was carefully laid down against the wood being careful not to damage the EAB larva and to reseal the gallery. Parafilm was then wrapped around the piece of wood to keep the bark pressed against it. For the purpose of this study this type of rearing until will be referred to as the "standard cup rearing unit" (Fig. 3.2). Once this artificial gallery was completed, the wood was placed into the emergence barrels, bug dorms, or into growth chambers. These groups were then placed into standard parasitoid rearing units with bolts of ash wood artificially infested with EAB larvae (Fig. 3.2), with no more than 40 units per one species. Units were constructed by placing artificially infested bolts into clear plastic cups, placing paper towels around the base of the bolt, filling the cup with roughly 5.08 cm of water, and placing a mesh covering over the cup which was held in place by a plastic lid with the center removed.

Water was placed into the cup to keep the ash wood from desiccating which risked killing the larvae. Water was refreshed as often as needed, and the paper towels near the base of the bolt prevented any emerged parasitoids from falling into the water and drowning. These units were then placed into the two growth chambers, with a maximum of 20 cups per species. The cups were evenly distributed inside the growth chambers, and were monitored daily for adult activity,

as well as emergence of subsequent generations. In the event of parasitoid emergence, the number of parasitoids that emerged, their sex, and development time were recorded.

Assessment of Parasitoid Phenology: Field Cage Study

In addition to the growth chamber study a field cage study was established at Ramsey Historic House and designed to assess the phenology of the introduced parasitoids of EAB in Tennessee. This study was designed to attain phenological data within the current year, as the growth chambers used temperature data from the previous year. This study was initiated when third-instar EAB larvae appeared in the field, because this stage is the optimal larval instar for the parasitoids to parasitize. Once second-instar EAB larvae appeared in the field parasitoids were requested from the USDA APHIS PPQ Biological Control Production Facility in Brighton, MI. This study was conducted twice over a two-year period (2016 and 2017) with the first run using 50 males and females, being distributed across 10 trees with five males and five females per tree, of the three introduced parasitoid species (*S. agrili, S. galinae, and T. plannipennisi).* The second run used 150 females and 90 males of each parasitoid species, with five females and three males of each species per tree. After requesting parasitoids, 30 trees were selected as host trees from young ash trees, approximately 5-8 cm diameter at breast height (DBH), growing in approximately 56-58 cm deep water, and showing early signs of possible decline (Fig. 3.3).

These 30 ash trees were then artificially infested with EAB larvae (5 larvae per tree) collected from the field in a similar fashion to the wood used in a related study (see Chapter IV). First, because these young ash trees in this site were standing in water, a Dremel® tool could not effectively be used to form artificial galleries as with the other studies. In this situation, a Dremel® gouging bit was used in a battery-powered drill. The bark was cut and peeled back just as in other studies, and the drill was used to form the galleries, which were then sealed using

several layers of parafilm. The galleries were sealed in this fashion because the larvae placed in them were fragile and perishable and could not be held for long periods prior to being placed in the galleries. Thus, galleries were formed and the larvae were placed into them on the same day they were collected. Larvae were collected for this study through the skinning of trees from sites infested with EAB. Second, the method used to place EAB larvae into the galleries also differed. In other studies, the bolts were placed on one side and the larvae were simply lifted up and laid inside. In this study using standing trees, larvae were inserted in a different manner. One person held the flap to the gallery open as another person used a paint brush to lift, place, and position the larva into the gallery. After ensuring that the underside of the larva was against the tree and facing down the tree, the person placing the larva then gently rolled the brush up the larva's body to nearly the tip of the larva. Then the person holding the flap open slowly closed the flap over the larva, to ensure it would not be crushed. After removing the brush from under the flap, the gallery was then sealed using two large (60.96 cm) zip-ties (this method was used only in the 2017 study), one placed near the base of the gallery flap and the other at the top of the flap. These zip-ties held the flap closed so the wood would not desiccate and potentially harm the larva. Zip-ties were chosen over parafilm, as parafilm dries in the sun, becomes brittle and breaks, allowing the gallery to open and desiccate as was learned in the 2016 study.

As many of the 30 trees as possible, with a minimum of 15 trees, were artificially infested with as many EAB larvae that could be collected on one day. To ensure that all larvae and conditions in the study were similar and to remove other variables, more trees were not artificially infested past the day of the initial infestation.

After trees were infested with EAB larvae, small sleeve cages were then placed around the trees (Fig. 3.4). Cages were wrapped around padded foam rings that were placed roughly

7.62 cm above and below the highest and lowest gallery on the individual tree. The rings allowed the tree cages to be slightly extended from the tree giving the parasitoids room to move and fly around without cage interference. The rings also kept the cage from adhering to the tree in the event of a rainstorm.

After parasitoids were received from the USDA APHIS PPQ Biological Control Production Facility, they were sexed, counted and placed into groups (five males and five females in the first study, and three males and five females in the second study). These groups were then released in their respective cages (10 trees per species). After parasitoids were released into these cages, the cages were monitored once each week to assess adult parasitoid activity and cage stability. At the end of the summer season, the trees were carefully felled, as to not damage the caged areas, cages, or their contents, with the cages still attached. The portions of the trees containing the cages were removed and taken to the laboratory, where the cages were removed and their contents examined for emerged parasitoids or adult EAB. The wood was then placed into emergence barrels similar to those described in Chapter IV and monitored for emergence of parasitoids or adult EAB. This wood was held in these barrels throughout winter in a shade house, to account for possible later emergence of *Spathius* parasitoid species which have a diapause phase in their life cycles.

Assessment of Emerald Ash Borer Phenology

To determine the life cycle of EAB in the southern United States, a study was designed to monitor the growth patterns of EAB in relation to the time of year in which they were observed. This study began by identifying locations with ash tree stands that still contained enough "healthy" trees to support long (two to four years) periods of study. Two such locations were identified at Doyle Farm (Fig. 3.5) and Oak Ridge National Laboratory (ORNL) (Fig. 3.6).

Sampling at each location was initiated in April 2016 and consisted of removing the bark from selected trees at each site every two weeks. However, it should be noted that the ORNL site was not sampled on the same frequency as the Doyle Farm site, with the ORNL site being sampled primarily in 2017. Three trees were selected to be sampled during each sampling trip so that samples were replicated and means could be calculated. This number of trees was chosen so that each sampling trip had a sufficient sample size while also leaving enough remaining trees to use throughout the duration of the study. Trees with EAB exit holes and epicormic shoots on the base of the trunk were selected as they had a higher probability of EAB infestation. Dead trees were excluded, but living trees with signs of decline (such as blonding, canopy thinning, bark splits, and epicormic shoots) were selected for potential sampling. After three trees were selected, each tree's bark was skinned, either while it was standing (Doyle Farm) or after being felled (ORNL), depending upon the site. Felled trees at ORNL were transported to the laboratory, while the bark of standing trees at the Doyle site was removed from the highest point reachable to the ground, and EAB numbers and life stages were assessed in the field (Fig. 3.7A). These trees also were left to fall afterward as they were in an isolated area. Trees at the ORNL site were felled and taken to the laboratory because their presence near roadways made them a hazard if they were simply left to fall on their own. Bark removal was conducted by using draw knives, which were used to make an initial cut in the bark and then used to peel the bark from the tree. This bark removal was done carefully as to not kill or damage the larvae beneath the bark. After the bark was removed from the trees, the number of larvae beneath the bark were counted and their instar was determined. Any potential parasitized larvae or parasitoid cocoons were also recorded (Fig. 3.7B). Larval stages were determined using pictorial guidelines (Gould et al.

2017). After these data were recorded, the DBH, as well as the area of skinned bark from each tree (cm^2), were recorded.

Results and Discussion

Assessment of Parasitoid Phenology: Growth Chamber Study

The F1 generation of parasitoids experienced a relatively successful emergence, though the total number of parasitoids were lower than the number of initial parasitoids received from the USDA APHIS PPQ Biological Control Production Facility. In the F1 generation, 17 *S. agrili* (2 males and 15 females) emerged with a 1:8.5 M:F (male to female) sex ratio. A total of 15 *S. galinae* (4 males and 11 females) emerged with a 1:3.75 M:F sex ratio. A total of 137 *T. plannipennisi* (35 males and 104 females) emerged with a 1:3.2 M:F sex ratio. Both *Spathius* species displayed development times ranging from approximately 45 to 48 days, while *Tetrastichus plannipennisi* displayed a development time of 44 days (Table 3.1).

The F2 generation of this group of parasitoids displayed a similar parasitism pattern as the previous generation with the number of emerged parasitoids lower than the parent generation. In the F2 generation only one species of parasitoid, *S. galinae*, successfully emerged: two males and two females (1:1 M:F sex ratio). The parasitoids that emerged from this generation displayed a development period of 56 days, a development time slightly longer than that of the previous generation. No emergence of F3 adults occurred.

This trend of lower emergence with each successive generation resulted primarily from a lack of parasitism (Table 3.2). This failure of parasitism was caused by a combination of parasitoids simply not being able to locate EAB larvae and parasitize them, as well as an approximate 50% mortality rate in the EAB larvae used to artificially infest the bolts used in the rearing process. Through the course of this study the fragility of the EAB larvae became more

apparent as many would not survive more than a few hours after being removed from ash wood or after being handled. This failure to parasitize EAB larvae caused a reoccurring issue when rearing parasitoids when each subsequent generation has fewer adults emerge.

