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Conifer Responses to a Stylet-Feeding Invasive Herbivore and Induction with Methyl Jasmonate: Impact on the Expression of Induced Defenses and a Native Folivore

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Abstract

1. Trees attacked by multiple herbivores need to defend themselves against dynamic biotic challenges; appropriate responses to one stressor can elicit hormonal responses that are antagonistic to another. Hemlock (*Tsuga canadensis*) infestation by hemlock woolly adelgid (HWA; *Adelges tsugae*) results in the accumulation of the defensive hormone salicylic acid (SA).
2. We explored the potential for HWA infestation to interfere with anti-folivore induced defense signaling and its implications for a native folivore (hemlock looper; *Lambdina fiscellaria*). Hemlocks were infested with HWA and/or sprayed with methyl jasmonate (MeJA); foliar defenses were analyzed and foliage quality for looper larvae was assessed.
3. Both treatments activated foliar defensive traits, including a HWA-mediated increase in peroxidase activity and accumulation of cell wall-bound phenolics and lignin, and a MeJA-mediated increase in lipoxygenase activity. The two treatments had an additive effect on other defensive traits and both treatments negatively affected looper performance.
4. These results suggest that SA and JA are not strictly antagonistic in conifers and that both have a role in anti-folivore defense signaling. Our study illustrates the need for a better understanding of hormone signaling, cross-talk, and induced responses in conifers.

Key Words conifers; SA-JA antagonism; induced defense signaling; stylet-feeders; defense induction

Introduction

Conifers (Pinaceae) often dominate temperate, alpine, and boreal forests in the northern hemisphere (Ralph *et al*., 2006). This family includes genera of major ecological and economic importance such as pine (*Pinus*), spruce (*Picae*), hemlock (*Tsuga*), and fir (*Abies*), and the ecological success of many conifer species is thought to be linked to their effective defenses against natural enemies (Bonello *et al*., 2006; Krokene, 2015). The energetic costs of these anti-herbivore responses make it important that plants be induced only when appropriate (Baldwin, 1998). In conifers, for example, the accumulation of terpene and phenolic metabolites induced by bark beetle (Coleoptera: Curculionidae) attacks can substantially improve the likelihood of host survival (e.g., Schiebe *et al*., 2012). Aside from a few specific systems (e.g., the pine processionary moth; *Thaumetopoea pityocampa*), most research addressing induced defense responses in conifers has focused on pine and spruce interactions with bark beetles; less attention has been paid to defense against other herbivorous insects (Ralph *et al*., 2006; Eyles *et al*., 2010).

When multiple herbivore species are present, the responses induced by one herbivore can affect co-occurring species. There are multiple examples of herbivores from different feeding guilds (e.g., leaf-chewing, stylet-feeding) indirectly affecting each other through their impact on plant physiology (e.g., Soler *et al*., 2012). The phytohormones jasmonic acid (JA) and salicylic acid (SA) play a central role in these induced plant defenses. Chewing insects such as caterpillars are generally thought to trigger the JA pathway, while stylet-feeding insects often elicit the SA pathway (Morkunas *et al*., 2011). Researchers have demonstrated positive interactions (cross-talk) and antagonism between these induced-response pathways that prevent plants from responding simultaneously to SA- and JA-elicited challenges (e.g., Kroes *et al*., 2015). However, this research has mostly been conducted using herbaceous model plants such as *Arabidopsis*,

tomato (*Solanum lycopersicum*), and tobacco (*Nicotiana tabacum*) (e.g., Preston *et al*., 1999; O'Donnell et al., 2003; Cipollini *et al*., 2004) (see Thaler *et al*., 2012).

