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

3

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20 Abstract

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

38

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

41

42 Introduction

43
44 Conifers (Pinaceae) often dominate temperate, alpine, and boreal forests in the northern
45 hemisphere (Ralph *et al.*, 2006). This family includes genera of major ecological and economic
46 importance such as pine (*Pinus*), spruce (*Piceae*), hemlock (*Tsuga*), and fir (*Abies*), and the
47 ecological success of many conifer species is thought to be linked to their effective defenses
48 against natural enemies (Bonello *et al.*, 2006; Krokene, 2015). The energetic costs of these anti-
49 herbivore responses make it important that plants be induced only when appropriate (Baldwin,
50 1998). In conifers, for example, the accumulation of terpene and phenolic metabolites induced by
51 bark beetle (Coleoptera: Curculionidae) attacks can substantially improve the likelihood of host
52 survival (e.g., Schiebe *et al.*, 2012). Aside from a few specific systems (e.g., the pine
53 processionary moth; *Thaumetopoea pityocampa*), most research addressing induced defense
54 responses in conifers has focused on pine and spruce interactions with bark beetles; less attention
55 has been paid to defense against other herbivorous insects (Ralph *et al.*, 2006; Eyles *et al.*, 2010).

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

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

68 Much less attention has been paid to woody plants. Although SA-JA antagonism has been
69 demonstrated in *Eucalyptus grandis* (Naidoo *et al.*, 2013), induced response signaling in woody
70 plants are likely mediated by signaling molecules that may be at least partly different from those
71 of herbaceous systems, and in ways that are more complex (Eyles *et al.*, 2010; Zhang *et al.*,
72 2010). For example, in Norway spruce (*Picea abies*), white-rot fungus (*Heterobasidion*
73 *parviporum*) infection leads to the parallel induction of both SA and JA pathways (Arnerup *et al.*
74 *et al.*, 2011), exogenously applied JAs can enhance pathogen resistance (Kozlowski *et al.*, 1999),
75 and exogenously applied SA can increase resistance against *Ips typographus* bark beetles (Krajnc
76 *et al.*, 2011). It is important to note, however, that hormone signaling complexity has been
77 reported and discussed in model herbaceous plant systems, as well (e.g., Kazan & Manners,
78 2008). Generally, however, the signaling hormones involved in woody plant responses, and their
79 interactions (i.e., cross-talk), remains largely unexplored and many aspects of these processes are
80 unknown (Eyles *et al.*, 2010; Zhang *et al.*, 2010). Furthermore, the indirect interactions between
81 herbivorous insects of different feeding guilds *via* alterations to induced defense responses in
82 woody plants is also largely unknown, especially for conifers.

83 Stylet-feeding arthropods (i.e., mites and insects) are major conifer pests in both
84 horticultural and forest settings (Cram *et al.*, 2012; Van Driesche *et al.*, 2013) and can be very
85 damaging during outbreaks (e.g., spruce spider mite [*Oligonychus ununguis*]; Furniss & Carolin,
86 1977; Monterey pine needle aphid [*Essigella californica*]; Hopmans & Elms, 2013). Knowledge
87 of mechanisms of induced resistance of conifers to stylet-feeding arthropods is relatively lacking
88 compared to other feeding guilds. Our understanding of how stylet-feeders indirectly interact

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

97 Several studies have investigated the metabolic and physiological effects of the invasive
98 hemlock woolly adelgid (HWA; *Adelges tsugae*) infestation on eastern hemlock (hemlock;
99 *Tsuga canadensis*). There is evidence that HWA feeding causes a hypersensitive-like response in
100 hemlock involving the foliar accumulation of hydrogen peroxide (H₂O₂; Radville *et al.*, 2011),
101 proline (Gómez *et al.*, 2012), and SA (Schaeffer *et al.*, 2018). Adelgid infestation also increases
102 emissions of methyl salicylate (MeSA), the volatile methyl ester of SA (Pezet *et al.*, 2013; Pezet
103 & Elkinton, 2014). These physiological effects indicate that HWA infestation induces a
104 hypersensitive-like, SA-linked response in the foliage of this conifer, and this reaction may
105 indirectly affect other herbivores by interfering with typical hormonal responses and induced
106 defenses in hemlock (e.g., Kroes *et al.*, 2015).

