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ORIGINAL ARTICLE

Abundance of volatile organic compounds in white ash phloem and emerald ash borer larval frass does not attract *Tetrastichus planipennisi* in a Y-tube olfactometer

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Abstract Many natural enemies employ plant- and/or herbivore-derived signals for host/prey location. The larval parasitoid *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) is 1 of 3 biocontrol agents currently being released in an effort to control the emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coloeptera: Burprestidae) in North America. To enhance its efficiency, allelochemicals that attract it need to be assessed. In this study, ash phloem volatile organic compounds (VOCs) of black, green, and white ash, and EAB larval frass were compared. Foraging behavior of *T. planipennisi* females in response to VOCs of white ash or frass from EAB larvae feeding on white ash phloem was tested using a Y-tube olfactometer. Results indicated that the 3 ash species had similar VOC profiles. EAB larval frass generally contained greater levels of VOCs than phloem. Factor analysis indicated that the 11 VOCs could be broadly divided into 2 groups, with α-bisabolol, β-caryophyllene, (*E*)-2-hexenal, (*Z*)-3-hexenal, limonene, methyl benzoate, methyl indole-3-acetic acid, methyl jasmonate, methyl salicylate as the first group and the rest (i.e., methyl linoleate and methyl linolenate) as a second. Abundance of VOCs in white ash phloem tissue and frass, nevertheless, did not attract *T. planipennisi* females. The concealed feeding of EAB larvae might explain the selection for detectable and reliable virbrational signals, instead of undetectable and relatively unreliable VOC cues from phloem and frass, in short-range foraging by *T. planipennisi*. Alternatively, it is possible that *T. planipennisi* is not amenable to the Y-tube olfactometer assay employed.

Key words *Agrilus planipennis*; biological control; *Fraxinus*; invasive species; semiochemical

Introduction

Since its discovery in 2002 in North America, emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coloeptera: Burprestidae), has destroyed tens of millions of ash trees (*Fraxinus* spp.) (Poland & McCullough, 2006; Kovacs *et al.*, 2010). The larval parasitoid *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) is 1 of 3 insect biocontrol agents from China currently being released in North America in an attempt to control this pest. The efficiency of biological control agents in suppression of host populations in large part depends on how effectively the agents can locate their hosts/prey. The cues most important in accomplishing this in the EAB-*T. planipennisi* system remain uncertain.

Many parasitoids exploit volatile organic compounds (VOCs) released by host plants in long-range host location (Geervliet *et al.*, 1998; Pareja *et al.*, 2009). Host

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plants release a blend of VOCs especially when damaged mechanically or by herbivorous hosts (Chen *et al.*, 2008; Chen & Poland, 2009). Many other parasitoids, together with other physical or chemical signals, utilize semiochemicals from host frass (or fecal waste) to locate hosts (Mattiacci *et al.*, 1999; Reddy *et al.*, 2002). Host-derived signals such as those from frass are thought to be more reliable albeit less detectable, than chemical signals from host plants (Vet *et al.*, 1990). The evolution of projectile frass ejection by the skipper caterpillar, *Epargyreus clarus* (Cramer) (Lepidoptera: Hesperiidae) is thought to be driven by the host location behavior of its natural enemies (Weiss, 2003).

Little is known about long- and short-range foraging cues employed by *T. planipennisi* females, except for evidence that they use vibration signals generated by feeding EAB larvae for short-range location of hosts (Ulyshen *et al.*, 2011) as they show no response to inactive larvae (Ulyshen *et al.*, 2010). Little is known about VOCs of black, green, and white ash phloem tissue or those of frass produced by EAB larvae feeding upon these species. The potential importance of these compounds to *T. planipennisi* females in short-range location of infested trees or hosts remains unexplored. On several occasions, however, we have observed *T. planipennisi* showing considerable interest in frass-filled EAB galleries exposed by removing bark from infested trees at field sites where *T. planipennisi* was released (M.D. Ulyshen, personal observation). In this study we compared VOCs of 3 ash species and corresponding EAB frass and tested the response of *T. planipennisi* females to a blend of VOCs using a Y-tube olfactometer. We hypothesized that phloem tissues of different ash species differ in VOC profiles given documented differences in foliar chemistry (Pureswaran & Poland, 2009; Chen & Poland, 2010) and head-space VOCs (Chen *et al.*, 2011b). We also hypothesized that frass VOC profiles are distinct from those of phloem due to changes associated with gut passage. We further hypothesized that *T. planipennisi* females, like many other parasitoid species, are attracted to ash phloem and frass.

