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Mechanisms of Antixenosis and Antibiosis of Ash Against Emerald Ash Borer

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MECHANISMS OF ANTIXENOSIS AND ANTIBIOSIS OF ASH AGAINST EMERALD ASH BORER

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I HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER MY SUPERVISION BY Chad M Rigsby ENTITLED Mechanisms of antixenosis and antibiosis of ash against emerald ash borer BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREEE OF Doctor of Philosophy

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ABSTRACT

Rigsby, Chad M. Ph.D. Environmental Sciences Ph.D. Program, Wright State University, 2016. Mechanisms of antixenosis and antibiosis of ash against emerald ash borer.

Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae), is an invasive forest pest causing widespread mortality of ash (Fraxinus spp.) in North America. Host resistance research and the development of resistant hosts offers a promising strategy for the long-term conservation of ash and management of EAB. Manchurian ash (*F. mandshurica*) shares an evolutionary history with EAB in Asia, resulting in its greater resistance relative to naïve North American ashes. In the following studies I investigate antixenosis and antibiosis mechanisms of resistant and susceptible ashes. Antixenosis in Manchurian ash was demonstrated by quantifying substantially lower oviposition on this species relative to North American ashes. The potential underlying mechanisms of antixenosis were addressed by profiling the bark and canopy volatile organic compounds (VOCs) emitted by susceptible black (*F. nigra*) and resistant Manchurian ashes and major species differences in VOC profiles were demonstrated.

To address antibiosis, the physiological responses of EAB larvae that had fed on Manchurian, white (*F. americana*), and green (*F. pennsylvanica*) ash were quantified. It was found that antioxidant and quinone-protective enzyme activities of larvae feeding on Manchurian ash were substantially higher, suggesting that larvae feeding on Manchurian ash experience relatively high levels of reactive oxygen species and quinone stress. Manchurian ash demonstrated substantially higher activities of defense-associated enzymes and reactions than black ash, especially phenolic-oxidizing enzymes. These

results support the conclusions of the larval physiology study that Manchurian ash appears to be able to generates greater amounts of quinone- and ROS-stress *in vivo* than North American ashes. Lastly, larval performance and bark phenolic chemistry and physiology were compared for Manchurian ash that were experimentally girdled or not. Girdling reduced larval performance by half but bark defenses did not differ by treatment indicating that decreases in larval performance are associated with factors other than a reduction in levels of host defenses.

It was concluded that Manchurian ash expresses antixenosis, which may be driven by the emission of certain volatiles. Also, that antibiosis appears to be related to the ability of Manchurian ash to generate an oxidatively stressful diet for larvae and larval success in compromised trees does not stem from a reduction in defense levels.

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DEDICATION

To my family who has shown me so much love and has been so incredibly supportive, especially my parents, Michael and Karen, as well as my fiancée and partner in life, Alexandra.

1 INTRODUCTION

1.1 INVASIVE SPECIES AND EMERALD ASH BORER

Invasive arthropods cause roughly $4.5 billion in damages per year (Pimentel et al. 2005), an estimate from 2005 which is likely a conservative estimate in 2016. It has been estimated that 2.5 invasive insect pests establish in North America every year due to international commerce and globalization (Aukema et al. 2010). Wood-boring insects are particularly devastating, costing federal, state, and local governments an estimated $2.5 billion per year which does not include roughly $960 million lost in residential property value and forest landowner timber loss (Aukema et al. 2011). Specifically, emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae), causes an estimated $1.7 billion annually (Aukema et al. 2011). EAB results in ash (*Fraxinus* spp.) mortality rates of over 99% in the field for major native North American ash species, resulting in millions of dead forest and landscape ash in the invaded range of EAB (Klooster et al. 2013; Herms and McCullough 2014). The related white fringetree, *Chionanthus virginicus* (Oleaceae), has also recently been reported to be a host to EAB in North America (Cipollini 2015), and this has been documented in several locations throughout the Midwest (Cipollini and Rigsby 2015). It has been estimated that roughly eight billion ash trees exist in the United States with a value of around $300 billion (Poland and

McCullough 2006; Sydnor et al. 2007). Hence, from a purely economic perspective EAB is, and will continue to be a devastating forest pest in North America. The ecological impacts of EAB in North America will also be profound as the loss of ash will result in widespread gap formation in forests with probable cascading effects on community composition and forest dynamics (Herms and McCullough 2014).

Massive ash dieback was reported in the greater Detroit area of Michigan in 2001 which was initially diagnosed as ash yellows disease (Herms and McCullough 2014). However in 2002, the cause of this ash dieback was identified as *Agrilus planipennis* (Haack 2002) and the beetle was found in other parts of the state and in neighboring Windsor, Ontario (Herms and McCullough 2014). Dendrochronological studies have found that EAB was most likely present in North America as early as the early 1990's and had begun attacking Detroit-area ash trees sometime around 1998 (Siegert et al. 2006). The likely source of the invasion is thought to be infested packing material from Asia (Cappaert et al. 2005) and molecular data point to China as being the source population (Bray et al. 2011). As of 1 March 2016, 15 years after the initial ash dieback in Detroit was reported, EAB has been confirmed in 25 states in the U.S. and two Canadian provinces, as far west as Colorado, as far south as Louisiana and Georgia, as far east as Massachusetts, and as far north as the Canadian provinces of Ontario and Quebec (www.emeraldashborer).

1.2 A ROLE FOR HOST RESISTANCE IN CONSERVATION AND MANAGEMENT

Interestingly, a 1966 horticultural report published in China described extensive white ash (*F. americana*) mortality in that country due to the infestation of an unknown species of *Agrilus* (Liu 1966). China is part of the native range of EAB where it is not considered a pest species, but rather a secondary colonizer of stressed or dying ash (Liu et al. 2003) and North American ash species planted in China routinely experience high mortality rates (Liu et al. 2003; Wei et al. 2004). This high mortality of North American ashes in China occurs in the presence of natural enemies. Duan et al. (2012) reported relatively high rates of larval mortality due to plant resistance in Asian ashes compared to North American ashes and higher rates of larval parasitism in North American ashes. This evidence suggests highly that EAB populations are not top-down controlled by predators and parasitoids which has been suggested for buprestids (Muilenburg and Herms 2012).

A comprehensive study of the various Canadian biocontrol programs since 1882 shows that the combined rate of biocontrol agent releases that "successfully eliminated" or "controlled" target invasive forest pest is roughly 37% on a per invasive forest pest basis and 9% on a per biocontrol agent released basis (MacQuarrie et al. 2016). That is to say, of all the invasive forest pests established in Canada since 1882, 37% of these have either been successfully eradicated or brought under control by way of biocontrol programs and 9 out of 100 biocontrol agents released either successfully eradicated the pest or brought the pest under control. Given the high monetary costs and relatively low success rates associated with biocontrol programs for invasive forest pests (MacQuarrie et al. 2016), the evidence that buprestid populations are bottom-up controlled (Muilenburg and Herms 2012), and the lack of evidence that parasitoid releases in North

America are reducing EAB populations or reducing ash mortality in infested areas (e.g. Duan et al. 2013) mandates that alternative approaches to host plant conservation and pest management must be considered in addition to biocontrol programs.

Rebek et al. published a seminal paper in 2008 confirming substantial interspecific variation in resistance between North American ashes and an Asian ash. These authors showed significantly lower mortality rates and exit hole densities for a cultivar of Manchurian ash (*F. mandshurica* cv. 'Mancana') relative to two cultivars of green (*F. pennsylvanica* cvs. 'Patmore' and 'Marshall's Seedless'), and a cultivar of white (*F. americana* cv. 'Autumn Purple') ash. This was the first study to demonstrate that a co-evolved, Asian species expresses a considerably more resistant phenotype than native North American species. This finding agrees with several other studies demonstrating that generally, little to no resistance is expressed in host species that lack a shared evolutionary history with an exotic insect (e.g. Nielsen et al. 2011). Therefore, Asian ashes are a source of resistance genes that can introgressed into North American species for re-planting efforts (Herms and McCullough 2014). Breeding programs are currently underway, attempting to take advantage of potentially resistant germplasm from native North American ashes (Koch et al. 2015).

The identification and characterization of the defense mechanisms of resistant ashes would aid in breeding efforts as this would allow for the development of molecular markers that can be used for targeted breeding (Herms and McCullough 2014). However, resistance can be a complex phenomenon. Painter (1951) originally described three categories of resistance: tolerance, non-preference (later re-named antixenosis by Kogan and Ortman [1978]), and antibiosis. It is hypothesized that tolerance to feeding is likely

an important component of resistance to wood-borers as these insects feed on high-value vascular tissue and the feeding activity of wood-borers disrupts photoassimilate transport (Villari et al. 2015). However, it can also be hypothesized that such high-value tissue would be heavily defended by way of antixenosis and antibiosis mechanisms.

1.3 ANTIXENOSIS

One of the original categories of resistance described by Painter (1951) was described as “non-preference”. Kogan and Ortman (1978) later re-named “antixenosis” to more accurately describe the non-preference reaction of insects to a resistant plant as well as to compliment the “antibiosis” terminology of Painter (1951). Ultimately, antixenosis described the category of plant defense where plants have chemical or physical traits that make it less likely that an herbivore will use the plant as a host (Painter 1951;1958; Kogan and Ortman 1978). Insects use olfactory, gustatory, tactile, and visual cues to make host-selection decisions and antixenotic traits have been described in many plant-insect systems for all of these cues, most of which have been described in agricultural systems (Smith 2005).

Though antixenotic mechanisms have been described for other sensory-based systems, much focus has been paid to volatile cues and olfaction in host selection research as it is thought to be the most important way insects find and select hosts (Bernays and Chapman 1994). Numerous studies have shown that the olfactory systems of insects are extraordinarily sophisticated and sensitive (e.g. Vosshall et al. 1999) and that their olfactory systems allow for their ability to discriminate between host and non-

hosts and between hosts of different quality (Lapis and Borden 1993; Gripenberg et al. 2010). Perhaps one of the well-documented phenomena associated with host selection discrimination is that many insects are repelled by volatiles emitted by plants that are being fed upon by conspecifics while attracted to plants that have no herbivore damage (e.g. Quiroz et al., 1997). This is presumably because the feeding activity of insects results in the induction of certain antibiosis mechanisms (discussed below) which would result in a poorer quality food source for offspring (Verheggen et al. 2013) and would attract natural enemies of the herbivore such as parasitoids (Paré and Tumlinson 1999; Kessler and Baldwin 2001).

Aside from discriminating between constitutive and induced plants *via* herbivore feeding, it has been shown in several insect-host systems that insects are capable of discriminating between hosts that are simply of better or poorer quality (Gripenberg et al. 2010). For example, eucalyptus longhorned borer, *Phoracantha semipunctata* (Coleoptera: Cerambycidae), consistently prefer to oviposit on Eucalyptus species that were of the highest quality for their offspring (Hanks et al. 1993). This is indicative of either the lack of volatile attractants (e.g. O'Neil et al. 2010) or the presence of volatile repellents (e.g. De Moraes et al. 2001) that may be emitted by non-preferred species. Sacchetti et al. (2015) reported that the preference hierarchy of sweet potato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), for three *Callistemon* host species reflected the increased emission rates of linalool and the decreased emission rates of 1,8-cineole, two monoterpenoids. In this study, the putative repellent, 1,8-cineole, was only emitted in trace amounts in the most preferred host, demonstrating the effects of a lack of repellents. This non-preference of poorer-quality hosts can be considered antixenosis (Smith 2005),

but also shows that antixenosis and antibiosis can be coupled in many systems (e.g. Klingler et al. 2005).

1.4 ANTIBIOSIS

Antibiosis can be defined as the category of plant resistance where plants employ mechanisms that deleteriously affect herbivores once they have chosen to feed on the plant (Painter 1951; Wise et al. 2008). Plants use a variety of antibiosis mechanisms including toxic secondary metabolites such as chemicals, proteins, mechanical defenses, or combinations of these (War et al. 2012). The antibiotic effects of these mechanisms can range from mild to lethal and even if an individual survives it may suffer from crippling effects such as reduced body size, fecundity, and extended development periods (Smith 2005). Furthermore, induced responses are a critical component of antibiosis, where certain signals such as herbivore feeding, salivary enzymes, or plant hormones (e.g. jasmonic acid [JA]) results in the expression of certain defenses (Ali and Agrawal 2012; War et al. 2012).

Secondary metabolites are defined as substances produced by plants that are not required for growth and development (Howe and Jander 2008), are typically associated with antibiosis, and can be constitutively stored or induced in response to herbivore attack (War et al. 2012). Major classes of metabolites implicated in host resistance are compounds such as terpenoids, characterized by isoprene monomers (Lange 2015), and phenolics, characterized by the presence of a phenol structure (Appel 1993). Furthermore, plants also utilize toxic (e.g. ribosome-inactivating proteins; Bertholdo-Vargas 2009) or

anti-nutritive (e.g. protease inhibitors; Broadway and Colvin 1992) proteins as antibiosis mechanisms. In many instances, enzymes and secondary metabolites, together, function as antibiosis mechanisms. For example, enzymes such as polyphenol oxidases (PPOs) and peroxidases (POXs) readily oxidize polyphenols into reactive quinones that can damage biomolecules such as proteins, nucleic acids, and lipids (Felton et al., 1992; Appel 1993; Summers and Felton 1994; Bi and Felton 1995). Furthermore, quinones can undergo redox cycles in insect midguts, causing substantial oxidative damage (Ahmad 1992). Additionally, two-component plant defenses that involve the separate storage of hydrolytic enzymes and their substrates that are then allowed to mix upon the rupturing of plant tissue, releasing toxins, has been described in many plants (Pentzold et al. 2014). This very mechanism has been described by Konno et al. (1998; 1999) in the privet tree, *Ligustrum obtusifolium*, which, like ash, is a member of Oleaceae. These authors describe a defense mechanism on privet where tissue damage allows β-glucosidase enzymes access to oleuropein substrate, hydrolyzing this securidoid into a potent protein denaturant.

Insects have a wide variety of mechanisms to counter the toxic effects of plant secondary metabolites such as detoxification (Pentzold et al. 2014). Detoxification mechanisms of specialist insects are believed to have evolved to be able to detoxify the specific defenses of their host plant (Jander 2014). Detoxification strategies can be classified into three general mechanisms: phase I, II, and III reactions (Dermauw and Van Leeuwen 2014). Phase I mechanisms are biotransformation reactions, such as cytochrome P450 monooxygenases (P450s), carboxylesterases (CarEs), and quinone reductases (QRs), that catalyze changes to the chemical structure of toxins. Phase II mechanisms are

conjugation reactions, such as glutathione-S-transferases (GSTs) and sulfotransferases (SULTs), where toxins are conjugated with endogenous substrates (Hodgson and Rose 2010). Lastly, phase III mechanisms are primarily performed by cellular transporters such as ATP-binding cassette (ABC) transporters that physically move toxins out of cells so they can be excreted (Hodgson and Rose 2010; Dermauw and Van Leeuwen 2014).

To counter the oxidative stress that can be associated with feeding on phenolic rich plant tissue, insects employ strategies that rely on antioxidant enzymes (e.g. Barbehenn 2002), and endogenous and/or exogenous free radical scavengers (e.g. Felton and Duffey 1992; Barbehenn et al. 2001). Two common antioxidant enzymes include catalases (CATs), responsible for the decomposition of hydrogen peroxide (H_2O_2) to H_2O, and superoxide dismutases (SODs) which are responsible for the decomposition of superoxide radicals ($\bullet O_2^-$) to H_2O_2 (Krishnan et al. 2007) and the activity of these enzymes is crucial in the relief of oxidative stress in insects (Barbehenn 2002). However, endogenous and exogenous free radical scavengers such as ascorbate and glutathione can be just as important or more important for some insects. Felton and Summers (1993) reported that the high activity of ascorbate oxidase (an enzyme responsible for oxidizing reduced ascorbate to dehydro-ascorbate) in the tissues of several plant species, results in reduced ascorbate being unavailable for *Helicoverpa zea* (Lepidoptera: Noctuidae) as a free-radical scavenger, and therefore increases the insect's vulnerability to oxidative stress.

1.5 PREVIOUS RESEARCH ON ASH RESISTANCE MECHANISMS

Prior to 2011 (when the research presented in this dissertation began), several studies had been performed primarily examining the antibiosis mechanisms of ash. Eyles et al. (2007) first identified and quantified the phenolic chemistry of three species: green, white, and Manchurian ash and these authors reported major differences in phenolic profiles. The existence of several coumarins, phenylethanoids, and lignans were detected in phloem of Manchurian ash and not in the phloem of North American species. Specifically, Manchurian phloem contained the phenylethanoids calceolarioside A and B, the lignans pinoresinol dihexoside and pinoresinol glucoside, and the hydroxycoumarins esculin, fraxin, fraxidin, fraxidin hexoside, mandshurin, and methylesculin. This was an encouraging result since there was such a distinction between native North American species and a very resistant, co-evolved species. The next year, Rebek et al. (2008) published their findings that Manchurian ash was indeed much more resistant than several native North American species.

In 2010, Mittapalli et al. published the results of a transcriptomic study (analysis of the differential abundance of all mRNA) that revealed the presence of several detoxification genes including several P450s and CarEs. Most importantly however, this study revealed a high number of trypsin and trypsin-like domains in the gut of EAB larvae. Trypsins are a type of serine proteases, which are a class of proteases (enzymes that hydrolyze proteins) (Zhu-Salzman and Zeng 2015). This finding suggests that EAB heavily rely on serine proteases for protein digestion and also suggests that the gut pH of EAB is likely basic since trypsins have basic pH optima (Mittapalli et al. 2010).

Several ash antibiosis studies were published in 2011. Bai et al. (2011) assessed the differential transcriptomic signatures of ash phloem and found that several genes

related to defense were differentially expressed between ash species. These authors reported that several genes for transcription factors and protein kinases were more highly expressed in Manchurian ash, but perhaps most notably that a Manchurian ash lipoxygenase (LOX) gene was more highly expressed compared to native North America ashes tested (Bai et al. 2011). LOXs can have direct or indirect negative effects on herbivores through either their involvement on JA synthesis (hormone associated with induced resistance in plants; Beckers and Spoel 2006) or by metabolizing toxic products ingested by herbivores (Felton et al. 1994). Also that year, Cipollini et al. (2011) reported the results of a comparative study quantifying the activities of several defense-associated enzymes and the phenolic chemistries of green, white, and Manchurian ash. These authors did not find clear differences in activities in any of the enzymes measured and their phenolic chemistry data supported the findings of Eyles et al. (2007). However, Cipollini et al. (2011) did report a significantly higher rate of browning of water extracts of Manchurian ash relative to the native North American species, presumably due to the oxidation of phenolics into quinones *via* the activity of PPOs. Also, these authors were able to show that the phenolic chemistry of the green ash cultivare 'Patmore' was similar to that of seedling green ash (wild source). This information was valuable as it showed that using ash cultivars in host resistance research could be extrapolated to ash in the landscape.

Also published in 2011, Rajarapu et al. reported the finding of three EAB antioxidant genes. First, a CAT gene was found to be relatively highly expressed in the midguts of larvae, but lower in other tissues (i.e. fatbody, Malpighian tubules, and cuticle). These authors also reported a SOD and glutathione peroxidase (GPX) gene that

were both more highly expressed in Malpighian tubules than other tissues. GPX is a POX that uses reduced glutathione (GSH) as a substrate to decompose H_2O_2, producing oxidized glutathione (GSSG) and H_2O. Lastly, Whitehill et al. (2011) published the results of a comparative proteomics experiment. Of the 33 proteins identified to be differentially expressed between ash species reported by the authors, of most interest were a putatively identified thylakoid-bound ascorbate peroxidase, a PR-10 protein related to the birch (*Betula*) family of allergens, an aspartic protease, and a phenylcoumaran benzylic ether reductase (PCBER). Ascorbate peroxidases reduce ascorbate, like ascorbate oxidases, by using it as a substrate to decompose H_2O_2 to H_2O, reducing its availability as a dietary antioxidant for herbivores which could make them more susceptible to oxidative stress (e.g. Felton and Duffey 1992). PR-10 proteins are mostly associated with resistance to pathogens, but their expression is regulated by methyl jasmonate (MeJA) in some plant species (Wang et al. 1999; Rakwal et al. 2001) and therefore can be associated with plant induced responses to herbivores and/or necrotrophic pathogens. Proteases are diverse in their biological functions which can range from direct effects on herbivores (Jiang et al. 1995) to the regulation of metabolic pathways (Simeos and Faro 2004). PCBER proteins are involved in neo-lignan synthesis (Gang et al. 1999), phenolic compounds that have been documented to inhibit feeding and growth (Miyazawa et al. 1994; Cabral et al. 2000; Garcia et al. 2000).

In 2012, Whitehill et al. published another investigation into the phenolic constituents of ash bark and phloem tissue. This study, however, included black (*F. nigra*), European (*F. excelsior*), and blue (*F. quadrangulata*) ashes in addition to the already characterized white, green (cv. Patmore), seedling green, and Manchurian ashes.

These authors reported that the phenolic chemistries of the closely related but phenotypically opposite (with regard to resistance) black and Manchurian ashes were quantitatively very similar. This study was the first to show similarities in the phenolic chemistries of a susceptible native and a resistant Asian species since previous research had only compared green and white ashes to Manchurian ash which are more distantly related to each other than black and Manchurian (Wallander 2008). These results suggested that phenolic chemistry alone may not explain major phenotypic differences between species with regards to EAB resistance. Additionally in 2012, Hill et al. published the results of an investigation into the nutritional attributes of the bark and phloem tissue of several ash species. These authors assessed many nutritional variables including water, sugar, starch, and protein content, percent carbon and nutrients, pH, levels of mineral nutrients, amino acids, and non-amino acid amines. The only significant differences between species reported by these authors were the tissue levels of tyramine, proline, and tyrosol which are hypothesized to have roles in wound healing, osmotic stress, and the synthesis of specific metabolites, respectively (Hill et al. 2012).

The last study that was performed before the following body of work was a 2014 study by Whitehill et al. These authors also assessed the phenolic chemistries and defense-associated enzyme activities of white, Manchurian, black, green (cv. Patmore), and wild green seedling ash. No differences in enzyme activities were detected between species and few differences were found between the phenolic chemistries of black and Manchurian ash, which further supported the conclusions of Whitehill et al. (2012) that phenolic chemistry may not explain major phenotypic differences between species with respect to EAB resistance. However, Whitehill et al. (2014) demonstrated deleterious

effects of trypsin inhibitor, and verbascoside on EAB larval survival and a negative effect of increasing lignin concentration on larval performance (i.e. growth) in artificial diet. These three substances were found to be induced upon MeJA application and could, at least in part, explain intraspecific variation in resistance in native North American species.

1.6 RESEARCH GAPS

One gap in the research that had been performed thus far has been that no studies thus far have directly addressed antixenosis. Previous studies addressing host resistance mechanisms have all focused on antibiosis, but antixenosis has not yet been considered. In order to assess antixenosis expression in resistant ash species, the decreased preference of EAB to resistant species must be demonstrated (i.e. non-preference) (Chapter 2). Previous research has hinted at the existence of antixenosis by showing that adult beetles prefer black, green, and white ash over Manchurian ash for feeding (Pureswaren and Poland 2009), but a preference for oviposition has not been shown. Wood-boring offspring are unable to switch hosts and therefore host placement is critical (Hanks et al. 1993; Hanks 1999), especially since the larval stage is the destructive stage of EAB. This, along with the 'Preference-Performance'/ 'Mother Knows Best' hypothesis (Jaenike 1978; Bernay and Graham 1988; Mayhew 2001; García-Robledo and Horvitz 2012), dictates that EAB females should oviposit on those hosts on which their offspring will best perform, regardless of whether or not the host is novel or co-evolved, which there is certainly precedent for (Desurmont et al. 2011).

After demonstrating antixenosis resistance in ash, the next logical step would be to attempt to identify the mechanisms (Chapter 3). EAB most likely uses a combination of visual, gustatory, and olfactory cues to find and hosts as evidenced by the trap-design and feeding preference research already performed (e.g. Crook and Mastro 2010) (Pureswaren and Poland 2009). However, as with past phenolic chemistry profiling, the volatile organic compound (VOC) profiles of Manchurian ash have never been directly compared to its most closely related North American conspecific (i.e. black ash). Additionally, past studies identifying and quantifying VOCs of different ash species have focused on the main constituents of VOC profiles (e.g. Crook et al. 2008). A logical place to begin would be to assess the VOC profile differences between these species and evaluate the effects of stress on VOC profiles and ovipositional preferences (Chapter 3).

With regard to antibiosis, one way to further elucidate resistance mechanisms of resistant ash species is to compare the biochemical and physiological responses of EAB larvae that were allowed to feed on resistant and susceptible hosts (Chapter 4). Rajarapu (2013) addressed the molecular responses of EAB to feeding and found that genes associated with chitin and peritrophic membrane (PM) metabolism and repair, CarE, UDP-glucosyltransferase (UGT), and sulfotransferase (SULT) genes were upregulated in larvae that fed on Manchurian ash relative to larvae that fed on green ash. These results suggest that certain compounds (e.g. iridoid glycosides), proteases, reactive oxygen species (ROS), and/or quinones could be damaging the PM. However, performing high-throughput gene expression analyses on non-model organisms of which no high-quality reference genome exists such as ash and emerald ash borer presents challenges (Armour et al. 2009; Ozsolak et al. 2010; Hong et al. 2011). Mapping short reads to incomplete

genome sequences results in reads that fail to align as well as false-positive errors (i.e. reads that align uniquely to a partial genome sequence but arise from elsewhere) (Hong et al. 2011). Because of this, information from molecular experiments should be confirmed at the enzyme activity and functional expression level in non-model organisms. No information exists on EAB responses to feeding on different hosts on the biochemical and physiological levels and if the same responses found by Rajarapu (2014) were independently confirmed it would provide additionally evidence for these types of defensive mechanisms in ash.

Cipollini et al. (2011) and Whitehill et al. (2014) quantified the activities of several defense-associated enzymes in susceptible North American and resistant Manchurian ash and found nothing that differentiated resistance phenotypes and found few activities that were induced by MeJA induction. However, these experiments were performed using a buffer pH that was slightly acidic (pH 6.8). The finding reported by Mittapalli et al. (2010) that EAB appears to heavily rely on trypsins for protein digestion suggests that the gut pH of EAB is basic. Additionally, these activities were quantified using a relatively narrow scope of substrates and it is possible that different ash species possess unique isozymes with different substrate affinities and/or kinetics. Additionally, certain defense-associated enzymes and activities of interest have not been quantified, for example, β-glucosidase (βG), LOX, and protein-denaturing activities. Therefore, to get a true representative comparison, enzyme activities need to be quantified at a pH more representative of the gut pH of EAB, activities should be quantified using more substrates, and other defense-associated enzymes and defense-associated processes need to be quantified (Chapter 5). This is especially important as previous work identifying

and quantifying phenolic profiles has not been able to explain major resistance phenotypic differences.

Lastly, Muilenburg et al. (2011) and D.N. Showalter (unpublished data) found that when girdled, the resistance of Manchurian ash is compromised below the girdle (i.e. higher larval survival and more extensive galleries). Therefore, girdling and inoculating Manchurian ash and correlating insect survival and performance with decreases in the levels of putative resistance mechanisms (e.g. enzyme activities) would allow for additional insights into the effective resistance mechanisms of Manchurian ash against EAB as well as reveal how EAB larvae are able to be more successful on stressed Asian ashes (Chapter 6).