In the F1 generation, 38 bolts were prepared for *S. agrili* with each bolt containing two EAB larvae. Of these 38 bolts, four produced parasitoids. Of these four parasitoid-producing bolts, 62.5% of the larvae in them (8) were parasitized, but the total number of larvae that were parasitized by *S. agrili* across all 38 bolts (76) was 6.41%. For the F1 generation of *S. galinae*, 26 total bolts containing two EAB larvae each were prepared for rearing. Of these 26 bolts, two had adult parasitoids emerge from them. Of those two parasitoid-producing bolts, 50% of the EAB larvae were parasitized, but the total number of EAB parasitized by *S. galinae* across all 26 bolts was 3.85%. Lastly for the F1 generation of *T. plannipennisi*, 40 bolts were prepared but only five bolts produced adult parasitoids. Of the five parasitoid-producing bolts, 60% of the larvae in them were parasitized, but the total number of larvae parasitized by *T. plannipenisi* across all bolts was 25.53%. In the F2 generation of *S. galinae*, only one of the five bolts prepared produced adult parastioids. Of this one parasitoid-producing bolt, 50% of the larvae were parasitized, but the total number of EAB larvae parasitized by *S. galinae* across all bolts in the F2 generation was only 10%.

The data for the second replication of this study have been collected; however, these data have not yet been analyzed and are not included in this thesis. This study was one half of a partner study, with the other half being conducted in New York. Because not all studies in both locations are complete, results of both studies have not been compared. Thus, implications of these data from both studies have not been determined.

One interesting phenomenon that was observed during the course of this study was the discovery of fully developed, yet dead aggregations of adult *T. plannipenisi* within the galleries of the rearing bolts (Fig. 3.8). In addition to this finding *T. plannipenisi* were observed dead beneath the bark of trees at the ORNL site during the 2017 EAB phenology study. The reason for the failure of these adult parasitoids to emerge is not known. One possible explanation is that while in the growth chambers the ash bolts desiccated causing the bark to contract and become too tough for parasitoid emergence. Another explanation is that the fluctuations of the temperature cycles from the field which were maintained in the growth chambers were too extreme for the parasitoids to survive resulting in them dying in the galleries. The explanation may explain why recoveries of these parasitoids have been so low in the southern United States.

Assessment of Parasitoid Phenology: Field Cage Study

In the first iteration of the field study, no parasitoids emerged from the trees across any of the three parasitoid species. This lack of emergence may have occurred due to high mortality of the EAB larvae used to infest the trees for this study. Initial plans were to seal the trees using parafilm similar to the ash bolts used in the parasitoid study; however, it was quickly discovered that the parafilm rapidly dried and began to break, allowing the sealed galleries to open and causing them to desiccate. Due to the time-sensitive nature of preparing the galleries for use in this study, paper staples were used to seal the galleries. These staples could not create an adequate seal and the galleries dried, leading to the possible death of the larvae used in the artificial infestation. This staple method was used only in the 2016 run of this study while zipties were used to seal galleries in the 2017 run. At the end of this study the wood used in it was held in emergence barrels to monitor for late emergence of parasitoids. This wood has not been fully analyzed for unemerged parasitoids or larval mortality, though no emergence of parasitoids or

adult EAB was observed. A second iteration of this study was conducted in 2017; however, data gathered from this study have not been analyzed and is not included in this document.

Assessment of Emerald Ash Borer Phenology

In 2016, first-instar EAB larvae were observed in May, with second-instar larvae occurring in June, third-instar appearing in June, and fourth-instar larvae appearing in July. This linear life cycle was primarily observed at the Doyle Farm sites, though a similar life cycle was displayed at the ORNL site. “Boring larvae” (fourth-instar larvae that have begun to bore deeper into the tree to overwinter) began to appear in August. Along with these occurrences, early instars were observed less and less as the season progressed and as the development of later instar larvae increased (Fig. 3.9). This type of developmental pattern is typical of an annual life cycle, and depicts a linear type of development with earlier life stages giving way to later life stages as the generation as a whole progresses with little overlap of larval instars (Fig. 3.10). This annual life cycle was also displayed in 2017 at both Doyle Farm and ORNL sites (Figs. 3.11 and 3.12, respectively). This annual life cycle has been observed persisting through multiple years as can be seen in Figure 3.13.

These results indicate that certain larval instars will be present only during a small window of time each year. Thus, if parasitoids are released at the wrong time, or if they emerge at the wrong time, the probability that larval hosts of a suitable size for them to parasitize is low. Understanding this phenological cycle, as well as the phenology of each parasitoid species, are important factors in deciding which parasitoids would be released in certain areas or regions. The importance of this concept can be seen now with the success of *T. planipennisi* in the northern United States and failure of the same parasitoids in the southern United States, as has been shown in this study (Duan et al. 2013).

The reason for the success of the parasitoids in the northern United States is that EAB in the north exhibits a 1½-year life cycle. This life cycle occurs when not every member of each generation can reach the same point of development by the end of the season. In the northern United States, this 1½-year life cycle occurs because not every larva matures, begins to bore at the end of the season, and overwinters in the gallery. In the spring, those larvae that were able to bore into the tree emerge as adults; however, those that were unable to bore continue their larval development and continue to feed until they become "boring larvae". They then bore into the wood, pupate, and emerge as adults later in the season. This development results in a staggered pattern of adult emergence, mating, egg laying, and the presence of all larval instars throughout the year. The ultimate cause of this staggered adult emergence is longer, more intense cold periods in the northern United States. This difference in temperature cycles can be seen in Figure 3.14, where, on average, Tennessee has 64 more days above 15.6° C than New York. Additionally, Tennessee consistently reaches 500 cumulative degree days (based on threshold of 50°F [28°C]), when EAB begin to emerge in the spring, about 50 days before New York (Fig. 3.15). Peak adult activity generally occurs at around 1,000 degree days, which also occurs approximately 45 days earlier in Tennessee than in New York (Fig.3.15) (Gould et al. 2017).

The availability of an array of larval stages throughout the year is significant because released or emerging parasitoids have a greater chance of finding a potential host at any point throughout the year in the northern United States as compared to parasitoids in the southern United States due to the staggering of generations. This difference in phenology may partially, if not entirely, explain the cause behind the success of some of the introduced parasitoids of emerald ash borer in the northern United States, and their failure in the southern United States. Parasitoids in the south have a small window of opportunity to find suitable hosts, meaning the

timing of their release will play a vital role in their ability to establish (Fig. 3.16). In contrast, parasitoids emerging in the north have a high likelihood of finding a potential host due to the variety of larval stages present throughout the year.

Summary

The introduced parasitoids of EAB can survive in the southern United States; however, they have not been able to establish and complete multiple generations from year to year. This inability to establish is caused by the phenological asynchrony of EAB and its introduced parasitoids in the southern United States. This asynchrony is evidenced by parasitoid phenology, where it was demonstrated that EAB larvae were parasitized, but the resulting progeny emerged later in the season when no suitable hosts were available. These data, however, still need to be compared to those data of the sister study in New York. Once analysis is completed, the second iteration of this study may provide further insights into the phenology of these introduced parasitoids as they relate to EAB.

EAB in the southern United States exhibits a different life cycle (i.e., an annual life cycle) than that found in the northern United States (i.e., a 1½-year life cycle). The life cycle of EAB in the south provides synchronized generations, meaning that certain larval instars are present only at certain times of the year. In contrast, the life cycle displayed in the north means that EAB has asynchronous generations providing a scattering of larval instars throughout the year. These differences in life cycles arise from temperature differences in the south and north, with the south having longer warm periods and the north having longer cold periods. This difference in life cycle is the primary cause behind the inability of the introduced parasitoids of EAB to establish in the south, while they have seen successful in the north. Understanding the phenology of these introduced parasitoids as well as EAB in its respective climate is vital to future biological control

of EAB. This research stands to broaden our understanding of these natural concepts as well as inform executive decisions on the biological control efforts against EAB in the United States.

CHAPTER IV

ASSESSMENT OF NATIVE PARASITOIDS OF EMERALD ASH BORER AND THEIR POTENTIAL FOR REARING AND RELEASE

Introduction

The emerald ash borer (EAB) (*Agrilus planipennis* Fairmare) (Coleoptera: Buprestidae), an invasive pest native to Eastern Asia, was first documented in the United States in 2002. As part of a national program sponsored by USDA APHIS to reduce the impact of EAB on ash, parasitoid species native to the same habitat as EAB have been reared, released, and evaluated in the eastern and southern United States. During evaluation of these introduced parasitoid species, several species of native parasitoids have been observed to parasitize EAB larvae (Duan et al. 2009, Cappaert and McCullough 2009, Hooie 2014, Hooie et al. 2015). These native parasitoid species, which include several members of the genera *Spathius*, *Atanycolus*, and others, tend to be generalists and parasitize the larvae of various native buprestid species. Research to understand how these native parasitoids, as well as introduced parasitoids, interact with EAB is vital to the effective and efficient use of these parasitoid species for optimal management of EAB.

Therefore, a study was designed to assess the incidence, diversity and abundance of native parasitoid species in eastern Tennessee, as well as assess the potential to rear and release native parasitoids as part of the biological control program against EAB. Questions to be answered in this study include: what species of native parasitoids are present, how abundant are these native parasitoids, can native parasitoid species be successfully recovered and reared, and how do these native parasitoids interact with the introduced parasitoids of EAB?

Materials and Methods

Collection of Native Parasitoids

To collect and assess native parasitoids, ash wood was collected from five sites in three counties in eastern Tennessee (Blount [two sites], Hamblen [one site], and Knox Co. [two sites]), where introduced parasitoids had previously been released (Fig. 4.1). It is important to note that these sites were selected because native parasitoids had previously been observed and/or collected at each of these sites. At each site, ash trees which were likely to be infested with EAB and, possibly, parasitoids, were identified. These trees were chosen by visually inspecting trees for signs or symptoms of stress or damage (thinning canopy, D-holes, epicormic shoots, blonding of the bark, and bark splits) caused by an infestation of EAB. For this study one tree exhibiting signs of decline or stress was selected at each site, only one tree was selected to ensure that an adequate number of trees were left to enable later portions of this study to be conducted. The selected tree was then felled and cut into sections (1 m long) and transported to the University of Tennessee East Tennessee Research and Education Center in Knoxville, TN.

