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Physiological responses of emerald ash borer larvae to feeding on different ash species reveal putative resistance mechanisms and insect counter-adaptations

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ABSTRACT

Emerald ash borer, *Agrilus planipennis* Fairmaire, an Asian wood-boring beetle, has devastated ash (*Fraxinus* spp.) trees in North American forests and landscapes since its discovery there in 2002. In this study, we collected living larvae from EAB-resistant Manchurian ash (*Fraxinus mandschurica*), and susceptible white (*Fraxinus americana*) and green (*Fraxinus pennsylvanica*) ash hosts, and quantified the activity and production of selected detoxification, digestive, and antioxidant enzymes. We hypothesized that differences in larval physiology could be used to infer resistance mechanisms of ash. We found no differences in cytochrome P450, glutathione-S-transferase, carboxylesterase, sulfotransferase, and tryptic BApNAase activities between larvae feeding on different hosts. Despite this, Manchurian ash-fed larvae produced a single isozyme of low electrophoretic mobility that was not produced in white or green ash-fed larvae. Additionally, larvae feeding on white and green ash produced two serine protease isozymes of high electrophoretic mobility that were not observed in Manchurian ash-fed larvae. We also found lower activity of β-glucosidase and higher activities of monoamine oxidase, *ortho*-quinone reductase, catalase, superoxide dismutase, and glutathione reductase in Manchurian ash-fed larvae compared to larvae that had fed on susceptible ash. A single isozyme was detected for both catalase and superoxide dismutase in all larval groups. The activities of the quinone-protective and antioxidant enzymes are consistent with the resistance phenotype of the host species, with the highest activities measured in larvae feeding on resistant Manchurian ash. We conclude that larvae feeding on Manchurian ash could be under quinone and oxidative stress, suggesting these may be potential mechanisms of resistance of Manchurian ash to EAB larvae, and that quinone-protective and antioxidant enzymes are important counter-adaptations of larvae for dealing with these resistance mechanisms.

1. Introduction

Emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is an invasive wood-boring insect introduced into North America from Asia, possibly during the early 1990s, where it is causing widespread mortality of ash (*Fraxinus* spp.) (Herms and McCullough, 2014). Recently, white fringetree, *Chionanthus virginicus* L. (Oleaceae), an ash relative, has also been documented as a larval host in North America (Cipollini, 2015). Larvae feed on the phloem, cambium, and outer sapwood layers, eventually girdling and killing susceptible hosts. Only a few studies have investigated mechanisms of resistance of angiosperm trees to wood-boring insects outside of the ash/EAB system (i.e. Dunn et al., 1990; Hanks et al., 1991, 1999; Muilenburg et al., 2011). This is especially concerning because of the potential economic and ecological impacts of exotic wood-borers (Aukema et al., 2010, 2011).

There is even less information available regarding physiological adaptations of wood-borers to counter host resistance mechanisms. Recent studies investigating the physiology, adaptations, and gene expression of phloem/xylem-feeding beetle species have made progress towards a better understanding of these systems (e.g. Crook et al., 2009; Geib et al., 2010; Scully et al., 2013, 2014). However, responses to feeding on different hosts are limited to a single study (i.e. Rajarapu, 2013). This author found that several glutathione-S-transferase (GST; EC 2.5.1.18) and cytochrome P450 monooxygenase (P450; EC 1.14.-.-) genes, as well

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as a β-glucosidase (EC 3.2.1.21) gene were expressed more highly in EAB larvae that had fed on green ash (*Fraxinus pennsylvanica*), a susceptible North American species, than those that fed on Manchurian ash (*Fraxinus mandschurica*), a resistant Asian species. Conversely, carboxylesterase (CarE; EC 3.1.1.1) and sulfotransferase (SULT; EC 2.8.2) genes, and genes associated with chitin metabolism, were more highly expressed in larvae that had fed on Manchurian ash.

Cytochromes P450 belong to an extremely important allelochemical detoxification enzyme family (Li et al., 2007), which oxidatively metabolize a wide variety of exogenous and endogenous substrates. GSTs are also major detoxification enzymes that have been shown to play a role in dietary tolerance of allelochemicals (Li et al., 2007). CarEs and SULTs also play detoxification roles (Li et al., 2007), and these genes were differentially upregulated in Manchurian ash-fed EAB larvae (Rajarapu, 2013). Rajarapu (2013) proposed that SULT contributes to detoxification of amines such as tyramine, which was found at greater concentrations in phloem of Manchurian ash relative to ash species more susceptible to EAB (Hill et al., 2012). Monoamine oxidases (MAOs) (EC 1.4.3.4) also metabolize tyramine, though MAOs have not been extensively studied outside their role in insect nervous systems (Sloley, 2004).

Faster browning (oxidation) rates of Manchurian ash phloem extracts, relative to EAB-susceptible ash species, have also been reported (Cipollini et al., 2011). Oxidation of phenolics produces toxic, reactive quinones that cross-link, denature, and reduce the quality of dietary proteins (e.g. Felton et al., 1992). This suggests that Manchurian ash may produce greater amounts of quinones or produce quinones more rapidly than susceptible ash species. However, EAB, like other insects, may be able to detoxify these quinones *via* quinone reductases (QRs; EC 1.6.99.2) that are induced by allelochemical consumption (Yu, 1987).

