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Moisture content and nutrition as selection forces for emerald ash borer larval feeding behaviour

YIGEN CHEN,[1] TINA CIARAMITARO[2] and THERESE M. POLAND[2] [1]Department of Entomology, Michigan State University, East Lansing, Michigan, U.S.A. and [2]USDA Forest Service, Northern Research Station, East Lansing, Michigan, U.S.A.

Abstract. 1. The exotic phloem-feeding emerald ash borer (EAB), *Agrilus planipennis*, has killed tens of millions of North American ash trees (*Fraxinus*) since its first detection in the U.S.A. in 2002. Ash trees are killed by larval feeding in the cambial region, which disrupts translocation of photosynthates and nutrients.

2. We observed that EAB larvae feed predominantly downwards in naturally grown green ash trees, a behaviour confirmed in greenhouse-grown black ash seedlings. Furthermore, biomass of larvae feeding downwards was greater than that for larvae feeding upwards.

3. We sought to determine the relative importance of four selection forces (i.e. gravity, moisture content, plant defence, and nutrition) in driving this downward feeding behaviour in this study. The gravity and plant defence (i.e. polyphenols) hypotheses were ruled out because even when seedlings were grown upside down, more EAB larvae moved upwards (towards the root area), and phloem tissue below the feeding site contained higher concentrations of defensive compounds than that above the feeding site.

4. The moisture content hypothesis was supported as phloem moisture above the feeding site decreased to levels reducing survivorship and biomass but was unaffected below. The nutrition hypothesis was also supported as the levels of 11 amino acids (mostly essential amino acids) below the feeding site were greater than those above. Furthermore, growth of larvae reared on an artificial diet deficient in protein and amino acids was worse than larvae reared in diet with complete ingredient or diet deficient in either protein or amino acids.

5. We conclude that moisture content and nutrients are two selective forces for the downward feeding behaviour of EAB larvae.

Key words. *Agrilus planipennis*, Buprestidae, Coleoptera, evolution, *Fraxinus*, natural selection, nutrients.

Introduction

Insect feeding behaviours (e.g. host plant selection and plant part specialisation within a plant) can be shaped by many biotic and abiotic factors. These biotic and abiotic factors fall broadly into the following five groups: gravity (Jander, 1963; Ramachandran, 1988), water content (Huberty & Denno, 2004; Schoonhoven *et al*., 2005), nutrition (Schoonhoven *et al*., 2005), allelochemicals (Dussourd & Denno, 1991; Chen *et al*., 2008), and natural enemy risk (Gonzáles *et al*., 2001; Kessler & Baldwin, 2002).

Many arthropods are known to possess gravity receptors in various parts of the body (Horn & Bischof, 1983; Horn & Föller, 1998) and their various behaviours are documented to be gravity-driven (Vollrath, 1986; Ramachandran, 1988). Although water is not traditionally considered as a nutrient, the nutritional value of water for survival and performance of insects is evident (Slansky & Scriber, 1985) and many phytophagous herbivores readily drink free water (Mellanby & French, 1958) in addition to acquisition of water from host plants. Nutrients, in particular protein or nitrogen, are limiting factors for arthropod survival (Slansky & Scriber, 1985; White, 1993), and given choices many phytophagous insects prefer high over low quality food, other conditions being equal (Schoonhoven *et al*., 2005). Plants are known to possess a diverse pool of plant secondary metabolites (also called plant defensive compounds) (Seigler & Price, 1976). The role of plant defence in the evolution of insect herbivore feeding behaviours has long been recognised (Bernays, 1998). Some

Correspondence: Yigen Chen, Department of Entomology, Michigan State University, East Lansing, MI 48824, U.S.A. E-mail: ygchen@msu.edu

insect feeding behaviours can be shaped by natural enemies. For instance, corn leaf aphid, *Rhopalosiphum maidis* Fitch (Hemiptera: Aphididae), selectively feed on nutritionally poor Johnsongrass [*Sorghum halepense* (L.) Pers.] whorls rather than on mature leaves, which are high in nutrients, to avoid parasitism by *Lysiphlebus testaceipes* Cresson (Hymenoptera: Braconidae) (Gonzáles *et al*., 2001).

The emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an exotic species first detected near Detroit Michigan, U.S.A. and Windsor Ontario, Canada in 2002, and is threatening the ash resource in North America (Haack *et al*., 2002; Poland & McCullough, 2006). EAB adults are known to utilise chemical signals originating from ash trees for host location (Rodriguez-Saona *et al*., 2006; Pureswaran & Poland, 2009). They have also been shown to exhibit phototaxis (Rodriguez-Saona *et al*., 2007; Chen & Poland, 2009). Relative to a wealth of information on EAB adult foraging behaviours, almost nothing is known about EAB larval feeding behaviours except that EAB larvae feed on the phloem and cambial region, which disrupts translocation of photosynthates and nutrients. The evolutionary forces that shape these behaviours are also unknown. However, distribution of EAB larvae and adult exit holes within trees in the field might indirectly indicate a downward (toward the root area) progression in feeding by EAB larvae (Cappaert *et al*., 2005). Early EAB eggs are observed to be laid on the bark in the tree canopy and later eggs are subsequently laid below previous ones (Cappaert *et al*., 2005).

In this study, we first observed that most EAB larvae fed downwards (moving towards the root area) in naturally grown green ash (*Fraxinus pennsylvanica* Marshall). This feeding behaviour was confirmed in greenhouse-grown black ash, *Fraxinus nigra* Marshall, seedlings. Furthermore, the biomass of larvae that fed downwards was greater than those that fed upwards (moving towards the canopy area). Four selection forces (i.e. gravity, moisture content, plant defence, and nutrition) for this feeding behaviour were hypothesised. We determined the water content, total polyphenols (a class of putative defensive compounds of ash trees) and amino acids of phloem tissue above and below the EAB feeding site. We then selectively tested the gravity, moisture content, and nutrition hypotheses based on the above chemical determinations. Hypotheses specifically tested were: (i) More EAB larvae will feed upward (toward the root area) when seedlings were grown upside down; (ii) EAB larvae will perform best when they are fed diets with moisture levels closest to the levels determined in phloem below the feeding site; and (iii) EAB larvae will experience lower mortality or higher biomass gain when they are reared on diet with higher nutritional qualities.

