Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Induces Twig Volatiles of Eastern Hemlock in a Forest Setting

Author(s): Joshua Pezet and Joseph S. Elkinton

Hemlock Woolly Adelgid (Hemiptera: Adelgidae) Induces Twig Volatiles of Eastern Hemlock in a Forest Setting

JOSHUA PEZET[1] AND JOSEPH S. ELKINTON[1,2,3]

ABSTRACT The hemlock woolly adelgid (*Adelges tsugae* Annand) is an invasive species causing high mortality of eastern hemlock (*Tsuga canadensis* (L) Carriere) in the forests of eastern North America. Recent findings revealed that sapling eastern hemlocks artificially infested with hemlock woolly adelgid in a plantation setting responded to the insect with an array of induced resin volatile changes. Here we determine if eastern hemlocks growing beneath a forest canopy respond to hemlock woolly adelgid infestation with the same patterns of constitutive and inducible volatile resin production as those plantation specimens. We inoculated previously uninfested branches of mature and immature hemlocks in a central New England forest with hemlock woolly adelgid. We then sampled twig tissue of infested and uninfested trees in late spring, early summer, and mid-autumn, after known intervals of adelgid activity when an induced response might be expected. We identified and quantified resin volatiles by gas chromatography–mass spectrometry. Constitutive levels of total monoterpenoids, sesquiterpenoids, and combined resin volatiles were all several-fold more abundant in forest trees than those previously measured in a plantation setting, pointing to further study of the influence of site factors on hemlock volatile production. Hemlock woolly adelgid infestation induced an array of changes in eastern hemlock's volatile profile, including many-fold increases in benzyl alcohol and methyl salicylate accumulation. Despite differences in constitutive concentrations of volatiles between the two sites, our findings verify that hemlock woolly adelgid elicits patterns of resin volatile induction in forest-grown eastern hemlocks quite similar to those previously observed in plantation grown trees.

KEY WORDS induced plant defense, terpene, forest insect, methyl salicylate

The hemlock woolly adelgid (*Adelges tsugae* Annand) has caused widespread mortality of eastern hemlock (*Tsuga canadensis* (L) Carriere) in many forests in eastern North America (Orwig and Foster 1998, McClure and Cheah 1999). The adelgid was first detected in Virginia more than half a century ago, with mitochondrial DNA suggesting a southern Japanese origin (Havill et al. 2006); it has now spread throughout most of the eastern U.S. portion of the hemlock's range (Preisser et al. 2008, Forest Health Protection Program 2011). In all but the northern region of the infestation, the adelgid's effect on hemlocks has been dramatic at even moderate densities: needle desiccation, branch mortality, and nearly total inhibition of new growth, often ending with tree mortality within a few years (McClure 1991a). Eastern hemlock is ecologically unique and valuable as the sole native shade-tolerant conifer in many regions of the eastern United States. Because the tree creates cool and moist microclimates in the midst of deciduous forests, it can function as a foundation species (sensu Ellison et al. 2005). The loss of entire hemlock populations due to adelgid infestation (Preisser et al. 2011) can substantially alter canopy structure, warming streams and soils, shifting litter decomposition and nutrient cycling patterns, and enabling early successional trees to proliferate, bringing profound and lasting changes to eastern forest ecosystems (Orwig et al. 2008, Gandhi and Herms 2010).

The impact of an herbivore on trees of coniferous forests can be mediated by either constitutive resin-based defenses (often principally involving volatile phytochemicals), an induced response, or both. The constitutive volatiles of wild populations and cultivars of eastern hemlock have been well characterized (Von Rudloff 1975b, McClure and Hare 1984, Lagalante and Montgomery 2003, Lagalante et al. 2007). Additionally, spatial and seasonal patterns of variation in a subset of eastern hemlock's volatile terpenoid array have been hypothesized to be strongly linked to patterns of feeding and development of colonizing adelgids (Lagalante and Montgomery 2003; Lagalante et al. 2006, 2007; Ingwell et al. 2009). In comparison, research to determine whether an inducible hemlock volatile response exists and mediates the damage incurred by hemlock woolly adelgid has until recently been limited to a single study. Broeckling and Salom (2003) demonstrated an increase in foliar monoter-

[1] Graduate Program in Organismic and Evolutionary Biology, University of Massachusetts, Amherst, MA 01003.
[2] Department of Environmental Conservation, University of Massachusetts, Amherst, MA 01003.
[3] Corresponding author, e-mail: elkinton@ent.umass.edu.

penoid emission rates from branch tips of artificially adelgid-infested plantation-grown eastern hemlocks compared with uninfested trees. However, infestation also lowered the ratio of new to previous year's foliar growth, and this ratio was strongly negatively correlated with volatile emission rate. This correlation revealed the possibility of a disparity between new and previous year's foliar volatile emission rates, rather than directly demonstrating an adelgid effect on hemlock secondary metabolism.

