Influences of Climate, Competition, and Novel Hosts on Parasitoids of Emerald Ash Borer, and their Establishment in Virginia and North Carolina

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Dissertation submitted to the faculty of the Virginia Polytechnic Institute and State University in partial fulfillment of the requirements for the degree of

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In

Entomology

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Abstract
(Academic)

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a species of wood boring beetle which feeds on the inner bark of ash trees, *Fraxinus* spp., and white fringe tree, *Chionanthus virginicus* L. In North America emerald ash borer feeding damages the trees vascular system, killing the tree in 1-6 years after initial infestation. Emerald ash borer's native range is north east China, the Russian Far East, and the Korean peninsula. In the mid-1990s emerald ash borer was accidentally introduced to Michigan from the Hebei and Tianjin city province regions of China. Since then, due to human-aided transport and natural spread, emerald ash borer now infests 35 states and five Canadian provinces. Studies in to Asia discovered several species of parasitic wasps which feed and reproduce on emerald ash borer; four of these species were approved for release in the United States. Three species which attack emerald ash borer larvae *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Spathius galinae* Belokobylskij and Strazanac (Hymenoptera: Braconidae), and one species which utilizes emerald ash borer eggs, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). This research focuses on the control of emerald ash borer using the three larvae-utilizing species of parasitic wasp. The first objective of this research focuses on the synchrony of emerald ash borer larvae with the early spring emergence of *S. agrili* and *S. galinae*. The first objective also assessed how two different cold temperature treatments

changed the emergence timing, and health of the wasps. The second objective assessed to determine the effects of competition between *S. agrili* and *S. galinae* when they were exposed to a single emerald ash borer larvae sequentially, and simultaneously. When exposed sequentially, the first wasp held the competitive advantage, but when exposed simultaneously *S. agrili* had a small advantage, but did not completely exclude *S. galinae.* The third objective focused on the potential for two larval parasitoids, *S. agrili* and *S. galinae* to parasitize emerald ash borer larvae in the novel host plant white fringe tree. We determined that both parasitoids are capable of finding and parasitizing emerald ash borer larvae within a non-ash host. Finally, we located 13 stands of emerald ash borer infested ash in Virginia and North Carolina in order to determine its life cycle, and overwintering life stage. We determined that emerald ash borer overwinters at different life stages, and has a more complex life cycle than previously reported. Additionally, all three species of larvae-utilizing parasitic wasp were recovered at field sites at least 1 year after release. These results all show promise for the biocontrol program, and indicate that biocontrol of emerald ash borer could be successful in Virginia and North Carolina.

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Abstract
(General Audience)

Emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) is a wood

boring beetle which feeds on the inner bark of ash trees, *Fraxinus* spp., and white fringe tree,

Chionanthus virginicus L. In the mid-1990s emerald ash borer was accidentally introduced to

Michigan from the Hebei and Tianjin city province regions of China. Since then, due to human-

aided transport and natural spread, emerald ash borer now infests urban and natural forests in 35

states and five Canadian provinces. North American ash trees did not evolve with emerad ash

borer, and have little resistance to their attack. A North American ash or white fringe tree

attacked by emerald ash borer dies in 1-6 years if left untreated. Systemic insecticide treatments

exist, but require annual treatment and are less effective than initially hoped. Additionally,

systemic insecticides are impractical to apply to forest ecosystems. Researchers traveled to the

Asia, and discovered several species of parasitic wasps which attack emerald ash borer. After

years of efficacy and host-exclusivity testing four species were released in the United States.

Three species which exclusively utilize emerald ash borer larvae *Tetrastichus planipennisi* Yang

(Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Spathius*

galinae Belokobylskij and Strazanac (Hymenoptera: Braconidae), and one species which utilizes

emerald ash borer eggs, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae). My

research focuses on the control of emerald ash borer using the three larvae-utilizing species of

parasitic wasp. Chapter two focuses on the life cycle of emerald ash borer larvae, *S. agrili* and *S.*

galinae during the early spring. We determined that both parasitoid species have similarly timed life cycles as EAB, indicating a good climate match in Virginia. Chapter three describes the interactions between *S. agrili* and *S. galinae* when competing for EAB larvae. We determined that the first species to attack the larvae has the advantage; and when adult wasps competed to find a larvae neither species completely dominated the other. The third objective focused on the potential for two larval parasitoids, *S. agrili* and *S. galinae* to parasitize emerald ash borer larvae in the novel host plant white fringe tree. We determined that both parasitoids are capable of finding and parasitizing emerald ash borer larvae within white fringe tree. Finally, we located 13 stands of emerald ash borer-infested ash in Virginia and North Carolina in order to determine its life cycle, and overwintering life stage. We determined that emerald ash borer overwinters at different life stages, and has a more complex life cycle than previously reported. Additionally, all three species of larvae-utilizing parasitic wasp were recovered at field sites at least 1 year after release. These results all show promise for the biocontrol program, and indicate that biocontrol of emerald ash borer could be successful in Virginia and North Carolina.

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people say animals can sense your stress, and will comfort you in times of need. Our cat Mal

does not do this, but at least he's a companion.

Attribution

Several colleagues aided in the writing, design and analysis of the research behind the following chapters of this dissertation. Their contributions are summarized here.

Chapter 2: Differences in early season emergence and reproductive activity between *Spathius agrili* (Hymenoptera: Braconidae) and *Spathius galinae*, larval parasitoids of the invasive emerald ash borer (Coleoptera: Buprestidae). Published in *Environmental Entomology*, nvz168, https://doi.org/10.1093/ee/nvz168

Ryan Meyer was an undergraduate research assistant in the Department of Entomology at Virginia Tech. Ryan is a coauthor on this paper, and assisted in experimental design and implementation of these experiments. He also contributed editorial comments.

Jian Duan, PhD is a research entomologist at the USDA ARS Beneficial Insects Introduction Research Unit in Newark, DE. Dr. Duan is a coauthor on this paper, assisted in the design, analysis, writing and editing.

Ben Slager, MS is a supervisory entomologist at the USDA APHIS in Brighton MI. Ben is a coauthor on this paper, and assisted in the design and implementation of these experiments. He also contributed editorial comments.

Scott Salom, PhD is a professor of forest entomology in the Department of Entomology at Virginia Tech. Dr. Salom is a coauthor on this paper, and assisted in experimental

design, writing, and editing. He was also the principal investigator for the grants supporting this research.

Chapter 3: Interspecific competition between idiobiont larval parasitoids of emerald ash borer. Submitted to the *Journal of Insect Behavior*. 2/6/2020

Jian Duan, PhD (USDA ARS BIIRU) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing.

Scott Salom, PhD (Department of Entomology, Virginia Tech) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing. He was also the principal investigator for the grants supporting this research.

Chapter 4: Responses of two introduced larval parasitoids of the invasive emerald ash borer (Coleoptera: Buprestidae) infesting a novel host plant, white fringe tree: Implications for biological control.

Jian Duan, PhD (USDA ARS BIIRU) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing.

Scott Salom, PhD (Department of Entomology, Virginia Tech) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing. He was also the principal investigator for the grants supporting this research.

Chapter 5: The lifecycle of emerald ash borer in Virginia, and recoveries of three introduced parasitoid species.

Jian Duan, PhD (USDA ARS BIIRU) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing.

Scott Salom, PhD (Department of Entomology, Virginia Tech) is a coauthor on this paper, and assisted in the experimental design, implementation, and editing. He was also the principal investigator for the grants supporting this research.

Mark Chivvis (Department of Entomology, Virginia Tech) is a coauthor on this paper, and aided in the location of sites, data collection and implementation.

Tim McCoy (Department of Entomology, Virginia Tech) is a coauthor on this paper, and aided in the location of sites, data collection and implementation.

Katlin Dewitt (Virginia Department of Forestry) is a coauthor on this paper, and aided in the location of sites, data collection and implementation.

Meredith Bean (Virginia Department of Forestry) is a coauthor on this paper, and aided in the location of sites, data collection and implementation.

Rachel Griesmer (Fairfax County Urban Forest Management) is a coauthor on this paper, and aided in the location of sites, data collection and implementation.

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Chapter One
Introduction and Literature Review

Emerald Ash Borer

      Emerald ash borer (EAB), *Agrilus planipennis* Fairemaire (Coleoptera: Buprestidae) is a

wood-boring beetle native to Asia. In both the native range and in North America, EAB

primarily feeds on ash trees (*Fraxinus* spp.), and has been reported to have a wider host range in

Japan and Korea, encompassing Manchurian walnut (*Juglans mandshurica var. sieboldiana*

Maxim.), wingnut tree (*Pterocarya rhoifolia* Sieb. & Zucc.), and Japanese elm (*Ulmus davidiana*

Planch) (Akiyama 1997). In North America, host range testing revealed the invasive population

of EAB does not attack any of these latter hosts (Haack and Petrice 2005). However, it is

capable of attacking and developing on another North American species, white fringe tree

(*Chionanthus virginicus* L.) (Haack et al. 2002, Cipollini 2015). EAB has also been reported to

feed on cultivated olive (*Olea europea* L.) in laboratory bioassays (Cipollini et al. 2017). In its

native range, EAB was found to attack healthy and unhealthy ash, however weak trees and edge

trees were the most vulnerable, and showed the most decline after infestation (Liu et al. 2003,

Wang et al. 2010). In North America, ash stands can suffer complete mortality within 6 years of

infestation, with lower ash density, mesic soil, and poor tree health corresponding to faster death

by EAB (Knight et al. 2013). North American ash trees natural range spans the continental

United States, and it is ornamentally planted in urban forests across the country (Fig 1). An

estimate of EAB damage to North American forests expected that between 2009 and 2019 EAB

would spread to 25 states, killing 37.9 million ash trees, and cost over $25 billion for removal

and replacement of urban ash alone (Kovacs et al. 2010). Currently, EAB has exceeded these

estimates in tree mortality, total invasive range; but total costs of EAB not been calculated (Emerald ash borer information 2020).

In most areas of China, EAB is univoltine or semivoltine requiring a minimum of one year for development (Xia et al. 2007). Genetic analysis by Bray et al. (2011) showed that EAB populations within North America matched populations within two regions of China, Hebei and Tianjin city provinces; pointing to a single introduction of EAB, or multiple introductions from a single Hebei or Tianjin population.

Lifecycle of emerald ash borer

During early spring, mated adult EAB females oviposit up to 7 times in sheltered locations on the bark of the ash. Individual female adults showed a potential for up to 140 eggs per adult (Wang et al. 2010).

Larvae eclose from the egg 12-19 days after oviposition, and proceed to chew through the bark and enter the phloem of the tree. As larvae develop through four instars, they burrow deeper into the cambial region. Fourth instar chew into the xylem in order to construct pupal chambers which they can overwinter in, with some larvae overwintering in feeding galleries in the phloem (Xia et al. 2007). This feeding process creates a series of serpentine shaped galleries in the phloem, which restricts and gradually eliminates the tree's ability to transport carbohydrates, eventually leading to tree mortality (Cappaert et al. 2005, Knight et al. 2013).

Larvae overwinter in their constructed pupal chambers within the xylem of the tree, or as larvae within the feeding galleries. Prior to dormancy, 4th instar tunnel outward starting the exit hole, then fill it with frass. When overwintering, the larvae curl into a J-shape, termed J-larvae. EAB larvae do not have an obligate diapause (Wang et al. 2010, Duan et al. 2013a). In the beginning of spring, larvae enter the pre-pupal stage and then pupate for 11-38 days. Adult

emergence occurs in early to late May, leaving a D-shaped exit hole, characteristic of Buprestidae (Wang et al. 2010, Haack 2002). In China, each living infested tree produced an average of 11.7 EAB adults, while dead trees produced 34.6 adults (Wang et al. 2010). In Russian field surveys, North American ash species consistently had higher densities of EAB larvae per m^2 than Russian ash species (Duan et al. 2012a).

Invasion of North America

EAB was initially discovered in Michigan in May of 2002 as an unknown buprestid reared out of ash logs by an Extension agent. By July, it was confirmed to be *A. planipennis*, which did not yet have a common name (Haack et al. 2002). Due to EAB's life cycle, where it remains dormant within the xylem of the tree, it can persist inside the tree even after felling and being chopped into firewood. Movement of firewood has been a primary mode of dispersal in the U.S. (Haack et al. 2002). To prevent this, the state of Michigan initially enforced an ash wood quarantine, which ultimately was not successful in preventing the spread (Haack et al. 2002). A federal quarantine on the movement of ash wood is currently in effect, but could end soon. A proposed rule by USDA APHIS in 2018 could end the federal quarantine on transport of domestic ash products, but has stalled in the legislative process after the comment period closed (Federal Register 83 FR 47310).

The life cycle of EAB may have contributed to difficulties in establishment of parasitoids due to synchronous overwintering in pupal galleries, which avoids parasitism in some locations (Gould personal communication).
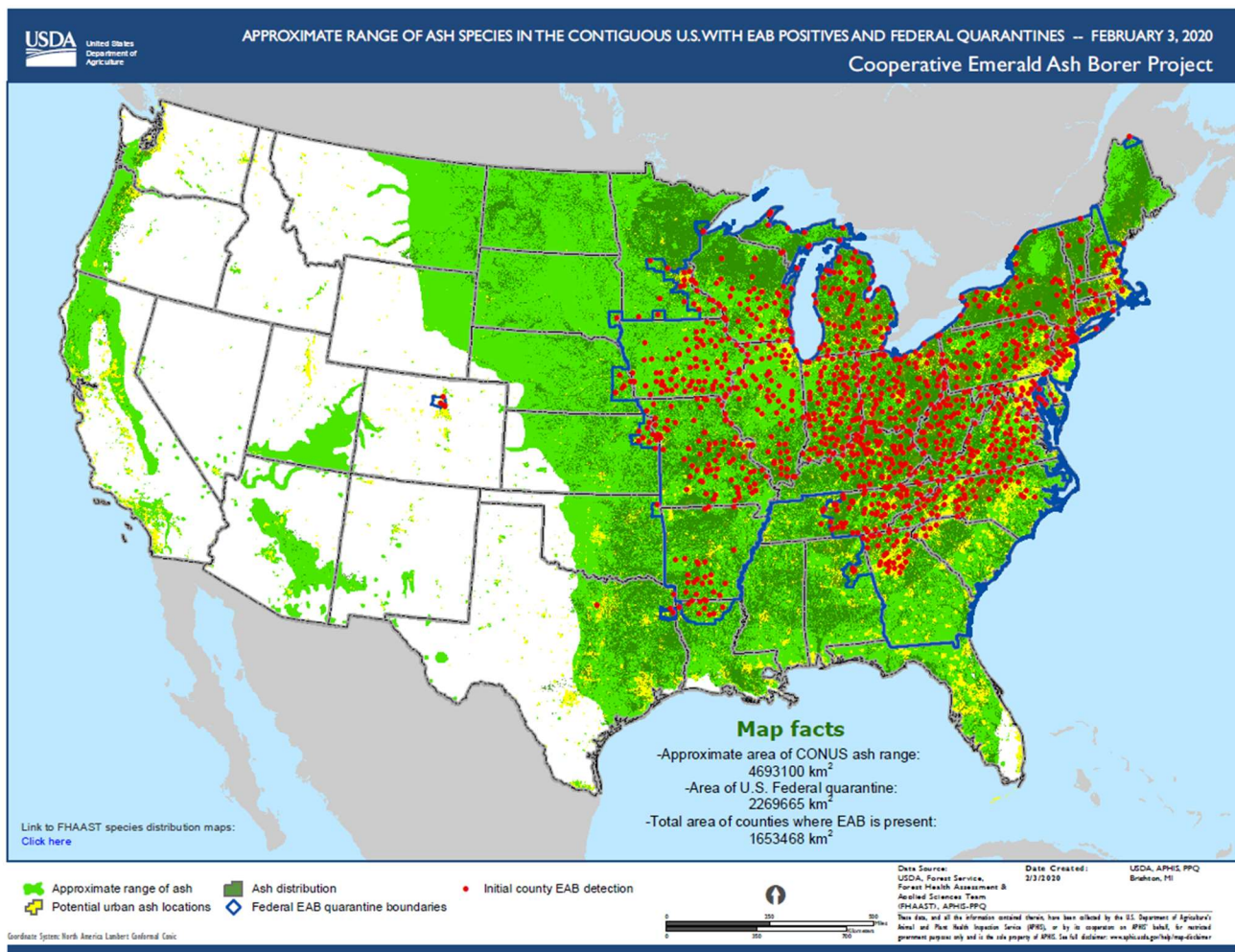
Fig 1. Range of North American ash, and the federal emerald ash borer quarantine map with initial county detections. (USDA Cooperative 2020) .

The Forest Health Assessment and Applied Sciences Team (formerly Forest Health Technology Enterprise Team), a unit within State & Private Forestry of the USDA Forest Service, has created a risk map to indicate likelihood of infestation (Fig. 2). Risk is based on host range, proximity to campgrounds, rest areas, and previous likelihood of detection.

Emerald Ash Borer (EAB) Risk 2014
Agrilus planipennis Fairmaire

EAB Risk 2014
- Low
- Moderate
- Moderate High
- High

Emerald Ash Borer (EAB) Risk 2013
EAB risk is a function of EAB detection likelihood, detection uncertainty, and potential introduction locations. Potential introduction locations include campgrounds, rest areas, and other locations identified by state cooperators. Introduction sites were given a high risk value and then combined with the 2014 EAB detection likelihood composite model. Risk values are classified into: Low, Moderate, Moderate High, and High classes.

0 50 100   200   300   400   500
Miles

Albers Equal Area Conic Projection

Map produced by FHTET, IL
Fort Collins, CO on 11-26-2013
File: EAB_Risk_2014.mxd
Project: EAB_2013

Climate

Origins of EAB were confirmed to be from the Hebei and Tianjin City provinces of China, where annual temperatures from 2015 ranged from -9 to 30°C (Weatherbase.com). Ambient temperature was found to significantly impact EAB egg hatching time, larval development time, and larval body mass (Duan et al. 2013a). Egg hatch was completely inhibited at temperatures below 12°C, and median hatching time decreased with increased temperature from 20 days at 20°C to 7 days at 35°C (Duan et al. 2013a). Larval growth rate was most dramatically decreased at 20°C [12.3 weeks to J-larvae], was only slightly increased by temperature changes from 25°C [7.1 weeks to J-larvae] to 30°C [6.3 weeks to J-larvae], and was

slightly decreased at 35°C [8.0 weeks to J-larvae](Duan et al. 2013a). This trend in larval

growth rate is similar by the trend in larval biomass, where at 20°C larvae increased biomass at a

slower rate [0.0050g per week] compared to at 25°C [0.0115g per week], and again slowing at

35°C [0.0077g per week] (Duan et al. 2013a). Supercooling points of EAB acclimatized pre-

pupae were found to reach -25°C, which suggests that winter mortality could restrict EAB

populations in a far northern climate (Venette and Abrahamson 2010). Research into

overwintering mortality in New England is currently underway (Jian Duan personal

communication).

Insecticide Control

Multiple insecticide products and application methods are used for control of EAB.

Imidacloprid, dinotefuran, and emamectin benzoate primarily have been used for control in

urban settings (Herms et al. 2009). Soil injections of imidacloprid and dinotefuran, as well as

trunk injections of emamectin benzoate have been shown to reduce the rate of canopy dieback in

green ash (Bick et al. 2018). However their capacity to protect trees from mortality due to EAB

has been mixed (Herms et al. 2014). Imidacloprid and emamectin benzoate are more likely to be

able to protect susceptible ash, and can be more economically viable than removal for urban trees

(Herms et al. 2014, McCullough and Mercader 2012).

Predation by Woodpeckers

Woodpecker predation of EAB accounts for a highly variable amount of larval mortality

(Lindell et al. 2008). In a study in Michigan, predation by woodpeckers was measured in

number of attacks m^2 of tree, which varied from zero to 37 attacks/m^2 across the study (Lindell et

al. 2008). In an unpublished Virginia study from 2014 to 2015, woodpecker predation accounted for 15-39% of all larvae surveyed, varying dramatically across sites and years. Infestation by EAB has been shown to impact population dynamics of three woodpecker species and a nuthatch. Populations of red-bellied woodpecker (*Melanerpes carolinus* L.) and white-breasted nuthatch (*Sitta carolinensis* Latham) were found to increase significantly, while downy (*Picoides pubescens* L.) and hairy woodpeckers (*Leuconotopicus villosus* L.) decreased significantly in highly impacted areas (Koenig 2013). Koenig et al. (2013) suggest that EAB infestation could also impact other avian species, however they also cautioned that this would be difficult to predict.

