The seasonality of two parasitoids (*Spathius agrili* and *Tetrastichus planipennisi*) of the emerald ash borer, *Agrilus planipennis*, and a survey for native natural enemies of the Emerald Ash Borer in eastern Tennessee

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Paris L. Lambdin, Major Professor

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The seasonality of two parasitoids (*Spathius agrili* and *Tetrastichus planipennisi*) of the emerald ash borer, *Agrilus planipennis*, and a survey for native natural enemies of the emerald ash borer in eastern Tennessee

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DEDICATION

To
Emily Rebecca Saunders Hooie
my wife
I can only hope I was as wonderful and supportive when you were writing your thesis as you were for me.
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ABSTRACT

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive species of bark borer native to eastern Asia whose primary habitat and food sources are trees in the genus *Fraxinus*. EAB is a major pest of all North American *Fraxinus* species and is responsible for mortality of millions of trees across its current North American range of 23 U.S states and 2 Canadian providences. After the discovery of EAB in Tennessee in 2010, parasitoid releases were started under the national EAB Biological Control Program. A research project was initiated in 2012 to 1) study the seasonality of the gregarious larval ectoparasitoid *Spathius agrili* Yang and the gregarious larval endoparasitoid *Tetrastichus planipennisi* Yang in the climate of eastern Tennessee, 2) determine the overwintering ability of the parasitoids in field releases, and 3) survey for potential native natural enemies of EAB. In 2013 a single generation of *S. agrili* developed from egg to pre-pupa in ca. 22 days before overwintering. Adult individuals of *S. agrili* from the same generation were found to have successfully overwintered in July 2014. No *T. planipennisi* successfully parasitized or overwintered. In 2013, *S. agrili* were successfully recovered from two field sites for the first time in eastern Tennessee after a single year of releases and successfully overwintering, indicating the ability of this species to establish. As in the previous study, no *T. planipennisi* were recovered. Three native parasitoids, *Spathius floridanus* Ashmead, an undetermined species of *Spathius*, and *Atanycolus cappaerti* Marsh & Strazanac, all known to be associated with EAB, were recovered at field sites. These recoveries represent the first documentation of these three native species associated with EAB in the southern U.S. These findings will help demonstrate the utility of *S. agrili* in the southern U.S. as a part of the national EAB Biological Control Program.
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CHAPTER I
INTRODUCTION AND LITERATURE REVIEW

Fraxinus

Distribution

The genus *Fraxinus* L. (Oleaceae) includes more than 45 species of deciduous flowering hardwoods, commonly referred to as ash, native to temperate regions of the Northern Hemisphere (GBIF 2014). In North America, 18 native species of ash exist ranging from southern Alaska to Texas. Of these, white ash (*Fraxinus americana* L.) and green ash (*Fraxinus pennsylvanica* Marsh) are the most widely distributed and are found in most forests in the eastern U.S. and Canada, as well as riparian lands in the Midwestern U.S. and urban environments across the continent (USDA NRCS 2014).

Description

Leaves of white ash are opposite odd-pinnately compound leaves (20 to 38 cm long usually with seven leaflets). The abaxial side of leaflets have white bleached-out hues. The flowers are in panicles near branch tips. Flowering begins when diameter at breast height (dbh) reaches 8 to 10 cm. Abundant flowering begins at 20 to 25 cm dbh. The fruit is a single-seeded, winged samara 10 to 15 mm long. This dioecious species may obtain heights of 18 to 21 m.

Leaves of green ash are odd-pinnately compound leaves that measure from 11 to 30 cm. Typically equipped with seven leaflets, the abaxial side of leaves are similar in color (green) to the adaxial side of leaves. As with white ash, flowering begins when (dbh) reaches 8 to 10 cm, while abundant flowering begins at 20 to 25 cm dbh. The fruit is a single-seeded, winged samara 10 to 15 mm in length. This dioecious species may obtain heights of 40 m.
Ecological Importance

North American ash contributes to a variety of environments and ecologies. Three species are most prevalent in eastern North America. White ash serves as a food source for many small birds and mammals, white-tailed deer, cattle, beaver, porcupine, and rabbits (Griffith 1991). It provides shelter for bats and woodpecker species. White ash, in combination with other hardwoods, is used in surface coal mine reclamation. Black ash (F. nigra Marsh) is a dominant tree in wet calcareous mixed woods especially in the northeastern U.S. (Gucker 2005a). It serves as a food source for moose and white-tailed deer, and is occasionally fed upon by beaver and rabbits. Green ash provides food and cover for beavers, bison, deer, game birds and nongame birds (Gucker 2005b). It provides thermal cover for stream and riparian environments and hosts species of swallowtails, sphinxes, and polyphemus moths.

Economic Importance

Green ash and white ash cultivars are highly valued as landscape and street trees because of their hardiness and ability to grow quickly (Cappaert et al. 2005b). For example, 14.4% of the total leaf cover in Chicago is provided by 600,000 ash trees which, in 2003, were valued at $231 million (Cappaert et al. 2005b, Federal Register 2003). Removal of ash trees across the U.S. would cost an estimated $20 to 60 billion not considering replacement of these trees. Ash is important in the commercial lumber industry as well, commonly used in the creation of a wide range of products from furniture and baseball bats to paper. Ash makes up ca. 7% of all saw timber in the eastern U.S. and represents an estimated $25 billion in stumpage value (Federal Register 2003, Poland and McCullough 2006).
Agrilus planipennis

The emerald ash borer (EAB), Agrilus planipennis Fairmaire (Coleoptera: Buprestidae), is an invasive species of bark borer native to eastern Asia whose primary habitat and food sources are trees in the genus Fraxinus. This exotic species was likely introduced into southeastern Michigan in the early to mid-1990s (Siegert et al. 2014). It was initially discovered near Detroit, MI in June 2002 (Cappaert et al. 2005b). Emerald ash borer is a major pest of Fraxinus species and is responsible for mortality of millions of trees across its current North American range of 23 U.S states and two Canadian providences (Cappaert et al. 2005b, USDA FS, 2014b). It was first detected in Tennessee in 2010 at a truck stop in western Knox County.

Effect on Fraxinus

Two years after its detection in the U.S., it was determined that more than 15 million ash trees had been killed or were dying due to damage by EAB in southeastern Michigan alone. Massive decline in ash populations have resulted in high economic costs (USDA FS 2014b).

Damage to the host tree occurs as EAB larvae excavate galleries in the phloem. These galleries are serpentine-shaped and packed with frass. Extensive feeding of multiple larvae disrupts translocation nutrients throughout the tree, creating a girdling effect which can lead to tree death after one to three years of infestation. Emerald ash borer infestations are difficult to detect until the tree exhibits the significant canopy dieback and woodpecker damage indicative of the final stages of decline (Cappeart et al. 2005b).

Distribution

Emerald ash borer has not been observed infesting non-Fraxinus species in North America (McCullough et al. 2004). However, as ash populations continue to decline, concern has been raised over the potential of EAB to infest other trees. Anulewicz et al. (2006) provided
EAB adults with various different hosts. Four North American ash species were chosen: green ash, white ash, black ash, and blue ash (*Fraxinus quadrangulata* Michaux); six non-ash hosts were chosen: privet (both *Lugustrum* and *Forestiera*), Japanese tree lilac (*Syringa reticulate* (Blume) H. Hara), American elm (*Ulmus americana* L.), black walnut (*Juglans nigra* L.), hickory (*Carya ovata* (Mill.) K. Koch), and hackberry (*Celtis occidentalis* L.). Branches from each species were used in cages for no-choice tests. EAB oviposited on all species tested. Larvae were able to develop to second instars before the branches desiccated on all ash species and privet. Larvae, attempting to feed on black walnut, Japanese tree lilac, American elm, and hackberry, died during their first instar. No feeding attempts were made on hickory (Anulewicz et al. 2006). In field studies using these same non-ash species as well as green and white ash, EAB adults occasionally landed and oviposited on vertically suspended logs of non-ash species, but laid fewer eggs than on ash logs (Anulewicz et al. 2008). Larvae on non-ash logs were unable to survive, although some galleries were found on walnut logs, the larvae had all perished in the first instar and the authors concluded that EAB likely feeds exclusively on *Fraxinus* in North America (Anulewicz et al. 2008).