Wood sections were then placed into cardboard emergence barrels (82 cm tall, 46 cm diameter) with metal rims to maintain the shape of the barrel (Fig. 4.2). These barrels were fitted with collection vials by using tin cutters and an X-Acto® knife to cut a hole into the cardboard lids of the barrels, then placing a plastic funnel into the hole with the tapered end facing out of the barrel. These funnels were hot glued onto the cardboard lids. Plastic collection vials were attached to these funnels by cutting a small hole into the vial's lid and placing the tip of the funnel through it. The vial lid and funnel were then hot glued together. Hereafter, this method will be referred to as the 'emergence barrels.' These barrels were stored on racks that held three rows of four barrels within a shade house. Wood was also placed into bug dorms (60 cm tall with

a 60 by 60 cm base), which are small plastic ventilated tents used to house insects (Fig. 4.3). The shade house was composed of a metal pipe framework covered in black plastic shade cloth (ca. 50%) on all four sides as well as the roof. This mesh allowed air to flow through the shade house to provide equitable temperature throughout the area, The roofs of the shade houses were also fitted with plastic tarps to provide shade to the interior of the shade house. After placement these barrels and bug dorms were monitored daily for emergence. Collection vials were checked daily for emerged parasitoids, and the inside of the barrels were inspected after the expected emergence time was completed to ensure no parasitoids were missed.

Upon emergence of adult parasitoids, they were collected, tentatively identified, counted, sexed, and held in containers (1 liter) with mesh covered lids. These containers were supplied with bolts of wood to provide resting areas for the parasitoids, as well as water and droplets of honey, until they could be placed into rearing units and exposed to their appropriate rearing methods (emergence barrels, bug dorms, and growth chambers). Various factors, such as bark thickness, larval size, and number of EAB larvae, that could affect parasitism were measured.

Hand trapping of parasitoids as a tool to assess parasitoid recovery also was used at each site. Hand collecting began during June and proceeded through July until native parasitoids were no longer observed in the field. Hand collecting was conducted by visiting each site, either from early to mid morning or mid to late afternoon, and inspecting the trunks of trees for potential native parasitoids. Upon finding a potential parasitoid, it was netted with a fine meshed, hand-held aerial net and then placed into a plastic vial. Collections ceased after no parasitoids could be found for a period of 20 minutes. These parasitoids were then taken to the laboratory, where they were sexed and identified. Upon determining this information, parasitoids were then placed into groups of the same species and into standard rearing units.

Rearing of Native Parasitoids

Three methods of rearing (emergence barrels, bug dorms, and growth chambers) were evaluated to assess which method is best suited for rearing native parasitoid species and to study the various factors which might affect their ability to parasitize EAB larvae and successfully emerge as adults. Adult parasitoids reared from larvae collected during completion of "***Collection of Native Parasitoids***" were used in this study. Parasitoids were then placed into standard parasitoid rearing units with bolts of ash wood artificially infested with EAB larvae, with no more than 40 units per one species. The development of standard parasitoid rearing units was described in Chapter III (Fig. 3.1 and Fig. 3.2). Units were constructed by placing artificially infested bolts into clear plastic cups, placing paper towels around the base of the bolt, filling the cup with roughly 5.08 cm of water, and placing a mesh covering over the cup which was held in place by a plastic lid with the center removed. These rearing units were then placed into growth chambers running programs built to simulate field recorded temperatures.

The barrel and bug dorm methods used to assess rearing of native parasitoids were identical to those described in the '***Collection of Native Parasitoids***'. The number and sex of newly-emerged parasitoids were recorded and they were then placed into either barrels or bug dorms as described earlier. The growth chamber method included the use of two Percival Scientific Intellus Ultra growth chambers. These growth chambers each contained three shelves and were able to run temperature cycles as well as light cycles. The light and dark cycles for the chambers were set to alternate every 12 hours, and the temperature was set to run at 30°C. Humidity was not an option for programming in these growth chambers, however, humidity was attempted to be maintained above 60% by leaving 3.78 liter containers of water to supply evaporated moisture. After recording the initial emergence data, such as the number of

parasitoids emerged, their sex, and the date, parasitoids were divided into groups of five (three females and two males) or as close to that ratio as possible. Adult groups were then placed into either emergence barrels or bug dorms with artificially infested wood or placed with artificially infested wood into plastic containers (1.9 liter) in a growth chamber. All groups were monitored daily for emergence. At the conclusion of this study all units were inspected to determine the number of EAB alive and dead, parasitized larvae, emerged parasitoids alive and dead, and the developmental time of the parasitoids. These inspections were done by skinning the bark from the wood and observing what was present beneath, looking for evidence of parasitism as well as determining the fate of the EAB larvae.

Due to the more controlled nature of the growth chamber study, several aspects that could potentially affect the effectiveness of these native parasitoids were evaluated. These aspects included: bark thickness and the number and size of EAB larvae used. Bark thickness was measured using a set of calipers when the artificial galleries were made. A more thorough understanding of how this factor could possibly affect the ability of parasitoids to parasitize their hosts is vital, because the thickness of the bark could hinder their ability to parasitize if the bark is too thick for them to penetrate with their ovipositors. The larval instar was determined using a field key. Understanding how larval size could affect the parasitoid's ability to parasitize also was vital information to enhance rearing these parasitoids, as larvae that are too small for them to detect will be of little use in rearing and larvae that are too large could be far enough in their development cycle to bore into the wood beyond the reach of the ovipositor. Larval size was studied by artificially infesting the wood with one to four larvae. It is important to understand how many larvae are optimal for the rearing of these parasitoids to make the best use of them as well as the parasitoids.

Results and Discussion

Collection of Native Parasitoids

No native parasitoids were recovered from the wood selected from the five sites. The only recovery of native parasitoids was done via the hand trapping method, and native parasitoids were only found to be present during the study period at the Miser Station Rd. site in Knox County. Collections of these parasitoids took place from mid-June through mid-July and ceased only when native parasitoids could no longer be found. Three parasitoid species were collected, Species A: 19 females and 10 males, Species B: 1 female, and Species C: 1 female. The identity of these parasitoids still need to be confirmed, however, it is likely that Species A is a member of the genus *Atanycolus*. What distinguished this site from the other sites is that Miser Station had the most advanced infestation, with the other sites showing fewer signs of decline or had already been deforested. The occurrence of these parasitoids at this site could indicate that these native parasitoids are more likely to inhabit sites with heavier infestations. If so they may also be present at sites with earlier or lighter infestations, however, they will likely not occur in large numbers. It is also possible that because EAB begin their infestation in the canopy and work their way down that the parasitoids will also be primarily located in the canopy, making them more difficult to recover or capture.

Rearing of Native Parasitoids

Due to a shortage of usable EAB larvae when native parasitoids were available, adults of Species A were placed into liter sized rearing units rather than the one previously described. These rearing units were constructed in the same fashion as the previous ones but consisted of two artificially infested ash bolts instead of one bolt and were placed into one-liter containers. The specimens of Species B and C were placed into standard cup rearing units. Due to the time-

restricted nature of developing these rearing units which became apparent throughout the course of this study, as well as the extremely low numbers of native parasitoids that were recovered for use, the assessment of variables (bark thickness, larval number and size) possibly affecting parasitism were not measured to give the parasitoids the best chance for successful parasitism. Bark thickness was not measured to maintain the freshness of the wood and bark of the bolt in hopes of providing the EAB larvae the best chance for survival inside the bolt, as the EAB larvae are fragile and easily damaged. The exact size of the EAB larvae was not measured because obtaining EAB larvae to use for this study is time-sensitive; however, the larval instar stage was determined as they were placed into rearing units. Second and third instar larvae were chosen as they fell within the optimal sizes for parasitism, this selection was done to give the parasitoids the greatest chance at success. As such any larvae that were available of the appropriate size, second to third instar, were used and the appropriate number, two larvae per bolt, was used. No parasitoid emergence was ever observed from these rearing units.

Summary

Native parasitoids were recovered in extremely low numbers with hand collections as the only recovery method to produce any results. Due to the time-consuming nature of developing the rearing units, as well as the low number of parasitoids available, the various factors which could have potential impact on the effectiveness of parasitism were not measured to provide the larvae the best chance of surviving so that they could be parasitized. A small number of rearing units were developed for these native parasitoids; however, no parasitism was observed from these units. Further studies are needed to properly assess the potential of the native parasitoids of EAB as biological control agents as well as the various factors which could be addressed to improve rearing methods.

The results of this study stand to broaden our understanding of how the environments in which parasitoids are released affect their survivability and effectiveness as biological control agents and also stand to broaden our possible management options through the potential use of native parasitoids. This knowledge and the strategies and programs which can be gleaned from it will be vital to the future of the biological control of EAB in the United States.