Methods

Experiment 1: EAB larval feeding direction in naturally grown green ash trees

Two moderately infested green ash trees (diameters at breast height ranged from 12 to 20 cm) from each of two sites (i.e. Legg Park, and Dansville area) in Ingham County, Michigan, U.S.A., were cut down and brought to the laboratory. Legg Park is a public recreation park of approximately 41.7 ha and the trees from Dansville area were cut from private property of approximately 16 ha. Each tree was divided into three ∼5 m sections (i.e. bottom, middle, and top) and the number of EAB galleries was recorded. EAB galleries could be broadly grouped into those with a larva or a pupa, and those empty (i.e. successfully emerged or predated by woodpeckers). The feeding directions of the first group galleries were recorded. Total trunk area of the bottom and middle sections of each tree was also calculated as $2 \times \pi \times R \times L$, where R and L denote the average of the radii of the two ends and length of each tree section, respectively. No EAB galleries were found on the top section of all trees and therefore the trunk area was not calculated.

Experiment 2: EAB larval feeding direction, test of gravity hypothesis, and effects of moving direction on phloem phytochemistry below and above the feeding site

Ash seedlings and EAB eggs. Black ash seedlings (2-year-old and ∼50–70 cm tall) were purchased from Lawyer Nursery Inc. (West Plains, Montana) in February, 2009, and stored in a dark room at 4 °C until potting. Seedlings were potted with TPOT2 tree pots (width: 15 cm; height: 41 cm; volume: 6.23 litre; Stuewe & Sons, Inc., Corvallis, Oregon) using Fafard #52 soil (BFG Supply Co., Burton, Ohio) as the planting medium. They were grown during April to May, 2009 (ca LD 14:10 h, 25 ± 2 °C) in a greenhouse located at the Tree Research Center, Michigan State University, East Lansing, Michigan, U.S.A. Seedlings were fertilised once every 3–4 days with approximately 2 litre of a nutrient solution containing 200 ppm nitrogen, 60 ppm phosphorus, 150 ppm potassium, 80 ppm calcium, 40 ppm magnesium, 60 ppm sulphur, 0.15 ppm copper, 4 ppm iron, 0.8 ppm manganese, and 0.32 ppm zinc.

In the laboratory, eggs were laid by field-collected EAB adults on evergreen ash, *F. uhdei* Wenzig Linglesh, sticks (diameter ∼1–2 cm). Age of the eggs was tracked (the day the eggs were deposited was considered as 0 days old). When eggs were 12 to 13 days old, they were removed with a knife, together with a thin layer of ash bark for experimentation.

Experimental set-up. The experiment was conducted in a green house in Tree Research Center. Seventy-two black ash seedlings of similar size (i.e. height and diameter of trunk) were selected. The seedlings were randomly assigned to two treatments (EAB and Control) approximately 4 weeks after potting. For the EAB treatment, six eggs of the same age were pinned to the main trunk of each seedling about 30 cm above the soil by gently pinning through the edge of the layer of attached ash bark using four #3 insect pins. For the control treatment, ash bark without eggs was pinned with the same number of insect pins. Six randomly selected seedlings from each treatment were sampled at 10-day-intervals beginning 10 days after eggs hatched and larvae began to feed on the EAB treatment (i.e. day 10, 20, 30, 40, 50, and 60).

Because EAB eggs generally hatch in our laboratory within 17–18 days at 25 ± 2 °C after oviposition, and the eggs used in the experiments were 12 to 13 days old, egg hatch did not occur until approximately 5 days after pinning. Hence, the first observations occurred 15 days after the eggs were pinned, corresponding to 10 days after egg hatching and initiation of feeding.

At each observation the trunk bark of each seedling was peeled carefully to check the number of EAB larvae, their development and the direction in which they were moving. Movement direction was classified as either downward (those below the pinning site when bark was peeled) or upward (those remain at and above the original pinning site). Trunk phloem samples were collected separately from directly above and below the feeding damage. Each treatment (EAB or Control) was originally replicated six times per observation time. For the EAB treatment, only those replicates in which at least one larva was observed were included in the EAB treatment data set, with an average of 1.7 larvae per seedling. Seedlings originally assigned to treatment EAB were considered Controls if no larvae were found. The final number of replicates for each treatment were as follows: day 10: $n_{\mathrm{EAB}} = 4$, $n_{\mathrm{Control}} = 8$; day 20: $n_{\mathrm{EAB}} = 6$, $n_{\mathrm{Control}} = 6$; day 30: $n_{\mathrm{EAB}} = 5$, $n_{\mathrm{Control}} = 7$; day 40: $n_{\mathrm{EAB}} = 6$, $n_{\mathrm{Control}} = 6$; day 50: $n_{\mathrm{EAB}} = 5$, $n_{\mathrm{Control}} = 7$; day 60: $n_{\mathrm{EAB}} = 3$, $n_{\mathrm{Control}} = 8$. Phloem samples were analysed to determine the contents of water, total polyphenols, and amino acids.

Test of gravity hypothesis. Significantly more EAB larvae moved toward the roots of ash seedlings during the previous experiment; therefore, to determine if this downward movement was due to gravity, 15–20 black ash seedlings of similar size were potted upside down. EAB eggs were pinned as described above. After 3–4 weeks, the bark of the seedlings was peeled and larval movement direction was recorded. The experiment was conducted in the same greenhouse as described above.

Water content determination. Phloem samples were weighed immediately following collection (fresh weight) and again after being lyophilised within 1 week of collection (dry weight) using a Modulyo® freeze dryer (Thermo Scientific, Pittsburgh, Pennsylvania). Water content of phloem was calculated as (fresh weight – dry weight) × 100%/fresh weight.

Extraction and determination of foliar polyphenols. Lyophilised samples were ground by a 475-A Wiley mill (Arthur M. Thomas Co., Philadelphia, Pennsylvania) and sieved through 20 mm mesh. Methods of total polyphenols extraction and determination are described in Chen and Poland (2009). Briefly, ground and weighed phloem tissue was extracted three times, 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. An external standard curve using gallic acid within the linear range was constructed to calculate sample concentration.