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2 OVIPOSITION PREFERENCE OF EMERALD ASH BORER AND THE EXPRESSION OF ANTIXENOSIS IN MANCHURAN ASH

2.1 INTRODUCTION

The forces acting on female oviposition choice have long been an important topic in behavioral and evolutionary biology and specifically in the context of plant-insect interactions (Gripenberg et al. 2010). The “mother knows best” hypothesis underlies key insect host-plant selection models (Jaenike 1978; Bernays and Graham 1988; Mayhew 2001), predicting that females will oviposit on hosts on which their progeny will optimally perform, which in turn will optimize their own fitness (Bernay and Graham 1988; García-Robledo and Horvitz 2012). Several authors, including Cunningham (2012), suggest that host quality is explained not just by factors affecting larval performance, but also by factors such as host abundance, number of possible adult feeding sites, insect learning, larval movement, and predator/parasitoid pressure. However, it is predicted that natural selection should select for females that are able to recognize host cues that help discriminate between better and poorer quality hosts for offspring (Craig and Itami 2008). While this hypothesis has often been addressed using insects and their co-evolved (or at least co-occurring) hosts, it can also be applied to insects interacting with novel hosts (García-Robledo and Horvitz 2012).

Oviposition preference-offspring performance relationships of wood-boring insects are poorly understood. However, adult oviposition preferences are especially important for wood-borers because host quality is a key determinant of larval performance and larvae are unable to switch to other hosts (Hanks et al. 1993; Hanks 1999). Hanks et al. (1993) found that under conditions of reduced larval competition, eucalyptus longhorned borer, *Phoracantha semipunctata* Fabricius (Coleoptera: Cerambycidae), preferred to oviposit on *Eucalyptus* species that were of the highest quality for their offspring. In contrast, Morewood et al. (2003) found that Asian longhorned borer, *Anoplophora glabripennis* Motschulsky (Coleoptera: Cerambycidae) preferred to oviposit on sugar maple (*Acer saccharum*) but larval survival was higher on red oak (*Quercus rubra*). However, *A. glabripennis* is rather unusual because it is polyphagous. Finally, Anulewicz et al. (2008) found that although female emerald ash borer (EAB), *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae) landed equally on white (*Fraxinus americana*) and green (*F. pennsylvanica*) ash logs and non-ash logs, they oviposited on the two ash species significantly more than on the non-ash species. They also found that EAB larvae could not survive on the non-ash species.

Emerald ash borer is an exotic, wood-boring beetle native to Asia that is causing widespread mortality of ash (*Fraxinus* spp.) in North America (Poland and McCullough 2006; Klooster et al. 2013), the economic costs of which are increasing annually (Aukema et al. 2011, Herms and McCullough 2014). Larvae feed on phloem and outer xylem of their hosts from late spring into fall (Wang et al. 2010). As it continues to spread unabated, EAB threatens susceptible North American ash species on a continental scale (Klooster et al. 2013). In its native range, infestations of Asian ash species are

usually isolated and associated with stress events, such as drought, suggesting that Asian ash species are inherently resistant; conversely, EAB does cause widespread mortality of North American ash planted in China (Wei et al. 2004; Herms and McCullough 2014). Consistent with this, a common garden study in North America revealed that EAB adult emergence and tree mortality was lower in a cultivar of an Asian species, Manchurian ash (*F. mandshurica*), than in cultivars of North American white and green ash.

The low resistance of green and white ash to EAB has been attributed to the lack of co-evolutionary history between these North American species and EAB, resulting in lack of effective defenses and higher larval performance (Rebek et al. 2008; Cipollini et al. 2011; Whitehill et al. 2011, 2012). However, it remains unclear whether the apparent resistance of Manchurian ash to EAB results from reduced oviposition preference (antixenosis), reduced larval performance (antibiosis), or some combination of the two.

In a two-year study, we evaluated oviposition preferences of wild EAB females by monitoring accumulation of ova on live trees of North American (green, white, and black) and Asian (Manchurian) ash in two common gardens, one in Dayton, OH located near the invasion front and the second in Novi, MI near the epicenter of the EAB invasion where ash mortality is much higher. In addition, we examined whether tree size or tree health affects attractiveness for oviposition. Lastly, we examined associations between oviposition and the number of exit holes on trees which would allow for conclusions about larval survival from the ovum to the adult stage. We predicted that adults would oviposit more heavily on novel, susceptible North American species than on the resistant coevolved Asian species.

2.2. METHODS

2.2.1 WSU common garden

The WSU garden was established in May 2007 with four-year-old ash saplings planted in a completely randomized design with trees on 3 m centers in a 9 x 7 row grid (Cipollini et al. 2011). The garden contains four species of ash: white ash (cv. 'Autumn Purple'), green ash (cv. 'Patmore'), Manchurian ash (cv. 'Mancana'), and black ash (cv. 'Fallgold'). White, green and Manchurian ash were part of the original planting in 2007, whereas black ash were planted as four-year-old saplings in 2010. All cultivars were obtained from Bailey Nurseries, Inc. (St. Paul, MN, USA). The wild green ash planted in the WSU garden were grown from seed from a wild population provided by the USDA Forest Service Northern Research Station, Delaware, OH, USA. Trees in this garden were not irrigated during this study.

This garden is located on the campus of Wright State University in Dayton, OH (N 39.780015 W 84.057911) on maintained grounds with mowed lawn throughout the garden, and is bordered by a 200-acre, old-growth forest to the east and north which contains approximately 10-15% wild ash. Ash trees in this forest and planted ornamentally nearby had EAB infestations ranging from no visual symptoms of EAB colonization (dieback, bark splitting, exit holes, etc…) to severe levels of symptoms, and a few dead individuals. Three trees in the WSU garden were confirmed infested with EAB prior to the 2012 season by way of exit holes. These included a black ash with approximately 55% dieback and two green ashes with approximately 10% and 0% dieback, which were all included in this study. All WSU ash were greater than 15.0 mm in diameter at breast height (DBH), except one that was 11.2 mm DBH (not included in

the study), and therefore were large enough to be colonized by EAB larvae. Ten white, nine green (‘Patmore’), nine green (wild), and 11 Manchurian ash were used in this study in 2012 and 2013. Six black ash were used in 2012, and five in 2013, due to the loss of one to EAB infestation (the same black ash individual with 55% dieback died by the 2013 season). Table 2.1 presents summary data of tree size and condition.

2.2.2 Novi common garden

The Novi common garden was established in April 2004 with four-year-old bare-root ash planted in a randomized complete block design with four blocks. Each block contained 20 green (cv. ‘Patmore’), 20 white (cv. ‘Autumn Purple’), and 20 Manchurian (cv. ‘Mancana’) ash trees for a total of 240 trees, obtained from Bailey Nurseries, Inc. Trees were planted in rows of 15, with four rows per block on 2 m centers and were not irrigated during this study. Twenty two Manchurian, 20 white, and 18 green ash at various stages of infestation were randomly selected for use in this study and were dispersed relatively evenly across blocks.

This experimental common garden is part of a larger ash research plantation, located on the most northern edge of the plantation (N 42.498330 W 83.461191), and has received past and current pressure from EAB. Trees in this experimental garden and in the larger plantation range from no signs or symptoms of EAB colonization to prominent signs and symptoms of EAB, which in some cases has led to tree mortality. Trees included in this study displayed only mild symptoms of infestation, if anything (Table 2.1).

The ash plantation at Novi is surrounded by a mixture of suburban development, woodlots, meadow and agricultural fields. The other gardens in the plantation include various species and cultivars of Asian, European, and North American species used in unrelated experiments and range in diameter at breast height from 50-135 mm. By 2012, almost all of the mature white and green ash growing wild or in developed areas in the vicinity of the garden had been killed by EAB (D. A. Herms, personal observation).

2.2.3 Tree Measurements and Ovum Collection.

2012 Season. Adult EAB activity was confirmed at the WSU garden on 26-May by observing new exit holes. To sample ova, trees were wrapped with four layers of heavyweight chef grade, 100% cotton cheesecloth on 30-May at the WSU garden and on 6 June at the Novi garden (the presence of adult EAB were visually confirmed at the time of wrapping at Novi). Cheesecloth was chosen for these experiments because prior observations suggested that EAB would oviposit on cheesecloth wrapped on trees in a passive manner, as they may on lichens and crevices of bark (V. Muilenburg, personal observation). Wrappings were cut into a long, double-layered strip and wrapped around trees with completely overlapping bands so that no bark was exposed and there were no less than four layers of cloth. Cloth was then secured to trees with zip-ties at the top and bottom. Because of the difference in age and size between the trees in the two gardens, WSU trees were wrapped from 0.5 to 1.0 m from the base of the tree and the Novi trees were wrapped from 1.0 to 1.5 m from the base of the tree along the main trunk (mean diameter at breast height ± 1 SE was 32.31 ± 1.51 mm for WSU garden and 93.72 ± 2.52 mm for the Novi garden).

Wrappings were initially placed on trees at the WSU garden on 30-May and were then removed and replaced 9 and 14 days later and removed without replacement 43 days later when the study ended on 12-July. Wrappings were initially placed on trees in the Novi garden on 6-June and removed and replaced 14 and 22 days later and removed without replacement 35 days later when the study concluded on 11-July. Upon removal, wrappings were placed in plastic bags, held on ice, and then stored at -20^{o}C for two to twelve hours to kill any other insects present. Additionally, after wrapping trees at both sites, canopy dieback was quantified visually according to the 0-100% canopy thinning/dieback scale of Smitley et al. (2008) and tree DBH was measured using digital calipers.

2013 Season. The 2013 experimental protocol was nearly identical to the 2012 protocol with a few differences. First, tree wrapping in the WSU garden occurred from 30-May through 17-July with collections 13, 20, 27, 34, 41, and 48 days after the initial wrapping. Tree wrapping at the Novi garden occurred from 6-June through 8-July with collections 7, 14, 21, and 28 days after the initial wrapping. Second, to exclude arboreal, non-flying predatory insects, an approximately 3 cm wide band of Tanglefoot® was applied to the bark immediately above and below the wrappings at both gardens. Finally, during mid-July of 2013, new exit holes, identifiable by light-colored wood immediately interior to the bark, were counted on tree trunks from zero to 1.6 m above ground level. Notably, one black ash in the control treatment in the WSU garden was killed by EAB activity during the 2012 season; this tree was not used for ovum collections during 2013 but was used for the exit hole analysis.

2.2.4 Statistical Analysis.

The effect of species, DBH, and percentage canopy dieback on cumulative oviposition was analyzed by a traditional Poisson regression model or a zero-inflated Poisson (ZIP) regression model in R (R Core Team, 2013). Regression models were selected by comparing the Poisson and ZIP models using Vuong's (1989) likelihood-ratio-based test for non-nested models which were conducted using the 'pscl' package in R (Zeileis et al. 2012). Once the appropriate regression model was selected, individual predictor variables were assessed using a chi-squared test based on the difference in log-likelihoods between a full model with all of the possible predictor variables to a model without the predictor variable of interest. Insignificant predictors were then eliminated from the model. The full (containing all predictor variables) and reduced models were then compared using a likelihood ratio test to confirm that the removed predictors did not significantly reduce the amount of variability explained in the reduced model. Manual inspection of data revealed two instances of trees with aberrantly high amounts of oviposition and/or canopy dieback (one individual tree each from the WSU garden in 2012 and from the Novi garden in 2013). In these cases, the model was estimated as explained above with the full complement of data (i.e., the complete datasets) and with the aberrant data removed ("modified" data; all data associated with the individual tree were removed from the set). Species differences were assessed by collapsing two species into one category and comparing the collapsed model to the final model by way of the chi-squared difference in log-likelihoods, which was performed for each species-species comparison.

This same Poisson regression model was used to assess the significant impact of species, DBH, canopy dieback, and oviposition for the previous year (2012) as predictors of the number of exit holes found on trees. This was performed for both the complete datasets as well as for "partial" datasets, which only included trees that had received ova in 2012 and/or any exit holes were detected in 2012 (i.e. any tree that had not received ova and no exit holes were detected were eliminated). Species comparisons could not be completed in models with the partial datasets due to the underrepresentation of Manchurian ash because no exit holes were detected and very little oviposition occurred on any trees of this species.

For simplification purposes, 'Patmore' and wild green ash were treated as distinct taxa in all analyses. Also, species differences were not determined for any of the manipulated datasets (i.e., "complete" and "partial"), rather only for the full datasets.

2.3 RESULTS

2.3.1 WSU Common Garden

2012. We recovered a total of 55 ova on 15 of the 45 trees (33%). Thirteen ova were found on white ash, 11 on green 'Patmore', 8 on green 'wild', 25 on black, and one on Manchurian ash. A summary of the DBH and % canopy dieback from this and all gardens in 2012 and 2013 is presented in Table 1. The ZIP regression model provided a better fit for these data than the Poisson regression model (Vuong, $V = -2.15$, $P = 0.02$). After comparison to the full model containing all of the predictor variables (species, DBH, and canopy dieback), DBH and species were found to be insignificant. After

removal of these insignificant predictor variables, the full and the reduced model were not significantly different and the only predictor retained in the reduced model was dieback ($\chi^2 = 12.91$, df = 1, $P < 0.001$). According to this fitted reduced model, increases in dieback were associated with increases in oviposition (ZIP; $\hat{\beta} = 4.10 \pm 0.53$). Though species as a specific predictor variable was insignificant, there were significant differences in oviposition between species with white ($\chi^2 = 6.63$, df = 1, $P = 0.01$), green 'Patmore' ($\chi^2 = 6.52$, df = 1, $P = 0.01$), green 'wild' ($\chi^2 = 6.07$, df = 1, $P = 0.01$), and black ($\chi^2 = 4.18$, df = 1, $P = 0.03$) ash all receiving more ova than Manchurian ash; however, there was no variation in ova accumulation among North American species ash (Figure 2.1A).

A single black ash tree experienced more severe dieback and substantially greater ova accumulation in the WSU garden during 2012 than the remainder of the trees. Roughly 22 EAB ova were recovered from this tree that also had approximately 55% canopy dieback, this observation was removed and the ZIP model was re-fit to the modified dataset. DBH, species as well as dieback were not able to predict oviposition. We were therefore unable to significantly predict oviposition preferences with this modified dataset using our predictor variables in the WSU garden in 2012 with the null model being insignificantly different from the full model.

2013. During 2013, a total of 95 ova were recovered from 24 of 44 trees (55%). Sixty-two ova were on white ash, 21 on green 'Patmore', seven on green 'wild', two on black and three on Manchurian ash. The ZIP regression model produced a better fit than the Poisson regression model and was selected, although they were not significantly different. Host species predicted oviposition ($\chi^2 = 40.35$, df = 4, $P < 0.001$) while DBH

and canopy dieback did not and thus were removed from the model (full and reduced models were insignificantly different). For this year, white ash received more ova than green 'Patmore' (χ^2 = 13.67, df = 1, P < 0.001), green 'wild' (χ^2 = 15.84, df = 1, P < 0.001), black (χ^2 = 20.48, df = 1, P < 0.001), and Manchurian ash (χ^2 = 34.07, df = 1, P < 0.001). Green 'Patmore' also received more ova than green 'wild' (χ^2 =3.90, df = 1, P = 0.05), black (χ^2 = 7.32, df = 1, P = 0.01), and Manchurian ash (χ^2 = 14.85, df = 1, P < 0.001). There were no other significant species comparisons (Figure 2.1B).

Exit Holes. A total of 20 exit holes were found on eight of the 45 trees with white ash having five, green 'Patmore' having four, green 'wild' having five, black having six, and Manchurian ash having no exit holes. The ZIP regression model provided a better fit for these data than the Poisson regression model and was therefore selected, although the two regression models were not statistically different. DBH (χ^2 = 6.26, df = 1, P = 0.01), canopy dieback (χ^2 = 4.69, df = 1, P = 0.03), 2012 oviposition (χ^2 = 8.99, df = 1, P < 0.01), and species (χ^2 = 11.78, df = 4, P = 0.02) were all significant predictors of the number of exit holes found on trees and the full model containing all of the predictor variables was retained as the selected model. The ZIP regression model showed that increases in ova accumulation in 2012 ($\hat{\beta}$ = 0.29 ± 0.11) and DBH ($\hat{\beta}$ = 0.28 ± 0.12) and decreases in dieback ($\hat{\beta}$ = -8.58 ± 4.59) were associated with increases in the number of exit holes on a tree in 2013. Manchurian ash (no exit holes were found on these trees) had significantly fewer exit holes than white (χ^2 =4.97, df = 1, P = 0.03), green 'Patmore' (χ^2 = 4.38, df = 1, P = 0.04), green 'wild' (χ^2 = 4.99, df = 1, P = 0.03), and black ash (χ^2 = 5.34, df = 1, P = 0.02) and none of the North American ashes differed from each other in numbers of exit holes. In the ZIP regression model using the partial dataset (i.e.,

including only the trees receiving at least one ovum and/or exit hole), canopy dieback, 2012 oviposition, and species were all unable to predict the number of exit holes while DBH ($\chi^2 = 5.24$, df = 1, $P = 0.02$) was able. Non-significant predictors were eliminated from the model and the full and reduced models were insignificantly different. In this ZIP regression model with the partial dataset, as DBH declined exit hole number increased ($\hat{\beta} = -0.14 \pm 0.05$). Table 2.2 summarizes these model building results.

2.3.2 Novi Common Garden

2012 Season. A total of 159 ova were recovered from 25 of 60 (42%) trees, with white and green ash receiving 65 and 93 ova, respectively, and Manchurian ash receiving one ovum. The ZIP regression model provided a better fit than the Poisson regression model (Vuong; $V = -2.76$, $P < 0.01$) and was therefore selected. Tree DBH and canopy dieback were both insignificant predictors of oviposition and were therefore dropped from the model, and the full and reduced models were not significantly different. Host species ($\chi^2 = 30.94$, df = 2, $P < 0.001$) was a highly significant predictor of ova accumulation and was therefore retained in the model. Green 'Patmore' ash received more ova than white ($\chi^2 = 10.87$, df = 1, $P < 0.01$) and Manchurian ash ($\chi^2 = 35.44$, df = 1, $P < 0.001$), and white ash received more ova than Manchurian ($\chi^2 = 29.62$, df = 1, $P < 0.001$) (Figure 2.2A).

2013 Season. Seventy-one ova were recovered from 15 trees (25%) in 2013 with green ash receiving 47, white ash receiving 20, and Manchurian ash receiving four ova. The ZIP regression model again best represented the data (Vuong; $V = -2.17$, $P = 0.02$).

Both DBH (χ^2 = 38.38, df = 1, P < 0.001) and canopy dieback (χ^2 = 35.64, df = 1, P < 0.001) were highly significant predictors of ova accumulation and accumulation was inversely related to both as the number of ova increased as DBH ($\hat{\beta}$ = -0.08 ± 0.02) and canopy dieback decreased ($\hat{\beta}$ = -14.24 ± 2.64). Species was also able to predict ova accumulation (χ^2 = 38.69, df = 2, P < 0.001). Green 'Patmore' received more ova than white (χ^2 = 48.26, df = 1, P < 0.001) and Manchurian ash (χ^2 = 56.36, df = 1, P < 0.001). White ash received fewer ova than green 'Patmore' but significantly more than Manchurian ash (χ^2 = 55.04, df = 1, P < 0.001) (Figure 2.2B). The final model therefore included all of the measured predictor variables.

There was also one tree at Novi in 2013 receiving an extraordinarily high number of ova (29), whereas the tree receiving the next highest number of ova received nine. This specific high observation was eliminated and the remaining data (i.e. the "modified" dataset) analyzed with the ZIP regression model. Species was not able to predict ova accumulation and was therefore dropped from the model. Canopy dieback (χ^2 = 3.83, df = 1, P = 0.05) was a significant predictor and DBH (χ^2 = 3.27, df = 1, P = 0.07) moderately predicted ova accumulation. DBH was retained in the model because if it was dropped, the full and reduced models would became significantly different (χ^2 = 11.82, df = 3, P < 0.01). The ZIP regression model showed that ova accumulation increased as both DBH ($\hat{\beta}$ = -0.03 ± 0.01) and canopy dieback (ZIP; $\hat{\beta}$ = -4.07 ± 2.14) decreased.

Exit Holes. A total of 24 exit holes were found on eight trees (13%) in this garden. White ash had eight exit holes, green had 16, and Manchurian had none. The data were best modeled by ZIP regression (Vuong; V = -3.11, P < 0.001). For the full dataset, DBH, canopy dieback, and 2012 oviposition were not able to predict the number of exit holes

and were eliminated from the model with no significant between the full and reduced models. Species ($\chi^2 = 9.93$, df = 2, $P < 0.01$), however, did predict the number of exit holes on a tree. Both green ($\chi^2 = 14.7$, df = 1, $P < 0.001$; total = 16) and white ($\chi^2 = 14.29$, df = 1, $P < 0.001$; total = 8) ash had more exit holes than Manchurian (total = 0). In the model fitted to the partial dataset, DBH, canopy dieback, 2012 oviposition, and species did not predict exit hole number and were eliminated from the model (full and reduced models were not significantly different) (Figure 2.3). Final models are presented in Table 2.2.

2.4 DISCUSSION

The "mother knows best" hypothesis predicts that insect herbivores will choose hosts for oviposition on which their offspring will best perform. Our results are consistent with this prediction as EAB females overwhelmingly deposited more ova on green and white ash than on Manchurian ash in both common garden plantations. This corresponds with patterns of adult feeding preferences (Pureswan and Poland 2009) and higher number of exit holes and tree mortality rates observed in previous studies for these species compared to Manchurian ash (Rebek et al. 2008). This hypothesis has also been supported by Desurmont et al. (2011) who found that oviposition preferences of females of the viburnum leaf beetle, *Pyrrhalta viburni* Paykull (Coleoptera: Chrysomelidae), corresponded with larval performance regardless of whether the host was native or novel. The limited neural abilities of insects (Bernays 2001) should allow them to recognize a co-evolved host much more quickly and efficiently than novel hosts in an invaded range, but we clearly saw the opposite in our study. These findings suggest the possibility of

unknown antixenotic interactions occurring between co-evolved species such as volatile oviposition deterrents (e.g. Gharalari et al. 2011). However, the possibility also exists that females are also choosing those host species on which they developed as larvae (the 'natal habitat preference induction' hypothesis) (Davis and Stamps 2004; Stamps and Davis 2006), but this remains unclear at this time.

North American ash species received significantly more ova than Manchurian ash, with the exception of 2012 at the WSU garden (when limited oviposition was observed). Most of the ova (38%) collected at the WSU garden during 2012 came from one black ash tree, a highly susceptible species, which drove trends in this garden during this season. After data associated with that specific tree were eliminated, the primary target of adults in 2013 was white ash. In 2013, the number of ova found on 'Patmore' green ash also increased relative to 2012 and it received significantly more ova than Manchurian, green 'wild', and black ash, but fewer ova than white ash. In the Novi garden, green and white ash were also consistently more preferred than Manchurian ash. In the Novi garden in 2013, a single green ash tree received most of the ova (41%), which drove species differences. However, this "outlier" is consistent with the highly susceptible nature of green ash (Rebek et al. 2008). When faced with several hosts in a common garden, EAB adults in our study oviposited more heavily on hosts on which larvae are most successful, in accordance with the predictions of the "mother knows best" hypothesis.

Research on EAB host location has been motivated by the need to develop effective monitoring methods (Crook and Mastro 2010). It is generally thought that specialists with a narrow host range use specific cues for host selection (Cunningham

2012). This is most likely the case with EAB, which appears to use a combination of visual and host volatile cues. Both sexes of EAB are attracted to blue and green light, and females are additionally attracted to red light (Crook et al. 2009). Monoterpenes and sesquiterpenes emitted from the bark of healthy and stressed green ash (Crook et al. 2008) are particularly attractive to mated females (Crook and Mastro 2010). However, there have been no olfactory studies thus far comparing the attractiveness of EAB females to host volatiles from different host species in the context of oviposition preferences, though this has been done in the context of host selection for adult feeding (Pureswaran and Poland 2009). Several possibilities for the differential preference of EAB among hosts could include the presence of a chemical super-stimulus (O'Neil et al. 2010) or the lack of chemical deterrents in North American ash that may be present in co-evolved Asian ash species (e.g. De Moraes et al. 2001)

The oviposition patterns found in this study corresponded with adult feeding preferences and larval colonization patterns observed previously in the field. Pureswaren and Poland (2009) found that EAB adults preferred to feed on green, white, and black ash foliage over Manchurian ash foliage. This suggests that palatability of leaves for adults is a good indicator of the quality of the phloem for larvae (Pureswaren and Poland 2009). Variation in ova accumulation between host species also corresponds to variation in exit hole numbers and tree mortality patterns observed in other studies. Anulewicz et al. (2007) reported greater canopy dieback for green ash than white ash in Michigan because green ash were attacked first, which corresponds with our findings that that green ash was somewhat preferred over white ash for oviposition in our experiments. However, Klooster et al. (2013) found no differences in mortality (both green and white ash

experienced > 99% mortality) between these two species in invaded forests along an invasion gradient ranging from no EAB to advanced EAB infestation. Importantly, Rebek et al. (2008) found that native green and white ash had higher exit hole numbers and higher mortality than Manchurian ash. While direct inoculation tests have shown that Manchurian ash is a lower quality host for larvae than black ash (e.g. Chakraborty et al. 2014), exit hole numbers and mortality patterns observed on different species in the field also clearly reflect EAB adult feeding and oviposition preferences.

Trees stressed by girdling are more attractive to adults than non-girdled trees (McCullough et al. 2009). Knight et al. (2012) found that healthy trees survived longer than declining trees, possibly indicating that declining trees were preferentially targeted by EAB. However, we found little evidence that oviposition rates increased with degree of canopy dieback. However, canopy dieback had a significant effect on oviposition in the WSU garden during the 2012 season, but not when the modified dataset without the extremely high oviposition and % dieback tree data were removed. Dieback also significantly affected oviposition in the Novi garden during the 2013 season both with and without the influential datum included the analysis. It is also important to note the limited amount of dieback of Manchurian ash observed in both gardens was most likely not due to EAB infestation. In both gardens, these trees showed no signs or symptoms of infestation and no received very limited oviposition pressure. We conclude that these trees were stressed by some other unknown factor.

Correspondence between ova accumulation in one year and number of exit holes in the next would be consistent with a positive correlation between oviposition preference and larval performance. McCullough et al. (2009) observed a significant relationship

between the number of captured adults and subsequent larval density in ash trees. Our results were mostly consistent with this pattern. No exit holes were recorded on Manchurian ash in either garden, and very few ova were collected from these trees. For susceptible trees at both gardens, accumulation of ova and the number of exit holes the following year were positively related, as predicted. This relationship was significant at the Wright State garden, but not at the Novi garden. These results indicate that, for the most part, the number of exit holes expected is related to the number of ova that accumulate on the trees, suggesting that larval mortality rates in the tree may be similar across susceptible hosts.

Our data, coupled with earlier observations, suggest that interspecific variation in ash resistance to EAB is likely attributable to both variation in phloem defenses against larvae (antibiosis) and factors influencing female oviposition preferences (antixenosis). This suggests that breeding for resistance should focus on traits that decrease attractiveness of trees to ovipositing females (*via* manipulation of volatile profiles, for example), as well as efforts to strengthen phloem defenses against larvae.

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Table 2.1. Mean ± 1 SE for DBH in mm and % canopy dieback (% CDB) for WSU and Novi gardens for both seasons. Data for % CDB are zero-inflated and Poisson-distributed. Green (P) = green ash (cv. 'Patmore'), Green (w) = green ash (wild source).

