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Interactive Influence of Leaf Age, Light Intensity, and Girdling on Green Ash Foliar Chemistry and Emerald Ash Borer Development

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Abstract Biotic and abiotic environmental factors affect plant nutritional quality and defensive compounds that confer plant resistance to herbivory. Influence of leaf age, light availability, and girdling on foliar nutrition and defense of green ash (*Fraxinus pennsylvanica* Marsh) was examined in this study. Longevity of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), adults reared on green ash foliage subjected to these factors was assayed. Mature leaves generally were more nutritious with greater amino acids and a greater ratio of protein to non-structural carbohydrate (P:C) than young leaves, in particular when trees were grown in shade. On the other hand, mature leaves had lower amounts of trypsin and chymotrypsin inhibitors, and total phenolics compared to young leaves. Lower defense of mature leaves alone, or along with higher nutritional quality may lead to increased survival and longevity of emerald ash borer feeding on mature leaves. Sunlight reduced amino acids and P:C ratio, irrespective of leaf age and girdling, and elevated total protein of young foliage, but not protein of mature leaves. Sunlight also dramatically increased all investigated defensive compounds of young, but not mature leaves. Girdling reduced green ash foliar nutrition, especially, of young leaves grown in shade and of mature leaves grown in sun. However emerald ash borer performance did not differ when fed leaves from trees grown in sun or shade, or from girdled or control trees. One explanation is that emerald ash borer reared on lower nutritional quality food may compensate for nutrient deficiency by increasing its consumption rate. The strong interactions among leaf age, light intensity, and girdling on nutrition and defense highlight the need for caution when interpreting data without considering possible interactions.

Keywords *Fraxinus pennsylvanica* · *Agrilus planipennis* · Abiotic factors · Foliar nutrition · Plant defense

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Introduction

Low nutritional quality and occurrence of plant secondary metabolites in many cases contribute to plant resistance to herbivory. For example, herbivores that feed upon plants with higher nutritional quality generally grow faster and perform better (i.e., have greater reproductive potential and longevity) than those reared on lower quality plants (Mattson 1980; Chen et al. 2008a. In addition, many plant allelochemicals adversely affect herbivore colonization and life histories (Ryan 1990).

Levels of nutrients and defensive compounds change in response to biotic and abiotic factors. One factor is age of leaves. Young and expanding tissues generally are believed to contain higher concentrations of nutrients because these tissues require more resources to support their rapid growth (Mattson 1980; Harper 1989). Regarding defenses, it is widely accepted that plant parts with higher fitness values should be better defended (Optimal Defense theory; McKey 1979). The greater fitness value of young expanding leaves over older leaves has been demonstrated experimentally in some plants (McKey 1979; Strauss et al. 2004), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Chen et al. 2008b).

Light is another factor that may affect plant nutritional and defensive compounds. Reduced light intensity results in low absorbance of solar energy in the light-dependent stage of plant photosynthesis. The resultant low storage of chemical energy may impair capture of CO_2 from the atmosphere and its conversion to photosynthates in the light-independent stage, and subsequent allocation of photosynthates to growth and defense. Plant response to a shaded environment is similar to 'carbon stress' described by the carbon-nutrient balance (CNB) hypothesis (Bryant et al. 1983) an idea that has increasingly lost credibility in recent years (Hamilton et al. 2001; Cipollini et al. 2002; Koricheva 2002; Nitao et al. 2002). According to this hypothesis, trees grown under shaded environments would be more carbon-limited and have lower amounts of carbon-based defensive compounds, while amounts of nitrogen-based defensive compounds would be higher.

Girdling, removal of a ring of bark and phloem around the outer circumference, is a practice used worldwide in agriculture, horticulture, and forestry, with various purposes (Noel 1970; Lahav et al. 1986; Mostafa and Saleh 2006). Girdling interrupts the transport of photosynthates to the roots and has a variety of physiological and biochemical effects on plants such as accumulation of carbohydrates above the girdle and a decline of carbohydrates below the girdle (Noel 1970; Roper and Williams 1989; Li et al. 2003; Mostafa and Saleh 2006). An accumulation of amino acids above the girdle has been reported in *Salix fragilis* twigs (Mittler 1958). Plants are also known to induce defensive chemicals following mechanical wounding and insect herbivory (Stout et al. 1998; Reymond et al. 2000; Lawrence and Koundal 2002), although the effects of girdling on the induction of defensive compounds are little known. Therefore, girdling might alter plant resistance to herbivory due to changes in nutritional qualities and defense.