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

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

118

119 **Materials and methods**

120

121 *Study System*

122 Hemlock is a structurally-dominant and ecologically-important conifer endemic to eastern North
123 America, a "foundational species" that creates unique and critical habitat for many terrestrial and
124 aquatic species (Snyder et al., 2002; Ellison *et al.*, 2005; Orwig *et al.*, 2008). Hemlock woolly
125 adelgid is an invasive stylet-feeding insect introduced to Virginia in the 1950s (Havill *et al.*,
126 2006). The invasion of eastern North America by HWA has caused widespread mortality of both
127 eastern and Carolina hemlock (*T. caroliniana*) and threatens to extirpate these species from their
128 native range. The life cycle of HWA specifically, and Adelgidae generally, are detailed
129 elsewhere (McClure, 1989; Havill & Footitt, 2007); Briefly, HWA is bivoltine, with a holocyclic
130 lifecycle in its native range but an obligate parthenogenetic lifecycle in its introduced range.
131 Although the first-instar 'crawler' phase can move along branches or be passively dispersed
132 between trees (McClure, 1990), adults are sessile, settling and feeding at the base of needles on
133 xylem ray parenchyma cells (Young *et al.*, 1995). Conversely, hemlock looper is native to
134 eastern North America and feeds on many tree species including eastern and Carolina hemlock

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

144

145 *Experimental Approach*

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

153 Half of the hemlocks were assigned randomly to the HWA treatment. Each tree in this
154 treatment was inoculated in late spring of 2015, 2016, and 2017 (timed to coincide with HWA
155 *progrediens* crawler emergence) using locally-collected (Mt. Tom State Reservation, MA, USA),
156 infested hemlock foliage and standard inoculation protocols (Butin *et al.*, 2007). Each potted
157 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

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

190

191 *Defense Responses*

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

201

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

204 conditioned), and 1 mM EDTA on ice for 20 min and the 10,000 g supernatant (5 min, 4°C) was
205 recovered and used as the source of enzymes. The guaiacol-oxidizing ($\epsilon_{470} = 26.6 \text{ mM}^{-1} \text{ cm}^{-1}$)
206 activity of peroxidase (POX) was quantified according to Cipollini et al., (2011). The activities
207 of chitinase (CHI) and lipoxygenase (LOX; $\epsilon_{234} = 23,000 \text{ M}^{-1} \text{ cm}^{-1}$; modifying to accommodate a
208 96-well microplate format) were quantified according to Rigsby et al., (2016).

209
210 *Secondary Metabolites and H₂O₂* For soluble phenolic metabolites (total soluble phenolics,
211 hydroxycinnamic acids, flavonoids, and proanthocyanidins), 200 mg tissue was twice-extracted
212 in 0.5 ml methanol for 24 hrs and the supernatants were pooled. Total soluble phenolic levels
213 were quantified using a modified Folin-Ciocalteu procedure described by Cipollini et al., (2011)
214 against a standard curve of gallic acid. Hydroxycinnamic acids were quantified with Arnou's
215 reagent against according to St-Pierre et al., (2013) against standard curve of chlorogenic acid.
216 Total flavonoids were quantified according to the procedure described by Chang et al., (2012)
217 against a standard curve of quercetin. Proanthocyanidin content was estimated according to the
218 acidified butanol method (Engstöm et al., 2014). The lack of affordable standards and issues with
219 using purified standards in the proanthocyanidin assay (Schofield et al., 2001) required that we
220 express tissue levels as $\text{Abs}_{550} \text{ g}^{-1} \text{ FW}$. Lastly, methanol soluble terpene levels were quantified
221 using H₂SO₄ according to Ghorai et al., (2012) against a standard curve of linalool.

