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

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HIGHLIGHTS

- White fringe tree could be a reservoir for EAB & parasitoids in Virginia.
- *Spathius agrili* & *Spathius galinae* can attack EAB in white fringe tree.
- Both species emerged sooner from white fringe tree than green ash.
- Fecundity, sex ratio and brood size were not affected by white fringe tree.

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ABSTRACT

Emerald ash borer (EAB), *Agrilus planipennis* is a buprestid species native to Asia, where it is a pest of ash trees, *Fraxinus* spp. Since 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 the potential for host range expansion. 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 under no-choice conditions. EAB larvae can be successfully parasitized in white fringe tree under laboratory conditions. These results indicate that *S. agrili* and *S. galinae* have potential to attack EAB larvae infesting 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/or and semivoltine life cycles which damage the host tree during the larval stage by feeding on the phloem tissue to the point that it effectively girdles and kills the tree (Wei et al., 2007; Tluczek et al., 2011). All North American native species of ash are

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susceptible, however blue ash (*F. quadrangulata Michx.*) is more resistant than other species (Tanis and McCullough, 2012). An estimate of damage from 2009 to 2019 indicated 17 million ash trees killed at a cost of over $25 billion (Kovacs et al., 2010). Emerald ash borer has spread beyond the range of which that estimate was based on, and has now killed hundreds of millions of ash trees in the invaded range in the U.S. (Emerald ash borer information, 2020).

Emerald ash borer has recently developed a new association with another North American native species, the white fringe tree (*Chionanthus virginicus* L.) (Cipollini and Rigsby, 2015). The native range of white fringe tree 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 that emerald ash borer has been utilizing white fringe tree simultaneously along with *Fraxinus* spp. in North America (Thiemann et al., 2016). Under both laboratory 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, with contrasting results (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) claim that white fringe tree may be an enemy free space for EAB, due to *T. planipennisi* failing to parasitize white-fringe-reared EAB in their study. These differences in results may be due differences in experimental design. Hoban et al. (2018) manually inserted healthy EAB larvae, whereas Olson and Rieske (2019) reared EAB from egg in white fringe bolts. All other species of introduced parasitoids have only been tested on EAB infesting ash (*Fraxinus* spp.) and the EAB biological control program has only considered the efficacy of parasitoids attacking EAB in ash.

Spathius agrili and *S. galinae* have similar reproductive strategies, and life histories. Both species are gregarious, larval ectoparasitoids, and attack late (3rd to 4th) instar EAB (Yang et al., 2010; Duan et al., 2014). Both species appear to agree with the optimal sex ratio theory (Charnov 1981), the proportion of female offspring increases with increasing host quality (Wang et al., 2007; Watt and Duan, 2014). Brood size is also similar between the two species; *S. agrili* have broods of 2–18 (average 9.1) cocoons per brood or parasitized host larva (Yang et al., 2010); similarly, *S. galinae* have broods of 8–16 ('typically' 8–12) cocoons per brood (Belokobylskij et al., 2012).

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 a single 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. (2012b). Each artificial gallery was carved to match the length, width, and height of each individual EAB larva, dissected alive from a bolt of tropical ash (described in section 2.3).

2.3. Host Larvae

In order to standardize host larva quality, all EAB larvae used in the following 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 layer of Parafilm was tightly wrapped around the lip of the cup, over the paper towel to prevent desiccation. Each bolt with a single inserted EAB larvae represent a single replicate.

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) × 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 ventilated clear testing arenas 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. For each parasitoid species (*S. agrili* or *S. galinae*) a treatment group (EAB inserted into white fringe tree), and control group (EAB inserted into green ash) were established. In each replicate, two naïve gravid females of the same species were added to the 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'. Development time (measured in days to emergence) was calculated from the median day of the 7 d parasitoid exposure period. Parasitism rate was calculated as EAB larvae scored 'parasitized' divided by total 'alive' and 'parasizited' EAB larvae from all exposure trials for each host plant type; dead EAB larvae were excluded due to the possibility of mechanical damage, or injury caused during the larval insertion process. Sex ratio was calculated as the proportion of female progeny (female progeny / all progeny); any replicates with only male offspring were excluded because of the likelihood of asexual reproduction as a result of haplodiploidy. Brood size was calculated after dissection (to account for unemerged adult parasitoids) as a sum of all adult parasitoids developed from a single EAB larva. No-choice treatment and control groups were repeated 61 times (see Fig. 1).

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 × 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. 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'. Parasitism rate and brood size were calculated as described in section 2.5. Sex ratio and time to emergence were not recorded. Two-choice treatments were repeated 17 times (see Fig. 2).

2.7. Statistical analyses

In no-choice testing, the analyses were performed separately by parasitoid species. Nominal logistic regression models were used to assess parasitism rate (i.e., the probability of each EAB larva being parasitized by test wasps) by tree species (as explanatory variable). We then evaluated the effect of host tree species (white fringe tree vs green ash control) using loglikelihood ration chi-square tests. We chose nominal logistic regression models for these analyses because our response variable, parasitism of host larvae, has a dichotomous (i.e., parasitized vs not parasitized) or binomial distribution.

