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Nutritional and Defensive Chemistry of Three North American Ash Species: Possible Roles in Host Performance and Preference by Emerald Ash Borer Adults

Yigen Chen¹ and Therese M. Poland²

Abstract

Black ash (*Fraxinus nigra*), green ash (*F. pennsylvanica*), and white ash (*F. americana*) are the three most abundant ash species in the northeastern USA. We compared emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), adult performance and preference among seedlings of the three ash species, and then related performance and preference to foliage nutritional quality and defensive compounds. Longevity of EAB adults reared on green and white ash was found to be greater than on black ash. EAB adult females also seemed to show feeding preference among the three species of ash trees because the total foliage area consumption was greater on green ash and white ash compared to black ash in dual-choice tests; however, the total mass of foliage consumed did not differ. The foliage of all ash species was high in nitrogen and in most macro- and micro-nutrients studied. The patterns of EAB performance and preference did not correspond to any of the individual chemical compounds tested (nitrogen, proteins, most macro- and micro-nutrients, or putative defensive compounds of ash seedlings). Nevertheless, greater longevity of EAB adults on green and white ash compared to black ash was probably related to unbalanced nutrients (total nitrogen/total non-structural carbohydrate ratio) of black ash. Putative defensive compounds (i.e., phenolics and protease inhibitors) did not contribute to EAB longevity in this study, probably because (1) EAB adults were able to excrete most of these compounds and (2) their effects were alleviated by high nitrogen levels. More research is needed to elucidate the interactions of nitrogen and carbohydrate levels, and the interactions of nutrient balance and defensive plant allelochemicals on EAB performance and preference.

The exotic emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), has killed tens of millions of ash trees (*Fraxinus* spp.) in the Great Lakes Region of North America since its discovery near Detroit Michigan, USA and Windsor Ontario, Canada in 2002 (Haack et al. 2002, Poland and McCullough 2006). All major eastern North American ash species are susceptible to emerald ash borer, including green (*F. pennsylvanica* Marshall), white (*F. americana* L.), black (*F. nigra* Marshall), and blue ash (*F. quadrangulata* Michaux) (Cappaert et al. 2005, Poland and McCullough 2006). An Asian ash species, Manchurian ash (*F. mandshurica* Ruprecht), was found to be more resistant than several cultivars of white and green ash (Rebek et al. 2008). Surprisingly, a hybrid cultivar of North American black ash × Manchurian ash was highly susceptible to EAB indicating that this cultivar was more similar to its North American parent in susceptibility to EAB. Efforts have been directed to elucidate mechanisms of resistance by Asian ash species to EAB, aiming to develop resistant ash varieties or hybrids (Eyles et al. 2007, Koch et al. 2008, Chen and Poland 2009a, b).

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Roles of nutritional quality and plant secondary compounds in plant resistance to insect herbivores have widely been implied or documented (Panzuto et al. 2001, Despland and Noseworthy 2006, Chen et al. 2008). Inter-specific differences among Manchurian ash, green ash, and white ash may contribute to Manchurian ash's relatively higher resistance to EAB infestation (Rebek et al. 2008) because some phenolic compounds (i.e., hydroxycoumarins, calceolariosides A and B) were only found in Manchurian ash phloem (Eyles et al. 2007). Green ash nutrients, defensive compounds, or their combination were suggested to be responsible for EAB adults' preference for mature leaves over young leaves (Chen and Poland 2009b). EAB adults were also found to prefer green ash foliage from seedlings that were grown in the sun or were stressed (girdled on the stem) to foliage from seedlings that were grown in the shade or were previously un-girdled, respectively (Chen and Poland 2009b). Levels of total non-structural carbohydrate (TNC) were found to be greater in foliage from seedlings grown in the sun or that were girdled, compared to seedlings grown in shade or ungirdled.

Black, green, and white ash trees are the three most abundant ash species in the northeastern USA (USGS 2009). Regarding EAB infestation (or female oviposition preference), Anulewicz et al. (2007) found that EAB preferred green over white ash, with blue ash being least preferred. However, Smith et al. (2006) found that green and white ash trees were killed with equal frequency in southeast Michigan forests, and black ash experienced the highest mortality during initial stages of invasion. In a comparison of feeding preference of EAB adults on leaves from six ash species in the laboratory conducted in metal screen cages, black ash, green ash, and white ash were preferred over Manchurian ash, blue ash, and European ash (*F. excelsior* L.), while no preference was detected among black ash, green ash, and white ash (Pureswaran and Poland 2009). In this study we compared the longevity of EAB adults reared on green, white or black ash and EAB feeding preference among these ash species. We then compared nutritional quality and defensive compounds among these ash species to investigate the underlying mechanisms for observed differences in longevity and feeding preference.

