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Intraspecific variation in *Fraxinus pennsylvanica* responses to emerald ash borer (*Agrilus planipennis*)

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Abstract The emerald ash borer (EAB; *Agrilus planipennis* Fairmaire) is a bark and wood boring beetle native to east Asia that was first discovered in North America in 2002. Since then, entire stands of highly susceptible green ash (*Fraxinus pennsylvanica* Marshall) have been killed within a few years of infestation. We have identified a small number of mature green ash trees which have been attacked by EAB, yet survived the peak EAB infestation that resulted in mortality of the rest of the ash cohort. Adult landing and feeding preference bioassays, leaf volatile quantification and EAB egg bioassay experiments were used to characterize potential differences in responses of these select "lingering" green ash trees relative to known EAB susceptible controls. Three selections were identified as being significantly less preferred for adult feeding, but no specific leaf volatile profile was associated with this reduced preference. Egg bioassays identified two ash selections that had significant differences in larval survival and development; one having a higher number of larvae killed by apparent host tree defenses and the other having lower larval weight. Correlation and validation of the bioassay results in replicated plantings to assess EAB resistance in the field is still necessary. However, the differences between lingering ash selections and susceptible controls measured by these bioassays indicate that more than one mechanism is responsible for the increased resistance to EAB that resulted in these selections surviving longer than their counterparts. Efforts to further increase ash resistance to EAB through use of these selections in a breeding program are underway.

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Introduction

The emerald ash borer (EAB, *Agrilus planipennis* Fairmaire) is a metallic bark and wood boring beetle native to east Asia (China, Japan, Korea, Mongolia, Eastern Russian). Asian ash species are not immune to EAB but do have a high level of resistance that results in a very low frequency of EAB successfully completing their life cycle. This frequency increases to a problematic level only when attacks occur in conjunction with other stressors (Wei et al. 2004). In contrast, North America ash species are highly susceptible which has resulted in unparalleled levels of ash mortality in urban and natural forests since the accidental introduction of EAB, estimated to have occurred in the 1990s (Herms and McCullough 2014; Siegert et al. 2014). Although EAB was first discovered in 2002 near Detroit, Michigan, USA and Windsor, Ontario, Canada (Haack et al. 2002), dendrochronological evidence indicates it was likely introduced over a decade prior to detection (Siegert et al. 2014). As of May 1, 2015, it has become established in 25 states in the USA and two provinces in Canada (www.emeraldashborer.info). This widespread infestation combined with the very high susceptibility to EAB in evolutionarily naïve North American ash species, has resulted in mortality rates reaching up to 99 % in some cases (Knight et al. 2013; Klooster et al. 2014).

Monitoring plots established in natural wooded areas revealed that a small number of mature ash trees, while attacked by EAB, survived the main wave of ash mortality (Knight et al. 2012). A targeted survey approach identified hundreds of surviving ash trees in areas long-infested by EAB (Knight et al. 2012; Marshall et al. 2013). In the study by Knight et al. (2012), only 2.6 % of ash trees survived out of the original population of 11,000 and only 1 % retained a healthy crown, defined as having a rating of one on a canopy condition scale of one (canopy full and healthy) to five (canopy has no leaves, epicormic sprouts may be present on the trunk; Smith 2006; Knight et al. 2014), 2 years after the ash mortality reached 95 %. These healthy trees are referred to as "lingering ash" because although they may simply represent the tail end of the mortality curve and still be killed by EAB in the future, they may also be trees with infrequently occurring phenotypes that make them less preferred by EAB or more resilient to EAB attack.

There are other known examples of allopatric resistance that occur without prior evolutionary contact in forest trees attacked by invasive insects. Resistance to the invasive beech scale insect, *Cryptococcus fagisuga* Lindinger, whose feeding activities render trees susceptible to fungal infection resulting in the development of beech bark disease, has been identified in American beech, *Fagus grandifolia* Ehrh. This resistance has been shown to be heritable and is the basis for development of American beech breeding programs (Koch et al. 2010; Koch 2010). In addition, both eastern (*Tsuga canadensis* (L.) Carrière) and Carolina hemlock (*Tsuga caroliniana* Engelm.) trees have been identified that survived heavy infestations by the hemlock wooly adelgid (*Adelges tsugae* Annand, Oten et al. 2014). The infrequently occurring resistant beech and hemlock trees are examples of naturally occurring variation within a species. Among the different ash species native to North American, there are indications that there is variation in the level of EAB-susceptibility or resistance between species. Although it has been shown that EAB can carry out its full life cycle in green, white (*Fraxinus americana* Linnaeus) and blue ash (*Fraxinus*

quandrangulata Michaux), canopy dieback and EAB attack density is significantly higher in green ash than in white ash located at the same sites and in white ash compared to blue ash when located at the same sites (Anulewicz et al. 2007, 2008; Tanis and McCullough 2012).