		Season			
		DBH (mm)		**% CDB**	
Garden	**Species**	**2012**	**2013**	**2012**	**2013**
WSU	Green (P)	25.5 (2.4)	30.7 (2.7)	0.9 (0.6)	14.7 (5.3)
	Green (w)	36.4 (3.3)	43.4 (3.9)	0.0 (0.0)	0.0 (0.0)
	White	25.4 (1.9)	30.8 (1.7)	0.0 (0.0)	0.6 (0.6)
	Black	22.3 (2.3)	24.9 (2.2)	11.6 (8.8)	16.6 (8.6)
	Manchurian	23.3 (1.2)	29.3 (2.6)	2.5 (1.8)	4.5 (2.1)
Novi	Green	98.6 (3.2)	110.9 (3.8)	10.8 (3.4)	17.2 (5.4)
	White	72.8 (2.6)	88.7 (2.6)	0.5 (0.3)	1.5 (0.6)
	Manchurian	69.7 (3.9)	84.2 (4.1)	3.9 (2.8)	8.2 (3.8)

Table 2.2. Summary of the final models selected for oviposition in each of the two gardens (WSU and Novi) for each season (2012 and 2013) as well as for exit holes found. The dataset used is indicated as the complete dataset (Complete), if an unusually high value was removed from the dataset (Modified), or if only individual trees either receiving at least one ovum during 2012 or with at least one exit hole in 2013 (Partial). Response variables are the cumulative oviposition per tree (Ovip) and the cumulative number of exit holes per tree (EH). Predictor variables are host species (H), tree DBH (DBH), canopy dieback (CDB), oviposition from 2012 (O), and all predictor variables (All). Not applicable is NA. Predictor variables retained have a $P < 0.05$ and predictor variables dropped have a $P > 0.05$. A model without predictor variables means that no predictors were significant and the resulting model only has an intercept.

				Predictors
Garden	**Season**	**Data**	**Response**	**Retained ($P < 0.05$)**
WSU	2012	Complete	Ovip	CDB
		Modified	Ovip	–
	2013	Complete	Ovip	H
	NA	Complete	EH	H, DBH, O
		Partial	EH	CDB
Novi	2012	Complete	Ovip	H
	2013	Complete	Ovip	H, CDB, DBH
		Modified	Ovip	CDB, DBH
	NA	Complete	EH	H
		Partial	EH	–

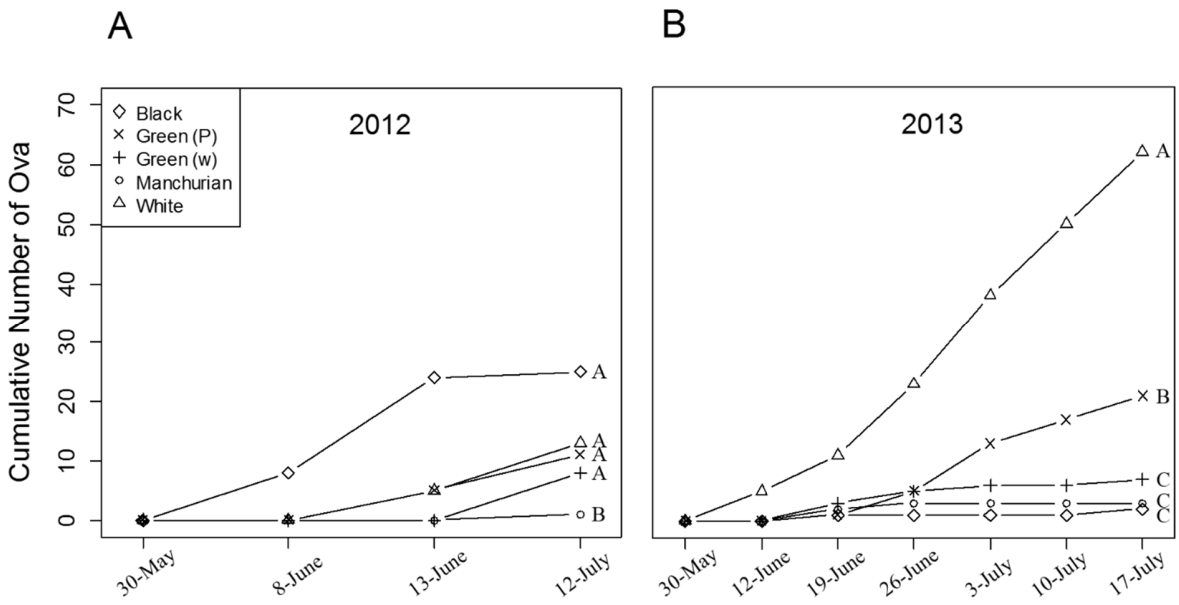

Figure 2.1. The cumulative number of EAB ova caught on wrappings through time in the WSU garden during the 2012 (A) and the 2013 (B) seasons. Different letters indicated species that have significantly different counts of cumulative ova by way of the χ^2-difference in log-likelihood between collapsed (containing species of interest) and not-collapsed (removing that species) models. Green (P) is green 'Patmore' and green (w) is green 'wild'. N = 6 for black, 10 for white, 9 for green (P), 9 for green (w), and 11 for Manchurian ash in 2012 and 2013, except N = 5 for black ash in 2013.

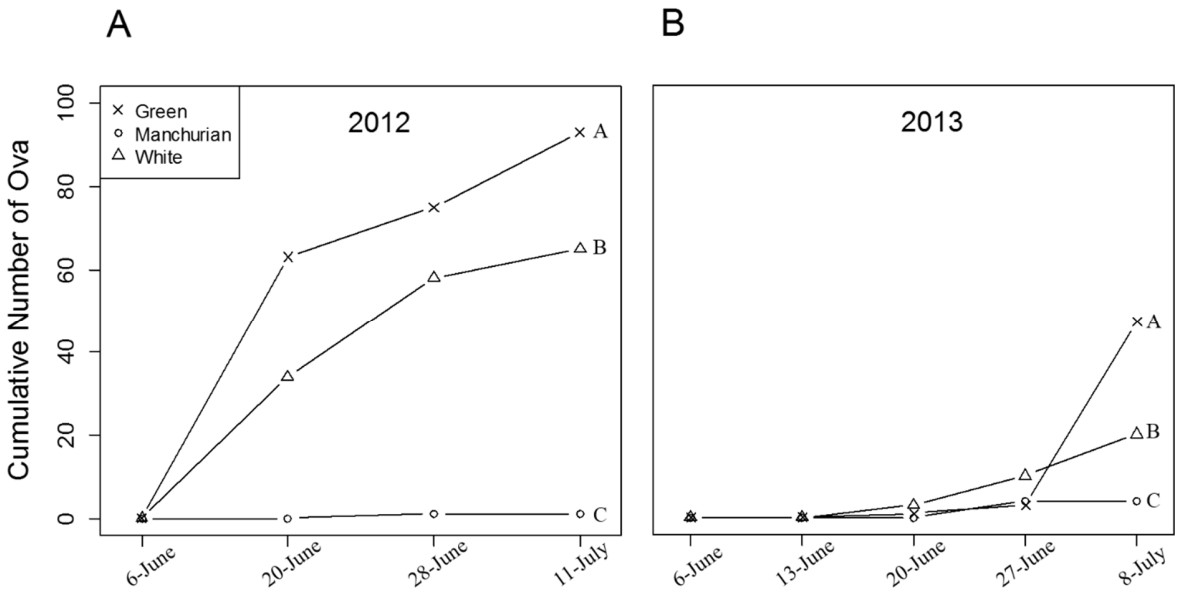

Figure 2.2. The cumulative number of EAB ova caught on wrappings through time in the Novi garden during the 2012 (A) and the 2013 (B) season. Different letters indicated species that have significantly different counts of cumulative ova by way of the $\chi 2$-difference in log-likelihood between collapsed and not-collapsed models. N = 20 for white, 18 for green, and 22 for Manchurian ash in 2012 and 2013.

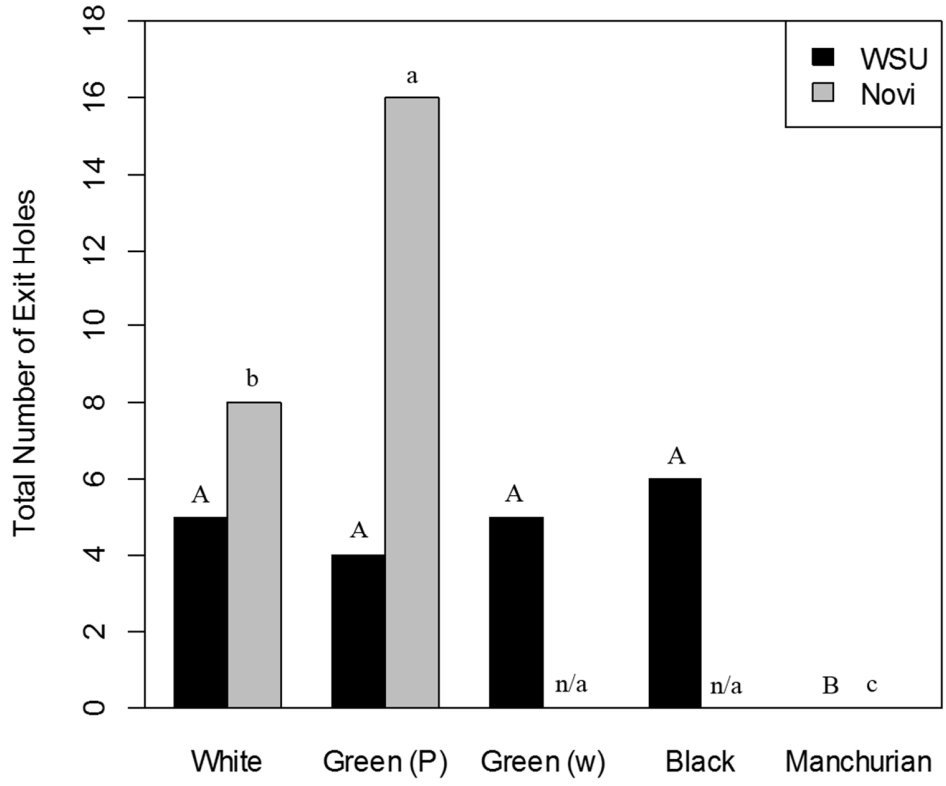

Figure 2.3. The total number of exit holes found on ash trees of each species in the WSU garden (black bars) and the Novi garden (grey bars). Species with significantly differing numbers of exit holes have different letters by way of the χ^2-difference in log-likelihood between collapsed and not-collapsed models. Upper case lettering compares species within the WSU garden and lower case lettering compares species within the Novi garden. Green (P) is green 'Patmore' and green (w) is green 'wild'. Green 'wild' and black ash are not planted in the Novi garden. N = 6 for black, 10 for white, 9 for green (P), 9 for green (w), and 11 for Manchurian ash in the WSU garden and N = 20 for white, 18 for green, and 22 for Manchurian ash in the Novi garden.

3 PROFILING OF CANOPY AND BARK VOLATILES OF CONSTITUTIVE AND INDUCED BLACK AND MANCHURIAN ASH

3.1 INTRODUCTION

Plants have evolved to defend themselves against herbivores. Three general strategies for how were proposed by Painter (1951; 1958), two of which were tolerance and antibiosis. The third mechanism is antixenosis which is characterized by non-preference (Kogan and Ortman 1978). Antixenosis mechanisms can be olfactory, visual, tactile, and/or gustatory (Smith 2005). With regard to olfaction, volatiles emitted from potential host can stimulate insect olfactory receptors and either cause positive chemotaxis (attractants), negative chemotaxis (repellents), or stop insect movement (arrestants) (Smith 2005). Ultimately, insects can use volatiles or volatile blends for host location and employ olfactory discrimination to determine resistant and susceptible hosts (e.g. Seifelnasr 1991; Lapis and Borden 1993; Gaum et al. 1994; Storer and van Emden 1995; da Costa et al. 2011).

It is hypothesized that specialist insects are strongly selected for efficient location and recognition of hosts and use specific cues for host selection (Cunningham 2012). Efficient location and recognition of acceptable hosts is especially important for wood-boring insects as wood-boring larvae cannot move to another host once deposited (Hanks et al. 1993; Hanks 1999). Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera:

Buprestidae), is an Asian-native, invasive wood-boring beetle in North America where it has devastated hundreds of millions of forest and landscape ash (*Fraxinus* spp.) (Herms and McCullough 2014). EAB is considered an ash specialist, though it has recently been found to attack and kill white fringetree (*Chionanthus virginicus*; Oleaceae) (Cipollini 2015). EAB has been shown to oviposit significantly less often on the co-evolved, resistant Manchurian ash than naive, susceptible North American ashes in common garden settings (Rigsby et al. 2014). This finding suggests that EAB uses discriminatory cues to evaluate host suitability and is able to discriminate between resistant and susceptible hosts in the landscape.

Much research exists on the chemical ecology of EAB, but most is typically in the context of creating better trapping and monitoring methods (e.g. Rodriguez-Saona et al. 2006; Crook et al. 2008). Several green leaf volatiles (GLVs), monoterpenes, and sesquiterpenes have been shown to generate antennal responses in EAB adults *via* coupled gas chromatography-mass spectrometry (GC-MS) or GC-flame ionization detection (FID) and gas chromatography-electroantennogram detection (GC-EAD) experiments (Rodriguez-Saona et al. 2006; Crook et al. 2008; de Groot et al. 2008). Additionally, six sesquiterpene compounds identified by Crook et al. (2008) elicited stronger and more consistent antennal responses from mated than from virgin females or males (Crook and Mastro, 2010). It has also been established that EAB adults are more attracted to stressed trees than healthy trees (McCullough et al. 2009; Jennings et al. 2014) and that volatile organic compounds (VOCs) from stressed or induced trees are more attractive to adult females than VOCs from control trees (Rodriguez-Saona et al. 2006).

However, despite the extensive research that has already been conducted on this general topic, specific volatile cues that attract mated females for oviposition have not been identified. Only one group of these authors (Rodriguez-Saona et al. 2006) used volatiles emitted from Manchurian ash (*F. mandshurica*) in their GC-FID/EAD experiments, a species co-evolved with EAB in Asia that displays a relatively high resistance phenotype (Rebek et al. 2008). Furthermore, no studies have addressed interspecific differences between Manchurian ash and its most closely-related North American congener, black ash (*F. nigra*) (Wallander 2008). Black and Manchurian ash are strikingly similar in their phloem phenolic chemistry yet have almost completely opposite resistance phenotypes (Whitehill et al. 2012; Chakraborty et al. 2014), with black ash receiving substantially more EAB eggs over the course of the summer oviposition season than Manchurian ash in common garden experiments (Rigsby et al. 2014), and its foliage being much more preferred by adults for feeding than Manchurian ash foliage (Pureswaren and Poland 2008). Additionally, direct inoculation experiments have shown that fewer EAB larvae survive, do not reach later instars, and are generally smaller when inoculated on Manchurian ash than susceptible North American species (Muilenburg et al. 2011; Chakraborty et al. 2014; Showalter et al. unpublished).

Collectively, these studies suggest that both antixenotic and antibiotic (characterized by adverse effects of plants on insects such as reduced growth and higher mortality; Painter 1951) mechanisms are potentially employed by Manchurian ash in its defense against EAB. While much research attention has been focused on antibiosis (e.g. Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2011; 2012; 2014; Chakraborty et al. 2014; Rigsby et al. 2015; *In Review*; and Chapter 6), relatively little research attention

has been directed at antixenotis and has only been peripherally addressed *via* past EAB chemical ecology studies (e.g. Rodriguez-Saona et al. 2006; Crook et al. 2008). The intention of this study is to directly address the potential underlying mechanisms of antixenosis by comparing the bark and canopy volatile organic compound (VOC) profiles of black and Manchurian ash trees, *via* GC-MS, that have been induced with methyl jasmonate (MeJA). MeJA is a derivative of the plant herbivore defense-associated hormone, jasmonic acid, and has been shown to mimic the volatile response of plants to herbivore feeding damage (Rodriguez-Saona et al. 2001; Gols et al. 2003; Rodriguez-Saona et al. 2006). We attempted to draw relationships between VOC profile characteristics, host induction, and host choice by quantifying EAB oviposition on trees from which VOC profiles were determined in a common garden environment. We had the specific hypotheses that (1) EAB oviposition would be substantially higher on black ash than Manchurian ash, (2) MeJA application would result in detectable changes in both bark and canopy VOC profiles and would increase oviposition, and (3) that oviposition could be predicted by certain VOC profile characteristics (e.g. increased emission of certain compounds).

3.2 METHODS

3.2.1 Ash Common Garden, Treatments, Monitoring for Oviposition, and Tree Health.

The ash plantation utilized for these experiments was established in November of 2012 using four year old bare-root stock (10-20 mm diameter at time of planting) from

Bailey Nurseries, Inc. (St. Paul, MN) planted in a randomized block design. Twelve Manchurian (cv. 'Mancana') and 12 black (cv. 'Fall Gold') ash trees were randomly selected, and half of the individuals from both species (six each) were subjected to the MeJA induction treatments. Trees were approximately the same size at the time of experimentation with no differences between species ($t = 0.557$, $P = 0.583$) in diameter immediately below the first set of branches of the main trunk (68.3 ± 0.9 mm). A 100 mM MeJA solution containing 0.01% Tween 20 was applied to all reachable surfaces of these trees until runoff on two occasions: 29 May and 12 June 2015 (Rigsby et al. *In Review*). Levels of oviposition were monitored as described by Rigsby et al. (2014). Briefly, cheesecloth was wrapped around an approximately 50 cm section of the main trunk. Cloth was removed and replaced at weekly intervals for five weeks from 5 June – 10 July and the removed cloth was visually inspected for EAB eggs. Lastly, individual trees were rated for their health condition where canopy dieback, bark splitting, and epicormic sprouting (classic symptoms of EAB infestation) were each rated on a scale of 0-5 with 0 being defined as non-symptomatic and 5 defined as severe, and these values were summed into a "Health Index".

3.2.2 Bark and canopy VOC Sampling

Field sampling of bark and canopy VOCs was performed similarly to the VOC collection procedure described by Böröczky et al. (2012). Briefly, a portable two-pump volatile collection system with a Teflon® sheet chamber (FEP100 fluoropolymer film; Dupont, Wilmington, DE, USA) positioned around a constructed wire frame was secured to the trunk with straps. Air was purified through charcoal and a SuperQ pre-filter (30 mg

SuperQ sorbent; Alltech Associates, Deerfield, IL, USA) and was introduced through the bottom of the Teflon® sheet chamber with one pump. The other pump pulled air from the top of the chamber and passed air through another SuperQ filter which adsorbed VOCs. Canopy collections were performed similarly except that the wire frame was not used. For these, a branch was wrapped with the Teflon® sheet that was secured to the branch with straps. The bark surface area sampled was calculated (bark) and the number of leaflets enclosed within the chamber (canopy) was counted so that emission rates could be normalized on those bases.

Bark VOC sampling was performed on 19 June 2015 (nine days after the second MeJA treatment. VOCs were collected for 3 hrs at a push flow rate of 0.75 (± 0.05) L/min and a pull flow rate of 0.5 (± 0.05) L/min. Flow rates were checked with variable area flow meters (Key Instruments, Trevose, PA, USA) and adjusted if needed between every sampling period. Inclement weather prevented us from sampling canopy VOCs on the same day so canopy sampling was performed on 24 June 2015 and samples were collected for 1 hr using the same flow rates. Filters on which volatile compounds had been collected were sealed with plumbers tape at both ends, wrapped in aluminum foil, and were overnight shipped to the Chemical Ecology Lab at Penn State University. SuperQ pre-filters used to purify air were washed with 600 µL acetone and Teflon® sheets were cleaned with 95% ethanol and kimwipes® between sampling periods in order to eliminate contamination. All Teflon® sheets and pump tubing were washed with scentless soap (Alconox®) and DI H_2O and wiped with ethanol between sampling dates, while wire frames were wiped with ethanol. Latex gloves were worn at all times when equipment was being handled.

3.2.3 GC-MS and Data Processing

Upon arrival at the Chemical Ecology Lab at Penn State University, SuperQ filters with adsorbed VOCs were eluted with 120 µl of a solution containing 100 ng nonyl actetate (internal standard) dissolved in a 1:1 mixture of hexane and dichloromethane (Burdick & Jackson, Morristown, NJ, USA). Eluted samples were analyzed by gas chromatography using an Agilent 6890 chromatograph (Agilent Technologies, Santa Clara, CA) coupled to an Agilent 5973 mass selective analyzer fitted with a Agilent HP-5MS bonded phase capillary column (0.25 mm x 0.25 µm x 30 m). Sample injections of 2 µL were made into an inlet operated in splitless mode with a split delay of 0.75 min and helium carrier gas flow of 0.8 mL/min. The oven was kept at an initial temperature of 60°C for one minute then increased to 150°C at a rate of 3°C/min, 20°C/min to 280°C, and held at 280°C for 5 min. Inlet and interface temperatures were 220°C and 280°C respectively. The mass selective analyzer was operated in electron impact mode using the default settings (ion source: 230°C, quadrupole: 150°C, and spectra generated at 70 eV). Initial identification of analytes was accomplished by matching of representative analyte spectra to reference spectra in the NIST 08, Adams (2012), and Joulain and König (1998) libraries as well as to spectra of authentic synthetic standards. Further confirmation of compound identities was accomplished by calculating retention indices using the Van den Dool and Kratz (1963) formula for temperature-programmed analysis and comparing to the retention index libraries of Adams (2012), MassFinder (Hamburg, Germany), Joulain and König (1998), as well as other published sources.

For the identification and quantification of analytes, a target compound identification method was created in ChemStation (Agilent Technologies, Santa Clara, CA) for compounds of interest manually chosen based on observation of differential emission between experimental treatments and the likelihood of being present in ash volatile emissions. Based on reference spectra obtained from authentic standards, well characterized essential oils, or clean occurrences in the ash VOC samples, an expected retention time, target ion, and relative abundance of up to three qualifier ions were manually chosen and imputed into the software. Automated peak integration, accumulation, and matching were performed through a user-specified algorithm in ChemStation and final selection of best-fit peak matches and peak integration was performed manually for each target compound. The resulting peak areas based on target ion responses from confirmed matches were transformed into approximated total ion peak areas using response factors determined from reference spectra. Absolute quantities of target compounds detected were approximated based on the instrument response to the internal standard, nonyl acetate. It is important to note that the absolute quantities of emitted volatiles are only approximate due to the fact that it was assumed that our instrument was equally sensitive to all analytes, which is not a safe assumption. However, relative quantities within each analyte are accurate despite the absolute quantities being rough estimates. For the purposes of analysis and interpretation, canopy emissions were expressed as pg/hr/leaflet and bark emissions were expressed as ng/hr/m^2.

3.2.4 Statistical Analyses of VOC Profiles

Canopy and bark VOC profiles were analyzed several ways. First, canopy and bark VOCs were placed into one or more of five categories: “whole profiles” consisting of every individual peak detected (but not necessarily identified) in GC chromatograms, GLVs, monoterpenes, sesquiterpenes, and antennally active compounds (AACs) consisting of compounds that have been shown by past studies to be antennally active in GC-EAD experiments. For each category of canopy and bark VOCs, a Permutational Multivariate Analysis of Variance (PERMANOVA; Anderson 2001) utilizing the R package: 'vegan' (Oksanen et al. 2012) was performed using species and MeJA-induction as predictors. PERMANOVA analyses were performed on the Euclidean distances of Hellinger-transformed peak areas with 10,000 permutations (Legendre and Gallagher 2001; Lieurance et al. 2015).

Secondly, differences in canopy and bark VOC category profiles were visualized *via* nonmetric multidimensional scaling (NMDS) using the ‘Gower’ dissimilarity index, also using the ‘vegan’ package in R. The ‘Gower’ dissimilarity index was chosen because it consistently gave the highest rank-order similarity of all possible dissimilarity indices available in the ‘vegan’ package and consistently estimated the NMDS model with the lowest stress statistic (< 0.2 for all ordinations). Lastly, VOC profile categories were analyzed by fitting emission rates of total internal standard-equivalent VOCs in a category (e.g. total VOCs, total GLVs, etc…) to an analysis of variance (ANOVA) model with species and MeJA-induction as predictors, removing insignificant predictors when appropriate.

3.2.5 Statistical Analyses of VOC Profile Constituents

A Monte Carlo re-sampling method was used to test for significant differences in the emission rates of individual compounds. An in-house F-distribution was constructed for each individual compound by calculating differences between the variance of datasets generated by random sampling from the mean variance of the original dataset (10,000 replicates). The F-critical value was found based on the 95% quantile value ($\alpha = 0.05$) which was compared to the F-value calculated *via* a fitted ANOVA model. Calculated F-values above the F-critical were used to directly calculate a P-value (number of Monte Carlo-generated F-values above F-value calculated by ANOVA fit divided by 10,000 total Monte Carlo-generated F-values). This process was only performed using volatiles that were consistently detected being emitted by both species (i.e. emitted by > 50% of individuals of both species). VOCs that were either not emitted by any trees of a species or emitted by ≤ 50% of trees of a species were not analyzed.

3.2.6 Statistical Analyses of Oviposition and Oviposition Relationships with VOCs and Tree Health

Oviposition data were statistically treated in the same manner as described previously by Rigsby et al. (2014). Briefly, data were fitted to a Poission regression and a zero-inflated Poisson (ZIP) regression model and the two models were compared *via* the Vuong's likelihood ratio-based test (Vuong 1989) for non-nested models using the 'pscl' package (Zeileis et al. 2008) in R. The model with the better fit, which was the ZIP model, was then used to calculate the χ^2 difference in log-likelihood functions for models with and without MeJA-induction as a predictor (the effect of species could not be

assessed because no eggs were recovered from cloth placed on Manchurian ash trees; see Results below).

To investigate the potential of emissions of individual VOCs and VOC categories (e.g. total GLV emissions, etc…) to predict oviposition, linear regression models were fitted using cumulative oviposition as the response vriable and emission rate of individual VOC or VOC category as the predictor variable. Tree health was also assessed as a predictor of egg counts by fitting a linear regression to these variables.

3.3 RESULTS

3.3.1 Identification of Compounds and Assignment of Compound Categories

Seventy-eight individual peaks were detected on all canopy and bark aeration samples throughout the sampling period (Table 3.1). Of these, five GLVs were detected and all were able to be identified and 13 monoterpenes or monoterpene ketones were identified, all of which were identified. Additionally, 37 sesquiterpenes or oxygenated sesquiterpenes were detected, 34 of which were able to be assigned an identification. Aside from these, 17 various other volatiles such as alkylbenzenes, aromatic alcohols, alkyl aldehydes, homoterpenes, phenylpropanoids, esters, alkanes, aromatic ketones, and diterpenoids were identified. Lastly, 10 volatiles were not able to be identified and these were not assigned to a category. We additionally detected 16 AACs reported by past authors (Table 3.2).

3.3.2 Whole Profile Characteristics

No species differences, MeJA-induction, or interaction effects were found for total canopy VOC, GLV, monoterpene, sesquiterpene, or AAC emissions (Figure 3.1A). However, significant species differences (ANOVA; $F = 5.184$, $df = 1$, $P = 0.0329$) for total bark monoterpenoid emissions and significant MeJA-induction effects (ANOVA; $F = 5.993$, $df = 1$, $P = 0.0228$) for total bark GLV emissions were detected (Figure 3.1B). PERMANOVA analyses of canopy (Table 3.3) and bark (Table 3.4) VOC profiles revealed that most categories of compounds differed significantly by species ($P < 0.05$) except for bark GLVs which was significantly affected by MeJA-treatment (Table 3.4). NMDS ordination showed reasonable species separations of canopy (Figure 3.2A-E) and bark (Figure 3.3A-E) VOC profiles with all ordinations considered acceptable (stress statistic < 0.2) but several ordinations were considered good (stress statistic < 0.1).

