Responses of two parasitoids, the exotic *Spathius agrili* Yang and the native *Spathius floridanus* Ashmead, to volatile cues associated with the emerald ash borer, *Agrilus planipennis* Fairmaire

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**HIGHLIGHTS**

- *Spathius* are attracted to host-associated volatiles of *A. planipennis*.
- Naïve *S. agrili* are attracted to: cues when associated with leaf tissue.
- Naïve *S. floridanus* are attracted to: an *A. planipennis* larva in stem tissue.
- *S. agrili* that decline to oviposit, avoid an *A. planipennis* larva in stem tissue.
- *S. agrili* respond to cues based upon physiological readiness.

**GRAPHICAL ABSTRACT**

**ABSTRACT**

Two parasitoids, the introduced specialist *Spathius agrili* Yang (Braconidae), and the native generalist *Spathius floridanus* Ashmead, have been proposed as biological control agents of the emerald ash borer, *Agrilus planipennis* Fairmaire (Buprestidae). However, little is known about their host-location behaviors. We evaluated responses to their host complex, *Fraxinus pennsylvanica* stem tissue, *F. pennsylvanica* foliage, and an *A. planipennis* larva within a stem. Experiments were conducted in a Y-tube olfactometer, using wasps reared on *A. planipennis* larvae in *F. pennsylvanica* stems. Naïve *S. agrili* were attracted to the entire complex, and to leaf tissue, relative to blanks. *S. agrili* were also more attracted to stems containing larva and leaf tissue together than leaf tissue alone. Naïve *S. floridanus* were attracted to larvae within stems, but nothing else. A further distinction is that *S. agrili* moved more, in the presence of foliage. Thus, *S. agrili* and *S. floridanus* appear to employ different host-location strategies. The former is attracted to host plant cues, which then elicit increased searching, whereas the latter is only attracted to infested tissue directly. We found no evidence that oviposition influences attraction by *S. agrili*, suggesting other forms of experience should be evaluated for potential sources of learned cues. Further, *S. agrili* that declined opportunities to oviposit oriented away from host-associated cues, suggesting distinct behavioral sequences occur by females that are not reproductively ready. Further understanding of host-location behavior may improve biological control by these parasitoids, by suggesting strategies for pre-release conditioning and providing tools for assessing post-release establishment.

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**1. Introduction**

The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an accidentally introduced invasive species indigenous to Asia. In North America, it was first discovered in
Detroit, MI (Haack et al., 2002), and it was verified from near Newberg, Wisconsin in August 2008. As of April 2014, there are confirmed infestations in 21 US states, two Canadian provinces, and 19 counties in Wisconsin (Emerald ash borer info, 2013). Population expansion can be attributed to several factors, including lack of coevolved tree defenses by North American Fraxinus (Herms et al., 2004), lack of natural enemies, inter- and intra-state movement of infested wood by humans, and its cryptic nature, which delays detection until several years after it has established (Cappaert et al., 2005; Siegert et al., 2007; Herms and McCullough, 2014).

In Asia, A. planipennis colonizes stressed and dead trees in the genus Fraxinus, specifically Manchurian ash, Fraxinus mandshurica Rupr., and Chinese ash, Fraxinus chinensis Roxb., and Fraxinus chinensis Roxb. subsp. rhynchophylla (Hance) (Chinese Academy of Sciences, 1986; Yu, 1992; Haack et al., 2003; Liu et al., 2003; Gould et al., 2005). In Japan and Korea, it has also been reported to colonize elm (Ulmus), walnut (Juglans), and wingnut (Pterocarya) (McCullough & Katovich, 2004). In North America, A. planipennis colonizes all Fraxinus spp. within its current distribution (Poland and McCullough, 2006). Emerald ash borer adults appear to prefer volatiles from ash species having particular chemical signatures (Rodriguez-Saona et al., 2006; Eyles et al., 2007), as well as stressed trees (Pureswaran and Poland, 2009). Manchurian ash has a different composition of phenolics and defensive proteins than that of North American white ash, Fraxinus americana L. and green ash, Fraxinus pennsylvanica L., but it is unknown whether this contributes to resistance (Cipollini et al., 2011; Herms and McCullough, 2014). It has been hypothesized that lignans and lignan-derivatives unique to Manchurian ash may contribute to its resistance to A. planipennis (Whitehill et al., 2012).