Materials and methods

Plants and insects

Black ash, *Fraxinus nigra* Marshall (Family: Oleaceae), green ash, *F. pennsylvanica* Marshall, and white ash, *F. americana* L., are the 3 most abundant ash species in the northeastern USA and were used in this study. Sources of ash trees and methods of ash phloem tissue and EAB larval frass collection were described in (Chen *et al.*, 2011a). Briefly, healthy (i.e., no visual exterior EAB symptoms) small-diameter (approximately 4–6 cm at 1.5 m height) trees belonging to the 3 species of ash were collected in Legg Park, Ingham CO., Michigan, USA, in January 2010. Five healthy black ash trees and 6 healthy green ash trees were cut from a low-lying area inside a mature riparian forest, whereas 6 healthy white ash trees were cut from a grove of open-grown conspecifics near the forest edge. A 15-cm section (approximately 4–6 cm in diameter) of trunk was cut from each tree. A 4th instar EAB larva was inserted head down into a narrow groove chiseled beneath a small bark flap peeled from the top end of each trunk section to encourage feeding in the direction with the greatest available host resource (Ulyshen *et al.*, 2010). The bark flaps were then held closed over the inserted larvae with thin strips of Parafilm M (Flinn Scientific, Inc, Batavia, Illinois, USA). After larval insertion, the trunk sections were held in an incubator (25 °C, ca. 75% humidity) for 5 d before collecting frass and phloem samples from each. Phloem samples were collected by peeling approximately 2 g of live cambial tissue from an area of each log section where larvae had not fed. Frass samples were collected from the larva feeding in the same log. Frass and phloem samples were lyophilized using a Modulyo freeze dryer (Thermo Scientific, Pittsburgh, Pennsylvania, USA). Lyophilized phloem samples were then ground in a 475-A Wiley mill (Authur M. Thomas Co., Philadelphia, Pennsylvania, USA), sieved through 20-mesh (opening: 0.85 mm) screen and prepared for extraction of VOCs. Because some larvae did not produce enough frass, the final numbers of replications for each treatment included in the statistical analyses were:

$$N_{\text{black ash\_phloem}} = 5,\ N_{\text{black ash\_frass}} = 3,$$
$$N_{\text{green ash\_phloem}} = 6,\ N_{\text{green\_frass}} = 5,$$
$$N_{\text{white ash\_phloem}} = 6,\ \text{and}\ N_{\text{white ash\_frass}} = 4.$$

Rearing of *T. planipennisi* followed the method described in (Ulyshen *et al.*, 2011). A laboratory colony of *T. planipennisi*, originally collected in 2008 from Liaoning Province of China, was used in this study. Only naive wasps that had not been presented with hosts were used in the experiment. They were 3–6-week old at the time of use and were presumed to have mated as mating activities were observed almost immediately after female emergence.