Extraction and analysis of phloem amino acids. Phloem samples were collected and prepared as in the preceding experiments. Approximately 50 mg phloem tissue contained in 1.5 ml microcentrifuge tubes was mixed with 600 μl of 25% (v:v) acetonitrile in 0.01 N HCl. The mixture was vortexed for 5–10 min and then placed in the microcentrifuge tubes at room temperature and held for 50 min. The mixture was then centrifuged at 10 000× ***g*** for 10 min. The top layer was filtered through a 0.45 μm Millex® syringe filter (SLHA 033 SS, Millipore Corp., Billerica, Massachusetts). The filtrate was prepared according to instructions provided with a Phenomenex® EZ:faast™ Free (Physiological) Amino Acid Analysis by GC-MS kit (Torrance, California). Amino acids in the filtrate were first purified by sorbents and a washing solution. The cleaned amino acids were then derived to their phenylisothiocyanate derivatives and analysed by means of a Thermo® TRACE GC Ultra™-DSQ II mass spectrometer (MS) (Thermo Scientific, Waltham, Massachusetts) using a ZB-AAA 10 m × 0.25 mm Amino Acid Analysis GC column. 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^{-1}. The MS 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^{-1}. Identification of individual amino acids was based on comparison of mass spectra in the samples with spectra of phenylisothyiocyanate 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.

Experiment 3: test of moisture content hypothesis

Because EAB larval feeding significantly decreased phloem water content above the feeding site while it did not affect that below the feeding site, we hypothesised that the downward feeding of EAB larvae was driven by optimal water content. Therefore, EAB larval development in relation to various water moisture levels was tested using artificial diet modified from Blossey *et al.* (2001) (see Appendix S1 for ingredients) for ease of manipulation of moisture levels. Moisture level was calculated as weight of de-ionised water divided by the sum of de-ionised water and all other ingredients in Appendix S1. Different moisture levels were generated by adjusting the weight of de-ionised water only. We selected 60% water moisture as an intermediate level for testing because that was the level found in control seedling phloem in experiment 1 and in the artificial diet used by the USDA Forest Service for rearing EAB larvae and larval parasitoids (East Lansing, Michigan). We also tested three additional moisture levels in 20% increments above and below 60% (i.e. 20, 40, and 80%). Fifteen pre-weighed EAB larvae ranging from the second to early third instar were individually placed in separate Petri dishes (4 cm diameter) packed with artificial diet of each moisture level. The 15 larvae in separate Petri dishes were considered a replicate. Each treatment was replicated three times (i.e. a total of 45 larvae per treatment). Mortality was monitored and biomass of surviving larvae was determined once a week for four continuous weeks. Only those larvae that survived the 4-week experimental period were used in biomass data analysis. The experiment was conducted in an environmental chamber with $25 \pm 2\ ^{\circ}$C and L:D 14 : 10 h.

Experiment 4: Test of the nutrition hypothesis

Four nutrition treatments were tested. The treatments were Control (complete formula as described in Appendix S1), No Yeast (with Yeast removed from formula in Appendix S1), No Casein (with Casein removed from the formula in Appendix S1), and Neither (formula with neither Casein nor Yeast). Yeast was the sources of amino acids and casein was the source of protein. Twenty pre-weighed EAB larvae ranging from the second to early third instar were individually placed in separate Petri dishes (4 cm diameter) packed with artificial diet of each nutrition level. The 20 larvae in separate Petri dishes were considered a replicate. Each treatment was replicated three times (i.e. a total of 60 larvae per treatment). Mortality was monitored and biomass of surviving larvae was determined once a week for four continuous weeks. Only those larvae that survived the 4-week experimental period were used in biomass data analysis. The experiment was conducted in an environmental chamber with $25 \pm 2\ ^{\circ}$C and L:D 14:10 h.

Statistical analysis

Experiment 1. The number of EAB larvae moving upwards and downwards was analysed with a χ^2 test with the 50% probability of moving either direction, separately for each site. The distribution of EAB galleries between the bottom and middle section of a tree was analysed with a χ^2 test with probability equal to the trunk surface area of each section. The ratios of the trunk surface of the bottom section to the middle section were 63.39 : 37.61, and 56.41 : 43.59 for Legg and Dansville, respectively.

Experiment 2. The number of EAB larvae that moved upward or downward was compared with a χ^2 test. Total polyphenols, water content, and individual amino acids were analysed as a repeated measure because data collected over time were likely correlated (PROC MIXED in SAS; Littell *et al*., 2006), separately for above the feeding site, below the feeding site and a ratio of below to above the feeding site. Total polyphenol, water content, and amino acid data were natural logarithm transformed before being subjected to analyses. The statistical model was $Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk}$, where Y_{ijk} denotes the measurement at time j on the kth subject assigned to treatment $i (i =$ EAB or Control; $j =$ 1–6, except water content data for which $j = 1$–3 in experiment 1; $k = 1$–8 in experiment 1), and μ, α_i, β_j, $(\alpha\beta)_{ij}$, and ε_{ijk} denote overall mean, treatment effects, observation effects, treatment and observation interactions, and error term, respectively. The covariance structures were selected based on fit statistics [i.e. -2 Res Log Likelihood, Akaike's information criterion (AIC), the AIC corrected (AICC), and Bayesian information criterion (BIC)]. The compound symmetry which assumes (i) equal variance at all observation times; and (ii) equal covariance between subjects (seedlings) at all pairs of observation times (type = cs in REPEATED statement in SAS) fit best with the data. Means among treatments were further separated by the protected LSD method when necessary. The number of EAB larvae that moved upward or downward in the *test of gravity hypothesis* was compared with a χ^2 test. The biomass of EAB larvae was analysed with a t-test. Only larvae from seedlings in which some larvae were observed moving upwards and some others moving downwards in the same seedling were included in the analysis.