Adult beetles emerge after approximately 450 degree-days (base 10 °C), fly into crowns of ash trees and feed on foliage (Yu, 1992). While mate attraction has a large visual component (Lelito et al., 2007), females also emit the lactone (3Z)-dodecen-12-olide, which combined with the ash stress volatile (3Z)-hexenol, enhances mate attraction at close range (de Groot et al., 2008; Grant et al., 2010; Silk et al., 2011). Females search for suitable hosts after one week, and oviposit 1 mm, oval-shaped eggs into bark crevasses. The larvae hatch after one week, bore into the cambium, feed and develop through four instars, and then move into the sapwood where they pupate (Yu, 1992; USDA APHIS, 2011). They are either univoltine or semivoltine, depending on temperature (Cappaert et al., 2005; Wei et al., 2007) and host condition (Haack, 2003).

Systemic pesticides and sanitation can reduce losses to high-value trees in urban environments (McKenzie et al., 2010; Mota-Sanchez et al., 2009; Smitley et al., 2010). However, there are currently no environmentally and economically viable options for managing this invasive species in forests. This suggests a need for biological control.

Three species of Asian, putatively host-specific, parasitic wasps have been released against A. planipennis, including the gregarious larval parasitoids Tetrastrichus planipennisi Yang (Hymenoptera: Euplodenidae) and Spathius floridanus Ashmead (Hymenoptera: Braconidae), and the egg parasitoid Oobius agrili Yang and Huang (Hymenoptera: Encyrtidae) (USDA APHIS, 2012). Several North American generalist species have been observed parasitizing A. planipennis in the field but their impacts are unknown (Duan et al., 2012). It is likely that reservoirs of these native parasitic wasps exist inside their common hosts, such as the bronze birch borer (Agrilus anxius Gory), two-lined chestnut borer (Agrilus bilineatus (Weber)), and bronze poplar borer (Agrilus liratus) Barter and Brown. Like A. planipennis in Asia, these species of Agrilus are associated with stressed trees (Anderson, 1944; Haack and Benjamin, 1982; Loech and Cameron, 1983; Dunn et al., 1986) and hence might attract similar natural enemies. Spathius floridanus Ashmead (Hymenoptera: Braconidae) is a native, larval idiobiont, ectoparasitoid that has been recovered from three families and multiple genera of wood boring beetles (Matthews, 1970; Marsh and Starzana, 2009). Its host range includes beetles colonizing at least seven host tree genera. It attacks A. anxius (Marsh 1979; Nash et al. 1951), A. bilineatus (J.S. Strazanac, personal communication), and A. planipennis (Duan et al., 2012). This contrasts with S. agrili, which has only been observed to parasitize Agrilus, almost exclusively A. planipennis, even under no-choice conditions in the laboratory, and is almost exclusively associated with Fraxinus in the field. Understanding the responses by these insects could potentially assist biological control of A. planipennis in at least two manners: (1) pre-release conditioning, and (2) improved monitoring.