Extraction and identification of VOCs

VOC extraction and identification followed the method described in (Chen *et al.*, 2008) with some

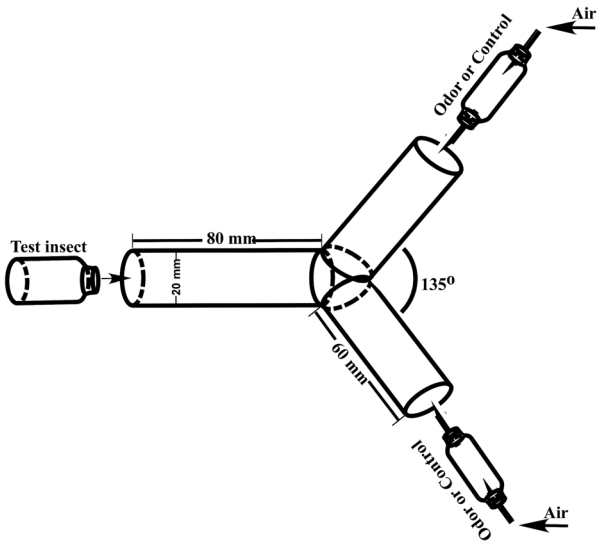

Fig. 1 Diagram of the Y-tube olfactometer used in the study.

modifications. Preweighed phloem and frass samples were extracted with 500 μL extraction solution made of H_2O, 1-propanol and HCl at 1 : 2 : 0.005 (v/v/v). Heptyl acetate (500 ng) was added as an internal standard. After vortexing for 30 sec, 800 μL dichloromethane ($MeCl_2$) was added and the mix was further vortexed for 30 sec before being centrifuged at 12 000 $\times$ *g* for 1 min. Samples were analyzed using a Thermo Scientific Trace gas chromatograph (GC) and DSQ-II mass spectrometer (MS) under positive ion chemical ionization (PICI) mode. The separation column was a Thermo TR-1MS column (30 m $\times$ 0.25 mm $\times$ 0.25 μm film). Methane was the reagent gas. Identification and qualification of VOCs was based on retention time and selected ions. Eleven VOCs were consistently detected. The selected ions (mass) for identification of these VOCs and retention times were: (*E*)-2-hexenal (99, 4.04 min), limonene (137, 6.29 min), methyl benzoate (118, 6.86 min), methyl salicylate (153, 7.94 min), (*Z*)-3-hexenal (159, 9.18 min), β-caryophyllene (205, 10.30 min), methyl jasmonate (225, 11.85 min), α-bisabolol (203, 12.32 min), methyl indole-3-acetic acid (190, 13.11 min), methyl linoleate (295, 14.99 min), and methyl linolenate (293, 15.02). Quantification was done with linear regression lines generated using commercially available compounds.

Behavior of T. planipennisi in Y-tube olfactometer

To test attractiveness of ash phloem and EAB larval frass to *T. planipennisi*, a Y-tube olfactometer (Fig. 1) was built. Due to limited availability of *T. planipennisi*, only white ash phloem and frass produced by EAB larvae feeding on white ash were tested. Two tests, ash phloem versus control and frass versus control, were conducted. The Y-tube olfactometer was held horizontally in both tests, which were conducted in a room illuminated with 8 fluorescent lamps (100 W). The room was at 25 $\pm$ 2 °C and approximately 75% relative humidity. In the phloem versus control test, 50 mg of ground phloem tissue was placed in one arm of the olfactometer and charcoal-filtered air flow (0.1 mL/min) was introduced. Only charcoal-filtered air flow was introduced in the other arm of the olfactometer and served as a control. One mated *T. planipennisi* female was released from the test vial and its orientation was observed for a maximum of 5 min. Outcomes were recorded as "Phloem" if the female travelled at least 10 mm into the arm containing phloem tissue, "Control" if the female travelled at least 10 mm into the empty arm and "No Choice" if the female did not move through the main (80 mm) arm within 5 min. Ten different females were tested each day for 3 consecutive days (a total of 30 females). Arms were cleaned with $MeCl_2$ and alternated between Phloem and Control after each trial to eliminate potential traces of phloem volatiles and chemicals from *T. planipennisi*. The experimental setup and procedure for the frass versus control test was the same as for the phloem versus control test except that 50 mg of frass produced by a 4th instar EAB larva was used instead of phloem tissue. Each *T. planipennisi* female was tested with frass from a different EAB larva.

