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Research paper

Impact of chronic stylet-feeder infestation on folivore-induced signaling and defenses in a conifer

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Our understanding of how conifers respond biochemically to multiple simultaneous herbivore attacks is lacking. Eastern hemlock (*Tsuga canadensis*; 'hemlock') is fed on by hemlock woolly adelgid (*Adelges tsugae*; 'adelgid') and by later-instar gypsy moth (*Lymantria dispar*; 'gypsy moth') caterpillars. The adelgid is a stylet-feeding insect that causes a salicylic acid (SA)-linked response in hemlock, and gypsy moth larvae are folivores that presumably cause a jasmonic acid (JA)-linked response. This system presents an opportunity to study how invasive herbivore–herbivore interactions mediated through host biochemical responses. We used a factorial field experiment to challenge chronically adelgid-infested hemlocks with gypsy moth caterpillars. We quantified 17 phytohormones, 26 phenolic and terpene metabolites, and proanthocyanidin, cell wall-bound (CW-bound) phenolic, and lignin contents. Foliage infested with adelgid only accumulated gibberellins and SA; foliage challenged by gypsy moth only accumulated JA phytohormones. Gypsy moth folivory on adelgid-infested foliage reduced the accumulation of JA phytohormones and increased the SA levels. Both herbivores increased CW-bound phenolics and gypsy moth increased lignin content when feeding alone but not when feeding on adelgid-infested foliage. Our study illustrates the importance of understanding the biochemical mechanisms and signaling antagonism underlying tree responses to multiple stresses and of disentangling local and systemic stress signaling in trees.

Keywords: biotic tree stress, conifer, defense induction, phenolics, phytohormones, terpenes.

Introduction

Plants are exposed to multiple stressors that vary in time, space and intensity, and responses to one type of stress can alter how plants respond to others (Eyles et al. 2007, Nguyen et al. 2016, Ohgushi 2005). These responses can involve interlaced biochemical pathways that are mediated by multiple hormones and other chemical, hydraulic and electrical signals (Huber and Bauerle 2016, Robert-Seilaniantz et al. 2011). The signaling molecules salicylic acid (SA) and jasmonic acid (JA) are often key regulators of plant defense (Buscaill and Rivas 2014, Dar et al. 2015, De Bruyne et al. 2014, Walling 2000, War et al. 2012, Xia et al. 2015). Both SA and JA defense responses can occur locally and/or systemically (Fürstenburg-Hägg et al. 2013) and are often non-additive (i.e., not simply the sum of both individual responses; Nguyen et al. 2016). Predicting plant responses to combined stresses thus remains challenging, especially in woody species whose responses are less understood and can differ from those found in herbaceous systems (Eyles et al. 2010). The intolerance of many woody plants to multiple assaults (Niinemets and Valladares 2006) underlines the importance of work addressing the biochemical underpinnings of woody plant responses to combined stressors.

Conifers (family Pinaceae) dominate alpine, boreal and some temperate forests in the northern hemisphere (Farjon 2001).

Research exploring conifer biochemical responses to stressors has primarily used pine (*Pinus*) or spruce (*Picea*) species as models. The family Pinaceae also includes nine other genera that, although ecologically and economically important, have received less research attention. Eastern hemlock (*Tsuga canadensis* L.; 'hemlock') is a canopy-dominant conifer endemic to eastern North America. It is considered a 'foundational species' that creates a unique and critical habitat for many terrestrial and aquatic species (Ellison et al. 2005, Orwig et al. 2008, Snyder et al. 2002). It is attacked by a diverse group of herbivores that includes hemlock woolly adelgid (*Adelges tsugae* Annand; 'adelgid'), an invasive stylet-feeding insect introduced from Japan (Havill et al. 2006). The adelgid has decimated hemlock populations in North America; chronic infestation can kill over 80% of mature trees within 4 years (Havill et al. 2016). Other hemlock herbivores include the folivorous larvae of the exotic invasive gypsy moth (*Lymantria dispar* L.; 'gypsy moth') and the native North American hemlock looper (*Lambdina fiscellaria* Guenée), scale insects (e.g., the exotic invasive elongate hemlock scale; *Fiorinia externa* Ferris) and spider mites (e.g., the native North American spruce spider mite; *Oligonychus ununguis* Jacobi).

The biochemical and physiological effects of chronic adelgid infestation on hemlock involve a hypersensitive-like response consisting of the accumulation of H_2O_2 (Radville et al. 2011), proline (Gómez et al. 2012), SA (Schaeffer et al. 2018) and increased methyl salicylate (MeSA) emissions (Pezet et al. 2013, Pezet and Elkinton 2014). These changes may affect the suitability of hemlock for later-arriving herbivores: Wilson et al. (2016) found that adelgid infestation increased hemlock looper larval growth and survival, while Kinahan et al. (2019) reported that later-instar gypsy moth larvae were more attracted to, and performed better on, adelgid-infested hemlock than on uninfested plants. The Kinahan et al. (2019) study was spurred by the observation that late-instar gypsy moth larvae were regularly seen roaming on the ground of our field site and differentially causing damage in adelgid-infested hemlocks compared with adelgid-free hemlock, an observation also reported by Pennsylvania state foresters (Donald Eggen, Pennsylvania Department of Conservation and Natural Resources; personal communication). Our research site is situated below an oak-dominated canopy that are presumably what early-instar larvae had been feeding on prior to relocating to hemlocks in the forest understory. Research following up on the Wilson et al. (2016) study found less dramatic impacts of adelgid on hemlock looper, but documented SA-induced increases in peroxidase activity and soluble phenolic accumulation (Rigsby et al. 2019). The characterization of phytohormone signaling and changes in specific secondary metabolites could provide further insight on hemlock defense signaling and responses to adelgid infestation.

We present the results of an experiment exploring how chronic adelgid infestation alters hemlock response to gypsy moth by addressing linkages between hormonal signaling and secondary metabolites. We predicted that adelgid infestation would induce the SA pathway (Schaeffer et al. 2018), gypsy moth feeding would induce the JA pathway and simultaneous stressors would yield non-additive responses in hormone and secondary metabolite accumulation. Importantly, neither adelgid nor gypsy moth is native to eastern North America and thus they lack an evolutionary history with this particular species of hemlock. Hemlock is thus unlikely to possess recently co-evolved responses against either herbivore, a fact that made detailed a priori predictions of its specific defense responses difficult.