The survival of a small number of green ash trees despite long-term EAB infestation may be due to natural variation within the species. Careful selection and breeding of these surviving trees could provide the opportunity to further improve ash resistance to EAB. Based on reports that North American ash species planted in Asia suffer high levels of mortality despite the presence of predatory insects (Liu et al. 2003; Wei et al. 2004), it has been suggested that increased levels of host resistance will likely be required to maintain ash as a component of forests in North America in addition to current efforts to control EAB populations through the release of parasitoids native to China (Duan et al. 2012; Herms and McCullough 2014).

A successful breeding program depends upon the ability to select superior performing parent trees. This requires an understanding of the specific characteristics or phenotypes that contribute to the extended survival time of a lingering ash. There are multiple points of interaction between the EAB and its ash host; adult beetles interact with leaves, twigs and branches while feeding and during mating, female adults interact with the main bole of the tree during oviposition, and larvae tunnel into ash outer bark, then continue through bark, phloem, cambium, and outer xylem (Poland et al. 2015). It is not known which point of the host–insect interaction is the most relevant to resistance or if multiple points of interaction may be important in additive or multiplicative ways for host resistance. Different points of interaction(s) may be important in different genotypes or species or at different pest densities.

Landing and feeding studies are well established host preference indicators and have previously been utilized for testing EAB adult preference in multiple species of ash (Puraswaran and Poland 2009). Adult beetles that prefer to feed on a specific genotype of ash may be more likely to mate and subsequently lay eggs on the same ash genotype. Although this has yet to be confirmed experimentally, studies have reported that ash foliage is highly utilized for mating behavior (Lelito et al. 2007; Jennings et al. 2014). Adult EAB have been shown to sense and respond to ash volatiles (Rodriguez-Saona et al. 2006; de Groot et al. 2008), indicating that volatiles may play a role in determining adult preferences. Unlike the mobile adult beetles which are able to fly from one tree to another, EAB larvae must survive until adulthood or fail to complete their life cycle in the tree on which the eggs are laid. Host defenses that interfere with larval development and pupation are therefore likely to be critical in ash defense against EAB. Mortality of larvae from apparent host defenses has been reported not only in Asian ash species that have co-evolved with the beetle but in green ash as well (Liu et al. 2007; Duan et al. 2012, 2013a, b).

In this report we use landing and feeding assays, characterization of leaf volatiles, and egg transfer bioassays on a small set of lingering ash selections, along with known EAB-susceptible controls, in an effort to identify specific traits or phenotypes that are likely to be associated with an increased ability to survive EAB infestation. The long term goal of this work is to develop a reliable screening technique that will allow us to distinguish between trees that have merely escaped infestation from trees that have reproducible, quantifiable phenotypes that allow them to better tolerate/survive EAB-infestation.

Methods

Plant selection and propagation

All ash genotypes included in one or more experiments are shown in Table 1. Green ash and Manchurian ash seeds for seedling sources and root stocks were purchased from commercial suppliers (see Table 1), scion wood of commercial cultivars was donated from commercial suppliers, and all other scion was collected by USDA Forest Service employees or cooperators in the field. Seedlings and grafts were between two and four years of age at the time the experiments were performed. Plants were grown at the USDA Forest Service facility in Delaware, OH as described in Rigsby et al. (2015). Ash selections and cultivars were propagated through grafting by hot callousing (either top cleft or side veneer grafts) or by bud grafting (Tubesing 1987; Carey et al. 2013), using commercial or FS grown seedlings (Table 1) as root stock. The *F. pennsylvanica* cultivar 'Summit' (PE-Sum) was chosen as the EAB-susceptible control based on a previous evaluation of its response to EAB attack in the field (Herms et al. 2015). The control was included in all experiments, along with the core group of experimental lingering ash selections, PE-L19, PE-L21, PE-L22, PE-L24 and PE-L36. Depending on availability, additional accessions (either seedlings, grafted lingering ash, alternate grafted controls, or alternate cultivars) were included in some experiments (Table 1).

Table 1 Trees included in the study

Short name	Species	Selection source	Source or provenance
MA-19	*F. mandshurica*	Grafted, wild selection	Heilongjian, China [imported by USDA Plant Introduction Station, Ames IA, accession #19150]
MA-Man	*F. mandshurica*	Grafted, "Mancana" cultivar	Morden Research Station, Canada
MA D891	*F. mandshurica*	Seedling from wild collected seed	China [import by Lawyer Nursery]
Penn 30742	*F. pennsylvanica*	Seedling from wild collected seed	Sheffield Seed Co.
Penn 40844	*F. pennsylvanica*	Seedling from wild collected seed	Sheffield Seed Co.
Penn 40791	*F. pennsylvanica*	Seedling from wild collected seed	Sheffield Seed Co.
Penn H880	*F. pennsylvanica*	Seedling from wild collected seed	Lawyer Nursery
Penn mix[a]	*F. pennsylvanica*	Mix of commercial seedlings	Commercial
PE-Sum	*F. pennsylvanica*	"Summit" cultivar	Summit Nursery, Stillwater MN
PE-12	*F. pennsylvanica*	Grafted, wild selection, USFS[b]	Ohio
PE-L15	*F. pennsylvanica*	Grafted, wild selection, USFS	Ohio
PE-L19	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L20	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L21	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L22	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L23	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L24	*F. pennsylvanica*	Grafted, wild selection, USFS	Michigan
PE-L36	*F. pennsylvanica*	Grafted, wild selection, USFS	Ohio