Parasitism

Four hymenopteran parasitoids of EAB have been introduced as biological control agents into the U.S. This includes one egg parasitoid, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) and three larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Spathius galinae* Belokobylskij and Strazenac (Hymenoptera: Braconidae). Field surveys of *T. planipennisi* have shown establishment, and since 2013 *T. planipennisi* has played a major role in EAB biocontrol (Duan et al 2013b, Duan et al. 2018). Additionally, native parasitoid species, including *Atanycolus* spp., have been found parasitizing EAB larvae at increasing frequencies over time (Duan et al. 2012b, Duan 2013c). *T. planipennisi, O. agrili* and native parasitoid species were outside of the scope of my laboratory research, but were observed in field collections.

Spathius agrili

     Spathius agrili is a larval ectoparasitoid discovered in the Tianjin Province of China in 2005 (Yang et al. 2005) (fig 3). Emergence of *S. agrili* occurs from late May to late July, asynchronously with EAB emergence. This results in *S. agrili* oviposition coinciding with later instar of EAB. This is important because *S. agrili* is an idiobiont parasitoid, paralyzing the EAB larval (Wang et al. 2007a, Yang S. 2013). Incapacitation of the EAB larvae was found not to occur immediately after oviposition, but rather upon egg eclosion, ca. 2 days after oviposition (Yang S. 2013). Development of *S. agrili* is dependent on EAB larval size; the clutch size, ratio of females to males, immature survival, and adult wasp body size (Wang et al. 2007b). Superparasitism was never observed by Wang et al. (2007b) in either field or laboratory setting, which is attributed to female *S. agrili* locating their hosts by vibration or sound of larval feeding which stops when paralyzed.

     Various rearing conditions were tested by Gould et al. (2011), to determine the effect of group mating, plant volatiles, temperature, and longevity under various conditions. They showed that group or singular mating had no statistically significant effect on reproductive potential or longevity, although sex ratio was skewed towards producing females in group mating (81% female) compared to singular mating (59% female). Presence of host plant volatiles did not have an effect on percentage of female *S. agrili* that oviposited, but did increase the number of clutches of eggs produced by each female *S. agrili* (Gould et al. 2011). Temperature differences from 25 to 28°C had no statistically significant effect on fecundity or longevity of adult *S. agrili* (Gould et al. 2011). Long-term chilling of *S. agrili* pupae at 10°C had detrimental effects on adult emergence and egg capacity, which escalated with amount of time spent at 10°C (Gould et al. 2011).

Spathius galinae

   Spathius galinae is a gregarious larval ectoparasitoid of EAB native to the Primorsky Krai of the Russian Far East and South Korea, where it attacks EAB within both native Asian and exotic North American host trees in their overlapping native range (Belokobylskij et al. 2012) (fig 3). It shares many life history traits with *S. agrili*, including specialization on EAB. Due in part to its native distribution it is likely a suitable climatic match for most of North America compared to the previously released parasitoids, developing in temperatures ranging from 20-30 °C (Belokobylskij et al. 2012, Federal Register 2015, Watt et al. 2016, Gould personal communication). *Spathius galinae* was found to generally parasitize 3rd and 4th instar EAB larvae, and occasionally 2nd instar, with 8-16 *S. galinae* larvae within any EAB larvae (Belokobylskij et al. 2012, Duan et al. 2012b). Although it is capable of parasitizing all life stages, 3rd and 4th instar were found to be parasitized at significantly higher rates than all other life stages, although *S. galinae* reared on J-larvae were found to have a large female-bias compared to other broods (Watt 2014). Due to its longer ovipositor it can parasitize EAB larvae in any size tree, regardless of bark thickness (Murphy et al. 2017).

   The development of *S. galinae* from egg to pupae in laboratory rearing conditions (25 ±1 °C , 16:8 L:D photoperiod) was completed by 50% of individuals in 10 days, and from egg to adult was completed 28-30 days by 50% of individuals (Duan et al. 2014). Longevity of males and females differed in laboratory conditions when reared as a group, with females outliving males (median survival of 7 weeks for female [maximum of 11 weeks], 6.5 for males [maximum of 8 weeks]). However, when reared in pairs, female and male longevity did not differ significantly (median survival of 7 weeks for females, [maximum of 15 weeks], 6 weeks for males [maximum of 9 weeks]) (Duan et al. 2014). All larvae of *S. galinae* exposed to

temperatures below 15°C entered diapause, indicating that the larval stage is the diapause inducing stage (Duan et al. 2014).
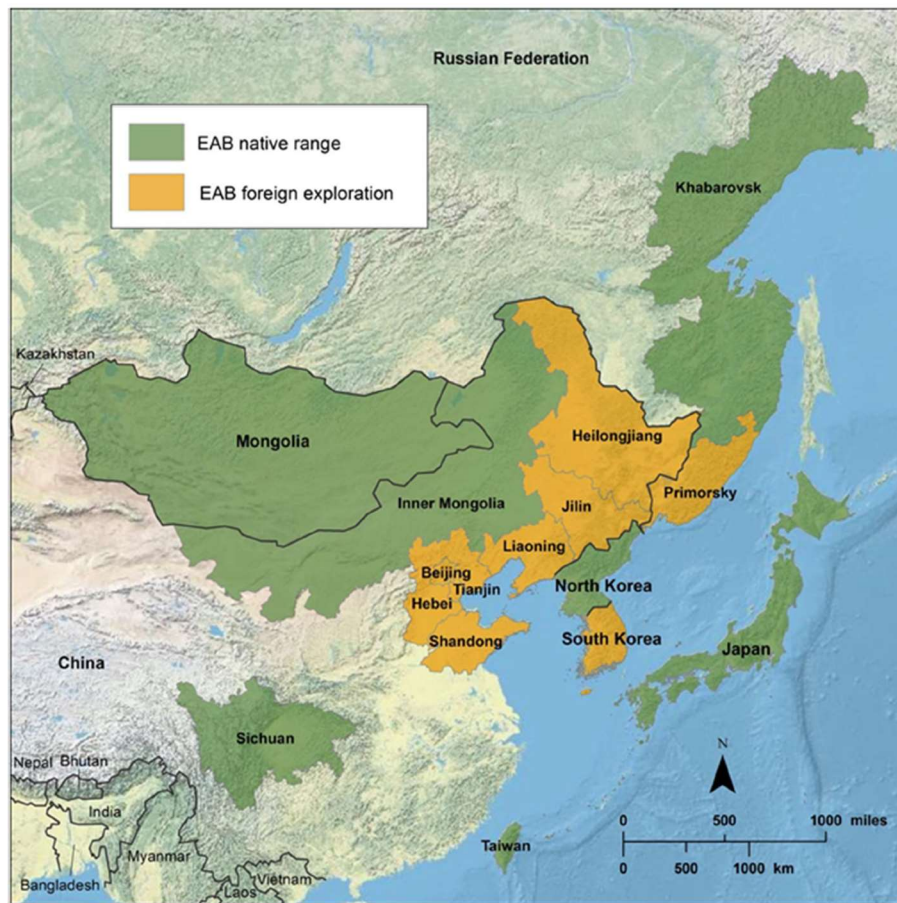
Figure 3. As published in Bauer et al. 2015. "Known native range of emerald ash borer, Agrilus planipennis, in Asia and other regions where exploration for EAB natural enemies has occurred since 2003. Service layer credits: United States National Park Service. Data source: https://sites.google.com/site/eduardjendek/world-distribution-of-agrilus-planipennis. Map created by United States Department of Agriculture, Forest Service, Northeastern Area State and Private Forestry, Office of Knowledge Management (Durham, New Hampshire, United States of America)."

The slow ash mortality (S.L.A.M.) project has used girdling trap trees and localized treatments to lower EAB larval density, which provides a complex potential management tactic for large ash stands (Mercader et al. 2015).

Interactions

Interactions among the four parasitoid biocontrol agents have been studied, but much remains to be learned. Interspecific competition may result in failure of a potential biocontrol agent to establish, or a suboptimal establishment. In a study by Yang et al. (2012), EAB larvae were exposed to *S. agrili* followed by *T. planipennisi* in dual-choice assays, resulting in multiparasitism when *T. planipennisi* was introduced 0-2 days after *S. agrili* had parasitized the larvae, but no parasitism when *T. planipennisi* was introduced after 4 days of *S. agrili* parasitism. They also exposed healthy EAB larvae to *S. agrili* and *T. planipennisi* simultaneously to determine competitive effects on parasitism rates, which resulted in higher *S. agrili* parasitism rates (76-79%), than *T. planipennisi* (5.3-9%). Additionally, progeny of *T. planipennisi* was significantly reduced in these groups (1.09-3.67 progeny per EAB larvae) compared to control groups (18.90-31.20 progeny per EAB larvae), while progeny of *S. agrili* were unaffected by exposure to *T. planipennisi*. In all cases of multiparasitism by *S. agrili* and *T. planipennisi*, only *S. agrili* emerged, and *T. planipennisi* larvae were found dead in the galleries (Yang et al. 2012).

Interactions between *Antanycolus* spp. and introduced parasitoids remains largely unknown due to difficulty rearing *Antanycolus* in laboratory settings (Duan personal communication 2015). Insecticide control using imidacloprid can be facilitated to work with biological control without impact on parasitoids (Davidson and Rieske 2016).

EAB Host Range Complications

As EAB expands its host range, complications arise in the biological control program. When pre-release evaluation was performed for *S. agrili* and *S. galinae*, EAB was believed to be host-specific to *Fraxnius* spp. (Yang et al. 2008, Duan et al. 2015). White fringe tree may act as a reservoir host and provide a partially enemy free space for emerald ash borer (Hoban et al. 2018, Olson and Rieske 2019). *Tetrastichus planipennisi* has been reported to be able to parasitize EAB inserted in white fringe tree (Hoban et al. 2018), but EAB that have developed from egg in white fringe tree may escape parasitism (Olson and Rieske 2019).

White fringe tree is been shown to be a suboptimal host for EAB. Larvae develop slower than in *Fraxinus* spp. and have higher mortality within white fringe tree (Cipollini and Rigsby 2015, Rutledge and Arango-Velez 2017). Despite this, white fringe trees have been attacked in areas simultaneously to ash trees (Thiemann et al. 2016).

Rationale and Objectives

Due to the origin of EAB's invasive population in Michigan EAB research had focused on norther US states. The lifecycle of EAB has been reported to vary between one and two years depending on host-tree stress (Tluczek et al. 2011). Unpublished data from USDA APHIS suggested that synchronous one-year life cycles were correlated with climate. Based on these unpublished reports, *T. planipennisi* and *S. galinae* were not recommended to be introduced south of the 40th parallel. Chapter two seeks to identify if Virginia climatic conditions are suitable for *S. galinae* or the recommended parasitoid species *S. agrili*. Chapter five seeks to vet the unpublished reports of EABs lifecycle in Virginia and North Carolina, and assess field releases of *S. agrili*, *S. galinae*, and *T. planipennisi* for recoveries & early signs of establishment.

If these two species are suitable for release in overlapping range, this will result in a novel interaction between the two congener species. *Spathius agrili* was collected from the Tiajin Province of China, whereas *S. galinae* was collected from the Primorsky Krai of Far East Russia (Yang et al. 2005, Belokobylskij et al. 2012). It is not known if their native ranges overlap, and interactions between the species have not been studied. Under laboratory rearing conditions it was anecdotally reported that *S. agrili* outcompeted *S. galinae* (J. Duan & K. Larson, personal communication). Chapter three seeks to verify these anecdoes, assessing the competition and potential multiparastisim between these two species.

Due to premtive reports of white fringe tree being a potential enemy free space for emerald ash borer (Olson and Rieske 2019), despite only *T. planipennisi* being tested. Chapter four seeks to assess the ability of *S. agrili* and *S. galinae* to parasitize EAB larve in white fringe tree under optimal rearing conditions.

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Chapter Two
Differences in early season emergence and reproductive activity between Spathius agrili (Hymenoptera: Braconidae) and Spathius galinae, larval parasitoids of the invasive emerald ash borer (Coleoptera: Buprestidae)

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Differences in early season emergence and reproductive activity between Spathius agrili (Hymenoptera: Braconidae) and Spathius galinae, larval parasitoids of the invasive emerald ash borer (Coleoptera: Buprestidae)

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Abstract

Both *Spathius agrili* and *S. galinae* are host-specific parasitic wasps introduced for biological control of emerald ash borer in North America. *Spathius agrili* is native to northeastern China and *S. galinae* comes from a more northern, colder climate in the Russian Far East. Their origin may lead to differing abilities to adapt to climate and their host in North America. We conducted both field and laboratory experiments to determine the timing of early season emergence and synchronization of each parasitoid species to their host in the U.S, and if manipulating prerelease conditions could affect emergence time. A cold acclimatization treatment prior to parasitoid emergence was assessed and compared to untreated control group reared with standard rearing protocols. Stands of naturally emerald ash borer-infested ash were sampled at two locations in Virginia throughout the experiment to determine when the parasitoid-susceptible life stage (third to fourth instar) occurred. Untreated *S. galinae* emerged approximately two weeks earlier than any other cohort, while cold acclimatized *S. galinae* emerged later than any other cohort. Emergence time of *S. agrili* was unaffected by cold acclimatization. Cold acclimatization treatment did not impact the parasitism rate of either species, nor did it have a multigenerational effects. Emergence time of the subsequent generation of *S. agrili* was delayed by cold acclimatization treatment, while *S. galinae* experienced no multigenerational effects. At Virginia field sites, susceptible EAB larvae were present during the emergence time of all four groups of parasitoids. Untreated *S. galinae* had the least overlap with any susceptible EAB larvae.

Introduction

The emerald ash borer (EAB), *Agrilus planipennis* Fairemaire, a pest of ash (*Fraxinus* spp.) trees native to Asia, was accidentally introduced to North America in the mid-1990's (Cappaert et al., 2005; Bray et al. 2011; Siegert et al. 2014). Since it was first discovered in 2002 in southeastern Detroit, Michigan and Ontario, Canada (Haack et al. 2002), this invasive beetle has established populations in over 35 US states and five Canadian provinces, killing millions of North American ash trees by 2019 (Emerald Ash Borer Information 2019). Currently, management of this invasive beetle in North America involves several options, including trunk injections of systemic pesticides, removal of infested trees, and biological control with specialized natural enemies (parasitoids) from the pest's native range (Bauer et al. 2008, Mercader et al. 2015). Because of the lack of efficient detection methods and difficulty of applying insecticides in natural forests, biological control is considered to be a critical long-term option for protection of ash stands in natural ecosystems (Duan et al. 2018).

Three hymenopteran parasitoids were introduced from northeast China, part of the pest's native range, in 2007 as biocontrol agents against EAB in the U.S. (Liu et al. 2007, Bauer et al. 2007, Bauer et al. 2008). These included two larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) (Yang et al. 2006) and *Spathius agrili* Yang (Hymenoptera: Braconidae) (Yang et al. 2005), and the egg parasitoid *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Zhang et al. 2005). To date, these three Chinese parasitoids have been released in 29 U.S. states as well as three of the five Canadian provinces invaded by EAB (Duan et al. 2018, MapBiocontrol 2019, Canadian Food Inspection Agency 2019). Releases of *S. agrili* and *T. planipennisi* occur either as mature adults, larvae, or pupae in ash bolts to be hung on infested ash trees depending on availability (USDA-APHIS/ARS/FS 2019). Recent field studies conducted in Midwest, mid-Atlantic and northeastern U.S. have shown that *T.*

planipennisi and *O. agrili* have established self-sustaining populations in many of the released areas in these regions (Duan et al. 2013, Duan et al. 2015, Davidson et al. 2016, Jennings et al. 2016). However, none of the published studies have confirmed the establishment of *S. agrili* from these regions (Hooie et al. 2015, MapBiocontrol 2019). Reasons for the failure of *S. agrili* to establish are not exactly known. Some hypotheses include a combination of biotic and abiotic factors, such as the asynchronization of adult parasitoid emergence with EAB larvae, or climatic conditions at release sites. Cold storage of mass reared adults has been attempted, which showed that a cold storage treatment at 10°C for three months or longer significantly decreased emergence, longevity, and fecundity of *S. agrili* (Gould et al. 2011).

Recently, another larval parasitoid, *Spathius galinae* Belokobylskij and Strazanac was collected in the Russian Far East, north of areas where *S. agrili* was collected in Tianjin province, China (Belokobylskij et. al. 2012, Duan et al. 2012). It was approved for release in the U.S. in 2015 (Federal Register 2015, Duan et al. 2015a, USDA-APHIS/ARS/FS 2019). Pre-release climate matching indicated that *S. galinae* is well suited to the climate of the northcentral and northeastern United States, where ash is abundant (USDA-APHIS/ARS/FS 2019, Duan et al. 2018). Releases of *S. galinae* also occur by two methods; adult release or larvae/pupae in ash bolts hung on infested ash trees (USDA-APHIS/ARS/FS 2019). In 2016, releases of *S. galinae* were made in several northeastern U.S. states, followed later by releases in the northcentral and Great Lake states (MapBiocontrol 2019). A recent field study conducted in the northeastern U.S. showed that this newly introduced EAB larval parasitoid successfully established self-sustaining populations with significantly increased abundance or density two years after the major field releases in six hardwood forests in Connecticut, Massachusetts and New York (Duan et al.

2019). However, little information is available on this parasitoid's ability to reproduce and establish in the mid-Atlantic and southern regions of the US, where EAB has expanded its range.

The two congener species, *S. agrili* and *S. galinae* share many life history traits that are promising for biological control. Both species are idiobiont larval parasitoids, paralyzing their host upon attack, have increased clutch size with increased host larval size, and show preference for third and fourth stage EAB larvae (Yang et al. 2006, 2010, Belokobylskij et al. 2012, Duan et al. 2014, Watt and Duan 2014). Differences between species arise in longevity of adults, fecundity, and sex ratio. *S. agrili* adults lived for an average of 8 wk with males living slightly longer (Gould et al. 2011), while *S. galinae* adults lived for a median 7 wk with females living slightly longer (Duan et al. 2014). Fecundity and sex ratios between these congener species differs as well. *S. agrili* has an average clutch size of 5.4 ± 0.2 eggs, and is capable of laying 51.2 eggs over her life with approximately three quarters female sex ratio (Gould et al. 2011). *S. galinae* has an between eight and 16 offspring per host, and is capable of producing up to an average 47 progeny over her life with a sex ratio (f:m) of 1.9:1 (Belokobylskij et al. 2012, Duan et al. 2014, Watt et al. 2016).

In the present study, we first determine the early-season adult emergence phenology of both these two congener species *S. agrili* and *S. galinae*, and assess a seven-day cold acclimatization as a method of delaying their emergence. Secondly, we evaluate if a seven-day cold acclimatization treatment impacts the longevity, parasitism rate, sex ratio, and/or fecundity of cold acclimatized wasps. Finally, we evaluate the subsequent generation, to ensure no multigenerational effects occurred. We hypothesized that a cold acclimatization treatment would alter the emergence time of early season adult parasitoids and affect the seasonal synchronization between parasitoid adults and suitable EAB larvae. We also hypothesized that the cold

acclimatization treatment would decrease longevity, parasitism rate, fecundity, and would have a multigenerational effect on reproduction.

Materials and Methods
Host Larvae for Fecundity and Reproduction Experiment

All EAB larvae used in the fecundity and reproduction experiments were 3rd or 4th instars reared at the USDA Beneficial Insect Introduction Unit (BIIRU) in Newark, DE. EAB larvae were reared in tropical ash, *F. uhdei* Wenz., bolts (1-2 cm diam, 20 - 25 cm length), using the methods described in Duan et al. (2012). Between three and seven larvae were reared per bolt, depending on bolt diameter and length. Larval-inoculated bolts were shipped to the Price's Fork Research Station (PFRC), in Blacksburg, VA in an insulated cooler weekly from May through September. The base of each bolt was wrapped in a paper towel soaked in distilled water, and all bolts were placed in a black plastic bag to prevent desiccation prior to use.

