Agricultural and Forest Entomology

Agricultural and Forest Entomology (2012), **14**, 324–330 DOI: 10.1111/j.1461-9563.2012.00572.x

Differential utilization of ash phloem by emerald ash borer larvae: ash species and larval stage effects

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Abstract

1 Two experiments were performed to determine the extent to which ash species (black, green and white) and larval developmental stage (second, third and fourth instar) affect the efficiency of phloem amino acid utilization by emerald ash borer (EAB) *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) larvae.

2 EAB larvae generally utilized green ash amino acids more efficiently than those of the other two species. For example, the concentrations of only six (two essential) and seven (two essential) amino acids were lower in frass from EAB that fed upon black and white ash than in the corresponding phloem, respectively. By contrast, concentrations of 16 (eight essential) amino acids were lower in the frass from EAB that fed upon green ash than in the phloem. In addition, in green ash, the frass : phloem ratios of 13 amino acids were lower than their counterparts in black and white ash.

3 The concentrations of non-essential amino acids glycine and hydroxylproline were greater in frass than in phloem when EAB fed on black ash, although not when EAB fed on green or white ash.

4 The concentration of total phenolics (a group of putative defensive compounds to EAB, expressed as antioxidant activity of acetone extraction) was high in EAB frass but even higher in the phloem samples when the data were pooled across ash species and EAB larval stages. This suggests EAB larvae may eliminate phenolics through a combination of direct excretion and enzymatic conversion of phenolics to nonphenolics before excretion. Because the ratio of frass total phenolics to phloem total phenolics in white ash was lower than the ratios in black and green ash, the ability to destroy phenolics or convert them to nonphenolics was greater when EAB larvae fed on white ash.

5 Fourth-instar EAB extracted phloem amino acids, including threonine, more efficiently than third-instar EAB. The different larval developmental stages of EAB did not differ in their apparent ability to destroy phenolics or convert them to nonphenolics.

Keywords *Agrilus planipennis*, amino acids, *Fraxinus*, nutritional ecology, phenolics, plant–insect interactions.

Introduction

Emerald ash borer (EAB) *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), an exotic species from Asia, has killed tens of millions of ash trees since it was first detected in North America in 2002 (EAB Info, 2010). Adults feed on ash foliage, whereas larvae feed in the cambial region beneath the bark, disrupting the translocation of photosynthates and nutrients. Although black (*Fraxinus nigra* Marshall), green (*Fraxinus pennsylvanica* Marshall) and white ash (*Fraxinus americana* L.), the three most abundant ash species in the north-eastern U.S.A., are all susceptible, a preference for green has been reported (Anulewicz *et al*., 2007). The underlying mechanism(s) for such preferences remain unknown, although volatile foraging signals (de Groot *et al*., 2008; Chen & Poland, 2009a), nutrition (Chen & Poland, 2009b, 2010) or defensive compounds such as phenolics (Eyles *et al*., 2007; Chen & Poland, 2009b) might be involved.

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By contrast to the fair amount of knowledge available on ash foliar chemistry (de Groot *et al*., 2008; Chen & Poland, 2009a, b), less information is available comparing the nutritional and defensive properties of phloem among different ash species (Eyles *et al*., 2007). Although EAB adults feeding on foliage excrete phenolics directly (Chen & Poland, 2010), it is not known whether the abilities of EAB larvae to extract amino acids or cope with plant secondary metabolites (i.e. phenolics) are affected by differences in nutritional and defence chemistry among the different ash species.

Phytophagous insects typically obtain nitrogenous nutrients, including essential and non-essential amino acids, from their host plants (Behmer, 2006). In addition to functioning as the building blocks of proteins, amino acids such as tyrosine and phenylalanine are indispensible for hardening of the insect cuticle (Chapman, 1998). The development of many insects is negatively affected by amino acid deficiency (Ito, 1972; Davis, 1975; Douglas, 1998). *Bombyx mori* L. (Lepidoptera: Bombycidae) failed to develop when 10 essential amino acids were absent from the diet and cocoons of pupae were smaller when diets deficient in proline were provided during the fifth instar (Ito, 1972). Similarly, *Ageneotettix deorum* (Scudder) and *Phoetaliotes nebrascensis* (Thomas) (Orthoptera: Acrididae) females preferred high- to low-proline diets (Behmer & Joern, 1994).