Not all trees were included in all experiments in the study

[a] A mix of the seedlings from the seed lots listed

[b] USFS is United States Department of Agriculture, Forest Service

All lingering ash accessions were selected in or near permanent monitoring plots located in southeast Michigan, USA described in Smith (2006) and Gandhi et al. (2014) or northwest Ohio, USA described in Knight et al. (2012). To be selected, a lingering ash tree had to have a healthy crown (canopy condition rating of one or two on a scale of one to five, Knight et al. 2014) and a DBH (diameter at breast height) of at least 23 cm. Scions from the lingering ash trees in the Michigan sites were collected and grafted in 2009, at which point the mortality of all ash trees greater than 10 cm DBH being monitored in 87 plots (only some of which included lingering ash) across all five sites ranged from 99 to 100 % (Table 2). The two lingering ash from the Ohio site were selected in 2008, where the average mortality levels of ash (>10 cm DBH) across nine monitoring plots was 97 % (Table 2). All lingering ash selections had a healthy crown and evidence of EAB infestation (such as exit holes, bark cracking or adventitious sprouts), but at a reduced level relative to the majority of the ash trees in the same stands that had declining crowns.

EAB collection, rearing, and egg production

EAB infested ash logs were cut in the winter dormant season and kept in cold storage (5 °C) until use. Logs were transferred as needed into 60-cm-long, 30-cm-diameter cardboard rearing tubes with screened end-caps and kept at 25 °C to allow EAB adult emergence as described in Chen and Poland (2009a, b). After emergence, EAB adults were transferred to separate rearing containers with four to six beetles of the same sex per container (one L plastic containers with a piece of window screen secured to the top with a rubber band). Adults were fed leaves of shamel ash (*Fraxinus uhdei* (Wenzig) Lingelsheim) to avoid preconditioning them to any species used in preference experiments. After 14 days, five sexually mature females and five sexually mature males were transferred to each breeding container and allowed to mate and lay eggs onto coffee filters (Meijer Brand, basket style white coffee filters, fits 8–12 cup coffee makers, Meijer, Inc., Grand Rapids, MI) placed over the window screen and attached to the tops of the containers with rubber bands (Abell et al. 2012).

Table 2 Stand mortality progression in long-term monitoring plots in sites where lingering ash selections were made (some lingering ash were located just outside of these plots)

Site code	Genotype	Total no. of ash in plots	Average % mortality								
			2004/2005	2006	2007	2008	2009	2010	2011	2012	2013
HM[a]	PE-L21	112	35	64	85	99	100[c]	100	98	98	100
IL[a]	PE-L19	165	58	92	96	98	99[c]	99	100	100	100
KEN[a]	PE-L22	404	49	88	97	99	99[c]	99	99	99	100
PON[a]	PE-L20 PE-L23	186	46	81	97	99	100[c]	100	100	100	100
PL[a]	PE-L24	159	58	87	90	97	99[c]	99	100	99	100
OO[b]	PE-L15 PE-L36	166	9	32	57	97[c]	100	100	100	99	100

[a] Site located in southeast Michigan

[b] Site located in northwest Ohio

[c] Year selected as lingering ash

Adult landing and feeding

A total of 10 landing and feeding preference experiments were performed over a 4-year period (2009–2011). Thirty beetles of the same sex were released into a 61 cm × 61 cm × 61 cm screen cage (Bioquip Produts, Rancho Dominguez, CA) or 83 cm × 53 cm × 36 cm clear plastic tub with perforated lid (Sterlite, Townsend, MA) containing leaves from six ash selections, including the control (PE-Sum), as described by Puraswaran and Poland (2009). One leaf per genotype (in a 30 ml glass vial of water) was randomly assigned to a tub, then randomly assigned to a position within the tub and was replicated five to eight times within each experiment. Based on results reported by Chen and Poland (2009a) that immature leaves have lower nutrition and higher feeding inhibitors, mature leaves were used by selecting the 3rd to 6th fully expanded pair of leaves from the apex. Eight total lingering ash selections were assayed, but because of the size of the cage and spacing of the leaves, only six leaves could be assayed at one time. Therefore, different combinations of lingering ash genotypes were assayed in each experiment. PE-Sum was included in each experiment as a known susceptible control and a Manchurian ash accession was included as an EAB-resistant control (Rebek et al. 2008; Duan et al. 2012). Two additional experiments without Manchurian ash examined the choices that EAB would face in the forest in North America. The number of beetles present on each leaf was quantified visually by observing beetles for 30 min every 2 h between approximately 12:00 and 18:00 on the first day and between 08:00 and 16:00 on the second day for a total of nine observation periods over 30 h. Leaves were weighed and scanned before and after feeding and the leaf area was measured before the start and again at the end of the experiment to calculate the proportion of each leaf consumed by EAB. Scanned images of leaves were analyzed using Winfolia software (Regent Instruments, 2003) to determine leaf area. Leaf areas were standardized by specific leaf weight to adjust for differences in leaf thickness in calculations of proportion consumed.