Materials and methods

Hemlock common garden planting and adelgid treatments

In early spring 2014, 350 herbivore-free hemlock saplings (0.5–0.7 m tall), which were grown from seed collected in Pennsylvania and had not been treated with insecticides, were purchased from Vans Pines Nursery (West Olive, MI, USA). It is important to note that additional variation in biochemical responses may have existed since these plants were not from a single genotype. The 320 healthiest of these trees were planted in five, 64-tree blocks (eight rows and columns with trees spaced 1–1.5 m apart), in the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, RI, USA) in April 2014. Trees were protected from herbivory and treatment cross-contamination with chicken-wire cages covered in mesh bags (90% light transmission; Agribon-15, Johnny's Selected Seeds, Waterville, ME, USA). Within each block, 16 trees were randomly selected for artificial infestation, performed every year at approximately mid-spring (timed with crawler emergence), by adelgid or elongate hemlock scale (*F. externa*). Briefly, we cut pest-infested stems from naturally growing hemlocks located less than 1 km from our experimental site. We used wire to secure this cut foliage to each hemlock in the adelgid treatment in order to infest these plants (see Butin et al. 2007 for detailed methods). Plants infested with elongate hemlock scale were not part of this study and were used in unrelated experiments. The 32 remaining trees in each block were left as uninfested controls and were sham-infested with herbivore-free foliage to control for inoculation-related disturbance. Only adelgid-infested trees and uninfested controls were included in this study. Trees averaged 1.5 m in height and 9-cm trunk diameter at the soil line; neither tree height nor trunk diameter differed between treatments (*t*-test; $P > 0.5$). The uninfested status of each control tree was confirmed via careful visual inspection of each tree prior to the removal of any foliage (see below for details). Adelgid densities on infested trees were ~0.75–1.0 adelgid cm^{-1} stem. Adelgid densities were estimated by counting the number of settled nymphs of the sistens that had broken aestivation and were actively feeding (indicated by the secretion of wool) on four 10-cm sections of

terminal stem growth per tree, and was done immediately prior to herbivory treatments in mid-October 2017 (see below for details).

Gypsy moth herbivory treatment

Egg masses of gypsy moth were obtained from the USDA APHIS Otis facility (Buzzards Bay, MA, USA) and reared in an incubator (16 h light/8 h dark cycle, 25 °C, and ~80% relative humidity) on artificial diet supplied by facility personnel until the fourth instar. Although we have repeatedly seen late-instar gypsy moth larvae consume hemlock foliage in both the field and lab (Kinahan et al. 2019), multiple attempts to rear early-instar gypsy moth larvae on hemlock foliage have failed (likely due to needle toughness; C.M. Rigsby, unpublished data). Therefore, six fourth-instar gypsy moth larvae were haphazardly selected (~50/50 sex ratio), starved for 4 h and placed on a haphazardly selected branch of a hemlock tree (biological replicate) that was bagged with nylon mesh (0.5 mm) and secured with zip ties. For bagged branches, the entire branch was bagged (all the way back to the main stem), which gave larvae enough space to prevent cannibalism. Branches on 12 adelgid-infested and 12 uninfested trees were bagged in this way, and an additional 12 adelgid-infested and 12 uninfested trees were bagged without gypsy moth larvae. This resulted in four treatment groups (adelgid/gypsy moth): −/−, −/+, +/− and +/+, with 12 biological replicates per treatment ($N = 48$). After 4 days (20 October 2017), each bag was removed and the branch was cut from the tree, wrapped in aluminum foil and immediately placed on ice for transport back to the lab where it was stored at −80 °C. Frozen foliage was ground into powder in liquid nitrogen using a mortar and pestle; 300, 100, 100 and 100 mg were partitioned into tubes for analyses of hormones, terpenes, phenolics and proanthocyanidins, respectively, and stored at −80 °C. When removing foliage from harvested branches for processing, we were careful to use only foliage from stem sections of the branch that contained settled adelgid and/or had noticeable damage to needles from gypsy moth. This was our only consideration for selecting foliage; as a result, our pooled tissue sample included herbivore-damaged needles of different ages and positions on the branch. This undoubtedly contributed to variation in our quantified biochemical responses as it is well documented that needle age impacts these in conifers (e.g., Nybakken et al. 2018). While we would have preferred to only use needles of a given age and branch position, our samples did not include enough material for us to perform such specific analyses. Because our experiment treated gypsy moth as a categorical factor (present, absent), we did not estimate gypsy moth damage beyond our visual confirmation that they had indeed fed on the sampled branches (i.e., partially or mostly consumed needles with obvious chewing damage on the margins). Foliage was removed haphazardly from different sections of control branches.

Quantification of phytohormones

The following 17 foliar phytohormones were extracted and quantified as described by Body et al. (2019): SA-defense signaling: SA, MeSA; JA-defense signaling: 12-oxophytodienoic acid (OPDA; JA precursor), JA, jasmonoyl isoleucine (JA-Ile; bioactive JA derivative), methyl jasmonate (MeJA); stress signaling: abscisic acid (ABA); gibberellins: gibberellin A1 (GA_1), GA_3, GA_4, GA_7; cytokinins: 6-(Δ2-isopentenyl) adenine riboside (iPR), 6-(Δ2-isopentenyl) adenine (iP), *trans*-zeatin riboside (*t*ZR), *trans*-zeatin (*t*Z); and auxins: indole-3-acetic acid (IAA), indole-3-butanoic acid (IBA). Frozen ground hemlock tissue was extracted in 15-ml falcon tubes with 2 ml of 2-propanol:H_2O:HCl (2:1:0.002; v:v:v) containing 50 µl of an internal standard mixture (stable isotope-labeled compounds: [2H6]-*cis*,*trans*-abscisic acid for negative ion and [2H6]-N6-isopentenyladenine for positive ion) to generate a final internal standard concentration of 12.5 µg l^{-1}. Homogenates were incubated in the dark for 30 min at 4 °C on a shaker at 80 r.p.m.. After incubation, phytohormones were partitioned into 1-ml dichloromethane and this phase was filtered [polytetrafluoroethylene (PTFE) membrane syringe filters; 0.22-µm pore size], transferred to a fresh tube and evaporated under a stream of nitrogen gas. The dichloromethane partitioning step was repeated, pooled with the residue from the previous extract and evaporated under nitrogen gas. After solvent evaporation, the residue was resuspended in 0.5-ml high-performance liquid chromatography (HPLC)-grade methanol and the extracts stored at −20 °C until the solid-phase extraction (SPE) and concentration step (<48 h). For the SPE and concentration of extracted hormones, a 96-well C_{18} SPE plate was conditioned with washes of 100% methanol, aqueous methanol (50%) and 0.1% (v:v) formic acid. Samples were applied to the SPE plate and eluted with 50-µL HPLC-grade methanol to concentrate the extracts by ~10-fold. Phytohormone samples were then stored in a 96-well microplate at −20 °C until being shipped to the Charles W. Gehrke Proteomics Center (University of Missouri, Columbia, MO, USA) on dry ice, where they were stored at −20 °C until further analysis.