Early Season Emergence.

All *S. agrili* and *S. galinae* cocoons used in this experiment were reared by the USDA APHIS PPQ Biological Control Production Facility, in Brighton, MI (afterwards referred to as the "APHIS Brighton Facility"). Green ash, *F. pennsylvanica* Marshall, bolts (12-15 cm, 2-4 cm dia.) were infested with EAB that were allowed to mature until the 3rd to 4th instar. Infested bolts were then subjected to gravid females of either *S. agrili* or *S. galinae* for seven days. Two treatment groups were established: The control group reared to late instar using the standard rearing condition of 28°C used by the APHIS Brighton Facility, and a group reared at 28°C then exposed to a cold acclimatization treatment of 10°C for 1 wk as late instar. Each treatment group was established for both *S. agrili* and *S. galinae*, for a total of four cohorts of *Spathius* spp.: cold

acclimatized *S. agrili*, cold acclimatized *S. galinae*, untreated *S. agrili*, and untreated *S. galinae*. 40 bolts of each cohort were produced, then shipped to Virginia Tech.

Each bolt was placed individually in a mesh-ventilated 3.78 liter square plastic container (Plastic Grip Jug 1 Gallon S-15711 ULine Plastics) in a brick of water-soaked floral foam (Oasis®). Containers were placed outdoor in the shade under a stand of 20-yr old conifer trees at PFRC (37.212273,-80.489594) on 8 March, 2017, and a tarp was hung above them to prevent rain water accumulation in the containers. Temperature was measured constantly using a HOBO® Pro v2 temperature monitor (Fig 1.). Containers were observed twice per week until any emergence was first observed, at which point they were observed daily. All observed wasps were sexed, and a subset were used for further experiments. After emergence ended and no parasitoids emerged for 7 days, bolts were observed twice weekly for any delayed emergence until 1 October, 2017.

Early Season Longevity, Parasitism Rate, Sex Ratio and Fecundity.

After emergence, pairs of male and female wasps were taken from each cohort and placed in a new mesh ventilated container (as described above), containing one to two bolts of tropical ash infested with a total of three to seven 3rd-4th instar EAB. The bottom 1-2 cm of each bolt were placed in saturated floral foam. Clover honey was streaked on top of the mesh ventilated lid to serve as a food source for the adult *Spathius*. Containers were placed in an incubation growth chamber (I-41LL, Percival Scientific) set to the average weekly day and night temperature of Blacksburg, VA (Fig 1.), matching photoperiod, and $60 \pm 10\%$ relative humidity in order to mimic field conditions. Containers were observed daily. After one week, the mated pair were removed from the container and were placed in a new container with new bolts of EAB-infested

tropical ash. This was repeated weekly until the female *Spathius* died. In the event the male died, the female was moved alone. Longevity of adult parasitoids was determined by observing these mating pairs date of death.

After paired adults were removed from a container, the container was observed daily for progeny emergence, until seven consecutive days of no emergence. Once emergence had completed bolts were dissected using a sharp utility knife to remove only the outermost layer of bark, revealing the EAB galleries and parasitoid cocoons. The fate of each EAB larvae was scored as healthy, parasitized (with presence of parasitoid cocoons or larvae), or killed by other (unknown) factors. To determine fecundity, any unemerged parasitoid adults, pupae, and larvae were counted and added to the count of emerged parasitoids.

Subsequent Generation Emergence.

Newly emerged adults were removed from the container on the day they were observed, and sexed. Emergence time was calculated as days since EAB larvae were exposed to paired adults until emergence of the new adult *Spathius* by individual. This information was tabulated by cohort to determine parasitism rate, and time to emergence by cohort.

Subsequent Generation Parasitism Rate.

After adults emerged, they were paired within cohort, and placed in a mesh ventilated plastic container containing one to two bolts of EAB-infested tropical ash (as described above). As with the previous generation, after one week the mated pair was removed, and placed in a new container with new bolts of EAB-infested tropical ash. This was repeated weekly until the female *Spathius* died. Once emergence had completed bolts were dissected, the fate of each EAB larvae was scored, and any unemerged parasitoids were counted (as described above).

Field Sampling of EAB Larvae

To determine EAB larval life stage in the field locally, two sites were located in Virginia, at Douthat State Park, Millboro, VA (37.89409, -79.8015) and Mid-County Park, Christiansburg, VA (37.17123, -80.4119) (MCP). Each site was visited once per month during the summer of 2017. One to two symptomatic trees were selected, felled and debarked in 1 m sections using methods described in USDA–APHIS/ARS/FS (2019). All EAB larvae present were collected and their lifestage was assessed. All larvae were brought back to the PFRC and observed for 3 wks for any endoparasitoid emergence.

Statistical Analyses

The nonparametric Kaplan-Meier survival platform (Kaplan and Meier 1958) was used to calculate the median emergence time and 95% confidence intervals for all four cohorts. Days since exposure to Virginia field conditions was used as the time to event, and the count of daily emergence used as the frequency of the event. This was performed following the protocol outlined in Duan et al. (2011). A log-rank and Cox proportional hazard test (based on the Kaplan-Meier survival platform) were performed to determine significance in differences between pairs of treatments (*S. agrili* cold-treated and untreated *S. agrili*, *S. galinae* cold-treated and untreated *S. galinae*, *S. agrili* cold-treated and *S. galinae* cold-treated, untreated *S. agrili* and untreated *S. galinae*). If no difference was found between cold acclimatization treatment and untreated, then treatment groups were pooled for comparisons between *S. agrili* and *S. galinae*. Due to multiple comparisons across all groups of cohorts, then four pairwise comparisons, the Bonferroni correction was used and the type I error rate (alpha) was set to 0.1.

Longevity of adult parasitoids was calculated in weeks using a one-way ANOVA to determine differences between treatments within each species, and between species. Due to a low number of individuals followed through their lifetime, cohorts were pooled by species, with

the assumption that cold acclimatization treatment had no effect on longevity. Sex ratio was determined by calculating the proportion of female to male progeny, any replicates with only male offspring were removed from the analysis due to haplodiploidy. The weekly replicates were used to determine parasitism rate by dividing the number of parasitized larvae by the total larvae provided, and fecundity based on a count of the parasitoid progeny per individual female. Data were then analyzed in a one-way ANOVA to determine differences between cold acclimatization treatment and untreated within species.

For assessment of the subsequent generation, The Kaplan-Meier survival platform was used again to determine differences between pairs of cohort treatments, as was performed for the previous generation emergence. Median emergence time and 95% confidence intervals were calculated through the survival platform. Log-rank and Cox proportional hazard test were performed between pairs to determine significance. Multigenerational effects on parasitism rate were assessed using a count of EAB larvae fate analyzed by a one-way ANOVA. Again, due to multiple comparisons across all groups of cohorts, then four pairwise comparisons, the Bonferroni correction was used and the type I error rate (alpha) was set to 0.1 for emergence analysis.

All data were analyzed in SAS JMP Pro 14.0.0 (SAS Institute 2019).

Results
Early Season Emergence

Emergence of cold acclimatized *S. agrili* began on 2 May, and completed emergence on 14 June. Untreated *S. agrili* emergence began on 5 May, and completed emergence on 16 June. Emergence of both cohorts of *S. galinae* began on 29 April and lasted until 16 June when emergence ended (Table 1 & Fig. 2). There were significant differences in emergence times

among the four cohorts (Log-Rank χ^2=128.038, df = 3, P<0.0001) (Table 1 & Fig. 2). Pairwise

comparisons showed that cold acclimatization treatment significantly delayed the emergence

time of *S. galinae* compared to untreated *S. galinae* (Log-Rank χ^2=91.856, df = 1, P<0.0001)

(Table 1 & Fig. 3). Emergence of untreated *S. agrili* was significantly later than untreated *S.*

galinae (Log-Rank χ^2=36.193, df = 1, P<0.0001) (Table 1 & Fig. 3). In contrast, cold

acclimatized *S. agrili* emerged earlier than cold acclimatized *S. galinae* (Log-Rank χ^2=20.723, df

= 1, P<0.0001) (Table 1 & Fig. 3). Cold acclimatization treatment did not affect emergence time

of *S. agrili* compared to untreated *S. agrili* (Log-Rank χ^2=5.01, df = 1, P=0.0252) (Table 1 &

Fig. 3).

Early Season Longevity, Parasitism Rate, Sex Ratio, and Fecundity

Both *S. agrili* and *S. galinae* cohorts were pooled regardless of treatment due to limited

replicates. A total of 21 pairs of *S. galinae* were set up, while a total of 10 pairs of *S. agrili* were

set up. Longevity did not differ between *S. agrili* and *S. galinae* (Table 2). Cold acclimatization

treatment did not affect parasitism rate for either *S. agrili* or *S. galinae* (Table 2). Due to lack of

differences among treatments, all treatments were pooled by species and no difference was

detected between parasitism rate of *S. agrili* and *S. galinae* (Table 2). Cold acclimatization

treatment did not affect sex ratio for either *S. agrili* or *S. galinae* (Table 2). When groups were

pooled regardless of treatment, sex ratio of *S. agrili* did not differ from *S. galinae.* It also did not

affect fecundity for either *S. agrili* or *S. galinae* (Table 2). When groups were pooled regardless

of treatment, fecundity of *S. agrili* was significantly greater than *S. galinae* (Table 2).

Subsequent Generation Emergence

There was an overall significant difference in subsequent generation emergence time among the

four cohorts (Log-Rank χ^2=92.9856, df = 3, P<0.0001) (Table 3 & Fig. 4). Pairwise comparisons

showed that the median emergence time of cold acclimatized *S. agrili* progeny was significantly longer than that of untreated *S. agrili* progeny (Log-Rank χ^2=15.5906, df = 1, P<0.0001) (Table 3 & Fig. 5). In contrast, there was no significant difference in emergence timing between cold acclimatized *S. galinae*, and untreated *S. galinae* (Log-Rank χ^2=1.30, df = 1, P=0.254) (Table 3 & Fig. 5).

Furthermore, there were significant differences in emergence time between the two parasitoid species. Cold acclimatized *S. agrili* had peak emergence significantly earlier than cold acclimatized *S. galinae* (Log-Rank χ^2=10.88, df = 1, P=0.0010) (Table 3 & Fig. 5). Untreated *S. galinae* progeny began emergence earlier than untreated *S. agrili* progeny, although both completed emergence in similar lengths of time (Log-Rank χ^2=17.84, df = 1, P<0.0001) (Table 3 & Fig. 5).

Subsequent Generation Parasitism Rate

There was no significant multigenerational effect of cold acclimatization treatment on the lifelong parasitism rate of the subsequent generation for either *S. agrili* (F = 0.1935, df = 1,74, P = 0.6613) or *S. galinae* (F = 0.0063, df = 1,56, P = 0.9368). When groups were pooled regardless of treatment parasitism rates did not differ between species (F = 0.4394, df = 1, 131, P = 0.5086) (Table 4).

EAB Larval Phenology in the Field

Very few EAB larvae were available in June, and at Douthat State Park. The majority of EAB sampled were still pupae. Total EAB larvae increased in July, with 3rd and 4th instar increasing to their maximum abundance. In August, the number of EAB larvae declined although some larvae

were still present (Fig. 6). At MCP, a similar pattern was observed, but with much lower overall numbers.

Discussion

Early season emergence of *S. agrili* and *S. galinae* differed under Virginia field conditions. The two species displayed differential responses to cold acclimatization. Median emergence of untreated *S. galinae* occurred on May 15th, approximately two weeks (11.5 – 14.5 d) earlier than *S. agrili*, and 25 d earlier than cold acclimatized *S. galinae*. The emergence time of *S. agrili* was unaffected by the cold acclimatization period, median emergence occurred on May 26th (cold acclimatized) and May 29th (untreated). The last cohort to emerge was cold acclimatized *S. galinae,* median emergence occurred on June 9th. With an average of 4 to 5 wk lifespan, both *S. agrili* and *S. galinae*, regardless of treatment, overlapped with 3rd and 4th instar EAB at both sampled field sites. Under our experimental conditions, *S. agrili* and cold acclimatized *S. galinae* emerged later in the season when more 3rd and 4th instar EAB are likely to be found in field conditions. Untreated *S. galinae* had very little overlap with available EAB larvae due to their earlier peak emergence.

Both *S. agrili* and *S. galinae* had shorter adult longevity than literature suggested. Both species of *Spathius* have similar adult longevity, 4.40-5.22 wks in this experiment, lower than their seven to eight wk longevity under laboratory rearing conditions (Gould et al. 2011, Duan et al. 2014). This could be due to differences in testing methodologies, or an effect of Virginia climate conditions.

Cold acclimatization had multigenerational effects on emergence time of *S. agrili*, but not on *S. galinae*. The subsequent generation of *S. agrili* emerged two days earlier when the parent generation was cold acclimatized. Emergence of *S. galinae* progeny was unaffected by cold

acclimatization. Cold acclimatization treatment did not affect longevity, sex ratio of progeny, or parasitism rate in either species. Differences were observed between the two species when cold acclimatized and untreated cohorts were pooled within species. The fecundity of *S. galinae* was lower than *S. agrili* which is consistent with previous literature of the both species (Gould et al. 2011, Belokobylskij et al. 2012, Duan et al. 2014, Watt et al. 2016).

Depending on the availability of EAB larvae at a given site, a cold treatment could be used to improve phenological synchronization between the phenology of the parasitoids and their host. With a better understanding of EAB phenology at individual sites, this early season release method could allow for infested bolts to be shipped to field sites earlier in the season, and aid in mass rearing. In the time since this experiment was completed, further climate matching has been completed on *S. agrili* and *S. galinae*, based on EAB overwintering lifestage across its invasive range (USDA-APHIS/ARS/FS 2019). The USDA now suggests release of *S. agrili* only south of the 40th parallel due to its failure to establish in the northern US, and *S. galinae* only north of it due to early spring larval availability (USDA-APHIS/ARS/FS 2019). As *S. galinae* establishes in the northern United States and Canada where *S. agrili* did not, each will contribute to the control of EAB as part of a complex of biological control agents in different geographical regions (Duan et al. 2019). As EAB biological control guidelines are updated with new information on EAB larval development parasitoid release timing is becoming more precise, and less likely to miss the critical period of larval availability. Therefore this method of early season release would not be suitable for *S. galinae* without a cold treatment. *S. agrili* could be released this way, because emergence occurred when 3rd and 4th instar EAB were present at both sites regardless of treatment.

Further studies could examine these patterns using true overwintering conditions, or in whole tree scenarios. Whole tree sampling in field scenarios should give the true representation of the overwintering survival and early season emergence of both species, and should be conducted in the future. Additional work should focus on longer cold exposures to simulate natural winters, a more varied temperature regime to observe impacts across a range of climate change conditions, as well as geographical ranges, and larger sample sizes if possible.

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Tables and Figures.
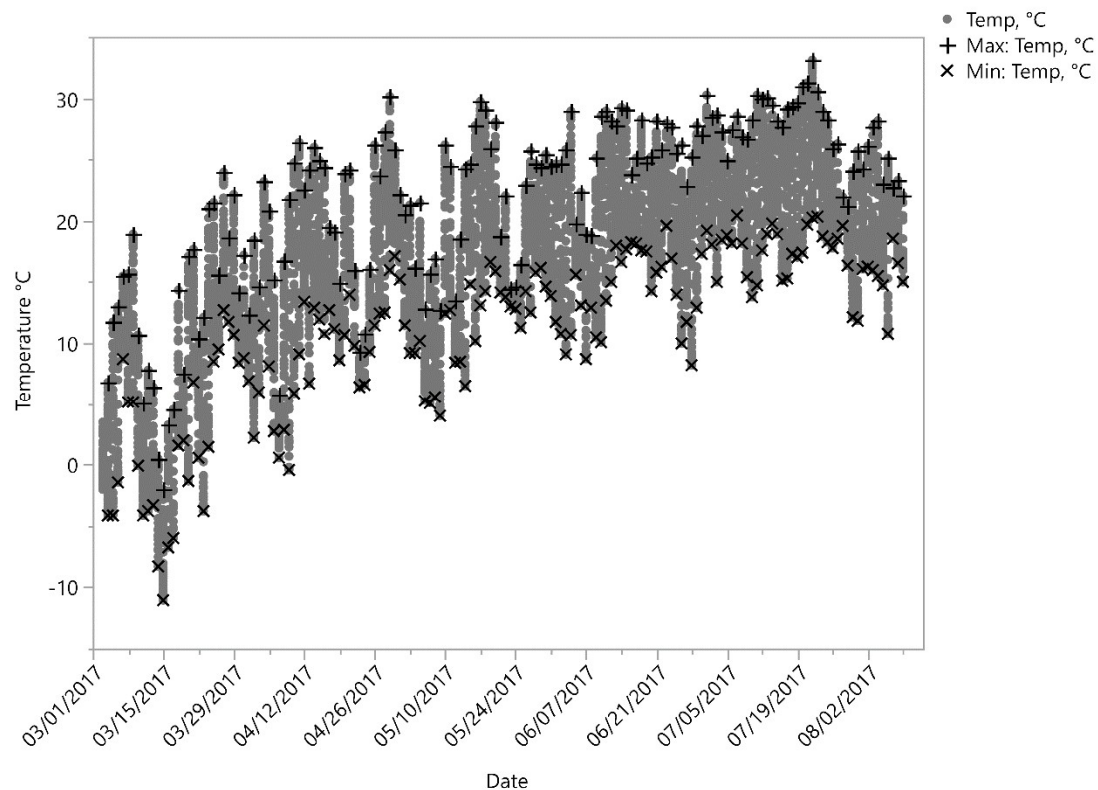
Figure 1. Daily temperature data from the hemlock stand where bolts were placed at Prices Fork Research Station, Blacksburg, VA. Temperature was recorded every 30 minutes. Daily high and low temperatures are indicated by + and x, respectively.
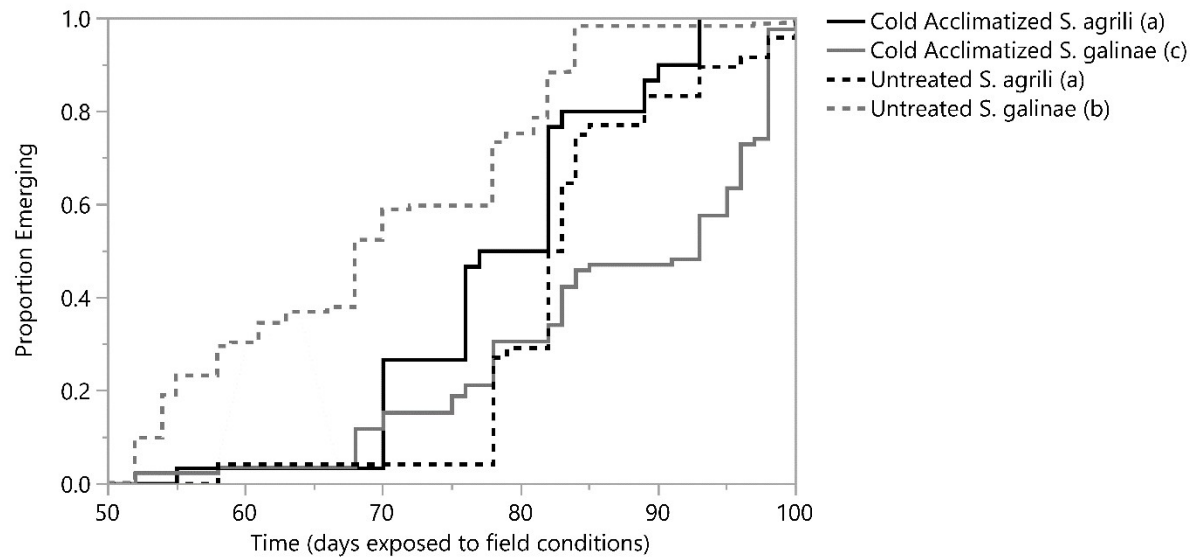
Figure 2. Daily emergence of *S. agrili* (shown in black) and *S. galinae* (shown in grey) adults over time. Cold acclimatization and untreated control groups Significant difference from pairwise comparisons is indicated by letter grouping in the legend (Log-Rank, P<0.01).
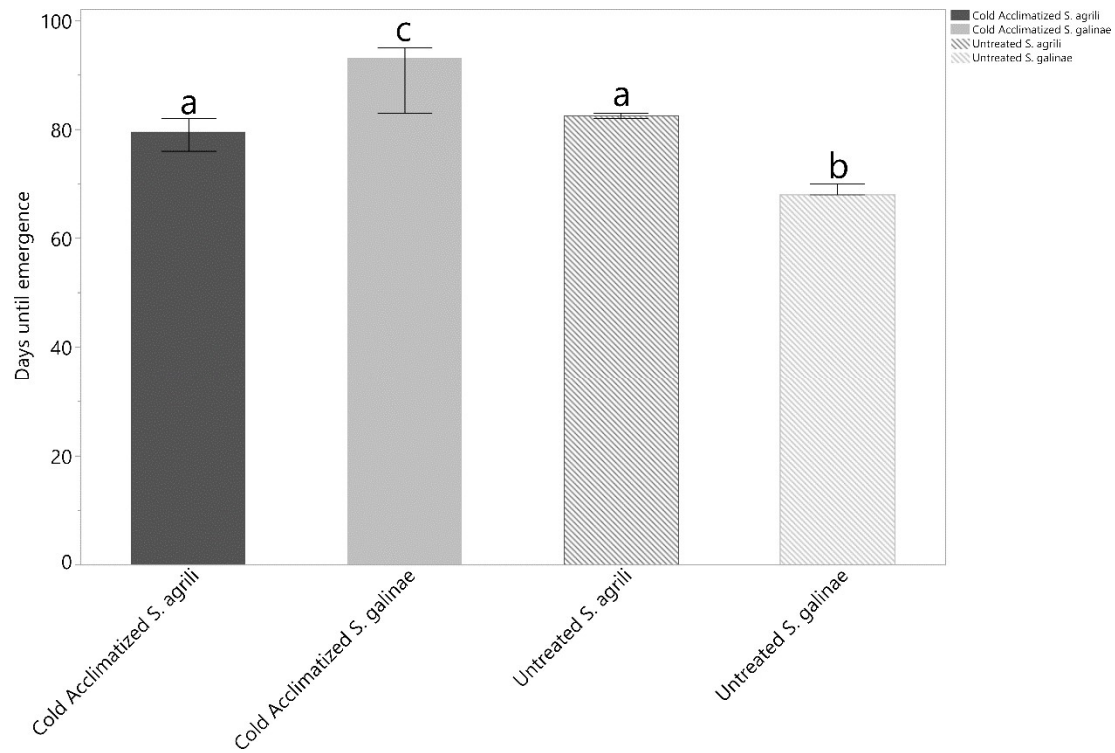
Figure 3. Median days to emergence for both cohorts of S. agrili and S. galinae (95% C.I. for the Kaplan-Meier survival curve). Significant difference is indicated by letter grouping (Log-Rank, P<0.01).