Statistical analysis

Unless otherwise indicated, statistical analyses were conducted using SAS (SAS Institute, 2009) with a critical threshold for significance of $\alpha = 0.05$. Normality and variance homogeneity were checked with Kolmogorov–Smirnov's *D* statistic and Levene's test, respectively. Because concentrations of different VOCs detected from the same sample were in one way or another correlated, VOCs were first analyzed with 2-way multivariate analysis of variance (MANOVA) with ash species as one factor (3 levels: black, green, and white ash) and sample type as the second factor (2 levels: phloem and frass). If significant effects (ash species, sample type, or interaction between ash species and sample type; determined by Wilk's λ) in MANOVA were detected, suggesting differences in overall VOC profiles, each VOC was then subjected to 2-way analysis of variance (ANOVA). Because VOCs of phloem and frass may be correlated, ANOVA is more conservative than paired *t*-tests in detecting significant differences.

Multivariate statistical tools such as principal components analysis have been used increasingly in studies of arthropod attractions to plant volatiles (van Dam & Poppy, 2008; Pareja *et al.*, 2009). Principal components analysis has the advantages of creating a small set of orthogonal variables through linear combinations of the original variables and interpreting the new set of variables based on initial loadings (Johnson & Wichern, 2007). In addition to the advantages of principal components analysis, factor analysis provides further transformation of the initial loadings into simpler structure which facilitates variable interpretation (Johnson & Wichern, 2007). Therefore, VOCs were subject to factor analysis (PROC FACTOR METHOD = PRIN ROTATE = VARIMAX in SAS) (SAS Institute Inc., 2009). The number of new variables to retain in the factor analysis was determined by following Chen (2013). New variables were later analyzed by 2-way ANOVA. If the interaction between ash species and sample type was significant, main principal components were further compared between ash species separately for each sample type, and between sample types for each ash species.

The numbers of *T. planipennisi* that made a choice between Control and Phloem (Control vs. Phloem trial) or between Control and Frass (Control vs. Frass trial) were analyzed by a χ^2 test with 50% probability of making each choice.

Results

VOC profiles of ash species and corresponding frass

Eleven VOCs were consistently detected in the phloem and corresponding frass of the 3 ash species, with methyl linoleate and methyl linolenate being the 2 most abundant (Table 1). MANOVA indicated significant effects of ash species, sample type and interactions on VOC profile ($P < 0.01$ in all case). Black and green ash phloem contained greater levels of methyl linoleate and methyl linolenate than white ash phloem (Table 1). Except methyl linoleate and methyl linolenate in green ash, concentrations of all VOCs in frass were greater than those of phloem tissues (Table 1).

Factor analysis reduced the 11 original variables (VOCs) into 2 main factors (Factor1 and Factor2) and these 2 factors explained 97.12% of variance contained in the original data (Table 2). All original VOCs except methyl linoleate and methyl linolenate weighed heavily on Factor1 (i.e., loadings were all greater than 0.6) which explained 84.94% of the variance contained in the original set of data. Factor2 weighed heavily on methyl linoleate and methyl linolenate. Factor1 VOCs were greater in frass than phloem tissue (Table 2). Frass from larvae fed black ash contained higher levels of Factor2 VOCs than frass from larvae fed either green ash or white ash, while there was no difference between frass from lavae fed the latter 2 ash species. Black and green ash phloem, however, contained greater levels of Factor2 than white ash phloem and no difference between phloem from black and green ash phloem was observed. Green ash phloem contained greater levels of Factor2 VOCs than corresponding frass, and there was no difference in Factor2 VOCs between frass and phloem of black and white ash species.