Leaf volatiles

Leaf volatiles were collected for a subset of nine of the genotypes used in the landing and feeding studies (Table 4). Foliar volatiles were collected from at least four trees of each genotype. A branch from each tree, matched for age of foliage by counting down the same number of fully expanded leaves from the apex, was selected for volatile collection. A large-size Reynolds oven bag (Reynolds Kitchens, Richmond, VA) was used to enclose the terminal part of each selected branch containing 6–12 compound leaves. A Super-Q volatile collection trap (Analytical Research Systems, Gainesville, FL) containing 30 mg Alltech Super-Q adsorbent material and a glass tube filled with activated charcoal were placed against the stem of the enclosed branch and the opening of the bag was secured tightly around the stem with a twist tie. The Super-Q trap was connected with plastic tubing to a battery-operated pump (Sensidyne, Clearwater, FL). Air was cleaned as it entered the bag through the charcoal tube, and headspace volatiles inside the bag were pumped out and absorbed through the Super-Q trap. After the volatile collections were complete, the aerated leaves were clipped, oven-dried, and weighed. Collected volatiles from the Super-Q were extracted and analyzed on a Thermo Scientific Trace Ultra Gas Chromatograph equipped with a DSQ-II Mass Spectrometer using methods described in Chen and Poland (2009a). The amount of each compound of interest was calculated in nanograms per gram dry foliar weight. All reagents and solvents used in this study were

purchased from Sigma-Aldrich (St. Louis, MO) or Fisher (Pittsburgh, PA). For each volatile compound, a qualitative value was assigned for each tree genotype based on the most frequent observation ('nd' if the volatile was not detected in most leaf samples of a genotype or 'p' present if most leaf samples had a detectable value for the compound). Occasionally in a case of a 'not detected' value, one or two leaf samples out of the set of 6–12 would have a trace amount detected but these were still considered 'not detected' and a notation was made. Similarly, some tree genotypes with a compound present in multiple leaf samples had an occasional leaf sample with a 'not detected' value, and if there were more than three of these a notation was made.

EAB egg transfer bioassays

Coffee filters with EAB eggs laid on them were removed from breeding containers every 2–3 days and kept in a growth chamber at 25 °C (16 h light, 8 h dark) for 10 days then transferred to 10 °C for up to 3 days until needed. Potted trees (2–3 years post-graft, 1.5–2.5 m tall) were brought from the outdoor growing facility 1 week prior to egg placement and placed in a randomized block design inside a greenhouse with temperature settings of 21 °C during the day and 15.5 °C at night. Supplemental grow lights were used between the hours of 06:00 to 10:00 and 16:00 to 20:00. Each genotype (Fig. 1; Table S2) was represented once in each of three replicates within the experiment. Four eggs were placed at each of four locations (sites) spaced at least 23 cm apart along the length of each tree, for a total of 16 eggs per tree. Egg attachment sites were consistently labeled from bottom (A) to top (D), and growth increment and diameter were recorded for each site. After inspecting eggs under a dissecting scope to rule out any damage or unusual color indicating poor viability, four individual strips with one egg on each strip were cut from the coffee filter and spaced evenly around the tree, then taped to the tree using common masking (painter's) tape. Gauze was wrapped lightly around the eggs to deter predation. Eight weeks after estimated egg hatch (16 days from date of oviposition, not including days in chill, Duan et al. 2013c), the trees were dissected and scored. Egg locations were
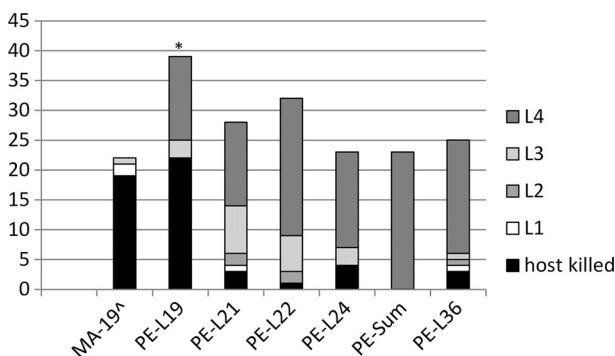

Fig. 1 Distribution of larval outcome for each genotype shown as a *stacked bar chart*. The multinomial distribution of outcomes for each genotype was compared to the control PE-Sum (as a statistical contrast) and only PE-L19 had a statistically significant result ($F_{1,241} = 12.85$, $p < 0.001$, designated *asterisk* on *graph*). All other contrasts to PE-Sum were not significant (PE-L21 $F_{1,241} = 0$, $p = 0.950$; PE-L22 $F_{1,241} = 1.10$, $p = 0.295$; PE-L24 $F_{1,241} = 1.07$, $p = 0.303$; PE-L36 $F_{1,241} = 0.19$, $p = 0.663$). *Note* that MA-19 data is from a parallel experiment and is graphed for comparison only and was not included in the statistical model