Phenolics are widely distributed in plants (Harborne & Baxter, 1993) and are oxidized to quinines in the insect gut by polyphenol oxidases after ingestion (Felton *et al*., 1989). The resultant quinines then bind to alkylatable amino acids (i.e. amino acids containing -SH, and -NH_2 functional groups) in proteins and form conjugations that are not subject to assimilation and thus diminish the nutritive value of the plants for the insect (Felton *et al*., 1992). Early-stage larvae of *Heliothis zea* Boddie (Lepidoptera: Noctuidae) fed phenolics such as chlorogenic acid, rutin or the aqueous phenolic extract from tomato foliage developed more slowly than those fed phenolic-free diets (Isman & Duffey, 1982). Phenolics such as gallotannins and proanthocyanidins in mountain birch (*Betula pubescens* ssp. *czereponovii*) also diminished the relative growth rate of *Epirrita autumnata* Borkhausen (Lepidoptera: Geometridae) (Ossipov *et al*., 2001).

The presence of plant defensive compounds such as phenolics may make free amino acids a major source of nutrients for phytophagous insects under conditions that promote protein conjugations by the phenolics (Felton *et al*., 1992). Ash species produce a variety of phenolics (Eyles *et al*., 2007). Therefore, in the present study, we assumed that the utilization of proteins as a nitrogen source would be limited in EAB larvae and we compared free amino acids in host plant tissues and in frass aiming to explore differences in the efficiency of utilization of three ash species by EAB larvae.

We first examined whether ash species and EAB developmental stage affect the efficiency of phloem amino acid utilization by EAB larvae. We hypothesized (utilization hypothesis) that: (i) phloem tissue would contain higher concentrations of amino acids than corresponding frass, indicating that EAB larvae efficiently extract amino acids from phloem, and (ii) EAB larvae would differ in their ability to extract amino acids from different ash species as a result of the reported adult feeding preferences (Chen & Poland, 2010), infestation preferences (Anulewicz *et al*., 2007) and chemical differences of different ash species (Chen & Poland, 2010) and at different EAB developmental stages because developmental stages usually affect the utilization of food (Scriber & Slansky, 1981). We then investigated whether ash species and EAB developmental stage affect the mechanisms for coping with phenolics. Some phytophagous insect species cope with plant allelochemicals by excreting plant toxins (Berenbaum, 1986), whereas others detoxify them (Ahmad *et al*., 1986). EAB adults feeding on foliage dispose of phenolics by direct excretion (Chen & Poland, 2010), although nothing is known about the mechanism(s) used by EAB larvae. We hypothesized (excretion hypothesis) that: (iii) the polyphenol content (expressed as antioxidant activity of acetone extract) of EAB larval frass would differ from corresponding phloem tissue, indicating an ability to dispose of dietary phenolics, and (iv) the efficiency with which EAB larvae dispose of phenolics would differ among ash species and among EAB instars, with later stages being more capable as a result of more developed detoxification systems (Sintim *et al*., 2009).

Although indices such as the consumption index, the efficiency of conversion of ingested food and the efficiency of conversion of digested food developed in 1960s (Waldbauer, 1968) are widely used to measure insect food consumption and utilization, these approaches measure the combined effects of all food components on insect growth. Because we were interested in tracking how individual components change in the process, these indices were not used in the present study.

Materials and methods

Sources of EAB larvae and ash trees

We used healthy EAB larvae at different developmental stages (second to fourth instar) and healthy (i.e. no visual outward EAB symptoms) small-diameter (approximately 4–6 cm at 1.5 m height) trees belonging to three species of ash (black, green and white). All trees and larvae were collected in Legg Park, Ingham Co., Michigan, in January 2010. Because of scarce and low EAB infestation on black and white ash trees in the area, all experimental EAB larvae were excised from two naturally grown mature green ash trees approximately 20 m apart. All stages of EAB larvae were present beneath the bark, as is generally true for trees infested by EAB for $\geq$ 1 years. Healthy green and black ash trees were cut from a low-lying area inside a mature riparian forest, whereas healthy white ash trees were cut from a grove of open-grown conspecifics near the forest edge. All EAB larvae and ash logs were refrigerated at 4 °C and with a relative humidity of 80% in the laboratory until needed and were used within several days of collection.

Experiment 1: Differential efficiency of phloem utilization in relation to ash species

We compared the abilities of EAB larvae to extract amino acids from phloem and cope with defensive compounds (i.e. phenolics) among excised black, green and white ash stems.

A 15-cm section (approximately 4–6 cm in diameter) of trunk was cut from each of six trees (i.e. replicates) per ash species. One larva was inserted head down into a narrow groove chiselled 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*., 2010a). The bark flaps were then held closed over the inserted larvae with thin strips of Parafilm M (Flinn Scientific, Inc, Batavia, Illinois). Only fourth-instar EAB larvae were used to guarantee adequate frass production to determine contents of amino acids and total phenolics (see below). After larval insertion, the trunk sections were held in an incubator (25 °C, approxmately 75% humidity) for 5 days 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 and phloem samples were lyophilized within 1 week of collection using a Modulyo freeze dryer (Thermo Scientific, Pittsburgh, Pennsylvania). Lyophilized phloem samples were then ground in a 475-A Wiley mill (Arthur M. Thomas Co., Philadelphia, Pennsylvania), sieved through 20-mesh screen and prepared for extraction of amino acids and phenolics.