Experiment 3 and 4. Mortality and biomass of EAB larvae were analysed as a repeated measure described in Experiment 2 with $i = 1$–4, $j = 1$–4, and $k = 1$–3. In addition, initial biomass of EAB larvae was analysed as a covariate of treatments when analysing biomass data and a different slope for each treatment was modelled as the data suggested. The analysis of covariance (ANCOVA) model was $Y_{ij} = \alpha_i + \beta_i X_{ij} + \varepsilon_{ij}$, where Y_{ij} was biomass of EAB larvae, X_{ij} was initial biomass of EAB, and β_i and α_i were slopes and intercepts of the regression lines, respectively. The compound symmetry variance–covariance structure (see assumptions above) was selected for the mortality data in Experiment 3 and biomass data in Experiment 4. The unstructured variance–covariance structure which assumes (i) unequal variance at all observation times; and (ii) unequal covariance between subjects (seedlings) at all pairs of observation times (type = un in REPEATED statement in SAS) was selected for biomass data in Experiment 4. Mortality and biomass data in Experiment 3, and mortality data in Experiment 4 were square root transformed. Biomass data in Experiment 3 were natural logarithm transformed.

Normality assumptions of the residuals of all models were met after the data transformation. If equal variance assumptions of the residuals of repeated measure models were not met by data transformation, they were accounted using GROUP = treatment option in SAS. Degree of freedom in the repeated measure analyses was calculated using Kenward–Roger method. In all analyses, the Type I error (rejecting the null hypothesis while the null hypothesis was true) was controlled at 0.05 level.

Results

Experiment 1: EAB larval feeding direction in naturally grown green ash trees

A total of 713 and 480 EAB galleries were observed in trees from Legg park and Dansville, respectively (Fig. 1a). An average of 78.63% (i.e. 560 galleries) and 55.27% (i.e. 258 galleries) of the galleries from Legg park and Dansville, respectively, contained larva or pupa and the feeding directions were determined (Fig. 1b). More EAB galleries were

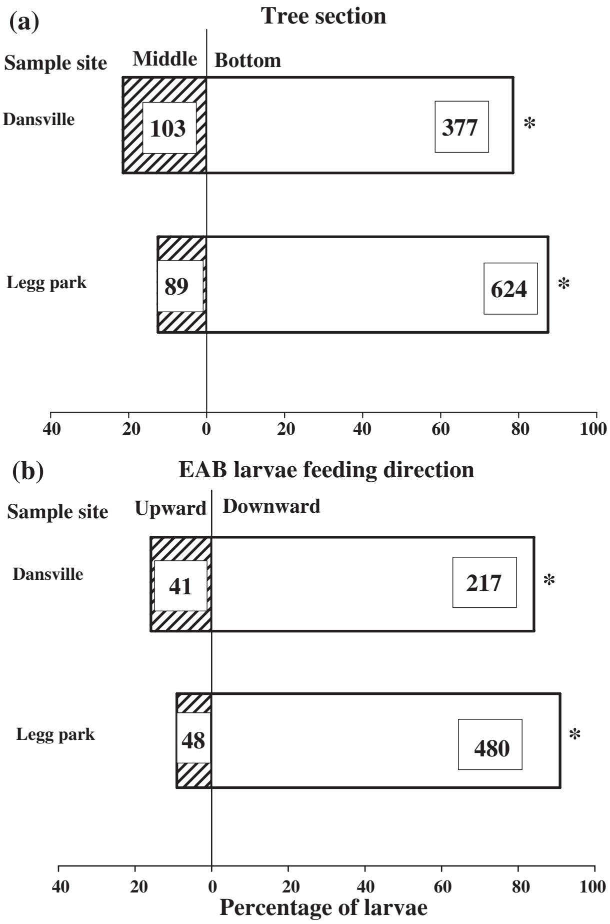

Fig. 1. Within-tree distribution (a) and feeding direction (b) of emerald ash borer (EAB) galleries. Numbers on the bar denotes number of EAB galleries moving to that direction or observed in that tree section. $*P < 0.0001$.

observed to move downwards in each location (Fig. 1b; Legg Park: $\chi^2 = 353.45$, $P < 0.0001$; Dansville: $\chi^2 = 120.06$, $P < 0.0001$). No EAB galleries were observed in the upper one-third section of the tree and more galleries were observed in the lower section compared to the middle section of the tree after accounting for the trunk surface area (Fig. 1a; Legg Park: $\chi^2 = 191.85$, $P < 0.0001$; Dansville: $\chi^2 = 95.62$, $P < 0.0001$).

Experiment 2: EAB larval feeding direction, test of gravity hypothesis, and effects of moving direction on phloem phytochemistry below and above the feeding site

A total of 46 larvae were found on 28 seedlings with 38 larvae moving downward and eight moving upward (Fig. 2a). The difference was significant ($P < 0.0001$). At least one EAB larva was observed to feed both upwards and downwards in five of the seedlings. The biomass of EAB larvae moving downwards ($n = 12$) on these five seedlings was significantly greater than for those moving upwards ($n = 5$) (Fig. 2b).