Methods and Materials

Ash seedlings and insects. Green ash, white ash, and black ash seedlings (2-yr-old and ca. 30-50cm tall) were purchased from Lawyer Nursery Inc. (West Plains, MT, USA) in February, 2009, and stored in a dark room at 4°C until potting. Seedlings were potted with TPOT2 tree pots (Width: 15cm; Height: 41cm; Volume: 6.23 L; Stuewe & Sons, Inc., Corvallis, OR, USA) using Fafard #52 soil (BFG Supply Co., Burton, OH, USA) as the planting medium. They were grown during April-May, 2009 (ca. 14L:10D, 25 ± 2°C) in a greenhouse located at the Tree Research Center at Michigan State University, East Lansing, MI, USA. Seedlings were fertilized once every 3-4 days with approximately 2 L of nutrient solution containing 200 ppm nitrogen, 60 ppm phosphorus, 150 ppm potassium, 80 ppm calcium, 40 ppm magnesium, 60 ppm sulfur, 0.15 ppm copper, 4 ppm iron, 0.8 ppm manganese, and 0.32 ppm zinc.

Ash trees naturally infested with EAB were felled at the end of 2008 and early 2009 in Ingham County, MI, cut into 90-cm long logs, and held in cold storage at 4°C. As beetles were required for experiments, logs were removed from cold storage to allow beetles to emerge in rearing tubes at 25°C. They were separated by sex and kept in 295 ml plastic beverage containers with an evergreen ash, *F. uhdei* (Wenzig) Lingleish, leaf in a vial of water for feeding. Containers with beetles were kept in growth chambers at 25°C with a photoperiod of 14L:10D, and > 70% relative humidity with fresh food that was replaced twice a week, until they were used in experiments.

EAB adult longevity in response to three ash species. In order to evaluate effects of three ash species on adult EAB longevity, three newly emerged EAB adults were placed in a sealed 295-ml plastic drinking cup where they were fed with excised leaves from either black, green, or white ash. A 5 × 7 cm area on the side of the cup was cut away and sealed with 1 × 1 mesh for ventilation. The experiment was a 3 (ash species) × 2 (EAB sex) factorial design. Three EAB adults of the same sex were placed in each cup. The petioles of excised compound leaves were placed into 20-ml glass vials filled with water before being placed into cups. Leaves were replaced with fresh leaves of the same ash species every 2-3 days. The leaves of different ash species were mature (approximately 2-3 weeks after full expansion) and matched for age and size at the time of foliage change to minimize foliage age effects on EAB longevity (Chen and Poland 2009b). Survival of EAB was monitored daily until all EAB were dead. Each treatment combination was replicated 5 times (cups). Cups were kept in chambers set at 25 ± 2°C and 70% relative humidity with a photoperiod of 14L:10D.

EAB adult feeding preference in response to three ash species. Dual-choice tests were conducted to determine EAB feeding preferences for different ash species. Tests were conducted in 17 × 6 × 12 cm (L × W × H) plastic cages (Pioneer Plastics, Inc., Dixon, KY, USA). Small holes (diam. = 0.2 cm) were punched in the cage lids for air circulation. The stems of excised leaves of the paired treatments (as described below) were inserted into 15 ml glass bottles filled with water and placed at opposite sides of the cage. Position of the treatments was randomly assigned. Five 3- to 4-d-old satiated virgin females were then released into the center of the cage and allowed to feed for 48 h. Three dual-choice preference experiments were conducted: (1) Black ash vs. White ash; (2) Black ash vs. Green ash; and (3) Green ash vs. White ash. As in the previous experiment, the leaves used in each assay were mature and matched for age and size. Foliage area consumed was quantified with a digital camera and a digital imaging software – Image Processing and Analysis in Java (ImageJ, version 1.34s, available in public domain <http://rsbweb.nih.gov/ij/>) by subtracting foliage area at the end of the experiment from initial foliage area. Fresh mass of foliage consumed during the experiment was calculated from separate linear equations between foliage area and foliage mass for each ash species. The specific leaf weight (SLW), which is an index of foliage density, was expressed as total fresh mass divided by total foliage area, g/cm². Each preference experiment was repeated 8 times in chambers set at 25 ± 1 °C and a photoperiod of 14L:10D.