Much less attention has been paid to woody plants. Although SA-JA antagonism has been demonstrated in *Eucalyptus grandis* (Naidoo *et al*., 2013), induced response signaling in woody plants are likely mediated by signaling molecules that may be at least partly different from those of herbaceous systems, and in ways that are more complex (Eyles *et al*., 2010; Zhang *et al*., 2010). For example, in Norway spruce (*Picea abies*), white-rot fungus (*Heterobasidion parviporum*) infection leads to the parallel induction of both SA and JA pathways (Arnerup *et al*., 2011), exogenously applied JAs can enhance pathogen resistance (Kozlowski *et al*., 1999), and exogenously applied SA can increase resistance against *Ips typographus* bark beetles (Krajnc *et al*., 2011). It is important to note, however, that hormone signaling complexity has been reported and discussed in model herbaceous plant systems, as well (e.g., Kazan & Manners, 2008). Generally, however, the signaling hormones involved in woody plant responses, and their interactions (i.e., cross-talk), remains largely unexplored and many aspects of these processes are unknown (Eyles *et al*., 2010; Zhang *et al*., 2010). Furthermore, the indirect interactions between herbivorous insects of different feeding guilds *via* alterations to induced defense responses in woody plants is also largely unknown, especially for conifers.

Stylet-feeding arthropods (i.e., mites and insects) are major conifer pests in both horticultural and forest settings (Cram *et al*., 2012; Van Driesche *et al*., 2013) and can be very damaging during outbreaks (e.g., spruce spider mite [*Oligonychus ununguis*]; Furniss & Carolin, 1977; Monterey pine needle aphid [*Essigella californica*]; Hopmans & Elms, 2013). Knowledge of mechanisms of induced resistance of conifers to stylet-feeding arthropods is relatively lacking compared to other feeding guilds. Our understanding of how stylet-feeders indirectly interact

with co-occurring herbivores (e.g., folivores) of conifers *via* changes in host quality is also limited. Mattson *et al*., (1989) reported that balsam twig aphid (*Mindarus abietinus*) density was inversely correlated with the survival and development of spruce budworm (*Choristoneura fumiferana*); Grégoire *et al*., (2015) found lower pupal weights in spruce budworm reared on trees that were symptomatic of balsam woolly adelgid (*Adelges piceae*) infestation. The authors of the latter paper hypothesized that this relationship reflected decreased foliar quality, although they could not detect clear relationships between specific adelgid symptoms, foliar secondary metabolites, and larval performance (Grégoire *et al*., 2014; Grégoire *et al*., 2015).

Several studies have investigated the metabolic and physiological effects of the invasive hemlock woolly adelgid (HWA; *Adelges tsugae*) infestation on eastern hemlock (hemlock; *Tsuga canadensis*). There is evidence that HWA feeding causes a hypersensitive-like response in hemlock involving the foliar accumulation of hydrogen peroxide (H_2O_2; Radville *et al*., 2011), proline (Gómez *et al*., 2012), and SA (Schaeffer *et al*., 2018). Adelgid infestation also increases emissions of methyl salicylate (MeSA), the volatile methyl ester of SA (Pezet *et al*., 2013; Pezet & Elkinton, 2014). These physiological effects indicate that HWA infestation induces a hypersensitive-like, SA-linked response in the foliage of this conifer, and this reaction may indirectly affect other herbivores by interfering with typical hormonal responses and induced defenses in hemlock (e.g., Kroes *et al*., 2015).

We present the results of research evaluating the ability of HWA to interfere with standard induced defense signaling and expression (tested by applying methyl jasmonate [MeJA] to plants with and without HWA) and assessing the plant-mediated impact of these treatments on a native folivore, hemlock looper (looper; *Lambdina fiscellaria*). The goals of this study were to (1) assess the impact of both SA-linked defenses *via* HWA infestation, and JA-linked defenses

via MeJA application, on the performance of a folivore, and to (2) determine whether HWA infestation alters the expression of JA-linked defenses and affects the negative impacts of JA-linked defense induction on folivores. We hypothesized that JA-linked responses are more appropriate anti-folivore defenses than SA-linked responses, and that HWA presence would attenuate the negative effects of JA-linked responses on looper larvae and on the expression of JA-linked defenses, presumably due to hormone signaling interference.