It has also been shown that EAB larvae differentially upregulate genes associated with digestion, including β-glucosidase, when feeding on susceptible green ash (Rajarapu, 2013). Several authors have reported reductions in the expression or activity of β-glucosidase in specialist insects feeding on host plants containing toxic glycosides (Pentzold et al., 2014), suggesting a potential adaptive mechanism aimed at decreasing the overall production of toxic products resulting from cleavage of the glucosidic bond. EAB may have this capacity since β-glucosidase genes were downregulated in larvae feeding on resistant Manchurian ash (Rajarapu, 2013), which contains several known phenolic glycosides (e.g. oleuropein and verbascoside) (see Whitehill et al., 2012, 2014).

Mittapalli et al. (2010) reported a high number of trypsin (a serine protease) and trypsin-like sequence domains in EAB larval midguts, but not other classes of proteases. This suggests that EAB is dependent on serine proteases (EC 3.4.21.-), and that interfering with them could be an effective host defense against EAB. Cipollini et al. (2011) and Whitehill et al. (2014) detected trypsin inhibitor activity in ash phloem extracts in radial diffusion assays, and Whitehill et al. (2014) tested the effects of soybean trypsin inhibitor (STI) on EAB larvae in bioassays with artificial diet. These authors reported that larval survival was not influenced at *in planta*-relevant trypsin inhibitor concentrations, though growth decreased in a dose-dependent manner. Ultimately, the relative importance of trypsin inhibitors as a mechanism of ash resistance to EAB needs further clarification.

Reactive oxygen species (ROS) of host origin can be highly damaging to insects, because they covalently bind to peritrophic membrane proteins or midgut cellular proteins and nucleic acids and cause lipid peroxidation (Bi and Felton, 1995). However, insect-produced antioxidant enzymes and free radical scavengers such as reduced glutathione (GSH) and ascorbate (Felton and Duffey, 1992) can protect herbivorous insects from ROS in their diet. Rajarapu et al. (2011) identified a superoxide dismutase (SOD; EC 1.15.1.1), a catalase (CAT; EC 1.11.1.6), and a glutathione peroxidase (GPX; EC 1.11.1.9) in EAB larvae. The high production of CAT in EAB larval midguts (Rajarapu, 2013) implies the presence of physiologically significant amounts of ingested H_2O_2 when feeding on ash phloem. GSH is an important electron donor in arthropods (Zhu-Salzman et al., 2008), acting as both an antioxidant and a co-substrate in enzymatically-driven antioxidant reactions. Glutathione reductase (GR; EC 1.8.1.7) reduces oxidized glutathione (GSSG) to GSH, regenerating it as an electron donor.

The goal of this study was to characterize the activities of detoxification, digestive, and antioxidant enzymes of EAB larvae when feeding on resistant Manchurian and susceptible white and green ash, which will improve understanding of resistance mechanisms of Manchurian ash to EAB, and the relative importance of larval physiological adaptations to these defenses. We predicted that enzyme activities of EAB larvae feeding on the resistant ash species reflect greater toxin exposure, as well as digestive and/or oxidative stress. Specifically, we predicted, based on previous gene expression experiments (Rajarapu, 2013), that larvae feeding on Manchurian ash would have higher CarE and SULT activities, and higher P450, GST, and β-glucosidase activities of larvae feeding on susceptible hosts. We also predicted that larvae feeding on Manchurian ash would have greater MAO activity because of the relatively high concentration of tyramine in Manchurian ash. Additionally, we predicted that the activity and production of trypsin isozymes would be influenced by unique trypsin inhibitors characteristic of the different ash species. Finally, we predicted that larval antioxidant enzyme activities and enzyme production would be greater in larvae feeding on Manchurian ash, due to the hypothesized ability of Manchurian ash to stress larvae via rapid oxidation of phenolics.

2. Materials and methods

2.1. Plants and insects

Larvae were obtained from two independent experiments, and differences in larval material utilized for enzyme analyses (i.e. age, instar, larval mass) reflect differences in experimental design. The experiment on responses of larvae to feeding on Manchurian and white ash was performed during the growing season of 2014, and the experiment on responses of larvae to feeding on green ash was performed during the growing season of 2013. For Manchurian ash-fed (Mf) and white ash-fed (Wf) larvae, 32 Manchurian ash (cv. 'Mancana') and 32 white ash (cv. 'Autumn Purple') trees (~2.5 cm basal diameter) were obtained from Bailey Nurseries, Inc. (Newport, MN), and grown outdoors in 58 L pots of mixed pine bark mulch and compost at the Ohio Agricultural Research and Development Center in Wooster, OH. Green ash-fed (Gf) larvae were collected from three replicate grafts of eight different green ash genotypes (total $n = 24$) that persisted in heavily EAB-infested natural areas in northeast Ohio and southwest Michigan. Green ash selections were propagated by grafting using either hot callus grafting (Carey et al., 2013) or bud grafting (Tubesing, 1987). Grafted trees were grown in an outdoor growing facility in 14.6 L containers in potting media consisting of Metro Mix® 510 (The Scotts Company, Marysville, OH) amended with 47 g Micromax Micronutrients (The Scotts Company, Marysville, OH), 376 g Osmocote® Plus 15–9–2 (The Scotts Company, Marysville, OH), and 700 g coarse perlite and 75 g aluminum sulfate per 2.8 cu. ft. bag. Potted green ash trees (2–3 years old, 1.5–2.5 m tall) were moved into a temperature-controlled greenhouse one week prior to inoculation.