In this study, we examined the effects of leaf age, light intensity, and girdling on green ash (*Fraxinus pennsylvanica* Marsh) resistance to emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), from a nutritional and defensive chemistry perspective. EAB is an exotic species first detected around Detroit Michigan, USA and Windsor Ontario, Canada in 2002, and is threatening the ash resource in North American (Haack et al. 2002; Poland and McCullough 2006). EAB attacks ash trees (*Fraxinus* spp.) of all species and sizes from large mature trees to trees that are 2 cm in diameter (Cappaert et al. 2005). Adults feed on foliage, and females lay eggs in bark cracks and crevices. The larvae tunnel in the cambial region disrupting the flow of nutrients and girdling the tree. Progress has been made on EAB host range (Anulewicz et al. 2007, 2008), biology (Bauer et al. 2004; Cappaert et al. 2005), behaviors (Rodriguez-Saona et al. 2006; Lelito et al. 2007), and chemical ecology (Rodriguez-Saona et al. 2006; Eyles et al. 2007; Crook et al. 2008; de Groot et al. 2008). EAB adults have been observed to prefer trees grown in open areas over those in the shady ones (Poland et al. 2005). Girdled trees are more attractive to EAB than non-girdled trees (Poland et al. 2005; McCullough et al. 2006, 2009). However, little is known to date about mechanisms that underlie these EAB behaviors and ash tree resistance to EAB, except that certain phloem phenolics might account for the relatively higher resistance of Manchurian ash (*F. mandshurica*) to EAB (Eyles et al. 2007). Elucidation of these mechanisms contributes to ash tree breeding programs to develop resistant varieties or hybrids.

We quantified the nutritional and defense compounds trypsin and chymotrypsin inhibitors, and total phenolics of green ash foliage, and we evaluated adult EAB performance reared on green ash foliage subject to various biotic and abiotic factors. Specifically we tested the following hypotheses: (1) young leaves have higher nutritional qualities and greater defensive compounds than mature leaves; (2) shaded trees have higher foliar nutrients and nitrogen-based defensive compounds (i.e., proteinase inhibitors) but lower carbon-based defensive compounds (phenolics), according to the CNB hypothesis; (3) girdled trees have elevated carbohydrate levels and defensive protease inhibitors and phenolics, but reduced P:C ratio; and 4) adult EAB will have greater longevity on leaves with higher nutritional quality and lower defense compounds.

Materials and Methods

Green Ash Green ash seedlings (2-yr-old and ca. 15–30 cm tall) were used for the ease of manipulation of the treatments. Seedlings were purchased from Lawyer Nursery Inc. (West Plains, MT, USA) in February, 2008, 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 L; Stuewe & Sons, Inc., Corvallis, OR, USA) using BACCTO® High Porosity Professional Planting Mix (Michigan Peat Co., Houston, TX, USA) and soil at a ratio of 3:1 as planting medium. Seedlings were grown during July–August, 2008 (ca. L16:D8 h) in a greenhouse set at approximately 25±2°C and supplemented with 400-Watt high-pressure sodium lamps from 10:00 pm to 6:00 am. The greenhouse is located on campus of Michigan State University, East Lansing, MI, USA. Osmocote 14-14-14 Classic fertilizer (Scotts Company LLC., Marysville, OH, USA) was mixed with the planting medium at ca. 4 kg m^{-3} soil. Seedlings were watered as need.

Approximately 5 weeks after potting when seedlings had 4–6 fully expanded compound leaves and 3–6 expanding leaves, seedlings were subjected to light intensity (2 levels:

Shade and Sunny) and girdling (2 levels: Girdled and un-girdled) treatments. Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes (Charlotte, NC, USA) covered with 80% TapeKnit shade cloth (American Nettings & Fabric Inc., Ferndale, WA, USA). The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%. The sunny treatment did not have a shade cloth cage. Two light intensity levels were assigned randomly to four whole plots arranged in a straight line. Twenty seedlings of similar size were randomly assigned to each of the four whole plots. Half (10) of the seedlings assigned to each whole-plot were randomly selected and girdled (i.e., a 2.5 cm band of phloem removed on the stem at a height of 15 cm from the soil), and the other half were left un-girdled. At the end of the experiments (2 weeks following these treatments), one young (Young: expanding leaves less than 3-weeks-old) or one mature (Mature: fully expanded leaves that were 4- to 6-weeks-old) compound leaf was randomly collected from each plant. Therefore, the experimental design was a completely randomized split-plot design with light intensity as whole plots. The split plots were girdling and leaf age arranged as a 2×2 factorial.

Insects EAB infested ash trees were felled at the end of 2007 and early 2008 and cut into 90-cm long logs. Logs were held in cold storage at 4°C. As beetles were required for experiments, they were allowed to emerge in rearing tubes at 25°C. Upon emergence they were separated by sex and used for bioassay.