Materials and methods

Study System

Hemlock is a structurally-dominant and ecologically-important conifer endemic to eastern North America, a "foundational species" that creates unique and critical habitat for many terrestrial and aquatic species (Snyder et al., 2002; Ellison *et al*., 2005; Orwig *et al*., 2008). Hemlock woolly adelgid is an invasive stylet-feeding insect introduced to Virginia in the 1950s (Havill *et al*., 2006). The invasion of eastern North America by HWA has caused widespread mortality of both eastern and Carolina hemlock (*T. caroliniana*) and threatens to extirpate these species from their native range. The life cycle of HWA specifically, and Adelgidae generally, are detailed elsewhere (McClure, 1989; Havill & Foottit, 2007); Briefly, HWA is bivoltine, with a holocyclic lifecycle in its native range but an obligate parthenogenetic lifecycle in its introduced range. Although the first-instar ‘crawler’ phase can move along branches or be passively dispersed between trees (McClure, 1990), adults are sessile, settling and feeding at the base of needles on xylem ray parenchyma cells (Young *et al*., 1995). Conversely, hemlock looper is native to eastern North America and feeds on many tree species including eastern and Carolina hemlock

(Wilson *et al*., 2016). This insect has been linked to the mid-Holocene decline of hemlocks in the northeastern United States (Foster *et al*., 2006) and widespread defoliation events in Maine in the early 1990s and eastern Canada in the 2000s (discussed in Wilson *et al*., 2016). Larval emergence occurs in the late spring and is timed to coincide with bud burst and the production of new foliage of its conifer hosts (Butt *et al*., 2010); late-instar larvae are, however, capable of feeding on older growth (Carroll, 1999). At outbreak densities, the feeding activity of late-instar larvae can cause rapid needle loss and kill mature trees within two years (Alfaro *et al*., 1999). These two herbivores co-occur in the northern portion of the HWA-invaded range and in the southern portion of the native range of the looper (Wilson *et al*., 2016).

Experimental Approach

Approximately 300 hemlock plants were purchased in the spring of 2015 as saplings (0.8-1.0 m in height) from Van Pines Nursery (West Olive, MI; derived from seed collected in Pennsylvania). All plants were previously herbivore-free and had not been treated with insecticides. Potted plants (7.6 liter/2 gallon pot size) were placed outside under shade cloth at The University of Rhode Island (URI; Kingston, RI, USA), regularly watered, and minimally fertilized (14:14:14 N:P:K Scotts Osmocote Controlled Release Fertilizer). Plants were overwintered outside under winter protection fabric (170 g $yard^{-2}$; Griffin Greenhouse Supplies).

Half of the hemlocks were assigned randomly to the HWA treatment. Each tree in this treatment was inoculated in late spring of 2015, 2016, and 2017 (timed to coincide with HWA progrediens crawler emergence) using locally-collected (Mt. Tom State Reservation, MA, USA), infested hemlock foliage and standard inoculation protocols (Butin *et al*., 2007). Each potted plant in the HWA received two branches (approximately 15-20 cm long) with densities ≥ 0.5

158 ovisacs cm^{-1}. Plants were annually infested with progrediens generation crawlers as part of
159 ongoing experimentation at URI and to generate an in-house source of HWA for use in unrelated
160 experiments. Additionally, reports of deleterious impacts of HWA on hemlock have been
161 reported mostly in the context of chronic infestation (Radville *et al*., 2011; Gómez *et al*., 2012;
162 Pezet *et al*., 2013; Pezet & Elkinton, 2014; Schaeffer *et al*., 2018; Wilson *et al*., 2018). The other
163 half of the hemlocks were assigned to the control (no HWA) treatment. To control for
164 mechanical disturbance, trees in the control treatment were 'sham inoculated' with HWA-free
165 hemlock foliage when trees in the HWA treatment were inoculated with infested foliage. To
166 insure that control trees remained free of HWA, both infested and uninfested plants were covered
167 with insect-proof mesh (AG-15 Insect Barrier; Agribon, Johnny's Selected Seeds, Waterville,
168 ME, USA; 90 %light transmission). At the time of experimentation, densities of adult
169 progrediens HWA (with ovisacs) were approximately 0.5 HWA cm^{-1} on infested trees and
170 control trees were confirmed HWA-free *via* visual inspection. No quantitative data on plant
171 growth or condition were taken, but visual inspection showed that infested plants were roughly
172 the same size as uninfested plants, but the foliage was not the characteristic bright-green of
173 healthy, uninfested plants such as those in the uninfested treatment.