Foliar macro- and micro-nutrients of three ash species. To investigate nutritional composition of black, green, and white ash foliage, macro- and micro-nutrients of the three ash species were determined. Because EAB adults prefer to feed on mature leaves, fully matured foliage was collected for assay approximately 5 wk after seedling potting. Freshly excised compound leaves were immediately dipped into liquid nitrogen for 2-3 seconds and brought to the laboratory in a cooler filled with ice. Leaves were then oven-dried at 60-70°C for 48 h. Dried foliage was later sent to A & L Great Lakes Laboratories, Inc. (Fort Wayne, IN, USA) for macro- (nitrogen, phosphorus, potassium, calcium, magnesium, sulfur, and sodium) and micro-nutrient (boron, zinc, manganese, iron, and copper) analyses. *n* = 6 replicate per ash species.

Total proteins and carbohydrates of three ash species. Methods of sample collection, and protein and carbohydrate (glucose, fructose, sucrose, starch) extraction and determination were described in detail in Chen and Poland (2009b). Briefly, mature leaves were cut and flash frozen in liquid nitrogen approximately 5 wk after seedling potting. Samples were then ground to fine powder with mortar and pestle in liquid nitrogen, and approximately 40-80 mg of ground plant tissue was used for protein and carbohydrate extraction. Total protein content was determined following the Bradford protein assay

and calculated with a standard curve established using bovine serum albumin. Glucose, fructose and sucrose content was determined using a glucose assay kit (Sigma-Aldrich, St. Louis, MO, USA) and calculated from a standard curve generated using D-glucose as a standard. The starch content was estimated in glucose equivalents. Total non-structural carbohydrate (TNC) was expressed as the sum of glucose and starch, because the amounts of fructose and sucrose determined following this method were negligible. Each treatment (species) was repeated 10 times, except for green ash which had 9 replicates. All chemicals used in this experiment and the following were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA).

Defensive chemistry of three ash species and EAB frass. Foliage samples were collected and prepared as in the preceding experiment. Methods of total phenolics, protease inhibitor extraction and determination were described elsewhere (Chen and Poland 2009b). Briefly, ground and weighed plant tissue was extracted for phenolics 3 times with 70% acetone at 4°C in the dark for 30 min. The supernatant after each centrifugation was pooled and the concentration [$\mu\text{mol g}^{-1}$ fresh weight (FW)] determined using a modified Prussian blue assay (Graham 1992). An external standard curve with gallic acid within the linear range was constructed to calculate sample concentration.

Trypsin and chymotrypsin inhibitors were extracted with a Tris-HCl buffer (pH 7.8) containing 7% polyvinylpyrrolidone, 1.67 mM phenylthiourea, 0.3 mM KCl, and 0.4 mM ascorbic acid (Stout et al. 1998). The mixture was centrifuged at 10,000 \times g for 10 min. The supernatant was used for assay. The trypsin and chymotrypsin inhibitor assays followed Walsh and Wilcox (1970), and Broadway (1993) using *N*- α -*p*-tosyl-L-arginine methyl ester (TAME) and *N*-benzoyl-L-tyrosine ethyl ester (BTEE) as substrates for trypsin and chymotrypsin determination, respectively.

In order to examine EAB adults' ability to dispose of phenolics by direct excretion, frass of EAB adults in the longevity experiment was collected for phenolic determination. The experimental design was a 3 (ash species) \times 2 (EAB sex) factorial with $n = 5$ replicates per treatment combination.

Statistical analysis. EAB longevity was analyzed by 3 (ash species) \times 2 (EAB sex) ANOVA. The longevity of the 3 individuals in each treatment combination (cup) was averaged before being subject to the analysis. Fresh mass of foliage consumed and foliage area consumed were analyzed by paired *t*-test, separately for each dual-choice test. SLW among species were analyzed by one-way ANOVA. Foliar macro- and micro-nutrients, total proteins, glucose, starch, TNC, protein:TNC ratio, ash foliage and EAB fecal phenolics, trypsin and chymotrypsin inhibitors were individually analyzed by one-way ANOVA. Protein:TNC ratio data were log transformed before analysis. Means of one- and two-way ANOVA were separated by student *t*-test at $\alpha = 0.05$. Comparisons of phenolics between ash foliage and EAB frass were conducted by paired *t*-tests, separately for ash species.

Results

EAB adult longevity in response to three ash species. Ash species significantly affected EAB adult longevity ($F = 3.39$; $df = 2, 24$; $P = 0.05$; Fig. 1). Data for male and female EAB adult longevity were pooled because EAB sex and the interaction between ash species and EAB sex did not affect EAB adult longevity. Longevity of EAB adults fed on white ash was about 30% greater than for EAB reared on green ash, which in turn was approximately 18% higher than for EAB reared on black ash.