Genetic analysis that compared seven populations of EAB collected in North America (six in U.S. and one in Canada) and 17 in eastern Asia (12 in China, five in South Korea, and one in Japan) was performed to determine the source population of the invasive EAB in North America (Bray et al. 2011). This analysis revealed that North American individuals carry a common mtDNA haplotype found in South Korea and China. Population assignment tests assigned 88% of North American EAB individuals collected to the Chinese providences of either Hebei or Tianjin City.
Emerald ash borer has also been reported in European Russia (Baranchikov et al. 2008). Specimens collected between 2003 and 2006 from a variety of areas within Moscow were identified as EAB in 2007. Field surveys conducted from 2008-2011 found EAB infesting both green ash and European ash (*Fraxinus excelsior* L.) in the Khabarovsk and Vladivostok regions (Duan et al. 2012b). While European ash seems to require more stress to succumb to EAB, it is still susceptible. EAB has been found in woodland forests south of Moscow. Surveys performed in July 2013 of ash along major roadways leading away from Moscow found that EAB travels an average of 30 to 41km annually. This rapid spread indicates human-assisted transport. The large ash component of these forests, combined with the ability to disperse great distances, means that EAB now has seemingly unhindered access to Eastern Europe (Gninenko et al. 2012). As of 2014, EAB has been found in Russian cities within 100km of Moscow such as Zelenograd, Klin, and Konakovo, but not yet in the city of Tver (145km away) (Orlova-Bienkowskaja 2014).

Riparian ash forests and large to medium diameter ash stands have a higher capacity to support dense populations of EAB (Crocker et al. 2009). Mortality in these forests appears to be higher than other ash habitats. Dense populations of EAB in these forests may influence the rate of the pest’s spread in North America (Crocker et al. 2009). While EAB infests all North American species of ash, it feeds most efficiently on green ash (Chen et al. 2012). Dispersal of EAB seems to decline rapidly after a distance of 100m when dispersed from artificial emergence points, with 90% of larvae recovered within this range (Mercader et al. 2009). However, over the course of one year, EAB have been observed to travel up to 638m from an emergence site (McCullough et al. 2005, Siegert et al. 2010).
Biology and Behavior

Biology and behavior of EAB was first studied in its native range in the Tianjin municipality, China (Wei et al. 2007). EAB-infested *Fraxinus velutina* Torrey were studied because *F. velutina* has been planted abundantly throughout the region due to its tolerance of salt. In this region, the annual average temperature was 12.1°C (range of -20.3 to 40.3°C) and annual rainfall was 500 to 700mm. EAB was determined to be univoltine with oviposition beginning in mid-May. Eggs hatch within 15.7 days on average in early June. Four instars were observed over a ca. 300 day larval generation. EAB overwintered as mature larvae in chambers excavated from sapwood. Larvae pupated in these chambers for ca. 20 days from early April to mid-May. Adult emergence began in early May, peaked in mid-May, and extended until early July with an average longevity of about 21 days (Wei et al. 2007, Wang et al. 2010b). Cannibalism was observed when larval gallery densities were high and tunnels crossed. Under such conditions, cannibalism rates were approximately 5% (Wang et al. 2010b).

Adult activity may range from May to September in Michigan (Brown-Rytlewski and Wilson 2005). In 2003, emergence was detected between the dates of June 5 to 13 after 242 to 307 growing degree days (GDD) (base temperature of 10°C) and continued until August 16 after 1139 GDD. In 2004, two emergences were recorded. The first emergence began on May 25 to June 1 at 229 to 270 GDD, respectively, and a second began on August 17 to 24. Observations continued until September 2 to 7 at 1064 to 1122 GDD, respectively, when no active adults were observed (Brown-Rytlewski and Wilson 2005). Adults fed on ash foliage for 5 to 7 days before mating. After this time, females continued to feed for up to seven days prior to oviposition. EAB could mate multiple times. Foliage feeding continued until death (Chamorro et al. 2012). EAB
prefer to oviposit on stressed trees with 40 to 50% canopy reduction, but will infest any North American ash species available (Jennings et al. 2014, Poland et al. 2005, Anulewicz et al. 2008).

In 2004, larvae that had developed from eggs deposited in lightly or moderately infested trees during the previous summer were in the second and third larval instars in April and did not complete development quickly enough to begin pupation. These two-year larvae were found in galleries beginning in wood from the previous year that was dead at the time of dissection and continuing into wood from the current year of growth (Cappaert et al. 2005a).

EAB larvae typically feed in a downward direction. Chen et al (2011) found that gravity and plant defenses were likely not the cause as the larvae feed upwards when the trees are grown upside-down. Moisture and amino acid levels are higher below the feeding point than above, suggesting that nutrients and moisture content are selective forces for the downward feeding behavior.

**Biological Control of Emerald Ash Borer**

While several insecticide methods have been recommended for EAB control, trees that exhibit more than 50% canopy dieback will not likely survive, regardless of treatment. Trees that exceed 63.5cm dbh tend to be unpredictable in treatment success, and multiple treatments must be made over the lifetime of the tree. (Herms et al. 2009, Mckenzie et al. 2010, McCullough et al. 2011). Because of the long-term costs associated with chemical control, biological control was considered a favored option for EAB control as successful establishment of a biocontrol agent can reduce control costs and substantially increase the overall effectiveness of possible combined control measures (Gould et al. 2013). Therefore, the USDA set aside funds for research on parasitoids of EAB (USDA 2012).
Surveys of EAB-infested wood in North America have yielded a variety of native parasitoid species that will attack EAB. A 2009 survey conducted in western Pennsylvania yielded several native Hymenopteran parasitoid species that attacked EAB: *Eupelmis pini* Taylor (Eupelmidae), *Dolichomitus vitticrus* Townes (Ichneumonidae), and two unidentified ichneumonids of the genus *Orthizema*. An accidentally introduced Asian eupelmid, *Balcha indica* Mani and Kaul, was also found to parasitize EAB. All five wasps cumulatively caused ca. 3.6% parasitism and may compliment classical biological control programs currently being implemented (Duan et al. 2009, 2011d). Field surveys in Pennsylvania revealed five additional species of parasitoids recovered from EAB: *Spathius laflammei* Provancher, *Atanycolus nigropyga* Shenefelt, two *Atanycolus* species, and an ichneumonid *Dolichomitus* sp. (Duan et al. 2013b). One prominent parasitoid species collected in this survey was *Atanycolus cappaerti* Marsh and Strazanac (Hymenoptera: Braconidae).

*Atanycolus cappaerti* is a native of North America and was first described from specimens collected in Michigan. It has been observed to attack EAB with parasitism rates as high as 71%. A solitary ectoparasitoid, it naturally feeds on larvae of other Buprestid borers such as *Agrilus liragus* Barter and Brown and *A. bilineatus* Weber. Due to populations of *Atanycolus* spp. increasing sharply in a study site recently invaded by EAB (Duan et al. 2011a), *A. cappaerti* is currently under investigation for its potential as an augmentative biocontrol agent of EAB (Cappaert and McCullough 2009).