Extraction and analysis of phloem amino acids was conducted as described by Chen *et al*. (2011). Pre-weighed frass and phloem tissue (approximately 10–20 mg) were mixed with 250 μL of 25% (v : v) acetonitrile in 0.01 N HCl in 1.5-mL microcentrifuge tubes. The mixture was vortexed for 5–10 min and held at room temperature for 50 min, and then centrifuged at 10 000 ***g*** for 10 min. The top layer supernatant was prepared in accordance with the instructions provided with an EZ:faast™ Free (Physiological) Amino Acid Analysis by GC-MS kit (Phenomenex, Inc., Torrance, California). Amino acids in the supernatant were first purified by sorbents and a washing solution. The cleaned amino acids were then derived to their phenylisothiocyanate derivatives and analyzed by means of a Thermo TRACE gas chromatograph UltraTM-DSQ II mass spectrometer (Thermo Scientific, Waltham, Massachusetts) using a ZB-AAA 10 × 0.25 mm Amino Acid Analysis gas chromatography column (Phenomenex, Inc.). Samples (2 μL) were injected in split mode (1 : 15) at an injection temperature of 250 °C. The oven temperature started at 110 °C and increased to 320 °C at 30 °C/min. The mass spectrometry temperature settings for source, quad and transfer line were 240, 180 and 310 °C, respectively. The ion scan range was 45–450 *m*/*z* at 3.5 scans/s. Identification of individual amino acids was based on a comparison of mass spectra in the samples with the spectra of phenylisothiocyanate derived from authentic amino acids standards provided by Phenomenex. Quantification of each amino acid was based on corresponding standard curves constructed from four different concentrations of each amino acid.

The methods of total phenol extraction and determination used in the present study are described by Chen & Poland (2009b). Briefly, ground and pre-weighed frass and phloem tissues were extracted three times, each with 70% acetone at 4 °C in the dark for 30 min. The supernatant after each centrifugation was pooled and its concentration (μmol g^{-1} dry tissue) determined using a modified Prussian blue assay (Graham, 1992). An external standard curve using gallic acid within the linear range was constructed to calculate the sample concentration.

Experiment 2: Differential efficiency of phloem utilization in relation to EAB larval development stage

The objective of this experiment was to compare the abilities of second-, third- and fourth-instar EAB to extract amino acids from phloem and cope with phenolics. A 15-cm section of trunk (approximately 4–6 cm in diameter) was cut from 18 green ash trees. Ash tree sections were randomly assigned to the three EAB instars (i.e. six replicates per instar). Larvae were inserted as described for Experiment 1.

Frass and phloem tissue collection and processing were the same as described for Experiment 1, except that samples were collected 7 days after larval insertion in an attempt to acquire adequate frass from the second-instar larvae. Extraction and determination of amino acids and total phenolics were performed as described in the preceding experiment.

Statistical analysis

For each ash species (Experiment 1) and EAB larval development stage (Experiment 2), individual amino acids and total phenolics were compared between phloem tissue and frass by paired *t*-tests because phloem tissue and frass samples were collected from the same sections of wood for each larval stage. The ratio of amino acid concentrations in frass to concentrations in phloem was used as an index of nutrient utilization efficiency, with lower ratios indicating greater efficiency. The ratio of total phenolics in frass to total phenolics in phloem was used as an index of defensive compound disposal efficiency, with higher ratios indicating greater efficiency. The ratios of amino acid concentrations and total phenolics between ash species were analyzed by one-way analysis of variance (ANOVA) (Experiment 1) or an independent *t*-test (Experiment 2; amino acids of phloem and frass of the second larval stage were not determined because insufficient frass was available). All data were ln-transformed to meet assumptions of normality (tested using Kolmogorov–Smirnov statistic *D*). Unequal variances of residuals after data transformation in the ANOVAS in Experiment 1 were accounted for by using the GROUP = treatment option in the REPEATED statement of PROC MIXED in SAS (SAS Institute, 2009). Means of ratios in ANOVAS were further separated by least significant difference tests if the overall hypothesis of no significant difference at $\alpha = 0.05$ was rejected.