Phytohormone identification and quantification was performed similarly to Body et al. (2019) via ultra-performance liquid chromatography-electrospray ionization-tandem mass spectrometry (UPLC-ESI-MS/MS). Ten microliters of each sample were injected into a UPLC-ESI-MS/MS system and phytohormones were separated by a reverse-phase C_{18} UPLC column on a Waters (Milford, MA, USA) Acquity H-class UPLC system coupled with a Waters Acquity TQ triple quadrupole MS/MS detector (Waters TQ Detector, Acquity Ultra Performance LC), controlled by the Waters Masslynx software. The binary solvent system used for phytohormone separation was 0.3-mM ammonium formate in water (negative-ion mode) or 0.1% formic acid in water (positive-ion mode) (mobile phase A), 0.1% formic acid in acetonitrile (mobile phase B), and

set as follows for a 10-min run: initial conditions were 2% B; gradient of 2–40% B over 5 min, ramp 40–98% B over 0.5 min, hold at 98% B for 2 min, rapid ramp (0.25 min) to and hold (2.25 min) at initial conditions. The column oven and autosampler temperatures were set to 50 and 10 °C, respectively; the solvent flow rate was set to 0.4 ml min^{-1}. For each phytohormone, Intellistart was used to generate a quantifying (best signal) transition (precursor > fragment pair) in which Q1 was set to 1 *m/z* filtering and Q3 set to 0.7. Optimized cone and collision energy voltages were then used to build a multiplexed multiple reaction monitoring method. The ion source in the MS/MS system was operated in the positive and negative ion modes with capillary voltages of 3.2 and 2.7 kV, respectively. The ionization sources and desolvation temperatures were set to 150 and 450 °C, respectively, with a nitrogen gas flow rate of 800 l h^{-1}. Phytohormone concentrations were normalized using the internal standard and expressed as microgram of standard per gram dry weight [μg g^{-1} dry weight (DW)] based on five-point standard curves ($R^2 \geq 0.99$). Fresh weights were converted to dry weights as described by Wilson et al. (2018).

Analysis of soluble phenolics

Phenolics were extracted in 0.5 ml 100% HPLC-grade methanol containing 0.5 mg ml^{-1} internal standard (butylated hydroxyanisole; BHA). The 20,000*g* supernatant was aspirated into a fresh tube and the tissue was extracted again with another 0.5 ml methanol, the 20,000*g* supernatants combined and filtered (PTFE membrane syringe filters; 0.22-μm pore size). Extracts were then used immediately for HPLC-UV analysis of soluble phenolics. The pellet was then used to extract and quantify cell wall-bound (CW-bound) phenolics and lignin as described by de Ascensao and Dubery (2003) and Villari et al. (2012), using gallic acid and spruce lignin (Sigma-Aldrich, St Louis, MO, USA) as standards, respectively. While more accurate methods exist for the quantification of both of these classes of compounds (e.g., LC–MS for proanthocyanidins, Flamini 2003; the acetyl bromide method for lignin, Moreira-Vilar et al. 2014), the methods used in these experiments have repeatedly been shown to detect treatment effects in similar experiments (e.g., Dalzell and Kerven 1998, Wallis et al. 2008). Proanthocyanidins were extracted similarly to soluble phenolics except that 70% acetone replaced methanol. Proanthocyanidins were quantified according to Engström et al. (2014) using butanol:HCl (95:5; v:v) and expressed as OD_{550} g^{-1} DW due to the lack of affordable standards and technical issues associated with using pure commercially available standards for this assay (Schofield et al. 2001). Spectrophotometric analysis was conducted using Greiner UV-Star® 96 well plates (Monroe, NC, USA) using a SpectraMAX M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA, USA) in the RI-INBRE facility (University of Rhode Island; Kingston, RI, USA).

Levels of foliar soluble phenolics were quantified with a Shimadzu Nexera-*i* HPLC equipped with a LC-2040C 3D photodiode array detector (PDA; set to scan 200–400 nm) located in the RI-INBRE facility. Phenolics were separated on a Luna reverse-phase C_{18} column (250 × 4.6 mm; 5-μm particle size) equipped with a 5-mm pre-column of the same stationary phase. The binary solvent system used for phenolic separation was 0.1% acetic acid (mobile phase A), 0.1% acetic acid in methanol (mobile phase B) and set as follows for a 48-min run: initial conditions were 5% B, hold at 5% B for 2.5 min; ramp 5–80% B over 40 min; rapid ramp (0.1 min) to 100% B, hold 100% B for 2.4 min; rapid ramp (0.1 min) to and hold (2.9 min) at initial conditions. The column oven and autosampler temperatures were set to 50 and 10 °C, respectively; the solvent flow rate was set to 1.0 ml min^{-1}. Because no information currently exists on the composition and identities of phenolic metabolites in hemlock tissues, phenolics were tentatively identified by matching peaks to the following external standards: apigenin, apigenin-7-glucoside, benzoic acid, caffeic acid, 3-caffeoylquinic acid (chlorogenic acid), *trans-p*-cinnamic acid, coniferyl alcohol, *p*-coumaric acid, coumarin, *trans*-ferulic acid, gallic acid, kaempferol, naringenin, quercetin, quercetin-3-*β*-*O*-glucoside, taxifolin, vanillin and vanillic acid (Sigma-Aldrich). These standards were chosen because they are commercially available, common phenolic metabolites reported in members of Pinaceae. Phenolics were quantified at 280 nm and peaks that could be matched to external standards based on retention time (RT) and UV spectrum were expressed as milligram of standard per gram dry weight (mg g^{-1} DW) based on five-point standard curves ($R^2 \geq 0.99$). Peaks that showed characteristic phenolic UV spectra but could not be matched to external standards were expressed as internal standard (BHA) equivalents.