In collaboration with scientists in China, several surveys have been performed to find natural parasitoids of the EAB from its native range. These studies have produced three potential parasitoids (*Spathius agrili* Yang [Hymenoptera: Braconidae], *Tetrastichus planipennisi* Yang [Hymenoptera: Eulophidae], and *Oobius agrili* Zhang and Huang [Hymenoptera: Encyrtidae]).
which are currently in use to suppress EAB populations (Ulyshen et al. 2010a, Duan et al. 2011b). Following an environmental assessment posted to the Federal Register (Federal Register 2007) and reviewed by researchers, land managers, and the general public, APHIS determined that release benefits would outweigh potential risks. A “Finding of No Significant Impact” was issued and releases began in 2007 after issuance of release permits (Bauer et al. 2008).

*Spathius agrili*, a gregarious larval parasitoid, was described in 2005 and was the first of the three parasitoids to be discovered. It was reared from EAB in China attacking ash trees native to North America (Yang et al. 2005). Its relationship with EAB was studied using regular forest surveys and laboratory observations. Long-term co-evolution has enabled *S. agrili* to emerge at a time when EAB with a pronotum and body wider than 1.5mm and a length of more than 12mm were available. These sizes are typical of third or fourth instar larvae and were most likely to be effective hosts (Wang et al. 2007). The ratio of females to males and total progeny increased when larger larvae were available (Wang et al. 2008). When provided 17 different wood-boring insect species, *S. agrili* only parasitized *A. planipennis*, indicating high host specificity (Yang et al. 2008). Subsequent host testing using only *Agrilus* species demonstrated that *S. agrili* will parasitize other hosts (*Agrilus bilineatus, A. anxius* Gory, *A. zanthoxyltumi* Li Meng Lou, *A. mali* Matsumura, and *A. inamoenus* Kerremans). However, parasitism rates are low, or development is inhibited in these species when compared to *A. planipennis* (USDA 2007). In its native range, *S. agrili* exhibits three to four generations per year and overwinters as a prepupa. Sexual reproduction is normal, but females can reproduce parthenogenetically. This species develops through five larval instars, while feeding on the hemolymph of the larval host. At 22 to 26°C, the generation time from egg to wasp was 27 to 28 days (Yang et al. 2010).
Host-seeking behavior of *S. agrili* begins with detection of volatiles released by stressed ash. The wasp detects vibrations given off by EAB larvae feeding by drumming on the bark with its antennae. It then probes beneath the bark with its ovipositor until contact with the larvae has been made. Paralytic venom is injected, possibly to inhibit movement or to avoid superparasitism, and oviposition begins (Wang et al. 2010a, Johnson et al. 2014).

Currently, the only approved method for monitoring release sites of parasitoids is the labor-intensive, destructive, and costly method of tree removal and dissection or the use of yellow pan traps for general collection of parasitoids (Gould et al. 2013). A male emitted pheromone has been identified that is attractive to male and female *S. agrili* (Cosse et al. 2012). Paper discs consisting of two halves of different colors were used to determine landing color preference for the parasitoid. Female *S. agrili* preferred to land on green, yellow, and white surfaces and avoided red, black, and purple. Color preference and pheromone data may lead to an effective pheromone trap for comparatively inexpensive trapping of *S. agrili* (Cooperband et al. 2013).

Currently, the efficacy of *S. agrili* in northern regions is in question. Lack of recoveries from releases in northern regions has led the USDA to alter its release guidelines. *Spathius agrili* will no longer be released above the 40th parallel as part of the EAB Biological Control Program (Gould et al. 2013).

*Tetrastichus planipennisi* is a gregarious endoparasitoid of primarily third and fourth instar EAB larvae described in 2006 from northeastern China (Yang et al. 2006). When exposed to actively feeding larvae of EAB and eight other buprestid species (*Agrilus anxius*, *A. bilineatus* Weber, A. *ruficollis* F., A. *subcinctus* Gory, A. sp., *Chrysobothris femorata* Oliver, C. *floricola* Gory, and *C. sexsignata* Say), five cerambycid species (*Neoclytus acuminatus* F., *Megacyllene robiniae* Forster, *Astyopsis sexguttata* Say, *Monochamus scutellatus* Say, an unknown species found
in maple), and one sawfly species (*Janus abbreviates* Say), *T. planipennisi* only parasitized EAB in ash branches. This discovery indicated high host specificity (Liu and Bauer 2007).

In laboratory studies, a single generation of *T. planipennisi* was found to take approximately four weeks. Adult males lived for an average of five weeks while females had an average of six weeks with a maximum of nine weeks. Females produced 23 to 26 progeny per week with a total lifetime realized fecundity of 57 per reproductively active female. It is suggested that *T. planipennisi* may have several generations in mid-Atlantic and Midwestern states where growing seasons average four to five months (Duan et al. 2011b).

In a laboratory study that compared the use of green ash to tropical ash, *Fraxinus uhdei* (Wenzig) Lingelsh, for *T. planipennisi* rearing, tropical ash was a more effective substrate as brood size of the parasitoid was increased significantly (Duan and Oppel 2012). *Tetrastichus planipennisi* may parasitize EAB from the first instar stage to prepupae. Host size was positively correlated to the number of progeny produced per host. However, this correlation was limited to third and fourth instar larvae as prepupae are typically in sapwood and possibly too deep for the parasitoid’s ovipositor to reach. Younger larvae may be parasitized, but at significantly reduced parasitism rates (Ulyshen et al. 2010b).

A comparative study of ovipositor length and oviposition capabilities between *T. planipennisi* and *Atanycolus* spp. determined that while *Atanycolus* spp. could oviposit successfully in bark up to 8.8mm thick (57.4cm dbh), successful oviposition by *T. planipennisi* required bark no thicker than 3.2mm (11.2cm dbh). Therefore, releases of *T. planipennisi* are recommended on trees with a dbh of 12cm or less. This guideline would largely limit *T. planipennisi* to early successional or regenerating ash stands. Parasitoids from EAB’s native
range with longer ovipositors, such as *Spathius* spp., may have more success in controlling EAB in older stands (Abell 2012).

Recoveries of *T. planipennisi* in some areas of release in the northern U.S. have been promising. *Tetrastichus planipennisi* were released into six forest sites in southern Michigan from 2007-2010. Each location consisted of a release site and a nearby control site. By 2012, parasitism levels increased from 1.2% to 21.2% for release sites and from 0.2% to 12.8% in control sites which led Duan et al. (2013a) to conclude that *T. planipennisi* is established and spreading in southern Michigan. A sudden decrease in EAB populations followed by an increase in parasitism of *T. planipennisi* and native *Atanycolus* spp. population in central Michigan indicate that these species are having a regulatory effect on EAB populations (Duan et al. 2014b).

*Oobius agrili* is an egg parasitoid of EAB discovered in 2004 in Jilin province, China (Zhang et al. 2005). Studies performed there in 2005 (Liu et al. 2007) demonstrated parasitism rates of 56.3% for July and 61.5% for August. The portion of the parasitized population that diapaused in August successfully overwintered, and later emerged in June with a parasitism rate of 28.6% (Liu et al. 2007).

*Oobius agrili* oviposit in EAB eggs ranging from newly laid to nine days old. Over her lifetime, a female will produce an average of 24 eggs with a daily maximum of five and a lifetime maximum of 62 eggs. In no-choice assays, *O. agrili* did not parasitize *Agrilus* eggs less than half the size of EAB eggs or eggs of cerambycids or lepidopterans. *Oobius agrili* did parasitize eggs of *Agrilus anixus*, *A. bilineatus* and *A. ruficollis*, all native species. When given a choice, however, *O. agrili* demonstrated a strong preference towards EAB eggs on ash as
opposed to the other *Agrilus* species on their host plants. This host specificity suggested that *O. agrili* would be a good candidate for biological control (Bauer and Liu 2007).