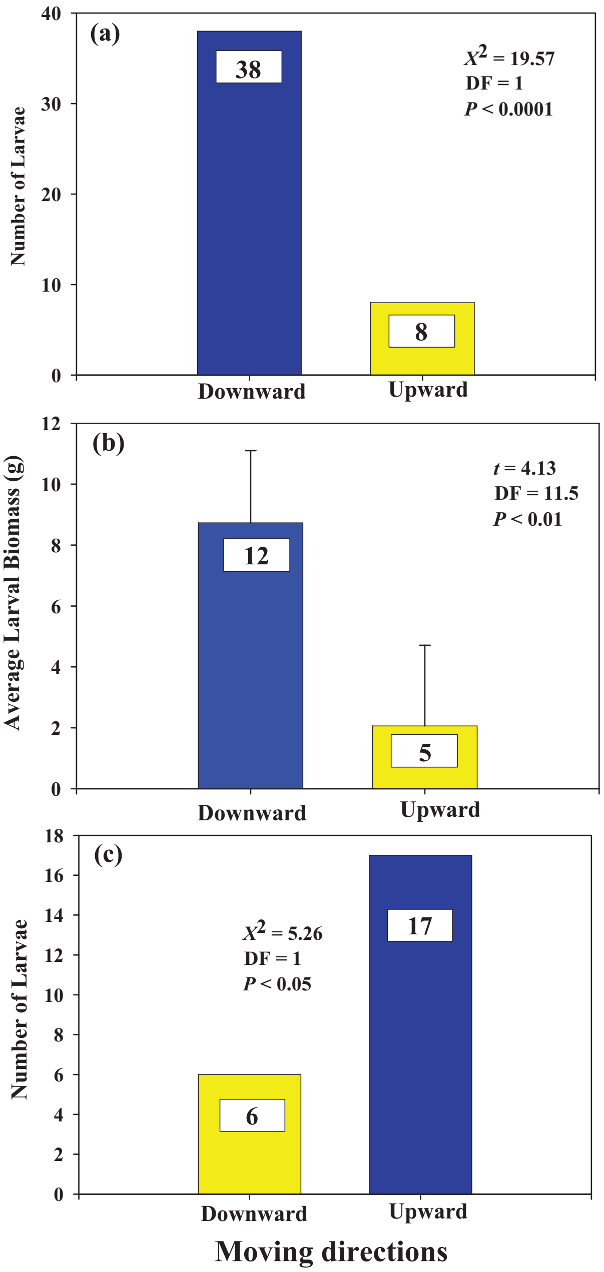

Fig. 2. Feeding direction and biomass of emerald ash borer (EAB) larvae in black ash seedlings grown normally (a) or upside-down (c). Numbers on the bar denote the number of larvae moving in that direction in (a) and (c) or included in the analysis (b).

A total of 23 EAB larvae were observed to feed either upwards (toward the roots, $n = 17$) or downwards (toward the canopy, $n = 6$) when black ash seedlings were potted upside down (Fig. 2c). The difference was statistically significant ($P < 0.05$).

EAB larval feeding in the trunk decreased total polyphenols in the phloem above the feeding site (Fig. 3a). Phloem polyphenols below the feeding site were not affected in the

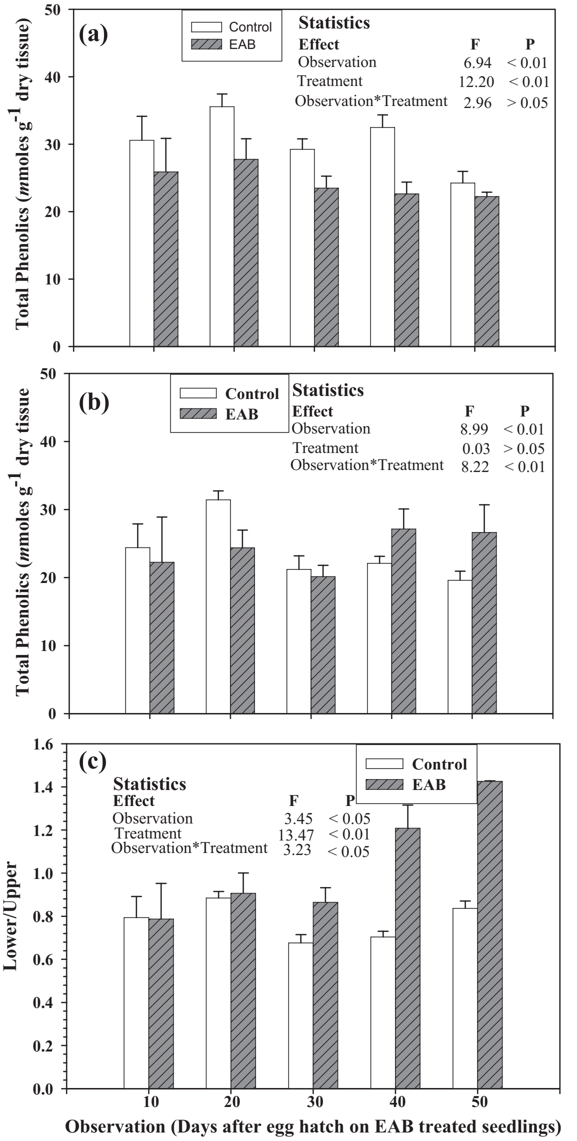

Fig. 3. Phloem polyphenols in response to emerald ash borer (EAB) larval feeding. (a) Above the EAB larval feeding site; (b) below the feeding site; (c) ratio of polyphenols below and above the feeding sites.

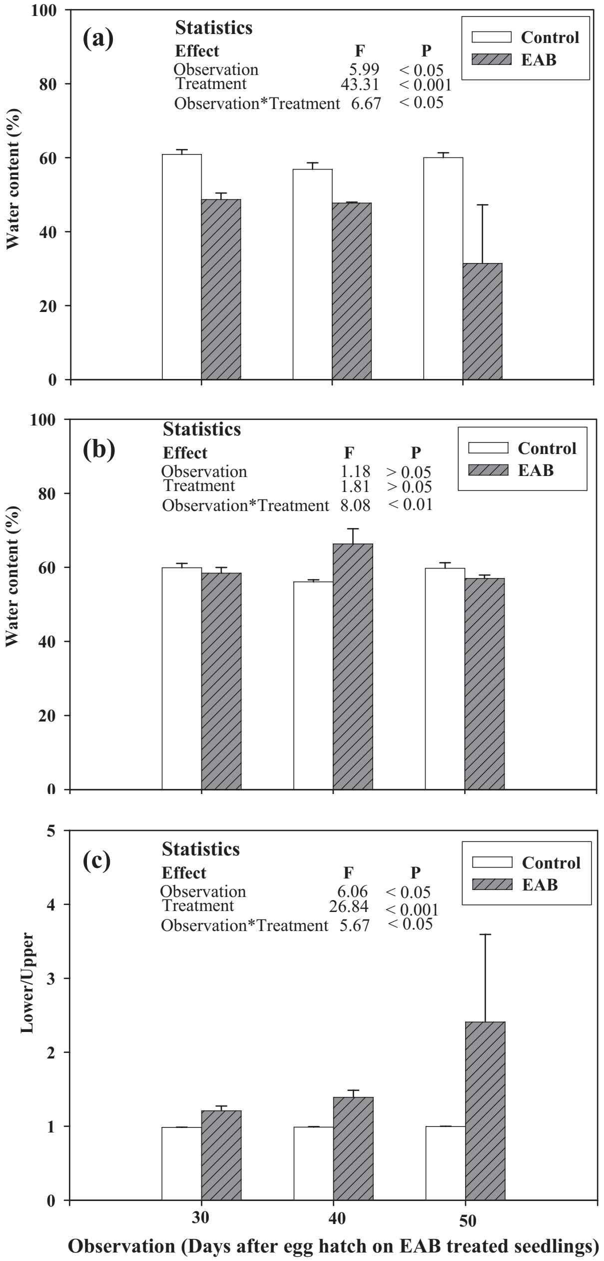

Fig. 4. Phloem water content (mean ± SE) in response to emerald ash borer (EAB) larval feeding. (a) Above the EAB larval feeding site; (b) below the feeding site; (c) ratio of water content below and above the feeding sites. Water content 10 and 20 days after egg hatch was not determined.

early stages of EAB larval feeding, while they were increased as feeding continued (Fig. 3b). As a result, phloem tissues below the feeding site contained greater total polyphenols than tissues above the feeding site in the later stages of larval feeding (Fig. 3c).