EAB eggs were obtained from the USDA-APHIS-PPQ Biological Control Rearing Facility (Brighton, MI) (Mf and Wf larvae), or the

USDA Forest Service Northern Research Station (East Lansing, MI) (Gf larvae) approximately 12–13 days after oviposition on coffee filters. Four eggs were placed at three sites on each tree stem, with each site spaced approximately 25 cm apart. Each inoculation site was then lightly wrapped with gauze to deter predators and reduce egg desiccation, as described in Chakraborty et al. (2014). The stem diameter at the first site above the soil line was 1.5–3.5 cm (average egg density = 330 eggs/m^2). Mf and Wf larvae were harvested 65–70 days, and Gf larvae 40–50 days, after estimated hatch date (based on date of oviposition on the coffee filters in the lab) by dissecting the trees and removing live, undamaged larvae. Larval instar was determined according to Loerch and Cameron (1983) and Chamorro et al. (2012), based on width of the head capsule, and then stored in individual 1.5 mL microcentrifuge tubes at −80 °C until extractions were performed.

To generate enough material for assays, all recovered Mf larvae were used to extract protein. Larvae feeding on Manchurian ash are more difficult to recover than larvae in susceptible species because they grow more slowly, are often much smaller, and have a much lower survival rate. This limited the number of larvae available for analysis. Of all Mf larvae, 12.5% were first instars, 10% were second instars, 32.5% were third instars, and 45% were fourth instars. Because larvae grew faster and survived better on white and green ash, not all larvae recovered were required to generate sufficient material for analyses, and proteins were extracted from a subset of randomly chosen larvae. Of these, all Wf larvae were fourth instars, and 13% and 87% of Gf larvae were third and fourth instars, respectively.

2.2. Extraction of larval proteins

Due to the difficulties in recovering Mf larvae, we were not able to dissect individual tissues (i.e., midguts), or group larvae from individual host trees as biological replicates. Therefore, larval tissue was cut with a sterile razor, head capsules and the last three posterior segments were discarded (except for first and second instar Mf larvae which were kept whole), and this tissue was pooled into masses of 100 mg to produce whole body extracts. No less than two larvae were used for each replicate, and typically more than two larvae were required to achieve 100 mg. Different instars were randomized to the degree possible (i.e., a third and fourth instar were pooled rather than two fourth instars, and tissue from a single larva was not used in more than one extract). Each pooled 100 mg sample was considered a "biological replicate" that was subsequently homogenized in 300 μL of 50 mM sodium phosphate buffer, pH 7.8, for 30 s on ice in a 1.5 mL microcentrifuge tube with a Teflon minipestle. The homogenate was then centrifuged at 10,000*g* (20 min, 2 °C) and the supernatant was placed in a fresh tube and used as the crude enzyme extract for all assays. Total soluble protein (Bradford assay), P450, and GST activity assays were performed immediately following the initial extraction. The remaining extract was frozen at −20 °C until use in individual enzyme activity assays or gels. All tests were performed within 4 weeks of the initial extraction, with one biological replicate from each host species used exclusively for the separation of proteins in native polyacrylamide gels (i.e. $n = 1$ each) in order to evaluate the differential production of functional proteins. The remainder of the biological replicates were used in activity assays (Mf, $n = 6$; Wf, $n = 16$; Gf, $n = 9$).

2.3. Equipment, reagents, and estimation of protein concentration

Standard round-bottomed 96-well polystyrene microplates (BD Bioscience, Billerica, MA) were used for all assays with absorbance readings at and above 340 nm using a SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA). Activity assays requiring optical density readings below 340 nm were performed in 1 mL quartz cuvettes (Fisher Scientific) and examined in a Spectronic® Genesys™ UV/Visible Spectrophotometer. A Bio-Rad (Hercules, CA) mini PROTEAN® 3 system was used for native PAGE. A White Light Transilluminator (FB-WLT-1417; Fisher Scientific, Hampton, NH) and light meter (LI-250 light meter; LI-COR, Lincoln, NE) were used for SOD activity assay and native gel staining. Images of gels were taken using a FUJIFilm Las-3000. Concentrated Bradford reagent was purchased from Bio-Rad (Hercules, CA). All other reagents and protein standards were purchased from Sigma (St. Louis, MO). Total soluble protein was measured using bovine serum albumin as a standard (Bradford, 1976).

2.4. Detoxification enzymes

Quantification of P450 activity was performed as in Rose et al. (1995) and was expressed as nmoles *p*-nitrophenol produced per min per mg protein (nmols/min/mg) using a standard curve of *p*-nitrophenol. GST activity was determined as described by Habig and Jakoby (1981). The extinction coefficient of 9.6 mM^{-1} cm^{-1} was used to express GST activity as nmols 1-chloro-2, 4-dinitrobenzene conjugated per min per mg protein (nmols/min/mg). CarE activity was quantified using the procedure described by Gong et al. (2013) using extract that was diluted 1:100 in assay buffer. The activity was expressed as nmoles of α-naphthol formed per min per mg protein (nmols/min/mg). Monoamine oxidase activity was assayed as in Holt et al. (1997) using tyramine as a substrate, and was expressed as the change in absorbance at 490 nm per mg protein per min (ΔAbs_{490}/mg/min). The sulfotransferase-mediated regeneration of 3′-phosphoadenosine-5′-phosphosulfate (PAPS) was assayed according to the protocol described by Gagné (2014). Activity was reported as nmoles *p*-nitrophenol produced per hour per mg protein (nmols/h/mg) using a standard curve of *p*-nitrophenol. *Ortho*- and *para*-QR activities were assayed as in Yu (1987) and Felton and Duffey (1992) using 1,2-naphthoquinone and 1,4-naphthoquinone as substrates, respectively. The extinction coefficient of 6.27 mM^{-1} cm^{-1} for NADPH was used to report these activities as nmols NADPH oxidized per min per mg protein (nmols/min/mg).