3.3.3 Individual Canopy VOCs

Of the 78 individual peaks detected, 14 canopy VOCs were found to be differentially emitted between species (Table 3.5). Eight of these compounds were consistently detected in collections from both species (emitted by > 50% of individuals) and found to be differentially emitted by species. The remaining six compounds were either found to be inconsistently detectable from one species and consistently detected from the other, or were not detected from one species but consistently detected from the other (Table 3.5). Of these compounds, only β-caryophyllene has been shown to be antennally active and was detected on a consistent basis in Manchurian ash (emitted by 8

of 12 trees), but was only emitted by 4 of 12 black ashes. However, those trees that did emit β-caryophyllene, black (68.5 ± 38.6 pg/hr/leaflet) and Manchurian (52.5 ± 16.9 pg/hr/leaflet) ash appeared to emit it at roughly the same rates.

3.3.4 Individual Bark VOCs

In total there were 19 compounds that were found to either be significantly differentially emitted by one species (five compounds), emitted by one species and not the other (11 compounds) or emitted by ≤ 50% of all individual of one species and consistently emitted by the other species (three compounds) (Table 3.6). Three of these compounds, linalool (10 of 12 trees), α-cubebene (11 of 12 trees), and α-humulene (7 of 12 trees) are AACs found to be consistently emitted by Manchurian but not by black ash bark (≤ 50% of trees). Alternatively, 7-epi-sesquithujene was inconsistently detected being emitted from Manchurian ashes (≤ 50% of trees) but consistently detected emitted from black ash (11 of 12 trees).

3.3.5 VOCs and Oviposition

No EAB eggs were detected on any Manchurian ash trees throughout the sampling period. Alternatively, 83% of black ash trees received eggs through the sampling period and MeJA induction significantly increased egg counts (χ^2 = 105.601, df = 1, P < 0.0001) where control trees received 27.8 ± 11.5 eggs while MeJA-treated trees received 68.3 ± 37.2 eggs. Additionally, oviposition was not significantly predicted by health index (linear regression; t = -1.995, P = 0.074), but there was a slight trend

towards greater egg counts on healthier trees. Total VOC, GLV, monoterpene, sesquiterpene, and AAC emission for both canopy and bark were unable to predict egg counts for black ash trees (linear regression; $P > 0.05$).

3.4 DISCUSSION

Though closely related, Manchurian and black ash have almost completely opposite resistance phenotypes towards EAB and evidence suggests that Manchurian ash possesses antixenotic traits that result in non-preference for feeding (Pureswaren and Poland 2009) and oviposition (Rigsby et al. 2014) in addition to antibiosis (Chakraborty et al. 2014). We attempted to identify volatiles putatively involved in antixenosis by comparing canopy and bark VOC profiles of these two species. We were able to identify 78 distinct peaks in GC-MS chromatograms. Of these, five GLVs, 13 monoterpenes, and 37 sesquiterpenes were identified along with 17 various other volatiles and 10 unidentified volatiles, and we detected 16 compounds that have been shown to be antennally active by past authors. Bark monoterpene emission rates were found to be significantly greater in black ash than Manchurian ash. Additionally, we detected major species differences in all compound categories for canopy emissions and for most of the compound categories for bark emissions *via* PERMANOVA analysis and NMDS ordination. We also detected differential emission by species for 14 canopy and 19 bark compounds. Lastly, though we did not detect significant MeJA-treatment effects on volatile profiles we did find that MeJA-treated black ash received significantly more EAB egg pressure through the season that control black ash and there was an insignificant, but slight trend of increased egg counts on healthier trees.

This research represents the first study to comprehensively profile Manchurian ash canopy and bark VOCs separately, as well as the first study to compare VOC profiles of Manchurian ash to its most closely related North American congener. Past studies have focused on major peaks on GC chromatograms, volatiles detected *via* EAD experiments, or specific classes of volatiles (e.g. Rodriguez-Saona et al. 2006; Crook et al. 2008; de Groot et al. 2008; Pureswaren and Poland 2009; Chen et al. 2011). Rodriguez-Saona et al. (2006) focused on the top 27 compounds (by IS-equivalent peak areas) which comprised over 95% of emissions and Crook et al. (2008) focused on sesquiterpenes that increased in emission rate 24 hrs after green ash trees were girdled. We did not detect several of the compounds reported by past authors did detect dozens of others (especially several sesquiterpenes). We found that the putatively important sesquiterpenes are substantially more abundant and diverse than noted by past authors and that the profile of sesquiterpenes being emitted is distinct between these two species, especially with regard to bark emissions.

The profiles of antennally active compounds also were distinct between the two species. Since they contain several AACs, EAB trap designs employ the use of commercially available oils, chiefly Manuka oil, a distillate from the New Zealand manuka tea tree (*Leptospermum scoparium*), and phoebe oil, a distillate of the Brazilian walnut tree (*Ocotea porosa*; sometimes placed in the genus *Phoebe*) (Crook and Mastro 2010). Douglas et al. (2001) detected 16 compounds from various Manuka oil samples throughout the North Island of New Zealand that we detected emitted from ash and Porter and Wilkins (1998) reported 17 compounds in Manuka oil under the trade name Manex that we also detected being emitted from ash. There is considerably less information

about the constituents of Phoebe oil, but Reynolds and Kite (1995) reported identifying almost twice the number of compounds in this oil (30) than authors have reported in Manuka oil and then reported an abundance of sesquiterpenoid constituents of phoebe oil.

Crook and Mastro (2010) posited that the reason that phoebe oil baited traps catch significantly more beetles than non-phoebe oil baited traps (i.e. no bait or Manuka oil only; Crook et al. 2008) is that phoebe oil contains 7-epi-sesquithujene, which was isolated from white ash and described by Khrimian et al. (2011) and which Manuka oil does not contain. Our data initially would appear to support this hypothesis as we found this compound to be emitted more consistently by black ash bark (11 of 12 trees) than Manchurian ash bark (four of 12 trees). However, the emission rates of those Manchurian ash trees that did emit 7-epi-sesquithujene were incredibly variable (46.60 ± 41.96 ng/hr/m^2; min/max = 0.59/172.23 ng/hr/m^2) and there was no relationship between black ash egg counts and the canopy or bark emission rates of 7-epi-sesquithujene. Our finding of a lack of/inconsistent emissions of 7-epi-sesquithujene is supported by Rodriguez-Saona et al. (2006) who analyzed Manchurian ash VOCs emitted from the entire plant and did not report detecting this compound. If this compound is truly as critical for EAB attraction as hypothesized by past authors, we were not able to detect evidence for it here.

In fact, we were unable to find clear evidence supporting the importance of a single compound or handful of compounds. However, we did detect the differential emission of several individual compounds in one species or another *via* Monte Carlo analysis. Interestingly, linalool and α-cubabene from the bark and β-caryophyllene from the canopy were three AACs found to be either not emitted (linalool and α-cubabene) or not consistently detected (β-caryophyllene) by black ash but consistently detected being

emitted from Manchurian ash. This evidence would perhaps call into question the assumption that these AACs are attractive, but suggests that some AACs may be repellent since antennal activity does not necessarily equate to “attractive” but can reveal repellents as well (e.g. Dube et al. 2011).

This evidence and our results could suggest that the scope of EAB/host interactions with regard to chemical attractants and repellents has perhaps been too narrow. Rather than focusing on one or even few AACs, sesquiterpenoids, monoterpenoids, and/or GLVs, it may be that several volatile constituents act antagonistically or synergistically to result in a behavior or host-use decision. For example, host plants of the sweet potato whitefly, *Bemisia tabaci* (Hemiptera: Aleyrodidae), emit dozens of VOCs containing both attractants and repellents, and whitefly preference appears to be related to the emission of an attractant (linalool) and a repellent (1,8-cineole) (Sacchetti et al. 2015). However, even though whiteflies were attracted to the host plant that emitted the most linalool and the least 1,8-cineole, they did not show a preference towards one of the other two host plants which emitted linalool:1,8-cineole ratios that were more than two orders of magnitude different (Sacchetti et al. 2015). This lack of discrimination strongly implicates roles for other VOC constituents. Furthermore, the emission ratios of certain constituents cannot be ignored as these have profound effects on herbivore attraction (Najar-Rodriguez et al. 2010), which is noted by Crook and Mastro (2010) for EAB trap design. Ratios of different constituent combination emission rates was not explored in this study, but could perhaps be extraordinarily important.

In North America, EAB is considered a specialist insect, only known to infest ash and white fringetree in its invaded range. (Herms and McCullough 2014; Cipollini 2015). It is hypothesized that due to the neural limitations of insects, only having to process a relatively limited amount of information will make specialists more efficient at finding hosts when compared to generalist insects (Bernays 2001; Craig and Itami 2008). Furthermore, the preference-performance or "mother knows best" hypothesis posits that adults will oviposit on those hosts on which their offspring will best perform, and therefore maximize the fitness of the adult (Jaenike 1978). Together, these hypotheses suggest that EAB adult females are selected for efficiently finding potential hosts of good quality. Rigsby et al. (2014) and our own egg count data demonstrate that females do, in fact, distinguish between hosts of better and poorer quality for their offspring and EAB egg counts were substantially higher on susceptible North American hosts than on Manchurian ash. However, the specific cues EAB adult females use for efficient host location and oviposition decisions remain to be uncovered.

Ultimately, the role of host volatiles in EAB attraction and antixenosis remains a complex, open, and important avenue for future research. Evidence suggests that Manchurian ash employs antixenosis mechanisms in its overall resistance strategy towards EAB and volatile attractants/repellents most likely play a large role in host choice decisions and preferences. We found that black and Manchurian ash actually differ substantially in their canopy and bark VOC profiles despite their close phylogenetic relationship (Wallander 2008) and similarity in phloem phenolic profiles (Whitehill et al. 2012; Chakraborty et al. 2014) and our data support the earlier findings of Rigsby et al. (2014) that EAB females prefer to oviposit on black over Manchurian

ash. Further experimentation is required to further identify specific volatiles and/or ratios of volatiles that play an important role in host selection for EAB.

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Table 3.1. GC-MS data, tentative identifications, compound categories, and identification of all volatile compounds detected from canopy and bark aerations of black and Manchurian ash. RT = retention time in minutes, RI = retention index, Target Ion = ion used to identify compound, RF = response factor determined from reference spectra used to approximate total ion peak areas. For compound categories, GLV = green leaf volatile, monoterp ketone = monoterpene ketone, oxygenated sesq = oxygenated sesquiterpene. For identification, S = standard match and L = library match. *N/A* = not applicable.

Peak	RT (min)	RI	Target Ion	RF	Tentative ID	Category	Identification
1	4.472	852	55	9.04	(*E*)-2-hexenal	GLV	S, L
2	4.508	854	67	5.23	(*Z*)-3-hexen-1-ol	GLV	S, L
3	4.710	865	56	4.03	*n*-hexanol	GLV	S, L
4	6.424	934	93	3.66	α-pinene	monoterpene	S, L
5	7.581	973	93	3.48	sabinene	monoterpene	S, L
6	7.700	977	93	3.64	β-pinene	monoterpene	S, L
7	8.115	991	93	4.08	myrcene	monoterpene	S, L
8	8.224	995	105	3.17	Unknown 1	alkylbenzene	*N/A*
9	8.636	1007	67	3.87	(*Z*)-3-hexenyl acetate	GLV	S, L
10	8.801	1011	93	4.35	δ-3-Carene	monoterpene	S, L
11	8.909	1014	43	3.68	hexyl acetate	GLV	S, L
12	9.303	1024	119	2.4	*p*-cymene	monoterpene	S, L
13	9.459	1028	68	6.28	limonene	monoterpene	S, L
14	9.528	1030	84	18.63	1,8-cineole	monoterpene	S, L
15	9.586	1031	79	5.28	benzyl alcohol	aromatic alcohol	S, L

16	9.771	1037	93	4.31	(*Z*)-β-ocimene	monoterpene	S, L
17	10.164	1047	93	4.94	(*E*)-β-ocimene	monoterpene	S, L
18	12.190	1100	93	8.7	linalool	monoterpene	S, L
19	12.377	1105	57	8.65	nonanal	alkyl aldehyde	S, L
20	12.888	1117	69	2.99	nonatriene	homoterpene	S, L
21	14.022	1143	67	4.34	(*Z*)-3-hexenyl isobutyrate	ester	S, L
22	15.848	1187	67	4.31	(*Z*)-3-hexenyl butyrate	ester	S, L
23	16.180	1194	110	9.12	Unknown 2	*N/A*	*N/A*
24	16.198	1195	120	3.47	methyl salicylate	phenylpropanoid	S, L
25	16.416	1200	57	4.53	dodecane	alkane	S, L
26	17.837	1233	82	4.49	(Z)-3-hexenyl isovalerate	ester	L
27	19.110	1263	133	2.95	*p*-ethyl acetophenone	aromatic ketone	L
28	19.464	1271	69	4.47	geranial	monoterpene	S, L
29	19.492	1271	120	2.67	ethyl salicylate	phenylpropanoid	S, L
30	20.374	1292	117	2.41	indole	aromatic	S, L
31	21.782	1325	82	4.8	(*Z*)-3-hexenyl tiglate	ester	S, L
32	22.842	1350	161	7.13	α-cubebene	sesquiterpene	S, L
33	23.515	1366	161	11.85	α-ylangene	sesquiterpene	L
34	23.569	1367	81	6.78	Unknown 3	*N/A*	*N/A*
35	23.602	1368	105	17.25	Unknown 4	sesquiterpene	*N/A*
36	23.945	1378	161	7.84	α-copaene	sesquiterpene	S, L
37	24.113	1381	119	7.07	2-epi-α-funebrene	sesquiterpene	L
38	24.562	1391	119	5.23	7-epi-sesquithujene	sesquiterpene	S, L
39	24.63	1393	93	13.26	β-elemene	sesquiterpene	S, L
40	24.852	1398	81	6.86	Unknown 5	*N/A*	*N/A*
41	25.205	1406	93	5.16	sesquithujene	sesquiterpene	L
42	25.447	1413	119	4.75	α-cedrene	sesquiterpene	S, L

43	25.597	1416	119	7.41	α-cis-bergamotene	sesquiterpene	L
44	25.753	1420	93	14.22	β-caryophyllene	sesquiterpene	S, L
45	26.039	1427	121	12.53	γ-elemene	sesquiterpene	L
46	26.152	1430	161	4.84	β-copaene	sesquiterpene	L
47	26.43	1437	119	6.46	α-trans-bergamotene	sesquiterpene	L
48	26.731	1444	69	7.74	sesquisabinene	sesquiterpene	L
49	26.926	1449	189	4.78	Unknown 6	sesquiterpene	*N/A*
50	27.114	1454	151	23.01	geranyl acetone	monoterp ketone	L
51	27.130	1454	93	4.71	α-humulene	sesquiterpene	S, L
52	27.299	1459	69	5.66	(*E*)-β-farnesene	sesquiterpene	S, L
53	27.681	1468	119	7.95	β-acoradiene	sesquiterpene	L
54	28.029	1476	189	8.65	Unknown 7	*N/A*	*N/A*
55	28.346	1484	132	10.58	α-curcumene	sesquiterpene	L
56	28.386	1485	105	11.08	aristolochene	sesquiterpene	L
57	28.458	1487	105	19.21	β-selinene	sesquiterpene	L
58	28.515	1488	161	15.84	eremophilene	sesquiterpene	L
59	28.818	1496	189	18.68	α-selinene	sesquiterpene	L
60	28.830	1496	93	9.9	(Z,*E*)-α-farnesene	sesquiterpene	S, L
61	29.037	1501	105	6.49	α-muurolene	sesquiterpene	L
62	29.360	1511	93	7.79	(*E*,*E*)-α-farnesene	sesquiterpene	S, L
63	29.570	1517	161	6.07	γ-cadinene	sesquiterpene	L
64	29.941	1528	161	7.79	δ-cadinene	sesquiterpene	L
65	30.326	1540	69	7.23	(*Z*)-nerolidol	oxygenated sesq	S, L
66	30.930	1558	59	13.58	elemol	oxygenated sesq	S, L
67	31.437	1574	69	8.04	(*E*)-nerolidol	oxygenated sesq	S, L
68	31.626	1579	105	5.07	(*Z*)-3-hexenyl benzoate	ester	S, L
69	31.961	1589	79	19.29	caryophyllene oxide	oxygenated sesq	L

70	32.007	1591	105	2.36	(*E*)-2-hexenyl benzoate	ester	L
71	32.934	1640	189	15.03	γ-eudesmol	sesquiterpene	L
72	33.236	1660	59	7.54	β-eudesmol	sesquiterpene	L
73	33.278	1663	59	13.15	α-eudesmol	sesquiterpene	L
74	33.429	1673	85	7.18	Unknown 8	*N/A*	*N/A*
75	33.791	1697	108	16.7	Unknown 9	oxygenated sesq	*N/A*
76	33.815	1698	43	10.92	Unknown 10	*N/A*	*N/A*
77	36.781	2093	255	7.39	abietatriene	diterpenoid	L
78	36.812	2103	91	15.77	abieta-8,12-diene	diterpenoid	L

Table 3.2. Antennally active compounds identified by past authors that were also identified from canopy and bark aerations from black and Manchurian ash in this study. GLV = green leaf volatile.

Tentative ID	Compound Class	Reference
(*E*)-2-hexenal	GLV	Rodriguez-Saona et al. (2006)
(*Z*)-3-hexen-1-ol	GLV	Rodriguez-Saona et al. (2006)
n-hexanol	GLV	de Groot et al. (2008)
(*Z*)-3-hexenyl acetate	GLV	Rodriguez-Saona et al. (2006)
hexyl acetate	GLV	Rodriguez-Saona et al. (2006)
(*E*)-β-ocimene	monoterpene	Rodriguez-Saona et al. (2006)
linalool	monoterpene	Rodriguez-Saona et al. (2006)
nonanal	Alkyl Aldehyde	Rodriguez-Saona et al. (2006)
nonatriene	homoterpenoid	Rodriguez-Saona et al. (2006)
α-cubebene	sesquiterpene	Crook et al. (2008)
α-copaene	sesquiterpene	Crook et al. (2008)
7-epi-sesquithujene	sesquiterpene	Crook et al. (2008)
β-caryophyllene	sesquiterpene	Rodriguez-Saona et al. (2006)
α-humulene	sesquiterpene	Crook et al. (2008)
eremophilene	sesquiterpene	Crook et al. (2008)/Cossé et al. (2008)
(*E*,*E*)-α-farnesene	sesquiterpene	Rodriguez-Saona et al. (2006)

Table 3.3. Predictors with significant effects on canopy volatile profiles as revealed by way of PERMANOVA analysis. PERMANOVA analysis was performed on the Hellinger-transformed peak areas for the whole VOC profiles (Whole Profiles), green leaf volatiles (GLVs), monoterpenes, sesquiterpenes, and antennally active compounds (AACs). Predictors not appearing in table were not significant ($P > 0.05$).

	Canopy			
Category	Predictor	F	df	P
Whole Profile	Species	7.34	1	0.0002
GLVs	Species	6.77	1	0.0078
Monoterpenes	Species	8.94	1	0.0004
Sesquiterpenes	Species	2.34	1	0.0209
AACs	Species	3.27	1	0.0381

Table 3.4. Predictors with significant effects on bark volatile profiles as revealed by way of PERMANOVA analysis. PERMANOVA analysis was performed on the Hellinger-transformed peak areas for the whole VOC profiles (Whole Profiles), green leaf volatiles (GLVs), monoterpenes, sesquiterpenes, and antennally active compounds (AACs). Predictors not appearing in table were not significant (P > 0.05).

	Bark			
Category	Predictor	F	df	P
Whole Profile	Species	12.97	1	0.0001
GLVs	MeJA	9.28	1	0.0026
Monoterpenes	Species	16.64	1	< 0.0001
Monoterpenes	Sp. x MeJA	2.81	1	0.0366
Sesquiterpenes	Species	38.70	1	< 0.0001
AACs	Species	13.35	1	< 0.0001

Table 3.5. Canopy emission rates ($\bar{x}$ ± 1 SE pg/hr/leaflet) of compounds found to be differentially emitted by black and Manchurian ash. * = antennally active compound, NCD = not consistently detected (emitted by ≤ 50% of trees within a species), ND = compound was not detected being emitted from any trees of a species, *N/A* = not applicable.

Tentative ID	Black ($\bar{x}$ ± 1 SE)	Manchurian ($\bar{x}$ ± 1 SE)	F-Critical	F-Value	P-Value
α-pinene	16.59 (1.23)	8.36 (1.08)	4.41	25.19	< 0.001
sabinene	9.08 (1.25)	1.40 (0.28)	5.22	37.07	0.001
β-pinene	6.43 (0.62)	3.55 (0.66)	4.32	10.19	0.005
1,8-cineole	15.58 (1.99)	9.49 (1.09)	4.17	7.15	0.014
methyl salicylate	155.78 (30.65)	8.82 (1.59)	5.18	22.93	0.005
β-caryophyllene*	NCD	35.15 (13.36)	*N/A*	*N/A*	*N/A*
α-trans-bergamotene	NCD	2.69 (1.34)	*N/A*	*N/A*	*N/A*
β-acoradiene	3.40 (1.60)	ND	*N/A*	*N/A*	*N/A*
γ-cadinene	ND	4.28 (1.83)	*N/A*	*N/A*	*N/A*
(Z)-3-hexenyl benzoate	15.85 (3.50)	75.50 (21.39)	7.28	7.57	0.047
β-eudesmol	ND	1.05 (0.34)	*N/A*	*N/A*	*N/A*
unknown	ND	42.67 (12.06)	*N/A*	*N/A*	*N/A*
abietatriene	3.72 (1.98)	544.00 (119.05)	5.05	20.61	0.004
abieta-8,12-diene	NCD	165.12 (53.74)	*N/A*	*N/A*	*N/A*

Table 3.6. Bark emission rates ($\bar{x}$ ± 1 SE ng/hr/m^2) of compounds found to be differentially emitted by black and Manchurian ash. * = antennally active compound, NCD = not consistently detected (emitted by ≤ 50% of trees within a species), ND = compound was not detected being emitted from any trees of a species, *N/A* = not applicable.

Putative ID	Black ($\bar{x}$ ± 1 SE)	Manchurian ($\bar{x}$ ± 1 SE)	F-Critical	F-Value	P-Value
linalool*	ND	5.44 (2.22)	*N/A*	*N/A*	*N/A*
geranial	3.10 (0.94)	ND	*N/A*	*N/A*	*N/A*
α-cubebene*	ND	3.55 (0.97)	*N/A*	*N/A*	*N/A*
2-epi-α-funebrene	17.45 (3.13)	ND	*N/A*	*N/A*	*N/A*
7-epi-sesquithujene*	23.58 (8.26)	NCD	*N/A*	*N/A*	*N/A*
β-elemene	ND	28.29 (2.85)	*N/A*	*N/A*	*N/A*
α-cedrene	2.72 (0.42)	0.41 (0.07)	5.02	17.94	0.0008
γ-elemene	12.89 (2.81)	ND	*N/A*	*N/A*	*N/A*
α-trans-bergamotene	29.31 (9.35)	140.27 (36.57)	5.44	9.08	0.0173
α-humulene*	NCD	2.84 (0.68)	*N/A*	*N/A*	*N/A*
Unknown 7	3.74 (1.19)	13.94 (2.89)	5.47	13.28	0.0039
α-curcumene	4.76 (1.40)	9.25 (1.77)	4.39	6.79	0.0202
aristolochene	ND	17.98 (2.78)	*N/A*	*N/A*	*N/A*
β-selinene	ND	40.93 (6.38)	*N/A*	*N/A*	*N/A*
α-selinene	NCD	23.98 (4.00)	*N/A*	*N/A*	*N/A*
γ-cadinene	ND	6.62 (0.90)	*N/A*	*N/A*	*N/A*
elemol	ND	8.38 (1.50)	*N/A*	*N/A*	*N/A*
γ-eudesmol	ND	7.19 (1.57)	*N/A*	*N/A*	*N/A*
abietatriene	1.97 (0.40)	4.57 (1.77)	4.12	18.12	0.0004

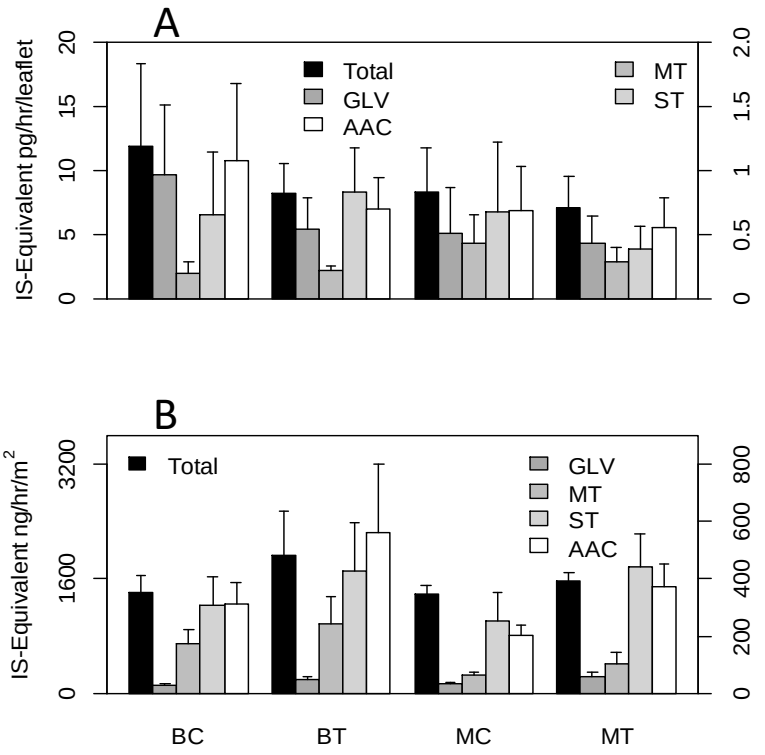

Figure 3.1. Emission rates of total volatile organic compounds (VOCs; black bars), green leaf volatiles (GLVs; dark gray bars), monoterpenes (gray bars), sesquiterpenes (light gray bars), and antenally active compounds (AACs; white bars) of canopy (A) and bark (B) by species/treatment combination. For canopy (A) emissions, total VOCs, GLVs, and AACs are plotted on the primary (left) y-axis and monoterpenes and sesquiterpenes are plotted on the secondary (right) y-axis. For bark (B) emissions, total VOCs are plotted on the primary y-axis and the remaining categories are plotted on the secondary y-axis. BC is control black ash, BT is MeJA-treated black ash, MC is control Manchurian ash, MT and MeJA-treated Manchurian ash

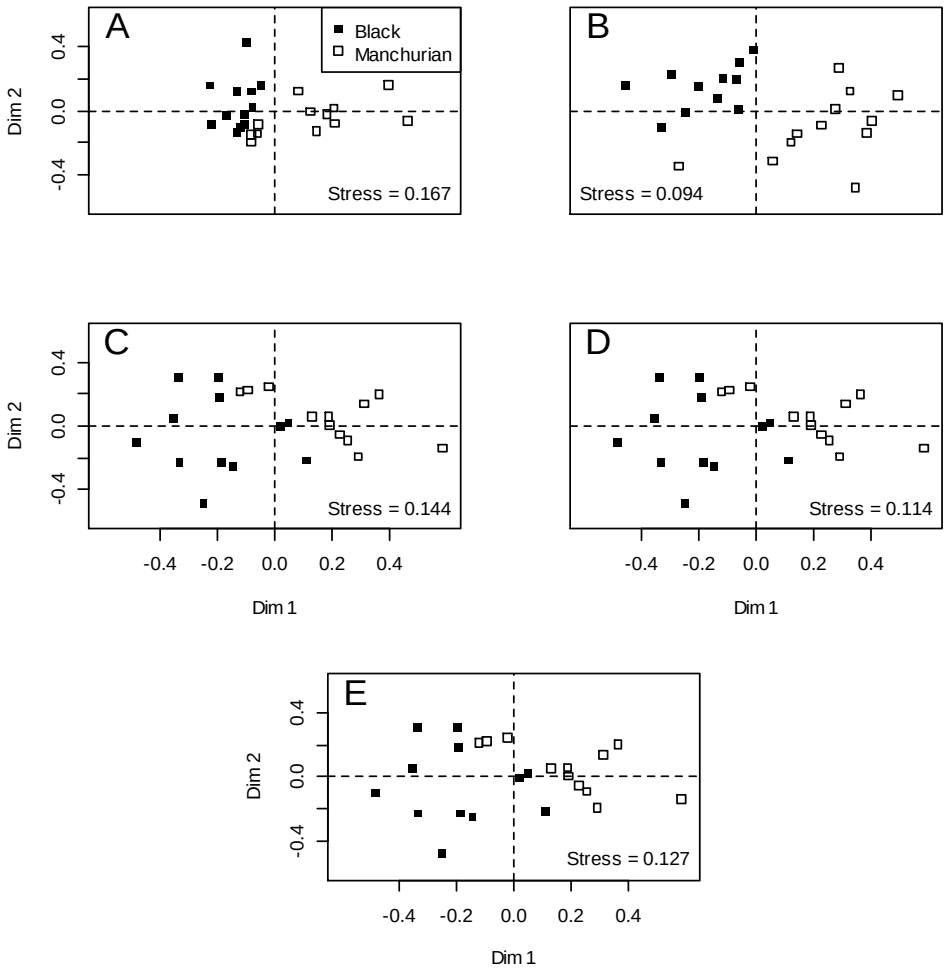

Figure 3.2. NMDS ordinations of canopy (A) whole VOC profiles, (B) green leaf volatiles (GLVs), (C) monoterpenes, (D) sesquiterpenes, (E) and antennally active compounds (AACs). Black squares are black ash and open squares are Manchurian ash trees. Black boxes are black ashes and white boxes are Manchurian ashes. In all cases, stress statistics were < 0.2 indicating an acceptable fit.