*Oobius agrili* was introduced into forested plots in Michigan. To monitor these releases, “sentinel EAB eggs,” consisting of a green ash bolt, 5cm diameter x 25cm long, wrapped snugly eight to ten times in a spiral with curling ribbon, that had been presented to gravid EAB which lay eggs underneath the ribbon were deployed. The eggs were counted and the bolts were placed in the field for seven days. These were then brought into a laboratory and examined for evidence of *O. agrili* parasitism. This method was effective at monitoring for *O. agrili* (Duan et al. 2012a, Duan et al. 2011c), and was used to demonstrate that *O. agrili* had successfully established in release stands in Michigan by 2010, but had not yet dispersed to neighboring control stands (Duan et al. 2012a).

Other species of EAB parasitoid from its native range have been considered for use as biological control agents. In addition to *T. planipennisi*, two species of parasitoid (*Atanycolus nigriventris* Vojnovskaja-Krieger and a previously undescribed *Spathius* species) were observed parasitizing third and fourth instar EAB larvae on both native ash species and green ash at cumulative parasitism rates of 0 to 8.3% and 7.3 to 62.7%, respectively. The *Spathius* species accounted for most of this parasitism (Duan et al. 2012b). This species was formally described as *Spathius galinae* Belokobylskij in 2012, from specimens collected in both South Korea and the southernmost coastal region of eastern Russia. *Spathius galinae* can be successfully reared on EAB in green ash in laboratory settings (Duan et al. 2014a) and will likely be more successful if released in colder regions of North America, such as Michigan, than does *S. agrili* (Belokobylskij et al. 2012).
RESEARCH OBJECTIVES

The focus of this research was to assess the seasonality of the two introduced larval parasitoids in eastern Tennessee and to survey for other natural enemies that may be attacking EAB. The objectives of this study were to: 1) determine the seasonality of the larval parasitoids *Spathius agrili* and *Tetrastichus planipennisi*, 2) determine the overwintering behavior of *S. agrili* and *T. planipennisi*, and 3) survey infested ash for the presence of native natural enemies that attack EAB in eastern Tennessee.
CHAPTER II
MATERIALS AND METHODS

Seasonality Studies

Experimental Site

The seasonality of the larval parasitoids *T. planipennisi* and *S. agrili* in eastern Tennessee, was assessed at an experimental site at the East Tennessee Research and Education Center (ETREC) Plant Sciences Unit Farm on Alcoa Highway (coordinates 35°54’11.19”, 83°57’26.87”) (Fig. 1). This site, established in April 2013, consisted of shade houses (6.1m x 6.1m) covered by tarps to reduce temperature variables introduced by exposure to direct sunlight (Fig. 2A).

Specimen Stock

Specimens of *S. agrili* and *T. planipennisi* used in this study were obtained from the USDA APHIS-PPQ EAB Biocontrol Laboratory in Brighton, MI. The methodology used at the laboratory to prepare specimens for shipment consisted of infesting bolts of ash, ca. 15cm long and 5cm in diameter, with EAB by placing EAB eggs, two per cm diameter, evenly spaced across the surface of the bolt. The bolts were positioned upright, in 1 to 2mm of water at 30 to 33°C; the eggs hatched and the larvae bored into the wood. When larvae, located underneath the bark, had developed into third instars, they were exposed to gravid females of one of the two parasitoid species that oviposit on or in the EAB larvae. The anticipated result was that the mean number of *S. agrili* and *T. planipennisi* per bolt would be ca. 30 or 140 individuals, respectively. The bolts, equipped with a means of attachment (a hole or a string for a nail), were removed and shipped to Room 151, Plant Biotech Building 2505 E.J. Chapman Dr. Knoxville, TN.
Figure 1. Study sites and emerald ash borer quarantine area in East Tennessee, 2012-2014.

Figure 2. A) A shade house covered by a tarp at the ETREC Plant Sciences Unit Farm used for barrel studies and B) barrels equipped with funnel traps used in life history study.
Green ash naturally infested with EAB larvae developing within galleries was collected from three EAB-infested locations in eastern Tennessee for use in seasonality studies: Vulcan Materials, site “V” (coordinates 35° 53’10.629", 84°14’ 24.6984”); The Mulch Company, site “M” (35°53’ 13.1676", 84°13' 27.4434”); and a private farm site located on Highway 11W (Rutledge Pike), site “R” (36°15' 5.1978", 83°35'16.6056”). These locations were determined to be suitable based on the presence of ash trees with signs of EAB infestation (record of EAB in the area, D-holes, bark splitting, woodpecker damage, canopy dieback, and epicormic shoots). Collected EAB-infested wood was stored in a laboratory building at the ETREC Plant Sciences Unit Farm for no more than two days before being used.

**Overwintering Ability**

The experimental design to assess overwintering ability consisted of two independent, completely randomized design tests for *S. agrili* and *T. planipennisi* (n=5 for each species). During the first two weeks of September, one to two EAB-infested trees at each of the three collection sites were felled and sectioned to obtain 30 EAB bolts (76cm long, 5-12cm diam.). Fifteen bolts were used for the *S. agrili* portion of the study and 15 for *T. planipennisi*. Ten plastic storage barrels (82cm height, 46cm diam.) equipped with ventilation holes (12.7cm diam.) covered with fine mesh (50 x 24 mesh) were placed in an upright position on the ground in the shade house based on the parasitoid’s behavioral preference of more vertical surfaces for oviposition. These barrels were arranged in two rows of five, and each set of five was designated to test either *S. agrili* or *T. planipennisi*. Emerald ash borer bolts from the three collection sites were distributed amongst the ten barrels with eight barrels receiving EAB bolts from all three sites and two barrels receiving two R EAB bolts and one V EAB bolt, due to a smaller number of useable trees at site M.
One parasitoid bolt of either *S. agrili* or *T. planipennisi* was placed upright into each barrel. A lid equipped with a funnel trap was placed on each barrel (Fig 2B). The funnel traps were monitored daily for parasitoids emerging from the bolts and the date, their numbers, and bolt number recorded. The barrels were monitored twice weekly until December 2013. While emergence was not expected until April 2014, daily monitoring of the barrels began in March. In July 2014 EAB bolts were dissected, and EAB bolt diameter, number of EAB larvae, EAB galleries, parasitoid individuals, and parasitoid life stage were documented. Based on these data, parasitism rates were calculated. A HOBO Pro v2 data logger device was placed in a randomly selected barrel of each parasitoid and another placed outside the barrel to assess the average daily temperature and humidity.

Data analysis of external and internal temperature and humidity data consisted of a paired t-test (*p* = 0.05) using SAS (SAS Institute 2006). Two-tailed Pearson correlations (*p* = 0.05) were performed in SPSS 22.0 (SPSS 2014) between the EAB bolt diameter, number EAB galleries, number of EAB larva, numbers of parasitoid eggs, number of parasitoid larvae, number of parasitoid prepupae, and total number of parasitoid individuals recovered to evaluate the association of these factors with the life history of *S. agrili* and *T. planipennisi*.