Table 1. **The summary of the Kaplan-Meier survival curve for early season emergence of** *S. agrili* **and** *S. galinae* **under cold acclimatization and untreated conditions.**

Group	Adults emerged	Median Time (d)	Median Time (calendar date)	Lower 95%	Upper 95%	25% Emerged	75% Emerged
Cold Acclimatized *S. agrili*	30	79.5a	May 26th	76	82	70	82
Cold Acclimatized *S. galinae*	85	93c	June 9th	83	95	78	98
Untreated *S. agrili*	48	82.5a	May 29th	82	83	78	84.5
Untreated *S. galinae*	381	68b	May 15th	68	70	58	79
Combined	544						

a. Significant differences in median emergence time are indicated by lettered grouping next to median time

Comparison between all groups indicated significant differences between groups.

Pairwise analysis between *S. galinae* treatments indicated cold acclimatization significantly delayed emergence

Pairwise analysis between untreated *S. agrili* and untreated *S. galinae* indicated *S. agrili* untreated emerged significantly later than *S. galinae* untreated

Pairwise analysis between cold acclimatized *S. agrili* and cold acclimatized *S. galinae* indicated that cold acclimatized *S. agrili*

emerged significantly later than cold acclimatized *S. galinae*.

Pairwise analysis between *S. agrili* treatments indicated no difference.

b. Groups without a factor (species or treatment) were not compared.

Table 2. The longevity, parasitism rate, sex ratio, and fecundity of the early season emergence of all four cohorts by treatment and pooled by species where no difference occurred.

Cohort	Longevity (Wks) [a]	Parasitism Rate (% attacked) [b]	Sex Ratio (% Female Progeny) [c]	Fecundity (Progeny/Adult) [d]
Cold acclimatized *S. agrili*	-	35.67 ± 7.30	59.94 ± 14.26	35.00 ± 14.97
Untreated *S. agrili*	-	28.48 ± 5.32	60.54 ± 12.75	33.00 ± 10.59
Cold acclimatized *S. galinae*	-	26.56 ± 4.64	59.51 ± 8.39	19.00 ± 3.99
Untreated *S. galinae*	-	20.86 ± 6.25	49.07 ± 12.51	14.40 ± 5.35
S. agrili (pooled)	5.22 ± 0.79	30.97 ± 4.05	60.28 ± 8.94	33.67 ± 5.87
S. galinae (pooled)	4.40 ± 0.61	24.54 ± 3.94	56.27 ± 7.04	17.35 ± 4.70

Pairwise analyses were only performed selectively based on biologically significant comparisons (ANOVA P<0.05).

a. Pairwise analysis indicated no significant difference (F = 0.6793; df = 1, 23; P = 0.4187)

b. Pairwise analysis between Cold acclimatized *S. agrili* and untreated indicated no difference (F = 0.6343; df = 1, 71; P = 0.4285).

   Pairwise analysis between Cold acclimatized *S. galinae* and untreated indicated no difference (F = 0.5362; df = 1, 75; P = 0.4663).

   Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated no difference (F = 1.2940; df = 1, 147; P = 0.2572).

c. Pairwise analysis between Cold acclimatized *S. agrili* and untreated indicated no difference (F = 0.0010; df = 1, 17; P = 0.9749).

   Pairwise analysis between Cold acclimatized *S. galinae* and untreated indicated no difference (F = 0.4802; df = 1, 28; P = 0.4943).

   Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated no difference (F = 0.1238; df = 1, 45; P = 0.7266).

d. Pairwise analysis between Cold acclimatized *S. agrili* and untreated indicated no difference (F = 0.0119; df = 1, 8; P = 0.9162).

   Pairwise analysis between Cold acclimatized *S. galinae* and untreated indicated no difference (F = 0.4758; df = 1, 13; P = 0.5034).

   Pairwise analysis between *S. agrili* (pooled) and *S. galinae* (pooled) indicated significant difference (F = 4.7071; df = 1, 22; P = 0.0417).
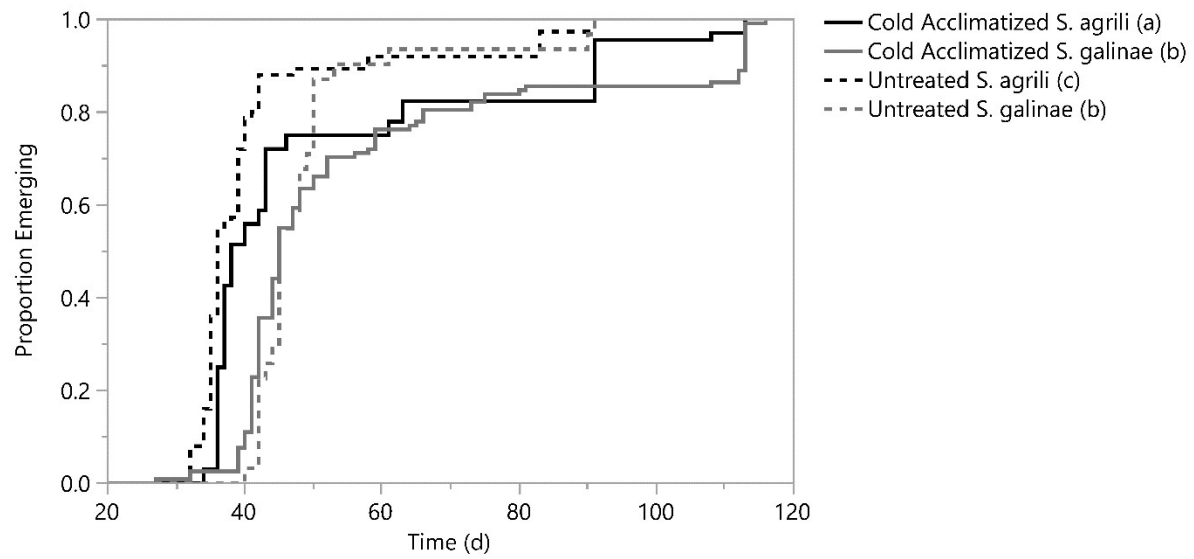
Figure 4. Daily emergence of all subsequent generation cohorts. *S. agrili* (shown in black), *S. galinae* (shown in gray), Cold-treated (shown in solid lines), and untreated (shown in dashed lines). Significant difference is indicated by letter grouping in the legend, (Log-Rank, P<0.01).
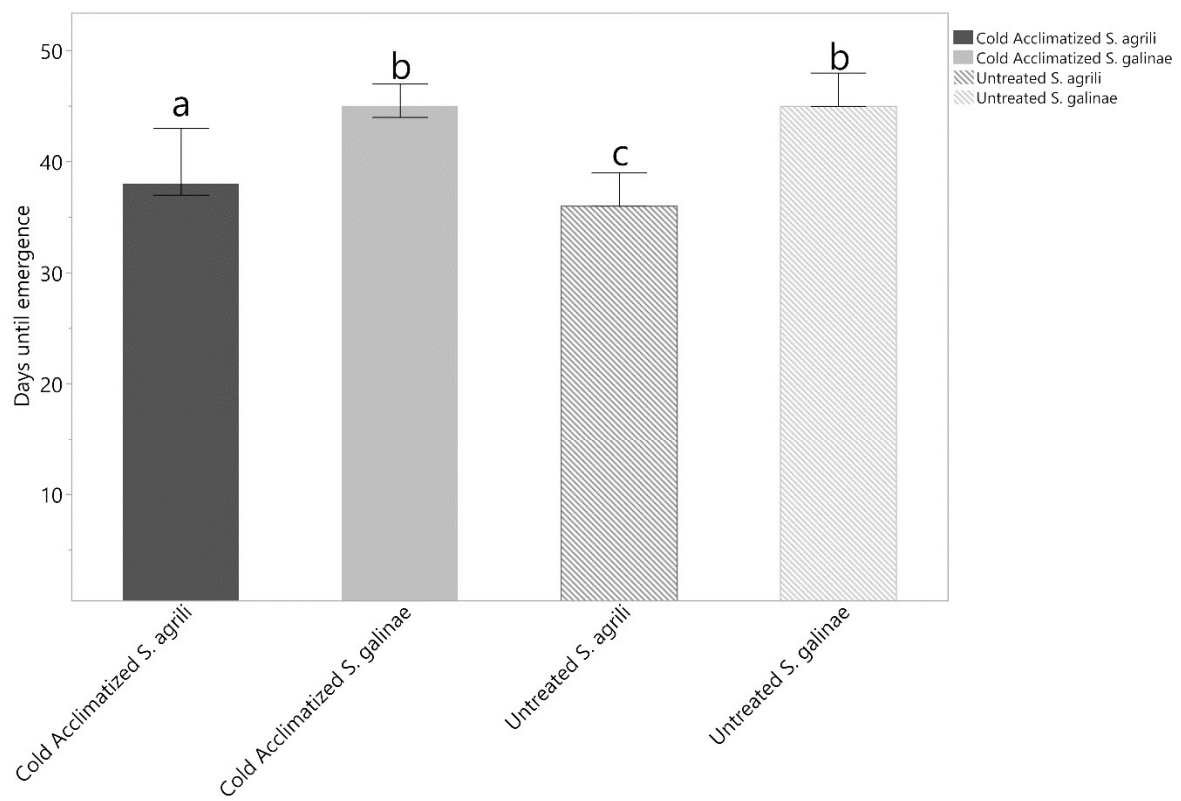
Figure 5. Median days to emergence for the subsequent generation of both cohorts of *S. agrili* and *S. galinae* (95% C.I. for the Kaplan-Meier survival curve). Significant difference is indicated by letter grouping (Log-Rank, P<0.01).

Table 3. Subsequent generation (progeny) emergence of *Spathius agrili* and *Spathius galinae* from parents under cold acclimatization and untreated conditions from the Kaplan-Meier survival curve.

Group	Adults Emerged	Median Time (d)	Lower 95%	Upper 95%	25% Emerged	75% Emerged
Cold Acclimatized *S. agrili*	68	38a	37	43	36.5	53.5
Cold Acclimatized *S. galinae*	118	45b	44	47	42	59
Untreated *S. agrili*	75	36c	36	39	35	40
Untreated *S. galinae*	31	45b	45	48	43	50
Combined	292					

a. Significant differences in median emergence time are indicated by lettered grouping next to median time (Log-Rank, $P<0.01$).

b. Groups without a factor (species or treatment) were not compared.

Table 4. The parasitism rate of the subsequent generation of *S. agrili* and *S. galinae*. No groups differed when compared using pairwise ANOVA (P<0.01).

Cohort	Parasitism Rate (% attacked)
S. agrili (pooled)	23.52 ± 3.67
Cold acclimatized *S. agrili*	22.01 ± 4.97
Untreated *S. agrili*	25.16 ± 5.17
S. galinae (pooled)	27.23 ± 4.21
Cold acclimatized *S. galinae*	26.66 ± 8.35
Untreated *S. galinae*	27.45 ± 5.22
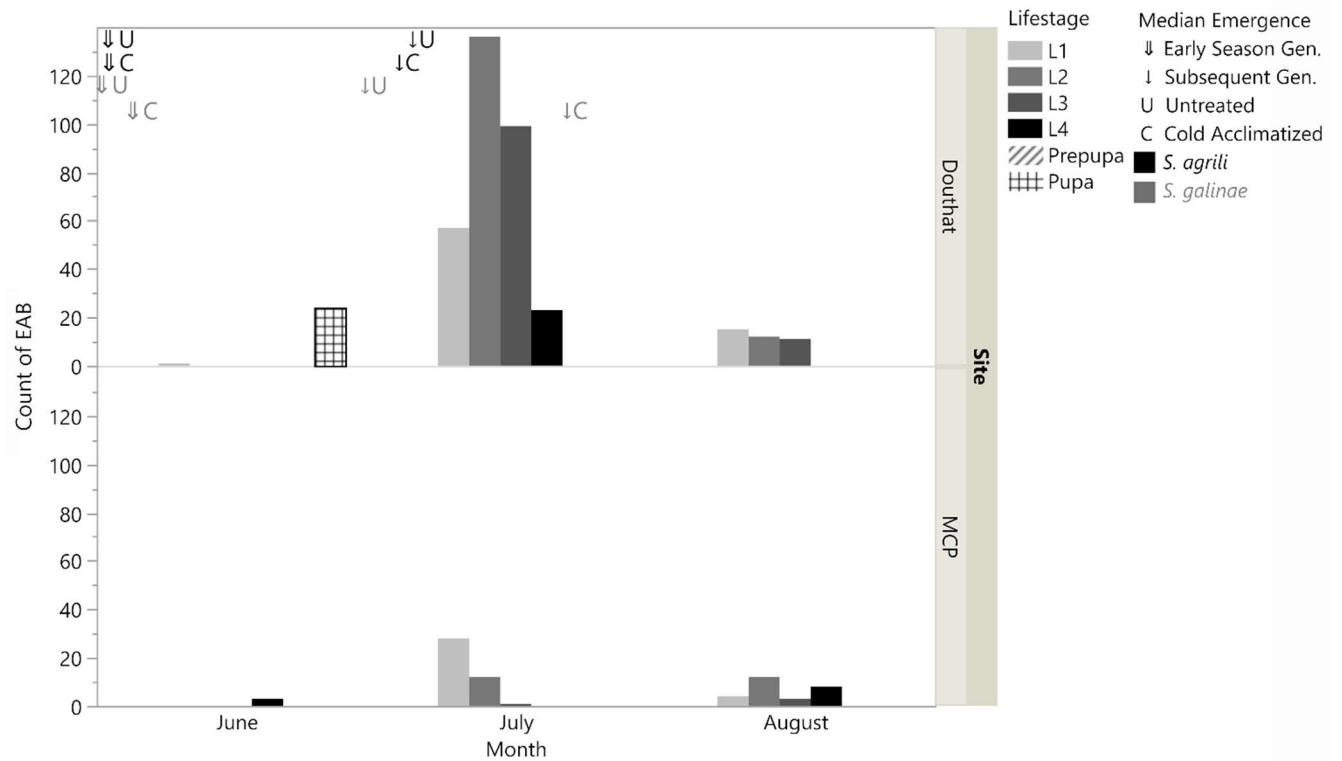
Figure 6. Sum of all EAB larvae sorted by lifestage from monthly sampling at each field site. Sampling at Douthat took place on 15 June 2017, 13 July 2017, and 16 August 2017. Sampling at MCP took place on 2 June 2017, 11 July 2017, and 14 August 2017. Median parasitoid emergence date is indicated above EAB larval count. Subsequent generation placement is based on the assumption that the parent generation emerges at the median emergence time, and there is no preoviposition period of the parent generation. Placement is an approximation, and is not to scale.

Chapter Three
Interspecific competition between idiobiont larval parasitoids of emerald ash borer

Abstract

New associations between parasitoid species is common in biological control, and interactions range from coexistence to competitive exclusion. *Spathius agrili* and *Spathius galinae* are two host-specific idiobiont larval parasitoids of the invasive emerald ash borer *Agrilus planipennis* that do not overlap in their native ranges. We conducted laboratory experiments to determine the results of competition between these two species. Competition between parasitoid larvae on a single host (intrinsic competition) and competition between adult parasitoids for oviposition sites (extrinsic competition) were evaluated. Successful multiparasitism did not occur in any trial, and intrinsic competition was determined by exposure order. Extrinsic competition favored *S. agrili*, but *S. galinae* was not excluded. Competition lowered parasitism rates for both species, but overall parasitism was not lowered.

Key Words: *Emerald Ash Borer, Competition, Novel interactions, Biocontrol, Parasitoids*

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Consent to participate. Not applicable.

Consent for publication. Not applicable.

Availability of data and material. Available upon request.

Code availability. Not applicable

Introduction

Interspecific competition in parasitoids can occur intrinsically between immature parasitoids on a

host, or extrinsically between adults searching for similar host resources (Godfray 1994, Mills

2006, Wang et al. 2008). Multiple species of parasitoids are often introduced in a classical

biological control program for an invasive pest (Smith 1929, Myers et al. 1989, Hajek 2004).

These parasitoids are selected for a high degree of host specificity with the invasive pest, and a

narrow host range (Debach and Rosen 1991). Even with a high degree of host specificity,

classical biological control can also result in new associations between parasitoid species that never interacted in their respective native ranges (Hajek 2004). Outcomes of these novel interactions can range from competitive exclusion, to coexistence, or result in failure of establishment of introduced natural enemies (Collier and Hunter 2001, Hajek 2004). Direct and apparent competition can also cause the exclusion of a species from a suitable habitat, especially when their niche, or host range is narrow (DeBach 1966, Mills 2006, Wang et al. 2008).

Emerald ash borer (EAB) *Agrilus planipennis* Fairimare (Coleoptera: Buprestidae) is an invasive beetle accidently introduced to Michigan in the 1990's from Asia where it is a native pest of ash (*Fraxinus* spp.) (Cappaert et al. 2005, Bray et al. 2011, Siegert et al. 2014). Since its introduction, EAB has caused widespread ash mortality across over 35 US states and five Canadian provinces (Emerald Ash Borer Information 2019). Chemical control options exist for residential or high value trees, but are not suitable for forest settings or ecosystem level control (Mercader et al. 2015). Four hymenopteran biological control agents of the emerald ash borer have been approved for release and introduced; two larval idiobiont exoparasitoids, *Spathius agrili* Yang (Hymenoptera: Braconidae) (Yang et al. 2005) and *Spathius galinae* Belokobylskij and Strazanac (Hymenoptera: Braconidae) (Belokobylskij et al. 2012); one larval koinobiont endoparasitoid, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) (Yang et al. 2006); and one solitary egg parasitoid, *Oobius agrili* Zhang (Hymenoptera: Encyrtidae) (Zhang et al. 2005). The two congener species, *S. agrili* and *S. galinae* share many life history traits that could lead to competitive interference between species. Both species are idiobiont larval parasitoids, paralyzing their host upon attack, and show preference for third and fourth instar EAB (Yang et al. 2006, 2010, Belokobylskij et al. 2012, Duan et al. 2014, Watt and Duan 2014).