Parasitic wasps are adept at locating cryptic hosts by exploiting chemical cues directly and indirectly associated with their development (Vet and Dicke, 1992; De Moraes et al., 1998; Boone et al., 2009). Since species of Agrilus tend to select stressed trees, it seems likely their natural enemies would be attracted to volatiles associated with tree stress, associated microorganisms (Madden, 1968; Sullivan and Berisford, 2004; Martinez et al., 2006; Boone et al., 2009), and/or infestation category. There is some evidence that S. agrili is attracted to volatiles, as it oriented toward cut leaves of Fraxinus velutina Torr. and F. pennsylvanica in laboratory assays (Yang et al., 2008). Ash bark with larval frass, A. planipennis larvae, and larval frass alone were also investigated, but results were inconclusive (Wang et al., 2010). The use of chemical signals for host location by S. floridanus has not been tested. To study attraction further, we introduced several more ecologically relevant conditions, including using intact leaflets of live Fraxinus americana seedlings, as opposed to excised foliage, F. pennsylvanica stem tissue, and an A. planipennis larva inside of stem tissue, as opposed to ash bark alone, and an A. planipennis larva alone. These components were tested singly and together against blanks, as well as against one another, for both wasp species, simulating choices encountered in nature. Our goal was to identify, which, if any, components of the Fraxinus–Agrilus complex were attractive to S. agrili and S. floridanus, their potential interactions, and their relative importance.

2. Methods

2.1. Culture of insects and plants

S. agrili and S. floridanus were received from lines maintained on A. planipennis in F. pennsylvanica at USDA APHIS EAB PPQ, Brighton, MI. These lines originated from Tianjin, China and locations within MI, respectively. Both lines have been supplemented with new introductions intermittently. Colonies were initiated by introducing wasps (5:1 female: male) into plastic containers with twigs containing 3rd-4th instar A. planipennis larvae (Gould et al., 2011) for two weeks. After this period, parasitoids were transferred to a new cup with honey streaked across the top, while the parasitized larva remained in the previous cup. All cups containing the two species of Spathius were kept inside a growth chamber at 25/22 °C on a 16 h day/8 h night cycle at 50–70% relative humidity.

Two-year old, greenhouse raised seedlings of F. pennsylvanica were acquired from Lawyer Nursery (Plains, MT) and stored at 4 °C until planting. Seedlings were planted singly in classic 600 pots (Nursery Supplies Inc., Chambersburg, PA) with Metro-Mix 360 (SunGro Horticulture, Bellevue, WA) potting medium in a greenhouse, prior to experiments. Seedlings were fertilized with Osmocote 14–14–14 and Peters Excel 21–5–20 according to manufacturer directions and as needed (Scotts Company LLC, Cranston, RI). Seedlings were held at a 16 h day/8 h night, light cycle, and 26.5 °C/22 °C temperature regimes, to emulate summer conditions.
Seedlings were watered daily for a week after planting, and then every other day thereafter, with tap water. Seedlings broke dormancy and began vegetative growth for at least two weeks.

2.2. Olfactometer conditions

Behavioral assays were conducted at 25 °C and 70% relative humidity in a horizontal glass Y-tube olfactometer (Fig. 1), within a laminar flow hood with overhead, fluorescent lighting. Purified air, sent via convoluted Teflon tubing from a VAS Systems air delivery system (VAS Systems, Rensselaer, NY), was passed across treatments before their presentation. Contingent upon the odor source, containment vessels were glass bell jars that sat over glass petri dishes in a basin of deionized water (Modified from: Ngi-Song et al., 1996), and/or were enclosed by Teflon leaf chambers (VAS Systems, Rensselaer, NY). Based on preliminary experiments, all trials were of 10-min duration, with the first minute considered an acclimation period. Also based upon preliminary experiments, wasps were allowed to acclimate to the room for at least 10 min before introduction into the arena (Johnson et al., 2013). Single (n ≥ 24), mated, ~7–14 day old ovipositionally naïve females were used and discarded after each trial. Odor sources were randomized using the sample() function in R (R Development Core Team, 2011) for every trial to prevent position bias. All glassware was cleaned with acetone and then hexane, and allowed to dry. Y-tubes were cleaned after every trial. Odor sources were replaced after four trials and all other glassware cleaned. All glassware was further washed with soap (Alconox, White Plains, NY) and water at the end of each assay.