Analysis of mono- and sesquiterpenes

Foliar mono- and sesquiterpenes were extracted from ground needles in 700-μL *n*-hexane containing 1-μL ml^{-1} m-xylene as internal standard by sonicating homogenates for 10 min in an ice bath. After sonication, the tubes were vortexed for 10 s and the 20,000*g* (5 min, 0 °C) supernatant was then transferred to a 2-ml glass autosampler vial which was capped with a PTFE-coated screw cap and stored at −30 °C until required for analysis (<1 week).

Mono- and sesquiterpene identification and quantification took place using a Shimadzu GC 2010 Plus gas chromatograph equipped with an AOC-20*i* autosampler and a flame ionization detector (GC-FID). Nitrogen was used as the carrier gas at a flow rate of 1.0 ml min^{-1} and an HP-5MS (30 m) column (30 m × 0.25 mm internal diameter; 0.25-μm film thickness). Terpene extract (2 μl) was injected using a split flow ratio of 30:1, and the following oven program was used: 40 °C for 5 min, increase by 3 °C min^{-1} to 225 °C, then increase by 25 °C min^{-1} to 280 °C, and held at 280 °C for 5 min.

The injector and detector temperatures were set to 260 and 300 °C, respectively. The tissue amounts of camphene, α-caryophyllene (a.k.a., α-humulene), β-caryophyllene, isobornyl acetate, limonene, linalool, myrcene, α-phellandrene, (−)-α-pinene and (−)-β-pinene were quantified using pure standards (Sigma-Aldrich). These external standards were selected because they are major foliar mono- and sesquiterpene species of hemlock and can comprise almost 90% of all mono- and sesquiterpenes in the foliage of this species (Lagalante and Montgomery 2003; C.M. Rigsby, unpublished data). Peaks matched to external standards based on RTs were expressed as mg g^{-1} DW.

Statistical approach

To assess the effect of adelgid infestation and gypsy moth herbivory on individual compounds, individual phytohormones, phenolic and terpene metabolites were statistically compared using an ANCOVA with adelgid presence, gypsy moth presence, and the interaction as predictors, and block and herbivory score as covariates. A reduced model was selected based on removing insignificant predictors and comparing full and reduced models with an ANOVA. The Benjamini–Hochberg false discovery rate-controlling procedure (Benjamini and Hochberg 1995) was used when testing the effects of treatments on single variables (i.e., individual hormones, phenolics and terpenes) because it is less stringent and more powerful than familywise error rate-controlling procedures such as the Bonferroni correction (e.g., Rigsby et al. 2017). We report Benjamini–Hochberg-adjusted *P*-values for single hormone and metabolite assessments.

Relationships between specific hormones and secondary metabolites were assessed *via* the correlative method described by Wallis et al. (2008), with the exception that all *P*-values were adjusted using the Benjamini–Hochberg method as described above. Correlations were computed between scaled levels of individual hormones and metabolites, as well as between hormones.

Results

Hormonal responses to herbivory

Phytohormone levels were significantly altered by herbivory by one or both insect species. Contents of three of the four quantified GAs were altered by herbivory. Adelgid increased GA_1 and GA_3 levels but reduced those of GA_7 (Figure 1). Gypsy moth feeding increased GA_7 levels when adelgid was absent, but not when adelgid was present (Figure 1c). Both herbivores elevated foliar SA contents when feeding individually, and their combined effect appeared to be somewhat additive (Figure 2a). In contrast, adelgid and gypsy moth had divergent impacts on foliar OPDA and JA levels: adelgid reduced the contents of both phytohormones and gypsy moth increased them (Figure 2b and c). As with GA_7 levels, however, the presence of adelgids prevented gypsy moth from inducing increases in foliar OPDA and JA contents. Gypsy moths also increased JA-Ile levels, the active form of JA; the presence of adelgid did not change the effect of gypsy moths on the contents of this hormone (Figure 2d). Neither herbivore, individually or in combination, affected the level of any of the other phytohormones we quantified (Table S1 available as Supplementary Data at *Tree Physiology* Online).

Phenolic metabolites and the impact of herbivory

Although we detected 25 peaks in methanol extracts of hemlock foliage, the total peak area of chromatograms (280 nm) was consistently dominated (≥95%) by 13 peaks (a representative chromatogram is presented as Figure S1 available as Supplementary Data at *Tree Physiology* Online). Our analyses focused on these 13 peaks (Table S2 available as Supplementary Data at *Tree Physiology* Online). Although we were unable to definitively match most of them to external standards, we provide tentative identifications based on RT and UV spectra. Peaks four (16.21 min RT; 1.8% of total peak area) and five (16.58 min RT; 65% of total) were tentatively identified as chlorogenic acids A and B, respectively (Figure S2 available as Supplementary Data at *Tree Physiology* Online). Peaks six (19.72 min RT; 2.8% of total) and seven (20.13 min RT; 1.1% of total) were tentatively identified as vanillin A and B, respectively (Figure S3 available as Supplementary Data at *Tree Physiology* Online). Peaks 11 (27.67 min RT; 1.2% of total) and 13 (37.69 min RT; 6.4% of total) were labeled as unidentified flavonoids A and B, respectively, on the basis of their characteristic two-peak banding pattern (Tsimogiannis et al. 2007; Figure S4a and b available as Supplementary Data at *Tree Physiology* Online, respectively). None of the contents of the 13 soluble phenolics that were quantified via HPLC-UV, or total proanthocyanidin content quantified through spectrophotometry ($\bar{x} \pm SE = 39.98 \pm 0.35$ OD_{550} g^{-1} DW) was affected by adelgid, gypsy moth or their interaction.

Both herbivores altered the contents of CW-bound phenolics and lignin. Each herbivore increased CW-bound phenolic levels when feeding alone and when feeding together their impacts appeared somewhat additive (Figure 3a). The pattern was different for foliar lignin levels: gypsy moth increased lignin levels, but only when adelgid was absent. Individually, adelgid appeared to affect lignin levels, but after *P*-values were adjusted *via* the Benjamini–Hochberg method, the effect of adelgid was only marginally significant (Figure 3b).