EAB adult feeding preference in response to three ash species. The relationships between foliage area (Y) and foliage mass (X) for black, green, and white ash could be well expressed as linear, $Y = a + bX$. The regression lines

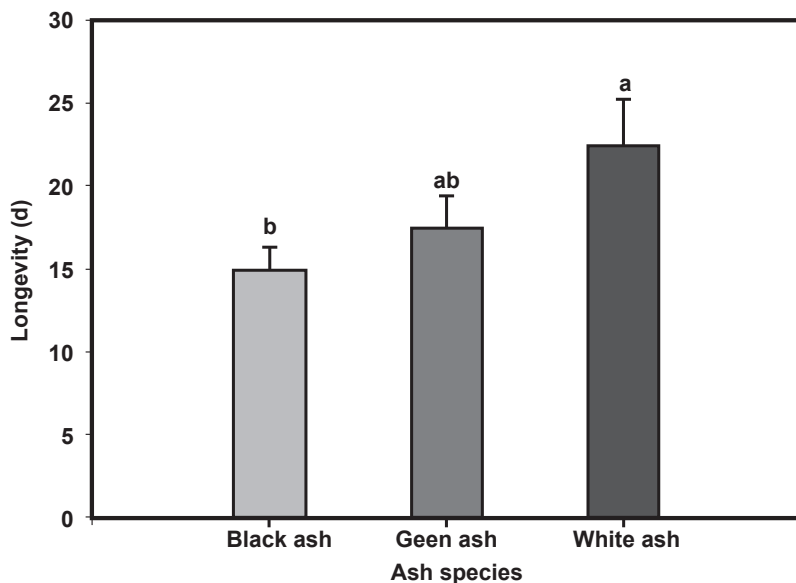


Figure 1. Longevity (mean \pm SEM) of EAB adults reared on three North American ash species in no-choice tests. Different lower-case letters above the bars denote significant difference at $\alpha = 0.05$. No significant difference between EAB sexes was found and as a result data over sex were pooled. $n = 30$ (15 males and 15 females) for each ash species.

for black ash, green ash, and white ash were $Y_{\text{black ash}} = -0.3127 + 0.0253 X$ (adj. $R^2 = 0.80$; $P_a > 0.05$; $P_b < 0.01$), $Y_{\text{green ash}} = -0.1328 + 0.0176 X$ (adj. $R^2 = 0.76$; $P_a > 0.05$; $P_b < 0.01$), and $Y_{\text{white ash}} = -0.0151 + 0.0117 X$ (adj. $R^2 = 0.74$; $P_a > 0.05$; $P_b < 0.01$), respectively. EAB females consumed a similar mass of foliage from each treatment during the 48 h assay in all the three dual-choice tests (Fig. 2A), although EAB adults ate significantly more white ash foliage in terms of foliage area compared to black ash foliage in the Black ash vs. White ash trial (Fig. 2B) and more green ash foliage was consumed than black ash foliage in the Black ash vs. Green ash trial (Fig. 2B) because the average specific leaf weight (SLW) of black ash ($0.0218 \pm 0.0007 \text{ g/cm}^2$) was significantly greater than that of green ash ($0.0165 \pm 0.0005 \text{ g/cm}^2$), which is in turn greater than that of white ash ($0.0116 \pm 0.0004 \text{ g/cm}^2$) ($F = 85.57$; $df = 2, 45$; $P < 0.0001$). The green ash and white ash foliage areas eaten in the Green ash vs. White ash trial did not differ (Fig. 2B).

Foliar macro- and micro-nutrients of three ash species. Foliar macro- and micro-nutrients of the three ash species are shown in Table 1. Regarding macro-nutrients, nitrogen content of the three ash species differed, with black ash the highest, green ash the intermediate, and white ash the lowest. Phosphorus of green ash foliage was the highest among the three. Magnesium levels were highest in black ash, which was followed by white ash and green ash in descending order. Sulfur amounts were highest in black ash, followed by green and white ash. No significant differences in potassium, calcium, and sodium among the three ash species were observed. For the 5 micro-nutrients, amounts of boron, zinc and copper differed among the ash species, those of manganese and iron did not.