174 Following the spring 2017 inoculation, twenty trees in the HWA treatment and twenty
175 trees in the control treatment were assigned randomly to one of two elicitor treatments (n = 10
176 per treatment): JA-induced (*via* MeJA) or constitutive (carrier solution only). MeJA was first
177 dissolved in a minimal amount of absolute ethanol (~ 0.5 ml) and then suspended in 0.1% (v:v)
178 Tween 20 carrier solution to produce a 1 mM concentration of MeJA (Sigma; St. Louis, MO).
179 This resulted in four 10-replicate treatments (40 total plants; used in bioassays and in chemical
180 analyses). The appropriate elicitor solution was applied with an atomizer until plants were

saturated once every week; preliminary experimentation determined the elicitor concentration used (Rigsby *unpublished data*). Two rounds of elicitor treatments were applied prior to the use of foliage in the bioassay (detailed below), and three rounds of elicitor treatments were applied during the bioassay. Elicitor applications were never made fewer than four days prior to the removal of foliage from plants and placing foliage in jars for the looper feeding bioassay. This was done to prevent any direct impact of MeJA on larvae. After five elicitor treatments, two randomly selected branches were removed from each plant, wrapped in aluminum foil and stored at -80°C for chemical analyses. Needle tissue was later separated from stems, ground in liquid nitrogen, partitioned into tubes (see below), and stored at -30°C until analysis.

Defense Responses

Equipment and Reagents We were interested in how our treatments broadly altered the chemistry and physiology of hemlock and therefore elected to utilize more general analytical methods. Bradford assay dye concentrate was purchased from BioRad (Hercules, CA, USA), and polyvinylpolypyrrolidone (PVPP; 25 μm average particle size) was purchased from The Vintner Vault (Paso Robles, CA, USA). All other reagents and standards were purchased from Sigma (St. Louis, MO). Spectrophotometric assays were performed in Greiner UV-Star® 96 well plates (Monroe, NC, USA). Plates were read using a SpectraMAX M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) in the RI-INBRE facility (University of Rhode Island; Kingston, RI).

Defensive Enzymes To extract native protein, 200 mg tissue was reacted with 1.5 ml 50 mM NaPO4 (pH 6.8) containing 10% (w:v) PVPP, 5% (w:v) Amberlite XAD4 resin (pre-

conditioned), and 1 mM EDTA on ice for 20 min and the 10,000 g supernatant (5 min, 4°C) was recovered and used as the source of enzymes. The guaiacol-oxidizing (ε_{470} = 26.6 mM^{-1} cm^{-1}) activity of peroxidase (POX) was quantified according to Cipollini et al., (2011). The activities of chitinase (CHI) and lipoxygenase (LOX; ε_{234} = 23,000 M^{-1} cm^{-1}; modifying to accommodate a 96-well microplate format) were quantified according to Rigsby et al., (2016).

Secondary Metabolites and H_2O_2 For soluble phenolic metabolites (total soluble phenolics, hydroxycinnamic acids, flavonoids, and proanthocyanidins), 200 mg tissue was twice-extracted in 0.5 ml methanol for 24 hrs and the supernatants were pooled. Total soluble phenolic levels were quantified using a modified Folin-Ciocalteau procedure described by Cipollini et al., (2011) against a standard curve of gallic acid. Hydroxycinnamic acids were quantified with Arnow's reagent against according to St-Pierre et al., (2013) against standard curve of chlorogenic acid. Total flavonoids were quantified according to the procedure described by Chang et al., (2012) against a standard curve of quercetin. Proanthocyanidin content was estimated according to the acidified butanol method (Engstöm et al., 2014). The lack of affordable standards and issues with using purified standards in the proanthocyanidin assay (Schofield et al., 2001) required that we express tissue levels as Abs_{550} g^{-1} FW. Lastly, methanol soluble terpene levels were quantified using H_2SO_4 according to Ghorai et al., (2012) against a standard curve of linalool.

Tissue pellets left over from the extraction of soluble phenolics were washed twice with methanol and cell wall-bound phenolics were extracted *via* esterification (de Ascensao & Dubery 2003) and quantified by way of the total phenolic content procedure described previously using gallic acid as standard. The tissue pellets were then subjected to the lignin extraction and quantification procedure described by Cipollini et al., (2011) using spruce lignin as standard.