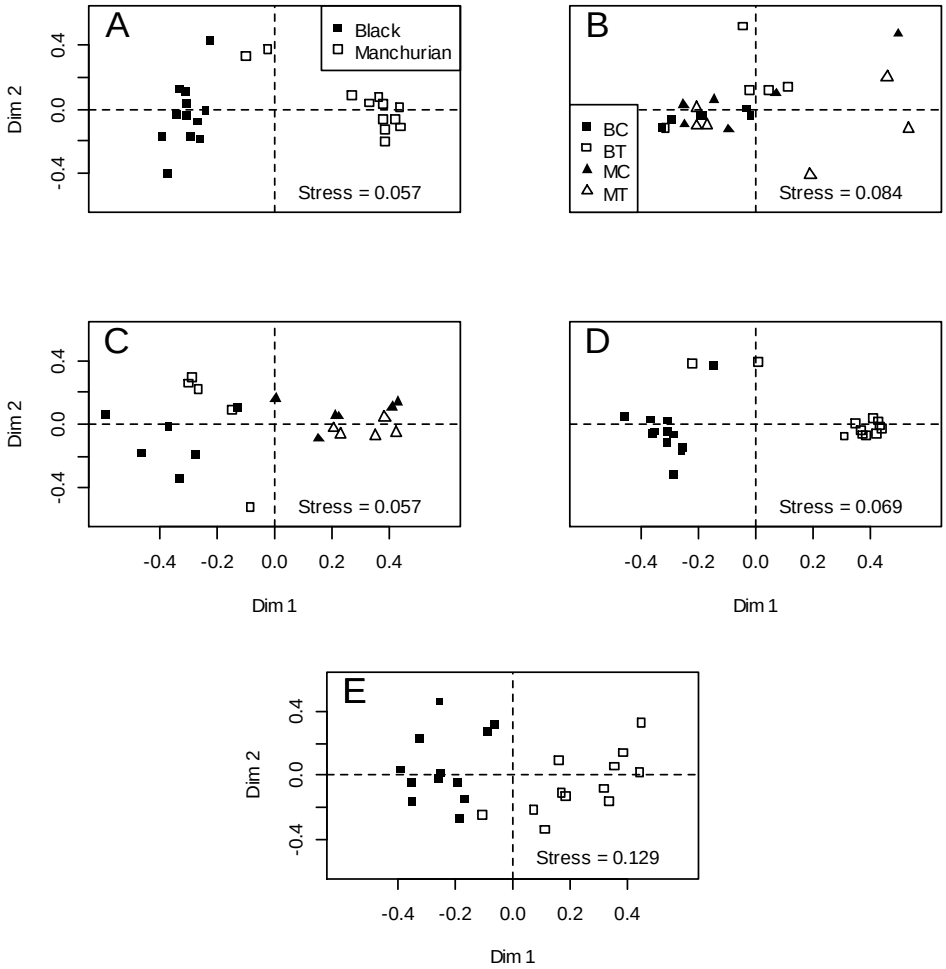

Figure 3.3. NMDS ordinations of bark (A) whole VOC profiles, (B) green leaf volatiles (GLVs), (C) monoterpenes, (D) sesquiterpenes, (E) and antennally active compounds (AACs). Black squares are black ash and open squares are Manchurian ash trees for plots A, D, and E. Black squares are control black ash (BC), open squares are MeJA-treated black ash (BT), black triangles are control Manchurian ash (MC), and open triangles are MeJA-treated Manchurian ash (MT). In all cases, stress statistics were < 0.2 indicating an acceptable fit.

4 PHYSIOLOGICAL RESPONSES OF EMERALD ASH BORER LARVAE TO FEEDING ON RESISTANCE AND SUSCEPTIBLE HOSTS

4.1 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 is 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 as a β-glucosidase (EC 3.2.1.21) gene were expressed more highly in EAB larvae that had fed on green ash (*F. pennsylvanica*), a susceptible North American species, than those that fed on Manchurian ash (*F. 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.

4.2 METHODS

4.2.1 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 to 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.

4.2.2 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 used in only 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 four 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).

4.2.3 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).

4.2.4 Detoxification Enzymes

Quantification of P450 activity was performed as in Rose et al. (1995) and was expressed as nmoles *p*-nitrophenol produced per minute 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 hr per mg protein (nmols/hr/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).

4.2.5 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).

4.2.6 Antioxidant Enzymes

Catalase 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).

4.2.7 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.

4.2.8 Statistical 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 ($\alpha \leq 0.05$), 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).

4.3 RESULTS

4.3.1. 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 α = 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.

4.3.2 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.

4.3.3 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 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 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.

4.4 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 (*F. 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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Table 4.1 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/hr/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 Materials and Methods) and are provided for comparative purposes.

	Activity						
Host	**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)

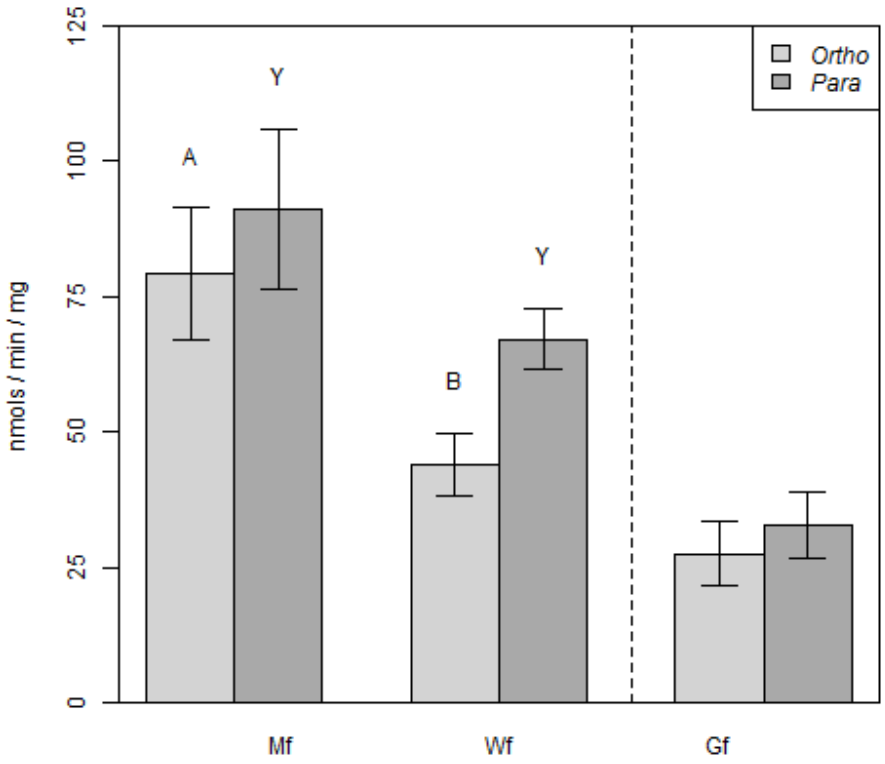

Figure 4.1. Mean *ortho*-quinone (*Ortho*; light grey bars) reductase and *para*-quinone (*Para*; dark grey bars) reductase activities (± 1 SE) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences within specific enzyme activity. Values for Gf larvae cannot be evaluated statistically (see Materials and Methods) and are provided for comparative purposes.

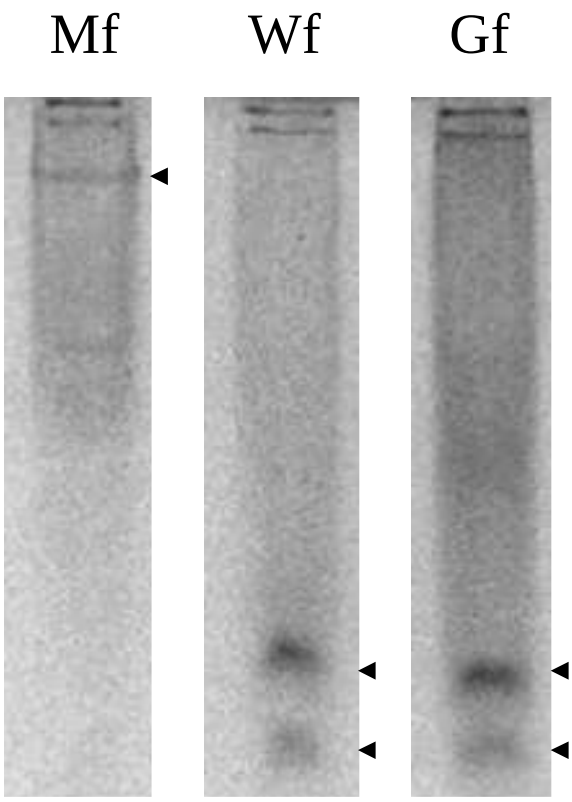

Figure 4.2. Native PAGE gel stained for serine protease activity with N-acetyl-DL-phenylalanine β-naphthyl ester (APNE) and Fast Blue B salt. Mf, Wf, and Gf indicate Manchurian ash-fed, white ash-fed, and green ash-fed larvae, respectively. Arrows indicate differentially expressed proteases between larval groups.

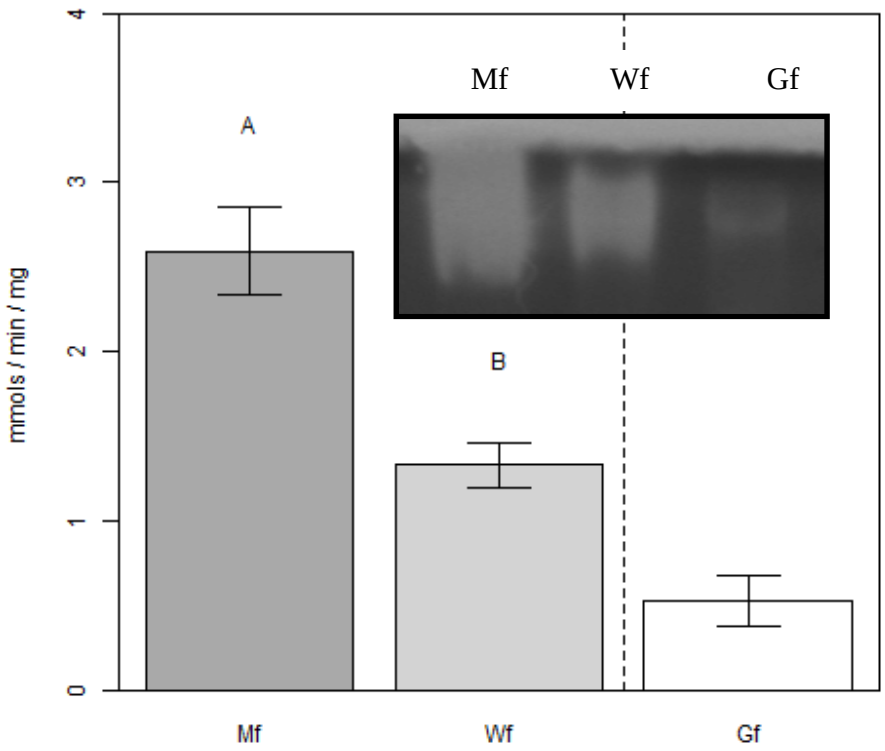

Figure 4.3. Mean catalase activity (± 1 SE) and catalase isozyme expression (insert) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences. Values for Gf larvae cannot be evaluated statistically (see Materials and Methods) and are provided for comparative purposes.

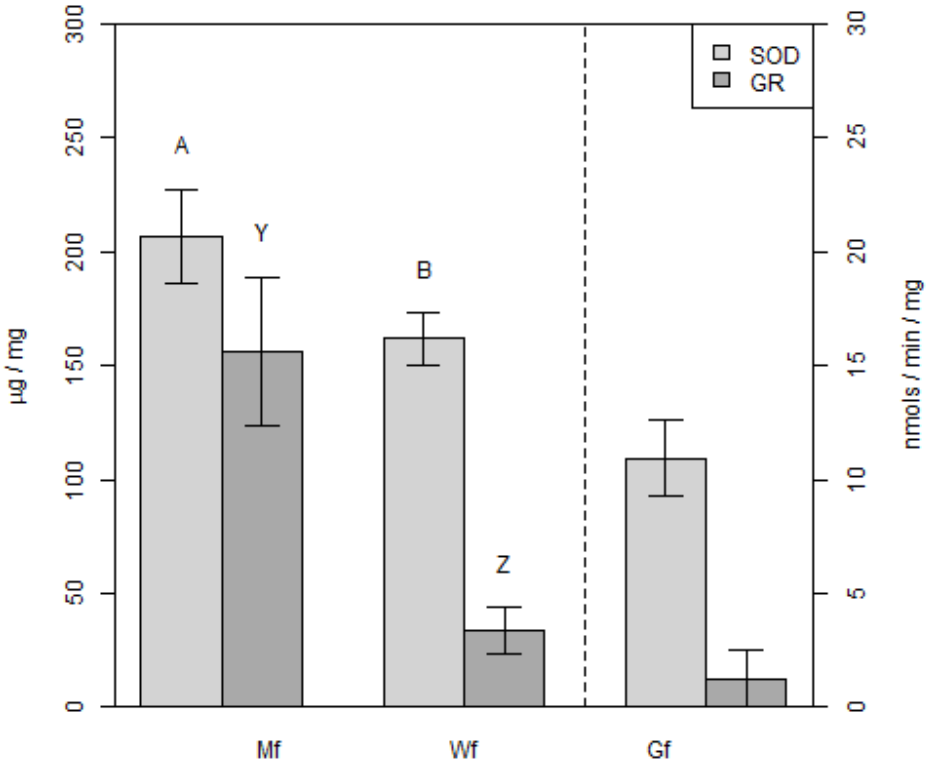

Figure 4.4. Mean superoxide dismutase (SOD; light grey bars; left Y-axis) and glutathione reductase activity (GR; dark grey bars; right Y-axis) (± 1 SE) of Manchurian ash-fed (Mf), white ash-fed (Wf), and green ash-fed (Gf) larval extracts. Unique letters indicate significant differences within enzyme type. Values for Gf larvae cannot be evaluated statistically (see Materials and Methods) and are provided for comparative purposes.

5 OXIDATIVE AND OTHER DEFENSE-ASSOCIATED ENZYME ACTIVITIES AND FUNCTIONAL EXPRESSION OF MANCHURIAN AND BLACK ASH

5.1 INTRODUCTION

Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae), is an Asian wood-boring beetle that has caused widespread mortality of ash (*Fraxinus* spp.) in North America (Herms and McCullough, 2014). Larvae feed primarily on phloem and resistance to this beetle has been studied primarily by comparing the co-evolved, resistant Manchurian ash (*F. mandshurica*) to the naïve North American and highly susceptible green (*F. pennsylvanica*), white (*F. americana*), and black (*F. nigra*) ash (reviewed by Villari et al., 2016). Comparison of phenolic profiles of constitutive (Whitehill et al., 2012), and induced (Whitehill et al., 2014; Chakraborty et al., 2014) phloem tissue of black and Manchurian ash has revealed few qualitative differences, reflecting their close phylogenetic relationship (Wallander, 2008). In fact, the phloem phenolic profiles of these two species have proved to be remarkably similar, which has complicated efforts to identify mechanisms responsible for interspecific patterns of resistance. However, comparison of these two species continues to provide a promising approach for identifying mechanisms of resistance of Manchurian ash to EAB, because they are so

closely related and chemically similar, yet differ so dramatically in their resistance to EAB.

Rigsby et al. (2015) reported that larvae that had fed on Manchurian, white, and green ash had similar activities of major detoxification enzymes (i.e. glutathione-*S*-transferases, cytochrome P450 monooxygenases, carboxylesterases, and sulfotransferases), which indicates that larval detoxification enzymes respond similarly to compounds present in both resistant and susceptible ash species. Phloem phenolic profiles of black and Manchurian ash differ from white and green ash, mostly due to the presence of certain coumarins, phenylethanoids and lignans, and absence of several flavonoids (Eyles et al. 2007; Cipollini et al. 2011; Whitehill et al. 2012). The relatively substantial differences in phloem phenolic chemistries between Manchurian ash and white and green ash, coupled with lack of differences in detoxification enzymes of larvae feeding on these hosts, suggest that direct toxicity of these metabolites may not play a central role in resistance. Given the similarities in phenolic profiles of Manchurian and black ash, the greater resistance of Manchurian ash to EAB may be explained by differential metabolism of phenolic compounds and/or the activities of defense-associated enzymes.

Unoxidized phenolics still may perform some defensive function. Trypsin inhibitor (TI) activity has been quantified using less purified protein extracts in past studies (Cipollini et al. 2011, Whitehill et al., 2014), but has been difficult to detect using more purified protein extracts (C.M. Rigsby, unpublished data). This suggests that the TI activity may be due to compounds other than proteins, e.g. phenolics, which have been shown to act as non-competitive trypsin inhibitors in several plant species (e.g. Shahwar

et al. 2012). Identifying the source of the observed TI activity is important, as EAB appears to rely heavily on serine proteases for protein digestion (Mittapalli et al., 2010; Rigsby et al., 2015). Rigsby et al. (2015) presented indirect evidence that EAB larvae that fed on different hosts had unique serine protease functional expression profiles that were associated with the resistance phenotype of the host.

Aside from the potential role of unoxidized phenolics as trypsin inhibitors, the pro-oxidant activity of phenolic acids, oxidized polyphenols, and phenoxyl radicals may lead to oxidative stress in herbivorous insects (Appel 1993; Summers and Felton 1994; Galati et al. 2002). Rigsby et al. (2015) reported that larvae feeding on Manchurian ash had higher activities of antioxidant and quinone protective enzymes (i.e. catalase, superoxide dismutase, glutathione reductase, and *ortho*-quinone reductase) presumably as defenses against pro-oxidant quinones and reactive oxygen species (ROS). Previous studies have not quantified ROS (e.g. H_2O_2) levels of ash bark, so it is unknown if higher ROS levels accumulate constitutively in Manchurian ash tissue. Water extracts of Manchurian ash brown faster than those of susceptible ash species (presumably due to oxidation of phenolics) (Cipollini et al. 2011), supporting the hypothesis that Manchurian ash has greater pro-oxidant activity.

Plant polyphenol oxidases (PPOs) and peroxidases (POXs) rapidly oxidize polyphenols in the presence of O_2 (PPOs) or H_2O_2 (POXs), and these oxidized phenoxyl radicals can form reactive *o*-quinones that have the potential to cross-link with proteins, reducing the quality of dietary protein and/or damaging midgut proteins (Felton et al., 1992). Phenoxyl radicals may also polymerize into polyphenolic polymers that are also toxic (Appel, 1993). Additionally, laccases (i.e. *p*-diphenol oxidases) and POXs play

major roles in lignin formation *via* the oxidation of monolignols (e.g. sinapyl alcohol) into monolignol radicals that spontaneously polymerize to form lignin (Wang et al., 2013). Lignification occurs in response to pathogen infection and wounding (Vance et al., 1980) and can contribute to herbivore resistance (Wainhouse et al., 1990). Interestingly, interspecific variation in host plant resistance is often associated with variation in PPO/laccase or POX activities (e.g. Goldwasser et al., 1999), rates of lignification, and the rate of wound periderm formation (e.g. Hebard et al., 1984). Manchurian, green, and white ash do not differ in constitutive phloem lignin levels (Cipollini et al., 2011). However, Manchurian ash typically has faster wound-healing and callus tissue formation than susceptible ashes (authors' personal observations). Whitehill et al. (2014) studied intraspecific variation in response of several ash species to MeJA application and found that increased bark lignin levels were correlated with decreased EAB exit hole density. Collectively, these studies suggest that differential oxidase activities may be more important than qualitative or quantitative variation in phenolic profiles in driving inter- and intraspecific variation in resistance of ash to EAB.

Variation in resistance could also result from differential activity of other defense-associated enzymes, in addition to oxidases. For example, the activation of oleuropein (a secoiridoid) by β-glucosidase into a toxic protein denaturant was found to differentiate resistant and susceptible varieties of privet (*Ligustrum obtusifolium*, Oleaceae; Konno et al., 1999) and olive (*Olea europaea*, Oleaceae; Spadafora et al., 2008). This same mechanism could be part of a defense response in ash as oleuropein and other iridoid glycosides are common in ash species (Whitehill et al. 2012), but β-glucosidase activity has not been quantified in ash. Plant chitinases (CHIs) are primarily associated with

pathogen resistance, but CHI genes can also be induced by insect herbivory (Zhu-Salzman et al., 2004) or application of insect regurgitant (Lawrence et al., 2008), where CHI enzymes would presumably degrade the peritrophic membrane. Corrado et al. (2012) reported that a CHI gene was upregulated in olive fruits in response to feeding by fruit fly larvae *(Bactrocera oleae*, Diptera: Tephritidae). A similar defense response may also occur in ash if feeding by EAB larvae induces higher activity of CHI, or if CHI enzymes of Manchurian ash are functionally more efficient than those of susceptible species. Lipoxygenase (LOX) has been shown to have direct effect on herbivores (Felton et al., 1994) and indirect effects in plant defense (War et al. 2012). Lipoxygenase catalyzes the hydroperoxidation of polyunsaturated fatty acids into fatty acid hydroperoxides, which are eventually enzymatically or chemically degraded into reactive aldyhydes, epoxides, and ROS (War et al. 2012). The oxidation of linoleic acid is a major function of LOX with respect to the synthesis of jasmonic acid (JA), which is a hormone associated with herbivore-induced resistance in plants (Feussner and Wasternack 2002).

The objective of this study was to test the hypothesis that Manchurian ash possesses higher constitutive or inducible activities of oxidative and other defense-associated enzymes than black ash, which may contribute to its higher oxidative stress imposed on feeding larvae and higher resistance to EAB. We used the exogenous application of MeJA, a derivative of JA, to assess inducible responses of these defense-associated enzymes and activities. The effect of JA can be mimicked by the exogenous application of MeJA (Erbilgin et al., 2006), which increased ash resistance to EAB in a previous study (Whitehill et al., 2014). Specifically, we quantified phloem H_2O_2 levels; assayed the activity of oxidative and other defense-associated enzymes (LOX, CHI, βG,

PPO, and POX); examined the differential expression of functional βG, PPO and POX isozymes; assessed the ability of ash PPO and POX enzymes to mediate protein cross-linking; and characterized the source of TI activity using protein, methanol, and water extracts where phenolics were either allowed to oxidize prior to assaying for activity, or protected from oxidation. We hypothesize that: (1) bark extracts of Manchurian ash will have higher oxidative and other defense-associated enzyme activities and/or the additional functional expression of isozymes than extracts of black ash, (2) Manchurian ash bark will have higher levels of H_2O_2 which is a ROS and a co-substrate for POXs, than black ash, (3) the increased activity of oxidative enzyme activities will result in the increased ability of water extracts to cause oxidative damage to proteins (i.e. cross-link proteins), and (4) that TI activity will be similar between species, but inducible by MeJA and that phenolics contribute heavily to TI activity

5.2 METHODS

5.2.1 Common Garden, Treatments, and Tissue Harvesting

A common garden containing 24 Manchurian cv. 'Mancana' ash and 24 black ash cv. 'Fallgold' was established in April 2011 at The Ohio State University's Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH, with trees planted in a randomized complete block design with three blocks. Trees were obtained as five-year-old bare root saplings from Bailey Nurseries, Inc. (St. Paul, MN) and were approximately the same size in May 2014 at the time of experimentation, with no species differences in stem diameter at 50 cm above the soil line ($\overline{X} \pm 1\ SE = 5.01 \pm 0.07 cm$) (*t*

= 0.72, P = 0.48). Twelve Manchurian ash trees were randomly selected and 11 healthy black ash trees were selected for use in the experiment, with six of the Manchurian and five of the black ash in turn randomly selected to be treated with MeJA, and the remaining trees left untreated. MeJA was applied exogenously on 29 July 2014 following methods similar to those of Whitehill et al. (2014). A 100 mM MeJA solution with 0.01% Tween 20 (v:v) in DI H_2O was applied to runoff directly to all reachable surfaces (i.e. trunk, branches, stems, leaves) using foam brushes. Preliminary experimentation demonstrated that the 1 M MeJA concentration used by Whitehill et al. (2014) was phytotoxic to our trees (denoted by extensive phloem browning and tissue death) and that a 100 mM MeJA concentration appeared to be enough to induce defenses with no obvious phytotoxicity. Experimental controls were painted at the same time with 0.01% v:v Tween 20 in DI H_2O. Three days later, on 1 August 2014, three to five branches from MeJA-treated and control trees were pruned from each tree, flash frozen in liquid N_2, and transported back to the lab at Wright State University where they were stored at -80°C until extractions were performed. Phloem tissue was cut from branches with a sterile razor directly into liquid N_2, ground using a mortar and pestle, and partitioned to different extracts.

5.2.2 Analytical Equipment and Reagents

LOX activity assays were performed in 1 mL quartz cuvettes (Fisher Scientific) and measured in a Spectronic® Genesys™ UV/Visible Spectrophotometer. All other enzyme activities were assayed using standard round-bottomed 96-well polystyrene microplates (BD Bioscience, Billerica, MA) with absorbance read using a SpectraMAX

190 microplate reader (Molecular Devices, Sunnyvale, CA). A Bio-Rad (Hercules, CA) mini PROTEAN® 3 system was used for electrophoretic separation of protein. Polyvinylpolypyrrolidone (PVPP; 25 µm average particle size) was purchased from The Vintner Vault (Paso Robles, CA). Ascorbate, dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), sodium dodecyl sulfate (SDS), and phenol were purchased from Fisher Scientific (Fair Lawn, NJ). Bradford assay dye concentrate was purchased from BioRad (Hercules, CA). All other reagents were purchased from Sigma (St. Louis, MO).