2.5. Digestive enzymes

β-Glucosidase activity was measured according to Konno et al. (1999) using the artificial substrate *p*-nitrophenyl β-glucopyranoside. Activity was reported as pmoles *p*-nitrophenol released per min per mg protein (pmols/min/mg). The BA*p*NAase activity of larval trypsins was assayed according to Saadati and Bandani (2011). A standard series of bovine trypsin was used to express the activity as μg bovine trypsin equivalents per mg protein (μg/mg).

2.6. Antioxidant enzymes

CAT activity was quantified by monitoring the rate of the disappearance of H_2O_2 (Mao et al., 2007). The linear portion of the curve and the extinction coefficient of 43.6 M^{-1} cm^{-1} were used to express activity as mmols H_2O_2 decomposed per min per mg protein (mmols/min/mg). SOD was quantified as in Hillstrom and Cipollini (2011) and was expressed as μg horseradish SOD equivalents per mg protein (μg/mg). GR activity was assayed as in Felton and Duffey (1992). The extinction coefficient of 6.27 mM^{-1} cm^{-1} for NADPH was used to report the activity as nmols NADPH oxidized per min per mg protein (nmols/min/mg).

2.7. Native PAGE gels

The native PAGE system described by Laemmli (1970) was utilized to separate proteins and retain enzyme activity. All gels were 0.75 mm thick and all stacking gels were 5% polyacrylamide. After loading, electrophoresis was performed at 200 V (constant voltage) until the dye front reached the bottom of the gel. For serine proteases, 120 μg of protein were separated using an 8% resolving gel and isozymes were identified by staining hydrolyzed N-acetyl-DL-phenylalanine β-naphthyl ester (APNE) with Fast Blue B salt (Hosseininaveh et al., 2009). Gels used to identify isoforms of antioxidant enzymes were pre-run for 10 min at 200 V to remove free persulfate ions that could inactivate these enzymes (Weydert and Cullen, 2011). For identification of CAT isozymes, 30 μg protein were separated using an 8% resolving gel and the gel was first equilibrated in assay buffer and then incubated with 20 U/mL horseradish peroxidase in 50 mL assay buffer at room temperature for 30 min. Then, 150 μL 30% H_2O_2 were added to the incubation mixture, and the gel was incubated for another 10 min. The solution was then decanted, the gel rinsed in DI H_2O, and the gel was rinsed twice with 0.5% (v:v) guaiacol in assay buffer (50 mL). Clear activity bands designated catalase isozymes where H_2O_2 had been degraded. For the SOD native gel, 30 μg protein were separated using a 10% resolving gel. The gel was stained in accordance with Weydert and Cullen (2011) and SOD species were differentiated by H_2O_2 inhibition (Kuo et al., 2013). After activity staining, staining solutions were decanted, gels rinsed in DI H_2O several times to remove excess stain, and images were taken immediately. Images of gels were analyzed using ImageJ (NIH) software to examine differences in enzyme staining intensity.

2.8. Data analyses

The Dixon test ('Outliers' package in R) (Komsta, 2011) was used to check enzyme activity data for outliers, which were removed from subsequent analyses. All enzyme activity assay data were validated for normality using a Shapiro–Wilk normality test, with the exception of SULT activity, which required a reciprocal transformation. The effect of host on enzymatic activity was assessed via a *t*-test ($\alpha \leqslant 0.05$), comparing Mf and Wf larvae. Gf larvae were not included in statistical analyses because they were not part of the same experimental design. Rather, they were used to document relative trends in their activities. All statistical analyses were performed in R (R Core Team, 2015).

3. Results

3.1. Detoxification enzymes

There were no significant differences between Mf and Wf larvae for P450 ($t = 0.143$, $p = 0.89$), GST ($t = 0.744$, $p = 0.46$), CarE ($t = 0.31$, $p = 0.76$), and SULT ($t = 0.347$, $p = 0.73$) activities, and activities for all three larval groups were very similar (Table 1). There were significant differences between Mf and Wf larvae for MAO activity ($t = 2.169$, $p = 0.04$), with Mf larvae having approximately 1.8 times higher activity than Wf larvae, though Mf and Gf larvae had similar activities (Table 1). The activity of *o*-QR was approximately 2.9 times and 1.7 times higher in Mf larvae than in Gf and Wf larvae, respectively, and the difference between Mf and Wf larvae was significant ($t = 2.838$, $p = 0.01$) (Fig. 1). The difference in *p*-QR activity between Mf and Wf larvae was significant at the $\alpha = 0.1$ level of significance, but not at the 0.05 level ($t = 1.888$, $p = 0.08$) (Fig. 1) with Mf activity being 1.4 times higher than Wf larvae. The *p*-QR activity of Gf larvae was again the lowest of the three groups, with Mf larvae having approximately 2.8 times higher activity.