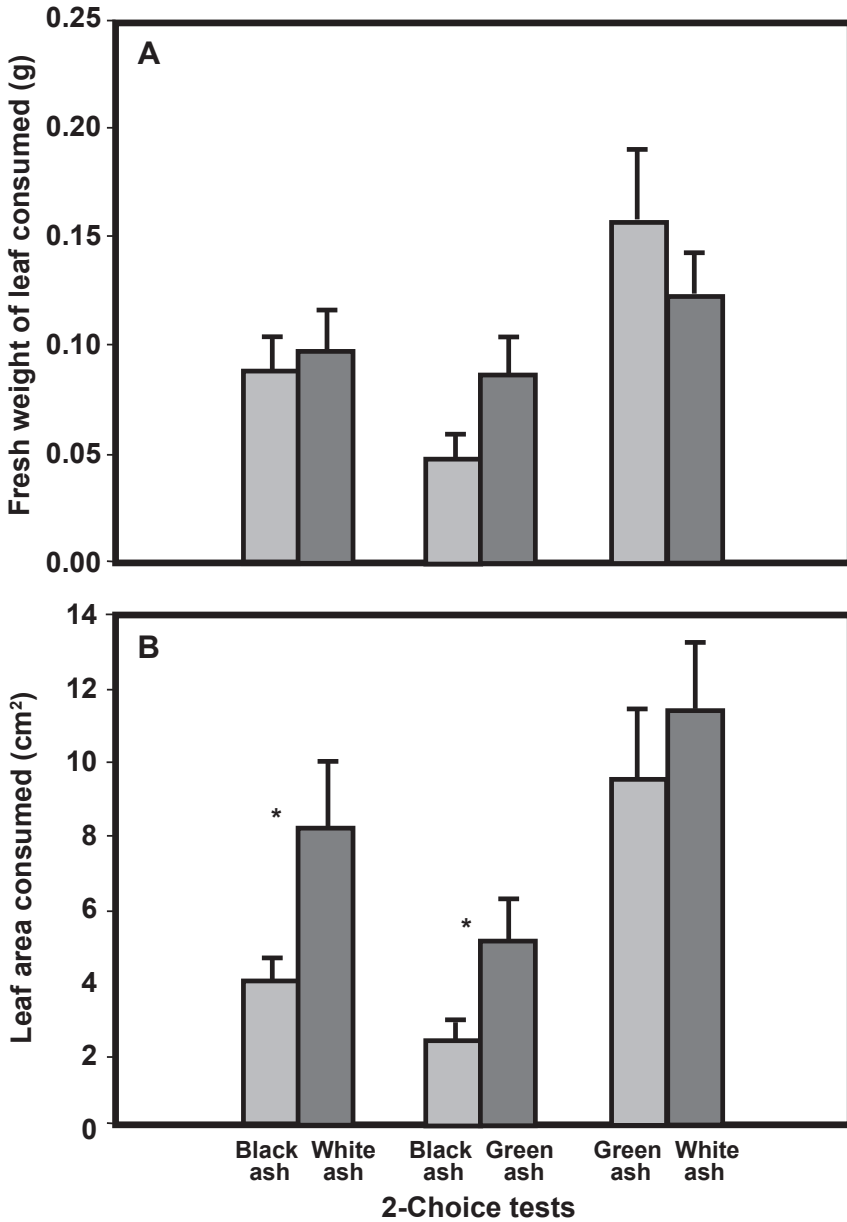


Figure 2. Fresh mass of foliage (A) and foliage area (B) (mean \pm SEM) consumed by EAB during dual-choice tests among three North American ash species over 48 h. Dual-choice tests: Black ash vs. White ash; Black ash vs. Green ash; and Green ash vs. White ash. * denotes significant difference at $\alpha = 0.05$. $n = 8$ replicates.

Total proteins and carbohydrates of three ash species. Total soluble proteins and carbohydrates of the three ash species are presented in Table 2. Total proteins of white ash were significantly greater than those of black and green ash ($F = 3.50$; $df = 2, 27$; $P < 0.05$). Glucose levels did not differ among ash species ($F = 1.71$; $df = 2, 27$; $P > 0.05$). Starch levels in green and white ash were marginally higher than in black ash ($F = 2.99$; $df = 2, 26$; $P = 0.07$). Green ash foliage contained higher TNC than white ash ($F = 4.34$; $df = 2, 26$; $P < 0.05$). Although statistically the difference in TNC between black ash and white ash was not significant, TNC levels were nearly 20% higher in white ash than in black ash. White ash had the highest protein:TNC ratio, followed by black and green ash ($F = 3.47$; $df = 2, 26$; $P < 0.05$).

Defensive chemistry of three ash species and EAB frass. Ash species differed in constitutive foliage phenolic levels (Fig. 3A). Phenolic levels in green ash were greater than in black or white ash ($F = 13.77$; $df = 2, 27$; $P < 0.01$). Black and white ash did not differ in total phenolics. The phenolic content in EAB frass ranged from nearly twice to over 2-fold higher than total phenolics in the foliage of ash species they fed upon (Fig. 3A). Frass from EAB adults reared on black ash foliage contained the greatest amount of total phenolics, followed by green and white ash. EAB males and females differed in their ability to excrete total phenolics, and female frass had higher amount of phenolics than male frass ($F = 4.83$; $df = 1, 24$; $P < 0.05$; Fig. 3B).