**Life History**

The experimental design consisted of two independent completely random design tests for *S. agrili* and *T. planipennisi* (*n*=3 for each species). During the first two weeks of September 2013, infested trees at each ash collection site were felled and sectioned to obtain 60 (76cm long, 5-12cm diameter) EAB bolts. From these, 30 EAB bolts were used for each of the *S. agrili* and *T. planipennisi* tests. Six cardboard barrels (120cm in length, 80cm diameter) were placed vertically in the shade house at the experimental site (Fig. 2B), and ten EAB bolts were placed
into each of the barrels. A parasitoid bolt was placed upright into each barrel, and a lid equipped with a funnel trap was then placed on each barrel. The funnel traps were monitored daily for parasitoids emerging from the bolts. When emergence was detected, trapped parasitoids were placed back into their respective barrel so that they would oviposit on EAB larvae. The funnel traps were then plugged to prevent further trapping. Three days after the first emergence (from parasitoid bolts), regular removal and dissection of EAB bolts began. The first five sets of EAB bolts were removed from each barrel in intervals of three days, and the remaining five sets of EAB bolts were removed in intervals of seven days. The observational period covered a total of 50 days. Twenty days after first emergence, daily monitoring for new adult emergence began. Each EAB bolt was removed, measured for diameter, and dissected to find EAB larvae and signs of parasitoids. Each EAB larva or group of parasitoids from a single host was removed and placed into individual vials in 95% ethyl alcohol. Each vial was labeled by date, tree, and barrel of origin. The developmental stage of the EAB larvae and parasitoid was identified and recorded. Percent parasitism and parasitoid to host ratios were also calculated from these data. A HOBO Pro v2 data logger device was placed in a randomly selected barrel of each parasitoid and another placed outside the barrel to assess the average daily temperature and humidity. Emergence and temperature/humidity data were used to determine growing degree days (GDD) for the development of the overwintering generation.

GDD will be calculated using the formula (Gould et al. 2013):

\[
GDD = \frac{(T_{max} + T_{min})}{2} - T_{base}.
\]

T = temperature in Celsius, Tmax = maximum daily temperature, Tmin = minimum daily temperature, Tbase (baseline temperature for determining GDD) = 10°C
Data analysis of external and internal temperature and humidity data consisted of a paired t-test (p = 0.05) using SAS (SAS Institute 2006). Analysis of EAB host, parasitoid development, and GDD data were performed using linear regression and Pearson correlation in SPSS 22.0 (SPSS 2014). Stepwise linear regression was used to select significant variables (p=0.05) associated with the life history of the parasitoids. The dependent variable was presence of *S. agrili* prepupae or *T. planipennisi* larvae or prepupae as these are the life stages in which the parasitoids overwinter. The independent variables were EAB bolt diameter, cumulative average temperature, maximum temperature, minimum temperature, GDD, cumulative relative humidity, maximum relative humidity, minimum relative humidity, number EAB galleries, and number of EAB larvae. The cumulative average temperature was calculated as the average temperature from the beginning of the study to the time of EAB bolt removal. The cumulative relative humidity was calculated in the same manner. The maximum and minimum temperatures and humidities were taken from the beginning of the study to the time of EAB bolt removal. Accumulated GDD were calculated for each EAB bolt removal. Significance was set at p = 0.05 and the adjusted R\(^2\) is reported. To further evaluate the association of these factors with the life history of *S. agrili* and *T. planipennisi*, two-tailed Pearson correlations (p = 0.05) were performed between the independent variables listed above and numbers of parasitoid eggs, number of parasitoid larvae, number of parasitoid prepupae, and total number of parasitoid individuals recovered.

**Overwintering Field Studies**

To determine the overwintering ability of *T. planipennisi* and *S. agrili* in natural conditions, several sites where EAB specimens were collected in traps in 2011 were examined for use in four separate tests for field releases as part of the National EAB Biological Control
Table 1. Sites used for parasitoid overwintering studies in eastern Tennessee.

<table>
<thead>
<tr>
<th>Site</th>
<th>County</th>
<th>Growth Stage</th>
<th>Ash component</th>
<th>Predominant ash species</th>
<th>Field release</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cactus Cove</td>
<td>Blount</td>
<td>Overstory</td>
<td>35%</td>
<td>white</td>
<td>yes</td>
</tr>
<tr>
<td>Haven Hill</td>
<td>Blount</td>
<td>Overstory</td>
<td>80%</td>
<td>white</td>
<td>no</td>
</tr>
<tr>
<td>Miser Station</td>
<td>Blount</td>
<td>Overstory</td>
<td>40%</td>
<td>white</td>
<td>yes</td>
</tr>
<tr>
<td>Cowan Park</td>
<td>Knox</td>
<td>Overstory</td>
<td>25%</td>
<td>green</td>
<td>yes</td>
</tr>
<tr>
<td>Rowe Farm</td>
<td>Claiborne</td>
<td>Overstory</td>
<td>60%</td>
<td>green</td>
<td>yes</td>
</tr>
<tr>
<td>Rutledge Pike</td>
<td>Grainger</td>
<td>Stump sprout clusters</td>
<td>80%</td>
<td>green</td>
<td>no</td>
</tr>
</tbody>
</table>
Program (Table 1). Tree and site characteristics were assessed in Spring 2012 and recorded for all sites in accordance with USDA requirements (Gould et al. 2013). Initial locations for site reconnaissance were based on EAB trap catches reported by Tennessee Department of Agriculture (Steve Powell, personal communication). All sites but Rutledge Pike were used for field releases of both *T. planipennisi* and *S. agrili* in 2012 and 2013.

**Cactus Cove Road Cages**

In October 2012, three white ash trees infested with EAB in a stand located on Cactus Cove Road (coordinates 35°50'24.98" N, 84°05'37.41" W) in Blount County, TN, were selected for an overwintering study based on the presence of EAB. Trees were topped at a height of ca. 5.75m using a Genie Z 45/22 articulating boom bucket truck. A large tree cage (ca. 7m tall, 5m diam. at base after Hakeem et al. 2011) was placed over the standing portion of each tree. The remainder of the trees were sectioned into 1m bolts and organized into smaller cages (1.5m tall, 1m x 1m base) by stem diameter and location within the tree (Fig. 3A). Both *S. agrili* and *T. planipennisi* were released into each of the cages (100 females and mixed males of each species in all cages).

In May 2013, the standing portion of the tree was felled and sectioned into 1m bolts and all wood material from cages was placed into cardboard barrels (120cm in length, 80cm diameter). These were housed horizontally in wooden frames inside a shade house at the ETREC Plant Sciences Unit Farm. Each barrel was equipped with a funnel trap mounted on its lid. Barrel traps were monitored biweekly starting in May 2013. Daily monitoring began when EAB adults were observed in the traps and continued until October. All *T. planipennisi* and *S. agrili* candidates were documented by date, barrel, and tree of origin. Each specimen collected was placed into vials containing 95% ethyl alcohol for specialist identification at the USDA APHIS-
Figure 3. A) Tree cages containing emerald ash borer-infested wood material and parasitoids at Cactus Cove and B) barrels containing wood material from Miser Station, Haven Hill and Cowan Park.
PPQ EAB Biocontrol Laboratory in Brighton, MI. These data will help determine the parasitoids’ overwintering success.

**Haven Hill, Miser Station, and Cowan Park Barrels**

In Spring 2013, three trees each from Haven Hill Road (coordinates 35°50'21.72" N, 84°05'38.29" W), Miser Station Road (coordinates 35°46'33.89" N, 84°07'28.84" W) in Blount County, TN, and Carl Cowan Park (coordinates 35°51'06.44" N, 84°05'36.67" W) in Knox County, TN were selected from available non-release trees (trees in release sites that did not have parasitoids directly released upon them). These were felled and sectioned into bolts (76cm long, 5-12cm diam.) and placed into plastic barrels (82cm height, 46cm diam.). These were housed horizontally in metal frames inside a shade house at the ETREC-Plant Sciences Unit Farm. Each barrel was equipped with a funnel trap mounted on its lid (Fig. 3B).

Barrel traps were monitored biweekly for parasitoid emergence beginning in early May 2013. Daily monitoring began when EAB adults were observed in the traps, and continued until October 2013. All *T. planipennisi* and *S. agrili* candidates were documented by date, barrel, tree, and site of origin. Each specimen collected was placed into vials containing 95% ethyl alcohol for specialist identification at the USDA APHIS-PPQ EAB Biocontrol Laboratory in Brighton, MI. These data will help determine the parasitoids’ overwintering success.