Results

Experiment 1: Differential efficiency of phloem utilization in relation to ash species

Nineteen amino acids (eight essential) were consistently detected in frass and phloem samples of black, green and white ash (Table 1). The three ash species differed quantitatively with respect to many phloem amino acids, with black and green ash generally having greater concentrations of four essential (i.e. isoleucine, leucine, phenylalanine, valine) and four non-essential amino acids (i.e. asparagine, aspartic acid,

Table 1 Mean ± SE amino acid concentrations (nmol g^{-1} dry tissue) of phloem and emerald ash borer larval frass from three ash species

	Black ash			Green ash			White ash		
Amino acid	Phloem	Frass	Ratio[a]	Phloem	Frass	Ratio	Phloem	Frass	Ratio
Essential amino acids									
Histidine	$18.6 \pm 2.8^{*A}$	6.5 ± 2.0	0.6 ± 0.1	$7.1 \pm 2.2^{**B}$	0.6 ± 0.4	0.2 ± 0.1	$8.5 \pm 1.4^{*B}$	2.2 ± 0.8	0.5 ± 0.1
Isoleucine	144.6 ± 40.0^{A}	83.8 ± 30.6	0.9 ± 0.1^{ab}	$93.8 \pm 12.8^{**A}$	14.7 ± 4.2	0.5 ± 0.1^{b}	42.3 ± 12.2^{B}	61.7 ± 17.9	1.1 ± 0.1^{a}
Leucine	190.8 ± 70.3^{A}	187.5 ± 74.6	1.0 ± 0.1^{a}	$275.5 \pm 37.8^{**A}$	42.2 ± 10.3	0.7 ± 0.0^{b}	48.5 ± 10.8^{B}	143.7 ± 41.4	1.2 ± 0.2^{a}
Lysine	60.4 ± 21.5	39.2 ± 10.0	1.0 ± 0.1^{a}	$28.7 \pm 5.0^{**}$	6.5 ± 2.5	0.5 ± 0.1^{b}	28.6 ± 7.2	26.1 ± 11.5	0.9 ± 0.2^{ab}
Phenylalanine	22.3 ± 6.4^{A}	15.6 ± 5.0	0.9 ± 0.1^{ab}	$11.7 \pm 2.0^{**AB}$	3.8 ± 1.5	0.5 ± 0.1^{b}	8.0 ± 1.1^{B}	13.2 ± 3.1	1.1 ± 0.2^{a}
Threonine	69.8 ± 44.3	53.3 ± 21.7	0.8 ± 0.2^{ab}	$89.1 \pm 11.4^{**}$	7.5 ± 2.0	0.4 ± 0.1^{b}	30.1 ± 10.3	31.1 ± 11.5	0.9 ± 0.1^{a}
Typtophan	$186.6 \pm 22.6^{**}$	21.0 ± 7.7	0.5 ± 0.1^{a}	$350.3 \pm 116.7^{**}$	4.6 ± 1.1	0.3 ± 0.1^{b}	$129.6 \pm 15.4^{**}$	8.9 ± 2.3	0.5 ± 0.0^{ab}
Valine	256.6 ± 63.1^{B}	158.7 ± 67.0	0.9 ± 0.1^{a}	$409.4 \pm 30.2^{**A}$	33.8 ± 5.3	0.6 ± 0.0^{b}	123.4 ± 25.0^{C}	99.3 ± 29.1	0.9 ± 0.1^{a}
Non-essential amino acids									
Alanine	$497.6 \pm 37.0^{*}$	120.2 ± 44.5	0.7 ± 0.1	$417.9 \pm 46.7^{**}$	44.4 ± 7.9	0.6 ± 0.0	$377.2 \pm 84.1^{*}$	83.4 ± 20.2	0.7 ± 0.1
Asparigine	40.0 ± 10.1^{AB}	39.5 ± 9.3	1.0 ± 0.1^{a}	$64.1 \pm 9.4^{**A}$	9.1 ± 2.9	0.5 ± 0.1^{b}	26.5 ± 3.9^{B}	22.7 ± 6.5	0.9 ± 0.1^{a}
Aspartic acid	$1326.2 \pm 151.8^{**A}$	70.7 ± 17.7	0.6 ± 0.0^{a}	$1330.4 \pm 292.7^{**A}$	19.7 ± 4.5	0.4 ± 0.1^{b}	$386.1 \pm 50.4^{**B}$	54.7 ± 10.0	0.7 ± 0.0^{a}
Glutamic acid	$168.7 \pm 14.6^{*}$	31.7 ± 10.1	0.6 ± 0.1	$145.4 \pm 27.7^{**}$	11.7 ± 3.1	0.5 ± 0.1	$116.6 \pm 14.6^{**}$	21.8 ± 4.0	0.6 ± 0.1
Glutamine	300.4 ± 69.4^{A}	284.4 ± 174.9	0.9 ± 0.1^{a}	$490.1 \pm 86.5^{**A}$	56.0 ± 28.2	0.5 ± 0.1^{b}	$110.7 \pm 14.0^{*B}$	32.4 ± 6.1	0.7 ± 0.1^{ab}
Glycine	16.8 ± 2.1	$88.6 \pm 37.1^{*}$	1.5 ± 0.2	14.2 ± 1.9	15.8 ± 3.3	1.0 ± 0.1	13.2 ± 1.6	38.1 ± 11.6	1.3 ± 0.1
Hydroxyproline	9.0 ± 1.9	$26.4 \pm 8.2^{**}$	1.5 ± 0.1^{a}	21.7 ± 8.7	7.9 ± 2.4	0.7 ± 0.2^{b}	20.4 ± 2.7	17.9 ± 3.2	0.9 ± 0.1^{ab}
Ornithine	5.9 ± 1.4	11.1 ± 2.5	1.4 ± 0.2	5.9 ± 0.9	7.5 ± 3.8	0.8 ± 0.3	4.4 ± 1.0	6.5 ± 1.5	1.2 ± 0.1
Proline	38.1 ± 10.3	236.1 ± 139.5	1.4 ± 0.2^{a}	$34.3 \pm 7.1^{*}$	11.5 ± 2.8	0.6 ± 0.1^{b}	26.9 ± 5.6	61.5 ± 21.3	1.2 ± 0.2^{a}
Serine	$94.3 \pm 15.2^{**A}$	15.4 ± 6.4	0.6 ± 0.1^{ab}	$55.8 \pm 10.3^{**B}$	4.5 ± 1.0	0.4 ± 0.1^{b}	24.9 ± 3.1^{C}	11.2 ± 3.8	0.7 ± 0.1^{a}
Tyrosine	109.2 ± 14.8	52.8 ± 19.9	0.8 ± 0.1	$117.1 \pm 23.1^{**}$	9.4 ± 3.1	0.4 ± 0.1	$129.6 \pm 29.0^{*}$	39.0 ± 12.2	0.7 ± 0.1