2.3. Selection and presentation of Agrilus–Fraxinus complex components

To determine which component(s) are attractive to S. agrili and S. floridanus, a series of hierarchal experiments was performed (Table 1) with components of the A. planipennis–Fraxinus complex, including stem tissue, ash leaf tissue, and an A. planipennis larva. Green ash stem tissue of ~5.1 cm circumference and ~12.7 cm length from 5 to 15 year old trees was cut and bored, and a live L3–4 instar A. planipennis larva was inserted under the bark and covered partially with Parafilm 12–48 h earlier. Leaf tissue consisted of an attached leaflet extending from a two year-old green ash seedling. Frass was usually present but could not be manipulated directly.

Subtractive elimination, starting with the entire complex relative to a blank, was used to determine attraction to Agrilus–Fraxinus complex components. Two treatments were applied in every experiment, a test treatment and a control.

Fig. 1. Glass Y-tube olfactometer used in experiments with S. agrili and S. floridanus. Treatment and control arms were randomized for each trial.
Table 1
Number of wasps that moved toward treatment or control arms, or made no choice, in a 10 min assay in a Y-tube olfactometer. a. *Spathius agrili*. Experiments 1-5 were conducted with naïve wasps. In experiment 6, wasps were either given an opportunity to oviposit and did (OY), were given an opportunity to oviposit and declined (OD), or were naïve (N); b: *Spathius floridanus* – Naïve.

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<th>Experiment</th>
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2.4. Effect of experience

Oviposition was manipulated to determine whether ovipositional experience affects attraction by *S. agrili* to infested stem tissue. Naïve *S. agrili* were singly assigned to plastic containers with honey streaked on the sides, and containing infested or non-infested ash tissue (described in 2.3). Wasps were kept in growth chambers (described in Section 2.1), for at least 48 h, and then used in experiments. Because oviposition by a wasp could not be confirmed until at least 1–2 weeks after exposure, groups of wasps were considered: those that were given a chance to oviposit and did (OY), those given a chance to oviposit but declined (OD), and naïve individuals not given an opportunity to oviposit (N). All wasps used in experience experiments on a given date were of the same age. Trials within an assay were randomized to prevent order bias between groups of wasps.

2.5. Data collection and analysis

The following behaviors were quantified visually and with a digital timer:

1. First choice: The first arm that a wasp entered after the acclimation period (Fig. 1).
2. Last choice: The location (arm or vestibule) of a wasp at the end of a trial.
3. Turnover: The number of times a wasp moved between the arms and vestibule of the Y-tube, and the directions of change.
4. Time spent in each location during the trial.

Additional data included date of trial, time of trial, age of wasp, and amount of time *A. planipennis* had spent in stem tissue before trial. Post-hoc determination of larval mortality and frass production was also performed.

Data from experiments conducted on different days were pooled for analysis. Trials wherein larvae died or had molted to late 4th instar were excluded from analysis. Wasps that did not enter an arm during the 10-min trial, or did not remain in an arm of the Y-tube after the 1-min acclimation period (i.e. the remaining 9 min) were deemed “no choice” and excluded from analysis.

All data were analyzed in R (R Development Core Team, 2011), with the null hypothesis being no treatment effect. Choice data were analyzed using Pearson’s chi-squared test. Total movement by experiment was analyzed using analysis of variance.

3. Results

3.1. Effect of host-associated volatiles on attraction of *S. agrili*

Naïve *S. agrili* were attracted to the entire *Agrilus*–*Fraxinus* complex ($\chi^2 = 9.256, p = 0.002$), and to leaf tissue alone ($\chi^2 = 4.167, p = 0.041$), relative to blank controls (Fig. 2a). Nearly three times as many wasps went to the full complex as to the blank, and over twice as many went to the leaf as to the blank. Complete statistics are in Table 1a. No other components were attractive by themselves. Wasps were also more attracted to the entire *Agrilus*–*Fraxinus* complex ($\chi^2 = 4.313, p = 0.038$) than to leaf tissue alone (Fig. 2a), by a factor of 1.6X. In the experiment testing ovipositional experience, wasps that were given the opportunity to oviposit but declined went to the blank control arm more often than to infested stem tissue, by a factor of nearly threefold ($\chi^2 = 10.373, p = 0.001$) (Fig. 2b).