5.2.3 Extractions and Protein, Phenolic, and H_2O_2 Estimations

Ash phloem tissue contains high levels of phenolic compounds and extracted protein is routinely of reduced quality (presumably due to quinone- and ROS-generation). Intensive preliminary experimentation with the goal of extracting high quality protein resulted in a slightly adjusted procedure from that described by Cipollini et al. (2011). Ground tissue was extracted in buffer (50 mM Na-PO_4, 1 mM each ascorbate, DTT, EDTA, and phenylmethylsulfonyl fluoride [PMSF], 7% PVPP, pH 7.0) for 1 hr at a 1 g to 5 mL ratio. Tubes were then centrifuged at 2,000 *g* for 10 min, the supernatant transferred to fresh tubes, and centrifuged again at 7,000 *g* for 10 min. Supernatant was acetone-precipitated and centrifuged at 16,000 *g* at 0°C for 15 min, the supernatant decanted, and protein pellets allowed to dry in a fume hood for 20-30 min. Pellets were then re-suspended in assay buffer (50 mM sodium phosphate, pH 7.8) and all steps were performed at 2°C unless otherwise noted. The pH of the extraction buffer was chosen in order to be as similar as possible to the pH of the re-suspension buffer without

compromising the polyphenol-adsorbing properties of PVPP, as this is compromised at pH values above 7.0 (Makkar et al., 1995). The pH of the re-suspension buffer was chosen so that it more closely reflected the presumed pH of EAB midguts based on the reliance of EAB on trypsins (Mittapalli et al., 2010) and for consistency with other studies (Rigsby et al. 2015). Protein was estimated according to Bradford (1976) using bovine serum albumin as standard. Water extracts were prepared similarly except that Milli-Q H_2O was used in place of buffer and there was no acetone-precipitation step. Water extract from a biological replicate was partitioned into four 1.5 mL tubes, two of which were placed directly at -20°C (designated "unoxidized" extracts, even though some unavoidable, low level of phenolic oxidation likely occurred during processing) and the other tubes were vortexed and allowed to oxidize overnight at 25°C before storage at -20°C (designated "oxidized" extracts). Methanol extractions and phenolic concentration estimations were performed according to Cipollini et al. (2011). Tissue H_2O_2 levels were quantified as described by Junglee et al. (2014) using KI and a standard curve of H_2O_2.

5.2.4 Defense-Associated Enzyme Activities

Protein extracts were used to assay the activity of all oxidative and other defense-associated enzymes. The activity of LOX was quantified according to Guo et al. (2012), using linoleic acid as substrate ($\varepsilon_{234\ nm}$ = 23,000 M^{-1} cm^{-1}). The activity of CHI was quantified using chitin azure as substrate (Pedraza-Reyes and Lopez-Romero, 1991) in a reaction mixture consisting of 20 µL extract and 980 µL substrate suspension. Chitin azure was chosen rather than the *p*-nitrophenyl-*β*-*N*-acetylglucosaminide employed by Whitehill et al. (2014) because this nitrophenyl-linked substrate is more specifically an

exochitinase substrate whereas chitin azure is a more general CHI substrate. β-glucosidase activity was assayed using *p*-nitrophenyl-β-glucopyranoside (*p*NβG) (Konno et al., 1999) and oleuropein as substrates. For the latter, the release of glucose was measured as in Siemens and Mitchell-Olds (1998). *p*NβG was used as a substrate to assay for general βG activity using a nitrophenyl-linked substrate, which was also used by Konno et al. (1999). Oleuropein was chosen because this secoiridoid is found in both ash species, and this compound and the β-glucosidase enzyme are part of the two-component defense system in privet (Konno et al. 1999) and olive (Spadafora et al. 2008). PPO activity was quantified according to Cipollini et al. (2011) using caffeic acid and catechol as substrates. Caffeic acid was chosen so that relative PPO activities of black and Manchurian ash could be compared to the relative activities reported by Cipollini et al. (2011) and Whitehill et al. (2014). Catechol was additionally used because preliminary experiments determined that this was a superior substrate to caffeic acid for ash PPOs. POX activity was quantified by following the co-oxidation of phenol and 4-aminoantipyrine (Carvalho et al., 2006), the oxidative polymerization of oleuropein at 485 nm (absorption peak determined by preliminary experimentation), and the oxidative polymerization of the monolignol analog syringaldazine (Lee et al., 2007). Preliminary experimentation (data not shown) showed that phenol and guaiacol (used in past studies) were equivalent substrates for POX. Oleuropein was used as a substrate for POX to discern whether this compound found in ash could be an acceptable substrate for POXs. Syringaldazine was chosen because it is commonly used to infer lignin polymerization capacity of POXs and laccases. Laccase activity was also tested with syringaldazine (Sollai et al., 2008) to assess any potential role of ash PPOs in monolignol

polymerization. For all defense-associated enzyme activity assays, samples were compared to a control reaction with protein extract replaced with buffer. For simplicity and comparability, enzyme assays and substrates in which extinction coefficients are not available or standards were not used (CHI, PPO, and POX activities), one unit of activity was defined as the change in absorbance of 0.001 (CHI = U/hr/mg; PPO and POX = U/min/mg) and only the linear portion of the curve of the change in absorbance was used to approximate activity.

5.2.5 Zymogram Analyses of Functional Protein Expression

The native PAGE system of Laemmli (1970) was used to detect functional proteins. Protein extracts (30 µg) were separated at 200 V (constant voltage) in 0.75 mm thick, 5% stacking and 10% resolving gels, equilibrated in cold assay buffer for 20 min, and rinsed with DI H_2O before staining. The method of Kwon et al. (1994) was used to identify βG enzymes using esculin and ferric chloride. This procedure was chosen because it is more economical than using a fluorescent substrate (i.e. methylumbellypheryl-β-D-glucoside) and esculin is a coumarin found in both black and Manchurian ash, though at greater levels in black ash. PPO enzymes were detected according to Marri et al. (2003) using catechol and *p*-phenylenediamine, which was added to the substrate solution because it is an anti-fade agent that prolonged substantially the staining intensity so that images could be taken. POXs were identified using guaiacol according to Camillo et al. (2013) (*p*-phenylenediamine was also added to this substrate solution for the same reasons). An additional zymogram was performed to identify unique ash POX isozymes using a 4% stacking and 6% resolving gel. The

purpose of this PAGE was to identify individual POX isozymes, and therefore wells were completely filled with protein extract in order to achieve the maximum amount of staining intensity (i.e. different wells were loaded with different amounts of protein). This was done to achieve improved staining intensity for black ash isozymes since they stained much more lightly than Manchurian ash isozymes in the initial gel. TI staining was performed according to Broadway (1993) using bovine trypsin, acetyl-phenylalanine-β-naphthyl-ester (APNE) and Fast Blue B salt. After staining, the solution was decanted, the gels rinsed in DI H_2O, and images were taken immediately. ImageJ software (NIH) was used to examine gel images to assess differences in staining intensity of bands.

5.2.6 Protein Cross-Linking Assays

The ability of unoxidized water extracts to cross-link proteins was determined by investigating shifts in mobility of soybean trypsin inhibitor (STI) bands in SDS-PAGE gels using methods modified from Leatham et al. (1980) and Konno et al. (1999). The STI standard that was used in these experiments is not a pure product and includes higher molecular weight peptides. The need for a purified protein was not critical, however, as the objective of the assay was to note the increased smearing of any peptide bands and the formation of high molecular weight polymers immobilized at gel interfaces that do not appear in control lanes.

Protein cross-linking mediated by PPOs was examined by mixing 250 µL of 3 mg/mL STI in assay buffer and 200 µL unoxidized water extract at 25°C. Protein cross-linking

mediated by POXs was examined similarly except that 1 µL 30% H_2O_2 was added to the reaction volume (4.8 mM H_2O_2). A 40 µL aliquot of reaction mixture was removed at designated time points chosen by preliminary experimentation (PPO = 0, 1, 3, and 9 hr; POX = 0, 15, 30 min, and 1 hr), subjected to electrophoretic separation, and stained with Coomassie blue. Water extracts used in these assays were from control trees that were diluted with Milli-Q H_2O to normalize phenolic concentrations.

5.2.7 Source of TI Activity and Quantification

TI activity against bovine trypsin of methanol, protein, and oxidized and unoxidized water extracts was quantified using N_{α}-Benzoyl-DL-Arginine-*p*-Nitroanilide (BA*p*NA) (Paulillo et al., 2012). A standard curve of STI was used to express activity as µg STI equivalents per mg protein (protein extracts) or per mg phenolics (all other extracts). Preliminary experiments showed that methanol used in methanol extracts did not inhibit bovine trypsin activity.

5.2.8 Statistical analyses

Outliers were identified via the Dixon test using the 'Outliers' package in R (Komsta, 2011), and were removed (one biological replicate removed for CHI activity). Response variables were tested for normality using a Shapiro-Wilk normality test. The significance of main effects of species and MeJA induction, and their interaction on the activity of enzymes were assessed *via* a two-way analysis of variance. In all cases, statistical significance was indicated by $\alpha \leq 0.05$. All analyses were performed in R (R

Core Team, 2015). Statistical analyses were not performed on the TI activity of oxidized water extracts due to the relatively small values detected as well as the data being severely zero-inflated.

5.3 RESULTS

5.3.1 H_2O_2 Levels

There was no effect of species ($F_{1,23} = 0.008$, $P = 0.931$) on H_2O_2 levels, but MeJA ($F_{1,23} = 14.166$, $P = 0.002$) increased levels by roughly 5% in both species, where control black and Manchurian tissue contained 285.5 ± 6.8 and 285.4 ± 3.5 nmoles/g FW, respectively, and MeJA-treated black and Manchurian ash tissue contained 299.9 ± 2.3 and 300.8 ± 3.5 nmoles/g FW, respectively. There was no species x treatment interaction for H_2O_2 levels ($F_{1,23} = 0.014$, $P = 0.907$).

5.3.2 LOX, CHI, βG, and PPO Activities and Native PAGE

The activity of LOX was significantly affected by both species and MeJA, while their interaction was not significant. Control and MeJA-treated black ash (1.39 ± 0.16 and 1.85 ± 0.22 µmoles/min/mg, respectively) were lower than control and MeJA-treated Manchurian ash (2.60 ± 0.19 and 3.30 ± 0.15 µmoles/min/mg, respectively). CHI, βG, and PPO activities were also higher in Manchurian than black ash, regardless of substrate, but MeJA had no effect on their activities (Tables 5.1 and 5.2). Native PAGE staining revealed only one βG enzyme in both species with the same electrophoretic

mobility and the pattern of staining intensity reflected enzyme activities (gels not shown). Two PPO bands were detected in PAGE of both black ash treatments, but no clear bands could be distinguished in the Manchurian ash samples. However, the Manchurian ash lanes were more heavily stained with PPO activity overall than the lanes containing black ash extract (Figure 5.1A) as evidenced by ImageJ analysis, concurring with enzyme activity data (control and treated black and control Manchurian ash lanes had 52%, 58%, and 90% of the staining intensity of the treated Manchurian ash lane, respectively).

5.3.3 POX Activities and Isozymes

Expression of POX activity was substantially greater in Manchurian than black ash extracts with all three substrates; MeJA had no effect (Tables 5.1 and 5.3). The POX-stained 10% acrylamide gel revealed only one distinguishable POX band in each of the extracts, and the staining intensity of the bands reflected activity assays (Figure 5.1B) as evidenced by ImageJ analysis (control and treated black and control Manchurian lanes had roughly 47%, 59%, and 86% of the staining intensity of the treated Manchurian ash lane, respectively). The black ash enzyme appeared to migrate farther, but distinct isozymes were not detected in either species in this gel. Zymogram staining using a 6% acrylamide gel revealed at least three distinct POX isozymes within each species, but no isozymes unique to either species could be detected (Figure 5.1C).

5.3.4 PPO- and POX-Mediated Protein Cross-Linking Activities of Water Extracts

PPO-mediated protein cross-linking was similar in both species (Figure 5.2A) with respect to both the time course and the intensity of the cross-linking. There was slight cross-linking in extracts of both species after three hours, but cross-linking was most evident at nine hours, as evidenced by the formation of a band of cross-linked protein just below the interface of the stacking and resolving gels, as well as general smearing of protein within the lane. The majority of POX-mediated protein cross-linking for both species took place within the first 15 min (Figure 5.2B). Manchurian ash extract cross-linked the STI standard much more intensely than the black ash extract, as evidenced by the appearance of a band of protein at the stacking gel interface that did not even migrate into the stacking gel. This band was undetectable in the black ash lanes. As detected in the PPO-mediated cross-linking gel, a band of cross-linked protein migrated just into the resolving gel and was roughly of the same intensity for both species in the POX-mediated cross-linking reactions.

5.3.5 Source, Species Differences, and Induction Effects on TI Activity

No TI activity was detected in protein extracts of phloem tissue (Table 5.4) and no proteins with TI activity were detected with native-PAGE staining (data not shown). The TI activity of methanol extracts was usually the highest of all extracts on a per mg phenolic basis (Table 5.4). TI activity of methanol extracts was not affected by species ($F_{1,23} = 2.610$, $P = 0.124$), MeJA ($F_{1,23} = 0.260$, $P = 0.616$), or their interaction ($F_{1,23} = 1.780$, $P = 0.199$). TI activity of unoxidized water extracts, however, did differ between species ($F_{1,23} = 18.500$, $P < 0.001$), with the magnitude of the difference influenced by MeJA induction as evidenced by a species x treatment interaction ($F_{1,23} = 5.680$, $P =$

0.028), coupled with no main effect of the MeJA treatment ($F_{1,23}$ = 2.440, P = 0.136). Overall, TI Activity was about 2x higher in unoxidized water extracts of Manchurian than black ash (Table 5.4). Furthermore application of MeJA induced 69% higher levels of TI activity in Manchurian ash, but had no effect on black ash (Table 5.4). TI activity was negligible in oxidized water extracts, with roughly half of the biological replicates of each species/treatment group expressing no activity, and the rest of the biological replicates expressing activity that was barely detectable (Table 5.4).

5.4 DISCUSSION

Manchurian ash is far more resistant to EAB than its most closely related North America congener black ash (Herms, 2015). However, previous studies have found that these species share remarkably similar phloem phenolic profiles (Whitehill et al., 2012; 2014; Chakraborty et al., 2014) and that drought-induced changes in phenolic profiles are not related to susceptibility (Chakraborty et al., 2014). Furthermore, EAB larvae feeding on hosts that differ substantially in their phenolic profiles (i.e. white, green, and Manchurian ash) showed no differences in the activities of major detoxification enzymes (Rigsby et al., 2015). Collectively, these patterns suggest that variation in EAB resistance of Manchurian and North American ashes is not due to differences in their respective phenolic profiles *per se*. Furthermore, Rigsby et al. (2015) observed that EAB larvae feeding on Manchurian ash had greater activities of antioxidant and quinone-protective enzymes. In this study, therefore, we focused on the activity of oxidative and other defense-associated enzymes as potential resistance mechanisms that differentiate Manchurian and black ash.

We found substantially higher POX activities in Manchurian ash protein extracts, as well as higher activities of CHI, βG, and PPO, although we did not detect unique functional isozyme expression between these species for any of the measured enzymes. LOX activity was also higher in Manchurian ash, and increased with MeJA treatment, as did phloem H_2O_2 levels. Manchurian ash had considerably greater POX-mediated protein cross-linking activity, and both species inhibited trypsin activity *via* phenolics rather than with proteinaceous trypsin inhibitors. Oxidative activation of phenolics is thought to substantially enhance their activity as anti-herbivore defenses (Appel, 1993) and the rapid oxidation of phenolics associated with higher oxidation enzyme activity in Manchurian ash, and greater MeJA inducibility of LOX and H_2O_2, could explain the higher activities of antioxidant and quinone-protective enzymes of EAB larvae feeding on this host (Rigsby et al., 2015). Coupled with the antinutritional effects of oxidized phenolics (Appel 1993), this quinone and ROS stress, mediated by oxidative enzyme, could explain why Manchurian ash is much more resistant to EAB than black ash, despite their very similar phenolic content and profiles. Responses of each of these enzymes are detailed below.

Protein extracts from Manchurian ash phloem expressed substantially higher POX activities than black ash extracts, and these differences were mirrored in native PAGE staining intensity (Figure 5.1B). These differences were not observed in previous studies with ash (Cipollini et al. 2011; Whitehill et al. 2014), perhaps because those assays were carried out at a slightly acidic pH rather than the basic pH used in this study, which was used because EAB relies heavily on trypsins for protein digestion (Mittapalli et al. 2010) which have basic pH optima. Preliminary experiments (data not shown) demonstrated

that POX activities were greatest at more basic pH, and that species differences were not detectable at neutral or acidic pH. Interestingly, all three isozymes detected were expressed in both species, indicating no differences in functional expression of POX isozymes. It is clear that Manchurian ash is able to polymerize phenolic substrates substantially faster than black ash on a per mg protein basis. What remains unclear is whether these differences are driven by POX enzyme quantity or efficiency, which warrants further studies in species comparisons in POX isozyme expression, substrate specificity, and enzyme kinetics.

Lignin has been shown to reduce larval survival and growth of *Dendroctonus micans* in a dose-dependent manner in Norway spruce and Sitka spruce (Wainhouse et al. 1990), and of the weevil *Pissodes strobi* (Coleoptera: Curculionidae) in Sitka spruce (Whitehill, et al 2015). We found that the rate of POX-catalyzed monolignol radical formation and polymerization was more than an order of magnitude greater in Manchurian ash than in black ash extracts. This suggests that lignin could accumulate much faster in Manchurian ash in response to larval feeding. Furthermore, the lack of detectable laccase activity in these extracts suggests that the contribution of non-POX oxidases to monolignol polymerization is negligible and that differences in monolignol polymerization are almost exclusively POX-mediated. Lignin decreased the growth of EAB larvae in artificial diet (Whitehill et al. 2014) and lignification is a critical process in wound periderm formation (Ginzberg, 2008), which is considered to be an important defense of trees against wood-borers (Muilenburg and Herms, 2012). Contrary to past studies that found no difference in constitutive lignin concentration of Manchurian and black ash bark (Cipollini et al. 2011; Whitehill et al. 2014), our findings support past

observations of substantially faster tissue browning and callus formation in Manchurian ash relative to susceptible species in response to EAB feeding (authors' personal observations).

We also observed higher PPO activities in Manchurian relative to black ash extracts, regardless of substrate, which could explain the more rapid browning rates of water extracts in this species reported by Cipollini et al. (2011). This, coupled with the findings of Rigsby et al. (2015) that larvae feeding on Manchurian ash had higher *ortho*-quinone reductase activities than those feeding on susceptible species, indicate that PPO-mediated quinone production is stronger in Manchurian ash than in susceptible ash species. Increased PPO activity and quinone production often contributes to increased herbivore resistance with increased mortality and decreased performance for many insects (e.g. Bhonwong et al. 2009). As was the case with ash POXs, differences in ash PPO activity required a basic pH in order to be observed, which is likely why species differences were not detected in previous studies (Cipollini et al., 2011; Whitehill et al., 2014).

POX-mediated protein cross-linking was also much more intense in Manchurian ash than in black ash extracts, suggesting that POX-mediated oxidative damage to biomolecules (e.g. proteins) is a putative defense mechanism that may be important in resistance to EAB. Protein cross-linking not only reduces the dietary quality of protein for herbivores (Felton et al., 1992), but enhances the cross-linking of structural components and generates impenetrable barriers for pathogens (Dowd, 1994). If feeding by EAB larvae on Manchurian ash results in a cascade of POX-mediated monolignol, phenolic, and protein cross-linking, as suggested by our data, it would explain the slow

growth and rapid encapsulation by callus of larvae feeding on Manchurian ash. Interestingly, PPO-mediated cross-linking did not vary between species, which was unexpected due to the significantly higher activity of PPO in Manchurian protein extracts.

We also detected higher activities of CHI, βG, and LOX in Manchurian relative to black ash extracts. Olive appears to employ CHIs as a resistance mechanism to fruit flies (Corrado et al. 2012), and the same defensive mechanism may be operative in ash. Expression of βG activity and staining intensity of this protein was higher for Manchurian than black ash. This enzyme could cleave secoiridoid glycosides that occur in ash, such as oleuropein and verbascoside (Eyles et al., 2007; Cipollini et al., 2011; Whitehill et al., 2012) to form toxic aglycones, as occurs for oleuropein in privet (Konno et al., 1999). Higher levels of verbascoside, also an iridoid glycoside, were induced in ash by application of MeJA, and verbascoside mortality of EAB larvae, in a dose-dependent manner, when incorporated in artificial diets (Whitehill et al., 2014). LOX activity was also greater for Manchurian than black ash, which is consistent with the higher expression of a LOX gene in Manchurian ash relative to black and green ash reported by Bai et al. (2011). LOX is often induced in plants by herbivores, and can decrease their performance (e.g. Hildebrand et al., 1986). The increased activity of the enzyme may also contribute to increased oxidative stress in EAB larvae when feeding on Manchurian ash.

TI activity was only detected reliably in methanol and unoxidized water extracts, suggesting that unoxidized phenolics were largely responsible for the activity. Phenolic compounds can act as protease inhibitors (e.g. Shahwar et al., 2012), although most studies have focused on proteinaceous protease inhibitors (e.g. Broadway 1993; Cipollini

and Bergelson 2000), which we did not detect in either species. Plant secondary metabolites generally act as non-competitive inhibitors (Shahwar et al., 2012; 2013), and ash phenolics may act in this way. Protein cross-linking also probably results in protein denaturation and inhibition of enzymes, including trypsins. Ultimately, the similarity in constitutive phenolic-based TI activity between the two species indicates that trypsin inhibition does not explain the large difference in resistance between them. However, Whitehill et al. (2014) reported that the addition of soybean trypsin inhibitor to artificial diet decreased survival and performance of EAB larvae so it is clear that larvae are susceptible to trypsin inhibition and that trypsin inhibition likely plays some role as a general defense mechanism.

Whitehill et al. (2014) found that application of MeJA enhanced resistance of black ash and other susceptible ash species to EAB. Other than to increase phloem H_2O_2 levels and LOX activity of both species and TI activity in in Manchurian ash, MeJA had little effect on enzyme activities in this study, perhaps because the concentration we applied was too low to induce greater activities, and/or because there was not adequate time for induced responses to occur prior to harvesting of tissues. H_2O_2 has several functions in stressed plants, including acting as a short-distance defense induction signal in responses to pathogen and herbivore attack (e.g. Levine et al., 1994; Cheeseman, 2007; Peiffer et al., 2009).

Finally, it is important to emphasize that the enzyme activities reported in this study are the first to be quantified at a pH that is more reflective of the putative physiological pH of EAB midguts (Rigsby et al., 2015). Extensive preliminary experimentation revealed that the activities and native PAGE staining profiles of several

enzymes for these two species became indistinguishable once the pH approached neutral and became acidic (data not shown), which was the pH used in past studies (i.e. pH 6.8; Cipollini et al., 2011; Whitehill et al., 2014). Of particular note was POX activity, which was essentially identical between species. In addition to increased protein quality in our extracts, the examination of enzyme activities at a relevant pH improved our ability to detect differences among species.

In summary, Manchurian and black ash are phylogenetically closely related and share very similar phloem phenolic profiles, yet Manchurian ash is much more resistant to EAB. The results of this study are consistent with the hypothesis that the higher resistance of Manchurian ash results from the greater activities of oxidation and defense-associated enzymes. Furthermore, these results are consistent with the observations of Rigsby et al. (2015) that EAB larvae experience much greater oxidative stress when feeding on Manchurian ash than when feeding on susceptible species. Higher activities of POX enzymes in Manchurian ash may contribute to EAB resistance *via* cross-linking of phenolics, monolignols, and proteins, thus decreasing nutritive quality of the host. Lipoxygenase, CHI, βG, and PPO activities were also much higher in Manchurian ash. Variation in rate and intensity of phenolic metabolism may thus be a more important determinant of EAB resistance than qualitative and quantitative variation in phenolic profiles.

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Table 5.1. Results of two-way ANOVA analysis of the effect of species, MeJA treatment, and their interaction on the activity of assayed enzyme activities. # denotes $F_{(1,22)}$ for CHI activity as one outlier was removed from the dataset. Bold F and P-values indicate significant effects.

Enzyme	Substrate	Factor	$F_{(1,23)}$	P
LOX	Linoleic Acid	Species	**54.22**	**<0.001**
		MeJA	**10.88**	**0.004**
		Species x MeJA	0.493	0.491
CHI#	Chitin Azure	Species	**6.555**	**0.019**
		MeJA	1.447	0.245
		Species x MeJA	0.000	0.990
βG	*p*NPG	Species	**5.016**	**0.037**
		MeJA	0.231	0.637
		Species x MeJA	0.082	0.778
	Oleuropein	Species	**6.496**	**0.023**
		MeJA	0.642	0.436
		Species x MeJA	0.183	0.675
PPO	Catechol	Species	**8.822**	**0.009**
		MeJA	1.115	0.307
		Species x MeJA	0.883	0.361
	Caffeic Acid	Species	**13.957**	**0.002**
		MeJA	2.567	0.128
		Species x MeJA	0.626	0.439
POX	Phenol	Species	**37.687**	**< 0.001**
		MeJA	0.726	0.405
		Species x MeJA	0.017	0.899
	Oleuropein	Species	**25.810**	**< 0.001**
		MeJA	0.150	0.704
		Species x MeJA	0.180	0.677
	Syringaldazine	Species	**27.761**	**< 0.001**
		MeJA	0.148	0.707
		Species x MeJA	0.020	0.889

Table 5.2. Mean activity (± 1 SE) of defense-related enzymes by species. CHI activity is presented as U/hr/mg, βG activity is presented as nmols/min/mg, and PPO activity is presented as U/min/mg. *p*NβG = *p*-nitrophenyl-β-glucopyranoside.

	Enzyme				
	CHI	βG		PPO	
	Chitin Azure	*p*NβG	Oleuropein	Catechol	Caffeic Acid
Black	3.0 (0.29)	0.08 (0.010)	4.2 (0.3)	22.3 (4.0)	3.8 (0.9)
Manchurian	4.8 (0.52)	0.11 (0.006)	5.8 (0.5)	44.6 (5.9)	10.5 (1.5)

Table 5.3. Mean activity (± 1 SE) of POX is presented as U/min/mg using three substrates: Phenol, oleuropein, and syringaldazine.

	POX Activity		
	Phenol	Oleuropein	Syringaldazine
Black	17.7 (4.8)	30.7 (4.6)	83.9 (14.5)
Manchurian	82.7 (9.0)	206.4 (34.4)	1,115.3 (175.1)

Table 5.4. Mean (± 1 SE) soybean trypsin inhibitor-equivalent activity of phloem extracts from different species/treatment combinations. Activity of protein extracts is expressed per mg protein while all other extracts are expressed per mg gallic acid-equivalent phenolics.