EAB larval feeding in the trunk decreased phloem water content above the feeding site (Fig. 4a). Phloem water content below feeding site was more variable and was increased only at 40 day after feeding (Fig. 4b). However, phloem tissues below the feeding site contained greater water content than tissues above the feeding site (Fig. 4c).

Twenty-three amino acids were consistently detected in the phloem tissue of black ash. They were alanine, α-aminobutyric acid, β-aminoisobutyric acid, asparagine, aspartic acid, glutamic acid, glutamine, glycine, glycine-proline, histidine, hydroxyproline, allo-isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. Glutamine, aspargine, aspartic acid, and alanine were typically the most abundant amino acids (Fig. 5 and Appendix S2), the least abundant included α-aminobutyric acid, β-aminoisobutyric acid, methionine, and histidine (Fig. 6

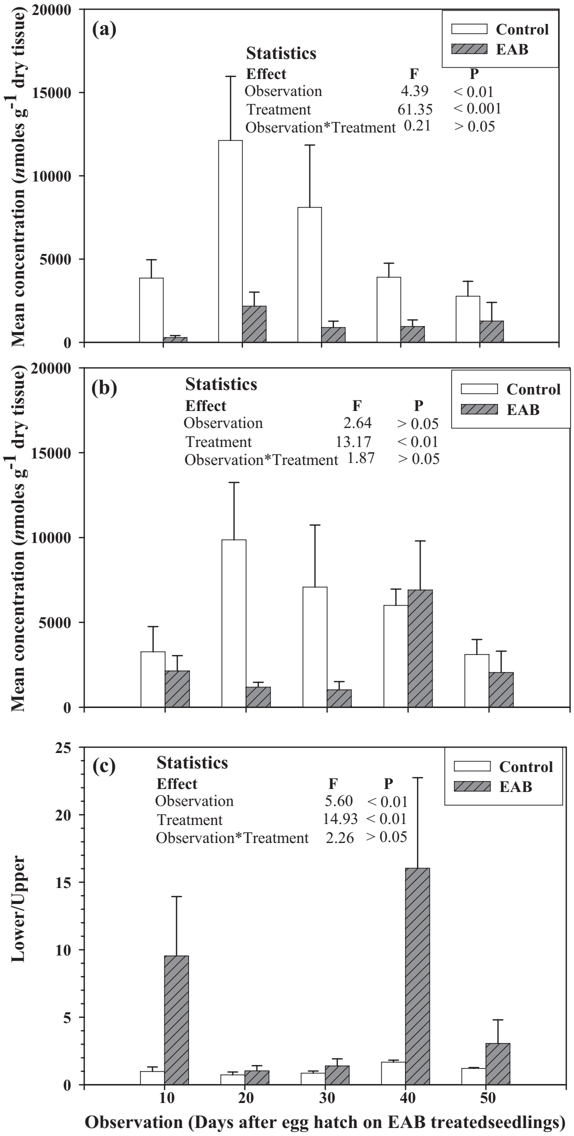

Fig. 5. Phloem glutamine (mean ± SE) in response to emerald ash borer (EAB) larval feeding. (a) Above the EAB larval feeding site; (b) below the feeding site; (c) ratio of glutamine below and above the feeding sites.

and Appendix S2). EAB larval feeding in the trunk decreased the concentration of almost all 23 amino acids above the feeding site (Figs 5a, 6a, and Appendix S2) and decreased most of the amino acids below the feeding site (Figs 5b, 6b, and Appendix S2). Concentrations of 11 phloem amino acids, among which eight are essential amino acids, were higher below the feeding site than above (Figs 5c, 6c, and Appendix S2).

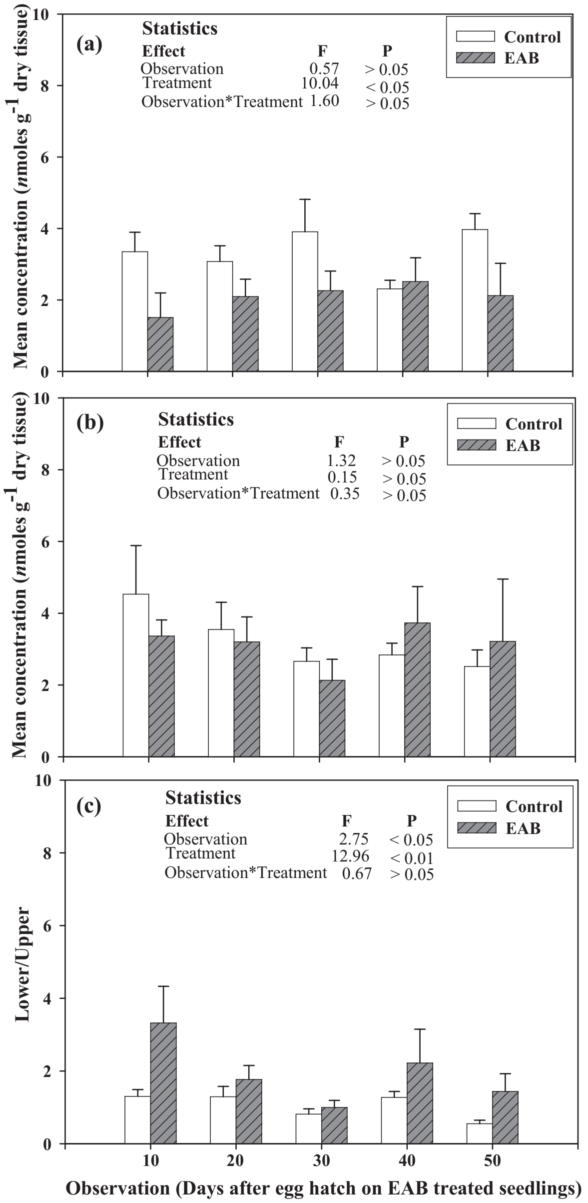

Fig. 6. Phloem methionine (mean ± SE) in response to emerald ash borer (EAB) larval feeding. (a) Above the EAB larval feeding site; (b) below the feeding site; (c) ratio of methionine below and above the feeding sites.