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APPENDIX

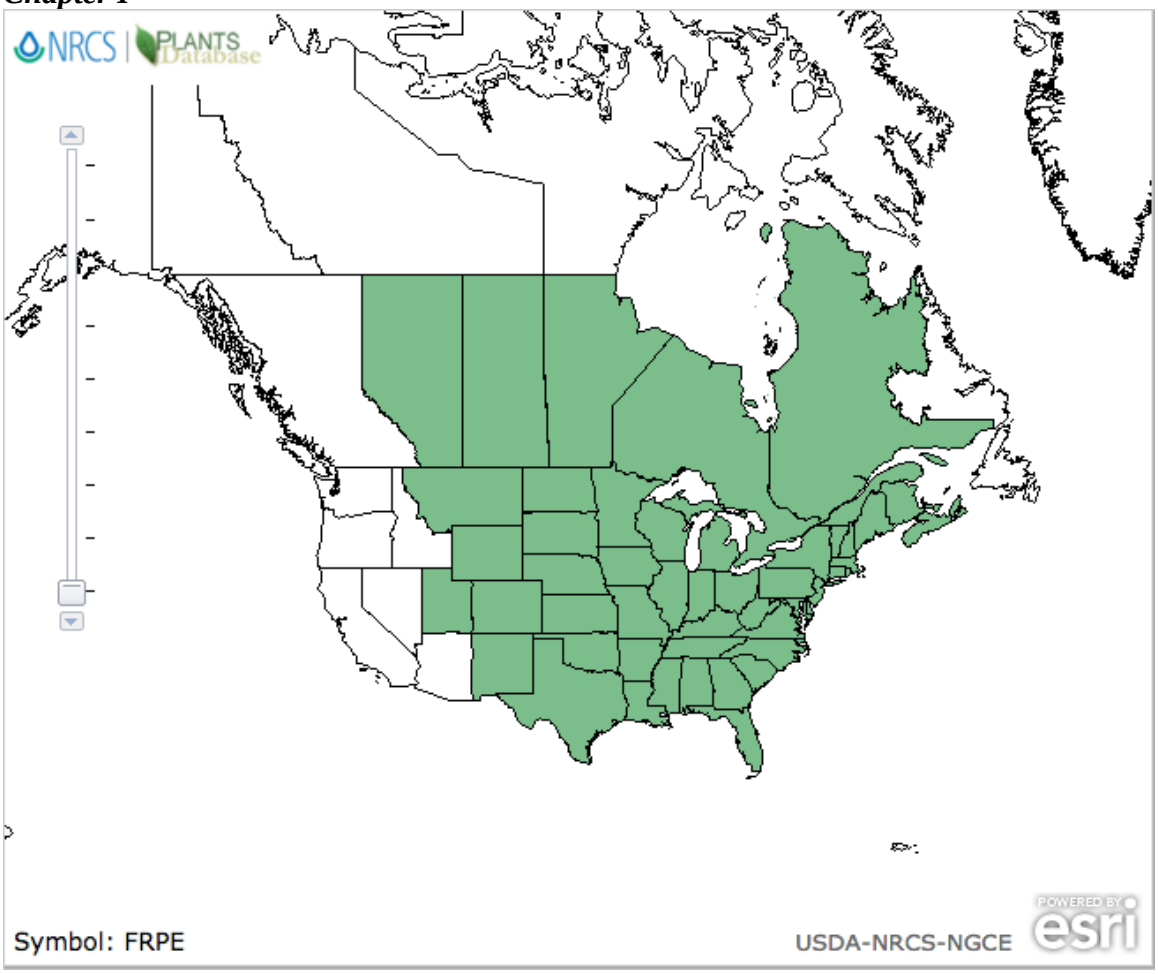

Figure 1.1. Distribution of green ash (*Fraxinus pennsylvanica*) in North America.

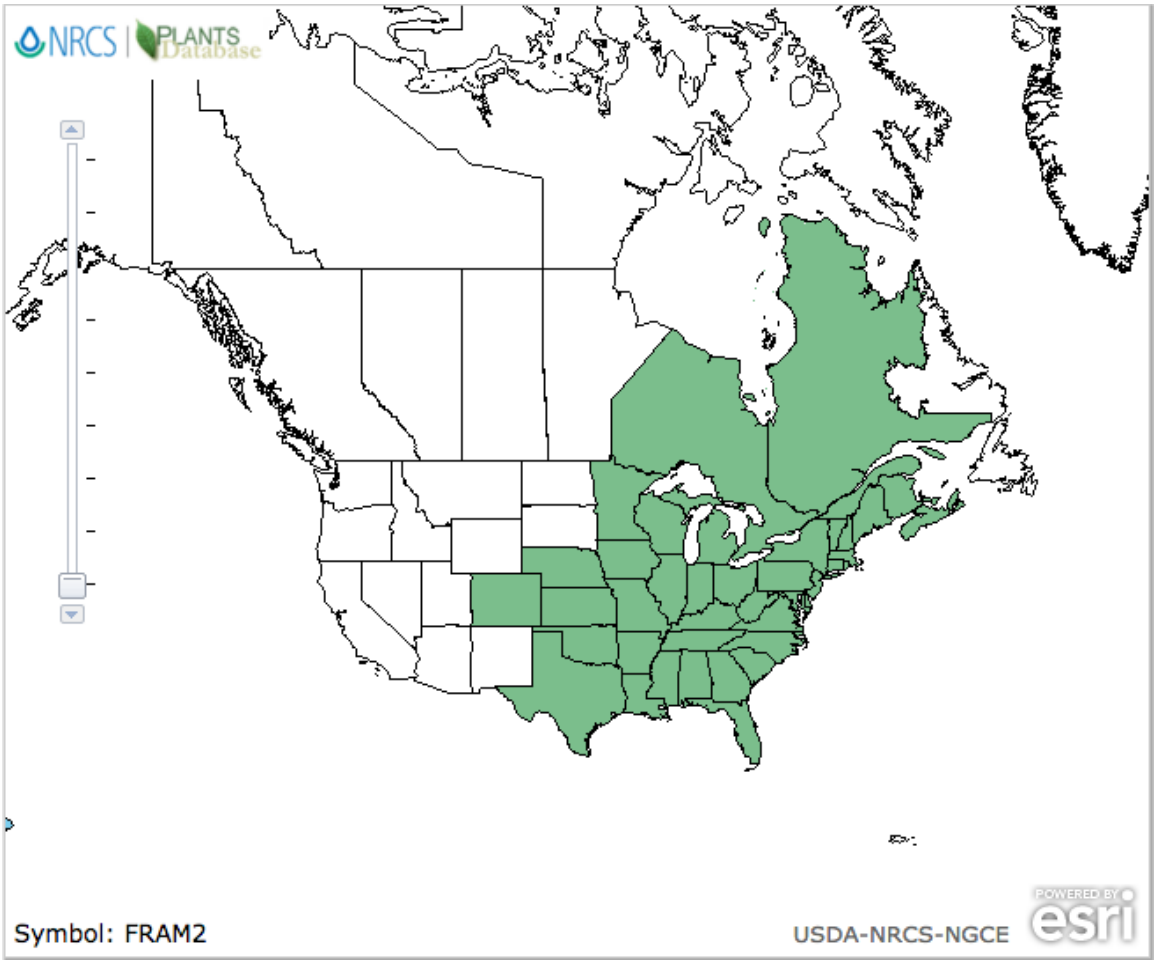

Figure 1.2. Distribution of white ash (*Fraxinus americana*) in North America.

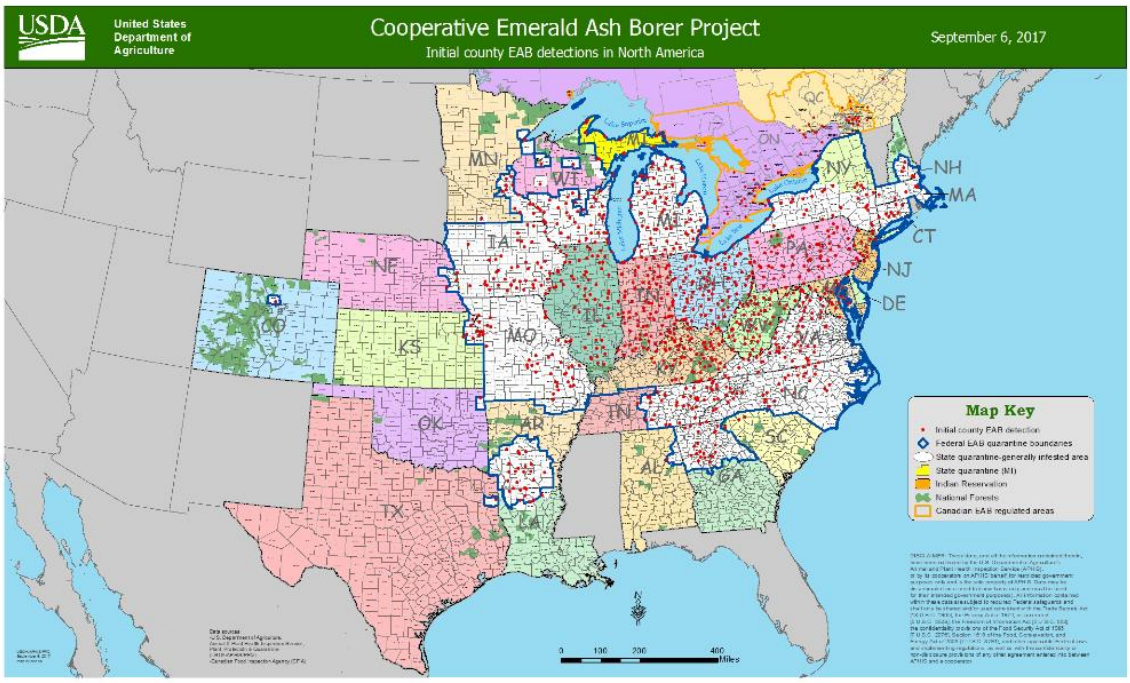

Figure 1.3. Distribution of emerald ash borer in the United States, September 2017.