	TI Activity			
	Protein	Methanol	Water Extracts	
	Extracts	Extracts	Unoxidized	Oxidized
Control Black	0	117.6 (8.3)	42.1 (9.5)	19.6 (4.2)
Treated Black	0	96.4 (15.2)	33.3 (6.0)	7.0 (4.6)
Control Manchurian	0	85.8 (9.4)	61.0 (16.5)	4.5 (4.5)
Treated Manchurian	0	93.4 (9.6)	103.5 (9.1)	9.5 (4.5)

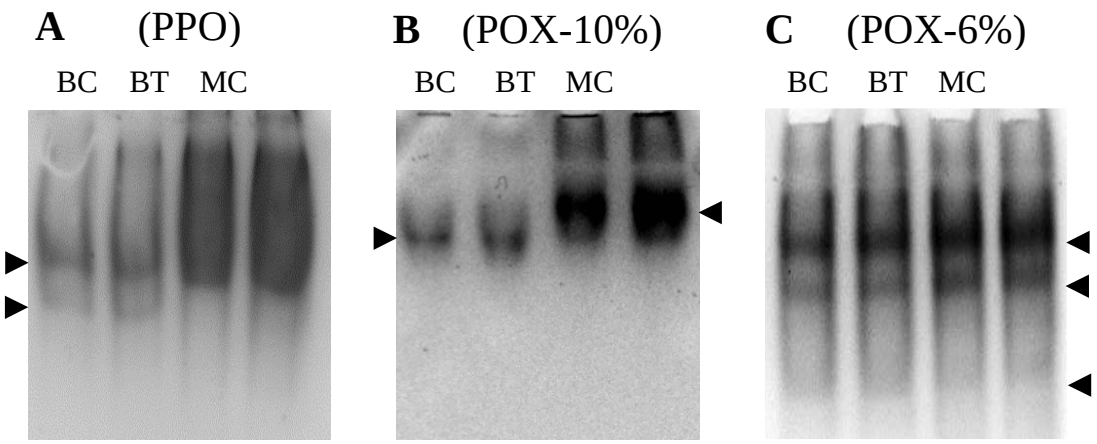

Figure 5.1. (A) Polyacrylamide gel stained with catechol and *p*-phenylenediamine to detect PPO activity. Distinct bands are only evident in black ash lanes (arrowheads) though staining intensity of lanes reflects relative values from PPO spectrophotometric activity measurements. Control and treated black and control Manchurian ash lanes had 52%, 58%, and 90% of the staining intensity of the treated Manchurian ash lane, respectively. (B, C) Polyacrylamide gel stained with guaiacol using 10% (B) and 6% (C) resolving gels to detect POX activity. Arrowheads indicate bands of activity (B) and individual isoenzymes (C). The staining intensity of control and treated black and control Manchurian ash lanes had roughly 47%, 59%, and 86% of the staining intensity of the treated Manchurian ash lane, respectively (B). BC= black ash controls, BT= MeJA-treated black ash, MC= Manchurian ash controls, and MT= MeJA-treated Manchurian ash.

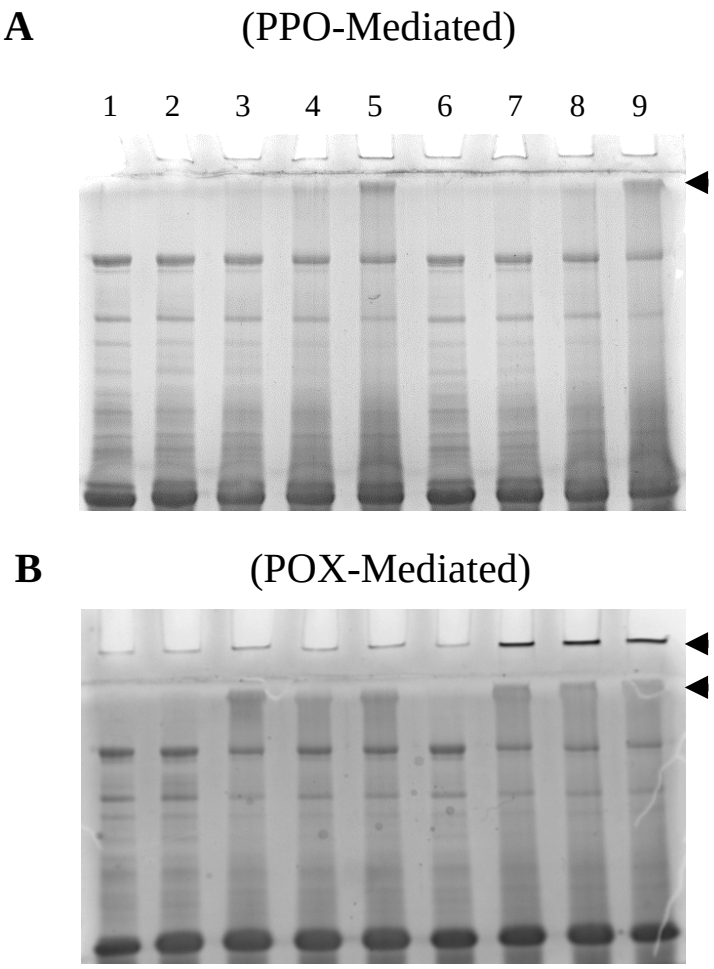

Figure 5.2. (A) PPO-mediated cross-linking of soybean trypsin inhibitor (STI) through time. Lane 1: STI standard at 9 hrs, lanes 2-5: black ash, lanes 6-9 Manchurian ash, lanes 2 and 6: 0 hrs, lanes 3 and 7: 1 hrs, lanes 4 and 8: 3 hrs, and lanes 5 and 9: 9 hrs. (B) POX-mediated cross-linking of soybean trypsin inhibitor through time. Lane 1: STI standard + H_2O_2 at 1 hr, lanes 2-5: black ash, lanes 6-9: Manchurian ash, lanes 2 and 6: 0 min, lanes 3 and 7: 15 min, lanes 4 and 8: 30 min, lanes 5 and 9: 1 hr. Arrows indicate the formation of bands of cross-linked protein.

6 AFFECT OF GIRDLING MANCHURIAN ASH ON PERFORANCE AND SURVIVAL OF EMERALD ASH BORER LARVAE AND ASSOCIATED PHYSIOLOGICAL CHANGES TO BARK TISSUE

6.1 INTRODUCTION

Emerald ash borer (EAB), *Agrilus planipennis* (Coleoptera: Buprestidae), is a devastating pest of ash (*Fraxinus* spp.) in North America where it has killed millions of forest and landscape trees since its discovery in 2002 (Herms and McCullough 2013). If left unchecked, the EAB infestation in North America could result in the elimination of an entire genus from the continent. Buprestid populations are generally thought to be bottom-up controlled by the availability of susceptible hosts (Muilenburg and Herms 2012; Herms and McCullough 2014). Furthermore, the relatively low rates of success of biocontrol programs dictates that multiple approaches to pest management and host conservation must be pursued. For example, the "successful" and "controlled" rate of biocontrol agent releases combined is roughly 37% per invasive forest pest and 9% per biocontrol agent released in Canada since 1882 (MacQuarrie et al. 2016). The fact that native North American ashes experience high mortality rates when planted in China (Wei et al. 2004; Liu et al. 2007) in the presence of natural enemies lends credence to this hypothesis and suggests that introduced biocontrol agents will likely not prevent widespread ash mortality in North America (Herms and McCullough 2014). However,

ash species that share an evolutionary history with EAB such as Manchurian ash (*F. mandschurica*) express relatively high levels of resistance (Rebek et al. 2008; Whitehill et al. 2012) and provide a reservoir of resistance genes that could be introgressed into North American ashes (Herms and McCullough 2014).

Putative resistance mechanisms of resistant ash species have been difficult to elucidate, but recently certain chemical compounds of interest and defense-associated, enzyme-catalyzed processes have been identified. The phloem phenolic chemistry of Manchurian ash and its most closely-related North American congener which is highly susceptible to EAB, black ash (*F. nigra*), are remarkably similar (Whitehill et al. 2012; Chakraborty et al. 2014). However, the phenolic compounds verbascoside (Whitehill et al. 2012), oleuropein (Whitehill et al. 2014), and pinoresinol and its derivatives (Chakraborty et al. 2014) are of interest as they have either been found in higher concentrations in Manchurian ash or concentrations increased in induced trees. Additionally, larvae fed on Manchurian ash have greater enzyme activities and functional expression of antioxidant and quinone-protective enzymes than those fed on white (*F. americana*) and green ash (*F. pennsylvanica*) (Rigsby et al. 2015). Furthermore, host defense-associated activities and functional expression of oxidative and defense-associated enzymes reported by Rigsby et al. (*In Review*) support earlier results, showing that Manchurian ash had significantly higher lipoxygenase (LOX), polyphenol oxidase (PPO), protein cross-linking, and especially peroxidase (POX) activities than black ash (*F. nigra*). Together these studies suggest that the resistance mechanisms of this co-evolved species are associated with not only the secondary metabolites, but their *in vivo* pro-oxidant activity.

Verbascoside and oleuropein are structurally similar phenylpropanoid glycosides (secoiridoids), and both have reported protein cross-linking and/or insecticidal activities (Konno et al. 1998; 1999; Muñoz et al. 2013; Whitehill et al. 2014). Bark verbascoside concentrations were consistently increased upon methyl jasmonate (MeJA) application in common garden experiments and had deleterious effects on larvae in artificial diet assays (Whitehill et al. 2014). Oleuropein was found at slightly greater concentrations in Manchurian ash than black ash (Whitehill et al. 2012) and has been shown to be an excellent substrate for Manchurian ash POX and β-glucosidase (βG) enzymes relative to black ash enzymes (Rigsby et al. 2014; Rigsby et al. *In Review*). Alternatively, pinoresinol derivatives are unique to Manchurian ash (Whitehill et al. 2012) or accumulate to a greater degree in Manchurian ash bark in response to EAB attack than black ash (Chakraborty et al. 2014). Ultimately, the bark phenolic profiles of Manchurian and black ash are remarkably similar despite the opposite resistance phenotypes of these species, but the importance of bark phenolics (1) most likely stems from their involvement in enzyme-catalyzed reactions (i.e. oxidation into reactive, pro-oxidant quinones), and (2) their usefulness as potential biomarkers of resistance.

Of primary interest are reactions mediated by oxidases and other defense-associated enzymes such as POX and PPO since larvae feeding on Manchurian ash show signs of quinone and oxidative stress (Rigsby et al. 2015). Investigations into oxidase reactions and activities have revealed significant species differences in phylogenetically and chemically similar ash species with opposite resistance phenotypes (Rigsby et al. *In Review*; D.N. Showalter, Unplublished Data). Other studies have detected few qualitative

differences in phloem phenolic profiles, but several phenolic species are thought to potentially influence resistance such as verbascoside (Whitehill et al. 2014),

In this study we attempted to build on previous work investigating host resistance mechanisms (Rigsby et al. *In Review*) and insect counter-adaptations (Rigsby et al. 2015). We attempted to isolate putatively-important factors in resistance by compromising the defense mechanisms of the resistant Manchurian ash and comparing them to Manchurian trees where defenses were not compromised (i.e. girdled vs. not girdled trees). We quantified bark phenolic compounds, with particular attention to oleuropein, pinoresinol, and pinoresinol derivatives. In addition, we quantified defense-associated and monolignol synthesis enzymatic activities, nutritional attributes, oxidative damage, and POX-mediated cross-linking activities of host bark tissue. Our hypothesis was that these putative defense mechanisms will be attenuated in girdled trees, allowing for their further identification, as well as increases in survival and performance of larvae inoculated on girdled hosts.

6.2 METHODS

6.2.1 Girdling, Inoculating, Harvesting, and Processing of Trees and Larvae

The same ash common garden described by Rigsby et al. (*In Review*) was used in this study. Briefly, the planting consisted of 24 Manchurian cv. ‘Mancana’ ash and 24 black ash cv. ‘Fallgold’, established in April 2011 at The Ohio State University’s Ohio Agricultural Research and Development Center (OARDC) in Wooster, OH. Trees were

planted in a randomized complete block design with three blocks with 16 trees per block (eight of each species). Trees were obtained as five-year-old bare root saplings from Bailey Nurseries, Inc. (St. Paul, MN) and were approximately the same size in May 2014 at the time of experimentation. Eleven Manchurian ash trees that had not previously been girdled were selected for experiments in this study, five of these were left as controls and six were girdled. To girdle, a 3 cm section of bark/phloem tissue directly below the first branch line was removed, which took place approximately 2 months prior to inoculating trees with EAB larvae. Epicormic sprouts were removed from the base of the tree whenever they appeared.

EAB eggs were used to inoculate similarly to Cipollini and Rigsby (2015). Ten eggs were cut from the coffee filter in which they were laid (obtained by personnel at the USDA-APHIS-PPQ EAB rearing facility in Brighton, MI) and glued to a sheet of Parafilm using non-toxic Elmers glue with the filter side facing the Parafilm. Three strips (30 eggs) were then attached to the trunk of a given tree below the girdle (girdled trees) or below the first branch (control trees) on 24-July 2015. Larvae were allowed to hatch, bore into the tree, and develop in trees for six weeks. After six weeks post-estimated hatch date, trees were cut at the soil line and placed in a cooler containing water so that logs would not dry out. Logs were transported to Wright State University where they were stored in this manner for less than 48 hrs. Logs were debarked and phloem tissue around larval galleries was immediately placed in liquid N, ground into a powder, weighed, placed in individual 50 mL conical tubes, and stored at -80^{o}C until required. Additionally, larvae were extracted and larval survival and mass were recorded. Each

larva was considered a technical replicate and all larvae were pooled from a single host tree and considered a biological replicate.

6.2.2 Equipment, Reagents, and Protein Concentration Estimation

Round-bottomed 96-well polystyrene plates used for spectrophotometric enzyme activity assays (BD Bioscience, Billerica, MA) and quantified using a SpectraMAX 190 microplate reader (Molecular Devices, Sunnyvale, CA). A Bio-Rad (Hercules, CA) mini PROTEAN® 3 system was used for SDS-PAGE. Images of gels were taken using a FUJIFilm Las-3000. Purified oleuropein (98%) and pinoresinol (98%) standards used in UPLC analysis was purchased from Sigma (St. Louis, MO) and Apin Chemicals (Milton, UK), respectively, and all reagents used were HPLC grade. Protein was estimated using concentrated Bradford reagent purchased from Bio-Rad (Hercules, CA) and bovine serum albumin (Sigma) as standard. HPLC grade methanol and acetic acid were purchased from Fisher Scientific (Pittsburg, PA). All other reagents were purchased from Sigma.

Quantification of known and unknown phenolic compounds was performed using a Waters Acquity H-class 1200 series ultra high performance liquid chromatograph (UPLC) equipped with a temperature-controlled autosampler, and a photodiode array detector (PDA) (Waters, Milford, MA). Separation of the analytes was carried out on an Acquity BEH C18 2.1×100 mm column, 1.7 µm particle diameter (Waters). Liquid chromatography-diode array detection-mass spectrometry (LC-DAD/MS) was employed to identify pinoresinol derivatives and confirm oleuropein and pinoresinol identity and

separation was performed using an Agilent 1290 Infinity UPLC system (Agilent Technologies, Inc, Santa Clara, CA) and metabolites were detected with an Agilent 1260 DAD in line with a hybrid Triple Quadrupole/Ion trap MS QTRAP 5500 from AB Sciex (Framingham, MA).

6.2.3 Metabolite Extractions, Identification, and Quantification

Phenolics were extracted following the methods of Eyles et al. (2007). Ground bark tissue (100 mg) was extracted for 24 hrs, twice, at 2ºC in 500 µL HPLC-grade methanol containing 500 µg/mL butylated hydroxyanisole (BHA) as an internal standard. The two extracts were then pooled into a fresh 1.5 mL microcentrifuge tube and stored at -20ºC until analysis.

The autosampler and column temperatures were set at 24 and 50 °C, respectively, and the injection volume was 0.8 µL. The binary mobile phase consisted of 0.1% acetic acid in water (solvent A), and 0.1% acetic acid in methanol (Solvent B), with a flow rate of 0.5 mL/min. Total run time was 11.14 min. The following linear gradient [cumulative run time (min), % solvent A] was used: 0.0, 95.0; 1.70, 85.0; 3.97, 70.0; 4.53, 60.0; 5.67, 40.0; 6,23, 10.0; 6.80, 0.0; 7.03, 95.0; 11.14, 95.0 . The scanning range was 210-400 nm. Data acquisition was performed using the Empower 3 software (Waters), and peak areas at 280 nm were integrated using the apex-track algorithm. Minimum detectable peak area was set to 11,000 peak area units, and peak area of each compound was corrected by dividing it by the peak area of the internal standard. Pinoresinol and oleuropein were identified using retention time and UV spectral matches to authentic standards, and

quantified using standard calibration curves. Pinoresinol derivatives, identified as described below, were quantified as pinoresinol equivalents. Six-point and seven-point standard curves ($R^2 > 0.999$) and relative standard error (< 3.2) were generated for oleuropein and pinoresinol, respectively. In both standard curves, three technical replicates were averaged for each concentration. To ensure consistency, standards and samples were run in the same session. Sample responses were quantified only if their peak area fell within the linear range of detector, and peak areas were converted to mg/g FW. Unknown phenolic compounds were quantified as internal standard-equivalent peak area.

The same column, instrumental conditions and linear gradient used in the Waters UPLC analyses were used for the LC-DAD/MS identification of pinoresinol, pinoresinol derivatives, and oleuropein and a 0.8 µL phenolic extract sample from a pool of samples was injected. First, UV spectral data were recorded from 210 to 400 nm at a sampling rate of 5 Hz with phenolic compounds being detected at 280 nm. Then, metabolites were detected through the mass spectrometer using the negative ion mode. MS parameters values, including curtain gas (30 psi), ionization (4500 V), temperature (550ºC), nebulizer gas (60 psi), heating gas (60 psi), collision activated dissociation (high), declustering potential (80 eV), and entrance potential (10 eV) were kept constant for the different surveys. The enhanced full-scan (EMS) survey was conducted for masses ranging from 100 to 1,000 *m/z* with a collision energy of 10 eV and a scan rate of 10,000 (*m/z*)/s. Information dependent acquisition (IDA) was used to obtain MS/MS spectra with a scan range from 100 to 1,000 *m/z*. IDA threshold was set up at 500,000 cps, and a dynamic exclusion was set to 10 s after two appearances in order to permit the detection

of co-eluting substances. Once a metabolite was above the IDA threshold, after EMS survey, its exact mass was determined by enhanced resolution (ER) survey at a scan rate of 250 (*m/z*)/s. In the meantime, the MS/MS spectrum of the compound was accessed by enhanced product ion (EPI) survey using a collision energy of 60 eV and a collision energy spread of 30 eV. The EPI scan rate was set up at 10,000 (*m/z*)/s. Both UV spectral and mass spectrometry data were acquired and processed using Analyst 1.6.1 software. Spectral match and multi-level fragmentation patterns were used to identify pinoresinol derivatives and confirm oleuropein and pinoresinol identity (Chakraborty et al. 2014) (Table 6.1). Retention times and PDA data of identified compounds were then compared with the Waters UPLC chromatogram, to match the corresponding peak.

6.2.4 Bark Tissue Protein Extractions and Defense-Associated Enzyme Assays

The bark protein extraction procedure described by Rigsby et al. (*In Review*) was modified slightly. Briefly, 2 g powder tissue was extracted at 2^{o}C for 1 hr in extraction buffer (50 mM Na-PO_4, pH 6.5, 10% [v:v] glycerol, 7% [w:v] PVPP,10 mM β-mercaptoethanol, and 1 mM each L-ascorbate, EDTA, and PMSF; PMSF was added immediately prior to extractions and every hour to the extraction buffer as required). Homogenates were then centrifuged at 2,000 *g* at 2^{o}C for 10 min, the supernatant transferred to fresh tubes, centrifuged again at 7,000 *g* at 2^{o}C for 20 min, and the supernatant was acetone precipitated. Protein pellets were then resuspended in 20% of the volume and these extracts were used for enzyme activity assays. Protein concentrations were immediately estimated *via* the Bradford (1976) method using bovine serum albumin and extracts were then stored at -80^{o}C. The slightly acidic pH of the extraction buffer was

chosen because PVPP is more effective at binding polyphenols at acidic pH (Makkar et al. 1995).

The activities of CHI, βG, and PPO were quantified as described by Rigsby et al. (*In Review*) using chitin azure (ΔAbs_{575}/hr/mg), *p*-nitrophenyl β-glucopyranoside (ε_{405} = 18.5 mM^{-1} cm^{-1}), and catechol (ε_{400} = 3,450 M^{-1} cm^{-1}) as substrates, respectively. POX activity was quantified with guaiacol (ε_{470} = 26.6 mM^{-1} cm^{-1}) (Cipollini et al. 2011) and syringaldazine (ε_{530} = 27 mM^{-1} cm^{-1}) (Rigsby et al. *In Review*). The capacity of host protein extracts to synthesize lignin monomers was quantified by assaying the activity of coniferyl aldehyde dehydrogenase (CAD; a key enzyme in the monolignol synthesis pathway) by monitoring the conversion of coniferyl aldehyde (ε_{400} = 6.27 mM^{-1} cm^{-1}) to coniferyl alcohol (Mansell et al. 1974). Briefly, 50 µL protein extract and 100 µL coniferyl aldehyde solution (in PAB; 500 µM final concentration) were allowed to incubate at 30°C for 2 min, then the reaction was initiated with 50 µL NADPH solution (in Milli-Q H_2O; 750 µM final concentration) and the decrease in absorbance at 400 nm was followed for 5 min. Enzyme activities were normalized on both a per mg protein and a per g fresh weight (FW) basis.

6.2.5 Bark Nutritional Attributes, Oxidative Damage, and Protein Cross-Linking Activity

Tissue powder (100 mg) was extracted on ice for 1 hr in 500 µL 50 mM Na-PO_4 buffer (pH 8.0) with 5% PVPP. The 10,000 x *g* supernatant was used to estimate glucose concentration *via* the glucose quantification procedure described by Siemens and

Mitchell-Olds (1998) and the Bradford (1976) assay was used to estimate protein concentrations. Total soluble sugars and starch were estimated using the spectrophotometric protocol described by Hill et al. (2012) using concentrated H_2SO_4 and phenol. Conjugated dienes were quantified in accordance with Summers and Felton (1994) by extracting 100 mg bark tissue in 1 mL 3:1 chloroform:methanol and recording the absorbance of the supernatant (ε_{234} = 29,500 M^{-1} cm^{-1}). Total protein disulfides (ε_{420} = 13,600 M^{-1} cm^{-1}) contents were also quantified according to Summers and Felton (1994) using the same 10,000 x *g* supernatant that was used for glucose and protein concentration assays.

The cross-linking activity of ash extracts was assessed using a modified method to that described by Rigsby et al. (*In Review*). A 1 mL volume of buffer (50 mM Na-PO_4, pH 8.0) containing 1 mg/mL purified soybean trypsin inhibitor (STI) and 5 mM H_2O_2 was used to extract 100 mg of powdered tissue (all samples). A volume was immediately removed (0 hrs) and reacted with SDS-sample buffer and heated to 95ºC for 5 min, then placed on ice. Another volume was removed after 2 hrs and also prepared for SDS-PAGE *via* the same procedure. A parallel reaction solution was carried through the procedure containing no host material as an additional control (0 and 2 hrs), and 15 µg of protein from each reaction solution was separated in SDS gels (5% stacking, 15% resolving). Protein cross-linking was denoted as described by Rigsby et al. (*In Review*).

6.2.6 Statistical Analyses

A logistic regression was used to assess the effect the girdling treatment on the proportion of surviving larvae and a linear regression was used to assess the girdling treatment on the endpoint mass of surviving larvae. A *t*-test was used to test the effect of girdling host enzymatic activities, nutritional attributes, and markers of oxidative damage in addition to the concentrations of pinoresinol and its derivitives and oleuropein. Principal components analysis (PCA) was performed on phenolic profiles to using the internal standard-equivalent peak areas of chromatogram peaks. Data were confirmed normal and outliers were identified and removed using the Shapiro-Wilk normality and Dixon tests, respectively ('Outliers' package in R) (Komsta 2011). All statistics were performed in R (R Core Team 2016).

6.3 RESULTS

6.3.1 Survival and Performance of Larvae

Larval survival increased by approximately 57% when inoculated below the girdle of girdled trees (37% surviving), which was a significant increase from controls (16% surviving) ($z = 3.339$, $P < 0.001$). Additionally, larval performance increased as the mean mass of surviving larvae (13.63 ± 1.60 mg) was roughly 63% greater than that of surviving larvae from control trees (5.09 ± 1.78 mg), which was statistically significant ($t = 3.407$, $P = 0.014$) (Figure 6.1).

6.3.2 Phenolic Metabolites and Profiles

A total of 40 individual peaks were identified by way of UPLC-PDA (Table 6.2). However, no clustering of treatment groups was detected in PCA ordination of phenolic profiles (Figure 6.2). Oleuropein, pinoresinol, and two pinoresinol derivatives (pinoresinol hexoside and pinoresinol derivative B) were identified from extracts, but there were no differences in tissue levels of any of these metabolites ($P > 0.05$) (Table 6.3).

6.3.3 Activity of Host Defense-Associated Enzymes

The girdling treatment had a significant effect on CHI activity on a per mg protein with activity being higher in girdled trees (Table 6.4), but was not affected on a per g FW basis (Table 6.5). The activities of βG and PPO were not significantly affected by girdling regardless of how activities were normalized (Tables 6.4 and 6.5). Girdling had a significant effect on POX activities quantified using both substrates on a per mg protein basis (Table 6.4), but only when using syringaldazine on a per g FW basis (Table 6.5). No effect of girdling was detected when quantifying POX activity using guaiacol on a per g FW basis (Table 6.5). Finally, CAD activity was significantly increased in girdled than in control host extracts on both a per mg protein and per g FW basis (Tables 6.4 and 6.5, respecitvely).

6.3.4 Nutritional Attributes, Oxidative Damage, and Protein Cross-Linking Activity of Bark

Tissue glucose levels were significantly increased in girdled host extracts than in extracts of controls while there were no girdling effects on total soluble sugar levels (Table 6.6). However, both total starch and total protein levels were significantly decreased in girdled relative to control trees (Table 6.6). Concentrations of conjugated dienes were not affected by girdling treatment, though levels were slightly higher in controls (Table 6.6). Protein disulfide concentrations were also not significantly affected by girdling on a per g FW basis but were higher in girdled host extracts on a per mg protein basis (Table 6.6). Additionally, there was qualitative differences in the POX-mediated cross-linking activities between treatments and protein appeared to cross-link to the same extent (data not shown).

6.4 DISCUSSION

The purpose of this study was to further identify the resistance mechanisms of Manchurian ash to EAB by directly inoculating healthy and compromised hosts *via* girdling. We were able to show that girdling results in the approximate doubling of larval survival and performance. We were unable to detect an effect of girdling on the tissue levels of the putatively important phenolic metabolites pinoresinol, pinoresinol A, pinoresinol B, and oleuropein and we were also unable to detect major phenolic profile differences by treatment. The activities of several enzymes including CHI, POX, and CAD were increased by girdling on a per mg protein basis, but these differences were no longer detected when activities were expressed on a per g FW basis, save for syringaldazine-POX and CAD activities. Tissue glucose levels were significantly increased by girdling, but girdling did not affect total sugar levels and significantly

decreased tissue starch and protein levels. Additionally, tissue disulfide levels were not affected by girdling, but disulfide levels per mg protein were significantly increased by girdling.

It is not surprising that girdling Manchurian ash results in increased survival and performance for EAB larvae as this insect infests dead and dying trees in its native range in Asia (Wei et al. 2004; Liu et al. 2007; Baranchikov et a. 2008). However, we have observed that larvae that have fed on Manchurian ash, girdled or not, are still substantially smaller with fewer survivors than larvae that have fed on susceptible North American species (e.g. Muilenburg et al. 2011; Rigsby et al. 2015; Author's unpublished data). We hypothesized that girdling the main trunks of these trees would result in the defenses of the tree to be compromised and this would allow for increased survival and performance of larvae. However, we were unable to detect clear evidence that putative mechanisms of resistance (i.e. PPO- and POX-mediated phenolic oxidation, polymerization, pro-oxidant activities, and reactive oxygen species [ROS] generation) were compromised in girdled trees. This could indicate that though conditions of the host have been altered to allow for increased larval success, the basic structure of Manchurian defenses are still functioning.