Since initial approval, these parasitoids have been released in over 28 states and three Canadian provinces (Duan et al. 2018, MapBiocontrol 2019, Canadian Food Inspection Agency 2019). Currently *T. planipennisi* and *O. agrili* have established self-sustaining populations in areas of the Midwest, mid-Atlantic, and northeastern US (Duan et al. 2013, Davidson and Rieske 2016, Jennings et al. 2016). No published study has reported establishment of *S. agrili* at any release site (MapBiocontrol 2019). *S. galinae* has established abundant self-sustaining populations at several sites in the northeastern US two years post-release, but it is too early to evaluate its establishment and impact in other recently released regions (Duan et al. 2019).

Prior studies have addressed competition for larval resources between *T. planipennisi* and *S. agrili* or *S. galinae*, which indicated that competition would likely be a minimal factor towards establishment in either case (Yang et al. 2012, 2013). Emerald ash borer larvae parasitized by *S. agrili* or *S. galinae* are susceptible to multiparasitism by *T. planipennisi* for 48 h after oviposition (Yang et al., 2012, 2013, Wang et al. 2015). Competition between *S. agrili* and *S. galinae* adults had been observed anecdotally at the USDA ARS Beneficial Insect Introduction Unit (BIIRU) in Newark, DE, which indicated a strong response favoring *S. agrili* (JJD unpublished data).

In the present study, we evaluate the potential competition either via interactions between their larvae on the same parasitized hosts (intrinsic competition) or via multiparasitism of the same host larva by adult parasitoids (extrinsic competition). Specifically, we aim to determine (1) if either species of *Spathius* can distinguish between healthy and parasitized hosts, (2) if multiparasitism can occur, and (3) if adult parasitoids compete for the same host larva for oviposition.

Materials and Methods
Host larvae and Substrate

All EAB larvae used in the competition experiments were 4th instar reared by the USDA ARS BIIRU in Newark, DE. Larvae were reared from eggs in tropical ash, *Fraxinus uhdei* Wenz., bolts (1 – 2 cm diam, 20 – 25 cm length) using the methods described in Duan et al (2012). In order to standardize larval density, and ensure an exact parasitoid to host ratio, 4th instar EAB were reared in tropical ash, dissected out live, then individually inserted into new bolts cut from green ash trees, *Fraxinus pennsylvanica*, Marshal. Bolts of green ash (1 – 2 cm diam, 15 cm length) were cut from naturally grown trees at the USDA BIIRU. A 3 – 4 cm flap of outer bark was peeled from each bolt, and an artificial EAB gallery was carved into the phloem tissue using a 3 mm gouge chisel (Palm Block Size #11, ChippingAway). A single larva was then inserted into each artificial gallery and covered with the bark flap, which secured with two thin Parafilm strips. The bottom 1 cm of each bolt was wrapped in tightly wound paper towel and secured in a small 113 ml sample cup (4 oz Medline Polypropylene Specimen Container, Medline) filled with saturated rock wool (Rockwool) using Parafilm to prevent desiccation. Inserted larvae were left to feed for 24 h and then checked for feeding activity prior to use in the experiments. Larvae that did not show any feeding activities 24 h after insertion were discarded. All competition trials were conducted in a clear butyrate tube (63 mm diam, 20 – 40 cm height, Thermoplastic Processes), which was friction-fit over the plastic sample cup containing the artificially infested bolt.

Adult Parasitoids

All adult parasitoids used in this experiment were naïve gravid females 2 – 3 wks post-emergence in order to maximize chances of parasitism. *Spathius galinae* were reared by the BIIRU in Newark DE according to procedures described in Duan et al. (2014), whereas *S. agrili*

were reared by the USDA APHIS PPQ Biological Control Production Facility in Brighton, MI (afterwards referred to as "APHIS Brighton Facility") according to method described in Gould et al. (2011). *S. agrili* adults was shipped overnight in an insulated cooler and acclimatized to experimental conditions for at least 48 h prior to use.

Intrinsic Competition

We used sequential exposures to evaluate intrinsic (larval) competition between *S. agrili* and *S. galinae*. For each experiment five groups were established based on four staggered time-between-exposures to the *S. galinae*, with a non-parasitized control group. To ensure a high rate of parasitism in a short exposure time a 10:1 parasitoid host ratio was used, based on the rearing methods outlined in Gould et al. 2011, and Duan et al. 2014. EAB larvae were exposed to *S. agrili* for 24 h. These parasitized larvae were divided into four treatment groups, based on staggered exposure to two *S. galinae* adults: 1) immediate exposure to *S. galinae*, 2) a one day delay, 3) a three day delay, and 4) a five day delay. At the corresponding time, a control group of non-parasitized EAB were exposed to *S. galinae*. Adults were left for seven days, then removed from the arena. A second round of this experiment was performed with the order of exposure reversed, exposing an EAB larva to 10 *S. galinae* for 24 h, exposure to two *S. agrili* at staggered times for one week. Emergence was observed for eight weeks.

Once eight weeks had passed, bolts were dissected using a sharp utility knife to peel the outer bark. Larval remains were dissected under a dissecting microscope for evidence of multiparasitism (parasitoid larvae). Each EAB larva was scored as alive (escaped parasitism), dead (unknown causes), parasitized by *S. agrili,* parasitized by *S. galinae*, or parasitized by both.

Extrinsic Competition

We used simultaneous exposures to evaluate extrinsic (adult) competition between *S. agrili* and *S. galinae*. Three groups of parasitoid exposures were created: 1) A control group of two *S. agrili*, 2) a control group of two *S. galinae*, 3) the competition group containing one *S. agrili* and one *S. galinae*. Each group was provided with one inserted EAB larvae in a green ash bolt for seven days. At the end of seven days the adults were removed, and each arena was observed three times per week for eight weeks. Once eight weeks had passed, bolts were dissected, and each EAB larvae was scored, using the methods described above.

Statistical Analysis

EAB fate during the intrinsic competition were analyzed using two sequential groupings, both using nominal logistic analyses. 1) Comparisons between all treatment groups and the unparasitized control group to determine if any treatment influenced parasitism success. 2) Comparisons between each treatment group to determine if the number of days until exposure by the second species influenced parasitism success.

EAB fate during the extrinsic competition was analyzed in four parts, all using logistic regression analyses: 1) Comparisons between the competition group and both control groups to determine differences in species-specific parasitism success; 2) Comparisons between the competition group and the *S. agrili* control group to determine differences in *S. agrili* parasitism success; 3) Comparisons between the competition group and the *S. galinae* control group to determine differences in *S. galinae* parasitism success; and 4) Comparisons between the

competition group and both control groups to determine parasitism success regardless of species. Data were analyzed using SAS JMP Pro 14.0.0 (SAS Institute 2019).

Results

Intrinsic Competition

In both exposure orders there was a significant difference in the rate of parasitism by the second species between pre-exposed larvae and control larvae (Fig. 1). In the exposure to S. agrili followed by S. galinae trials, exposure to S. agrili almost completely excluded S. galinae (df = 8; $P < 0.0001$) (Table 1). Number of days until exposure to S. galinae did not influence parasitism rate (df = 2, $P = 0.6310$). Exposure to S. galinae followed by S. agrili decreased parasitism rate by S. agrili, but it was still able to parasitize larvae regardless of days since exposure to S. galinae (df = 1; $P < 0.005$) (Table 1). Number of days until exposure to S. agrili did not influence the outcome (df = 3, $P = 0.1015$).

Extrinsic Competition

Species-specific success was impacted by competition between *S. agrili* and *S. galinae* adults (df = 4, $P < 0.0001$) (Fig 3.). Competition lowered parasitism success by *S. agrili* compared to *S. agrili* only control (df = 3, $P = 0.0345$) (Table 2). Competition also lowered parasitism success by *S. galinae* compared to *S. galinae* only control (df = 3, $P = 0.0008$) (Table 2). Competition did not affect the overall parasitism rate of EAB larvae (df = 1, $P = 0.9794$) (Table 2).

Discussion

Extrinsic competition occurred between *S. agrili* and *S. galinae*, lowering species-specific parasitism of both species. There was no significant difference in success of either species, and

neither species outcompeted the other. Intrinsic competition did not occurred, because pre-exposure to a parasitoid prevented any parasitism by a subsequent species. In previous studies of competition between either *S. agrili* or *S. galinae* and *T. planipennisi*, an endoparasitoid of EAB, multiparasitism occurred rarely in a brief window after initial attack (Yang et al. 2012, 2013). Multiparasitism between these species was possible due to *T. planipennisi* not paralyzing larvae after oviposition, and *Spathius* relying on feeding vibrations to locate hosts (Ulyshen et al. 2011, Yang 2012, 2013). Because both species of *Spathius* paralyze their host larva upon oviposition, competition was unlikely to occur, and never occurred in our experiments. Based on this, intrinsic competition between *S. agrili* and *S. galinae* is extremely unlikely to occur.

When extrinsically competing for a single EAB larva, both species together were less successful than when they were alone. Both species were successful at equal rates, and overall parasitism rate was not negatively affected by competition. A nominally higher ratio of *S. agrili* succeeded, although this difference was not statistically significant. This is a positive result for EAB biological control, and indicates that these two species can coexist in regions where they overlap. Neither species completely outcompeted the other, even in single larva experiments where competition could easily exclude one or the other. A limited number of trials were also performed using EAB larvae inserted into white fringe tree, a novel host of EAB. Due to limited plant material the number of replicates was not statistically significant. In these competition trials both *S. agrili* and *S. galinae* were successful at parasitizing EAB larvae.

Competition between congener parasitoids could result in competitive exclusion from a number of advantages. The outcome of competition could be determined by the early-acting-superiority (Wang et al. 2003), life history strategies (Wang and Messing 2004), or host-feeding on parasitized hosts (Collier and Hunter 2001). In the case of *S. agrili* and *S. galinae* it appears that

the competitive advantage of the faster acting species dictated the winner of the competition. In other wood boring beetle systems, niche partitioning mitigated negative competitive effects (Hanks et al. 2001). Unfortunately in the case of *S. agrili* and *S. galinae* they both occupy the same niche of parasitizing 3rd to 4th instar EAB larvae (Yang et al. 2006, 2010, Belokobylskij et al. 2012, Duan et al. 2014, Watt and Duan 2014). *Spathius galinae* has a longer ovipositor than *S. agrili* (Yang et al. 2006, Belokobylskij et al. 2012). Compounding the niche overlap, neither *S. agrili* nor *S. galinae* is restricted by bark thickness, despite a difference in ovipositor length (Gould et al. 2011, Murphy et al. 2017).

Even though competitive interference reduced the individual success of both *S. agrili* and *S. galinae* in this experiment, the ecological implications of this interaction may be limited. In field settings EAB larval density can be as high as 46 EAB larvae per m^2 at peak infestation (Duan et al. 2015), making competition for limited larval resources of limited relevance to success. With no instances of successful multiparasitism under this extremely host-limited scenario, there will be no measurable intrinsic competition effects in the field. Future research will observe these trends in field conditions to determine if competition affects success and establishment, and if niche partitioning across bark thickness occurs.

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Tables and Figures

Table 1 Parasitism rate of *S. agrili* and *S. galinae* when exposed to already parasitized larvae

Replicate	% Parasitism (*S. galinae*)	% Parasitism (*S. agrili*)
No delay	0	18.2
One day delay	0	10.0
Three day delay	5.9	20.0
Five day delay	0	18.2
Unparasitized control	42.9*	80*

Asterisk indicates significant difference from other groups within column (Logistic Regression, $P<0.05$)

Table 2 Parasitism rate of EAB larvae to *S. agrili* and *S. galinae* when under extrinsic competition

	S. agrili % Parasitism	*S. galinae* % Parasitism	Overall % Parasitism
Competition	33.3*	16.67*	54.17
S. agrili control	58.33	--	58.33
S. galinae control	--	54.55	54.55

Asterisks indicate groups which differed from control within column (Logistic Regression, P<0.05)
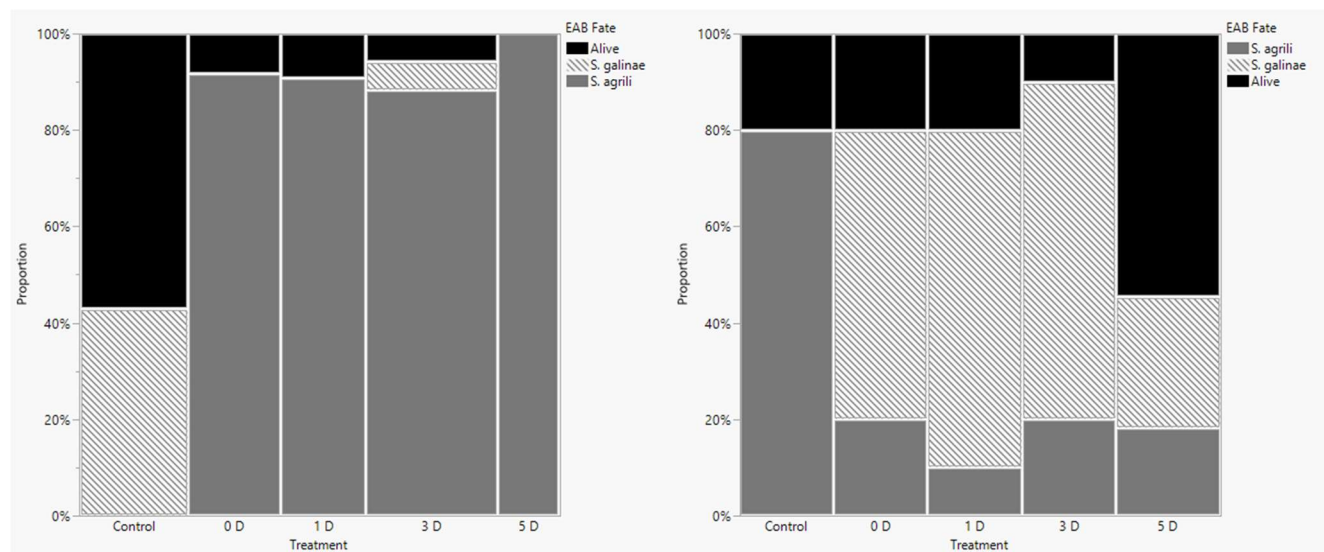
Figure 1 The fate of EAB larvae from intrinsic competition trials, pre-exposure to *S. agrili* (left), and pre-exposure to *S. galinae* (right). Bar color indicates the fate of EAB larvae, gray indicating parasitism by *S. agrili*, diagonal shaded indicating parasitism by *S. galinae*, and black indicating a larvae that escaped parasitism. All groups differed from the control, and in both cases the pre-exposed species was more successful; indicating early-acting competitive superiority dictates success.
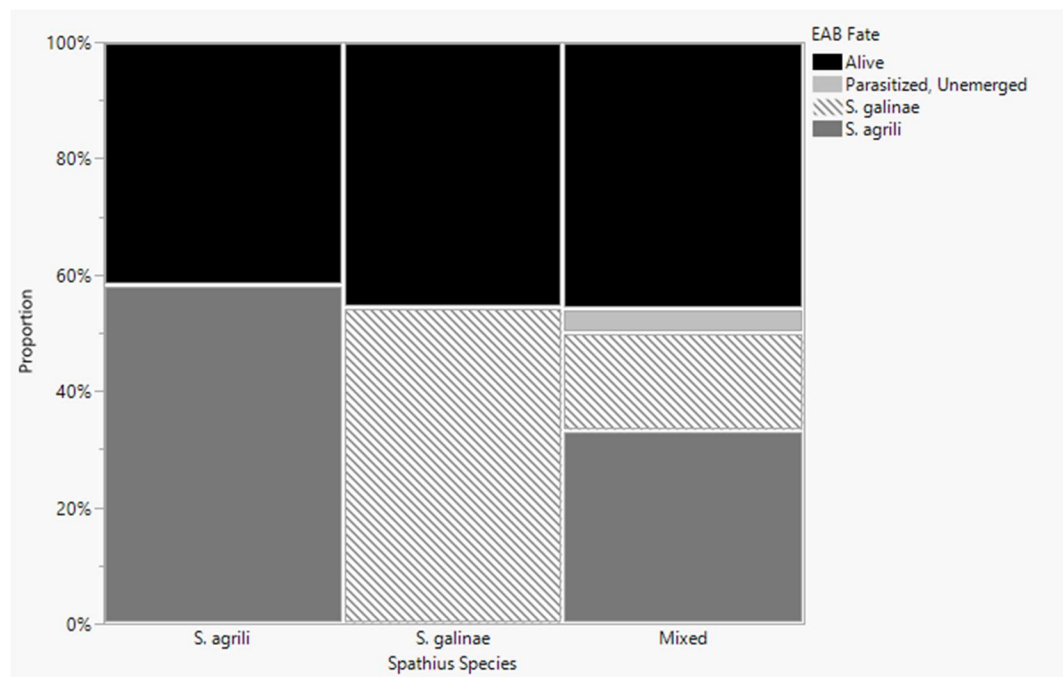
Figure 2 The fate of EAB larvae in the extrinsic competition experiment. "Parasitized,

Unemerged" refers to an unidentified *Spathius* pupae which did not develop to an identifiable

life stage.

Chapter Four
Responses of two introduced larval parasitoids of the invasive emerald ash borer (Coleoptera: Buprestidae) infesting a novel host plant, white fringe tree: implication for biological control

Abstract

Emerald ash borer (EAB), *Agrilus planipennis* is a buprestid species native to Asia, where it is a pest of ash trees, *Fraxinus* spp. After it was accidentally introduced to the United States in the 1990s, this beetle has become one of the most destructive invasive pests of North American ash trees. In 2015 it was found attacking the white fringe tree, *Chionanthus virginicus*, indicating a broader host range. This study evaluates the responses of two introduced larval parasitoids, *Spathius agrili* and *S. galinae*, to EAB larvae infesting this novel host plant. Third to fourth instar EAB larvae reared with tropical ash in the laboratory were inserted into green ash and white fringe tree bolts. Infested bolts were exposed to gravid females of both *Spathius* spp. under no-choice and *S. galinae* under choice testing conditions. No-choice testing indicated no difference in parasitism rate on EAB larvae between white fringe and green ash for either parasitoid species. Two-choice testing with *S. galinae* also indicated no difference in parasitism rate when green ash was an option. Sex ratio and brood size were unaffected by host substrate for EAB, but both species emerged sooner on EAB in white fringe tree. EAB larvae can be found and parasitized in white fringe tree under laboratory conditions. These results indicate that *S. agrili* and *S. galinae* have potential to attack EAB larvae developing in white fringe tree.

1. Introduction
1.1 Emerald Ash Borer

As an invasive species spreads from its native range, it forms new associations with native species in its invasive range (Hajek 2004). These associations add greater levels of complexity to the control of the invasive pest species. Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire

(Coleoptera: Buprestidae), is a devastating invasive species accidently introduced from Northeast Asia to North America in the early 1990's, which was initially believed to be exclusive to ash trees (*Fraxinus* spp.) (Cappaert et al., 2005; Bray et al. 2011; Siegert et al., 2014). Since its initial detection in 2002 (Haack et al. 2002), it has spread through natural and human-assisted means to over 35 states and five Canadian provinces, and reaches from Nova Scotia to Texas (Canadian Food Inspection Agency 2019, Emerald Ash Borer Information 2020). Emerald ash borer has univoltine and semivoltine life cycles which damage the host tree during the larval stage by feeding on the phloem tissue to the point that it girdles the tree (Wei 2007, Tluczek et al. 2011). An estimate of damage from 2009 to 2019 indicated 17 million ash trees killed at a cost of over $25 billion (Kovacs 2010). Emerald ash borer has spread beyond the range of which that estimate was based killed hundreds of millions of ash trees (Emerald ash borer information 2020).