Effects on F. pennsylvanica Growth and Leaf Chlorophyll Contents To examine the effects of shade and girdling on green ash growth, heights (from soil to meristem) of green ash seedlings were determined at the onset of treatments and again at the end (2 weeks following treatments). Because leaf chlorophylls are the most important photosynthetic pigments of higher plants and are positively correlated to photosynthetic potential (Filella et al. 1995; Markwell et al. 1995; Richardson et al. 2002), leaf chlorophyll content was measured by using a Minolta SPAD-502 (Konica Minolta Sensing, Inc., Japan) at the end of experiments. Chlorophyll content (μmol m^{-2}) was calculated according to a standard curve for corn (*Zea mays*) plants, chlorophyll (μmol m^{-2})=10 $^{(M^{\wedge}\ 0.261)}$, where M is the chlorophyll meter reading (Markwell et al. 1995). The experiment was a split-plot design as described above.

Effects on F. pennsylvanica Nutritional Chemistry Seedlings were grown as described above. Approximately 2 weeks after treatments, one mature (3–4 weeks after full expansion) or one young (within 2 weeks following sprouting; ca. 20–50% size of mature leaflets) compound leaf from each tree was cut and flash frozen in liquid nitrogen. Samples were brought to the laboratory in a cooler containing dry ice, and 2–4 leaflets of the collected compound leaf were ground to fine powder with mortar and pestle in liquid nitrogen. The ground plant tissue was stored at −20°C until bioassayed.

Approximately 40–80 mg of ground plant tissue were used for extraction. Extraction of total soluble protein, amino acids, and soluble non-structural carbohydrate (glucose, fructose, and sucrose) followed Bi et al. (2003) and Chen et al. (2009). Extraction of non-soluble non-structural carbohydrate (starch) followed Marquis et al. (1997) and Chen et al (2009). Total protein content was determined following the Bradford protein assay, and calculated with a standard curve established by using bovine serum albumin (Ni et al. 2001). Amino acid content was colorimetrically determined using the cadmium (Cd)-ninhydrin procedure (Doi et al. 1981). The amino acid content was calculated from a standard curve generated using glycine as a standard. Glucose, fructose, and sucrose contents were determined by using 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. All chemicals used in this experiment and the following were purchased from Sigma-Aldrich (St. Louis, MO, USA), or Fisher Scientific (Pittsburgh, PA, USA).

Effects on F. pennsylvanica Protease Inhibitors and Total Phenols Leaf samples were collected and prepared as in the preceding experiment. Trypsin and chymotrypsin inhibitors extraction followed Stout et al. (1998). Ground and weighed plant tissue was mixed with a Tris-HCl buffer (pH 7.8) containing 7% polyvinylpolypyrrolidone, 1.67 mM phenylthiourea, 0.3 mM KCl, and 0.4 mM ascorbic acid. The mixture was centrifuged at 10,000×g for 10 min. The supernatant was used for assay.

The trypsin inhibitor assay followed Walsh and Wilcox (1970), and Broadway (1993). Bovine trypsin (0.2 mg ml^{-1} in 1 mM HCl) was mixed with an equal volume of plant extract and incubated at room temperature for 10 min. Eighty microliters of the mixture was added to 3 ml substrate solution [1.04 mM *Nα-p*-tosyl-L-arginine methyl ester (TAME) in 0.05 M Tris-HCl buffer]. The activity of trypsin that remained after reaction with trypsin inhibitors in the sample was monitored at 247 nm for 3 min with a Shimadzu UV Mini-1240 spectrophotometer at 25°C and was converted to amount of trypsin from a standard curve using Bovine trypsin within the linear range (0~0.1 mg ml^{-1}). The amount of trypsin inhibitors was expressed as amount of inhibited trypsins, and was calculated by subtracting the trypsins remaining after the

reaction from the amount of trypsin (8 μg) used in the beginning of assay.

The chymotrypsin inhibitor assay followed Walsh and Wilcox (1970), and Broadway (1993). *Nα*-Tosyl-Lys-chloromethylketone (TLCK)-treated bovine chymotrypsin (0.2 mg ml^{-1} in 1 mM HCl) was mixed with an equal volume of plant extract and incubated at room temperature for 10 min. Eighty microliters of the mixture were added to 3 ml substrate solution containing 0.5 mM *N*-benzoyl-L-tyrosine ethyl ester (BTEE), 5% methanol, and 0.05 M Tris-HCl buffer. The activity of chymotrypsin that remained after the reaction with chymotrypsin inhibitors was monitored at 256 nm for 3 min with a Shimadzu UV Mini-1240 spectrophotometer at 25°C and was converted to amount of chymotrypsins from a standard curve using Bovine chymotrypsin within the linear range (0~0.1 mg ml^{-1}). The amount of chymotrypsin inhibitors was expressed as amount of inhibited chymotrypsins, and was calculated by subtracting chymotrypsins remaining after the reaction from the amount of chymotrypsin (8 μg) used in the beginning of assay.