[a]Ratio of frass amino acid to phloem amino acid. Statistically significant difference between phloem and frass amino acids from the same species of ash: $^{*}\alpha = 0.05$ and $^{**}0.01$, respectively. Different upper- and lowercase superscript letters indicate a significant difference in phloem amino acids and ratio between species, respectively, using a least significant difference mean separation procedure ($n_{\text{black ash, frass}} = 5$; $n_{\text{black ash, phloem}} = 6$; $n_{\text{black ash, ratio}} = 5$, except for threonine, in which $n = 2$; $n_{\text{green ash, frass}} = 6$; $n_{\text{green ash, phloem}} = 6$; $n_{\text{green ash, ratio}} = 6$; $n_{\text{white ash, frass}} = 5$; $n_{\text{white ash, phloem}} = 6$; $n_{\text{white ash, ratio}} = 5$, except for ornithine and threonine in which $n = 4$).

glutamine, serine) than white ash. Black ash phloem tissue contained greater concentrations of histidine and tryptophan (essential amino acids), and alanine, aspartic acid, glutamic acid and serine (non-essential amino acids), although it contained lower concentrations of glycine and hydroxyproline (non-essential amino acids) than the corresponding frass produced by EAB larvae. Green ash phloem tissue had higher concentrations of all amino, acids except glycine, hydroxyproline and ornithine, than the corresponding frass samples. White ash phloem samples had higher concentrations of seven (i.e. histidine, typtophan, alanine, aspartic acid, glutamic acid, glutamine and tyrosine) of 19 amino acids than the corresponding frass samples. The frass : phloem ratios of 13 amino acids (seven essential and six non-essential) of green ash were generally lower than those of black or white ash.

No significant difference in total phenolics between phloem and frass for any ash species was detected (Fig. 1A). However, when the data were pooled across all three ash species, phloem samples contained greater total phenolics than frass samples (Fig. 1A). The ratio of total phenolics in frass to total phenolics in the phloem of white ash was lower than the ratios for black or green ash and the difference in ratios between black and green ash was not significant (Fig. 1B).

Experiment 2: Differential efficiency of phloem utilization in relation to EAB larval development stage

Sixteen amino acids (six essential and ten non-essential) were consistently detected in the phloem samples of green ash and in frass produced by third- and fourth-instar larvae feeding on the phloem (Table 2). For third instars, the concentrations of typtophan, aspartic acid and tyrosine were greater in the phloem than in the corresponding frass; whereas, for fourth instars, concentrations of 8 (i.e. threonine, typtophan, valine, alanine, asparagine, aspartic acid, glutamic acid and serine) of the 16 amino acids were greater in the phloem than in the corresponding frass. The ratio of threonine in frass to threonine in phloem was greater for third instars than for fourth instars.

Differences in total phenolics between phloem and frass were not significant, for any EAB developmental stage (Fig. 2). However, when the data were pooled across the EAB developmental stages, phloem samples contained greater total phenolics than frass samples (Fig. 2). There were no significant differences in the ratios of frass : total phenolics among the EAB larval developmental stages (data not presented).