**Rowe Farm Pan Traps**

Rowe Farm (coordinates 36°28'05.87" N, 83°26'09.89" W) in Claiborne County, TN had previously been used as a field release site in 2012. However, this site was not used for releases in 2013, as significant ash decline rendered the site unsuitable for continued releases. To assess the overwintering of released parasitoids that may have survived on remaining live ash at this location, ten yellow pan traps (Gould et al. 2013) were placed throughout this site.
Figure 4. A) A pan trap used for parasitoid monitoring at Rowe Farm and B) tree cages over ash stump sprout clusters at Rutledge Pike.
in early summer of 2013 and monitored for both native and released parasitoids (Fig. 4A). Each pan trap consisted of a metal L-shaped shelf brace with two nested plastic yellow serving bowls (105ml). The lower bowl was fixed to the brace with plastic ties. The upper bowl was set inside the lower and charged with diluted (50% DI water) low-tox antifreeze. The brace was then fixed ca. 2m up the tree with wood screws.

The contents of the bowls were collected weekly from May to November 2013. Parasitoids suspected to be *S. agrili* or *T. planipennisi* were separated from collected material, and documented by pan location and date. Parasitoids were placed into vials containing 95% ethyl alcohol for specialist identification at the USDA APHIS-PPQ EAB Biocontrol Laboratory in Brighton, MI. These data will help determine the parasitoids’ overwintering success.

**Rutledge Pike Cages**

On June 13, 2013, three clusters of green ash stump sprouts at a location on Rutledge Pike (36°15'05.19" N, 83°35'16.61" W) in Grainger County, Tennessee were selected for study. Each cluster had at least four stems measuring 5 to12cm dbh. This is the ideal size used for parasitoid production (Gould et al. 2013). Each cluster of trees was trimmed to a height of ca. 6m from ground level. Between June 13 and 19, 2013, large cages (ca. 7m tall, 5m diam. at base) were placed over each of the three tree clusters (Fig. 4B) and labeled as #1 to 3. On 26 June 2013 EAB adults were received from the USDA APHIS-PPQ EAB Biocontrol Laboratory. These adults were sorted into three sets (40 female, 33 male per set) and were released into each cage to create an artificial infestation. On August 21, 2013, adult *S. agrili* and *T. planipennisi* were released into the cages (100 females and mixed males of each species in all three cages). These were allowed to overwinter until April 17, 2014 when the cages were removed and stump sprouts larger than 5cm diam. were felled and sectioned into 76cm-length sections. Half of these were
stored in plastic barrels (82cm height, 46cm diam.) equipped with funnel traps at ETREC Plant Sciences Unit Farm for overwintering until 2015 (ongoing research). The remaining wood was dissected to extract EAB and parasitoids to determine occurrence of parasitism, parasitism rates and parasitoid overwintering success.

**Determining Seasonality**

Information from these field study observations, as well as the generation lengths, percent parasitism, overwintering ability, GDD, and life history data observed in the ‘Seasonality Studies’ were evaluated to obtain a more complete understanding of the seasonality of the parasitoids being released in this region. A general schematic of the seasonality of the parasitoids recovered was generated. This information will provide a greater understanding of the impact of local climate conditions on the development of these parasitoids and their potential for establishment in this region.

**Native Natural Enemy Monitoring and Collecting**

In a survey to document potential native enemies of EAB in eastern Tennessee, parasitoids other than *T. planipennisi* or *S. agrili* species found emerging from the test barrels or in pan traps used in overwintering studies, were collected. These were placed into vials containing 95% ethyl alcohol and documented by date, site, and barrel/pan trap for identification.

**Database and Voucher Preservation**

All data (order, family, genus, species of all potential native enemies of EAB identified, number of specimens collected within each species category, and the location the specimens collected) were entered into an excel database. Specialists Juli Gould at USDA APHIS Otis, MA and Jon Lelito at the USDA APHIS-PPQ EAB Biocontrol Laboratory in Brighton, MI verified
species identification of representative specimens. All voucher specimens were cataloged and placed into the University of Tennessee’s insect museum in Knoxville, Tennessee.
CHAPTER III
RESULTS AND DISCUSSION

Seasonality Studies

Overwintering Ability

Adults of *S. agrili* and *T. planipennisi* emerged from infested bolts supplied by the EAB Biocontrol Laboratory that were placed in overwintering barrels on September 24, 2013. No emergence of subsequent generations of *S. agrili* or *T. planipennisi* occurred in 2013. No *S. agrili* or *T. planipennisi* had emerged in the barrels by July 2014, so dissection of wood material was conducted on July 7, 2014 (ca. 1400 GDD after beginning of study, ca. 950 GDD after January 1, 2013) to determine parasitism and parasitism rates. When the wood material was dissected, prepupae, pupae, and live adults of *S. agrili* were found in EAB galleries, indicating that *S. agrili* had successfully overwintered and one brood (i.e., live active adults) was preparing for emergence. The prepupal *S. agrili* probably would not have emerged until Fall 2014 or Spring 2015. These *S. agrili* had successfully parasitized 42.1% of EAB larvae (n=19) (Table 2). No *T. planipennisi* of any live stage were found in dissected wood material. The reason(s) for failure of *T. planipennisi* to successfully complete its life cycle is unknown.

Temperature data recorded by HOBO loggers indicated that there was no significant difference \( t = -0.511, \text{df} = 1, 255, p = 0.61 \) between mean external and internal barrel temperatures \( \bar{X}_{\text{internal}} = 11.17°C \pm 0.49 \), \( \bar{X}_{\text{external}} = 11.07°C \pm 0.63 \). As the conditions in the barrels used in this overwintering study were equal to outside conditions, it is reasonable to conclude that *S. agrili* could overwinter in Tennessee in a standing tree.

Mean humidity was significantly different \( t = -15.971, \text{df} = 1, 255, p = 0.0001 \) between external and internal barrel relative humidity \( \bar{X}_{\text{internal}} = 67.36% \pm 0.70 \), \( \bar{X}_{\text{external}} = 76.05% \pm 0.71 \). While these values are significantly different, they are not so dissimilar to cause a
Table 2. Individuals of *S. agrili* found on parasitized emerald ash borer (n=8) in overwintering study.

<table>
<thead>
<tr>
<th>Individual</th>
<th>Parasitized EAB larvae</th>
<th>Life stages of <em>S. agrili</em></th>
<th>Total per EAB larvae</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>pre-pupae</td>
<td>pupae</td>
</tr>
<tr>
<td>Individual 1</td>
<td>0</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Individual 2</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Individual 3</td>
<td>0</td>
<td>0</td>
<td>6</td>
</tr>
<tr>
<td>Individual 4</td>
<td>0</td>
<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Individual 5</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Individual 6</td>
<td>1</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Individual 7</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Individual 8</td>
<td>4</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
<td>11</td>
<td>17</td>
</tr>
</tbody>
</table>
significant negative biological effect. The EAB Biocontrol Laboratory keeps the humidity in their growth chambers >70% (Jon Lelito, personal communication). However, this humidity level is used for parasitoid rearing and is not indicative of natural humidity requirements of the parasitoids. Humidity likely had no effect on the study.

Pearson correlation analysis identified several factors associated with *S. agrili* overwintering ability (Table 3). A positive correlation was documented between the number of *S. agrili* prepupae and total *S. agrili* and the number of EAB galleries. All stages of *S. agrili* and total *S. agrili* were positively correlated with total number of EAB larvae recovered, indicating that access to higher EAB populations will have a positive effect on the parasitoid’s ability to successfully parasitize and overwinter. A greater understanding of these factors may allow for more effective deployment of parasitoids in release sites by favoring sites with larger EAB infestations.