Emerald ash borer has recently developed a new association with another North American native species, white fringe tree (*Chionanthus virginicus* L.) (Cipollini and Rigsby 2015). White fringe trees native range is limited to the south eastern United States; however it is a commonly stocked nursery plant across the United States (USDA NRCS 2020). Dendrochronological evidence suggest emerald ash borer has been utilizing white fringe tree simultaneously along with *Fraxinus* spp. in North America (Thiemann et al. 2016). Under both lab and field conditions EAB causes mortality in white fringe tree (Cipollini and Rigsby 2015, Thiemann et al. 2016). While it is a viable host, in white fringe tree EAB experiences higher larval mortality and slower development than in North American ash species (Cipollini and Rigsby 2015, Olson and Rieske 2019).

1.2 Biological Control

Four hymenopteran parasitoids have been introduced from parts of EAB's native range as biological control agents against this invasive pest (Liu et al. 2007, Bauer et al. 2008, Belokobylskij et. al. 2012, Duan et al. 2012a). Three species were introduced in 2007 from northeast China, including two larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) (Yang et al. 2006) and *Spathius agrili* Yang (Hymenoptera: Braconidae) (Yang et al. 2005), and one egg parasitoid, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) (Zhang et al. 2005). One larval parasitoid species, *Spathius galinae* Belokobylskij and Strazanac, was introduced in 2015 from the Russian Far East (Belokobylskij et al. 2012). Of these species only *T. planipennisi* has been tested on emerald ash borer larvae feeding in white fringe tree (Hoban et al. 2018, Olson and Rieske 2019). Hoban et al. 2018 showed successful parasitism by *T. planipennisi* on EAB manually inserted into white fringe tree. Conversely Olson and Rieske (2019) suggests that white fringe tree may be an enemy free space for EAB when larvae are naturally infested. All other species have only been tested on EAB infesting ash (*Fraxinus* spp.) and until this point the EAB biological control program has only looked at the rearing and efficacy of parasitoids in ash. This experiment seeks to assess whether either *S. agrili* or *S. galinae* can parasitize emerald ash borer larvae in white fringe tree, under laboratory rearing conditions.

2. Materials and methods
2.1 Preliminary Field Sampling

In June of 2018 five ornamental planted white fringe trees in Blacksburg, VA, and three ornamental planted white fringe trees in Newark, DE were surveyed for signs of EAB infestation (branch dieback, D-shaped exit holes, or epicormic sprouts). Any branches with signs of

infestation were cut, and then brought to the USDA ARS Beneficial Insect Introduction Research Unit (BIIRU) in Newark, DE where they were dissected to determine if EAB were present.

2.2 Plant Material

Bolts of green ash (*F. pennsylvanica*) and white fringe tree (1-2 cm diam, 12-14 cm length) were cut from trees grown at the USDA ARS BIIRU. Each bolt was soaked in a 10% bleach solution for 30 min, then scrubbed with a soft-bristled brush under tap water to prevent mold growth. Bolts were then paired to match size as closely as possible. A 3-4 cm flap of outer bark was peeled from each bolt, and an artificial EAB gallery was carved into the phloem tissue using a 3 mm gouge chisel (Palm Block Size #11, ChippingAway) using methods described in Duan et al (2012).

2.3 Host Larvae

All EAB larvae used in the fecundity and reproduction experiments were 3rd or 4th instars reared at the USDA BIIRU in Newark, DE. Emerald ash borer larvae were reared in tropical ash, *Fraxinus uhdei* Wenz., bolts (1-2 cm diam, 10-25 cm length) using the methods described in Duan et al (2012b). In order to ensure larvae of high quality, larvae were dissected out of the tropical ash bolts and paired by size. Paired larvae were then inserted into the paired white fringe tree and green ash bolts (described in section 2.2). Each flap was closed, and the bottom of the bolts were placed 1 cm into saturated floral foam (Aquafoam) to prevent desiccation. Larvae were left to feed for 24 h. Larvae were judged to be healthy and feeding if the artificial gallery was packed with frass, which indicated the larvae had begun feeding on phloem tissue. Any larvae not showing feeding signs were discarded. The bottom 1 cm of each bolt was wrapped in tightly wound paper towel and secured in a 113 ml sample cup (4 oz, Medline Polypropylene

Specimen Container, Medline) filled with saturated rock wool (Rockwool). A tightly wrapped

layer of Parafilm was used to prevent desiccation during the experiment.

2.4 Parasitoids

Both species of parasitoids used in this experiment, *S. agrili* and *S. galinae,* were sourced from

laboratory colonies kept by the USDA at the Brighton Rearing Facility and the BIIRU,

respectively. Adult parasitoids were collected in ventilated clear plastic containers 6 cm (h) x 16

cm (diam), clover honey was streaked onto the mesh ventilation to provide a food source, and

water was provided through a cotton dental wick (Richmond Dental, Charlotte, NC). Parasitoids

were housed in environmental chambers (Percival Scientific) at 25 °C, and 16:8 (L: D) for a

minimum of 48 h post-eclosion. All females used in this experiment were presumed to be gravid

due to 48 h spent with males, and both species have been observed mating immediately after

eclosion (Yang et al., 2010; Duan et al., 2014).

2.5 No-Choice Testing

No-choice testing took place in an environmental chamber at conditions described in section 2.4.

All no-choice tests took place in a ventilated clear testing arena constructed from a capped mesh-

ventilated clear butyrate tube (63 mm diam, 20-40 cm height, Thermoplastic Processes). The

tube was friction-fit over the plastic sample cup containing the artificially infested bolt

(described in section 2.4) to create the arena (Fig. 1). A green ash control group and white fringe

experimental group were established for both *S. agrili* and *S. galinae*. In each treatment, two

naïve gravid females were added to each arena for 7 d. Clover honey was streaked on the mesh

ventilation to provide a food source. After adult parasitoids were removed, emergence of adult

progeny was observed three times per week. Six wk after initial exposure, bolts were dissected

and the fate of each EAB larvae was scored as 'alive', 'parasitized', or 'dead'. Total progeny number was calculated after dissection to account for unemerged parasitoids.

2.6 Two-Choice Testing

Two-choice testing took place in ventilated arenas constructed from a 500 ml cup base, and a closed top acrylic cylinder lid (20 cm height x 12 cm diam., Consolidated Plastics, Stow, OH) which contained a 4 cm layer of saturated floral foam at the bottom. Paired infested bolts of white fringe tree and green ash were placed centrally in the arena, approximately 1 cm deep in the saturated floral foam (Fig. 2). Due to limited EAB larval availability, and lack of field establishments of *S. agrili*, only trials of *S. galinae* were performed using two-choice testing. Two gravid female *S. galinae* were added to each arena for 7 d. Clover honey was streaked on the mesh ventilation to provide a food source. After adult parasitoid removal, emergence of adult progeny was recorded three times per week. After six weeks bolts were dissected, and the fate of each EAB larvae was scored as 'alive', 'parasitized', or 'dead'. Total progeny number was calculated after dissection to account for unemerged parasitoids.

2.7 Statistical Analyses

Data from the no-Choice tests were analyzed separately by parasitoid species. Dead EAB larvae were excluded due to the possibility of mechanical damage, or injury caused during the larval insertion process. Parasitism rates from both no-choice and choice tests were analyzed using nominal logistic regression to compare white fringe tree to the green ash control.

Days to emergence and sex ratio were analyzed separately with one-way ANOVA to determine differences between host plant effects. Sex ratio was calculated as the proportion of female to male progeny; any replicates with only male offspring were excluded because of the

likelihood of asexual reproduction as a result of haplodiploidy. All statistical analyses were performed in JMP Pro 15 (SAS Institute).

3. Results

Field samples of the two yard-planted white fringe trees on private property on McCoy Rd in Blacksburg, VA identified apparent symptoms of EAB infestation - branch dieback, D shaped exit holes, and epicormic sprouts. Dissection of the stems confirmed presence of late instars of feeding, but no associated parasitism. No other stand or planting showed symptoms of EAB feeding. Survey of several stands of white fringe trees in Newark, Delaware did not identify any apparent symptoms of EAB infestation.

The rate of parasitism from the no-choice tests in the laboratory did not differ between green ash and white fringe tree for either species in any test (Table 1). The rate of parasitism of EAB by both *Spathius* species in the no-choice test were numerically higher in green ash, but not statistically different. In two-choice testing, parasitism rate of EAB by *S. galinae* was numerically higher in white fringe tree, but not significantly different. Overall, over 60% of the EAB larvae were parasitized in all of the assay treatments.

The time to emergence differed between green ash and white fringe tree for both *S. galinae* and *S. agrili* (Table 2). Both species emerged significantly earlier from larvae within white fringe tree than larvae within green ash; *S. agrili* emerged 3.55 ± 1.05 d earlier, and *S. galinae* emerged 6.41 ± 0.82 d earlier. Sex ratio did not differ between green ash and white fringe tree for either species (Table 2). Both *S. agrili* and *S. galinae* have a female-biased sex ratio in all trials, which was numerically lower in white fringe, but not significantly different from green ash. Brood size for *S. agrili* and *S. galinae* did not differ between white fringe tree

and green ash (Table 2). *Spathius agrili* had nominally larger broods in green ash, and *S. galinae* had nominally larger broods in white fringe, but these differences were not significant.

4. Discussion

As EAB's host range expands to white fringe tree, and potentially other species including cultivated olive (Cipollini et al. 2018), there is a concern that the biocontrol program would be ineffective on novel host plants of EAB. Findings of our no-choice tests indicated that host attack (parasitism) rate and progeny fitness of neither *S. agrili* nor *S. galinae* were negatively impacted by the host's new association with the white fringe tree. Both parasitoid species were capable of attacking and successfully developing from EAB larvae infesting white fringe tree in no-choice testing. The sex ratio and clutch size remained the same in white fringe tree for both *S. agrili* and *S. galinae*. Both parasitoid species emerged earlier from EAB within the white fringe tree compared to green ash. Results from our two-choice testing of *S. galinae* also showed no difference in EAB parasitism rate and parasitoid progeny fitness when both host plant were present in the same arena, further suggesting that white fringe tree may not negatively impact EAB parasitism by *S. galinae*.

Host seeking behavior of the larval parasitoids can be tied to volatile compounds produced by the larval host, the host plant, or specific compounds only produced by the feeding-damaged host plant (Cipollini and Peterson 2018, Strand and Obrycki 1996, Turlings et al. 1990). Previous research suggested that plant volatile compounds play a critical role in host seeking behavior of *S. agrili* with significant attraction to *Fraxinus* spp. (Yang et al. 2010). *Spathius agrili* was previously shown to be attracted to volatile compounds produced by EAB, as well as volatiles from *Fraxinus* (Johnson et al. 2014).

Further research should consider how host searching is influenced by white fringe tree volatiles, and complex of volatile compounds produced by EAB larval feeding. Differences in emergence time may indicate other developmental differences between *Spathius* adults reared in white fringe tree bolts. Future research should consider health measurements, including adult longevity, fecundity, and time to oviposition. Field sampling near past parasitoid release locations should include surveys for white fringe tree. Felling and debarking white fringe tree could determine if parasitism is occurring in the novel host under field conditions. Further research is planned to infest whole trees with EAB larvae in order to determine if these patterns hold up in a more natural setting.

In our study, we standardized larval host quality using artificially inserted late instar EAB larvae throughout all the treatments. This testing method may in fact have limited the real tri-trophic effect of the host plant substrates on the parasitoids via the direct effect on host larval development. Host development rate and quality as influenced by the quality of the larval host's plant or diet directly affect the health of the parasitoid offspring (Harvey and Gols 2011, Wang et al 2007, Watt and Duan 2014). Several published studies have shown that white fringe tree is a suboptimal host for EAB larvae compared to North American ash hosts (Cipollini and Rigsby 2015, Rutledge and Arango-Velez 2017). While EAB can complete its life cycle in white fringe tree, larval development is slower (Rutledge and Arango-Velez 2017). Lower larval quality and size in the novel host could reduce the efficacy of *S. agrili* and *S. galinae* in naturally infested white fringe tree. The widespread distribution of this novel host plant could lead to geographically isolated populations of EAB, or parasitoids. These isolated populations could serve as reservoirs for reintroduction to the natural ecosystem as ash regrows.

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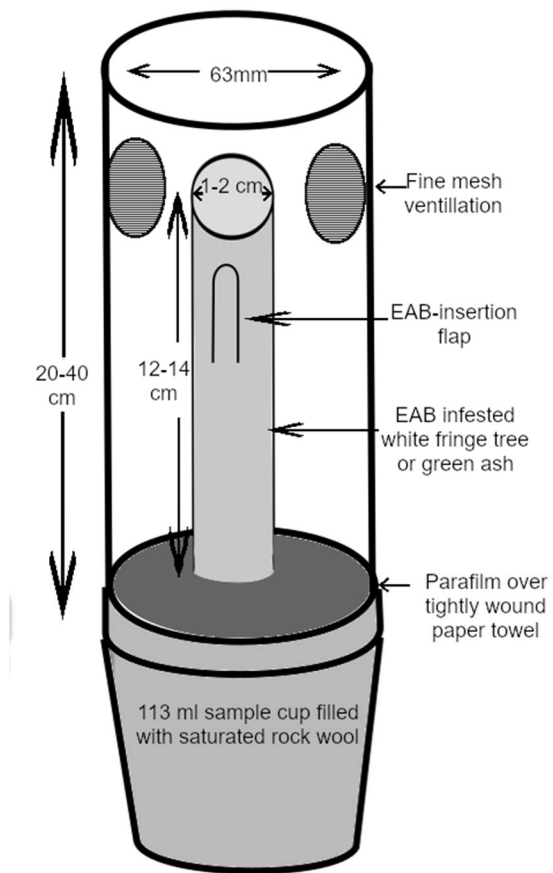
Figure 1: Diagram of the no-choice testing arena for host plant suitability for *S. agrili* and *S. galinae*.
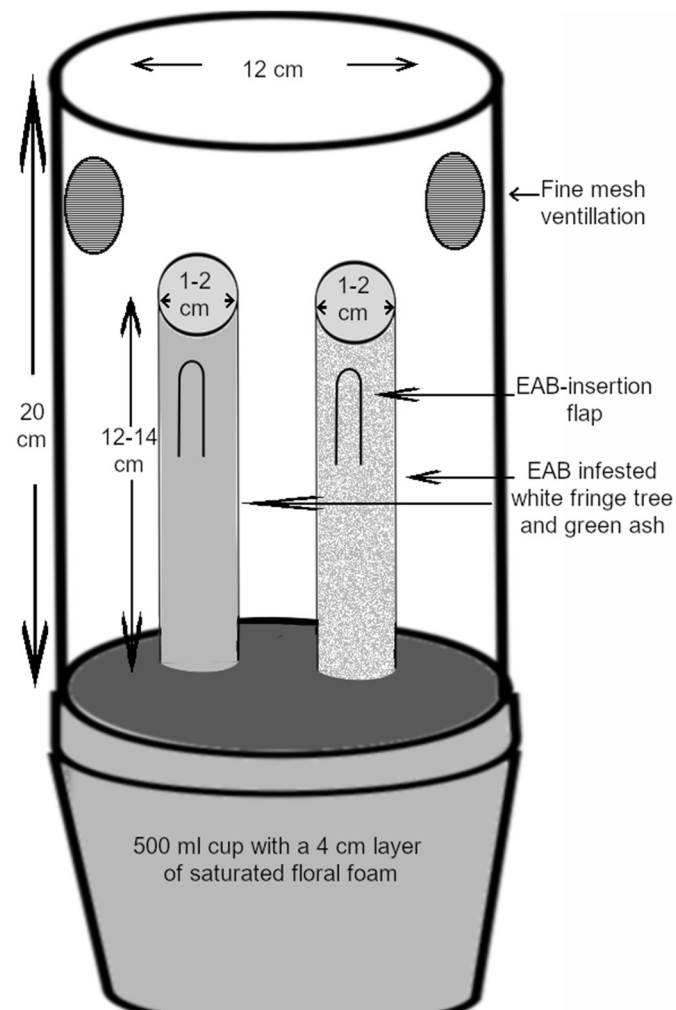
Figure 2: Diagram of the two-choice testing arena for host plant suitability for *S. agrili* and *S. galinae*.

Table 1. Percent parasitism by *S. agrili* and *S. galinae* utilizing emerald ash borer larvae in white fringe tree & green ash under no-choice and choice conditions

EAB Host Species	% Parasitism (No-Choice *S. agrili*) [1]	% Parasitism (No-Choice *S. galinae*)[2]	% Parasitism (Two-Choice *S. galinae*) [3]
White fringe tree	61.1 ± 7.7 (n = 36)	53.1 ± 8.5 (n = 32)	58.8 ± 12.4 (n = 17)
Green ash	74.5 ± 6.5 (n = 51)	69.4 ± 6.9 (n = 49)	52.9 ± 12.4 (n = 17)

Analyses were performed within column (Logistic Regression, P<0.05)

[1] Analysis showed no significant difference ($\chi^2 = 2.18$; df = 1; P = 0.140)

[2] Analysis showed no significant difference ($\chi^2 = 1.76$; df = 1; P = 0.185)

[3] Analysis showed no significant difference ($\chi^2 = 0.12$; df = 1; P = 0.730)

Table 2. Time to emergence, sex ratio, and brood size of *S. agrili* and *S. galinae* from emerald ash borer larvae within white fringe tree and green ash.

EAB Host Species	*S. agrili* Time to emergence (d)[1]	*S. galinae* Time to emergence (d)[2]	*S. agrili* sex ratio (% Female Progeny)[3]	*S. galinae* sex ratio (% Female Progeny)[4]	*S. agrili* Brood Size (# Progeny per host larvae)[5]	*S. galinae* Brood Size (# Progeny per host larvae)[6]
White fringe tree	39.5 ± 0.81 * (n = 149)	40.6 ± 0.67 * (n = 105)	83.0 ± 3.5 (n = 19)	67.2 ± 5.2 (n = 15)	6.47 ± 0.69 (n = 23)	6.65 ± 0.54 (n = 26)
Green ash	43.1 ± 0.67 (n = 223)	47.0 ± 0.46 (n = 225)	87.8 ± 2.7 (n = 29)	77.1 ± 3.7 (n = 30)	6.03 ± 0.54 (n = 37)	7.28 ± 0.43 (n = 40)

Analyses were all performed within column. Asterisks indicate results which significantly differed from control.

[1] Analysis showed significant difference (F = 11.4; df = 371; P < 0.005)

[2] Analysis showed significant difference (F = 61.6; df = 329; P < 0.0001)

[3] Analysis showed no significant difference (F = 1.14; df = 46; P = 0.291)

[4] Analysis showed no significant difference (F = 1.56; df = 44; P = 0.218)

[5] Analysis showed no significant difference (F = 0.27; df = 59; P = 0.607)

[6] Analysis showed no significant difference (F = 0.81; df = 65; P = 0.370)

Chapter Five
The lifecycle of emerald ash borer in Virginia, and recoveries of three introduced parasitoid species

Abstract

Emerald ash borer, *Agrilus planipennis*, EAB, is a devatstating invasive pest of North American ash. Native to Asia, EAB was introduced to North America accidentally in the mid-1990s, but went undetected until 2002. Since then it has spread to 35 states and five Canadian provinces, and has caused widespread ash mortality. Biological control of EAB has been attempted utilizing four species of parasitic wasps an egg parasitoid, *Oobius agrili* and three larval parasitoids, *Tetrastichus planipennisi*, *Spathius agrili*, and *Spathius galinae*. This research focuses on the lifecycle of emerald ash borer in Virginia and North Carolina, and the release and recovery of the three larval parasitoids. Thirteen field sites across Virginia and North Carolina were located, and trees at each site were felled and debarked to determine EABs life cycle. A subset of sites were visited during the summer months to determine the summer phenology of EAB. Parasitoids were released, and monitored using destructive and non-destructive sampling methods. We discovered that the life cycle of EAB in Virginia and North Carolina is more complex than initially reported, and that EAB undergoes both a one-year and two-year lifecycle. The overwintering life stage of EAB was not synchronous across all sites, nor within site. Non-destructive sampling methods, yellow pan traps and larval sentinel logs were not successful at recovering parasitoids. Destructive felling and debarking recovered all three species of larval parasitoid at field sites in Virginia and North Carolina.