marked on the tree and then eggs were examined with a hand lens or dissecting microscope to note their condition. 'Bad egg' was noted as an outcome in cases when eggs were unhatched, which was often accompanied by the presence of mold or other damage to the egg. Eggs that had obviously hatched (exit hole on underside of the egg and/or frass inside egg) were noted as 'good eggs'. All sites with good eggs were dissected, using grafting knives or carving tools, beginning at the point where each larva entered the tree. Larval galleries were carefully followed until the larva was found or the gallery ended. The outcomes scored were non-overlapping and comprehensive (all larvae were assigned to one and only one outcome) and included host defense killed, unrecovered, and live larvae by instar (L1, L2, L3 and L4). Larval instar was determined based on the head capsule width (Cappaert et al. 2005; Chamorro et al. 2012). In cases where the gallery ended in callus filled or brown tissue, and there was no evidence of bacterial or fungal infection such as a foul smell or the presence of fungal mycelium, larvae were considered killed by apparent host defenses even if distinct larval tissue was no longer discernable (Duan et al. 2012, 2014). In this experiment, no evidence of bacterial or fungal infection was observed in any of the dead larvae. Larvae killed by host defenses in lingering ash selections were similar in appearance to larvae killed in resistant ash species observed in separate egg bioassay experiments (Fig. S1). The unrecovered larvae category included those that hatched but did not appear to enter the tree, as well as any instances where fewer larvae were recovered than the number of hatched eggs. Living larvae extracted from the tree without sustaining any damage (125 out of 137 total larvae) were weighed.

Statistical analysis

Minitab 16.4.2 (Minitab Inc. 2013) was used for initial exploratory data analysis including summary statistics, graphing and distributional analysis. Generalized linear models were fitted using SAS or SAS Enterprise Guide (version 9.4 or 6.1 respectively, SAS Institute 2002–2013) and are described specifically for each dataset below. Alternate models were compared and the best fitted model was chosen for interpretation based on minimizing Akaike Information Criteria (AIC), observation of plots of standardized residuals, and meaningful biological context of the factors. Tests and post hoc tests (contrast or least squares means (LSM) comparisons) were only computed for the best fit model. All test statistics and associated degrees of freedom and p values are reported in the text or tables.

Adult landing data were summed over nine time points (covering 30 h) in order to eliminate repeated measures correlations and possible time series auto-correlations in the landing data (auto-correlation was not apparent in exploratory data analysis). The summed data were then transformed using the Freeman–Tukey variance stabilizing transformation for count data (which normalized the data for analysis), then modeled using Enterprise Guide (calling Proc Mixed) with a variance components covariance structure and REML estimation method. Leaf area consumed over 30 h was calculated as the proportion of each leaf consumed, and transformed to normalize using the square root function. Models for both adult landings and proportion of leaf area consumed included a random factor of cage nested within experiment due to the necessity of combining across experiments and years caused by the limit of six leaves per cage. A Wald test for significance of the random factor was interpreted before evaluating the fixed factors (only to gauge the magnitude of any blocking effect as it is essential to the model structure). The models tested the fixed factors leaf genotype, EAB sex, and differences in leaf size (binned into four categories). LSM's for both landing and proportion of leaf area consumed were calculated and compared to a control level. For post hoc comparisons (LSM's) of genotype, the control level was PE-

SUM, included as a control in all experiments across the 3 years. The Dunnet–Hsu method was used to adjust p values for multiple comparisons when calculating differences of the LSM's for both landing and feeding.

Larval outcome data were modeled as a multinomial response using a cumulative logit function (as the link function in PROC GLIMMIX, maximum likelihood estimation with Laplace approximation). The variables site, diameter, and increment showed high correspondence (data not shown) resulting in model fitting difficulties when more than one of these factors were included in the model, so only site was used. Additional fixed factors considered were genotype and genotype × site interaction. Replicate was considered a random factor and was tested using the Wald test (only to gauge the magnitude of the effect as it is essential to the model structure). Contrasts were fit for comparison of genotype differences. A similar generalized linear model was fit for larval weights using Enterprise Guide (calling PROC GLM) with the same experimental factors used to model larval outcome. Differences of the LSMs were computed using the horticultural selection 'Summit' as the control with p values adjusted using the Dunnet–Hsu method to correctly evaluate significance of multiple comparisons.

Results

Adult landing and feeding

Differences in EAB landings on leaves were attributed to leaf genotype, leaf size, and EAB sex. Overall, at any given time point there were more adults observed on the sides of the cages than on any given leaf. The best fit model for the adult landing data included leaf genotype, EAB sex, and leaf area category as fixed effects. The random factor, cage within experiment was not significant ($Z = 0.61$, $p = 0.272$), indicating that combining over experiments was appropriate. Genotype was significant ($F_{18/297} = 2.68$, $p < 0.001$) but no differences in LSM's between genotypes and the control (PE-Sum) were significant. Leaves were selected for maturity, not size, and the initial leaf area (each leaf was assigned to one of four categorical values) was significant ($F_{3/297} = 4.15$, $p = 0.007$). Differences of LSM's showed that the largest leaves were statistically different from the smallest ($F_{297} = 2.89$, $p = 0.012$); more EAB landed on the larger leaves. Sex was significant ($F_{1/297} = 5.26$, $p = 0.023$), with males found on the leaves more than females. Sex and size differences were not analyzed further since genotype is the principle factor of interest and post hoc differences between genotypes were not discernable.