Table 1
Mean activity levels (±1 SE) of cytochrome P450 (P450; nmols/min/mg), carboxylesterase (CarE; nmols/mg/min), glutathione-S-transferase (GST; nmols/min/mg), sulfotransferase (SULT; nmols/h/mg), monoamine oxidase (MAO; ΔAbs_{490}/min/mg), β-glucosidase (β-GLUC; pmols/min/mg) and the tryptic BA*p*NAase activity (μg/mg) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larvae. Different letters indicate significant differences between Mf and Wf larvae within specific enzyme activity. Values for Gf larvae cannot be evaluated statistically (see Section 2) and are provided for comparative purposes.

Host	Activity						
	P450	CarE	GST	SULT	MAO	β-GLUC	BA*p*NAase
Mf	0.279 (0.047)	0.035 (0.005)	137.3 (14.6)	0.504 (0.046)	1.78 (0.19)^a	1.91 (0.17)^b	13.34 (2.51)
Wf	0.287 (0.034)	0.038 (0.004)	128.8 (8.2)	0.773 (0.147)	0.98 (0.22)^b	4.70 (0.83)^a	15.46 (1.19)
Gf	0.329 (0.071)	0.039 (0.003)	123.6 (6.9)	0.294 (0.049)	1.44 (0.36)	10.84 (2.37)	24.26 (6.58)

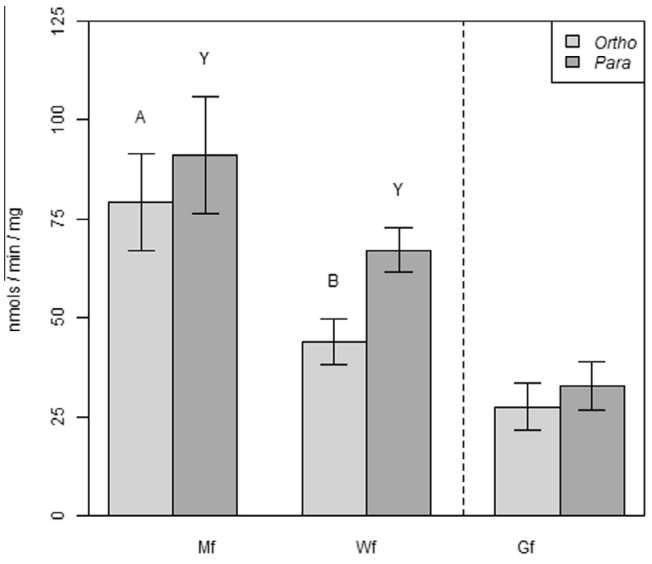

Fig. 1. Mean *ortho*-quinone (*ortho*; light gray bars) reductase and *para*-quinone (*para*; dark gray bars) reductase activities (±1 SE) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences within specific enzyme activity. Values for Gf larvae cannot be evaluated statistically (see Section 2) and are provided for comparative purposes.

3.2. Digestive enzymes

The activity of β-glucosidases was approximately 2.5 and 5.7 times higher in Wf ($t = 2.103$, $p = 0.05$) and Gf larvae (Table 1) than in Mf larvae, respectively. The difference in tryptic BA*p*NAase activity between Mf and Wf extracts was not significant ($t = 0.860$, $p = 0.40$). Gf extracts had approximately 1.8 times higher BA*p*NAase activity than Mf larvae (Table 1). Serine protease staining revealed distinct differences in bands between larvae that had fed on different species (Fig. 2). Serine proteases generally appeared in groups of high and low electrophoretic mobility. Two proteases of low electrophoretic mobility were evident in larval extracts from all three hosts. One additional protease of low mobility was evident in Mf extracts that did not appear in Wf and Gf extracts, and two proteases of high mobility that appeared in Wf and Gf extracts but not in Mf extracts.

3.3. Antioxidant enzymes

Mf larvae displayed significantly higher CAT activity than Wf larvae ($t = 5.671$, $p < 0.001$), with Mf larval CAT activity nearly double the activity of Wf larvae. Mf and Wf larvae also had 4.9- and

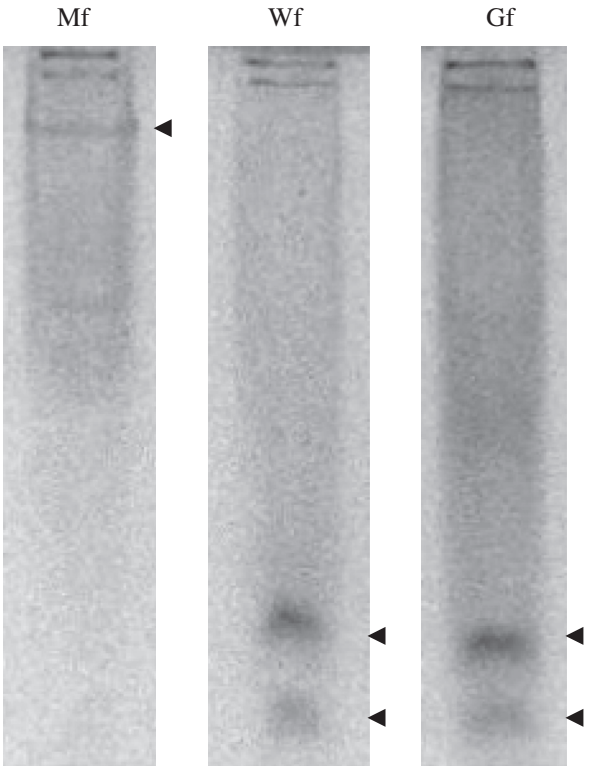

Fig. 2. Native PAGE gel stained for serine protease activity with N-acetyl-DL-phenylalanine β-naphthyl ester (APNE) and Fast Blue B salt. Mf, Wf, and Gf indicate Manchurian ash-fed, white ash-fed, and green ash-fed larvae, respectively. Arrows indicate differentially expressed proteases between larval groups.