Total phenol extraction followed Hagerman (1988) and Cork and Krockenberger (1991). Determination of total phenol followed Graham (1992). Briefly, ground and weighed plant tissue was extracted ×3, each time with 1 ml 70% acetone at 4°C in the dark for 30 min. The supernatant after each centrifugation was pooled and its concentration [m*mol* g^{-1} fresh weight (FW)] determined using a modified Prussian blue assay. An external standard curve with gallic acid within the linear range was constructed to calculate sample concentration.

EAB Performance in Response to Green Ash Foliage To evaluate effects of leaf age, shade, and girdling on green ash foliar nutritional and defensive chemistry, adult EAB longevity reared on foliage subject to these treatments was assayed. One newly emerged adult EAB male and one female were placed in a sealed 295-ml plastic drinking cup where they were fed excised compound leaves from different treatments (treatments were as described above for the previous experiment). A 5×7 cm area on the side of the cup was cut away and sealed with 1×1 mesh for ventilation. The petioles of excised leaves were placed into 20-ml glass vials filled with water before being placed into cups. Leaves were exchanged with fresh leaves of the same treatment every 2–3 days. Survival of EAB was monitored daily until all EAB were dead. Each treatment was replicated ten times (cups). Cups were kept in chambers set at 25±2°C and 70% relative humidity with a photoperiod of L14:D10h.

Statistical Analyses All experiments were completely randomized split-plot designs with light intensity as whole plots, and girdling and leaf age (arranged as a 2×2 factorial) as split plots. Leaf cholorphyll, foliar total proteins, amino acids, TNC, protein:TNC ratio, trypsin inhibitors, chymotrypsin inhibitors, total phenolics, and EAB longevity were analyzed by PROC MIXED in SAS (SAS Institute 1999) with the interaction of light intensity and its replication as a random effect. Protein:TNC ratio data were log transformed before being subject to analysis. Survival of EAB adults was analyzed with repeated measure ANOVA (PROC GLM in SAS) with date as the repeated measure. Comparison was separately conducted between Young and Mature leaf treatments, between Sunny and Shady treatments, and between Control and Girdled treatments. In all analyses, the type I error α was controlled at 0.05.

Results

Effects on Green Ash Growth and Leaf Chlorophyll Content Green ash seedling height was not affected by light intensity or girdling (data not shown). Effects of leaf age, light condition, and wound status on leaf chlorophylls are shown in Table 1. Mature leaves had higher leaf chlorophyll than young leaves. For mature leaves, exposure to sunny conditions decreased leaf chlorophyll levels, while girdling had no effect. For young leaves, sunny conditions decreased leaf chlorophyll levels of girdled trees, but had no effect on chlorophylls of control trees; girdling decreased chlorophylls of trees under sunny conditions, while it did not affect chlorophylls of trees under shady conditions.

Effects on Green Ash Foliage Nutritional Chemistry Total proteins, amino acids, TNC, and P:C in relation to leaf age, light, and girdling treatments are shown in Table 2. Leaf age and girdling did not affect the amount of proteins. Exposure to sunny conditions increased proteins of young but not mature leaves. Mature leaves had significantly greater amino acids than young leaves, regardless of light and girdling treatments. Shading increased amino acid quantity, while girdling did not affect amino acids.

Leaves from trees grown under sunny conditions had higher TNC levels, irrespective of leaf age and girdling. Girdling increased TNC levels when trees were grown under sunny conditions, but not under shady conditions. Mature leaves had greater TNC than young leaves when trees were under sunny conditions. In contrast, young leaves had greater TNC content under shady conditions.