Experiment 3: Test of water content hypothesis

Mortality of EAB larvae reared on artificial diet with various moisture levels are shown in Fig. 7a. Mortality was the lowest when moisture level was 60%, followed by 40%, 80%, and 20% in descending order ($F_{3,32} = 10.03$, $P < 0.0001$). Biomass of surviving EAB larvae was highest when moisture level was 80%, followed by 60%, then 40% and 20% in descending order (Fig. 7b).

Experiment 4: Test of the nutrition hypothesis

Mortality of EAB larvae reared on artificial diet with various nutrition levels are shown in Fig. 8a. Interaction between treatment and observation was not significant ($F_{9,19.9} = 0.95$,

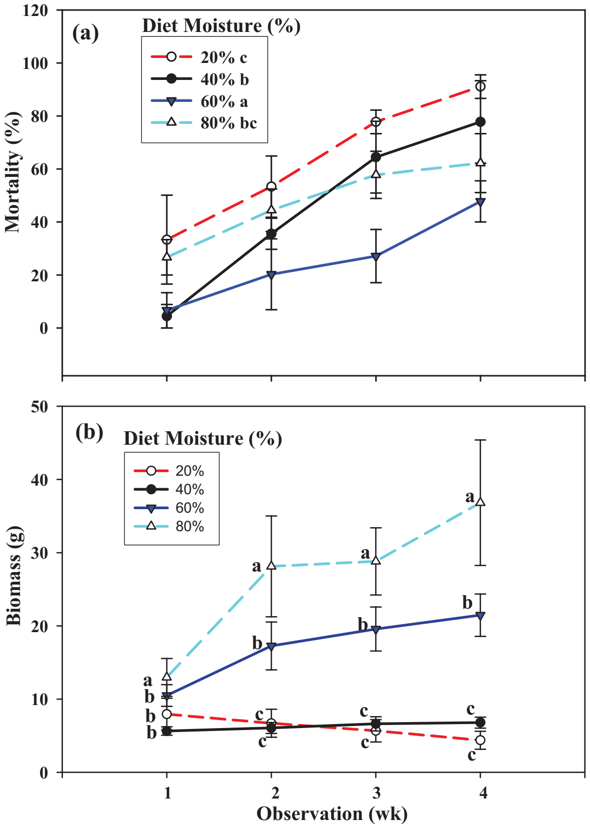

Fig. 7. Mortality (a) and biomass (b) (mean ± SE) of emerald ash borer (EAB) larvae reared on artificial diet with various moisture levels. Moisture levels followed by different lower-case letters in (a) denote significant difference at $\alpha = 0.05$ level (data over all observations were combined because no significant interaction between moisture level and observation was detected). Different lower-case letters next to biomass averages in (b) within the same observation denotes significant difference at $\alpha = 0.05$ level.

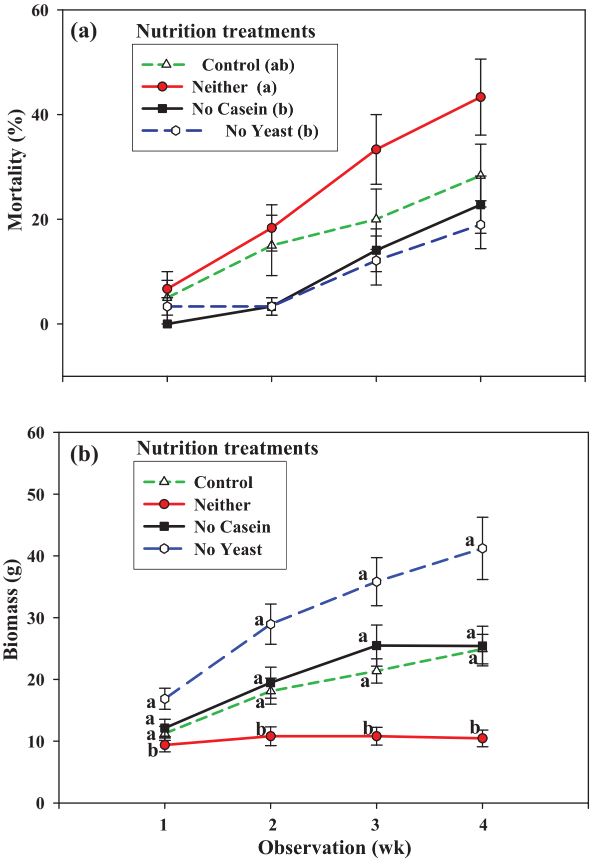

Fig. 8. Mortality (a) and biomass (b) (mean ± SE) of emerald ash borer (EAB) larvae reared on artificial diet with various nutrition levels. Moisture levels followed by different lower-case letters in (a) denote significant difference at $\alpha = 0.05$ level (data over all observations were combined because no significant interaction between nutrition level and observation was detected). Different lower-case letters next to biomass averages in (b) within the same observation denotes significant difference at $\alpha = 0.05$ level.

$P > 0.05$). Mortality was the highest when diet contained neither casein (protein source) nor yeast (amino acid source) ($F_{3,4.28} = 9.86$, $P < 0.05$). Biomass of surviving EAB larvae was shown in Fig. 8b. Significant interaction between treatment and observation was detected ($F_{9,111} = 15.32$, $P < 0.0001$). Biomass was lowest when diet contained neither casein nor yeast ($F_{3,104} = 16.70$, $P < 0.0001$), and differences among the other three treatments were not significant.