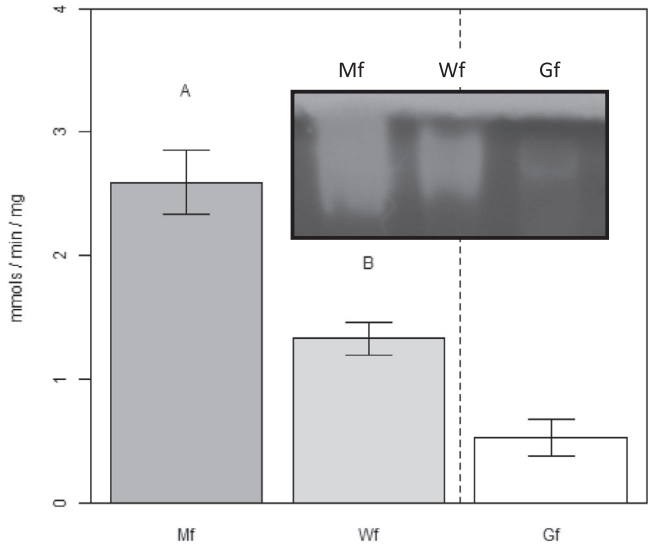

Fig. 3. Mean catalase activity (±1 SE) and catalase isozyme expression (insert) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences. Values for Gf larvae cannot be evaluated statistically (see Section 2) and are provided for comparative purposes.

2.5-fold greater CAT activity than Gf larvae, respectively (Fig. 3). CAT staining revealed a single band of relatively low electrophoretic mobility (Fig. 3, inset) common to all three larval groups, but was much higher in abundance in Mf larvae than in Wf (55% the intensity of Mf) or Gf (11% the intensity of Mf) larvae. Band staining of Wf larvae was intermediate, whereas bands from Gf larvae stained relatively faintly, reflecting patterns of lower CAT

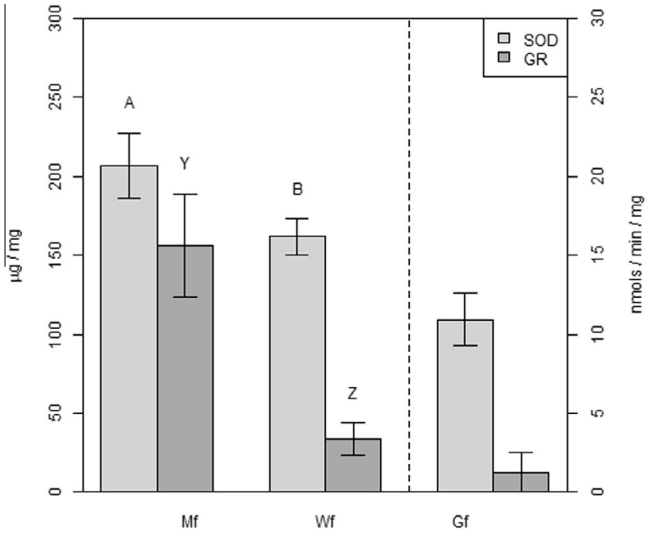

Fig. 4. Mean superoxide dismutase (SOD; light gray bars; left *Y*-axis) and glutathione reductase activity (GR; dark gray bars; right *Y*-axis) (±1 SE) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences within enzyme type. Values for Gf larvae cannot be evaluated statistically (see Section 2) and are provided for comparative purposes.

activity of extracts (Fig. 3). The SOD activity of Mf extracts was significantly higher than that of Wf extracts ($t = 2.045$, $p = 0.05$), with Mf larvae producing 1.3 times higher activity than Wf larvae, while SOD activity of Gf larvae was even lower (Mf activity was 1.9-fold higher) (Fig. 4). Staining for SOD proteins revealed one CuZnSOD band of relatively intermediate electrophoretic mobility (data not shown), which stained with roughly the same intensity in all three larval groups (Wf ~ 92% of Mf, Gf ~ 90% of Mf). GR activity was also significantly different between Mf and Wf larvae ($t = 4.77$, $p < 0.001$), with Mf larvae having 4.7 times greater activity than Wf larvae; GR activity of Gf larvae was again the lowest of the three groups (Fig. 4), with Mf larvae having 12.4 times higher activity than Gf larvae.