**Life History**

As in the ‘Overwintering Ability’ test, adults initiated emergence from both *S. agrili* and *T. planipennisi* bolts supplied by the EAB Biocontrol Laboratory that were placed into overwintering barrels on September 24, 2013. Dissection of wood material at regular intervals began on September 27, 2013. The final set of wood sections were dissected on November 11, 2013.

*Spathius agrili* successfully parasitized 23.3% of EAB larvae (n=60). Eggs of *S. agrili* were recovered 3 to 15 days after adult emergence, larvae were recovered 9 to 15 days after adult emergence, and pupae were found 22 to 50 days after adult emergence (Fig. 5). Demonstrating that *S. agrili* individuals were preparing for overwintering. No *T. planipennisi* of any life stage
<table>
<thead>
<tr>
<th>Variables</th>
<th>Prepupae</th>
<th>Pupae</th>
<th>Adults</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Correlation coefficient</td>
<td>p-value</td>
<td>Correlation coefficient</td>
<td>p-value</td>
</tr>
<tr>
<td>Diameter of EAB bolts</td>
<td>0.035</td>
<td>0.903</td>
<td>-0.14</td>
<td>0.619</td>
</tr>
<tr>
<td>Galleries of EAB$^{1}$</td>
<td>0.712</td>
<td>0.003**</td>
<td>0.442</td>
<td>0.099</td>
</tr>
<tr>
<td>Total EAB larvae</td>
<td>0.830</td>
<td>&lt;0.001**</td>
<td>0.616</td>
<td>0.014*</td>
</tr>
</tbody>
</table>

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

1-Number of galleries of Emerald Ash Borer
Figure 5. Average *S. agrili* per EAB in life history test barrels: growing degree days (in brackets under ‘Days post-adult emergence from bolts’) calculated from time parasitoids were placed in barrels.
were found in dissected wood material. Some aspect of the barrel conditions could have affected their parasitizing ability or development; if so, the specific cause remains unknown.

Mean temperature was significantly different ($t = -14.618$, $df = 1, 36$, $p = 0.0001$) between external and internal barrel temperatures (internal $\bar{x} = 13.24^\circ C \pm 0.81$, external $\bar{x} = 12.21^\circ C \pm 0.86$). However, both means are similar in value, and it is possible that while the temperatures are statistically different, they are not biologically different as both means are below optimum parasitoid development temperatures (Gould et al. 2013). Therefore, it is reasonable to conclude that the data collected from this study is applicable for discerning the life history of the parasitoids in the local climate.

Mean humidity was significantly different ($t = 11.623$, $df = 1, 36$, $p = 0.0001$) between external and internal barrel relative humidity (internal $\bar{x} = 75.58\% \pm 1.07$, external $\bar{x} = 81.35\% \pm 1.04$). While these values are significantly different, they are similar enough as to not be biologically significant. Thus, the internal humidity was above optimal levels for parasitoid development.

Stepwise regression identified total number of EAB larvae ($R^2 = 0.286$) as the only variable significantly associated with recovery of *S. agrili* prepupae (Table 4). As prepupae are the overwintering stage of *S. agrili*, and the parasitoid can survive winter conditions (as indicated in the previous section), this analysis indicates that having access to a greater number of hosts is an important factor for survival of *S. agrili* in the field.

A greater understanding of these factors can greatly assist in the effective targeting of *S. agrili* in release sites. Sites with higher potential host counts are favorable. However EAB infestations move lower on trees as population density increases. Parasitoids are limited by the length of their ovipositors to smaller diameter portions of the tree with thinner bark. Further
Table 4. Variables that influenced recovery of *S. agrili* prepupae selected by stepwise linear regression ($R^2 = 0.286$).

<table>
<thead>
<tr>
<th>Coefficients $^a$</th>
<th>Unstandardized Coefficients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Model</td>
<td>B</td>
</tr>
<tr>
<td>1</td>
<td>(Constant)</td>
</tr>
<tr>
<td></td>
<td>Number of EAB Larvae</td>
</tr>
</tbody>
</table>

$^a$ Dependent Variable: Number of prepupae
studies should be initiated to determine the most effective infestation densities in which to apply *S. agrili* to an area.

Pearson correlation analysis identified several factors that affected recovery of *S. agrili* individuals (Table 5). Recovery of *S. agrili* prepupae was negatively associated with cumulative average temperature, minimum temperature and minimum relative humidity, while positively associated with GDD and days post-initiation of the study. Recovery of *S. agrili* eggs and larvae was negatively associated with maximum relative humidity.

As this study occurred during the fall, the decreasing temperatures, accumulation of degree days, and accumulation of time all correlated with development of *S. agrili* to prepupae. This analysis indicates that over the course of the 50-day study, *S. agrili* individuals developed at roughly the same rate, as no eggs or larvae were found after 15 days, likely reaching prepupal stage after ca. two weeks and then preparing to enter diapause. As the parent generation for these *S. agrili* and those from the ‘Overwintering Ability’ test emerged on the same date, it is likely that these individuals would have survived overwintering.

**Overwintering Field Studies**

**Cactus Cove Road Cages**

One female *S. agrili* adult was recovered from the Cactus Cove Road Cage study. It was found in the collecting head of a barrel on April 22, 2013. This recovery is the first incidence of *S. agrili* successfully overwintering from outdoor cage conditions. Modifications to the methodology for this experiment may lead to increased recoveries of the parasitoid. A similar study, based on this test and performed in the same site the following year has currently yielded ca. 38 *S. agrili* individuals (unpublished data).
### Table 5. Correlations of host and environmental factors and recovery of developing stages of *S. agrilli*.

| Variables | Egg | | | | Larva | | | | | | Prepupa | | |
|-----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
|           | Correlation coefficient | p-value | Correlation coefficient | p-value | Correlation coefficient | p-value |
| Temperature | | | | | | | | | | | | | | |
| Cumulative average on date of recovery | 0.171 | 0.367 | 0.237 | 0.208 | -0.571 | 0.001** |
| Max. on date of recovery | 0.012 | 0.948 | 0.163 | 0.389 | 0.288 | 0.123 |
| Min. on date of recovery | 0.148 | 0.434 | 0.191 | 0.311 | -0.567 | 0.001** |
| GDD$^1$ | -0.131 | 0.490 | -0.103 | 0.587 | 0.461 | 0.010* |
| Relative humidity | | | | | | | | | | | | | | |
| Cumulative average on date of recovery | -0.332 | 0.178 | -0.332 | 0.178 | 0.064 | 0.800 |
| Max. on date of recovery | -0.529 | 0.024* | -0.529 | 0.024* | 0.334 | 0.175 |
| Min. on date of recovery | 0.296 | 0.233 | 0.296 | 0.233 | -0.572 | 0.013* |
| General | | | | | | | | | | | | | | |
| Diameter of EAB bolts | 0.172 | 0.362 | -0.098 | 0.607 | -0.058 | 0.760 |
| Galleries of EAB$^2$ | 0.097 | 0.615 | -0.100 | 0.607 | 0.298 | 0.117 |
| Days post-initiation | -0.162 | 0.392 | -0.156 | 0.410 | 0.539 | 0.002** |

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

1-Growing Degree Days (GDD) calculated as $(T_{max}+T_{min})/2$-Tbase and initiated at start of study.
2-Number of galleries of emerald ash borer
No *T. planipennisi* individuals were recovered over the course of this study. *Spathius agrili* diapauses during winter, requiring an accumulation of GDD before it emerges. *Tetrastichus planipennisi*, however, does not diapause during winter; it emerges when the temperature increases above freezing (0°C) (Hanson et al. 2013, Jon Lelito, personal communication). Because of this overwintering behavior, the short warm periods common to southern winters may cause *T. planipennisi* to emerge prematurely from the shelter of the tree and die when the temperature drops again. The cold-warm-cold cycle can repeat itself multiple times over one winter potentially having a devastating effect on *T. planipennisi* populations.