Girdling generally decreased P:C. The reduction in P:C was especially obvious in young leaves grown under shady conditions and in mature leaves grown under sunny conditions. Under sunny conditions, P:C was almost

Table 1 Leaf age, shade, and girdling of *Fraxinus pennsylvanica* on leaf chlorophyll content (mean±SEM μmol m^{-2})

Light	Control		Girdled[a]	
	Young[b]	Mature[c]	Young	Mature
Shady[d]	287.6±23.2	518.6±15.6	294.3±14.1	524.7±26.6
Sunny	323.3±28.7	499.5±17.0	221.9±14.4	428.6±24.8
Statistics				
Source	DF	F	P	
Light	1	6.37	< 0.05	
Wound	1	7.03	< 0.01	
Leaf age	1	196.71	< 0.01	
Light*Wound	1	9.47	< 0.01	
Light*Leaf age	1	1.70	> 0.05	
Wound*Leaf age	1	0.25	> 0.05	
Light*Wound*Leaf age	1	0.27	> 0.05	

[a] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%.

doubled in young leaves from girdled trees compared to mature leaves from girdled trees. P:C of control trees under sunny conditions was not affected by leaf age. On the contrary, under shady conditions, mature leaves had greater P:C compared to young leaves, regardless whether trees were girdled or not. Shading elevated P:C by more than double compared to leaves from trees under sunny conditions, irrespective of leaf age and girdling.

Table 2 Foliar proteins, amino acids, and total non-structural carbohydrate (TNC) content (mean±SEM) of *Fraxinus pennsylvanica* seedlings under different treatments

Light			Proteins(mg g^{-1} FW[a])		Amino acids (μmol g^{-1} FW)		TNC (mg g^{-1} FW)		Protein:TNC ratio (w/w)	
	Wounding		Young[b]	Mature[c]	Young	Mature	Young	Mature	Young	Mature
Shady[d]	Control		13.7±1.4	14.8±0.7	6.6±1.0	16.9±1.6	11.9±1.2	8.2±0.7	1.3±0.2	1.9±0.2
	Girdled[e]		9.7±0.7	14.0±1.1	4.8±1.1	18.5±1.1	14.7±1.4	9.2±0.8	0.7±0.1	1.6±0.1
Sunny	Control		14.5±1.5	14.9±1.3	0.8±1.3	8.1±1.6	24.9±1.8	21.0±2.4	0.6±0.1	0.8±0.1
	Girdled		15.5±1.8	13.8±0.6	0.3±0.7	7.2±1.4	32.0±3.1	51.2±4.3	0.5±0.1	0.3±0.0
Statistics										
Source		*DF*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Light		1	3.63	> 0.05	33.49	< 0.01	39.15	< 0.01	25.80	< 0.01
Wound		1	2.05	> 0.05	0.21	> 0.05	41.13	< 0.01	33.50	< 0.01
Leaf age		1	1.50	> 0.05	128.98	< 0.01	0.95	> 0.05	8.55	< 0.01
Light*Wound		1	1.92	> 0.05	0.13	> 0.05	27.37	< 0.01	1.54	> 0.05
Light*Leaf age		1	4.03	< 0.05	8.61	< 0.01	14.63	< 0.01	25.08	< 0.01
Wound*Leaf age		1	0.11	> 0.05	0.90	> 0.05	11.20	< 0.01	2.18	> 0.05
Light*Wound*Leaf age		1	2.53	> 0.05	1.2	> 0.05	15.17	< 0.01	12.89	< 0.01

[a] fresh weight

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%

[e] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil.

Effects on Green Ash Foliage Defensive Compounds Trypsin and chymotrypsin inhibitors, and total phenolics of *F. pennsylvanica* foliage in response to light, girdling, and leaf age are shown in Table 3. The level of trypsin inhibitors was more than 30% greater in young leaves than in mature leaves. Light exposure and girdling had no significant influence on levels of trypsin inhibitors. The interaction between light and leaf age on chymotrypsin inhibitors was significant. Under sunny conditions, young leaves had more than three times higher chymotrypsin inhibitor levels, compared to mature leaves. However, under shady conditions, the difference in chymotrypsin inhibitors was not significant. Growth under sunny conditions more than doubled chymotrypsin inhibitor levels of young leaves, but had no significant effect in mature leaves.

The amount of total phenols in foliage from the sunny treatment was more than twice as high as in foliage from the shade treatment. Light exposure interacted with leaf age: under sunny conditions, young leaves had more than twice the level of total phenols compared to mature leaves; however, under shady conditions, mature leaves had greater total phenols than young leaves. Girdling decreased total phenolics of young leaves under shady conditions, while it elevated total phenolics of mature leaves under sunny conditions.

EAB Performance in Response to Green Ash Foliage Subject to Age, Shade, and Girdling Survival of EAB adults feeding on green ash foliage of various treatments over time is shown in Fig. 1A–C. Survival of EAB grown on mature leaves was greater than that on young leaves (Fig. 1A). There was no significant difference in survival of EAB feeding on foliage grown under sunny compared to shady conditions (Fig. 1B), or foliage from girdled vs. control trees (Fig. 1C). Similarly, only leaf age significantly influenced EAB adult longevity, which was greater on older leaves (Fig. 1D).