222 Tissue pellets left over from the extraction of soluble phenolics were washed twice with
223 methanol and cell wall-bound phenolics were extracted *via* esterification (de Ascensao & Dubery
224 2003) and quantified by way of the total phenolic content procedure described previously using
225 gallic acid as standard. The tissue pellets were then subjected to the lignin extraction and
226 quantification procedure described by Cipollini et al., (2011) using spruce lignin as standard.

227 Needle H₂O₂ levels were estimated according to the KI procedure described by Rigsby et
228 al., (2016) using H₂O₂ as a standard curve.

229

230 *Looper Bioassay*

231 In early spring 2017, we obtained looper eggs from a colony maintained at the Canadian Forest
232 Service's Laurentian Forestry Centre (Québec City, QC, Canada). Movement of the eggs from
233 Canada to the United States, and our subsequent work with them, was covered under APHIS
234 permit P526P-14-01875. The eggs were placed on arrival in a growth chamber (15°C, 75% RH,
235 16L/8D cycle) and monitored daily for hatching. Upon hatching, a 15-cm stem section was
236 clipped from each of the treated plants and stuck in a moistened piece of floral foam within a 0.8
237 L Ball Mason jar. Each plant provided all of the foliage for a given jar throughout the experiment
238 and contained both current-year foliage and foliage produced in past years. The APHIS permit
239 necessary to work with these larvae required that they be contained in a biological control
240 facility, and the potted plants used in these experiments were too large to bring into the facility
241 and be placed in environmental chambers. This necessitated the use of clipped foliage in jars
242 rather than larvae being directly placed on plants. Larvae were assigned randomly to jars as they
243 hatched until each jar contained six looper larvae. Each jar was covered with a fine white mesh
244 (0.5 mm; nylon) to allow ventilation but prevent escape. Jars were kept in the growth chamber,
245 changing their position daily within the growth chamber to account for possible microclimatic
246 differences. Each jar was cleaned weekly by adding a new stem section, replacing the floral
247 foam, and removing all waste from the jar. Foliage was never placed into jars within 48 hrs of
248 being sprayed with elicitor.

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

254

255 *Data Analysis*

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

266

267 **Results**

268

269 *Hemlock Foliar Defense Responses*

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

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

274
275 *Metabolites* Adelgid infestation and MeJA application significantly impacted all classes of
276 soluble phenolics (Table 1). HWA and MeJA both tended to have an additive effect on all
277 phenolic categories; the HWA x elicitor interaction was nonsignificant for all of the soluble
278 phenolic classes (Table 1). HWA infestation increased the cell-wall-bound phenolic content of
279 foliage, but there was no effect of MeJA or the HWA x elicitor interaction (Table 1). Adelgid-
280 infested plants also contained more lignin, and although there was no main effect of MeJA, there
281 was an interactive effect between HWA infestation and MeJA application on lignin content
282 where MeJA application appeared to attenuate the HWA-caused increase in lignin. Methanol-
283 soluble terpene content of foliage was not influenced by HWA infestation or elicitor treatment
284 with terpene content remaining constant between treatment combinations ($P > 0.05$ for all; Table
285 1). Lastly, needle H_2O_2 content was elevated by HWA infestation and decreased by MeJA, but
286 there was no significant interactive effect. The H_2O_2 content of foliage was highest in the
287 infested-control treatment and lowest in the uninfested-MeJA treatment (Table 1).

288
289 *Herbivore Responses*

290 HWA infestation reduced the survival of looper larvae over time ($F_{1, 434} = 5.49$, $P = 0.0196$; Fig.
291 1A), and there was a trend (albeit insignificant; $P = 0.0999$) towards HWA increasing pupal
292 weight ($F_{1, 36} = 2.86$, $P = 0.0999$; Fig. 1B). While MeJA did not affect larval survival ($F_{1, 434} =$
293 0.73 , $P = 0.39$; Fig. 1A), it did decrease weight at pupation ($F_{1, 36} = 7.26$, $P = 0.0107$; Fig. 1B).