Herbivory impacts on foliar mono- and sesquiterpenes

We were able to match each of the 10 external standards to peaks based on RTs of known hemlock terpene metabolites (Table S3 available as Supplementary Data at *Tree Physiology* Online). Isobornyl acetate was the most abundant of the identified peaks, representing 46% of the total area of

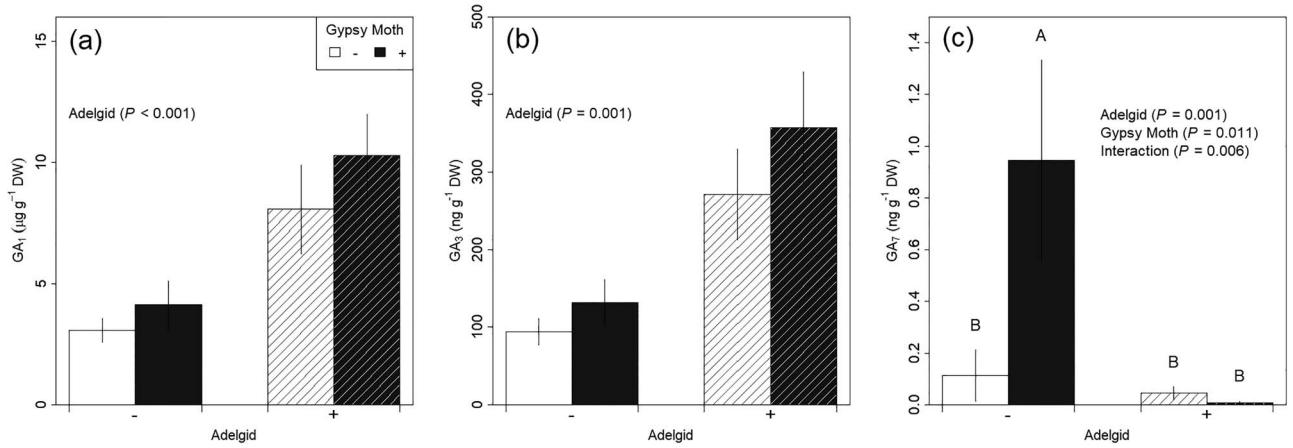

Figure 1. Levels (mean ± SE; treatment $n = 12$) of three GAs by treatment combination: (a) GA_1, (b) GA_3 and (c) GA_7 of young hemlock trees. Light bars are treatments without gypsy moth ('–'), while dark bars are treatments where gypsy moth larvae fed on the plant ('+'). '+' and '–' along the *x*-axis refer to foliage on which adelgid was present (hatched bars) or absent, respectively. Significant effects of treatments are included in plots (Benjamini–Hochberg-corrected *P*-values); different letters indicate significant differences in the case of a significant interactive effect.

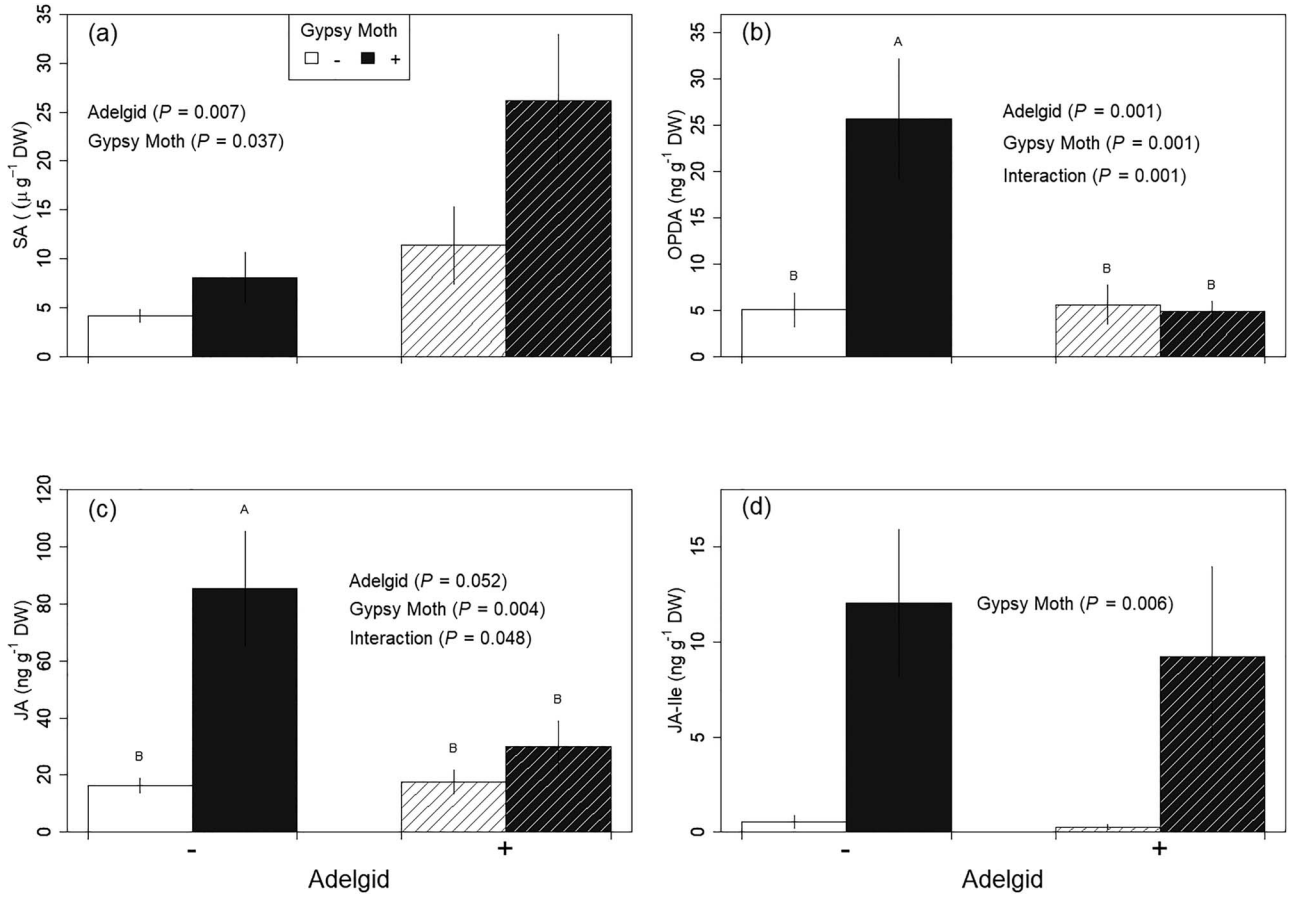

Figure 2. Levels (mean ± SE; treatment $n = 12$) of four defensive hormones by treatment combination: (a) SA, (b) OPDA, (c) JA and (d) JA-isoleucine (JA-Ile). Light bars are treatments without gypsy moth ('–'), while dark bars are treatments where gypsy moth larvae fed on the plant ('+'). '+' and '–' along the *x*-axis refer to foliage on which adelgid was present (hatched bars) or absent, respectively. Significant effects of treatments are included in plots (Benjamini–Hochberg-corrected *P*-values); different letters indicate significant differences in the case of a significant interactive effect.