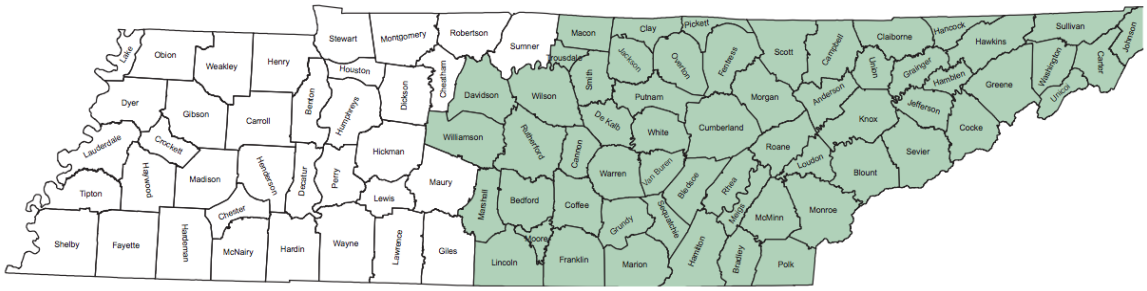

Figure 1.4. Counties quarantined for emerald ash borer in Tennessee, September 2017.

Figure 1.5. Damage caused by feeding by larvae of emerald ash borer (i.e., larval galleries).

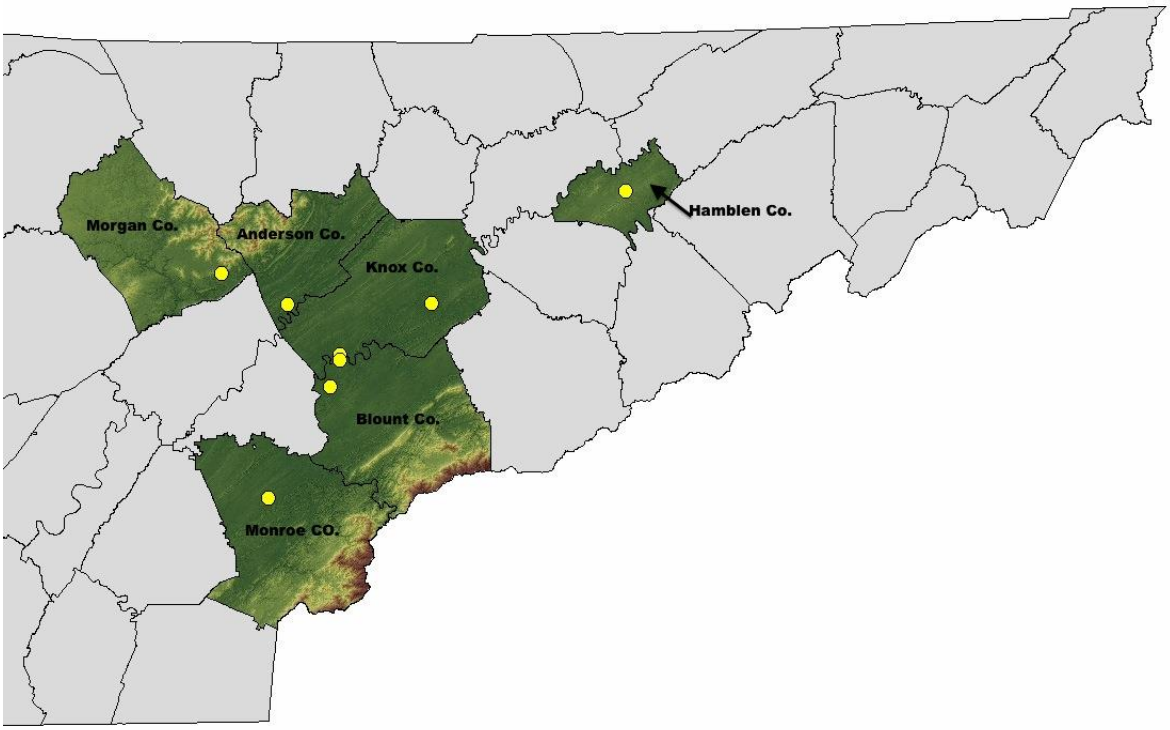

Figure 2.1. Locations of emerald ash borer parasitoid releases in eastern Tennessee, 2012-2015.

Figure 2.2. Installation of a yellow pan trap to monitor native and introduced parasitoids of emerald ash borer

Table 2.1. Sites where introduced parasitoids of emerald ash borer were released from 2012-2014 in eastern Tennessee; sites were monitored three years after releases began via yellow pan traps.

Site	County	GPS Coordinates	Release Year	Year Pan Trapping Began	*O. agrili*	*S. agrili*	*T. planipennisi*
Carl Cowan Park	Knox	Lat. 35.85175 Lon. -84.09357	2012	2015	0	2,854	4,464
			2013		627	857	6,236
			2014		4,314	1,025	3,194
Haven Hill Rd.	Blount	Lat. 35.85175 Lon. -84.09441	2012	2015	0	3,305	4,453
			2013		877	1,082	6,267
			2014		3,931	1,025	3,194
Miser Station Rd.	Blount	Lat. 35.7759 Lon. -84.12459	2012	2015	0	2,875	4,480
			2013		377	857	6,253
			2014		4,137	1,025	3,194
Frank Lorino Park	Hamblen	Lat. 36.22309 Lon. -83.25226	2013	2016	100	1,270	1,800
			2014		5,529	3,648	17,381
Ramsey Historic House	Knox	Lat. 35.96701 Lon. -83.5877	2013	2016	0	390	1,800
			2014		5,679	3,559	19,647
Total*					25,571	23,772	82,363

***Total number represents the total number of each parasitoid species released at these five sites**

Table 2.2. Parasitoids collected from 2015 pan traps installed in sites (Carl Cowan Park, Haven Hill Rd., and Miser Station Rd.) where parasitoids were released in eastern Tennessee (see Table 2.1).

Month	*Tetrastichus planipennisi*	*Atanycolus* spp.	*S. agrili*	*S. elegans*	*S. floridanus*	*Spathius* spp.
April	0	0	0	0	0	0
May	0	0	0	0	0	8
June	0	0	0	0	0	3
July	0	0	0	1	6	1
August	0	4	1	0	27	3
September	0	5	0	1	32	6
October	0	0	0	0	2	0

Shaded columns represent exotic species

Table 2.3. Parasitoids collected from 2016 pan traps installed in sites (Carl Cowan Park, Haven Hill Rd., Miser Station Rd., Frank Lorino Park, and Ramsey Historic House) where parasitoids were released in eastern Tennessee (see Table 2.1).

Month	*Tetrastichus planipennisi*	*Atanycolus* spp.	*S. agrili*	*S. elegans*	*S. floridanus*	*Spathius* spp.
April	1	0	0	0	0	0
May	0	2	0	0	2	0
June	0	5	0	0	5	0
July	0	1	0	1	0	1
August	0	0	1	0	0	1
September	0	0	0	0	0	0
October	0	1	0	0	2	0

Shaded columns represent exotic species

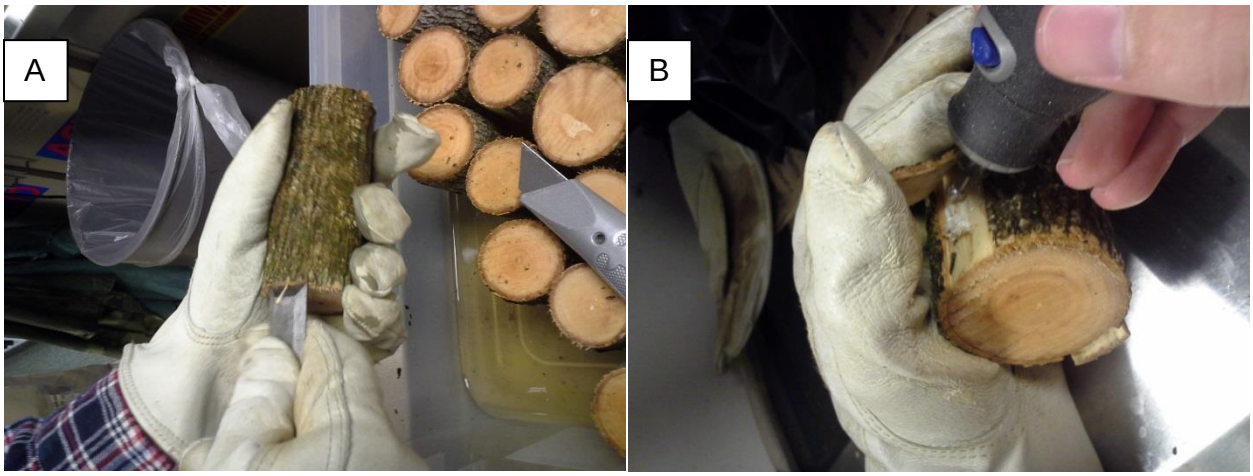

Figure 3.1. A chisel (A) was used to peel bark from a bolt of ash wood and a Dremel® tool (B) to form a gallery in an ash bolt.

Figure 3.2. Standard parasitoid rearing units used in growth chamber study.

Figure 3.3. Ramsey Historical House site, where the field cage study was conducted, 2016-2017.

Figure 3.4. Sleeve cages used to assess parasitism of emerald ash borer larvae in the field cage study; sleeves were suspended by foam tubing to allow the cage to be suspended away from the tree.

Figure 3.5. The primary study site for the emerald ash borer phenology study, Doyle Farm, Knox County, 2016-2017.

Figure 3.6. A second site for the emerald ash borer phenology study, Oak Ridge National Laboratory, Anderson County, 2016-2017.