**Miser Station, Haven Hill and Cowan Park Barrels**

No *S. agrili* or *T. planipennisi* were recovered from the material collected from Miser Station, Haven Hill, or Cowan Park. These sites had been used for parasitoid releases for only one year at the time the trees were felled. Parasitoid populations may not have had the opportunity to establish and increase to detectable levels on the trees selected for felling. It is also possible that the parasitoids were unsuccessful in either parasitizing or overwintering, but this explanation is unlikely considering the successful recovery of *S. agrili* from the Rowe Farm site (see ‘Rowe Farm Pan Traps’)

**Rowe Farm Pan Traps**

The Rowe Farm pan traps yielded one female *S. agrili* adult on August 12, 2013. This recovery is the first incidence of recovery of *S. agrili* in an open release site part of the EAB Biological Control Program in the southern U.S., and indicates that *S. agrili* can overwinter and potentially become established. No *T. planipennisi* specimens were recovered, possibly due to its overwintering behavior.
Rutledge Pike Cages

Neither *S. agrili* nor *T. planipennisi* successfully infested EAB larvae in Rutledge Pike cages. While EAB were successfully introduced to the Rutledge Pike cages; only cage 3 was effectively infested with 30 EAB larvae recovered. Cages 1 and 2 had two and zero EAB, respectively. For all larvae recovered development seemed to have been slowed, and no larvae had developed past the second instar (based on head capsule diameter). It is unknown why the EAB larvae developed slowly or why only one cage was successfully infested. This slow development is likely the cause of the unsuccessful parasitism within the cages, as both parasitoids prefer the larger and more developed hosts of the third and fourth instars (Gould et al. 2013).

**Determining Seasonality**

Based on general observations over the course of the overwintering and seasonality studies and communication with the USDA APHIS EAB Biocontrol Laboratory, a general seasonality schematic of *S. agrili* was generated (Fig 6). *Spathius agrili* exhibits multiple broods throughout the year. Up to three have been observed in laboratory settings (Jon Lelito, personal communication). For this reason, prepupae may be observed throughout the year and adults may be observed from late spring into late fall. Because prepupae are most prevalent throughout the year, they may be the best for diagnostic assessments of parasitism when dissecting ash. Additional studies to determine brood number in the southern U.S. are warranted to further elucidate the biology of this species.

**Native Enemy Monitoring and Collecting**

The Haven Hill Cage and the Rowe Road pan trap studies yielded specimens of the native parasitoid *S. floridanus* Ashmead and an unknown *Spathius* species. Both identifiers
Figure 6. Estimated seasonality of life stages of *S. agrili* based on field observations and controlled studies, Tennessee 2013-2014.
suspect that these unknown specimens are one of the 19 known *Spathius* species native to North America (Marsh and Strazanac 2009). The Cactus Cove study also yielded confirmed specimens of *A. cappaerti* Marsh & Strazanac (Table 5). Both *S. floridanus* and *A. cappaerti* are known to parasitize EAB (Cappaert and McCullough 2009; Duan et al. 2012c). The presence of native parasitoids that attack EAB may have promising implications for the EAB Biological Control Program in the southern U.S. Methods for mass rearing of native parasitoids may be developed, so that they can be incorporated into releases in select areas infested with EAB. Also, native parasitoids may lend themselves to rearing in field insectaries, possibly reducing costs and labor of rearing insects in laboratory facilities. Further study of these insects and their interactions with EAB should be undertaken.
Table 6. Parasitoids (Hymenoptera: Braconidae) collected from emerald ash borer parasitoid release sites in Tennessee, 2013 (for each collection record one individual was recovered).

<table>
<thead>
<tr>
<th>Species</th>
<th>Date</th>
<th>Location</th>
<th>Native</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Atanycolus cappaerti</em></td>
<td>21 May</td>
<td>Haven Hill</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>23 May</td>
<td>Haven Hill</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Spathius agrili</em></td>
<td>22 Apr</td>
<td>Haven Hill</td>
<td>No</td>
</tr>
<tr>
<td></td>
<td>12 Aug</td>
<td>Rowe Farm</td>
<td>No</td>
</tr>
<tr>
<td><em>Spathius floridanus</em></td>
<td>29 May</td>
<td>Haven Hill</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>1 Jun</td>
<td>Haven Hill</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>7 Jun</td>
<td>Rowe Farm</td>
<td>Yes</td>
</tr>
<tr>
<td><em>Spathius sp.</em></td>
<td>23 May</td>
<td>Haven Hill</td>
<td>Probable</td>
</tr>
<tr>
<td></td>
<td>29 May</td>
<td>Haven Hill</td>
<td>Probable</td>
</tr>
<tr>
<td></td>
<td>1 Jun</td>
<td>Haven Hill</td>
<td>Probable</td>
</tr>
<tr>
<td></td>
<td>29 Jul</td>
<td>Rowe Farm</td>
<td>Probable</td>
</tr>
</tbody>
</table>

*All specimens are morphologically similar and suspected to be the same species.*
Conclusions

The data collected from seasonality tests indicate that *S. agrili* are capable of developing and successfully overwintering when exposed to sufficient numbers of EAB hosts. The further recovery of *S. agrili* individuals from field release sites further indicates its potential success as a biocontrol agent. This information can be used to develop criteria for future parasitoid releases in this region. *Spathius agrili* is no longer being released north of the 40th parallel because of its questionable efficacy in colder climates (Gould et al. 2013). The discovery of *S. galinae*, a species likely well suited to colder regions, has threatened to remove *S. agrili* from the EAB Biological Control Program entirely. However, these recoveries indicate that *S. agrili* should be kept on as a release agent, allowing USDA APHIS to deligate resources and ultimately save money that can be used to further the biological control program elsewhere. The continued spread of EAB in the southern U.S. demonstrates the need for *S. agrili* to remain a part of the EAB Biological Control Program.

The recovery of *S. agrili* from the Cactus Cove Road Cages, and further recoveries of ca. 38 *S. agrili* from similar ongoing studies, may reveal a relatively inexpensive means to create field insectaries. Parasitoids could potentially be mass reared with minimal input when compared to laboratory facilities. The release of parasitoids into cages placed over targeted EAB-infested trees could allow for denser and larger populations of parasitoids to be released at once, potentially increasing their parasitism rates when.

*Tetrastichus planipennisi* does not seem to be effective in eastern Tennessee. Establishment of this parasitoid in northern release sites shows that it should certainly continue being released. However, it is likely best to discontinue releases in regions that often have winter temperatures fluctuating freezing, such as Tennessee.
In 2014, three more sites in eastern Tennessee became eligible for parasitoid establishment monitoring in sites used in this study will continue to be monitored. Additionally, the presence of native parasitoids that are known to attack EAB may have promising implications for the EAB Biological Control Program in the southern U.S. Native parasitoids could be reared and released as augmentative biological control agents to supplement the classical biological control program currently ongoing.

Native parasitoids may also lend themselves to rearing in field insectaries, such as the tree cages, possibly reducing material and labor costs of rearing the insects in laboratory facilities. Further study of these insects and their interactions with EAB should be undertaken. The incorporation of native parasitoids into EAB management with establishment of *S. agrili* may ultimately lead to control of EAB populations in the southern U.S.
LIST OF REFERENCES


GBIF (Global Biodiversity Information Facility). 2014. *Fraxinus*. Biodiversity occurrence data. GBIF Data Portal, data.gbif.org,


McCullough, D. G., T. M. Poland, A. C. Anulewicz, P. Lewis, and D. Cappaert. 2011. Evaluation of *Agrilus planipennis* (Coleoptera: Buprestidae) control provided by emamectin benzoate and two neonicotinoid insecticides, one and two seasons after treatment. *J. Econ. Entomol.* 104:1599–1612.


VITA

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