Discussion

We found that EAB larvae differed in the efficiency by which they extract many essential and non-essential amino acids from the three ash species studied, being most efficient when feeding on green ash. Only six (two essential) and seven (two essential) amino acids were present at lower concentrations in the EAB frass in black and white ash than in the corresponding phloem, respectively, compared with 16 (eight essential) in green ash. In addition, the frass : phloem ratios of 13 green ash amino acids were lower than their counterparts in black and white ash, indicating a higher nutrient utilization efficiency when feeding on green ash. Because the concentrations of most amino acids in green ash were comparable with those in black ash and greater than those in white ash, these results further indicated that more amino acids in absolute values were utilized by EAB larvae

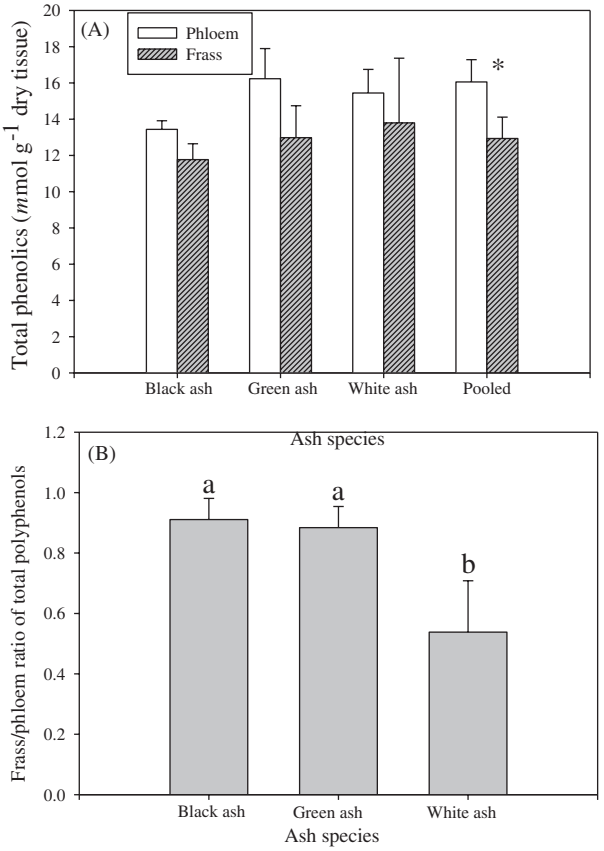

Figure 1 Total phenolics (mean ± SE) of phloem tissue of three ash species and emerald ash borer larval frass (A) and ratio of total phenolics in frass to total phenolics in phloem (B). *Significant difference in (A) between phloem tissue and frass when the data were pooled across ash species at $\alpha = 0.05$. Different lower-case letters above the means indicate a significant difference between ash species at $\alpha = 0.05$. Replicates in (A): $n_{\text{black ash, frass}} = 3$; $n_{\text{black ash, phloem}} = 3$; $n_{\text{green ash, frass}} = 6$; $n_{\text{green ash, phloem}} = 6$; $n_{\text{white ash, frass}} = 5$; $n_{\text{white ash, phloem}} = 5$; $n_{\text{pooled, frass}} = 14$; $n_{\text{pooled, phloem}} = 14$. Replicates in (B): $n_{\text{black ash}} = 3$; $n_{\text{green ash}} = 5$; $n_{\text{white ash}} = 3$.

feeding on green ash. Phytophagous insects have developed different pre- and post-ingestive strategies to survive in an environment with limited nutrients and almost ubiquitous plant allelochemicals that deter feeding and are anti-nutritive or toxic. For example, to overcome nutrient deficiency, some insects may choose host plants with a higher nutritional quality (Chen *et al*., 2008) or practice omnivory (Polis & Strong, 1996). Diet mixing is common for many insect herbivores with respect to mitigating nutrient imbalance (Bernays & Minkenberg, 1997). Post-ingestive strategies include the evolution of insensitive target sites and different categories of detoxifying enzymes in the gut to counteract the negative effects of allelochemicals (Ahmad *et al*., 1986; Berenbaum, 1986). These findings may account for the preference exhibited by EAB for green ash in the field (Anulewicz *et al*., 2007).

The concentrations of glycine and hydroxylproline were greater in frass than in phloem when feeding on black ash, although this was not the case when feeding on green or white ash. These results suggest that EAB larvae have difficulty absorbing these amino acids when feeding on black ash only. Although glycine and hydroxyproline are non-essential amino acids, they play very important roles in insect survival (Rapport *et al*., 1980; Konno *et al*., 1997). For example, glycine in insect guts is known to decrease the precipitation of proteins by digestibility reducers such as tannins and phenolics (Konno *et al*., 1997).