Green ash foliage had almost 70% higher levels of trypsin inhibitors than white ash, which in turn contained 3-fold higher levels of trypsin inhibitors than black ash (Fig. 4). The levels of chymotrypsin inhibitors in black and white ash were greater than in green ash (Fig. 4).

Discussion

EAB adults lived longer when they fed on green and white ash foliage than when they fed on black ash, and they also seemed to prefer green and white ash over black ash in this study. Although most of the macro-nutrients (i.e., nitrogen, phosphorus, magnesium, and sulfur) differed among black, green, and white ash, the better performance and preference of EAB on green ash and white ash over black ash might not be caused by any of the macro-nutrients alone, because the amounts of all these nutrients in black ash foliage were generally either the highest (i.e., total nitrogen, potassium, magnesium, and sulfur) among the three ash species or not statistically different (i.e., sodium) from those of the other two species (Table 1). Phosphorus levels in white ash and green ash foliage were the lowest and the highest, respectively. However, the performance and preference of EAB on these two species did not differ. Ash species also differed in most of the micro-nutrients (i.e., boron, zinc, and copper). The patterns of EAB performance and preference did not parallel those of micro-nutrients, and the micro-nutrients levels in black ash foliage were never the lowest.

Proteins are generally considered the main source of nitrogen. High total soluble protein level in white ash might in part contribute to the greater performance and preference of EAB. However, nutrient balance, in particular, the protein:TNC ratio might be a more important determinant in insect feeding preference on artificial diets (Bede et al. 2007, Behmer 2009). Most insects studied in the laboratory, in particular generalist insects, chose diets with protein:TNC ratios greater than 1 over those less than 1 (Bede et al. 2007). The protein:TNC ratios of black ash and white ash in this study were greater than 1, while the ratio of green ash was lower than 1. Additionally, protein:TNC ratio of white ash foliage was significantly higher than that of green ash, and no significant differences were detected between white ash and black ash, or between black ash and green ash. Therefore, the protein:TNC ratio alone can not explain the lower performance of EAB adults reared on black ash foliage. Furthermore, the reliability of the protein:TNC ratio as an indicator of nutrient balance in

Table 1. Foliar macro- and micro-nutrients (mean \pm SEM) of three North American species.

Ash species	Macro-nutrients							
	Nitrogen (%)	Phosphorus (%)	Potassium (%)	Calcium (%)	Magnesium (%)	Sulfur (%)	Sodium (%)	
Black ash	5.96 \pm 0.34a	0.39 \pm 0.02b	2.79 \pm 0.18a	0.58 \pm 0.07a	0.43 \pm 0.04a	0.33 \pm 0.02a	0.01 \pm 0.00a	
Green ash	4.55 \pm 0.56ab	0.57 \pm 0.07a	2.59 \pm 0.20a	0.54 \pm 0.07a	0.29 \pm 0.04b	0.29 \pm 0.02ab	0.01 \pm 0.00a	
White ash	3.85 \pm 0.14b	0.29 \pm 0.02b	2.19 \pm 0.16a	0.75 \pm 0.08a	0.36 \pm 0.03ab	0.22 \pm 0.01b	0.01 \pm 0.00a	
Normal range ¹	1.80 ~ 2.00	0.10 ~ 0.17	1.50 ~ 2.70	1.50 ~ 2.00	0.35 ~ 0.47	0.20 ~ 0.30	--	
Micro-nutrients								
	Boron (ppm)	Zinc (ppm)	Manganese (ppm)	Iron (ppm)	Copper (ppm)			
Black ash	19.00 \pm 2.32a	21.83 \pm 1.38b	66.50 \pm 10.37a	80.00 \pm 4.27a	2.17 \pm 0.31ab			
Green ash	10.67 \pm 0.92b	31.17 \pm 3.25a	106.67 \pm 22.93a	73.67 \pm 6.91a	1.00 \pm 0.00b			
White ash	22.50 \pm 1.43a	19.50 \pm 1.38b	66.67 \pm 14.10a	84.17 \pm 9.93a	3.50 \pm 0.67a			
Normal range ¹	48 ~ 59	10 ~ 125	75 ~ 100	50 ~ 200	13 ~ 17			

¹Normal ranges of mid-summer ash trees provided by A & L Great Lakes Laboratories, Inc. Different lower-case letters following means within a column denote significant difference at $\alpha = 0.05$. $n = 6$ replicates for black, green, and white ash.

Table 2. Foliar proteins, glucose, starch, total non-structural carbohydrate (TNC), and protein to TNC ratio (means \pm SEM) of three North American ash species.