Introduction

Emerald ash borer (*Agrilus planipennis*) (Coleoptera: Buprestidae), EAB, is an invasive pest of ash (*Fraxinus* spp.) introduced to North America in the mid 1990's from a part of its natural range in China (Bray et al. 2011, Siegert et al. 2014). Genetic analysis indicated that the invasive population of

emerald ash borer is most closely related to EAB populations in the Hebei and Tianjin City provinces of China (Bray et al. 2011). Emerald ash borer was first reported in North America in southern Detroit Michigan where it was discovered in a stand of dying ash (Haack et al. 2002) and quickly spread to Windsor, Ontario, Canada (Canadian Food Inspection Agency 2020). Spread through natural and anthropogenic means, including firewood transport (Haack et al. 2010, USDA APHIS 2010), EAB currently infests 35 states and five Canadian provinces (Emerald Ash Borer Information 2020). Within Virginia emerald ash borer was initially detected in Fairfax County in 2003 and was eradicated, but rediscovered in 2008 (Virginia Department of Forestry 2020). By 2019 EAB had spread to 88 counties within Virginia causing widespread ash mortality (Virginia Department of Forestry 2020).

All North American ash species and white fringe tree (*Chionanthus virginicus*) are susceptible to EAB attack, and have little host plant resistance to feeding damage (Anulewicz et al. 2008, Rebek et al. 2008, Cipollini et al. 2015). Without population regulation caused by limited host resources or natural enemies capable of controlling populations, emerald ash borer populations can grow unrestricted causing tree mortality in 1-6 years (Jefferies 1984, Bauer et al. 2008, Rebek et al. 2008, Duan et al. 2012, Herms et al. 2014). Emerald ash borer in Hebei, and Tianjin City provinces of China has a primarily univoltine life cycle (Wang et al. 2010). In both its native and invasive range, EAB emerges as adults in late-April to mid-May, mate, oviposit on the bark of host trees. First instar eclose approximately two-three weeks later and larvae burrow into the phloem layer (Cappaert et al. 2005, Wang et al. 2010, Chamorro et al. 2012). While feeding within the phloem, larvae undergo four instars then burrow into the sapwood of the host tree to create a pupal chamber, folding their bodies into a "J" shape called the J-larval stage (Cappaert et al. 2005). In Tianjin City province, China and northern parts EABs invasive range, EAB synchronously overwinters as prepupae within the sapwood (Wang et al. 2010), whereas in North America overwintering life stage has been reported to vary with latitude, and stage of infestation (Cappaert et al. 2005, Sobek-Swant et al. 2012).

Currently four natural enemies from EAB's native range have been introduced for biological control, an egg parasitoid, *Oobius agrili* Zhang and Huang (Hymenoptera: Encyrtidae) and three larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Spathius galinae* Belokobylskij and Strazenac (Hymenoptera: Braconidae). Three species from northeast China, *O. agrili*, *T. planipennisi* and *S. agrili* were initially introduced in 2007, and *S. galinae*, from the Primorsky Krai of the Russian Far East was approved for release in 2015 (Yang et al. 2005, 2006, Zhang et al. 2005, Liu et al. 2007). All three species of larval parasitoids attack 3rd to 4th instar EAB larvae, preferentially attacking larger larvae (Yang et al. 2010, Duan et al 2011, 2014). Recent field studies have identified established populations of *T. planipennisi* and *O. agrili* in northeastern, mid-Atlantic, and Midwestern States (Duan et al. 2013, 2015, Davidson et al. 2016, Jennings et al. 2016). No published study has confirmed establishment of *S. agrili* from these regions (Hooie et al. 2015, MapBiocontrol 2020), although field-cage trials suggest overwintering should be possible under Michigan and Maryland conditions (Ulyshen et al. 2011). *Spathius galinae* was recovered during field studies in six hardwood forests in Connecticut, Massachusetts, and New York, with increasing populations over two years, indicating establishment (Duan et al. 2019). Field trials of early season emergence of *S. agrili* and *S. galinae* indicated that both species would remain synchronous with EAB larvae under Virginia field conditions (Ragozzino et al. 2020). Current USDA recommendations suggest release of *S. agrili* only south of the 40th parallel due to early spring larval availability, and *T. planipennisi* only north of the 40th parallel due asynchronous life stages of overwintering larvae (USDA-APHIS/ARS/FS 2020). Our objectives for this research is to provide a detailed picture of the life cycle of EAB in Virginia, quantify the mortality factors impacting EAB, and assess introduced parasitoid establishment and recoveries.

Materials and Methods
Site Selection

Field surveys were conducted at ash stands in naturally forested locations across Virginia, and one artificially planted location in North Carolina.

Eight sites were located in Virginia, containing EAB infested ash (*Fraxinus* spp.) and at least 10% ash trees over 5 cm DBH (Table 1, Fig. 1). Cooperators at Virginia Department of Forestry, and Fairfax County Urban Forest Management located five additional sites (Table 1). All field sites, except GOL, contained a mix of primarily deciduous hardwood species, including ash (*F. pennsylvanica* Marsh, *F. americana* L.), maple (*Acer rubrum* L.), boxelder (*Acer negundo* L, sweetgum (*Liquidambar styracifula* L.), oak (*Quercus* spp.), poplar (*Populus* sp.), tulip tree (*Liriodendron tulipifera* L.), elm (*Ulmus* spp.), and cherries (*Prunus serotina* Ehrh., *P. virginiana* L.), and some coniferous species including pine *(Pinus* spp.*)* and spruce (*Picea* spp.). One site, MCP contained <5% black ash (*F. nigra* Marsh). Trees at GOL were monocultures of row planted green ash (*F. pennsylvanica* Marshall) trees. Adjacent to the ash stand was a row planted stand of loblolly pine (*Picea taeda* L.), and an agricultural field of tobacco (*Nicotiana* spp.).

Parasitoid Releases

During the summer of 2013, *T. planipennisi* were shipped as infested bolts from the USDA ARS rearing facility in Brighton, MI (Brighton) for release at two field sites in southern Virginia (NF and STRV) (Table 2). In 2016, *T. planipennisi* were shipped from Brighton as infested bolts for release at four additional sites across Virginia and North Carolina (MCP, DOU, LEE, and GOL). In 2017 adult *S. agrili* from Brighton and *S. galinae* from the USDA ARS Beneficial Insect Introduction Research Unit (BIIRU) in Newark, DE were released at GOL (Table 2). Field cooperators at Virginia Department of Forestry, and Fairfax County Urban Forestry Management released infested bolts of *T. planipennisi* from Brighton at CRN, CBS, FLT, CUSF, and WSF from 2016 to 2018 (Table 2).

Monitoring

Monitoring was performed using non-destructive, and destructive sampling techniques. Larval sentinel logs were prepared by artificially infesting a bolt of green ash (5-9 cm dia. x 15-25 cm length) with susceptible EAB larvae, as described in Duan et al. (2012). A 3-4 cm long flap of outer bark was peeled from the bolt, and an artificial gallery was carved in the phloem using a 3 mm gouge chisel (Palm Block Size #11, ChippingAway). This was repeated three to five times per bolt, depending on diameter. A field collected fourth instar EAB was inserted into an artificial gallery, then covered with the flap of bark and secured with Parafilm. Bolts were hung at release sites from infested ash trees for seven days before being collected and placed in a Percival growth chamber to allow parasitoids to develop. After 6 weeks bolts were dissected to assess any unemerged parasitoids or remaining EAB larvae.

Yellow pan traps were placed on symptomatic ash trees approximately 5, 10, and 15 m from the release points within the stand using a protocol modified from the Emerald Ash Borer Release and Recovery Guidelines (USDA APHIS 2015). Yellow pan traps were collected once per week in 2016, and biweekly in 2017.

Destructive sampling by felling and debarking infested ash trees occurred during the year of initial releases at DOU, MCP, LEE, and GOL. Otherwise, all sampling took place at least 1 year post-initial release (Table 2). To determine EAB overwintering lifestage and voltinism, each site was visited annually during February-April. To determine EAB development during summer field conditions, DOU and MCP were visited biweekly during June, July, and August 2017. GOL was visited only during August 2017, due to site restrictions. At each site, symptomatic trees were selected, felled and debarked in 1 m sections using methods described in USDA–APHIS/ARS/FS (2020). Additional trees were felled until a minimum of 100 EAB larvae were sampled per visit. All EAB galleries present were scored by EAB lifestage and fate (alive, exited as an adult, parasitized, killed by disease, killed by woodpecker, killed by other factors). Galleries which showed signs of age (calloused or decaying phloem tissue) were additionally scored as "old". Parasitized and predated larvae were recovered, and

parasitoids and predators were identified morphologically. Any larvae not killed in sampling were brought back to the Prices Fork Research Center in Blacksburg, Virginia, or the USDA BIIRU, and observed for 3 wks for any parasitoid emergence. Total phloem area (y) of each tree felled was calculated using a second-order polynomial model ($y = 0.024x^2 - 0.307x + 2.63$) as a function of tree DBH (McCullough & Siegert 2007).

Statistical analyses

Data were divided by season into winter sampling, and summer monthly sampling. Parasitism susceptibility was then analyzed using a one-way ANVOA to determine significance of site as an effect ($P<0.05$) and a Student's t-test ($P<0.05$) was used to determine significance between sites. For analysis of parasitism 1st and 2nd instar EAB were excluded, as they are not susceptible to parasitism. EAB fate was tabulated by tree, then percent parasitism by species, and percent mortality by each scored factor (woodpecker predation, disease, other) was calculated. Larval fate was then analyzed using a one-way ANOVA ($P<0.05$) and Student's t-test ($P<0.05$). Parasitism susceptibility for summer sequential sampling was analyzed individually for each site to determine differences by month using a one-way ANOVA and Student's t-test ($P<0.05$). Statistical analyses were performed in SAS JMP Pro 15 (SAS Institute 2020).

Results
Winter Sampling

Parasitism rate by introduced and native species, and woodpecker predation all varied by field site (Table 3). Introduced parasitoids were recovered at seven sites CRN, CUSF, DOU, GOL, LEE, MCP, and NF (Table 3). Introduced parasitoids were recovered at least one year after the final release of parasitoids at five of these sites, CRN, CUSF, DOU, GOL, and NF (Table 2). Of the 11 sites at which *T. planipennisi* was released, they were recovered at five, DOU, GOL, LEE, MCP, and NF. Only DOU and NF had recoveries of *T. planipennisi* later than 1 year after latest release (Table 3).

Spathius agrili was released at six sites, and recovered at three, CRN, CUSF, and GOL, all later than 1 year after latest release (Table 3). *Spathius galinae* was only released at GOL, where it was recovered at least one year after latest release (Table 3).

Parasitism by native species occurred at 10 sites CLV, CRN, CUSF, DOU, FLT, GOL, HNNH, LEE, NF, and STRV. *Atanycolus* spp. (Hymenoptera: Braconidae) were found at every site with native parasitoids other than CRN. The remaining native parasitoid species were *Balcha indica* Mani and Kaul (Hymenoptera: Eupelmidae) found at CRN and FLT, Phasgonophora sulcata Westwood (Hymenoptera: Chalcididae) found at DOU, or other species that could not be identified at DOU, HNNH, NF, and STRV (Table 3).

Woodpecker predation was highly variable, ranging from 11% at STRV to 67% at CBS of all EAB killed (Table 4). Neither disease nor other mortality factors differed significantly by site, accounting for a mean $3.22 \pm 7.28\%$ and $5.94 \pm 11.90\%$ of EAB larvae, respectively.

EAB density did not differ significantly by site (Table 3), despite being highly variable by tree. The proportion of overwintering EAB susceptible to parasitism differed by site (Table 4). The overwintering life stage of EAB larvae differed by site, most overwintered as pupae or prepupae except for HNNH which overwintered as 4th instar, and NF which overwintered as J-larvae (Table 4). Emerald ash borer overwinters as 2nd instar to pupae at all sites other than LEE (Table 4), indicating univoltine and semivoltine populations at those sites. Despite low larval availability during overwintering months at DOU, *T. planipennisi* was still recovered years after release.

Summer Sampling

Neither larval sentinel logs, nor yellow pan traps collected any parasitoid species of EAB, native or introduced. Felling and debarking was successful at surveying for parasitoid.

Summer felling and debarking revealed EAB density per sq. m of phloem area was lower at MCP than DOU (Table 5). Native parasitoids were not recovered at any site during summer sampling (Table 5). Emerald ash borer life stage, and parasitoid susceptibility varied by month at an individual site (Fig. 2, Table 6). DOU had no susceptible larvae in June, but susceptible larvae significantly increased in in July and August. MCP had significantly fewer susceptible larvae in June and July compared to August. Due to site inaccessibility, GOL was not sampled in June or July, but the majority of EAB larvae were susceptible to parasitism in August. There was no variation in other mortality factors between DOU, GOL, and MCP (Table 5).

Discussion

All three species of introduced parasitoids were recovered at least one year after final release. Recoveries of *S. agrili* occurred at sites in CRN, CUSF, and GOL. These recoveries stretched a distance of 360 km, and involved releases occurring in different years and with different site conditions. CUSF is within a seasonal wetland dominated by sycamores, with primarily mature ash trees (29-41 cm DBH) and had little ash regrowth. CRN is also a seasonal wetland, however it has sapling to pole sized ash trees (7-11 cm DBH) and had ash regrowth, and was dominated by oaks. GOL is an upland site, with artificially row planted pole sized ash trees (12-15 cm DBH), with no regrowth due to mowing. Recoveries at these highly varied sites add to the body of knowledge that establishment by *S. agrili* is possible in southern US states, despite its failure to establish in the northern US. *Spathius agrili* may be useful as part of the complex of parasitoids released against EAB in the southern US. If *S. agrili* establishes self-sustaining populations at these sites and is continued to be used as a biocontrol agent against EAB, then as global temperatures warm it could be reintroduced to northern parts of EABs invasive range. Additionally at GOL, *S. galinae* was recovered one year after the final release, indicating that it may be a viable biological control agent south of the 40th

parallel. Recoveries of *T. planipennisi* longer than one year after final release only occurred at two sites, NF and DOU. However, dissections of *T. planipennisi* infested release bolts at CRN, CBS, CUSF, and FLT indicated that far fewer adult *T. planipennisi* emerged than were estimated by the USDA Brighton Facility which rears parasitoids for release (unpublished data).

Overwintering EAB life stage varied site to site. Only four sites had less than 10% EAB larvae available to parasitoids in late winter, indicating that an asynchronous two-year life cycle occurs at the majority of southern sites. This, combined with fewer-than-expected *T. planipennisi* adults released may have resulted in falsely negative results regarding the capability of *T. planipennisi* to establish south of the 40th parallel. At DOU, a site with only 6% of live overwintering larvae as 3rd or 4th instars, *T. planipennisi* was recovered three years after its final release. This indicates that *T. planipennisi* should be capable of overwintering at other sites with a small proportion of asynchronous EAB larvae. A wider sample of field conditions allowed for a clearer picture to form regarding the overwintering life stages, and points of failure in the establishment of *T. planipennisi*.

Limitations of this research include non-repeated sampling at some field sites, and other factors not being categorized into more descriptive criteria. Non-destructive trapping methods were ineffective at capturing any parasitoid species. Other research has indicated that yellow pan traps and larval sentinel logs can be effective tools to capture the parasitoids of emerald ash borer (USDA–APHIS/ARS/FS 2019, Jian Duan personal communication), and failure to recover parasitoids using these methods may have been due to user error. Larval sentinel logs dessicated quickly, and larvae suffered high mortality. Due to inexperience, it was unclear if mortality was due to mechanically damaging larvae upon reinsertion, larval health prior to reinsertion, or desiccation in the field. Future work should continue to monitor these field sites, and assess parasitoid populations for establishment. Releases of parasitoids should reassess the infested bolt release method to ensure parasitoids are being released, or shift to releasing adult wasps. Future research should consider expanding to additional

field sites as EAB expands its invasive range within Virginia and North Carolina to include more diverse areas where ash trees are present.

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Tables and Figures

Table 1. Emerald ash borer field sites in Virginia and North Carolina. Sites 1-8 were located by Virginia Tech. Sites 9 & 10 were located by Virginia Department of Forestry. Sites 11-13 were located by Fairfax County Urban Forest Management. All sites contained at least 10% ash.

| Site Number	Site Name	Site ID	Latitude	Longitude	Town	State
1	Mid-County Park	MCP	37.171649	-80.410365	Christiansburg	VA
2	Douthat State Park	DOU	37.89169	-79.801357	Clifton Forge	VA
3	Leesylvania State Park	LEE	38.584983	-77.261101	Woodbridge	VA
4	Cherry Research Farm	GOL	35.373598	-78.044468	Goldsboro	NC
5	Clover	CLV	36.850872	-78.670828	Clover	VA
6	Staunton River State Park	STRV	36.702921	-78.660659	Fairview Acres	VA
7	News Ferry Road	NF	36.665978	-79.030425	Terrys Corner	VA
8	Hannah Road	HNNH	37.009681	-78.750828	Harrisburg	VA
9	Whitney State Forest	WSF	38.67013	-77.80794	Warrenton	VA
10	Cumberland State Forest	CUSF	37.51608	-78.31946	Union Hill	VA
11	Cub Run Stream Valley Park (North)	CRN	38.89779	-77.469361	South Riding	VA
12	Cub Run Stream Valley Park (South)	CBS	38.8974	-77.467336	South Riding	VA
13	Flatlick Stream Valley Park	FLT	38.8861	-77.4258	Chantilly	VA
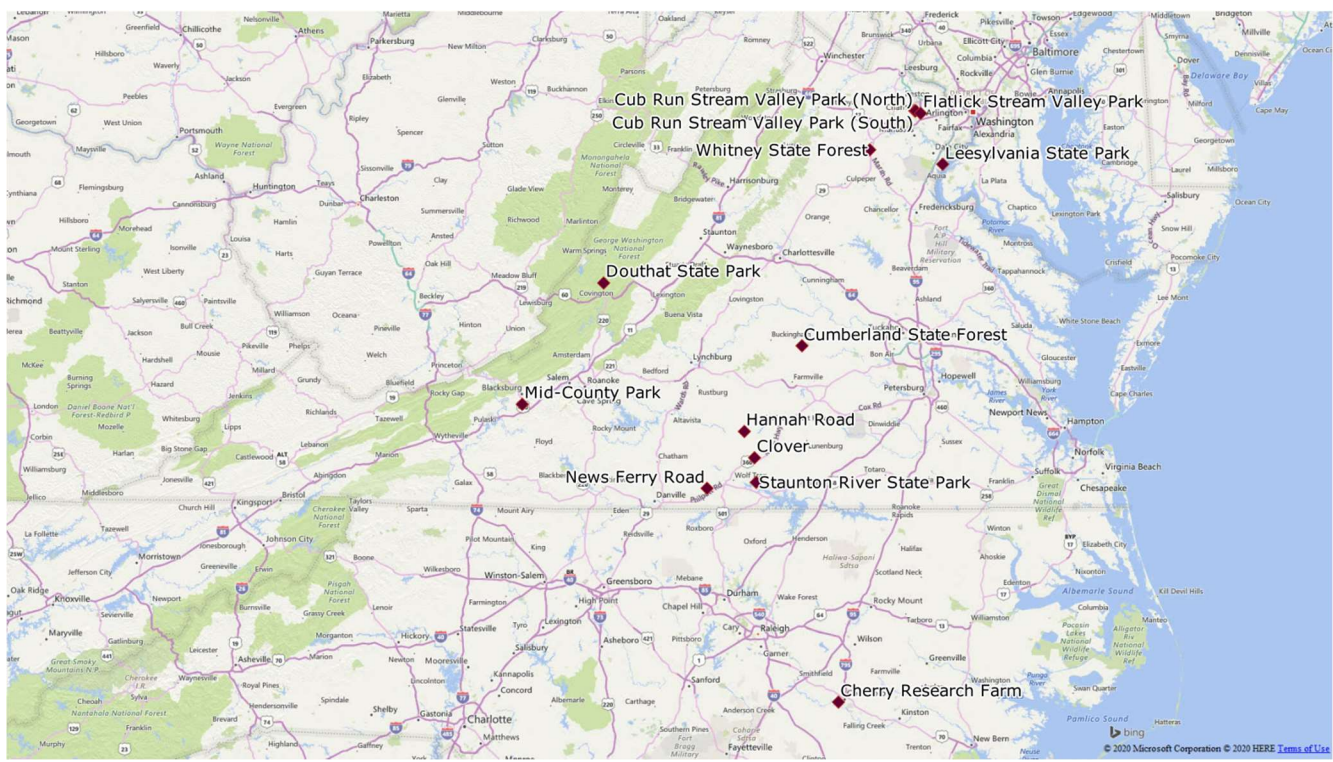
Figure 1. A map of emerald ash borer field sites in Virginia and North Carolina, as detailed in table 1.