Discussion

More EAB larvae fed downwards (toward the root area) than upwards (toward the canopy area) in both field-grown trees (Fig. 1b) and greenhouse-grown black ash seedlings (Fig. 2a). Furthermore, the biomass of EAB larvae that fed downwards was greater than those that fed upward (Fig. 2b). Five broad factors (i.e. gravity, moisture content, nutrition, plant defence, and natural enemy) might be driving this feeding behaviour.

Because most EAB larvae moved upwards (toward the root area) when black ash seedlings were grown upside down (Fig. 2c), gravity appears unimportant in determining the direction in which EAB larvae feed. Phloem tissue above the feeding site in EAB damaged seedlings in this study contained an average water content of 45% compared to phloem tissue below the feeding site (~60%) which was similar to the water content of phloem in control seedlings (Fig. 4). When EAB larvae were reared on artificial diet with manipulated moisture levels, they performed best on diet with a moisture level of 60%; moisture levels both above (80%) and below (20 and 40%) 60% negatively affected EAB larval growth and survival. This clearly indicates the importance of moisture level for survival and development of EAB larvae feeding in the cambial area of a tree.

Amino acids are structural units for proteins, which are involved in virtually every cellular process. EAB larval feeding quantitatively decreased most phloem amino acids both above and below the feeding site (Figs 5, 6, and Appendix S2). However the magnitude of decrease in phloem amino acids below the feeding site was lower and as a result concentrations of 11 amino acids below the feeding site were greater than the same compounds above the feeding site (Figs 5c, 6c, and Appendix S2). Eight of the 11 amino acids are among the 10 essential amino acids (i.e. histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, valine) which cannot be synthesised by most arthropods on their own and must be acquired from plants or with the help of endosymbionts such as bacteria or yeasts (Douglas, 1998). The detrimental effects of essential amino acid deficiency on insect growth are well known (Fraenkel & Printy, 1954; Davis, 1975). EAB larvae are currently not known to have endosymbionts. As a result, the growth of EAB larvae would be expected to be more severely affected if they feed on phloem above than below the feeding site of cambial tissues and they would be expected to feed downwards where these amino acids are more abundant. As expected no biomass gain in EAB larvae feeding on diet with neither protein (casein) nor amino acids (yeast) was observed while positive biomass gain in their counterparts reared in complete diet or diet deficient in protein only or yeast only was detected (Fig. 8b). EAB larvae appeared to perform equally well in all later three nutrition treatments, indicating that EAB larvae can utilise both protein and amino acids.

Polyphenols are widely observed in the plant kingdom (Harborne, 1994) and are feeding deterrents to many herbivorous insects (Lane *et al*., 1985; Harborne, 1994). Polyphenols are the only known group of plant defensive compounds in ash species (Kostova & Iossifova, 2007). Ash tree phloem contains a wide variety of polyphenols and hydroxycoumarins which might confer resistance to Manchurian ash (*Fraxinus mandshurica* Rupr.) against EAB infestation (Eyles *et al*., 2007). Nevertheless, the directional feeding of EAB toward the roots is unlikely due to changes of polyphenols because: (i) black ash is equally susceptible to white ash (*Fraxinus americana* L.) and green ash (*F. pennsylvanica*) (Cappaert *et al*., 2005), which do not contain hydroxycoumarins (Eyles *et al*., 2007); (ii) black ash phloem below the EAB feeding site contained higher polyphenols compared to phloem above the feeding site, although polyphenols in the phloem of both positions were decreased by larval feeding (Fig. 3); (iii) the majority of polyphenols in ash phloem was directly secreted by EAB larvae through faecal frass (Chen & Poland, 2010).

Woodpeckers (Cappaert *et al*., 2005; Lindell *et al*., 2008) and the larval parasitoids, *Tetrastichus planipennisi* Yang (Hymenoptera: Eulophidae) and *Spathius agrili* Yang (Hymenoptera: Braconidae), introduced from China (Liu *et al*., 2007), are the main natural enemies of EAB larvae. Woodpeckers typically use visual and vibrational cues (e.g. holes in the trees), or systematic foraging behaviours to locate hidden prey in the tree (Lima, 1984; Flemming *et al*., 1999; Newell *et al*., 2009). Parasitoids of concealed hosts such as EAB larvae usually use vibratory cues to locate hosts (Lawrence, 1981; Meyhöfer *et al*., 1997; Fischer *et al*., 2001). *Tetrastichus planipennisi* uses vibratory sounds produced by EAB larval chewing to distinguish host suitability (Ulyshen *et al*., 2011). Their impacts on larval feeding behaviour were not specifically tested in this study, and further research is needed to determine how they may interact with other selective forces.

In summary most EAB larvae were observed to feed toward the root area in the field and when black ash seedlings were potted normally in the greenhouse. Of the tested four broad factors (i.e. gravity, moisture content, nutrition, and plant defence) that might be driving this directional feeding, moisture content and nutrition were proved to be two selective forces for this observed behaviour. EAB was speculated to have been introduced into North America in the 1990s (Poland & McCullough, 2006) and its association North America hosts is relatively short. However, insects in general are well known to possess various apparatuses to assess their environment (Bernays, 1998), natural selection will favour EAB larvae that can detect water content and nutrition changes caused by their feeding. Corresponding to pattern of changes of phloem chemistry and water content caused by EAB larval deeding, to maximise growth or performance of their progeny, selection will probably shape oviposition behaviour of EAB adult females in two ways (not mutually exclusive from each other): first, more eggs will be laid in the lower sections of a tree; and second, eggs will be progressively laid downwards within a tree. The first prediction was confirmed (Fig. 1a and Timms *et al*., 2006) and the second one was indirectly supported by the evidence that early EAB eggs were observed to be laid on the bark in the tree canopy and later eggs were subsequently laid below previous ones (Cappaert *et al*., 2005).

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Supporting Information

Additional Supporting Information may be found in the online version of this article under the DOI reference: 10.1111/j.1365-2311.2011.01278.x

Appendix S1. A table showing the ingredients of emerald ash borer artificial diet used in the laboratory study and the steps to make the diet.

Appendix S2. Figures showing the effects of emerald ash borer larval feeding on 19 amino acids.

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