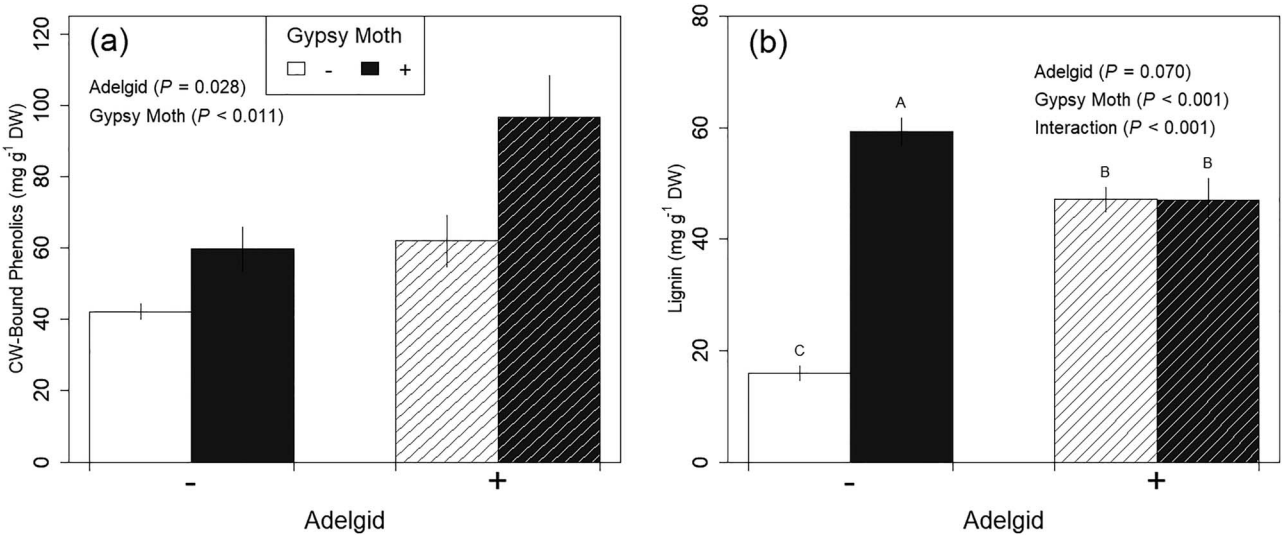

Figure 3. Tissue levels (mean ± SE; treatment $n = 12$) of (a) CW-bound phenolics and (b) lignin by treatment combination of young hemlock trees. Light bars are treatments without gypsy moth ('–'), while dark bars are treatments where gypsy moth larvae fed on the plant ('+'). '+' and '–' along the *x*-axis refer to foliage on which adelgid was present (hatched bars) or absent, respectively. Significant effects of treatments are included in plots (Benjamini–Hochberg-corrected *P*-values); different letters indicate significant differences in the case of a significant interactive effect.

identified peaks, followed by α-pinene (20% of area) and camphene (8% of area). The remaining peaks ranged between 4.1 (α-phellandrene) and 0.31% (linalool) of the total area. As with soluble phenolics, neither herbivore nor their interaction affected the contents of any of the 10 terpene metabolites (Table S3 available as Supplementary Data at *Tree Physiology* Online). A representative GC-FID chromatogram is presented as Figure S5 available as Supplementary Data at *Tree Physiology* Online. No terpene compound quantified *via* GC-FID was affected significantly by adelgid, gypsy moth or their interaction.

Relationships between hormones and secondary metabolites

We detected several corollary relationships between phytohormones, chiefly between bioactive GAs and defense/stress phytohormones and between components of the JA pathway (Table 1). Specifically, GA_1/GA_3 levels were positively correlated with each other and with SA levels, while GA_4/GA_7 contents were correlated with each other and with JA contents. There were negative relationships between GA_1/GA_3 and OPDA/JA levels and between GA_4/GA_7 and SA levels. The only phytohormone-secondary metabolite relationships detected were between components of the JA pathway and lignin: OPDA, JA and JA-Ile levels were all positively correlated with lignin levels (Table 1).

Table 1. Significant Pearson correlations of all pairwise comparisons of phytohormone and secondary metabolite levels of hemlock foliage.

Compound 1	Compound 2	Sign	P	*P*-Value
GA_1	GA_3	+	0.97	<0.0001
GA_1	SA	+	0.82	<0.0001
GA_3	SA	+	0.82	<0.0001
GA_4	GA_7	+	0.72	<0.0001
GA_4	OPDA	+	0.59	<0.0001
GA_4	JA	+	0.59	<0.0001
GA_4	JA-Ile	+	0.51	0.0094
GA_7	OPDA	+	0.76	<0.0001
GA_7	JA	+	0.56	0.0017
GA_7	JA-Ile	+	0.62	<0.0001
JA	OPDA	+	0.61	<0.0001
JA	JA-Ile	+	0.73	<0.0001
JA	Lignin	+	0.48	0.0195
JA-Ile	OPDA	+	0.75	<0.0001
JA-Ile	Lignin	+	0.61	<0.0001
Lignin	OPDA	+	0.52	0.0017
GA_1	GA_4	–	0.60	<0.0001
GA_1	GA_7	–	0.66	<0.0001
GA_1	OPDA	–	0.44	0.0454
GA_1	JA	–	0.44	0.0502
GA_3	GA_4	–	0.60	<0.0001
GA_3	GA_7	–	0.63	<0.0001
GA_3	JA	–	0.48	0.0215
GA_4	SA	–	0.59	<0.0001
GA_7	SA	–	0.74	<0.0001

Discussion

Plants respond to attack by multiple herbivores via the activation of one or more defense pathways. Understanding the complex nature of these responses requires well-replicated experiments that explore relevant ecological interactions in order to see how stressors interact. Although such scenarios have been explored in herbaceous plants, our understanding of how stressors and defense responses interact in woody species is unknown outside of a few model systems (e.g., bark beetles and conifers). In this study, we explored the impact of adelgid, gypsy moth