Table 2. Releases of *T. planipennisi*, *S. agrili* and *S. galinae* between 2016-2018

Site Name	Coordinates	Species	Number Released	Method	Date Released	Year of Sampling
NF	36.665978, -79.030425	*T. planipennisi*	591	Adults	7/17/2013	2014, 2015
NF	36.665978, -79.030425	*T. planipennisi*	102	Adults	7/29/2013	2014, 2015
NF	36.665978, -79.030425	*T. planipennisi*	548	Adults	8/9/2013	2014, 2015
NF	36.665978, -79.030425	*T. planipennisi*	217	Adults	8/17/2013	2014, 2015
STRV	36.702921, -78.660659	*T. planipennisi*	757	Adults	7/17/2013	2014, 2015
STRV	36.702921, -78.660659	*T. planipennisi*	120	Adults	7/29/2013	2014, 2015
STRV	36.702921, -78.660659	*T. planipennisi*	582	Adults	8/9/2013	2014, 2015
STRV	36.702921, -78.660659	*T. planipennisi*	186	Adults	8/17/2013	2014, 2015
LEE	38.58, -77.26	*T. planipennisi*	1005	Infested Bolts	7/13/2016	2016
DOU	37.89, -79.80	*T. planipennisi*	1010	Infested Bolts	7/19/2016	2016, 2017, 2019
DOU	37.89, -79.80	*T. planipennisi*	1033	Infested Bolts	7/26/2016	2016, 2017, 2019
MCP	37.17, -80.41	*T. planipennisi*	1034	Infested Bolts	8/2/2016	2016, 2017, 2019
DOU	37.89, -79.80	*T. planipennisi*	1050	Infested Bolts	8/10/2016	2016, 2017, 2019
MCP	37.17, -80.41	*T. planipennisi*	1004	Infested Bolts	8/25/2016	2016, 2017, 2019
LEE	38.58, -77.26	*T. planipennisi*	1009	Infested Bolts	9/1/2016	2016
GOL	35.37, -78.04	*T. planipennisi*	1004	Infested Bolts	9/8/2016	2016, 2017, 2019
GOL	35.37, -78.04	*S. agrili*	212 females*	Adults	8/22/2017	2016, 2017, 2019

GOL	35.37, -78.04	*S. galinae*	215 females*	Adults	8/22/2017	2016, 2017, 2019
CUSF	37.51608, -78.31946	*T. planipennisi*	403	Infested Bolts	6/20/2017	2019
CUSF	37.51608, -78.31946	*S. agrili*	203	Adults	6/17/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	564	Infested Bolts	6/17/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	351	Infested Bolts	6/19/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	348	Infested Bolts	6/25/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	472	Infested Bolts	8/2/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	838	Infested Bolts	8/29/2018	2019
CUSF	37.51608, -78.31946	*T. planipennisi*	482	Infested Bolts	9/6/2018	2019
CRN	38.89779, -77.469361	*S. agrili*	368	Adults	6/2017	2019
CRN	38.89779, -77.469361	*T. planipennisi*	453	Infested Bolts	6/2017	2019
CRN	38.89779, -77.469361	*S. agrili*	258	Adults	6/2018	2019
CRN	38.89779, -77.469361	*T. planipennisi*	523	Infested Bolts	6/2018	2019
FLT	38.8861, -77.4258	*S. agrili*	191	Adults	6/13/2017	2019
FLT	38.8861, -77.4258	*T. planipennisi*	455	Infested Bolts	6/2017	2019
FLT	38.8861, -77.4258	*S. agrili*	250	Adults	6/2018	2019
FLT	38.8861, -77.4258	*T. planipennisi*	508	Infested Bolts	6/2018	2019
CBS	38.897400, -77.467336	*T. planipennisi*	453	Infested Bolts	6/13/2017	2019
CBS	38.897400, -77.467336	*S. agrili*	363	Adults	6/13/2017	2019
CBS	38.897400, -77.467336	*T. planipennisi*	523	Infested Bolts	6/20/2018	2019
CBS	38.897400, -77.467336	*S. agrili*	258	Adults	6/20/2018	2019
WSF	38.67013, -77.80794	*T. planipennisi*	855	Infested Bolts	7/25/2017	2019
WSF	38.67013, -77.80794	*S. agrili*	249	Adults	7/25/2018	2019
WSF	38.67013, -77.80794	*T. planipennisi*	348	Infested Bolts	7/25/2018	2019

*A 3F:1M sex ratio for released parasitoids was assumed. All females were assumed gravid upon release.

Table 3. Emerald ash borer density & mortality factors by site. Woodpecker (WP) represents % of all larvae predated. Percent parasitism (P) excludes 1st and 2nd instar from the total EAB value, as they are not susceptible to parasitism.

Site	Mean EAB Density[1]	Total EAB Sampled	Total EAB Susceptible to Pars	% WP Predation (Mean)[2]	% Mortality (Other)[1,4]	% Mortality (Disease)	% Parasitism (Native Spp.)[2]	% Parasitism (Introduced Spp.)[2]	% Parasitism (*T. planipennisi*)[2]	% Parasitism (*S. agrili*)	% Parasitism (*S. galinae*)
CBS	4.38 ± 20.88	55	53	67.78 ± 10.69^A	9.67 ± 2.96^A	0.00	0.00^{BC}	0.00^C	0.00^B	0.00	--
Clv	42.22 ± 8.19	3371	3325	27.01 ± 4.25^D	3.18 ± 1.18^B	9.20 ± 1.92	1.90 ± 1.21^{BC}	0.00^C	--	--	--
CRN	10.05 ± 20.88	105	100	58.22 ± 10.69^{AB}	1.67 ± 2.96^{AB}	5.27 ± 4.83	1.39 ± 3.04^{BC}	2.02 ± 3.50^{BC}	0.00^B	2.03 ± 0.84**	--
CUSF	25.44 ± 36.16	458	458	11.20 ± 18.51^{CDE}	1.52 ± 5.13^{AB}	13.42 ± 8.37	4.55 ± 5.27^{ABC}	7.57 ± 6.07^{ABC}	0.00^B	7.58 ± 1.45**	--
DOU	50.31 ± 22.87	424	412	43.31 ± 13.09^{ABCD}	0.27 ± 3.63^B	8.33 ± 5.92	4.43 ± 3.73^{ABC}	0.27 ± 4.29^{BC}	0.266 ± 3.82^{B3}	--	--
FLT	4.13 ± 20.88	42	40	51.19 ± 10.69^{ABC}	0.00^B	0.00	9.92 ± 3.04^A	0.00^C	0.00^B	0.00	--
GOL	12.95 ± 18.08	171	170	18.61 ± 9.89^{DE}	0.00^B	11.63 ± 4.47	3.57 ± 2.82^{ABC}	11.36 ± 3.24^{AB}	2.19 ± 2.89^B	3.30 ± 0.77**	5.86 ± 0.93**
Hnnh	27.15 ± 8.19	1841	1802	34.76 ± 4.43^{CD}	2.57 ± 1.23^B	4.23 ± 2.00	0.48 ± 1.26^C	0.00^C	--	--	--
Lee	19.04 ± 51.14	120	120	20.83 ± 26.18^{ABCDE}	0.00^{AB}	0.00	15.00 ± 7.45^{ABC}	11.67 ± 8.58^{ABC}	11.67 ± 7.64^{AB}	--	--
MCP	16.88 ± 19.33	262	235	19.09 ± 9.89^{DE}	0.68 ± 2.74^B	0.00	0.00^{BC}	14.29 ± 3.24^A	14.29 ± 2.89^A	--	--
NF	41.40 ± 9.50	2320	2308	43.84 ± 4.86^{BC}	4.04 ± 1.34^{AB}	4.62 ± 2.19	0.62 ± 1.38^C	0.23 ± 1.59^C	0.23 ± 1.42^B**	--	--
StRv	37.93 ± 11.73	2902	2844	11.18 ± 6.01^E	6.09 ± 1.66^{AB}	8.82 ± 2.71	5.19 ± 1.71^{AB}	0.00^C	0.00^B	--	--
WSF	18.45 ± 25.57	438	427	41.63 ± 13.09^{ABCD}	1.70 ± 3.62^{AB}	0.28 ± 5.91	0.00^{BC}	0.00^C	0.00^B	0.00	--

[1] EAB dsenisty based on (Total EAB /m^2 Phloem Area)

[2]Indicates significant difference by site on the column by letter groupings (Student's t test, P<0.05).

[3]Indicates a parasitoid recovery greater than 1 year after last releases.

[4]Other factors include encapsulation by host plant defenses, and any other factor that was not apparent.

Table 4. Lifestage of healthy emerald ash borer during overwintering sampling, percent of larvae susceptibile to parasitism indicates potential for *T. planipennisi* to successfully establish. Overwintering life stage of EAB indicates a semivoltine or univoltine population. All sites other than Lee have multiple life stages overwintering, indicating semivoltine populations.

Site	Majority Lifestage	% of Live Larvae Susceptible to Parasitism*	Total Overwintering $2^{nd} - 4^{th}$ instar	Total Larvae Sampled
CBS	--	--	13	55
Clv	Pupa	10.20 ± 0.86^{DF}	1490	3371
CRN	Prepupa	20.00 ± 6.06^{CD}	30	105
CUSF	Pupa	0.49 ± 1.64^{H}	406	458
DOU	Pupa	6.06 ± 2.89^{EFGH}	132	424
FLT	--	--	8	42
GOL	Adult**	2.70 ± 5.45^{EFGH}	37	171
Hnnh	4th Instar	41.76 ± 1.24^{B}	716	1841
Lee	Pupa	0.00^{GH}	56	120
MCP	Pupa	10.82 ± 2.38^{DEF}	194	262
NF	J-Larvae	29.04 ± 0.99^{C}	1126	2320
StRv	Prepupa	6.35 ± 0.67^{EG}	2410	2902
WSF	Prepupa	75.00 ± 3.46^{A}	92	438

*Indicates significance within column one-way ANOVA (P<0.05), lettering indicates groupings (Student's t-test P<0.05).
**Was sampled in late April in 2018 and pupation had completed.

Table 5. Life Table Summary data from June, July and August sampling at DOU and MCP. Only August sampling at GOL.

Site	Total EAB Sampled	Mean EAB Density (Total EAB / m² Phloem Area)*	% WP Predation (Mean)	% Mortality (Other)	% Mortality (Disease)	% Parasitism (Native Spp.)	% Parasitism (Introduced)	% Parasitism (T. planipennisi)	% Parasitism (S. agrili)	% Parasitism (S. galinae)
DOU	534	29.26 ± 6.54^A	2.03 ± 6.00	0.00 ± 1.17	0	0.00	0.00 ± 0.77	0.00	--	--
GOL	212	15.88 ± 7.66^{AB}	0.52 ± 7.35	0.00 ± 1.43	0	0.00	0.43 ± 0.94	0.00	0.43 ± 0.19	0.00
MCP	87	4.98 ± 6.86^B	9.26 ± 6.00	1.85 ± 1.17	0	0.00	1.19 ± 0.77	1.19 ± 0.75	--	--

*Indicates significant difference within column, letterings indicates groupings (Student's t test, P<0.05)

Table 6. Larval availability and percent of larvae susceptible to introduced parasitoids at two sites by month (all non-healthy larvae were excluded).

Site	Total EAB Sampled (June)	Total Susceptible Larvae (June)	% Susceptible Larvae (June)	Total EAB Sampled (July)	Total Susceptible Larvae (July)	% Susceptible Larvae (July)	Total EAB Sampled (August)	Total Susceptible Larvae (August)	% Susceptible Larvae (August)
DOU	181	0	0 ± 2.93[B]	315	122	38.73 ± 2.22[A]	38	11	28.95 ± 6.40[A]
GOL	--	--	--	--	--	--	212	180	84.9 ± 2.45
MCP	18	3	16.67 ± 8.27[B]	41	1	2.44 ± 5.47[B]	28	12	42.86 ± 6.63[A]

*Sites were analyzed by row for significant difference over sampling time (Student's t-test P<0.05).

**GOL was not sampled in June or July due to restricted access to the site, and as such, could not be analyzed over time.
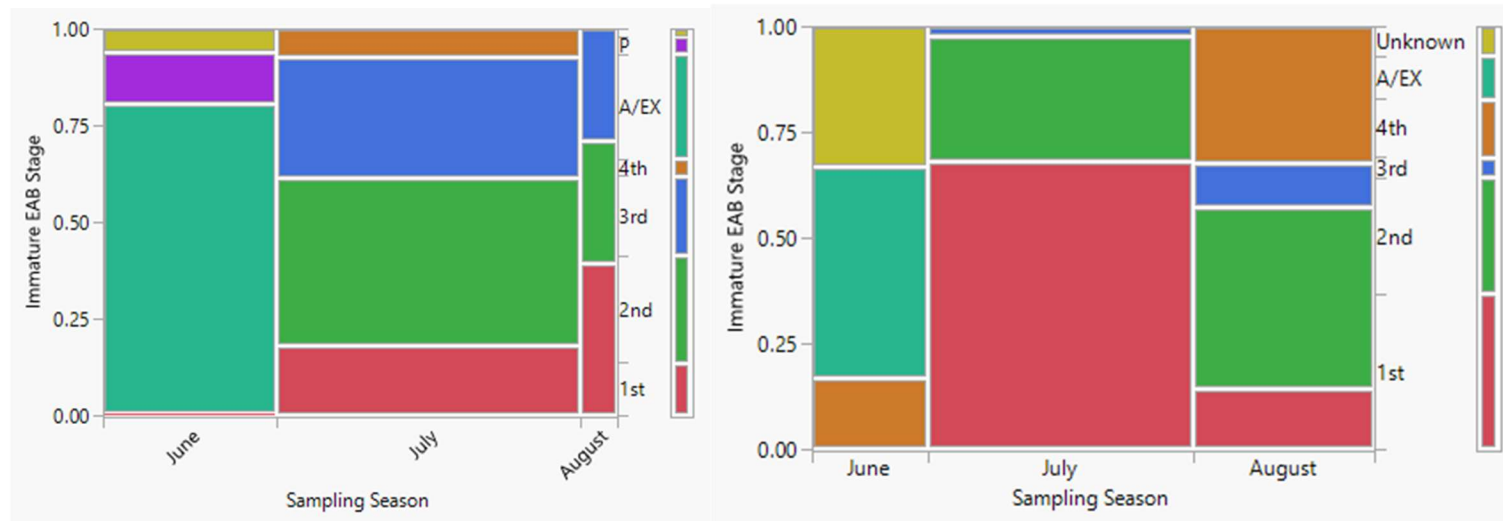
Figure 2. Summer Larval Development by Site.

DOU (left) MCP (Right). Width of bars are proportional to number of larvae in the sample, but does not reflect site-site comparisons.

Lifestage of EAB is indicated by color, 1st, 2nd, 3rd and 4th indicating instar, P indicating pupa, and A/EX indicating an EAB gallery

terminating in an adult EAB exit hole.

Chapter Six
Conclusions

   The research detailed in chapters two, three, four, and five describes the phenology and life history of the invasive emerald ash borer *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), and the successes and complications of its biological control in Virginia and North Carolina. The biological control of any invasive species requires accurate knowledge of the phenology of the pest and the associated parasitoids. This research documents the phenology of emerald ash borer and two larval parasitoids, *Spathius agrili* Yang (Hymenoptera: Braconidae), and *Spathius galinae* Belokobylskij and Strazenac (Hymenoptera: Braconidae) in Virginia and North Carolina. Furthermore, the precedeing chapters detail two complications of biological control; competition between congener species, and parasitism of emerald ash borer within a novel host.

   As part of this research, three species of larval parasitoids of emerald ash borer, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae), *Spathius agrili* and *Spathius galinae*, were released in Virginia and North Carolina (chapter 5). Felling and debarking of trees, larval sentinel logs, and yellow pan traps were used to recover parasitoids, but only felling and debarking resulted in parasitoid recoveries. This research stretches from 2013 to 2019, across twelve field sites spanning Virginia and one field site in North Carolina. Winter field surveys revealed dramatically different EAB phenologies, population densities, and mortality factors occurring across Virginia. Winter sampling recovered all three species of introduced parasitoids, native parasitoids, and showed evidence of woodpecker predation. Summer field sampling showed the larval development of EAB, which will aid in future parasitoid release timing. Emerald ash borer has been previously reported to undergo two different life cycles, a one year cycle with EAB diapausing within the sapwood as prepupae, or a two year cycle with EAB diapausing as larvae in the phloem. Portions of the EAB population across Virginia

underwent both life cycles within a single site, but the proportion of the population that did so varied site by site (chapter 5). Previously, EAB was reported to undergo only a one-year synchronous life cycle south of the 40th parallel. Reportedly, all EAB emerged in early May, mated, then oviposited in bark crevices. After eclosion larvae fed on susceptible host plant phloem through August before digging a pupation chamber in the sapwood of the host tree. Larvae then overwintered as dormant 4th instar (J-larvae) or pupae. My results conflict with those reports, and show that emerald ash borer populations are asynchronous, and undergo one and two-year life cycle in Virginia (chapter 5).

Early spring phenology studies indicated that *S. agrili* and *S. galinae* could both be viable for release under Virginia field conditions (chapter 2). Both species emerged earlier than EAB was available to them, but survived long enough to overlap with the susceptible EAB population at two Virginia field sites (chapter 2, chapter 5). Subsequent generations overlapped with susceptible EAB at every field site (chapter 5). A brief cold acclimatization prior to Virginia field exposure caused different responses in the emergence patterns of *S. galinae* and *S. agrili*. Emergence of *S. galinae* was significantly delayed by cold treatment, but had no multigenerational effect. However emergence of *S. agrili* was not impacted by cold treatment, but emergence of their subsequent generation was delayed. This study revealed differences in emergence patterns of the species from infested release bolts but also demonstrated the variability in the number of parasitoids released from infested bolts. Despite differences in emergence, both *S. agrili* and *S. galinae* emereged synchronously with EAB populations in Virginia, indicating both have potential to survive if released.

With the knowledge that *S. agrili* and *S. galinae* could coexist in field conditions, we investigated the new association between them. To do so, we simulated low host density conditions to observe intrinsic and extrinsic competition between *S. agrili* and *S. galinae*. We exposed 4th instar EAB larvae to *S. agrili* and *S. galinae* simultaneously (extrinsic), and staggered (intrinsic). We determined that neither species completely outcompetes the other in either intrinsic or extrinsic competition (chapter 3). During extrinsic competition, *S. agrili* succeeded more frequently than *S.*

galinae, but did not exclude it. Multiparasitism never occured between *S. agrili* and *S. galinae* under any trial condition. Total parasitism of EAB larvae was not impacted by competition.

We researched the impact of emerald ash borers expanding host range on the biological control agents, *S. agrili* and *S. galinae*. Previous literature reported mixed results of parasitoid exposure to emerald ash borer feeding on its novel host, white fringe tree *Chionathus virginicus* L. Conflicting research reported white fringe tree as a potential enemy free space from *T. planipennisi* (Hoban et al. 2018, Olson and Rieske 2019). We exposed gravid adult *S. agrili* and *S. galinae* to EAB artificially inserted into white fringe tree bolts under choice, and no-choice conditions (Chapter 3). Our results indicate that emerald ash borer feeding in white fringe tree was equally susceptible to parasitism by *S. agrili* and *S. galinae* under laboratory conditions (Chapter 4).

Finally, the biological control of emerald ash borer faces an uphill battle against a devastating pest. Biological control needs to nimbly respond to new information as EAB is found infesting new novel host plants, and expanding its invasive range further south and west. Future research should continue to research parasitoid interactions with white fringe tree under whole tree & field conditions.

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