Behavior of T. planipennisi in a Y-tube olfactometer

Significantly more *T. planipennisi* females made a choice than those did not in the Control vs. Phloem trial (24 vs. 6; $\chi^2 = 10.8, P < 0.01$). Among the 24 *T. planipennisi* females that made choices, 45.83% females chose Control and 54.17% chose Phloem and the difference was not significant ($\chi^2 = 0.17, P > 0.05$). Significantly more *T. planipennisi* females made a choice than those that did not in the Control versus Frass trial (29 vs. 1; $\chi^2 = 26.1$, $P < 0.001$). Among the 29 *T. planipennisi* females that made choices in this trial, 48.28% females chose Control and 51.72% chose Frass. The difference was not significant ($\chi^2 = 0.03, P > 0.05$).

Discussion

Black, green, and white ash phloem tissue was similar in VOC profiles, except for quantities of methyl linoleate and methyl linolenate (Tables 1 and 2), which function as brood pheromone in honeybees, *Apis mellifera* L. (Hymeonptera: Apidae) (Le Conte *et al.*, 1990) and an attractant for a parasitic mite, *Varroa jacobsoni* Oudemans (Mesostigmata: Varroidae) (Le Conte *et al.*, 1989). Green ash foliage contained relatively lower levels of head space VOCs, compared to black and white ash (Pureswaran & Poland, 2009). Methyl linoleate and methyl linolenate, however, were not investigated in Pureswaran and Poland (2009) and their roles in EAB and natural enemy interactions remain unstudied. VOCs were generally more abundant in EAB larval frass than in ash phloem (Tables 1 and 2).

When foraging in the wild, parasitoids generally utilize a blend of VOCs (Novartis Foundation, 1999). The importance of the ratio of these VOCs has increasingly been recognized (Novartis Foundation, 1999; Reddy *et al.*, 2002; Crook & Mastro, 2010). In this study, the major factor (Factor1; explained 84.94% variance of the original data set) generated from the factor analysis indicated that 9 VOCs (i.e., all except methyl linoleate and methyl

Table 1 Concentrations (mean ± 1SE μg/g dry tissue) of volatile organic compounds in black, green, and white ash phloem and frass produced by emerald ash borer larvae feeding on these ash species.

Compound	Black ash		Green ash		White ash		Statistics† (*P* value)		
	Phloem ($n = 5$)	Frass ($n = 3$)	Phloem ($n = 6$)	Frass ($n = 5$)	Phloem ($n = 6$)	Frass ($n = 4$)	Ash species‡	Sample type§	Interaction¶
α-bisabolol	17.95 ± 1.86	24.73 ± 0.97	16.92 ± 1.42	28.47 ± 10.41	17.24 ± 2.77	28.78 ± 4.50	>0.05	<0.05	>0.05
β-caryophyllene	6.04 ± 0.32	9.42 ± 0.93	5.26 ± 0.35	8.91 ± 2.06	4.08 ± 0.19	8.18 ± 0.89	>0.05	<0.01	>0.05
(*E*)-2-hexenal	20.46 ± 1.23	30.80 ± 3.58	17.57 ± 1.35	36.46 ± 9.55	17.77 ± 0.88	35.36 ± 3.72	>0.05	< 0.01	>0.05
(*Z*)-3-hexenal	6.57 ± 0.40	9.98 ± 1.11	5.69 ± 0.43	11.45 ± 3.01	5.56 ± 0.27	10.90 ± 1.15	>0.05	<0.01	>0.05
Limonene	2.78 ± 0.16	4.00 ± 0.43	2.59 ± 0.27	4.83 ± 1.28	2.47 ± 0.13	4.55 ± 0.47	>0.05	<0.01	>0.05
Methyl benzoate	5.91 ± 0.36	8.82 ± 0.99	5.05 ± 0.38	10.07 ± 2.59	4.87 ± 0.24	9.70 ± 1.06	>0.05	<0.01	>0.05
Methyl indole-3-acetic acid	2.90 ± 0.16	4.35 ± 0.47	2.52 ± 0.18	5.30 ± 1.28	2.48 ± 0.12	5.08 ± 0.50	>0.05	<0.01	>0.05
Methyl jasmonate	6.94 ± 0.41	10.43 ± 1.18	6.06 ± 0.47	12.21 ± 3.19	5.94 ± 0.29	11.84 ± 1.28	>0.05	<0.01	>0.05
Methyl linoleate	34.99 ± 2.95	63.77 ± 9.10	32.82 ± 2.38	31.27 ± 7.41	13.02 ± 0.77	33.27 ± 8.31	<0.01	<0.01	<0.05
Methyl linolenate	66.28 ± 5.41	125.02 ± 14.33	61.91 ± 5.02	57.50 ± 12.98	22.55 ±1.67	57.91 ± 15.63	<0.01	<0.01	<0.01
Methyl salicylate	7.75 ± 0.55	11.28 ± 1.26	6.59 ± 0.50	13.31 ± 3.45	6.51 ± 0.32	12.83 ± 1.37	>0.05	<0.01	>0.05