Needle H_2O_2 levels were estimated according to the KI procedure described by Rigsby et al., (2016) using H_2O_2 as a standard curve.

Looper Bioassay

In early spring 2017, we obtained looper eggs from a colony maintained at the Canadian Forest Service's Laurentian Forestry Centre (Québec City, QC, Canada). Movement of the eggs from Canada to the United States, and our subsequent work with them, was covered under APHIS permit P526P-14-01875. The eggs were placed on arrival in a growth chamber (15^{o}C, 75% RH, 16L/8D cycle) and monitored daily for hatching. Upon hatching, a 15-cm stem section was clipped from each of the treated plants and stuck in a moistened piece of floral foam within a 0.8 L Ball Mason jar. Each plant provided all of the foliage for a given jar throughout the experiment and contained both current-year foliage and foliage produced in past years. The APHIS permit necessary to work with these larvae required that they be contained in a biological control facility, and the potted plants used in these experiments were too large to bring into the facility and be placed in environmental chambers. This necessitated the use of clipped foliage in jars rather than larvae being directly placed on plants. Larvae were assigned randomly to jars as they hatched until each jar contained six looper larvae. Each jar was covered with a fine white mesh (0.5 mm; nylon) to allow ventilation but prevent escape. Jars were kept in the growth chamber, changing their position daily within the growth chamber to account for possible microclimatic differences. Each jar was cleaned weekly by adding a new stem section, replacing the floral foam, and removing all waste from the jar. Foliage was never placed into jars within 48 hrs of being sprayed with elicitor.

We conducted weekly survival assays by removing all foliage and floral foam from the jar and transferring living larvae into clean jars with new foliage and floral foam. Larvae were monitored until pupation, at which point the date of pupation was noted and the pupa weighed. Data on the six looper larvae per jar was averaged to generate a per-jar mean for each of the 40 replicates.

Data Analysis

Plant chemical and physiological parameters were analyzed *via* a two-way analysis of variance (ANOVA) with HWA, MeJA application, and the interaction as predictors. If a significant interaction was found, a Tukey test was used to separate means. For the bioassay experiment, looper survival, pupal weight, and time to pupation were statistically treated similarly to Wilson *et al*., (2016). Briefly, data were inspected for normality (Shapiro-Wilk test) and homoscedacity (Bartlett's test) (all response variables satisfied these requirements), and then a repeated measures-ANOVA was used to analyze the effect of HWA, MeJA application, and their interaction. The effect of the same predictors on time to pupation and pupal weights were analyzed using a two-way ANOVA. The statistical program R was used for all analyses (R Development Core Team, 2017).

Results

Hemlock Foliar Defense Responses

Defensive/Antioxidant Enzyme Activities Adelgid infestation increased the activity of both POX and CHI, but not LOX (Table 1). Elicitor application increased the activity of CHI and LOX, but

not POX (Table 1), and there was no significant HWA x elicitor interaction for any enzyme activity (Table 1).

Metabolites Adelgid infestation and MeJA application significantly impacted all classes of soluble phenolics (Table 1). HWA and MeJA both tended to have an additive effect on all phenolic categories; the HWA x elicitor interaction was nonsignificant for all of the soluble phenolic classes (Table 1). HWA infestation increased the cell-wall-bound phenolic content of foliage, but there was no effect of MeJA or the HWA x elicitor interaction (Table 1). Adelgid-infested plants also contained more lignin, and although there was no main effect of MeJA, there was an interactive effect between HWA infestation and MeJA application on lignin content where MeJA application appeared to attenuate the HWA-caused increase in lignin. Methanol-soluble terpene content of foliage was not influenced by HWA infestation or elicitor treatment with terpene content remaining constant between treatment combinations ($P > 0.05$ for all; Table 1). Lastly, needle H_2O_2 content was elevated by HWA infestation and decreased by MeJA, but there was no significant interactive effect. The H_2O_2 content of foliage was highest in the infested-control treatment and lowest in the uninfested-MeJA treatment (Table 1).