EAB feeding (analyzed as proportion of leaf area consumed) was found to differ by cage within experiment and by leaf genotype. The random (blocking) factor cage within experiment, was significant ($Z = 2.54$, $p = 0.006$) and examination of the parameter estimates for the cages shows two cages in one experiment had less than typical feeding (data not shown). Since these were only two of sixty-two cages, we considered it appropriate to interpret the fixed effects over all cages. Genotype was significant ($F_{18/289} = 7.01$, $p < 0.001$) while leaf area category was not significant ($F_{3/289} = 1.98$, $p = 0.117$). Contrasts of LSM between all the genotypes and the susceptible control, PE-Sum revealed that three lingering ash selections (PE-L15, PE-L22 and PE-L24) and the seedlot PE-30742 had significantly less leaf area consumed (Table 3). However, no significant differences in the proportion consumed (compared to PE-SUM) were found for four additional seedlots

	Genotype	Estimate[a]	Difference versus PE-Sum[a]	Adj. p value
Table 3 Least squares means of leaf proportion consumed for each genotype and test against control, PE-Sum	PE-Sum	0.386	n/a	n/a
	Man D891	0.218	−0.169	<0.001*
	MA-19	0.585	0.198	<0.001*
	MA-Man	0.470	0.084	0.314
	Penn 30742	0.260	−0.126	<0.001*
	Penn 40791	0.263	−0.124	0.886
	Penn 40844	0.346	−0.041	0.999
	Penn H880	0.319	−0.067	0.299
	Penn mix	0.407	0.021	0.999
	PE-12	0.346	−0.040	0.659
	PE-L15	0.286	−0.100	0.004*
	PE-L19	0.294	−0.092	0.078
	PE-L20	0.289	−0.097	0.725
	PE-L21	0.311	−0.076	0.221
	PE-L22	0.279	−0.107	0.014*
p values are adjusted using Dunnett–Hsu method with 289 *df*	PE-L23	0.274	−0.112	0.465
* *p* < 0.05	PE-L24	0.282	−0.105	0.016*
[a] On the transformed data scale	PE-L36	0.312	−0.074	0.996

(including PE-mix, a mixture of four different seedlots) and a wild selection from Ohio that was EAB infested at the time it was collected (PE-12, $p = 0.658$).

Three accessions of the EAB-resistant species *F. mandshurica* were compared with the EAB-susceptible control PE-Sum in order to better understand variation within this EAB resistant species (Table 3). The genotype MA-19 ($p < 0.001$) had a significantly higher proportion of leaf area consumed, while the proportion of MA-Man ($p = 0.314$) was not significantly different from the control. Conversely, the seedling MA-D891 ($p < 0.001$) was significantly lower than PE-Sum in the proportion of leaf area consumed.

Volatiles

To determine if variation in leaf volatile profiles correlated with adult feeding preferences, leaf volatiles were collected from the head space of ash leaves, quantified using GC/MS (Table S1) and qualitatively scored for each genotype (Table 4). Comparison of the qualitative volatile scores (present or absent) between individual lingering ash and the control (PE-SUM), revealed that only tetradecane was present in all three lingering ash selections and absent in PE-Sum. However, tetradecane was also present in other susceptible selections including PE-12 (a wild selected EAB-susceptible tree) and PE-mix (a mixture of seedlings from various seed lots). Despite the detection of intraspecific variation in leaf volatiles, no specific volatile profile associated with adult feeding preferences was identified.

EAB egg transfer bioassays

Genotype had a significant effect on larval outcome (instar or mortality) in the egg bioassays, but neither replicate nor egg placement site were significant (Table 5). The

Table 4 Volatiles results

ID	Apinene	Chexylac	Limonene	Cocimene	Nonanal	Dimeth	Dodec	Bicyclop	Trideca	Copaene	Caryoph	Tetradec	Farnesen	Pentadec
PE-12	P	P	P	nd[b]	P	P	P	P	P	P	nd	P	nd[b]	P[c]
PE-L15	P	nd[b]	P	nd[b]	P	nd[a]	P	P	P	nd[b]	nd[b]	P	nd[a]	nd[b]
PE-L19	P	P	P	P	P	nd[b]	nd[b]	P	P	nd[a]	nd	P	nd	P[c]
PE-L21	P	P[c]	P	P	P	P	P	P	P	P[c]	nd	P	nd	P
PE-L22	P	P[c]	P	P[c]	P	P[c]	P	P	P	P[c]	nd[b]	P	nd	P
PE-L24	P	P	P	P	P	nd[b]	P	P	P	P[c]	nd	P	nd	P
PE-L36	P	P[c]	P	P	P	P	P	P	P	P	nd	P	P[c]	nd[b]
Penn mix	P	P[c]	P	P[c]	P	P[c]	P	P	P	P	nd	P	nd[b]	P[c]
PE-Sum	P	P	P	P	P	P[c]	P	P	P	P	nd[a]	nd[b]	nd[a]	P[c]
Penn overall	P	P	P	P	P	P	P	P	P	P	nd	P	nd	P