Discussion

Effects of Leaf Age on Plant Nutritional Quality and Defense Young and growing tissues generally are believed to contain higher quantities of nutrients (Mattson 1980; Harper 1989; White 1993), because these tissues require more resources to support their rapid growth. Total soluble protein accounted for less than 1.6% of the fresh weight of leaf tissues in this study, which might indicate the scarcity of nitrogen sources for arthropods feeding on green ash foliage. The amounts of total soluble protein did not differ between young and mature leaves (Table 2). However, mature leaves had greater levels of amino acids than young leaves, which is contrary to what is typically believed. Amino acid levels in plants and animals are in many cases

Table 3 Trypsin and chymotrypsin (mean±SEM) of *Fraxinus pennsylvanica* seedlings foliage under different treatments

Light		Trypsin inhibitors (mg g^{-1} FW[a])		Chymotrypsin inhibitors (mg g^{-1} FW)		Total phenolics (mmol g^{-1} FW)	
	Wounding	Young[b]	Mature[c]	Young	Mature	Young	Mature
Shady[d]	Control	15.5±1.7	14.5±2.8	2.3±0.4	2.7±0.3	17.4±2.7	21.2±1.5
	Girdled[e]	20.7±2.5	16.3±3.5	2.2±0.6	2.7±0.7	10.0±1.2	20.7±1.1
Sunny	Control	17.8±2.1	10.8±3.5	4.9±1.4	1.6±0.5	52.8±4.8	22.2±1.6
	Girdled	21.9±2.6	12.1±3.3	5.5±1.1	1.7±2.0	57.9±5.5	27.4±1.2
Source	*DF*	*F*	*P*	*F*	*P*	*F*	*P*
Light	1	0.05	> 0.05	1.56	> 0.05	0.00	> 0.05
Wound	1	2.87	> 0.05	0.04	> 0.05	2.39	> 0.05
Leaf age	1	9.46	< 0.01	4.77	< 0.05	11.89	< 0.01
Light*Wound	1	0.06	> 0.05	0.06	> 0.05	0.01	> 0.05
Light*Leaf age	1	2.45	> 0.05	7.57	< 0.01	5.45	< 0.05
Wound*Leaf age	1	0.74	> 0.05	0.02	> 0.05	0.64	> 0.05
Light*Wound*Leaf age	1	0.01	> 0.05	0.03	> 0.05	0.00	> 0.05

[a] fresh weight

[b] Young and expanding leaves

[c] Mature leaves: ca. 3–4 weeks after full expansion, and not senescent

[d] Shade treatment was a 1.2×0.7×1.1 m (L×W×H) cage built with 1.9 cm (inner diam) PVC pipes covered with TapeKnit shade cloth. The shade cloth reduced the amount of sunlight available to the trees by approximately 70–80%

[e] A 2.5 cm band of phloem removed from stem at a height of 15 cm from the soil.