†Volatile organic compounds were first analyzed by a multivariate analysis of variance. Because significant effects (ash species, sample type, or interaction between ash species and sample type; determined by Wilk's λ) were detected, individual volatile organic compound was further analyzed by an analysis of variance.

‡Three levels: black, green, and white ash.

§Two levels: phloem and frass.

¶Interaction between ash species and sample type.

Table 2 Factor analysis of concentrations of volatile organic compounds in black, green and white ash phloem and frass.

Compound	Loadings[†]					
	Factor1 (84.94%)			Factor2 (12.18%)		
α-Bisabolol	**0.6778**[‡]			0.2499		
β-caryophyllene	**0.8148**			0.5380		
(*E*)-2-hexenal	**0.9446**			0.2576		
(*Z*)-3-hexenal	**0.9346**			0.2895		
Limonene	**0.9334**			0.2449		
Methyl benzoate	**0.9324**			0.3011		
Methyl indole-3-acetic acid	**0.9482**			0.2534		
Methyl jasmonate	**0.9382**			0.2750		
Methyl linoleate	0.2928			**0.9492**		
Methyl linolenate	0.2317			**0.9647**		
Methyl salicylate	**0.9398**			0.2702		
Statistics[§]	Ash species[¶]	Sample type[††]	Interaction[‡‡]	Ash species	Sample type	Interaction
P value	>0.05	<0.01	>0.05	<0.01	<0.05	<0.01

[†]Volatile organic compounds were analyzed by factor analysis with the principal component method and Varimax rotation.
[‡]Weights greater than 0.5 were bold.
[§]The 2 major factors were further analyzed by analysis of variance.
[¶]Three levels: black, green, and white ash.
[††]Two levels: phloem and frass.
[‡‡]Interaction between ash species and sample type.

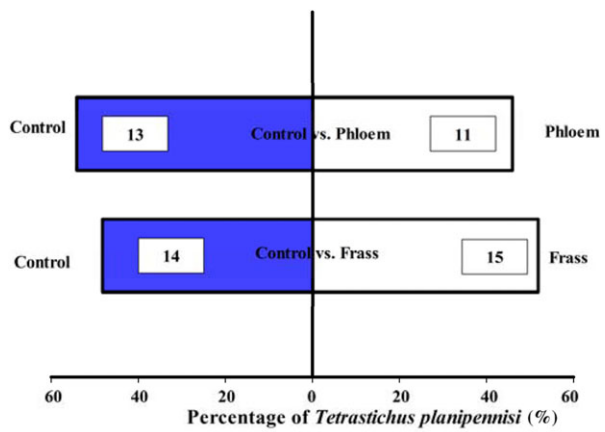

Fig. 2 Percentage of *Tetrastichus planipennisi* females that chose between Control and Frass or between Control and Phloem in a Y-tube olfactometer. Numbers on the bars denote the numbers of *T. planipennissi* females that made a choice. A total of 30 *T. planipennisi* females were tested for each test.

linolenate) were important in the VOC blend (each VOC weighed heavily on Factor1). Therefore, testing of *T. planipennisi* female foraging using phloem and frass were pragmatic.