Development time in no-choice bioassays, and brood size in both choice and no-choice bioassays were analyzed using one-way ANOVAs to determine differences between host plant effects. Normality and

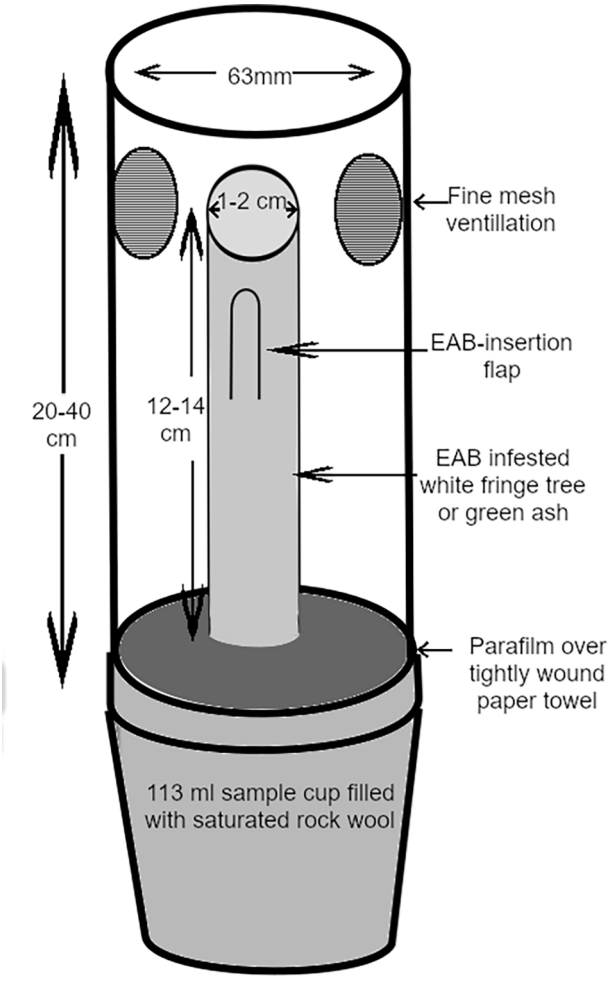

Fig. 1. Diagram of the no choice testing arena for host suitability.

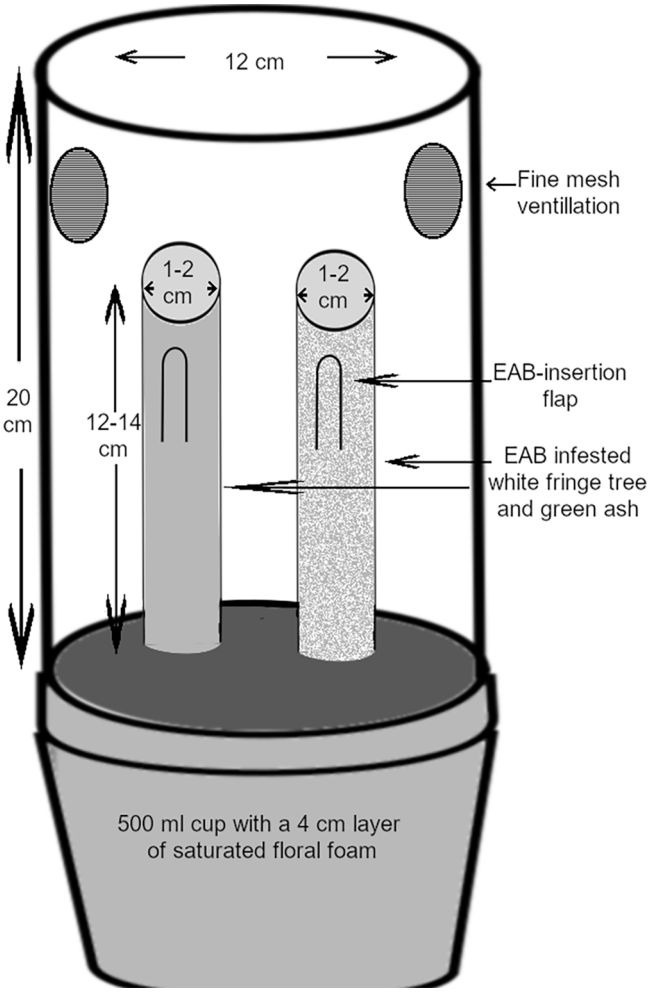

Fig. 2. Diagram of the two choice testing arena for host plant suitability.

homoscedasticity were assessed by the residual plots. Sex ratio in both no-choice and choice bioassays were assessed using a contingency table, and analyzed using a likelihood ratio Chi-square test. 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.

Parasitism success 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). 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 3.89 ± 1.52 d earlier, and *S. galinae* emerged 6.54 ± 1.14 d earlier (Table 2).