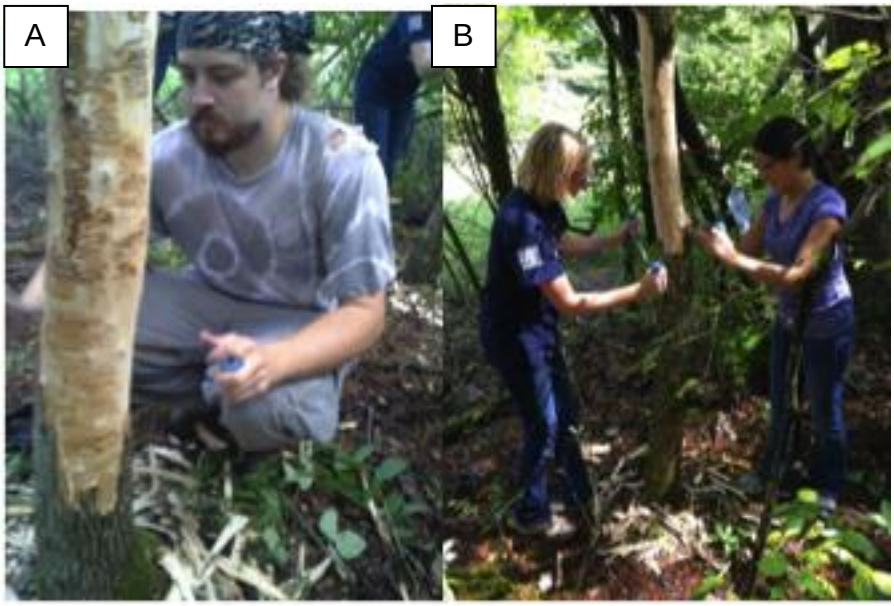

Figure 3.7. Bark removal from trees (A) to assess emerald ash borer phenology (B).

Figure 3.8. Fully developed, dead adult *T. plannipenisi* within an emerald ash borer gallery on an ash bolt.

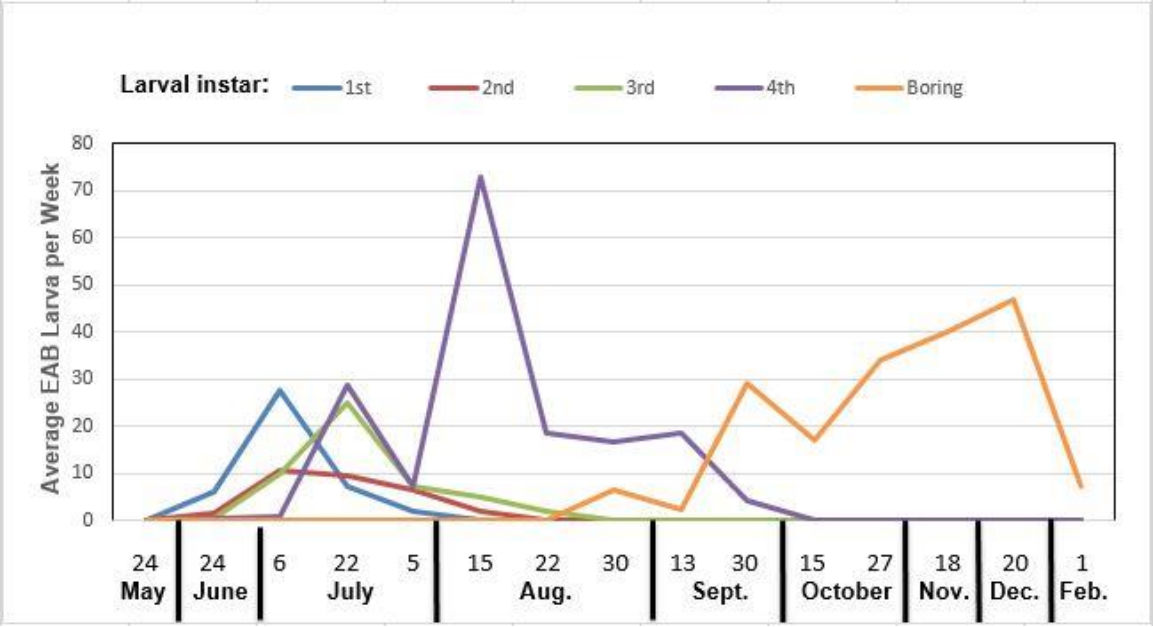

Figure 3.9. Phenology of emerald ash borer at Doyle Farm, 2016.

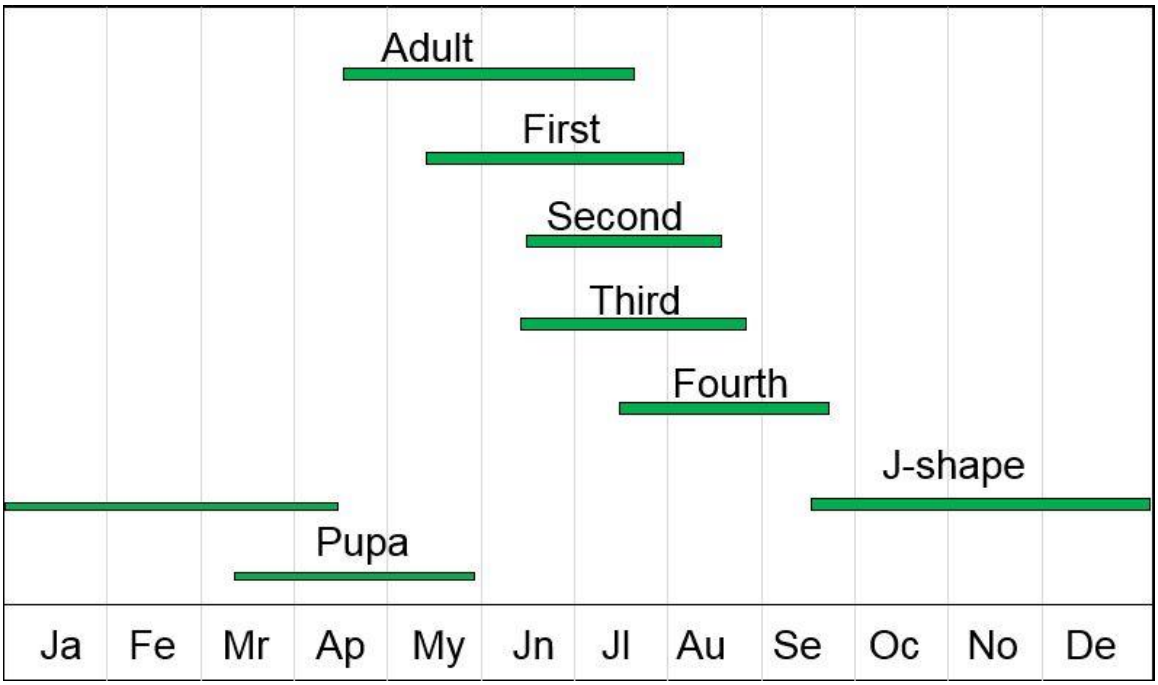

Figure 3.10. Annual life cycle of emerald ash borer in eastern Tennessee, 2016 and 2017.

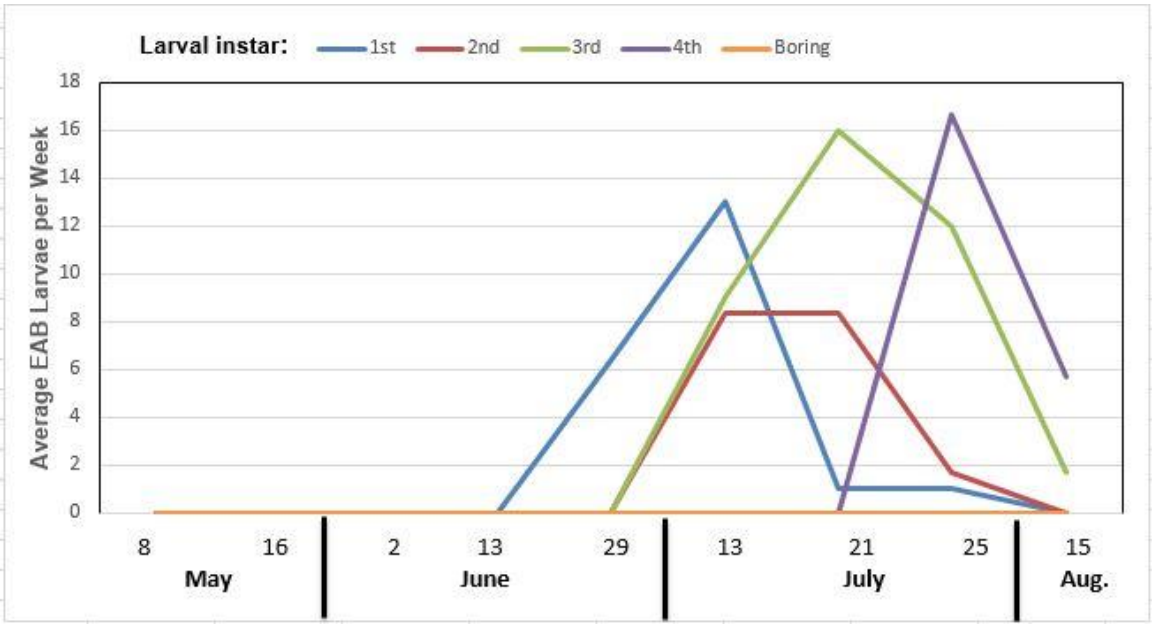

Figure 3.11. Phenology of emerald ash borer at Doyle Farm, 2017.