294 The HWA x elicitor interaction affected neither larval survival nor pupal weights ($P > 0.05$).

295 Time to pupation was not affected by any predictor variable ($P > 0.05$).

296

297 **Discussion**

298

299 We found that changes in hemlock physiology associated with an invasive herbivore and with

300 elicitor application affected both secondary chemistry and the response of a native

301 herbivore. Although our initial hypothesis of HWA/MeJA (i.e., SA/JA) antagonism was

302 generally not supported, the physiological responses of hemlock that we observed appear partly

303 mediated by both SA and JA pathways. Such antagonistic responses are important since plants

304 often must respond to simultaneous or sequential challenges (Ponzio *et al.*, 2013). Moreover, our

305 results are consistent with the ability of stylet-feeding insects to manipulate plant physiology *via*

306 induced defenses linked to this cross-talk in ways that can dramatically alter host quality for

307 other herbivores (e.g., Inbar *et al.*, 1999). Historically, there has been little research specifically

308 addressing JA-SA cross-talk and indirect herbivore effects in woody plants. The hemlock-HWA

309 system provides an excellent model for better understanding these indirect interactions as chronic

310 HWA infestation results in SA induction and a hypersensitive-like response in its host (Radville

311 *et al.*, 2011; Gómez *et al.*, 2012; Pezet *et al.*, 2013; Pezet & Elkinton, 2014; Schaeffer *et al.*,

312 2018). We had expected that both HWA infestation (SA induction) and MeJA (JA induction)

313 would induce changes in hemlock chemistry and physiology and would affect looper

314 performance, but that simultaneous challenge would result in hormonal signaling interference

315 that would compromise the induction and expression of appropriate anti-folivore defenses,

316 ultimately positively influencing looper larvae.

317 We found certain defensive traits to be distinctly elicited by one treatment, some of
318 which were predictable. LOX activity was positively affected by MeJA application, for example,
319 and HWA infestation had a positive impact on H₂O₂ accumulation. These traits are associated
320 with their respective signaling responses as LOX has a direct role in JA synthesis (Beckers &
321 Spoel, 2006) and H₂O₂ accumulation is associated with SA signaling both upstream and
322 downstream of SA (Herrera-Vásquez *et al.*, 2015). Intriguingly, POX activity and cell wall-
323 bound phenolic and lignin accumulation were positively affected only by HWA infestation.
324 Peroxidases use H₂O₂ as a co-substrate to polymerize phenolics and monolignols, which serve to
325 scavenge H₂O₂ (Tenhaken, 2014). The extent to which the HWA-mediated increase in POX
326 activity, cell wall-bound phenolic, and lignin accumulation is an antioxidant response to H₂O₂
327 accumulation or an SA-linked anti-herbivore response remains to be determined. We also found,
328 however, that certain defensive traits were not strictly regulated by one induction treatment or
329 the other, and these responses appeared to be additive rather than antagonistic (i.e., CHI activity
330 and soluble phenolics). One defensive trait (methanol-soluble terpene content) was not
331 influenced by either treatment, though this is not necessarily surprising as it has been shown that
332 conifers may not accumulate foliar terpenes following herbivore attack (e.g., Litvak & Monson,
333 1998). Additionally, the use of methanol to extract terpenes, as per this assay method (Ghorai *et*
334 *al.*, 2012), may limit the interpretation of the results of this assay as methanol is a relatively poor
335 solvent for non-polar terpene species.