Qualitative score of volatiles produced by each genotype *P* present, *nd* not detected/zero/absent. apinene = α-pinene, chexylac = *cis*-hexeyl acetate, cocimine = *cis*-ocimene, dimeth = dimethyl-3-methylene-2-vinylcyclohexane, dodec = dodecane, ciclyclop = bicyclopentyl-2-one, trideca = tridecane, caryphy = caryophyllene, tetradec = tetradecane, farnesen = α-farnesene, pentadec = pentadecane

[a] One leaf had a detectable (non-zero) value

[b] Two leaves had detectable (non-zero) values

[c] Four or more leaves had non-detectable (zero) values

Table 5 Test of factors significant in GLM model of egg outcome for the EAB egg transfer experiment

Parameter	Statistic	df	p value
Rep	Z = 1.10	3	0.137
Genotype	F = 5.21	5/226	<0.001
Site	F = 2.78	15/226	0.087

model that best fit the data based on AIC included the fixed effects tree genotype and site, along with the random factor, replicate (Table 5). Contrasts of multinomial outcomes between the lingering ash selections and the EAB-susceptible control PE-Sum showed that only PE-L19 was significantly different ($p < 0.001$) with 22 apparently host defense killed larvae while PE-Sum had none. An EAB-resistant Manchurian ash selection (MAN-19) was included in a separate experiment performed in parallel, and while not included in the model, the data is shown in Fig. 1 for comparison purposes. The number of host defense response killed larvae for PE-L19 (22) was similar to the 19 found in MAN-19, while PE-L19 had a large number of late instar larvae (17) compared to only one in MAN-19 (Fig. 1).

Weights of the recovered larvae were analyzed for effects of genotype or other experimental factors using the best fitting general linear model that included replication, site, genotype and larval instar as fixed factors (Table 6). Larval outcome and genotype were significant in explaining recovered larvae weights. Comparison of LSM between lingering ash genotypes and the susceptible control demonstrated that the lingering ash genotype PE-L22 had significantly lower larval weight ($p = 0.017$, Table S2).

Discussion

Previous studies have demonstrated that adult preferences in landing and feeding between ash species can be determined using choice bioassays (Puraswaran and Poland 2009), but this is the first study attempting to tease out preferences between different genotypes within the same ash species. Our experiments show a statistically significant genotype effect on landing choice, but were not sufficient to separate the individual genotypes by difference in LSM. Adult feeding may be a better way to assess cumulative adult preference for specific leaf tissue, because it integrates all of the adult EAB behavior over the 36 h experiment. Three lingering ash genotypes plus seedlings from the unselected commercial seed lot Pen 30742 were less preferred by adult beetles for feeding, indicating these seedlings may also have a lingering ash phenotype. The rank order of the three Manchurian ash accessions based on proportion consumed was consistent with results from an independent adult feeding study that compared accessions of Manchurian ash, black ash and their hybrids (data not shown) which provided an indication of the fidelity of the assay. Replicated field

Table 6 Test of fixed effects for larval weights analysis

Parameter	F value	df num	df denom	p value
Larval stage	23.74	3	111	<0.001
Site	0.97	3	111	0.412
Genotype	5.08	5	111	<0.001
Rep	0.38	2	111	0.686

trials of the lingering ash genotypes are being established to further assess their relative EAB-resistance phenotypes and determine if the field results correlate with the bioassay results.

The lower adult feeding preference for some lingering ash selections may indicate a mechanism for resistance. Genotypes that are less preferred by adults for feeding may have fewer eggs laid on them as a result. For example, blue ash is less preferred than white ash in adult feeding choice bioassays (Puraswaran and Poland 2009) and has also been reported to sustain significantly lower levels of EAB damage and mortality compared to white ash when both species are located at the same sites (Anulewicz et al. 2007, 2008; Tanis and McCullough 2012). Several studies have shown that adult EAB preferentially land on stressed trees, but that healthy trees, or severely stressed and dying trees, are less attractive (McCullough et al. 2009; Crook and Mastro 2010; Marshall et al. 2012). Jennings et al. (2014) reported finding more EAB eggs, adults, mating pairs and ovipositing females on trees that were intermediately stressed based on crown condition. Chen and Poland (2009b) demonstrated that adult EAB preferred to feed on foliage from stressed (girdled) green ash rather than from non-stressed trees. Taken together, these studies likely indicate that even though tree health may influence adult EAB behaviors, oviposition patterns correspond with adult feeding preferences, supporting the idea that trees that are less preferred for adult feeding may be more resistant to EAB.