3.2. Effect of host-associated volatiles on attraction of *S. floridanus*

Naïve *S. floridanus* were significantly attracted to stem tissue with a feeding larva relative to a blank control ($\chi^2 = 10.667, p = 0.001$) (Fig. 2c). There was a trend towards attraction to stem tissue against a blank (over 2X), but this was non-significant ($\chi^2 = 3.240, p = 0.072$) (Fig. 2c). All other components showed no significant treatment effects. Complete statistics are in Table 1b.

3.3. Effects of host-associated volatiles on movement by *S. agrili* and *S. floridanus* within, and between, various locations of olfactometer

Means of all movements between locations within the olfactometer (Table 2a) varied among experiments with different treatments for *S. agrili* (df = 8, $F = 9.54$, $p < 0.001$). The largest mean movement was 17.8 times, in the presence of leaf tissue against a blank. Wasps exposed to stem tissue with or without a larva, prior to an experiment, moved less, 2.4 (OY), 5.3 (OD), 3.8 (N) than...
those that assayed against stem tissue without exposure 8.6, when presented with stem tissue with a feeding larva against a blank (Table 2a). Means of all movements between locations within the olfactometer (Table 2b) by *S. floridanus* were different between experiments (df = 4, $F = 2.61$, $p = <0.04$). The most movements, 4.3, were observed when *S. floridanus* was presented stem tissue with a feeding larva against a blank (Table 2b). Overall, *S. agrili* showed more movement than *S. floridanus*. Additional details are in Johnson (2013).

### 4. Discussion

These results indicate that *S. agrili* is attracted to the complex of odors associated with *A. planipennis* and its intact host plant, that the most potent source of volatiles is from leaves, that foliar volatiles are required for attraction, and that the attraction to *F. pennsylvaniaeca* leaves is heightened by the presence of *A. planipennis* boring in a stem. Attraction to foliar volatiles in our assays was consistent with observations in previous studies (Yang et al., 2008; Wang et al., 2010), but differed in that females of *S. agrili* demonstrated attraction to attached leaves, and interestingly, showed a lower degree of attraction than in the prior detached-leaf assays. Females of *S. floridanus* are likewise attracted to volatiles of this host complex, but to different sources. These insects were most attracted to an *A. planipennis* larva feeding within *F. pennsylvaniaeca* stems, but not to foliage. Future work should evaluate the extent to which these different strategies may relate to the wasps’ specialist vs. generalist life histories.

We did not find evidence of learning, despite it having been demonstrated in other systems, including in bioassays using similar conditions (Vet and Groenewold, 1990; Vet and Dicke, 1992; Allison and Hare, 2009). Other life history events such as

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**Fig. 2.** Movement by wasps toward odor sources in Y-tube olfactometer during 10-min assays. Percent entering treatment (right, dark bars); and control (left, open bars) are shown. Treatments with *, **, or *** are significant at $p < 0.05$, 0.01, or 0.001, respectively. (a) Naïve *Spathius agrili*; (b) *Spathius agrili* that were naive (N), were given an opportunity to oviposit and declined (OD), or were given an opportunity to oviposit and did (OY); (c) Naïve *Spathius floridanus*. 

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### a. Naïve *Spathius agrili*

![Graph a. Naïve Spathius agrili](image)

### b. *Spathius agrili* given an ovipositional opportunity

![Graph b. Spathius agrili given an ovipositional opportunity](image)
emergence from the host, as well as other cues, should be examined for sources of learning. Our observations on wasps that had been given an opportunity to oviposit but declined may provide some insight into ovipositional readiness. These insects not only showed no attraction to host-associated cues, but actually showed increased movement towards blanks. This suggests that when

![Diagram](image)

**Table 2**

Number of times wasps changed locations among treatment (t) and control (c) arms, and vestibule (v), in a Y-tube olfactometer during 10-min assays. All data are standardized by the number of replicates. Wasps had no access to treatment material. Each time a wasp changed position is tallied in the sequence prior-next. a: *Spathius agrili*; b: *Spathius floridanus*.