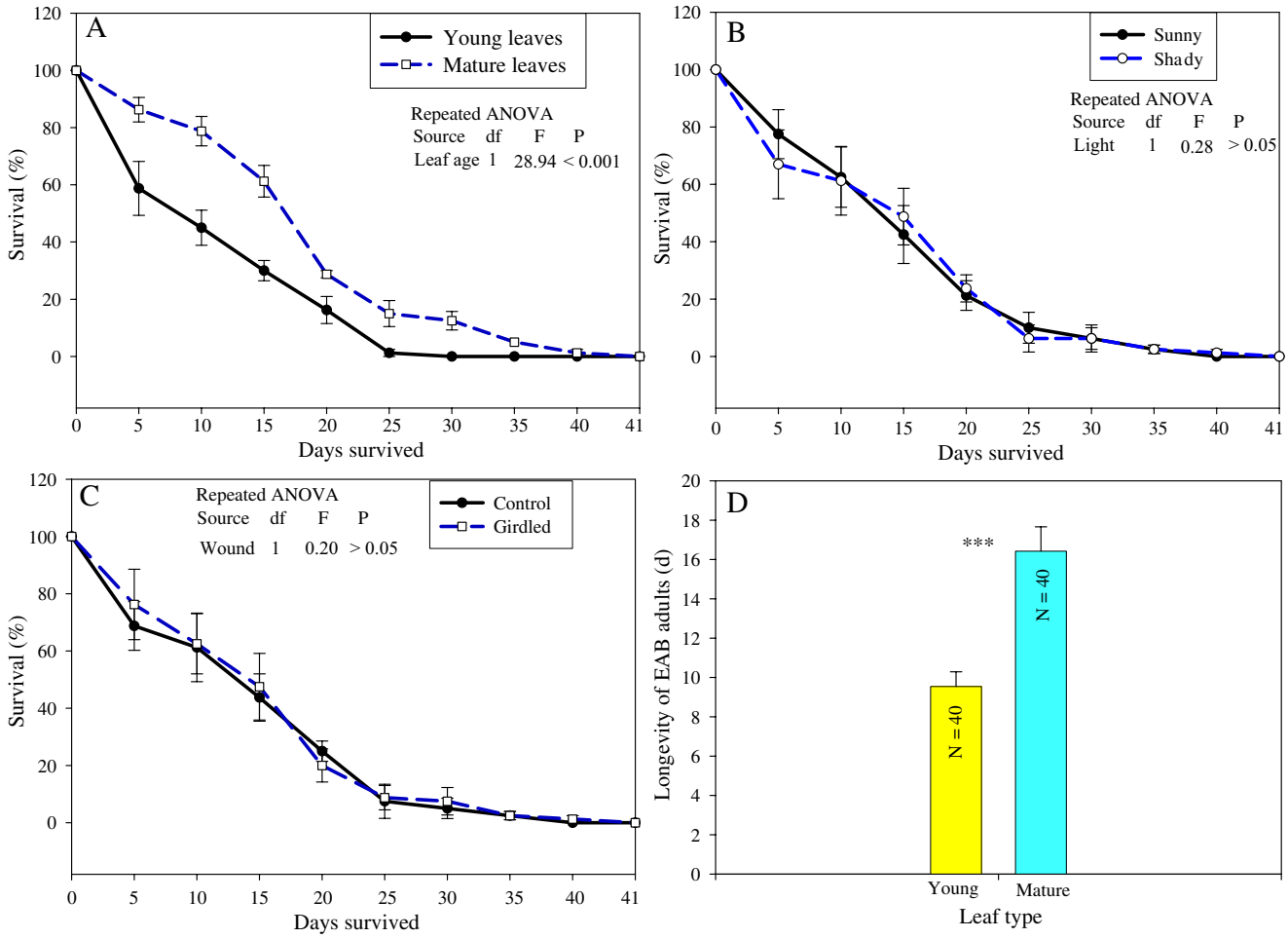

Fig. 1 Survival (means±SEM %) of Emerald Ash Borer (EAB) adults over time (**A**–**C**) and longevity (**D**). Newly emerged EAB were used for bioassay. Survival data were analyzed with repeated (time) ANOVA. Comparison was separately conducted between Young and Mature leave treatments, between Sunny and Shady treatments, and between Control and Girdled treatments. Young leaves: young and expanding leaves; Mature leaves: ca. 3–4 weeks after full expansion, and are not senescent. Standard errors of some data points do not appear because they are either smaller than symbols representing these data points or they are zero. *** $P<0.001$

elevated by stress (Good and Zaplachinski 1994; Bischof 1996; Dadmarz et al. 1998). We were not aware of any stress other than shade and girdling that the experimental seedlings experienced in this study. Therefore, higher amount of total amino acids in mature than young green ash leaves may be intrinsic. Nutrient balance, especially the ratio of protein to digestible carbohydrate (P:C) also has been shown to be important for the development of many insects; several of them chose higher P:C over lower P:C when given choices in the laboratory using artificial diets (Lee et al. 2002; Bede et al. 2007). The effects of leaf age on P:C interacted with the effect of light exposure: under sunny conditions, young leaves had greater P:C than mature leaves, especially in girdled trees, because young leaves had less TNC than mature leaves. On the other hand, under shady conditions mature leaves had higher P:C compared to young leaves, because older leaves had less TNC than young leaves. Interactive effects of leaf age and light condition on tannin concentration have been observed in *Inga oerstediana* seedlings (Nichols-Orians 1991). Thus, light condition may need to be considered when studying the effect of leaf age on nutritional qualities and defensive chemistry.

Young leaves had greater levels of trypsin and chymotrypsin inhibitors, and total phenolics than mature leaves in this study (Table 3). This is *consistent* with Optimal Defense theory, predicting that plant parts with higher fitness value should be better defended (McKey 1979). The greater fitness value of young expanding leaves over older leaves has been experimentally demonstrated in other study systems (McKey 1979; Strauss et al. 2004), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Chen et al. 2008b).