The differential absorption of amino acids from different ash species likely results from interactions of amino acid transport systems, either symport, uniport or facilitated diffusion, with some unique allelochemicals being present in different ash species. Green and white ash trees differ in their phenolic profiles (Eyles *et al*., 2007). Allelochemicals inhibit amino acid uptake in other systems. For example, cyclochampedol, a flavonoid from acetone extracts of the bark from *Arctocarpus champeden* (Family: Moraceae) trees, also inhibits absorption of leucine in the midgut of the silkworm, *Bombyx mori* L. (Lepidoptera: Bombycidae) in the presence and absence of potassium (Parenti *et al*., 1998).

Phenolics are widely distributed in many plant families (Harborne & Baxter, 1993) and generally denature plant proteins and thus reduce the nutritive value of plants. Therefore, evolving efficient strategies to cope with phenolics would benefit EAB larvae. The results obtained in the present study suggest that EAB larvae may employ two strategies with different mechanisms to cope with phenolics. First, numerically higher phloem total phenolics than corresponding frass total phenolics but a statistically insignificant difference between and frass total phenolics of same ash species, irrespective of ash species (Fig. 1A) and EAB instar (Fig. 2), indicated that direct excretion was the single most important elimination method. Second, more phenolics were detected in phloem than in frass after the data were pooled across ash species (Fig. 1A) and EAB instars (Fig. 2), suggesting that enzymatic conversion of phenolics into nonphenolics is also important. Four major categories (i.e. oxidases, hydrolases, transferases and reductases) of detoxifying enzymes are widely observed in herbivorous insects (Ahmad *et al*., 1986). Further studies are needed to determine which of these is/are involved in the ash-EAB system. Regardless, the ability to convert phenolics to nonphenolics depended upon the ash species, and was greater when EAB larvae fed on white ash compared with black and green ash (i.e. because the ratio of frass total phenolics to phloem total phenolics of white ash was lower than the ratios for black and green ash). These differences may be attributable to a differential induction of detoxifying enzymes in response to different allelochemical profiles of the three ash species. Green and white ash trees differ in phenolics profiles (Eyles *et al*., 2007) and detoxification enzymes are known to be selectively induced (Terriere, 1984).

Fourth instars extracted phloem amino acids more efficiently than third instars, including threonine, an essential amino acid and an important component of proteasomes that are actively involved in degrading proteins (Kisselev *et al*., 2000). The more efficient utilization of amino acids by fourth instars might contribute to greater *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) progeny when the parasitoid was reared on fourth instars than on younger instars (Ulyshen *et al*., 2010b). All EAB instars in the present study eliminated phloem phenolics mainly through direct excretion. Although some phloem phenolics

Table 2 Mean ± SE amino acid concentrations (nmol g^{-1} dry tissue) of green ash phloem and frass of third and fourth instars of emerald ash borer feeding on this species[a]

	Third instar			Fourth instar		
Amino acid	Phloem	Frass	Ratio[b]	Phloem	Frass	Ratio[2]
Essential amino acids						
Isoleucine	91.1 ± 50.2	72.5 ± 23.6	1.2 ± 0.3	163.6 ± 44.2	30.6 ± 12.0	0.6 ± 0.2
Leucine	106.9 ± 47.9	107.1 ± 31.9	1.1 ± 0.2	221.7 ± 57.6	41.1 ± 14.9	0.6 ± 0.2
Phenylalanine	20.3 ± 8.9	13.8 ± 3.2	0.8 ± 0.2	11.8 ± 1.7	2.5 ± 1.5	0.4 ± 0.2
Threonine	26.9 ± 6.3	27.0 ± 8.7	1.0 ± 0.1[a]	41.8 ± 3.1**	1.6 ± 1.6	0.1 ± 0.1[b]
Typtophan	594.5 ± 169.6**	10.8 ± 2.8	0.4 ± 0.0	229.8 ± 54.7**	2.5 ± 1.5	0.2 ± 0.1
Valine	233.6 ± 88.7	82.2 ± 27.7	0.9 ± 0.2	331.4 ± 57.4*	29.5 ± 9.3	0.6 ± 0.1
Non-essential amino acids						
Alanine	242.8 ± 56.3	77.1 ± 22.5	0.8 ± 0.1	358.5 ± 52.4*	55.2 ± 23.5	0.6 ± 0.1
Asparigine	251.5 ± 117.5	23.8 ± 12.7	0.5 ± 0.2	64.4 ± 26.3*	6.7 ± 4.3	0.3 ± 0.2
Aspartic acid	734.0 ± 268.7*	40.9 ± 13.2	0.6 ± 0.1	823.3 ± 191.0*	16.1 ± 5.4	0.4 ± 0.1
Glutamic acid	130.0 ± 56.8	20.6 ± 5.4	0.7 ± 0.1	85.3 ± 13.6*	11.1 ± 7.3	0.3 ± 0.2
Glutamine	141.4 ± 54.9	25.5 ± 16.2	0.7 ± 0.2	188.2 ± 52.7	12.7 ± 4.8	0.5 ± 0.2
Glycine	14.3 ± 1.7	40.8 ± 14.2	1.3 ± 0.2	10.4 ± 2.2	9.2 ± 3.7	0.8 ± 0.3
Hydroxyproline	19.8 ± 9.6	10.3 ± 5.1	0.7 ± 0.3	13.6 ± 4.7	11.8 ± 4.3	1.0 ± 0.1
Proline	15.2 ± 5.9	36.2 ± 57.4	1.9 ± 0.6	22.2 ± 5.2	18.1 ± 4.8	0.9 ± 0.2
Serine	26.0 ± 7.1	4.4 ± 1.6	0.5 ± 0.2	19.4 ± 3.8**	0.6 ± 0.6	0.1 ± 0.1
Tyrosine	228.8 ± 66.0*	21.4 ± 8.0	0.6 ± 0.1	70.5 ± 4.6	9.7 ± 5.6	0.4 ± 0.1