<table>
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| 5 | + | + | + | – | – | – | 0.163 | 1.913 | 1.150 | 1.738 | 0 | 1.150 | 0.013 | 6.125 | – | + | + | – | – | – | 0.163 | 1.913 | 1.150 | 1.738 | 0 | 1.150 | 0.013 | 6.125 |

a Stem tissue of *Fraxinus pennsylvanica*.
b Leaf tissue of *F. pennsylvanica*.
c Larva of *Agrilus planipennis*.

c. Naive *Spathius floridanus*

![Diagram](image)

**Fig. 2 (continued)**
wasps are not yet physiologically ready to oviposit, their search for other resources, such as nectar, may elicit different orientation sequences. Future studies should address this by studying how unmated wasps (or those having no eggs), or starved wasps, respond to stimuli previously shown to be attractive.

Time data may reflect how long it takes various species of female wasps to respond to, or process stimuli (Wajnberg, 2006). A decrease in mean time spent searching, as in experiments 1–3b, may reflect assessment by a female S. agrili of host-preservation (Johnson, 2013). As cues become more complex, they provide more information, possibly allowing the parasitoid to assess host-presence more rapidly (Vet and Dicke, 1992; Wajnberg, 2006). The observed trend may not follow for experiments 4 and 5 because this control arm was not blank. These data may indicate the amount of time various species of Spathius spend investigating host-associated cues before giving up (Charnov, 1976; Wajnberg, 2006). The different trends between the wasps’ choice of which source of volatiles to follow, versus how long to remain in an olfactometer arm, may arise from the experimental condition of wasps not being able to actually contact a host, or oviposit, following their movement toward host-associated odors.

The highest mean turnovers for S. agrili and S. floridanus occurred when presented leaf tissue alone relative to a blank control, and infested ash stem relative a blank control, respectively. Exposure to leaf tissue volatiles may induce increased movement by S. agrili, facilitating location of short-range cues or vibrations by concealed hosts. Unattractive components such as non-infested ash stem tissue might also elicit increased movement, but perhaps orient the wasps away from locations that do not contain hosts.

Comparisons between S. agrili and S. floridanus can contribute to our knowledge of relatively specialist versus generalist life history strategies. Female S. agrili are attracted to leaf tissue volatiles and exhibit heightened search activity in their presence. Increased movement may facilitate location of a larval host, which was shown to be attractive in the presence of leaf volatiles. The more generalist S. floridanus is attracted to stem tissue containing its natal host, and also displays increased movement in its presence. Perhaps the generalized attractiveness of a feeding host larva is offset by the presence of ash volatiles, which in the case of S. floridanus appear to be not innately attractive, as they are for S. agrili. Spathius agrili and S. floridanus use different cues to locate their hosts at a distance, but in close range, appear to increase movement to locate additional attractive cues.

Understanding attraction to components of the Agrilus–Fraxinus complex by S. agrili and S. floridanus may improve biological control of A. planipennis. As cues involved in learning are better understood, pre-release conditioning and manipulation of oviposition could increase wasp efficacy. Additional, attractive components might aid trap development, biocontrol agent selection, and interpretations of establishment or failure. Further work on host-location behaviors for S. agrili and S. floridanus is needed. Identification of Agrilus–Fraxinus complex volatiles that induce attractive responses would assist in pre-release conditioning and monitoring. We do not know whether S. agrili responds as strongly (or at all) to other species of Fraxinus, whether it can differentiate between them, and whether wasps can differentiate between healthy or stressed trees. Understanding the host-range and preference of S. floridanus might also aid biocontrol, especially if a natal host alters the preferences of S. floridanus. Future research on these topics should provide additional guidance for biocontrol of A. planipennis, and hopefully allow better targeting of the pest and training of biocontrol agents.

Acknowledgments

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