336 One of the more important and interesting results of this study, confirming the findings of
337 previous researchers (Radville *et al.*, 2011), is not only that hemlock accumulates H₂O₂ when
338 infested with HWA, but also that H₂O₂ did not accumulate when plants were sprayed with
339 MeJA. Hydrogen peroxide has a variety of functions in plants in addition to being a co-substrate

340 for POX enzymes (Cheeseman, 2007), including roles in stress response-signaling (Orozco-
341 Cárdenas *et al.*, 2001; Morkunas *et al.*, 2011; Petrov & Van Breusegem, 2012). For example,
342 H₂O₂ accumulation resulted in the identification of 82 H₂O₂-responsive proteins in leaves of
343 seedling hybrid poplars (*Populus simonii* × *Populus nigra*) (Yu *et al.*, 2017). Hydrogen peroxide
344 has also been shown to both amplify and antagonize SA signaling/accumulation (Peleg-
345 Grossman *et al.*, 2010; Petrov & Van Breusegem, 2012). The ultimate implications and impacts
346 of H₂O₂ accumulation in hemlock foliage remain unknown, but are likely consequential as H₂O₂
347 accumulation could have any of the described effects in hemlock or others. Furthermore, the
348 interaction between H₂O₂ and JA, and specifically the fact that JA pathway activation (*via* MeJA
349 application) results in a reduction in H₂O₂ levels regardless of HWA infestation, suggests that
350 antioxidant mechanisms are part of JA pathway elicitation.

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

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

376

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383

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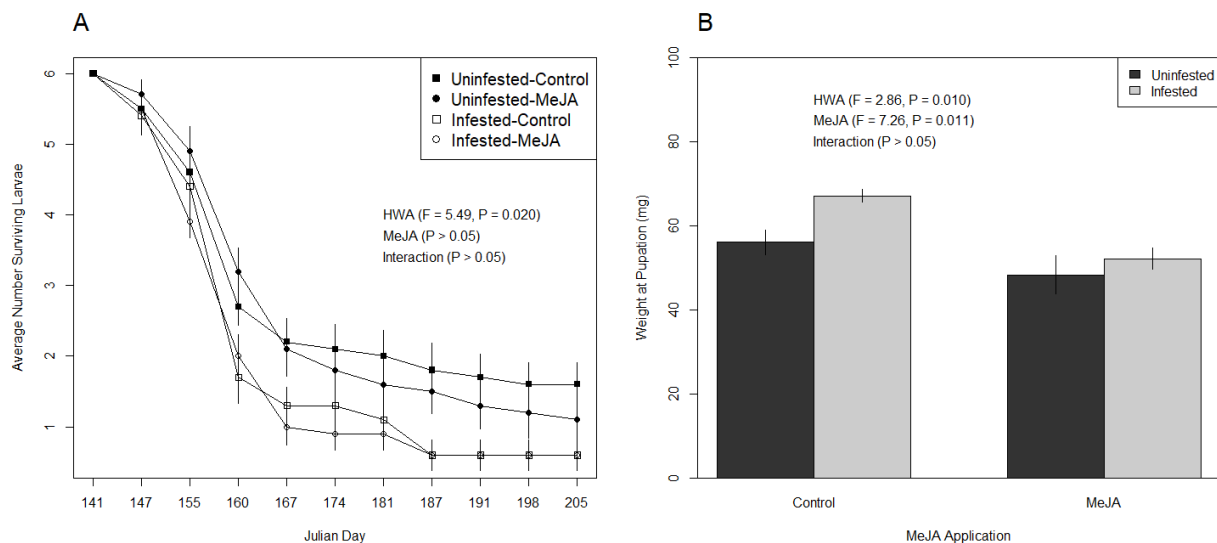
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596 Figure 1. Response of hemlock looper to HWA infestation and MeJA application. (A) Average
 597 number surviving looper larvae (± 1 SE) through time that fed on foliage of plants from the four
 598 treatments. (B) Mean pupal weight in mg (± 1 SE)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



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

Response Variable	Uninfested		Infested		HWA- Infestation		MeJA- Application		Interaction	
	Control	MeJA	Control	MeJA	<i>F</i> _{1,36}	<i>P</i>	<i>F</i> _{1,36}	<i>P</i>	<i>F</i> _{1,36}	<i>P</i>
Enzyme Activities										
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 ₂ O 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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