Contrary to our expectations, the VOCs from white ash phloem or frass from EAB adults fed upon white ash did not attract *T. planipennisi* females in our Y-tube olfactometer assays. A similar lack of attraction of *Spathius agrili* Yang (Hymenoptera: Braconidae), an idiobiont ectoparasitoid of EAB, to EAB larvae and frass has been reported (Wang *et al.*, 2010). However, Johnson *et al.* (2014) found that *S. agrili* was attracted to ash leaves alone or to the host complex of ash leaves and an EAB larva boring in an ash stem. The lack of significant response by *T. planipennisi* to EAB frass in particular is inconsistent with the aforementioned behavioral observations in which *T. planipennisi* females appeared to show considerable interest in frass-filled EAB galleries exposed on infested trees in the field. It is possible that *T. planipennisi* was not amenable to the conditions of our Y-tube olfactometer assays.

Alternatively, the nonpreference of *T. planipennisi* to frass and phloem over clean air in our Y-tube bioassays might be explained by the feeding ecology of EAB larvae, at least over the short range. Although ash phloem and EAB larval frass are rich in VOCs they are not readily accessed by *T. planipennisi* because of the concealed feeding of EAB larvae. Except for minute entrance holes made following egg hatching and cracks in the bark resulting from callus formation, bark typically provides a solid layer of protection for developing EAB larvae. During feeding EAB larvae form serpentine galleries that

zigzag generally downwards (i.e., toward root area), following an increasing gradient of nutrients and water (Chen *et al.*, 2011a). This feeding behavior makes frass VOCs unlikely cues by which to pin-point exact host locations. Vibrational stimuli, which are known to be used by a wide range of parasitoids including parasitoids of wood borer, stem borer, fruit borer, and cocooned hosts (Lawrence, 1981; Meyhöfer & Casas, 1999; Broad & Quicke, 2000), may be more important to *T. planipennisi* in locating hosts over a short range (Ulyshen *et al.*, 2011). The ectoparasitoid, *S. agrili*, reportedly uses vibrational signals for host location (Wang *et al.*, 2010). Compared to volatile signals (i.e., VOCs) released from phloem and frass, vibrational signals may be more reliable as short-range cues. As a result, evolution might have selected the use of reliable vibrational signals by *T. planipennisi*, rather than less detectable and relatively unreliable signals due to the concealed feeding ecology of EAB larvae.

Due to limited availability of *T. planipennisi*, responses of *T. planipennisi* to phloem tissue of black ash and green ash and frass collected from EAB larvae feeding upon the phloem were not tested in this study. Quantities of methyl linoleate and methyl linolenate in phloem and frass of EAB larvae feeding on these ash species differed among these 3 ash species (Table 1). Although concentrations of methy linoleate and methyl linolenate in frass that was produced by EAB larva feeding on white ash phloem were over 2 fold than those of white ash phloem, neither frass nor phloem was attractive to *T. planipennis* in the study (Fig. 2). This might suggest that these 2 volatiles along and/or in combination at this range did not entice *T. planipennis*. The attractiveness of these 2 volatiles along or in combination with other compounds to *T. planipennisi*, however, remains to be elucidated.

While we found no evidence for the use of EAB larval frass cues in short-range host location, efficacy of biological control of EAB might be improved by utilization of long-range host location cues, for example, those from ash leaves. *Spathius agrili* has been shown to be attracted to leaves of green ash and velvet ash (*F. velutina*) (Wang *et al.*, 2010; Johnson *et al.*, 2014). Therefore, further studies should be directed at examining the attractiveness of ash leaves to *T. planipennisi*.

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Disclosure

The authors have no conflicts of interest to disclose.

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