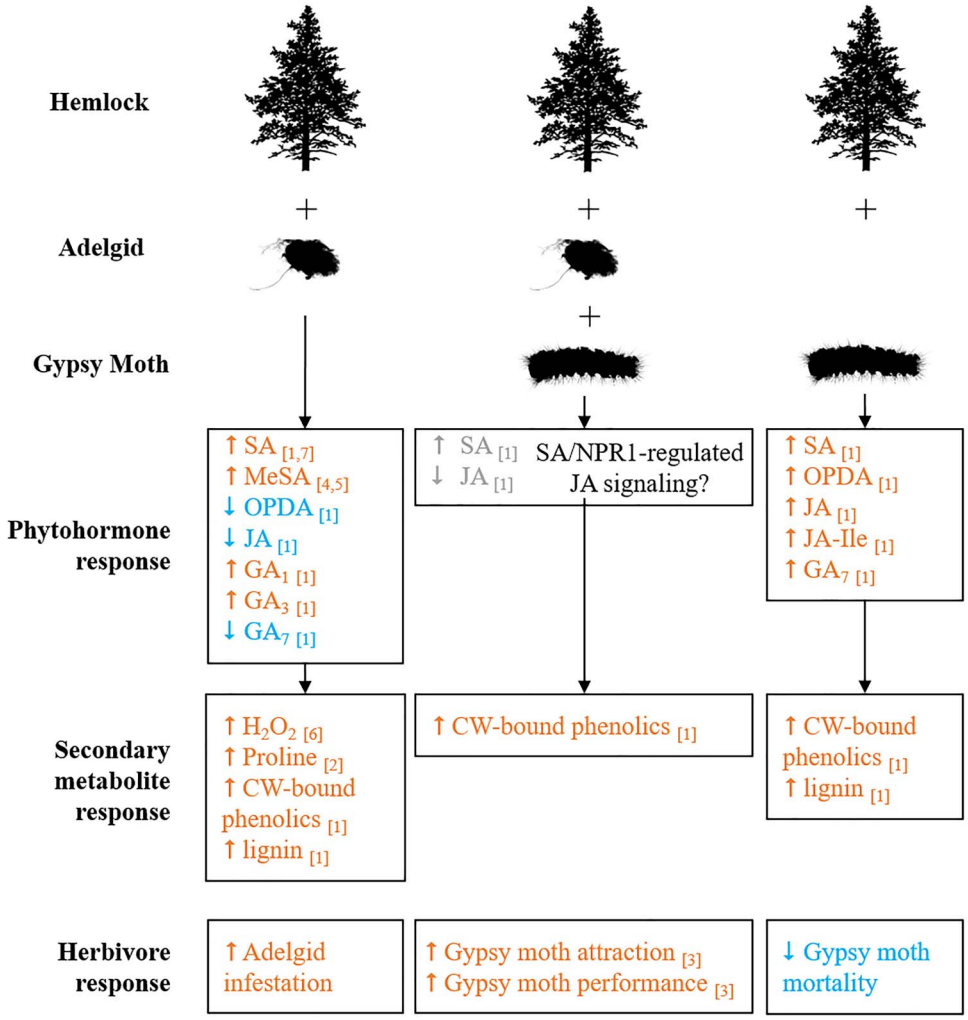

Figure 4. Summary of the response of eastern hemlock *T. canadensis* to infestation by hemlock woolly adelgid (*A. tsugae*, adelgid; left) and folivory by gypsy moth larvae (*L. dispar*, right) individually or in response to the infestation by the adelgid followed by gypsy moth larvae (middle). This figure includes results presented [1] in this study, [2] Gómez et al. 2012, [3] Kinahan et al. (2019), [4] Pezet et al. 2013, [5] Pezet and Elkinton 2014, [6] Radville et al. 2011 and [7] Schaeffer et al. 2018. Color key: blue, decrease in levels; orange, increase in levels; gray, potential mechanisms. Abbreviations: GA_1, gibberellin A1; GA_3, gibberellic acid; GA_7, gibberellin A7; NPR1, NONEXPRESSER OF PR GENES 1.

and their interaction on a wide range of defense responses in hemlock. Overall, we found that adelgid infestation induced the accumulation of SA, GAs and a few secondary metabolites. Gypsy moth increased the levels of both SA and JA, a single GA, lignin and select phenolic classes. Throughout our results, prior infestation by adelgid inhibited the gypsy moth-induced changes in JA, a single GA and lignin levels, while levels of other GAs remained unchanged (Figure 4). These results confirm that adelgid feeding interferes with JA accumulation and may provide a mechanistic explanation for increased host quality for folivores.

Salicylic acid and JA interact antagonistically and crosstalk between these pathways helps fine-tune defenses toward specific stressors (Beckers and Spoel 2006). This crosstalk can also prevent the induction of appropriate defenses to specific pests if one pathway is elicited but a later response requires the induction of the other pathway (e.g., Kroes et al. 2015). Interestingly, we found that adelgid affected JA levels both alone and as part of an interactive effect with gypsy moth. Folivory induced OPDA and JA accumulation in the absence of adelgid infestation but not in its presence (Figure 2b and c). This finding is consistent with the SA/NONEXPRESSER OF PR GENES 1 (NPR1)-regulated mechanism of JA signaling suppression shown in model plants, where the SA-induced monomerization of NPR1 suppresses JA biosynthesis genes (Beckers and Spoel 2006, Caarls et al. 2015) and antagonizes JA-responsive genes (Beckers and Spoel 2006). This interpretation is supported by higher JA-Ile levels in both the adelgid and gypsy moth trees; SA/NPR1 is not known to antagonize the JASMONATE RESISTANT 1 (JAR1)-mediated conjugation of JA and isoleucine into JA-Ile. These data are consistent with the hypothesis proposed by Schaeffer et al. (2018) and Rigsby et al. (2019) that adelgid infestation activates SA-dependent defenses

that protect the insect from JA-based defense responses. While demonstrated in model herbaceous plants such as *Arabidopsis thaliana* (e.g., Kroes et al. 2015), the role of these phytohormones in response to different stresses (e.g., pathogen infections, herbivory) and the biochemical mechanism behind their crosstalk is less definitive in woody plants (e.g., Arnerup et al. 2011, Body et al. 2019, Kozlowski et al. 1999, Krajnc et al. 2011).