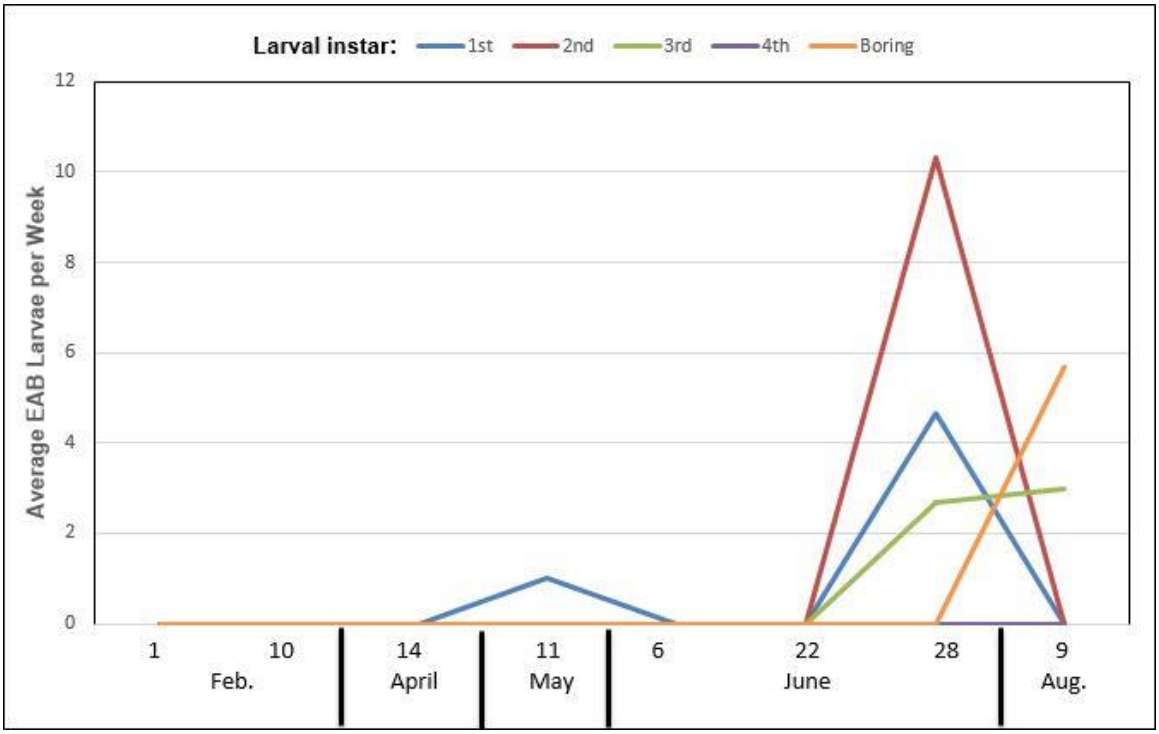

Figure 3.12. Phenology of emerald ash borer at Oak Ridge National Laboratory, 2017.

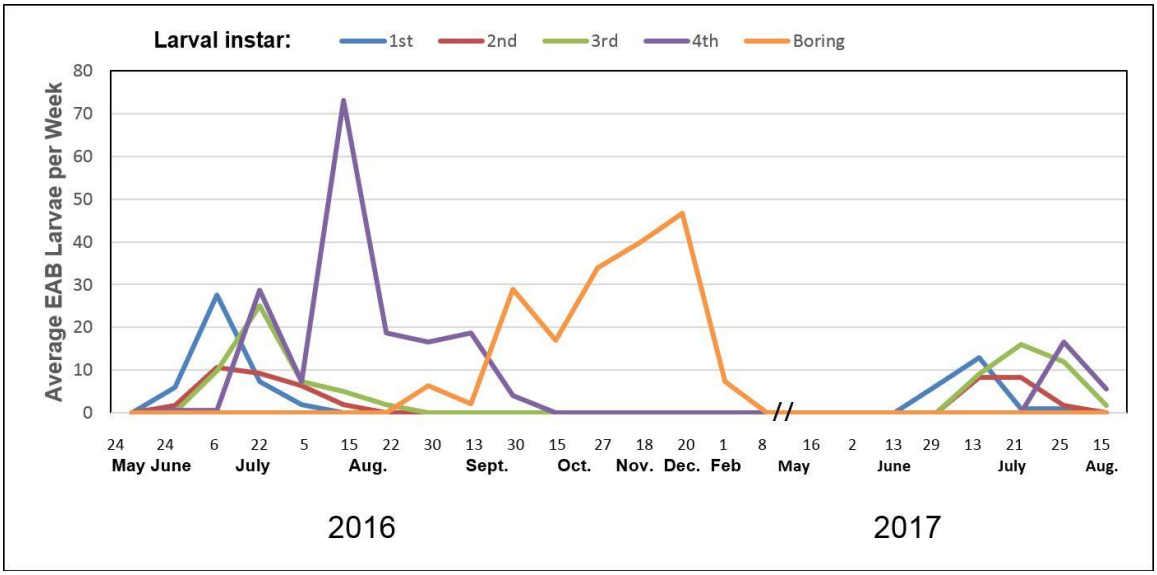

Figure 3.13. Combined phenological data of emerald ash borer at Doyle Farm, 2016 and 2017.

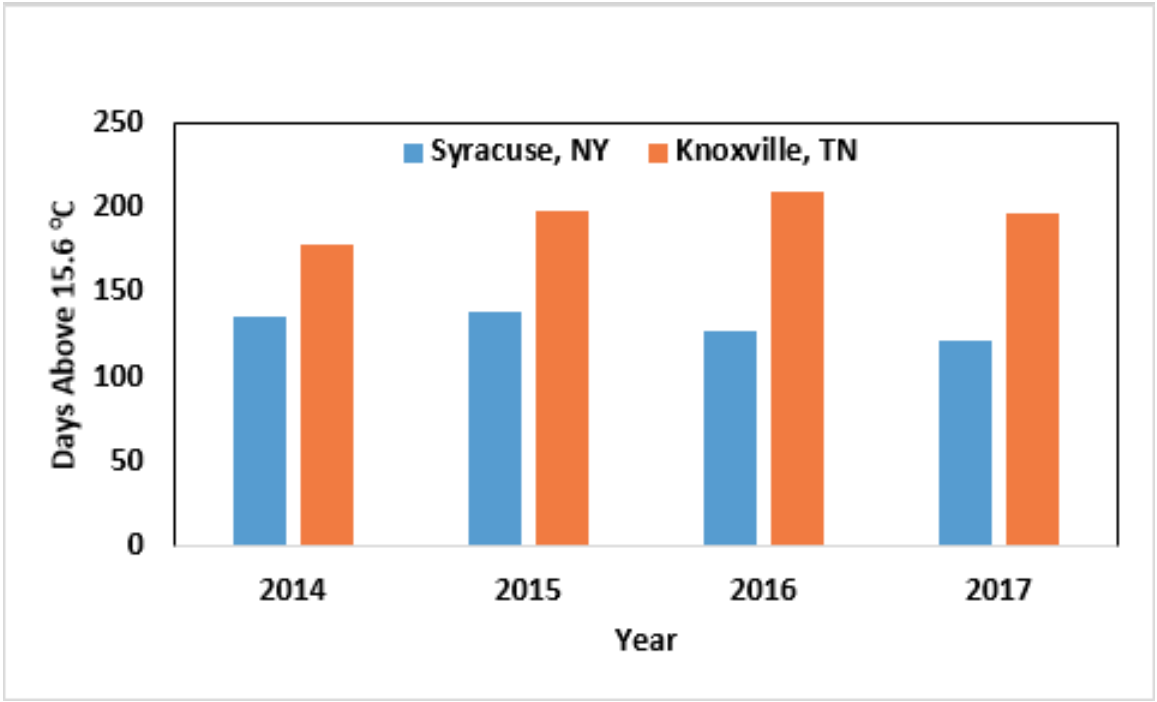

Figure 3.14. Number of days above 15.6° C in New York and Tennessee, 2014 through 2017.