Herbivore Responses

HWA infestation reduced the survival of looper larvae over time ($F_{1, 434} = 5.49$, $P = 0.0196$; Fig. 1A), and there was a trend (albeit insignificant; $P = 0.0999$) towards HWA increasing pupal weight ($F_{1, 36} = 2.86$, $P = 0.0999$; Fig. 1B). While MeJA did not affect larval survival ($F_{1, 434} = 0.73$, $P = 0.39$; Fig. 1A), it did decrease weight at pupation ($F_{1, 36} = 7.26$, $P = 0.0107$; Fig. 1B).

The HWA x elicitor interaction affected neither larval survival nor pupal weights ($P > 0.05$). Time to pupation was not affected by any predictor variable ($P > 0.05$).

Discussion

We found that changes in hemlock physiology associated with an invasive herbivore and with elicitor application affected both secondary chemistry and the response of a native herbivore. Although our initial hypothesis of HWA/MeJA (i.e., SA/JA) antagonism was generally not supported, the physiological responses of hemlock that we observed appear partly mediated by both SA and JA pathways. Such antagonistic responses are important since plants often must respond to simultaneous or sequential challenges (Ponzio *et al*., 2013). Moreover, our results are consistent with the ability of stylet-feeding insects to manipulate plant physiology *via* induced defenses linked to this cross-talk in ways that can dramatically alter host quality for other herbivores (e.g., Inbar *et al*., 1999). Historically, there has been little research specifically addressing JA-SA cross-talk and indirect herbivore effects in woody plants. The hemlock-HWA system provides an excellent model for better understanding these indirect interactions as chronic HWA infestation results in SA induction and a hypersensitive-like response in its host (Radville *et al*., 2011; Gómez *et al*., 2012; Pezet *et al*., 2013; Pezet & Elkinton, 2014; Schaeffer *et al*., 2018). We had expected that both HWA infestation (SA induction) and MeJA (JA induction) would induce changes in hemlock chemistry and physiology and would affect looper performance, but that simultaneous challenge would result in hormonal signaling interference that would compromise the induction and expression of appropriate anti-folivore defenses, ultimately positively influencing looper larvae.

We found certain defensive traits to be distinctly elicited by one treatment, some of which were predictable. LOX activity was positively affected by MeJA application, for example, and HWA infestation had a positive impact on H_2O_2 accumulation. These traits are associated with their respective signaling responses as LOX has a direct role in JA synthesis (Beckers & Spoel, 2006) and H_2O_2 accumulation is associated with SA signaling both upstream and downstream of SA (Herrera-Vásquez *et al*., 2015). Intriguingly, POX activity and cell wall-bound phenolic and lignin accumulation were positively affected only by HWA infestation. Peroxidases use H_2O_2 as a co-substrate to polymerize phenolics and monolignols, which serve to scavenge H_2O_2 (Tenhaken, 2014). The extent to which the HWA-mediated increase in POX activity, cell wall-bound phenolic, and lignin accumulation is an antioxidant response to H_2O_2 accumulation or an SA-linked anti-herbivore response remains to be determined. We also found, however, that certain defensive traits were not strictly regulated by one induction treatment or the other, and these responses appeared to be additive rather than antagonistic (i.e., CHI activity and soluble phenolics). One defensive trait (methanol-soluble terpene content) was not influenced by either treatment, though this is not necessarily surprising as it has been shown that conifers may not accumulate foliar terpenes following herbivore attack (e.g., Litvak & Monson, 1998). Additionally, the use of methanol to extract terpenes, as per this assay method (Ghorai *et al*., 2012), may limit the interpretation of the results of this assay as methanol is a relatively poor solvent for non-polar terpene species.