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 no-choice trials, which was numerically lower in white fringe, but not significantly different from green ash.

Brood size of *S. agrili* and *S. galinae* did not differ between white fringe tree and green ash in choice or no choice trials (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. The field survey results indicated that EAB is utilizing white fringe tree in Virginia, and developing to maturity. Our results show that both *S. agrili* and *S. galinae* can parasitize EAB larvae within white fringe tree under no-choice, and two-choice conditions. Our no-choice tests indicated that host attack (parasitism) and reproductive activity of both *S. agrili* and *S. galinae* were not 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 brood 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. Due to the long parasitoid exposure time, this result does not indicate phenological development time, rather it illustrates there may be a difference between the host plants. Emergence of *S. galinae* is inhibited by bark thickness, and harder, drier bark (Duan et al., 2014). Higher host-plant density was posited as a factor in the failure of *T. planipennisi* to parasitize EAB within white fringe tree (Olson and Rieske. 2019). We speculate that the difference in emergence time may be a result of the differences in host-plant density, bark thickness, and/or other host-plant factors inhibiting adult emergence from the green ash bolt. One cause could be a result of increased time to create an emergence hole in the bark due to increased bark density or thickness. Results from our two-choice testing of *S. galinae* also showed no difference in EAB parasitism rate and parasitoid reproductive activity 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*.

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).

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 plants 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 fitness 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 biomass is smaller and development slower than in green or white ash trees (Rutledge and Arango-Velez, 2017). We utilized inserted EAB larvae in order to test parasitism under ideal circumstances. Emerald ash borer can develop to adulthood in white fringe tree (Cipollini and Rigsby, 2015). However, 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.

Multiple North American tree species have been studied as reservoir hosts for EAB and its associated parasitoids. Blue ash is resistant to emerald ash borer (Tanis and McCullough, 2012), and has previously been hypothesized to act as a reservoir for *T. planipennisi* in areas of high ash mortality (Peterson et al., 2015). The widespread distribution of this novel host plant could lead to geographically isolated populations of EAB, or parasitoids. Based on our results and others (Hoban et al., 2018; Peterson et al., 2015), we propose that these isolated populations could serve as reservoirs for reintroduction to the natural ecosystem as ash regrows. Phenological research suggests that both *S. galinae* and *T. planipennisi* are climatically suited to the northeastern U.S. (Jones et al., 2020). While white fringe tree is not native to the northeastern U.S., it is commonly planted ornamentally (USDA, NRCS, 2020). The differences in geographic distribution of reservoir host plants may aide in the establishment of *S. agrili, S. galinae*, and *T. planipennisi* across North America.

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). Bark thickness was only shown to affect the parasitism success of *S. agrili* or *S. galinae* in large tree with a minimum bark thickness of 4 mm (Gould and Ayer, 2011; Murphy et al., 2017).

Further research should consider how host searching is influenced by white fringe tree volatiles, and complex of volatile compounds produced

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 for no-choice & choice conditions.

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]	*S. galinae* Choice Brood Size (# Progeny per host larvae)[7]
White fringe tree	35.61 ± 0.84* (n = 160)	40.61 ± 0.67 * (n = 105)	78.64 ± 3.59 (n = 160)	67.01 ± 7.06 (n = 97)	6.47 ± 0.69 (n = 23)	6.65 ± 0.54 (n = 26)	6.80 ± 0.866 (n = 10)
Green ash	39.50 ± 0.68 (n = 106)	47.15 ± 0.47 (n = 218)	84.38 ± 3.59 (n = 378)	77.52 ± 7.06 (n = 218)	6.03 ± 0.54 (n = 37)	7.28 ± 0.43 (n = 40)	7.33 ± 0.91 (n = 9)

Analyses were all performed within column. Asterisks indicate results which significantly differed from green ash control.
[1] Analysis showed significant difference (F = 12.88; df = 265; P = 0.0004).
[2] Analysis showed significant difference (F = 62.6; df = 322; P < 0.0001).
[3] Analysis showed no significant difference (χ^2 = 1.384; df = 1; P = 0.239).
[4] Analysis showed no significant difference (χ^2 = 3.778; df = 1; P = 0.0519).
[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).
[7] Analysis showed no significant difference (F = 0.17; df = 18; P = 0.677).

by EAB larval feeding. Environmental factors that influence host habitat seeking should be considered, to determine if *Spathius* can locate white fringe tree under field conditions. Differences in emergence time may indicate other developmental differences between *Spathius* adults reared in white fringe tree bolts. Additionally, direct comparisons between white fringe tree, and green ash bolts should be done simultaneously with future work. Comparisons should include bark thickness, phloem density, and C:N ratio using methods from Olson and Rieske (2019). Future research should consider measurements of adult parasitoid 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.

CRediT authorship contribution statement

Max Ragozzino: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Visualization. **Jian J. Duan:** Conceptualization, Methodology, Formal analysis, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Scott Salom:** Conceptualization, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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