Reduced oviposition alone may explain the correlation of adult feeding preference and EAB attacks, or alternately reduced fecundity and increased larval mortality may play a role (either separately or in combination). None of the lingering genotypes had both significantly higher larval mortality combined with significantly lower adult feeding preference, but one genotype, PE-22, combined significantly less adult feeding with significantly lower larvae weights. Lower larval weight is correlated with decreased adult fecundity in many insect species, so this observation may indicate a relationship between adult feeding preference and fecundity (Honěk 1993; Keena 2002; Wang et al. 2002). The finding that some lingering ash genotypes are less preferred by adults for feeding than EAB-susceptible controls may explain why these trees were able to survive longer than their counterparts under high levels of EAB infestation.

Leaf volatiles are sensed and used by EAB to locate host trees in natural forests (Crook et al. 2008; de Groot et al. 2008; McCullough et al. 2009; Crook and Mastro 2010). Variation in patterns of leaf volatiles between trees may be associated with adult preferences in landing or feeding, particularly if compounds that activate the antennae of EAB are uniquely present or absent in different genotypes. However, our study found no relationship between differences in landing or feeding and volatiles. Given the findings of EAB preferences for stressed trees (Chen and Poland 2009b, McCullough et al. 2009), comparisons of leaf volatiles induced in lingering ash and controls by wounding or EAB larval and adult feeding may be warranted. Despite the lack of differences in constitutive leaf volatiles, differences in induced volatiles may occur as a result of different levels of EAB larval or adult feeding on lingering ash. The reduced levels of feeding in lingering ash may be low enough that they do not trigger the typical changes in leaf volatile profiles/chemistry that have been documented to increase EAB preferences to wounded trees (Rodriguez-Saona et al. 2006; Chen and Poland 2009a, b), which could also be a factor contributing to the lower EAB attacks observed on lingering ash.

Host selection is particularly important for wood-boring insects such as EAB because once an egg is laid, the larva is committed to survive or die in the host tree. In insect–host interactions, resistance can be due to reduced host preference, to reduced larval or adult insect development, fitness and survival (Schiers and De Bruyn 2002). The egg bioassay

allows a direct comparison of larval performance between lingering ash selections and known susceptible controls, independent of host selection. Two lingering ash selections were identified that were significantly different from the EAB-susceptible control. The percentage of apparent host defense killed larvae was significantly higher in PE-L19 while larval weight was significantly lower in PE-L22 compared to the EAB-susceptible control. Larval outcome (instar or mortality) and larval weight are both potential indicators of overall larval performance, and the reduced larval growth and development observed in some lingering ash selections could explain why they were able to survive EAB infestation longer than the surrounding ash population.

EAB mortality due to host defenses decreases as larval density increases, presumably because defenses have been substantially weakened or overcome by stress imposed by larval feeding (Duan et al. 2010). Optimal egg density for rearing EAB larvae on green ash was reported to be less than 200 eggs per m^2 of phloem area because this density is low enough to minimize competition and cannibalism yet high enough that no mortality due to host defenses were reported (Duan et al. 2013b). The egg densities in our bioassay ranged from an estimated 259 to 619 eggs per m^2 of bark surface area (calculated using site diameter to estimate area), well above densities reported in conjunction with larval mortality due to host defenses (Duan et al. 2012) and within the density ranges where no host defense killed larvae were observed (Duan et al. 2013b). Therefore, few if any host defense killed larvae would be expected at the egg densities used in our experiment, supporting the finding that the significantly higher amount of host defense killed larvae in PE-L19 are indicative of a genotype effect. Furthermore, in our model only genotype was found to be a significant factor for larval outcome while site, which incorporated stem diameter and therefore egg dose differences, was not significant.

Lingering ash were selected because they were documented to have survived and maintained a healthy crown after stand mortality in the surrounding ash population reached 97–99 % from intense EAB pressure. Although clearly the selected lingering ash trees were not immune to EAB, they often were the last remaining live ash trees in the park or natural area where they grew (Table 2), indicating they may have a higher level of resistance to EAB. Three of the five lingering ash selections included in both the adult preference and egg bioassay experiments were found to be significantly different from the EAB-susceptible control. PE-L24 was less fed upon, PE-L19 had greater host defense killed larvae, and PE-L22 was less fed upon and had significantly lower larval weight. An additional genotype, PE-L15, was only included in the adult preference bioassays and was also found to be significantly less fed upon. These results indicate that both host selection and host defense mechanisms play a role in EAB-tolerance in the surviving lingering green ash. Oviposition preference was not directly tested in either screen, but is a logical mechanism of EAB resistance to be evaluated in future experiments with lingering ash selections. Careful selection and breeding may lead to further gain in resistance to EAB, particularly if the genes that play a role in reduced host preference and reduced larval performance can be combined.

Our results indicate that both adult feeding bioassays and egg bioassays measuring larval survival and development may be useful early screening techniques to identify ash selections with phenotypes most relevant to improved EAB-resistance in the field. Efforts are currently underway to validate the bioassay results in replicated field plantings. Continued monitoring and preservation of ash trees that fit the criteria of a lingering ash (DBH > 25 cm, stand ash mortality >97 %) is recommended and may lead to the identification of additional EAB-resistant selections of North American ash species that can be incorporated into breeding programs.

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