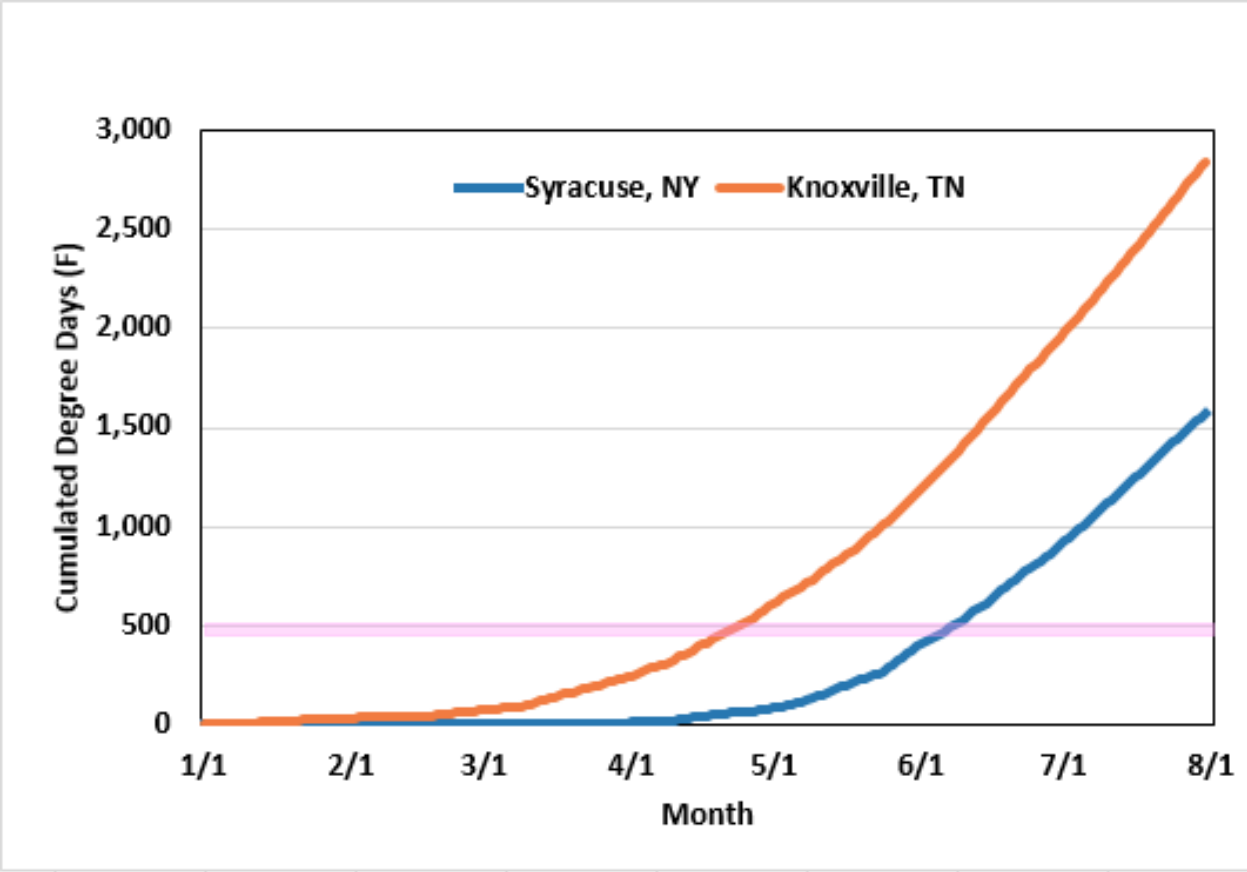

Figure 3.15. Mean cumulative degree days based on a threshold of 50°F (10°C) in New York and Tennessee, 2014-2017.

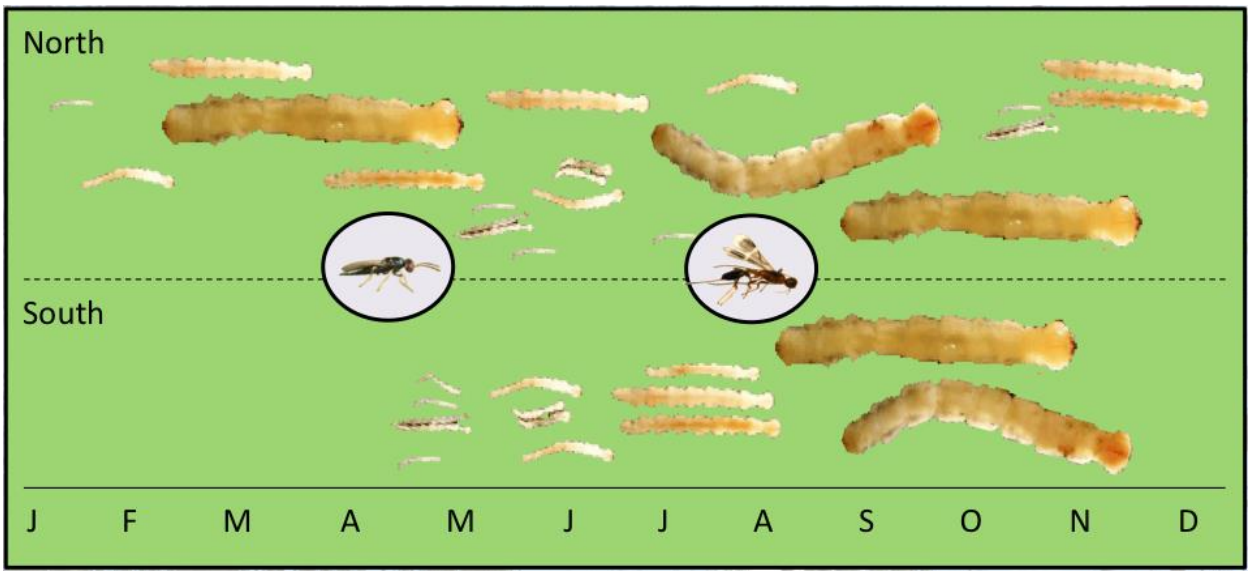

Figure 3.16. Schematic illustrating differences in phenology of emerald ash borer in the northern and southern United States, as well as anticipated emergence times of adult parasitoids.

Table 3.1. Development time and progeny production of F1 and F2 generations of parasitoids reared from emerald ash borer in the first growth chamber study.

Species	Development time (d)	No. Male	No. Female	Total No.	Ratio (M:F)
F1 Generation					
S. agrili	47.75±5.97	2	15	17	1:8.5
S. galinae	45.5±7.5	4	11	15	1:3.75
T. planipennisi	44	35	104	137	1:3.2
F2 Generation					
S. galinae	56	2	2	4	1:1

± Represents the standard deviation across the number of days in the emergence period.

Table 3.2. The number of rearing bolts producing parasitoids and the subsequent percent parasitism of emerald ash borer larvae.

Species	Bolts w/ Emergence	% EAB Larvae Parasitized	
		Emergence Bolts	Total
F1 Generation			
S. agrili	4(38)	62.5	6.41
S. galinae	2(26)	50.0	3.85
T. planipennisi	5(40)	60.0	25.53
F2 Generation			
S. galinae	1(5)	50.0	10.0

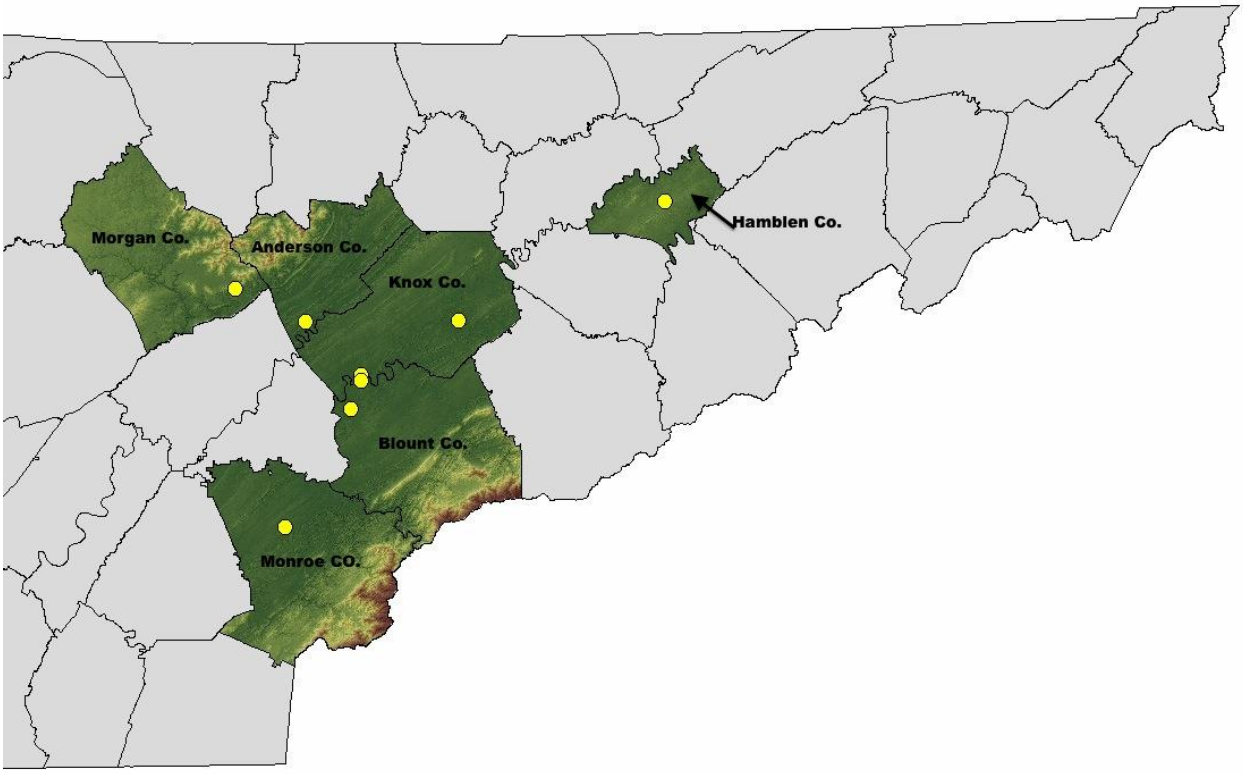

Figure 4.1. Location of study sites to assess native parasitoids of emerald ash borer; introduced parasitoid species had previously been released at each site, and native parasitoid species had been recovered.

Figure 4.2. Emergence barrels on barrel rack.

Figure 4.3. Bug dorm used in studies.

VITA

James Forest Palmer was born on February 12, 1993 at the University of Tennessee Medical Center in Knoxville, Tennessee to his parents Scott and Sheila Palmer. He grew up in Jefferson County, Tennessee, where he attended The King's Academy from Kindergarten throughout High School. From August 2011 to May 2015 he attended Carson-Newman University in Jefferson City, Tennessee, where he obtained a Bachelor of Science degree in Environmental Science.

James began a Master of Science degree in the Department of Entomology and Plant Pathology at the University of Tennessee in August of 2015 and concluded his program in May of 2018.

James is fascinated with anything science related and takes joy in expanding his own horizons as well as the horizons of other.