4. Discussion

The objective of this study was to elucidate physiological responses and putative adaptations of EAB larvae to host resistance mechanisms of Manchurian ash, and to illuminate the role of specific groups of larval enzymes in this interaction. In order to address these objectives, the activities and production of selected detoxification, digestive, and antioxidant enzymes of larvae having fed on resistant Manchurian ash cultivar 'Mancana' were compared to larvae having fed on the susceptible white ash cultivar 'Autumn Purple'. We found that extracts of Mf larvae had significantly higher MAO, *o*-QR, CAT, SOD, and GR activities than Wf larvae, while β-glucosidase activity was significantly higher in Wf larval extracts. Additionally, we found that Mf larvae uniquely produced a single serine protease of low electrophoretic mobility, while both groups of larvae feeding on susceptible hosts produced two serine proteases of high electrophoretic mobility. We also found that the staining of a single CAT enzyme mirrored the CAT enzymatic activity measured in all three larval group extracts, with the highest production and activity in Mf larval extracts. These results suggest that resistance mechanisms of Manchurian ash to EAB include oxidation of phloem phenolics and the production of ROS in higher amounts than in white and green ash. Conversely, because some hosts are more oxidatively stressful than others, key adaptations of larvae appear to involve the detoxification of quinones, as well

as relief from oxidative stress (Cipollini et al., 2011; Rigsby et al., unpublished results). Additionally, the differential production and activity of serine proteases and β-glucosidase could represent adaptive responses to unique trypsin inhibitors of different host species, and the ingestion of toxic phenolic glycosides, respectively.

MAO activity was higher in Mf than in Wf larval extracts, though activity in Gf larvae was more similar to that of Mf larvae than Wf larvae. This is perhaps evidence of the greater capacity for Mf larvae to degrade tyramine relative to Wf larvae, and reflects the greater tyramine concentrations reported in Manchurian ash phloem tissue compared to susceptible species (Hill et al., 2012). MAOs play important roles in the degradation of amine neurotransmitters (Gilbert et al., 2000) and serve other purposes, such as in cuticle sclerotization (Sloley, 2004). However, MAO expression in most insects appears limited to the Malpighian tubules (Roeder, 2005), though low levels of activity have been reported in the central nervous system of some insects (e.g. Sloley and Downer, 1984). More recently it was demonstrated by Cabrero et al. (2013) that tyramine acts as a diuretic in the Malpighian tubules of *Drosophila melanogaster*. It is possible that the consumption of tyramine, if not detoxified, could act as a diuretic in larvae resulting in water loss and dehydration, and/or that tyramine consumption could result in deleterious behavioral changes (Rajarapu, 2013), since it is a neuroactive compound in insects (Roeder, 2005; Lange, 2009). Ultimately, it is unknown whether tyramine ingested by Manchurian ash-feeding larvae is toxic, and if so, it remains unclear what the relative importance of SULT and MAO may be in the detoxification process.

Activities of *o*-QR and *p*-QR were generally higher in Mf than in Wf larval extracts, indicating that larvae experience a greater degree of stress from reactive quinones when feeding on resistant Manchurian ash than when feeding on susceptible white ash. Yu (1987) first documented QR enzymes in insects and their induction in response to feeding on selected plant allelochemicals. Later, Felton and Duffey (1992) demonstrated the importance of QRs as a constituent of the quinone-protective system in midguts of *Helicoverpa zea*. Phenolic compounds can induce oxidative stress *in vivo* that can lead to higher mortality and reduced growth (Summers and Felton, 1994). Furthermore, these pro-oxidant phenolics can undergo deleterious redox cycles in the midgut (Ahmad, 1992). This phenomenon may contribute to resistance of Manchurian ash to EAB. It is noteworthy that while both *o*- and *p*-QR activities were elevated in Mf larvae, only *o*-QR activity was significantly higher in Mf larvae relative to Wf larvae. Interestingly, while there are many phenolics that can be oxidized directly into *o*-quinones, there are none in ash that can be immediately oxidized into *p*-quinones, which typically require additional enzymatic and/or chemical reaction steps to be synthesized (e.g. juglone synthesis from α-hydrojuglone-glucoside in walnut; Strugstad and Despotovski, 2012). Yet, *p*-QR activity was slightly higher than *o*-QR activity in extracts from all three larval groups. Therefore, the source of *p*-quinones that could be reduced by larval *p*-QR remains unclear.

β-Glucosidase activity was highest in Gf, intermediate in Wf, and lowest in Mf larval extracts, which conforms to patterns of gene expression reported by Rajarapu (2013) in larvae recovered from Manchurian and green ash. The low activity in Mf larvae could be an adaptive response to the ingestion of toxic phenolic glycosides, which is common in specialist insects consuming defensive glycosides (reviewed by Pentzold et al. (2014)). Several phenolic glycosides are present in ash phloem, including oleuropein and verbascoside (Whitehill et al., 2012) that could be activated by β-glucosidase. Oleuropein, which was found in greatest concentration in Manchurian ash (Whitehill et al., 2012), cross-links strongly with protein once activated by plant β-glucosidases in extracts from privet (Konno et al., 1999), while verbascoside decreased larval survival of EAB in an artificial diet bioassay (Whitehill et al., 2014). Clearly, the potential role of these compounds in ash resistance to EAB merits further investigation.

Tryptic BA*p*NAase activities were higher but more variable in Gf larvae relative to Mf and Wf larvae. This variation may be due to the diversity of host genotypes fed on by Gf larvae in this study compared to the single genotype used for the other two groups. However, activity staining following separations in native PAGE gels revealed three isozymes differentially produced in Mf larvae and those larvae that had fed on susceptible hosts. The similarity in enzyme activity coupled with differential isozyme production likely indicates either a compensatory adjustment of protease production based on host species, or differential inhibition of certain proteases by each host, presumably due to unique trypsin inhibitors in phloem tissue. Such regulation of digestive proteases has been demonstrated in several insects (e.g. Chikate et al., 2013), but have never been studied in buprestids. Furthermore, Mf larvae may incur a physiological cost when upregulating inhibitor-insensitive proteases. However, costs and benefits of phenotypically plastic protease expression in insects have not been well-studied (Zhu-Salzman and Zeng, 2015).