Several enzyme activities were increased by girdling on a per mg protein basis, but when normalized on a per g FW basis most activities were not differentiable by treatment, save for syringaldazine-POX and CAD activities. CAD is not a defense-associated enzyme *per se*, but it is an important enzyme in the monolignol synthesis pathway where it catalyzes the conversion of cinnamyl-aldehydes to alcohols that are monomeric precursors of lignin (Trabucco et al. 2013). The increased activity of CAD on

both a per mg protein and per g FW basis indicates that these trees are likely responding to the girdle wound which is typically reported by authors assessing CAD activity and expression and lignin deposition in wounded plants (Hawkins and Boudet 2003; Deflorio et al. 2011; Cheng et al. 2013; Choi et al. 2016). Manchurian ash has a very strong wound healing response and most Manchurian ashes that are girdled one season are able to completely heal the girdle over with callus tissue by the next growing season (author's personal observation). In the lignin synthesis pathway the activity of syringaldazine-POX represents the final step of monolignol oxidation, spontaneous polymerization into lignin, and lignin deposition (e.g. Quiroga et al. 2000) and POX gene expression also typically increases in due to wounding or pathogen infection (e.g. Deflorio et al. 2011). This activity was also significantly greater in girdled trees than controls on both a per mg protein and per g FW basis. With both control and girdled trees being inoculated (and therefore having live, actively feeding larvae), this indicates that the plant could be targeting wound healing rather than larval encapsulation with the increased activity of CAD and syringaldazine-POX.

Reductions in bark starch levels in girdled trees below the girdle are typically reported (e.g. Li et al. 2003; Maier et al. 2010) and we detected an approximately 27% reduction in available starch in girdled trees. We also found increased glucose levels but a lack of difference in bark soluble sugar levels between control and girdled trees. One possible explanation for these findings is that girdled trees are mobilizing their starch reserves, equilibrating sugar levels for metabolic processes and utilizing glucose for primary metabolism below the girdle. Jordan and Habib (1996) reported this process in peach trees, where the mobilization of carbohydrates below the girdle was used to

maintain soluble sugar content below the girdle. We would hypothesize that once starch resources are exhausted trees would no longer be able to use those reserves as a sugar source and glucose and sugar levels would begin to decrease as well.

We could find no other studies reporting reductions in total soluble protein levels below a girdle, but these findings could also be explained the reduction in resources below the girdle. Bark tissue contains decreased levels of nitrogen during the growing season (Wildhagen et al. 2010) and though protein synthesis does occur in the phloem tissue of angiosperms (Lin et al. 2009; Ham and Lucas 2013), phloem protein essentially adheres to traditional "source/sink" dynamics model (Fisher et al. 1992). It is conceivable that phloem tissue further away from the canopy typically acts as a protein and nitrogen sink and without access to protein, amino acids, and nitrogen from sources above the girdle, these resources would eventually diminish. This reduction in protein levels, not only total protein but also damaged protein as indicated by total disulfides/mg protein, also demonstrates that increases in defense-associated enzyme activities on a per mg protein basis are, for the most part, canceled out by reductions in protein when normalized on a per g FW basis. Because of this, larvae would be experiencing essentially the same levels of host defenses per g tissue consumed in both control and girdled trees.

It is clear that without changes in phloem phenolic profiles or levels of specific metabolites between Manchurian and black ash (Whitehill et al. 2012; Chakraborty et al. 2014) or between girdled and control Manchurian ashes (this study), direct toxicity of phloem phenolics and phenolic composition does not contribute to interspecific variation in resistance and does not appear to contribute to intraspecific variation in resistance in

Manchurian ash. We detected few differences in defense-associated enzyme activities between treatments on a per g FW basis which clearly distinguished Manchurian and black ash in past experiments (Rigsby et al. *In Review*). These findings suggest that reductions in host defenses in stressed and weakened Manchurian ash were not contributing to increased larval survival and performance in these experiments. Rather, increased survival and performance appears to be associated with other physiological changes to the host tissue. However, we were unable to find evidence for a specific mechanism for increased larval success. While glucose levels in host tissue were significantly increased by girdling, total sugar levels were unchanged, and starch and protein levels decreased. Together, this would not suggest that increased nutritional quality of tissue, allowing for larvae to access resources that allow them to overcome host defenses, would be occurring.

One factor that we did not address in these experiments, however, was the relative levels of ROS of the host tissue. ROS accumulation (specifically H_2O_2) typically occurs at and around wound sites in plants and can function as a signal for the expression of defense-associated genes (Orozco-Cárdenas et al. 2001; Cheeseman 2007) and there is a reasonable expectation that H_2O_2 will accumulate near larval feeding and girdle sites. There is indirect evidence of this as catalase activity of larvae feeding on Manchurian ash was significantly greater than that of larvae feeding on white and green ash (Rigsby et al. 2015). Catalase is responsible for the degradation of H_2O_2 to H_2O and its increased activity in Manchurian-fed larvae suggests greater oxidative stress due to H_2O_2 in these larvae. If host tissue H_2O_2 levels or H_2O_2 generation were decreased or impaired in some way by girdling, it would represent the removal of a significant hurdle for larvae to

overcome. One interesting observation that has never been explained is the substantially greater tissue levels of tyramine in Manchurian ash relative to North American ashes (Hill et al. 2012). Tyramine could have a role to play in H_2O_2 generation as it could be a substrate for monoamine oxidases and its oxidation would produce H_2O_2 (e.g. Zhang et al. 2012). If tyramine is in fact used to generate H_2O_2 it is possible that girdling could result in the depletion of this resource as well, but this hypothesis would certainly need to be addressed.

To summarize, we hypothesized that girdling would attenuate putative host defenses and allow for increased larval survival and performance. We were able to successfully increase larval survival and performance on Manchurian ash by way of girdling and inoculating larvae on host tissue below the girdle. However, phloem phenolic chemistry was not significantly affected and defense-associated enzyme and protein cross-linking activities were not substantially different on a per g FW basis. Furthermore, the nutritional quality of phloem tissue did not appear to change for the benefit of larvae. These data suggest that mechanism(s) other than a girdle-produced reduction in defense levels are responsible for increased larval success. Future research should address other potential mechanisms of this increased larval success in girdled Manchurian ash such as a reduction in tissue ROS levels and/or ROS generation.

6.5 REFERENCES

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Table 6.1. Chromatographic, UV, and mass-spectral data of oleuropein, pinoresinol and two pinoresinol derivatives isolated from phloem of *Fraxinus mandschurica*.

Peak number[1]	RT	$[M-H]^-$	Main fragments *m/z*[2]	λ_{max} (nm)	Assigned identity
18	4.718	357	151.1; 136.1; 91.9; 162.2	226, 276	Pinoresinol hexoside
26	5.526	357	151.1; 136.1; 91.9; 162.2	228, 278	Pinoresinol derivative B
36	6.020	539	377.2; 307.2; 275.2; 403.1; 121.0; 139.0	232, 280	Oleuropein
38	6.184	357	151.1; 136.1; 91.9; 162.2	276	Pinoresinol

[1] Peak numbers correspond to the numeration reported in Table 6.2; [2] Main fragments are reported in order of decreasing abundance; RT = Retention time (min)

Table 6.2 Chromatographic and UV data of all phenolic compounds isolated from phloem of *Fraxinus mandschurica*. RT = retention time (min) and (sh) = shoulder.

Peak number	RT	λ_{max} (nm)
1	2.080	279
2	2.465	290 (sh), 334
3	2.648	221, 275
4	2.721	221, 275
5	2.943	224, 290 (sh), 334
6	3.054	257
7	3.175	224, 290 (sh), 334
8	3.228	279
9	3.458	255, 299
10	3.517	219, 265
11	3.582	232, 346
12	3.744	325
13	3.891	231, 325
14	4.045	292, 334
15	4.180	267
16	4.221	266
17	4.451	250, 325
18[1]	4.718	226, 276
19	4.781	284
20	4.900	221, 273
21	5.013	277
22	5.098	279
23	5.168	229, 315 (sh)
24	5.219	220, 248 (sh), 328
25	5.439	223, 324
26[2]	5.526	228, 278
27	5.620	296 (sh), 329
28	5.665	285 (sh), 329
29	5.700	279
30	5.744	219, 248 (sh), 328
31	5.795	266, 324
32	5.837	286 (sh), 328
33	5.864	330
34	5.907	282 (sh), 328
35	5.954	280 (sh), 327
36[3]	6.020	232, 280
37	6.077	278
38[4]	6.184	276
39	6.315	225, 248 (sh)
40	6.369	231, 280 (sh)

[1]Identified as pinoresinol hexoside; [2]Identified as pinoresinol derivative B; [3]Identified as oleuropein; [4]Identified as pinoresinol

Table 6.3. Mean (± 1 SE) Manchurian ash bark tissue levels of putatively important phenolic metabolites (mg/g FW) by treatment.

	Metabolite Levels (mg/g FW)			
Treatment	Pinoresinol Hexoside	Pinoresinol Derivative B	Pinoresinol	Oleuropein
Control	1.67 (0.29)	4.55 (0.20)	0.53 (0.06)	3.88 (0.58)
Girdled	1.80 (0.14)	4.67 (0.26)	0.44 (0.04)	3.46 (0.27)

Table 6.4. Mean (± 1 SE) activities of CHI, βG, PPO, POX (both substrates) and CAD of control and girdled Manchurian ash on a per mg protein basis with results of *t*-tests (*t* and P values).

		Activity (mg protein)			
Enzyme	Substrate (Units)	Control	Girdled	*t*	P
CHI	Chitin Azure (ΔAbs_{575}/hr/)	1.59 (0.14)	3.72 (0.57)	2.964	0.018
βG	Oleuropein (µmoles/min/)	11.03 (1.57)	15.60 (1.97)	1.757	0.113
PPO	Catechol (µmoles/min/)	1.61 (0.40)	1.78 (0.36)	0.252	0.807
POX	Guaiacol (µmoles/min/)	16.43 (1.80)	33.38 (2.51)	5.263	< 0.001
	Syringaldazine (µmoles/min/)	130.95 (52.38)	878.04 (234.09)	2.838	0.019
CAD	Coniferyl Alcohol (µmoles/min/)	0.43 (0.06)	2.19 (0.28)	4.924	0.001

Table 6.5. Mean (± 1 SE) activities of CHI, βG, PPO, POX (both substrates) and CAD of control and girdled Manchurian ash on a per mg protein basis with results of *t*-tests (*t* and P values).

		Activity (g FW)			
Enzyme	Substrate (Units)	Control	Girdled	*t*	P
CHI	Chitin Azure (ΔAbs_{575}/hr/)	2.69 (0.42)	4.36 (0.95)	0.060	0.954
βG	Oleuropein (µmoles/min/)	20.04 (3.26)	17.79 (2.65)	0.102	0.921
PPO	Catechol (µmoles/min/)	2.77 (0.33)	2.16 (0.66)	0.256	0.804
POX	Guaiacol (µmoles/min/)	28.31 (3.11)	38.43 (5.38)	1.820	0.102
	Syringaldazine (µmoles/min/)	303.74 (113.59)	1,057.88 (323.51)	2.199	0.056
CAD	Coniferyl Alcohol (µmoles/min/)	0.71 (0.11)	2.49 (0.37)	3.732	0.006

Table 6.6. Mean (± 1 SE) Manchurian ash bark tissue levels of glucose, soluble sugars, starch, soluble protein, conjugated dienes, and total disulfide bonds by treatment with results of *t*-tests (*t* and P values). Total disulfide bond levels are presented on both a per g FW and a per mg protein basis.

Substance	Units	Control	Girdled	*t*	P
Glucose	mg/g FW	5.61 (0.54)	8.87 (1.09)	2.516	0.033
Sugars	mg/g FW	69.52 (4.30)	67.28 (4.92)	0.335	0.745
Starch	mg/g FW	147.23 (9.13)	107.95 (7.06)	3.461	0.007
Protein	mg/g FW	1.65 (0.16)	1.13 (0.11)	2.728	0.023
Dienes	mmoles/g FW	0.72 (0.07)	0.53 (0.09)	1.614	0.141
Disulfides	mmoles/g FW	0.31 (0.05)	0.43 (0.08)	1.229	0.250
	mmoles/mg protein	0.20 (0.03)	0.41 (0.08)	2.237	0.052

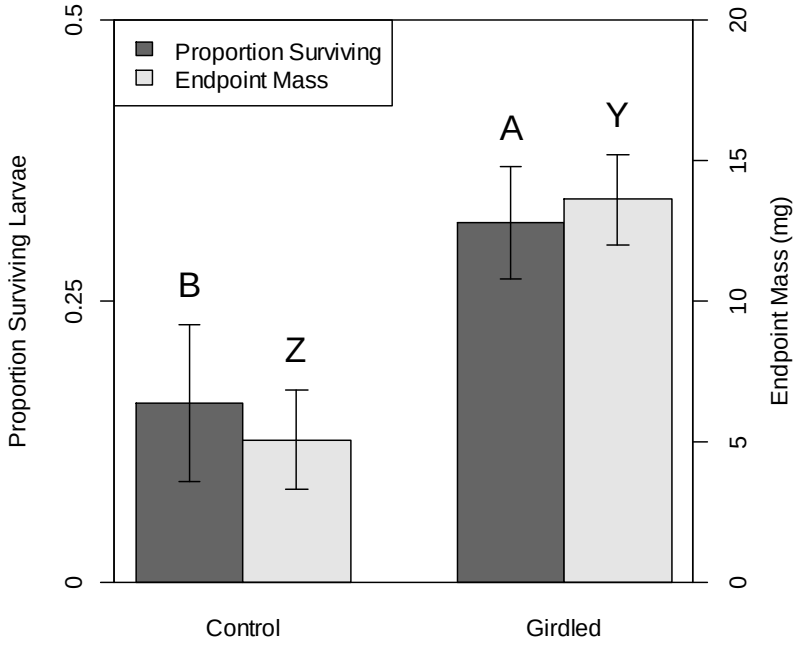

Figure 6.1. Mean proportion of surviving larvae (± 1 SE) after incubation period (primary y-axis, dark gray bars) and mean endpoint mass of surviving larvae in mg (± 1 SE; secondary y-axis, light gray bars) by treatment group. Different letters indicate significant differences. Treatment effects on proportion surviving larvae was analyzed via a logistic regression and treatment effects on endpoint mass of surviving larvae was analyzed by way of a *t*-test.

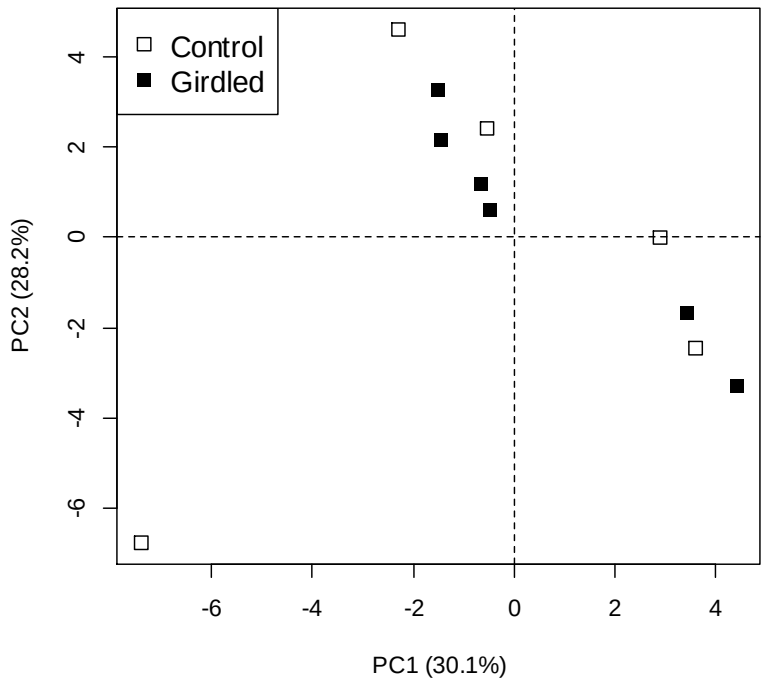

Figure 6.2. The first (x-axis) and second (y-axis) principal components of a PCA model fit to bark phenolic profiles of control and girdled Manchurian ash, explaining 30.1% and 28.2% of the variation in phenolic profiles, respectively. Open squares are control and closed squares are girdled trees.

7 CONCLUSIONS AND SUGGESTIONS FOR FUTURE RESEARCH

7.1 INTERSPECIFIC VARIATION IN ANTIXENOSIS AND MECHANISMS

The primary goal of this research was to further identify and characterize the mechanisms of antibiosis as well as to shed light on potential antixenosis mechanisms of Manchurian ash. In chapter 1 (Rigsby et al. 2014), we demonstrated a clear ovipositional preference of emerald ash borer (EAB) adult females towards susceptible native North American hosts over the co-evolved, resistant Manchurian ash. Along with the finding that EAB adults prefer to feed on these same susceptible hosts over Manchurian ash (Pureswaran and Poland 2009), these data are suggestive that an antixenosis mechanism could be actively expressed in resistant species. Antixenosis mechanisms can be expressed via olfactory, tactile, gustatory, or visual cues (Smith 2005), however, since it is believed that olfaction is likely the most important for long-range host-finding for herbivorous insects (Bernays and Chapman 1994), in Chapter 2 we focused on the differential emissions of volatile organic compounds (VOCs) between black and Manchurian ash. We found that the VOC profiles of these two species, unlike bark phenolic profiles, are extraordinarily unique and though several individual compounds were differentially emitted by species from the canopy and bark, few individual compounds stood out. However, a handful of antennally active compounds were differentially emitted by species.

Relatively little research has attempted to directly address putative antixenosis mechanisms of ash to EAB and therefore this is a wide open and potentially fruitful area of research. It is clear that resistant Manchurian ash is much less preferred for host use by EAB adults, but without a characterized mechanism of antixenosis it is unclear as to whether this non-preference stems from a trait of the plant (i.e. antixenosis) or if non-preference stems from the recognition by adults that Manchurian ash is a poorer quality host for larvae. In this context, differential VOC profiling of resistant and susceptible species should continue. In addition to this, olfactometer experiments could shed light on the attractiveness of whole-profiles or individual volatiles. Compounds found to be differentially emitted by black and Manchurian ash in Chapter 3 could be tested in olfactometer assays to evaluate their attractant or repellent activities. Furthermore, non-volatile antixenotic mechanisms should be evaluated, especially adult gustatory cues. Existing data show that adult feeding and ovipositional hierarchies essentially mirror each other, but no evidence exists that implies a causal relationship between adult feeding and oviposition. Additionally, volatile compounds have been shown in several systems to have roles in antibiosis and this is perhaps worthy of investigation. For example, cotton emission of caryophyllene oxide inhibits *Heliothis virescens* larval growth (Stipanovic et al. 1986).

7.2 MECHANISMS OF INTERSPECIFIC VARIATION IN ANTIBIOSIS

These studies were able to make significant strides in our understanding of interspecific variation in antibiosis and the mechanisms that drive this variation. We clearly demonstrated in Chapter 5 (Rigsby et al. *In Review*) that relative to black ash,

Manchurian ash is able to oxidize phenolic substrates and polymerize lignin monomers significantly faster. The oxidation of phenolics can be problematic to insects for two reasons: first, oxidized phenolics (i.e. quinones) react with biomolecules (Summers and Felton 1994). For instance, quinones covalently bind to protein, inhibiting their activity and reducing their nutritional quality for the insect (Appel 1993). Indeed, protein cross-linking assays qualitatively showed that the POX-mediated protein cross-linking activity of Manchurian ash is stronger than black ash. Secondly, the process of phenolic oxidation can produce reactive oxygen species (ROS) that can then initiate deleterious redox cycles (Barbehenn 2002). For instance, the intermediate in the polyphenol oxidase (PPO)-catalyzed oxidation reaction is the phenolate ion which can donate an electron to molecular oxygen (O_2) to form the highly reactive superoxide anion radical (O_2^-) and in the presence of hydrogen peroxide (H_2O_2) can, in turn, form reactive hydroxyl radicals (•OH). Hydroxyl radicals can oxidize biomolecules such as lipids which will catalyze self-perpetuating cycles of lipid oxidation (Bi and Felton 1995). Without adequate quinone-protective and antioxidant mechanisms to prevent the deleterious effects of quinone and ROS generation, damage to the midgut and specifically the peritrophic matrix ensues, which can result in lesions and cellular damage (Barbehenn 2002).

Data from Chapter 4 (Rigsby et al. 2015) on the physiological responses of EAB larvae to feeding on phenotypically different hosts support the hypothesis that variation in resistance phenotype can be explained by the greater ability of Manchurian ash to cause a quinone and oxidatively-stressful diet for larvae than susceptible hosts. Differences in other enzyme activities were detected (e.g. β-glucosidase), however the significantly higher activities and functional expression of *ortho*-quinone reductase (*o*-

QR), superoxide dismutase (SOD), catalase (CAT), and glutathione reductase (GR) of those larvae that fed on Manchurian ash relative to those that fed on susceptible species (white and green ash) are what stood out in these experiments. Though direct measures of oxidative stress were unable to be performed and we therefore cannot speak to the levels of damage and stress experienced by these larvae, it is clear that quinone-protective and antioxidant mechanisms are induced to a much greater extent in larvae feeding on Manchurian ash. Thus, the conclusion that these larvae are under substantially greater amounts of stress is supported by observations that the induction of quinone-protective and antioxidant mechanisms is often reported in conjunction with increases in oxidative stress (e.g. Micheal and Subramanyam 2013).

The two studies described above provide solid evidence for linking the interspecific variation in resistance to differences in the host's ability to generate quinones and oxidative stress. However, further investigation of ash oxidase enzymes and genes are warranted. Our native PAGE experiments failed to detect isozyme differences between black and Manchurian ash, but this technique cannot differentiate increases in staining intensity being due to greater amounts of enzyme present in the gel from increases due to enzyme efficiency. In order to determine this, Manchurian and black ash PPOs and POXs can be purified and thoroughly characterized (e.g. Michaelis-Menten kinetics, isoelectric points, amino acid sequences, crystal structures, phenolic substrate affinities, etc.) and antibodies can be raised against purified ash oxidases and western blots performed. The purpose of these experiments would be to determine if Manchurian oxidases are more active because there is simply more enzyme present or increased activity stems from more efficient enzymes. Information from these experiments can be

directly applied to the genetic modification of North American ashes for replanting efforts. For example, the alteration of transcription factors can be performed so that PPOs and POXs are overexpressed which would likely increase resistance in North American species. As a notable example, transgenic *Populus* that overexpresses PPO has been shown to more resistant to certain species of lepidopterans (Wang and Constabel 2004; Barbehenn et al. 2007). Additionally, peroxidase genes should be identified and characterized so that techniques like RNAi could be used to assess the defensive function of POXs in Manchurian ash.

7.3 MECHANISMSM OF INTRASPECIFIC VARIATION IN ANTIBIOSIS

Intraspecific variation in resistance, however, proved to be more complicated than interspecific resistance. The experiments for Chapter 6 attempted to draw relationships between larval success and host chemical and enzyme-based defenses as well as nutritional factors of the bark tissue by inoculating girdled and control trees and quantifying larval survivorship and performance. Unfortunately no clear patterns emerged for any of the factors that were quantified. No differences were detected in bark phenolic profiles and few differences were detected for enzymatic defenses and nutritional attributes. POX activities were significantly greater for girdled trees on a per mg protein basis, but these differences were virtually eliminated when activity was expressed on a per g fresh weight (FW) basis. This was because girdled tissue had significantly lower levels of soluble protein that resulted in similar activities when they were normalized on a per g FW basis. Glucose levels were increased in girdled tree tissue which could have been due to the mobilization of starch, which was reduced in girdled

tree tissue, but total sugar levels were not different. One activity that remained significantly increased in girdled tissue regardless of normalization was that activity of cinnamyl alcohol dehydrogenase (CAD) which converts cinnamyl aldehyde to cinnamyl alcohol immediately prior to polymerization into lignin polymers *via* POX (Choi et al. 2016). However, the higher activity of this enzyme could be interpreted as being consistent with a wound healing response as opposed to be a response towards the insect. Manchurian ash, in fact, has a very strong wound healing response as trees that were girdled in the previous year had completely healed over girdles the next year (personal observation).

Addressing intraspecific variation in resistance from here will be more complicated than interspecific variation because it does not appear to be as clearly defined as interspecific variation. Two things that we were not able to address in Chapter 5 were bark ROS-generation *in vivo* and the physiological responses of larvae to feeding on girdled and control Manchurian ash. It is possible that though oxidase enzyme activities were not significantly different by treatment, ROS generation by other mechanisms may be inhibited by girdling. The following is speculative, but I believe these experiments actually warrant further investigations into tyramine. Bark tyramine levels have been shown to be higher in Manchurian ash than susceptible species (Hill et al. 2012) and the activity of monoamine oxidase (MAO) was found to be significantly greater in Manchurian ash-fed larvae relative to larvae that fed on susceptible species (Rigsby et al. 2015).

There are three hypothesized defense-associated mechanisms that tyramine can have a major role: first, tyramine can be directly toxic to larvae, secondly, tyramine in ash

bark tissue could be involved in the formation of wound periderm, and third, tyramine itself could be a source for ROS-generation in the midgut of larvae as one of the products of the oxidation of tyramine by MAO is H_2O_2 (Holt et al. 1997). Girdling severs the connection between the canopy and the lower parts of the tree, eliminating photoassimilate transport and if the limited amount of bark nitrogen is mobilized and re-allocated to protein and/or lignin synthesis at the wound site, little to no nitrogen (in the form of tyramine) would be available to serve a defensive function (*via* one or more of the three above hypotheses). Therefore, I suggest further investigations into the potential role of tyramine in the form of diet bioassays and looking for ash genes responsible for incorporating tyramine into cell walls. Additionally, intraspecific variation in resistance could also be explained by differences in volatile terpenoid synthesis. Preliminary experiments (Chapter 3) showed that bark VOC emissions were substantially reduced below the girdle of Manchurian ash. Constituents of bark VOC profiles below the girdle have yet to be identified but it is possible that certain volatiles could have roles in antibiosis.

7.4 CONCLUSIONS FOR FUTURE RESEARCH FOCUS

From a more general perspective, while both antixenosis and antibiosis warrant further investigation, I do believe that a particular emphasis should be placed on antibiosis. First, since the very definition of antibiosis implies deleterious effects on the herbivore (Smith 2005), the existence of antibiosis in the North American landscape would have the added benefit of increasing the number of tree-killed larvae and therefore help reduce populations. It is also hypothesized that natural selection cannot result in an

organism that has both strong antibiosis and antixenosis (Abrahamson and Weis 1997; Mauricio et al. 1997; Wise et al. 2008). An organism that has evolved strong antixenosis would therefore have no selection pressure for strong antibiosis because relatively few herbivores would choose to use it as a host. Likewise there would be no selection for individuals with strong antixenosis on those organisms already expressing strong antibiosis because herbivores choosing to use that particular host would have relatively deleterious effects. Direct inoculation of larvae on Manchurian ash shows that this species displays formidable, but not complete antibiosis and therefore, while antixenosis could certainly be contributing to resistance in the North American landscape where no antixenosis or antibiosis exists, I believe antibiosis is relatively more important than antixenosis.

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