Ash species	Total proteins (mg g ⁻¹ FW)	Glucose (mg g ⁻¹ FW)	Starch (mg g ⁻¹ FW)	TNC (mg g ⁻¹ FW)	Protein: TNC ratio (w/w)
Black ash	10.96 \pm 0.82b	10.32 \pm 0.77a	0.78 \pm 0.36b	11.09 \pm 0.86b	1.07 \pm 0.15ab
Green ash	11.59 \pm 1.24b	12.91 \pm 1.31a	3.72 \pm 1.33a	16.42 \pm 1.70a	0.74 \pm 0.10b
White ash	16.29 \pm 2.25a	11.16 \pm 0.89a	2.03 \pm 0.66ab	13.19 \pm 1.22ab	1.33 \pm 0.21a

Different lower-case letters following means within a column denote significant difference at $\alpha = 0.05$. n = 10 replicates per species, except for green ash which n = 9.

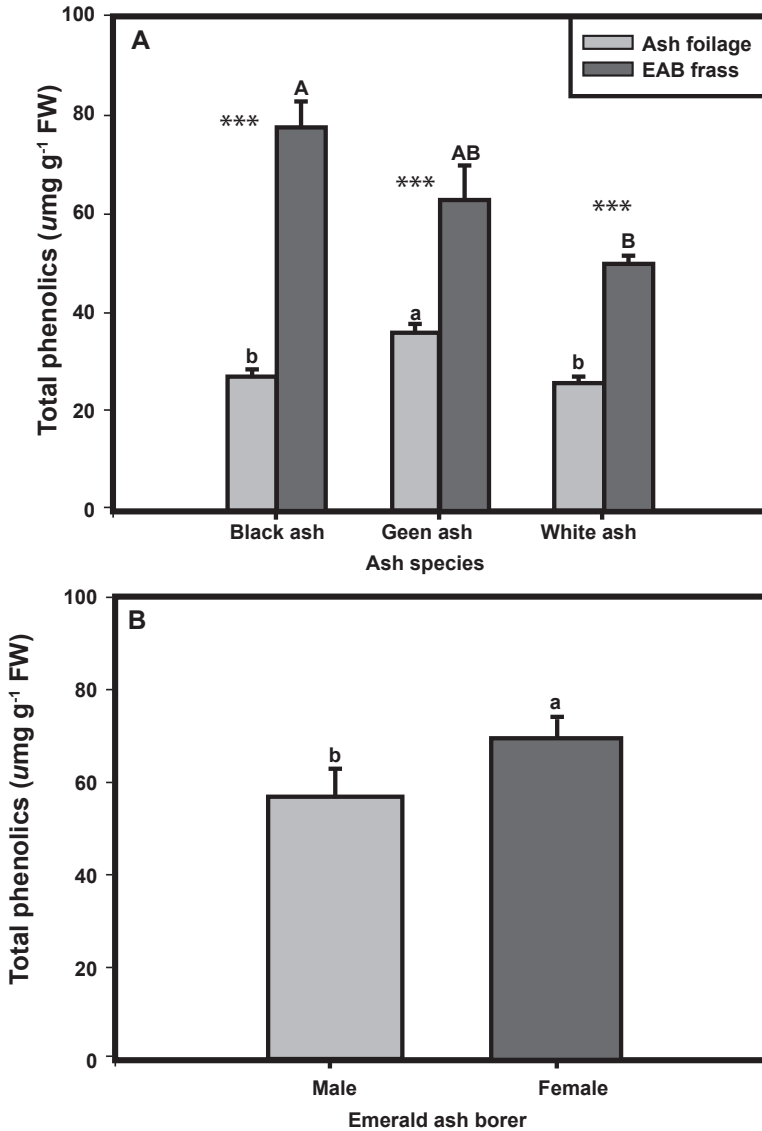


Figure 3. Total phenolics (mean \pm SEM) of three North American ash species and EAB adult frass. (A) Phenolics of ash species and EAB adult frass ($n = 10$); (B) Phenolics of male and female EAB adult frass ($n = 15$). Different lower-case letters denote significant difference among three ash species (A) and between male and female EAB frass at $\alpha = 0.05$; Different upper-case letters denote significant difference of frass phenolics from EAB adults (males and females pooled) reared on three ash species at $\alpha = 0.05$; *** indicates $P < 0.001$.