Activity of all three antioxidant enzymes assayed in this study was higher in Mf extracts, which indicates that larvae feeding on Manchurian ash could be under higher levels of oxidative stress than when feeding on the susceptible hosts. The substantially higher CAT activity observed in Mf larvae relative to susceptible hosts is suggestive that the H_2O_2 accumulation or production is greater in Manchurian ash. The elevated activity of SOD in Mf larvae relative to Wf larvae suggests that superoxide radicals may also be more abundant in Manchurian ash. We detected a single CAT and CuZnSOD enzyme using native PAGE activity staining, which confirms the findings reported by Rajarapu et al. (2011) using gene expression techniques. In our study, biological replicates of Mf larvae contained both third and fourth instars. However, the majority of extracted protein in each biological replicate was from fourth instars. Furthermore, Rajarapu et al. (2011) found that CAT and SOD gene expression remained unchanged through larval development. Hence, the differences in CAT and SOD enzyme activity and production that we observed are likely attributable to differences in host species rather than instar, although we cannot completely exclude the latter possibility.

Quinones can catalyze the formation of ROS in the insect digestive tract (Krishnan et al., 2007), which can cause oxidative damage to the midgut, proteins, lipids, and nucleic acids and inhibit absorption of nutrients (Bi and Felton, 1995). In the absence of a sufficient oxidative stress-relief system, ROS can severely impair the digestive system (Krishnan et al., 2007). Accordingly, we observed that the abundances and activities of various digestive enzymes were impaired in insects feeding on Manchurian ash. Additionally, the importance of the availability of GSH can be inferred from the activity of GR. Mf larvae could be oxidatively stressed (inferred from CAT and SOD activities) and we failed to detect differences in GST activity between larval groups. This indicates that GSH is more important as a non-enzymatic antioxidant or as a co-substrate for GPX. It has also been reported that thiols such as GSH decrease the net production of quinones via polyphenol oxidase activity (Negishi and Ozawa, 2000).

Past experiments have shown that Manchurian ash has significantly higher extract browning rates than susceptible ash species (Cipollini et al., 2011). Additionally, experiments comparing the activities of quinone-generating enzymes, ROS-generation, protein cross-linking, and other defensive mechanisms of Manchurian ash to the closely-related but susceptible North American native black ash (*Fraxinus nigra*) have revealed that Manchurian ash provides an oxidatively more stressful, quinone-rich substrate for EAB larvae

(Rigsby et al., unpublished results). From these experiments, polyphenol oxidases and, specifically, peroxidases are substantially more active in Manchurian ash than black ash (Rigsby et al., unpublished results). These data correspond well with the results reported here.

We did not detect differences between larvae having fed on different hosts in the activities of several of the detoxification enzymes assayed (i.e. P450s, CarE, GST, and SULT). This could indicate that both the resistant and susceptible host species contain compounds that required detoxification by these enzymes or that these assays, performed with standard substrates, did not target all of the relevant enzyme isoforms. For example, P450s are a large and diverse superfamily of enzymes and *O*-demethylation is only one of the activities of P450s. Prior research on EAB P450 gene expression and molecular docking suggests that P450s play a role in the detoxification of certain ash phenolics (Rajarapu, 2013). However, in order to truly address the functional role of these detoxification enzymes in adaptation to host defenses, a much more targeted study is needed that focuses on differential expression as well as diverse enzymatic activities of detoxification genes. It should be stressed that these results do not mean that these detoxification enzymes are not important in this interaction, but rather that these enzymes together respond similarly regardless of host species with these standard substrates.

To summarize, physiological responses of larvae feeding on EAB-resistant Manchurian ash indicate that they could be experiencing higher levels of oxidative stress, presumably due to higher levels of ROS and reactive quinones, than larvae feeding on susceptible North American species. Based on our results, we propose that resistance of Manchurian ash to EAB results from the presence of enzymes that oxidize its induced and constitutive phloem phenolic profiles to a much greater (or unique) degree than in susceptible white and green ash, ultimately resulting in decreased growth and survival of EAB. We observed little variation between host plants in the activity of most larval detoxification enzymes that we assayed, with the exception of MAO and *o*-QR activity, indicating that ingested toxins may be metabolized similarly in all three larval groups, or that relevant activities of these enzymes remain to be determined. Finally, our results were consistent with a compensatory response of presumably digestion-associated β-glucosidases and serine proteases, suggesting a potential fitness cost associated with decreased nutrient acquisition from the diet of the insect. We therefore conclude that resistance of Manchurian ash to EAB likely results, in part, from the oxidation of dietary phenolics and the generation of ROS either *in planta* or *in insectum*. Limitations in the material available to us (i.e. our limited number of Mf larvae) prevented further analyses in this study. However, future investigations should involve the further identification and characterization of the differentially produced serine proteases (e.g. purification, mass spectrometry, and gene expression), the identification, substrate specificity, and expression of important detoxification enzymes, and the further characterization of important quinone- and ROS-protective enzymes and free radical scavengers.

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