One of the more important and interesting results of this study, confirming the findings of previous researchers (Radville *et al*., 2011), is not only that hemlock accumulates H_2O_2 when infested with HWA, but also that H_2O_2 did not accumulate when plants were sprayed with MeJA. Hydrogen peroxide has a variety of functions in plants in addition to being a co-substrate

for POX enzymes (Cheeseman, 2007), including roles in stress response-signaling (Orozco-Cárdenas *et al*., 2001; Morkunas *et al*., 2011; Petrov & Van Breusegem, 2012). For example, H_2O_2 accumulation resulted in the identification of 82 H_2O_2-responsive proteins in leaves of seedling hybrid poplars (*Populus simonii* × *Populus nigra*) (Yu *et al*., 2017). Hydrogen peroxide has also been shown to both amplify and antagonize SA signaling/accumulation (Peleg-Grossman *et al*., 2010; Petrov & Van Breusegem, 2012). The ultimate implications and impacts of H_2O_2 accumulation in hemlock foliage remain unknown, but are likely consequential as H_2O_2 accumulation could have any of the described effects in hemlock or others. Furthermore, the interaction between H_2O_2 and JA, and specifically the fact that JA pathway activation (*via* MeJA application) results in a reduction in H_2O_2 levels regardless of HWA infestation, suggests that antioxidant mechanisms are part of JA pathway elicitation.

The effects of our treatments on hemlock foliar defenses and the ultimate impacts on looper larvae were mixed. Our hypothesis that JA-linked responses are appropriate anti-folivore defenses was supported; our hypothesis that HWA infestation would interfere with standard anti-folivore (i.e., JA) induced defense signaling and would attenuate the negative effects of JA-linked responses on looper was not supported. For example, MeJA application reduced looper pupal weights, but did not affect looper survival, while HWA did not significantly impact pupal weights or larval survival. This suggests that induced defense signaling is more nuanced than simple JA-SA antagonism in hemlock, and that both hormones likely have roles. The notion that extensive JA-SA cross-talk exists in plant biotic stress response signaling is not novel (e.g., Smith *et al*., 2009), but these findings highlight the complex nature of this cross-talk and how additional complexity can be introduced when plants are attacked by multiple herbivores (Nguyen *et al*., 2016).

In this study, we demonstrated that HWA induces defense responses involving phenolic metabolites and antioxidant/defensive proteins, that these responses are not necessarily the same in MeJA-induced plants, and that some responses were additive (e.g., phenolics). The treatment-associated physiological effects on hemlock foliage had mixed effects on looper larval performance, where survival was negatively impacted by HWA infestation and MeJA application negatively impacted pupal weight. Our results only partly supported our initial hypotheses that JA-linked responses are more appropriate anti-folivore defenses, and that HWA infestation would benefit folivores by interfering with standard anti-folivore (i.e., JA-linked) hormonal signaling. It is possible that the infestation level of our plants (0.5 HWA cm^{-1}), while ecologically relevant, may not have been enough to result in our hypothesized effects. Our results illustrate how HWA-mediated plant defense induction can alter the suitability of this conifer for other co-occurring herbivores but also emphasize the need to further study multi-stress interactions and physiological antagonism in conifers.

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Figure 1. Response of hemlock looper to HWA infestation and MeJA application. (A) Average number surviving looper larvae (± 1 SE) through time that fed on foliage of plants from the four treatments. (B) Mean pupal weight in mg (± 1 SE) of hemlock looper larvae fed foliage of plants from the four treatments.