Effects of Light or Shade on Plant Nutritional Quality and Defense Exposure to sunlight elevated total proteins of

young green ash leaves, whereas it did not affect proteins of mature leaves in the study. The increased proteins in young leaves under sunny conditions might be partly attributable to trypsin and chymotrypsin inhibitors because the pattern of protein changes in young leaves under sunny conditions parallels that of trypsin and chymotrypsin inhibitor levels (discussed below). In contrast, exposure to sunlight decreased amino acid levles and P:C ratio, irrespective of leaf age and girdling. The lower P:C of leaves in the sun is due to a greater rate of increase in TNC in these leaves rather than an increase in total proteins. Although sunlight exposure reduced leaf chlorophyll levels (Table 1), it brought about net accumulation of TNC in foliage grown in the sun. On the other hand, although shading increased leaf chlorophyll content in compensation for lower sunlight, it brought about a net decline of TNC. Sunlight sharply elevated amounts of chymotrypsin inhibitors (nitrogen-based defensive chemicals) and total phenolics (carbon-based defensive compounds) of young leaves, but not mature leaves. A similar pattern of light effects on tannin levels of young and mature leaves has been observed in *Inga oerstediana* (Nichole-Orians 1991). The tannin levels of young *I. oerstediana* leaves grown to maturity in 20% of full sunlight was increased compared to young leaves grown to maturity in 2% of full sunlight. However, light intensity did not change tannin content of already mature leaves. The increase in chymotrypsin inhibitors for young leaves in the sun was *inconsistent* with the predictions of the CNB hypothesis that argues that trees grown under sunny environments should have lower nitrogen-based defensive but higher carbon-based defensive compounds (Bryant et al. 1983), while the elevation of total phenolics in young leaves in the sun followed the CNB predictions. The fact that defensive compounds, both carbon- and nitrogen-based, were elevated only in young leaves, highlights the need for caution when interpreting data without considering possible interactions such as leaf age. This also indicates the need to consider as many interactions as possible when developing hypotheses.

Effects of Girdling on Plant Nutritional Quality and Defense Girdling reduced leaf nutritional quality, in particular of young leaves grown in the shade and of mature leaves grown in the sun. The reduction of P:C was mainly due to accumulated TNC. Girdling is known to elevate TNC amounts above the girdled sites in many plant species including white ash, *F. Americana* (Noel 1970; Roper and Williams 1989; Li et al. 2003). The interactive effects of girdling, leaf age, and sunlight intensity on TNC indicated that the effects of girdling on foliage TNC were dependent on leaf age and growing conditions of seedlings. The greater photosynthetic capacity of mature leaves over young leaves grown in the sun (higher leaf chlorophyll levels; Table 1) might explain the accumulation of TNC in mature leaves from girdled trees in the sun. However, the increased accumulation of TNC in young leaves from girdled trees in the shade is contradictory to the higher photosynthetic capacity of mature leaves over young leaves from girdled trees grown in the shade, which is also indicated in Table 1. One explanation is that girdling makes TNC in young leaves grown in the shade more demanding, and TNC synthesized in mature leaves is exported to young leaves. No significant changes in protease inhibitors and total phenolics were observed following girdling. This is surprising since a variety of plant defensive chemicals are known to be induced after mechanical wounding and insect herbivory inflicted on leaves (Stout et al. 1998; Chen et al. 2008b). One possible explanation is that plants may respond differently to foliar damage and phloem injury.

EAB Adult Performance in Response to Green Ash Foliage Subjected to Various Treatments Increased EAB adult longevity was observed only on mature leaves compared to young and expanding leaves. This was probably attributable mainly to weaker defense (lower protease inhibitors and total phenolics) of mature leaves. Greater amino acids and P:C ratio of mature leaves, especially, higher P:C ratio in the shade, might also confer better performance of EAB adults on mature leaves. Reduction of light elevated amino acid and P:C ratio. However, EAB longevity was not affected by light intensity. A similar pattern was observed between girdled and control trees in that girdling decreased the nutritional quality of foliage, but EAB performance was not affected. The failure to detect EAB performance difference between low and high nutritional food might be explained by compensatory feeding of EAB adults that fed upon low quality foliage, since defensive compounds between these treatments were not significantly affected. Compensatory feeding of many herbivores on low quality host plants has been documented (Mattson 1980; Scriber and Slansky 1981).

In summary, leaf age, light exposure, and girdling interactively affected green ash nutritional and defensive compounds. Both nutritional quality and defensive compounds might contribute to EAB adult performance in this study and feeding preferences reported elsewhere (Poland et al. 2005; McCullough et al. 2006). However, the role of TNC in EAB preferences deserves special attention because the accumulation of TNC in foliage from girdled trees and trees grown in the sunny areas parallels EAB adult preference to these trees over corresponding un-girdled trees and trees grown in the shady areas. Additionally, although girdling and sun exposure altered nutritional and defensive chemistry of green ash foliage, EAB adult performance in the no-choice tests was not affected. The underlying mechanisms need further investigation. Eluci-

dation of these mechanisms contributes to ash tree breeding programs to develop resistant varieties or hybrids.

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