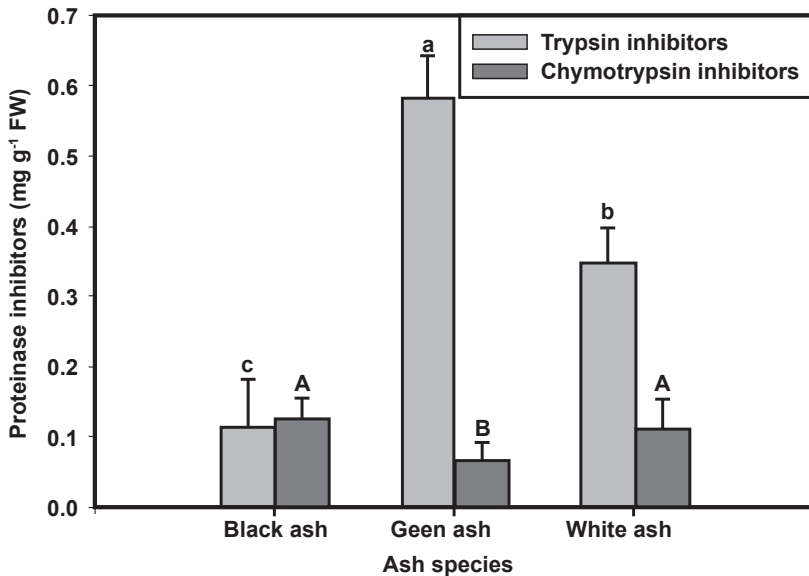


Figure 4. Trypsin and chymotrypsin inhibitors (mean \pm SEM) of three North American ash species. Different lower- and upper-case letters denote significant differences among three ash species in trypsin and chymotrypsin inhibitors at $\alpha = 0.05$, respectively. $n = 10$ replicates for black, green, and white ash.

studies involving live plants may need to be further examined. Proteins in live plants may include defensive enzymes and non-defensive proteins. Almost all diet self-selection studies so far were conducted with artificial diets in the laboratory and the proteins used were completely non-defensive proteins (e.g., casein; Waldbauer and Friedman 1991, Woods 1999). Many defensive proteins and enzymes are induced by insect herbivore feeding (Chen et al. 2009a, b), and are included in the total protein determination.

The shorter longevity of EAB adults on black ash might be caused by more unbalanced nutrients (total protein/TNC) of black ash than green and white ash. At first glance, the worse performance of EAB adults on black ash foliage seems contradictory to the general belief that nitrogen is a limiting factor for plant and animal growth (Mattson, Jr. 1980, White 1993) and the importance of nutrient balance in insect feeding preference (Behmer 2009). However, as shown in Table 1, the foliar total nitrogen of all three species in this study was very high (i.e., nearly or over 2-fold the levels of normal ranges of ash trees and higher than many of the naturally grown tree species). Furthermore, black ash had the highest contents of total protein while it had the lowest levels of TNC (Tables 1 and 2). Therefore, under conditions where nitrogen is not limiting, the more unbalanced nutrients of black ash foliage might impair EAB performance.

Phenolics were suggested to be involved in Manchurian ash's relatively higher resistance to EAB infestation because some phenolic compounds (i.e., hydroxycoumarins, calceolariosides A and B) were only found in Manchurian ash phloem (Eyles et al. 2007), compared to green ash and white ash. Phenolics and protease inhibitors might also partially contribute to EAB adults' shorter longevity on young green ash leaves and preference for mature foliage (Chen and

Poland 2009b). Neither phenolics nor protease inhibitors seemed to contribute to EAB adult longevity in this study. The following two reasons may support the claim. First, most of the phenolics and protease inhibitors might be directly excreted or detoxified by EAB detoxification enzymes. As shown in Fig. 3, EAB frass contained approximately 2-fold or more phenolics compared to ash foliage, regardless of ash species. Furthermore, EAB females are more capable of excreting phenolics than males. Many phytophagous insects are also known to possess well-developed detoxification systems that help insects reduce or eliminate toxicity of plant allelochemicals (Ahmad et al. 1986). Second, the defensive roles of phenolics might be alleviated by high nitrogen availability or optimal nutrient balance. An excellent example is the strong interaction of nutrients with plant allelochemicals found by Simpson and Raubenheimer (2001). When the migratory locusts, *Locusta migratoria* L. (Orthoptera: Acrididae), were fed upon diets with optimal protein to carbohydrate ratio, the otherwise negative effects of tannic acid (a carbon-based polyphenol) on their performance disappeared.

In summary, EAB adults in this study performed better on and preferred green ash and white ash than black ash. The better performance and preference on green and white ash might be due to relatively more balanced nutrients of green and white ash foliage. The negative effects of the putative defensive compounds (phenolics and protease inhibitors) of ash seedlings might be alleviated by high nitrogen levels. As a result, the patterns of these plant allelochemicals did not parallel with EAB performance and preference.

Acknowledgments

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