The positive relationships between the contents of bioactive GAs and components of the JA pathway were intriguing. Many studies have demonstrated antagonism between GA-mediated growth and JA-induced defense via the interaction of DELLA and jasmonate ZIM domain (JAZ) repressor proteins. DELLA proteins are plant growth repressors that share the highly conserved sequence of amino acids (aspartic acid [D], glutamic acid [E], leucine [L], leucine [L], alanine [A]), whose degradation is stimulated by GAs (Sun and Gubler 2004) and JAZ proteins are JA-linked defense repressors whose degradation is mediated by JA (Huot et al. 2014). Growth and defense are ultimately mediated by the binding of DELLA and JAZ proteins, with growth promoted by DELLA suppression and defense promoted by JAZ suppression (Wasternack and Hause 2013). Work using DELLA loss-of-function *Arabidopsis* mutants suggests that these proteins modulate SA/JA signaling, promoting the JA and subjugating the SA pathway (Navarro et al. 2008). These interactions between GA and JA pathways occur downstream of both hormones, and there are no known mechanisms by which the accumulation of one could limit the accumulation of the other. DELLA proteins are also known to be central hubs for phytohormone crosstalk in plants, having characterized interactions with brassinosteroids, ABA, auxins, ethylene, cytokinins and strigolactone in addition to SA and JA responses (Davière and Achard 2016). The accumulation of bioactive GAs could have unknown impacts on typical hormone signaling responses. It is also possible that GAs have a role in hemlock defense (e.g., Morkunas et al. 2011). In poplar, for example, GAs have been correlated with heritable aphid resistance (Body et al. 2019). Although adelgid infestation substantially reduces growth and new foliage production (Wilson et al. 2018), GA accumulation could benefit the insect *via* the mobilization and translocation of resources to adelgid settlement sites.

Surprisingly, levels of ABA and cytokinins were unaffected by our treatments and uncorrelated with other hormones or metabolites. Abscisic acid is known to respond to both piercing and chewing insects (Appel et al. 2014, Body et al. 2019, Schaeffer et al. 2018). The lack of ABA accumulation in this study could indicate that our hemlock trees may not have been osmotically stressed by chronic adelgid infestation (e.g., Gonda-King et al. 2014) and that perhaps adelgid densities were not sufficient enough to result in osmotic stress. Due to the interaction among phytohormone signaling and biosynthesis and the potential role of cytokinins in tree resistance to aphids (Body et al. 2019), we expected an alteration of cytokinin levels and do not readily have an explanation as to why levels of cytokinins did not respond to either herbivore.

Cell wall-bound phenolics accumulated significantly in response to both herbivores and lignin accumulation was dependent on treatment combination. Lignin contents showed a similar pattern except that the effect of adelgid on lignin accumulation was only marginally significant after *P*-value adjustment. Phenolics, including CW-bound phenolics, polyphenolics (e.g., lignin) and terpenes, are important anti-herbivore and -pathogen metabolites in conifers (Raffa et al. 2017). Co-regulation of metabolites and the simultaneous accumulation of specific classes of metabolites have been demonstrated in these plants (e.g., Keefover-Ring et al. 2016, Wallis et al. 2008). The co-accumulation of lignin with JA pathway components suggests that lignin accumulation is an integral component of the JA-mediated induction response, presumably resulting in tougher foliage that makes feeding difficult for herbivores (Cipollini 1997).

The lack of substantial shifts and co-regulation in terpene and soluble phenolic profiles of hemlock plants to our treatments ran contrary to our hypothesis that each stressor would generate its own response and that simultaneous stresses would generate a non-additive response in secondary metabolite composition. This is especially curious in the light of the observed changes in several phytohormones. Precedent does exist for a lack of response of foliar monoterpenes to stress or elicitation (e.g., Heijari et al. 2005). Increases in terpene emission rates could stabilize tissue terpene pools despite increased terpene synthesis (e.g., Litvak and Monson 1998) and adelgid infestation has been shown to increase terpene emission rates of hemlock (e.g., Broeckling and Salom 2003). It is more difficult to offer a hypothesis for the lack of a response of soluble phenolic compounds, especially since Rigsby et al. (2019) found that soluble phenolic levels increased as a result of adelgid infestation and MeJA application. Phenolic turnover rates (e.g., oxidation) or polymerization to cell walls, which we did measure and found substantial increases, could help explain unaltered soluble phenolic pools, but this is still an unusual result. It is important to note that we quantified CW-bound phenolics using a published spectrophotometric procedure (de Ascensao and Dubery 2003) rather than via HPLC (e.g., Bonello and Blodgett 2003). This did not allow us to obtain information on CW polymerization of specific phenolic species, which could have provided further insights into phenolic turnover and use of hemlock in response to these herbivores. Similarly, we quantified only mono- and sesquiterpene metabolites that we knew to be major terpenes of this species, and did not (i) perform any stereochemical analyses or (ii) identify or quantify diterpene resin acids. These more detailed analyses were unfortunately beyond our capabilities but could have provided additional insights in specific defense responses of hemlock.

In this study, we demonstrated the differential and non-additive impact of adelgid infestation on the phytohormone and defense response of hemlock to gypsy moth. The lack of JA pathway induction in the presence of adelgid might partly provide a mechanistic basis for the findings of increased gypsy moth (Kinahan et al. 2019) and hemlock looper (Wilson et al. 2016) performance on adelgid-infested hemlock. The observed responses of SA and JA are also consistent with hypotheses offered in earlier research (e.g., Schaeffer et al. 2018). These experiments show that adelgid infestation increases host quality for folivores and that this increased performance may partly be explained by hormone signaling antagonism. Much remains unknown in this specific system however, including any alterations in nutritional quality and the potential role of GAs in the inhibition of JA pathway induction and resource mobilization. The unanticipated accumulation of GAs could have major implications regarding the mediation of increased folivore attraction and performance on hemlock. This system thus provides an excellent opportunity to study how chronic stress imparted by adelgid impacts the signaling and the chemical/biochemical responses of a woody plant to another herbivore.

Organismal- and landscape-scale information suggests that woody plants generally respond poorly to simultaneous stressors (Haavik et al. 2015, Krokene 2015, Niinemets and Valladares 2006). Despite our understanding of how multi-stress scenarios impact woody plants in the field, a substantial gap in our understanding of biochemical mechanisms remains; our study illustrates the importance of understanding how conifers, and woody plants in general, biochemically respond to multiple stresses and the importance of research in non-model, woody plants. An enhanced understanding of these interactions will ideally allow for increased predictability and better management practices of forest and ornamental woody plant resources.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Conflict of interest

None declared.

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