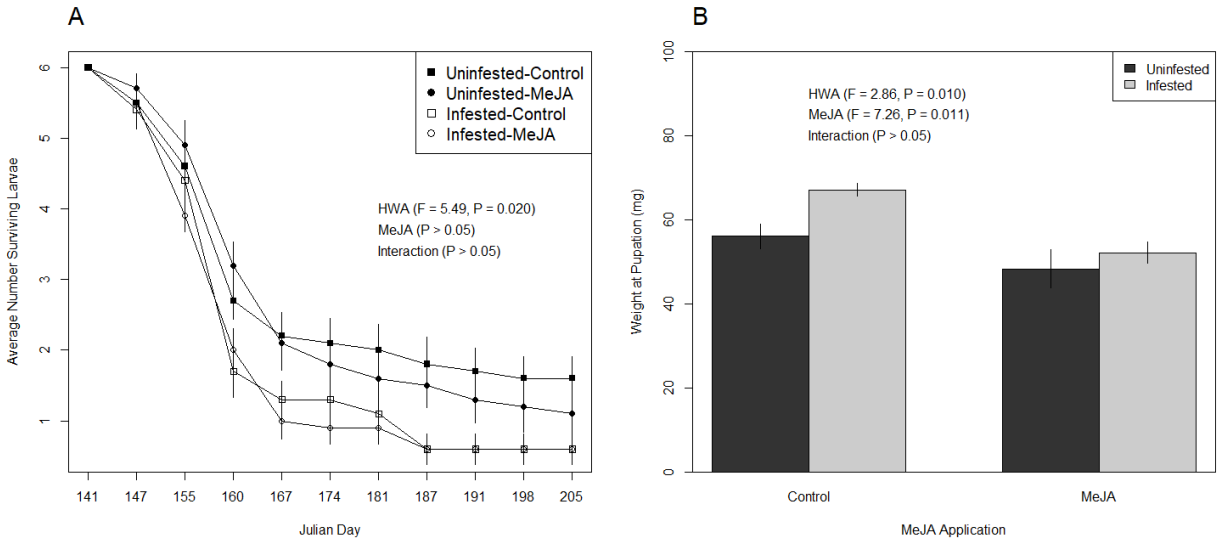

Table 1. The effect of HWA-infestation, MeJA-application, and the interaction on enzyme activities and metabolites that were quantified. *F*- and *P*-values (significant values are in bold) are the results of a two-way ANOVA using HWA-infestation, MeJA-application, and the interaction as predictor variables. Different letters indicate significant differences between treatment combinations according to a post-hoc Tukey test, and no letters indicate no significant treatment differences.

Response Variable	Uninfested		Infested		HWA-Infestation		MeJA-Application		Interaction	
Enzyme Activities	Control	MeJA	Control	MeJA	$F_{1,36}$	*P*	$F_{1,36}$	*P*	$F_{1,36}$	*P*
Peroxidase (POX)	157.2 (19.2)	182.6 (40.7)	329.4 (67.3)	393.1 (122.1)	6.8	**0.013**	0.4	0.547	0.1	0.795
Chitinase (CHI)	0.22 (0.04)^{b}	0.33 (0.05)^{b}	0.62 (0.10)^{a}	0.86 (0.10)^{a}	33.6	**< 0.001**	5.1	**0.030**	0.7	0.422
Lipoxygenase (LOX)	74.2 (15.3)^{ab}	92.5 (10.1)^{ab}	69.4 (6.9)^{b}	126.3 (20.4)^{a}	1.1	0.313	7.1	**0.011**	1.9	0.179
Metabolites										
Total Soluble Phenolics	78.1 (4.8)^{c}	101.8 (3.3)^{b}	99.5 (5.8)^{b}	131.0 (6.4)^{a}	23.6	**< 0.001**	27.9	**< 0.001**	0.6	0.457
Hydroxycinnamic Acids	35.8 (1.5)^{b}	54.9 (4.5)^{a}	47.6 (3.9)^{ab}	58.3 (2.6)^{a}	4.3	**0.046**	19.3	**< 0.001**	1.5	0.232
Flavonoids	50.6 (2.2)^{b}	66.1 (2.0)^{a}	62.1 (3.4)^{a}	70.1 (1.6)^{a}	10.7	**0.002**	24.2	**< 0.001**	2.5	0.124
Proanthocyanidins	0.6 (0.1)^{c}	1.4 (0.1)^{b}	1.2 (0.17)^{b}	1.8 (0.2)^{a}	11.7	**0.002**	22.4	**< 0.001**	0.2	0.650
Cell Wall-Bound Phenolics	10.0 (1.5)^{b}	13.4 (1.8)^{ab}	22.1 (6.0)^{a}	18.1 (2.1)^{ab}	6.7	**0.014**	0.0	0.919	1.3	0.258
Lignin	3.8 (0.2)^{b}	4.5 (0.2)^{ab}	4.9 (0.3)^{a}	4.5 (0.2)^{ab}	7.3	**0.011**	0.4	0.532	5.8	**0.021**
Methanol-Soluble Terpenes	2.8 (0.4)	2.5 (0.1)	3.1 (0.1)	2.8 (0.2)	1.9	0.177	1.8	0.186	0.0	0.918

H_2O_2	65.7 (11.9)^{ab}	15.2 (3.3)^{b}	133.6 (39.1)^{a}	39.9 (13.6)^{b}	4.3	**0.047**	13.1	**0.001**	1.2	0.285

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