[a]Amino acids of phloem and frass of the second-instar larvae were not determined because insufficient frass was available.
[b]Ratio of frass amino acid to phloem amino acid. Statistically significant difference between phloem and frass amino acids of the same instar: *$\alpha = 0.05$ and **0.01, respectively. Different lowercase superscript letters after means denote a significant difference in ratio between instars ($n_{3rd,\ frass} = 4$; $n_{3rd,\ phloem} = 4$; $n_{3rd,\ ratio} = 4$, except for hydroxyproline in which $n = 3$; $n_{green\ ash,\ frass} = 4$; $n_{green\ ash,\ phloem} = 4$; $n_{green\ ash,\ ratio} = 4$, except for phenylalanine in which $n = 3$).

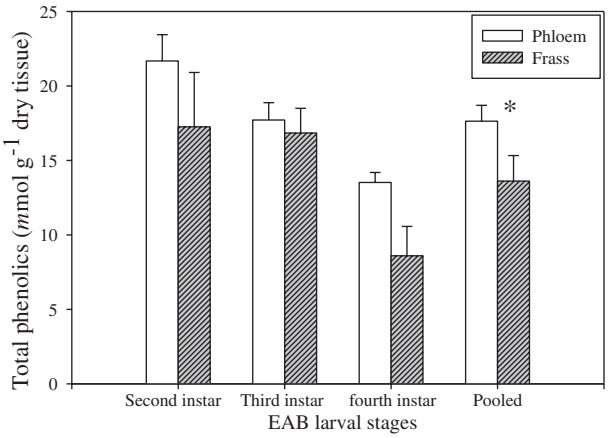

Figure 2 Total phenolics (mean ± SE) of green ash phloem tissue and corresponding frass of emerald ash borer (EAB) larvae of three developmental stages. *Significant difference between phloem tissue and frass when the data were pooled across the three developmental stages at $\alpha = 0.05$; $n_{2nd,frass} = 6$; $n_{2nd,phloem} = 6$; $n_{3rd,frass} = 6$; $n_{3rd,phloem} = 6$; $n_{4th,frass} = 6$; $n_{4th,phloem} = 6$.

were converted or destroyed in the EAB gut, the extent to which this took place did not differ among the EAB instars.

The use of the Prussian blue assay may have hindered our ability to fully determine the mechanism(s) by which EAB larvae cope with phenolics. The Prussian blue assay is widely used to estimate total phenolics based on antioxidant activity, and antioxidants other than phenolics such as ascorbic acid might interfere with these estimates (Asami *et al*., 2003). Future studies will aim to compare the individual phenolics of ash phloem with those of frass.

In summary, EAB larvae generally utilized green ash amino acids more efficiently than those of black and white ash. EAB larvae may eliminate phenolics through a combination of direct excretion and enzymatic conversion of phenolics to nonphenolics before excretion. Because the ratio of frass total phenolics to phloem total phenolics in white ash was lower than the ratios in black and green ash, the ability to destroy phenolics or convert them to nonphenolics was greater when EAB larvae fed on white ash. Fourth-instar EAB extracted phloem amino acids, including threonine, more efficiently than third-instar EAB.

Acknowledgements

We thank Drs Deguang Liu (University of California, Davis), Vanessa Muilenburg (Ohio State University) and six anonymous reviewers for providing insightful comments that significantly improved an early draft of the manuscript. The research was supported by the USDA Forest Service, NA FHP.

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Accepted 25 February 2012
First published online 4 April 2012