It is now well known that constitutive and inducible resin chemical defenses are common strategies many conifers have evolved to respond to herbivores and pathogens (Hudgins et al. 2004, Zulak and Bohlmann 2010). Research on the regulation of pest defense in the well-studied genera of Pinaceae: *Pinus* (pines), *Picea* (spruces), and *Abies* (firs), has revealed that jasmonic acid or ethylene-dependent signaling pathways are predominantly active. These biosynthetic mechanisms result in primarily terpenoid-based defenses against the defoliators, bark beetles, and wood borers that commonly feed on these conifers (Hudgins and Franceschi 2004, Keeling and Bohlmann 2006). Herbivores from different feeding guilds, however, often elicit distinct plant responses (Kaloshian and Walling 2005). Stylet feeders of the Aphidoidea, a group that includes serious pests of conifers (Hain et al. 1991, McClure and Cheah 1999, McKinnon et al. 1999, Zvereva et al. 2010), activate salicylic acid (SA)-dependent biosynthetic pathways in many angiosperms that have significant overlap with those used in pathogen defense (Moran and Thompson 2001, Martinez et al. 2003, Zhu-Salzman et al. 2004). In light of the influence of these herbivore defense mechanisms in other plant taxa, it is relevant to investigate whether gymnosperms such as eastern hemlock are capable of mobilizing SA-dependent signaling in response to ecologically important piercing–sucking herbivores.

Recently, we and our colleagues obtained the first conclusive evidence of an insect-inducible hemlock volatile response (Pezet et al. 2013) by confirming that eastern hemlocks in a northeastern U.S. plantation setting responded to adelgid feeding with induced increases or decreases of several classes of twig and needle volatiles. We had hypothesized that an herbivore as devastating to its host as hemlock woolly adelgid would induce a terpenoid-based resinosis like that observed in many of the pines, spruces, and firs. We found instead that the insect elicited relatively minor changes in terpenoids—with an overall terpenoid decrease in twig tissue, where the adelgid feeds—but dramatically increased several nonterpenoids, particularly benzenoid volatiles associated with pathogen defense and systemic acquired resistance in many angiosperms. Our findings suggest a SA-driven insect defense syndrome may be active in adelgid-infested eastern hemlocks.

Often, the hemlocks of greatest concern are those in natural forest settings where, in contrast to plantations with full sun and amended soils (conditions similar to those experienced by many ornamental eastern hemlock trees), the availability of soil nutrients, mois-

ture, and light is patchy and heterogeneous. Spatial and temporal variation in nutrient access or climate affect the expression of both constitutive and inducible resin defenses in pine species (Lombardero et al. 2000; Sampedro et al. 2010, 2011). The degree to which this is the case for wild forest-grown eastern hemlocks, however, remains unexplored. In addition, tree age or ontogenetic stage have been shown to influence levels of putative conifer resin defenses and observed insect or pathogen resistance. We found studies revealing variously that younger trees (Karban 1990, Bauce et al. 1994) or older trees (Zagory and Libby 1985, Wainhouse et al. 2009) were more heavily defended among pine, fir, and spruce species. Research papers on eastern hemlock's resin secondary metabolism variously report immature saplings (Broeckling and Salom 2003) or mature trees (Lagalante et al. 2006) as their subjects, or remain unspecified as to this point. Because the influence of hemlock age on resin chemistry remain unknown, we investigated the effects of adelgid infestation on twig resin volatile profiles of both sapling and mature eastern hemlocks growing wild in a hemlock dominated stream valley of central New England. Previous studies revealed the majority of adelgid-elicited changes in accumulated volatiles took place in eastern hemlock twigs, rather than the needles (Pezet et al. 2013), and document considerable seasonal variation in the tree's volatile levels (Lagalante et al. 2006). Therefore, to establish how hemlocks in conditions typical of northeastern U.S. forests respond to adelgid infestation, we measured accumulated resin volatiles in twigs of infested and uninfested trees in the days following active adelgid feeding or commencement of aestivation, respectively, when an induced phytochemical response would be most likely detected, if present.

Materials and Methods

Study System. Eastern hemlocks in the northeastern United States undergo bud break in May, and the initially green, flexible young growth shoots finish their elongation in midsummer. By then, both twigs and needles have hardened and become visually more similar to adjacent previous year's growth.

The hemlock woolly adelgid is bivoltine in eastern North America, as in its southern Japanese range of origin (McClure and Cheah 1999, Havill et al. 2006). In the northeastern states, first-instar progrediens generation "crawlers" settle in April (before bud break) on mature, previous year's twigs just below the needle abscission seam, and feed with a stylet bundle on xylem ray parenchyma cells in the twig (Young et al. 1995). The insect is sessile for the remainder of development, and adults complete oviposition in June; their progeny (nymphs of the "sistens" generation) emerge, settle primarily on young current year's foliage, and enter a period of diapause for the duration of the summer. In early fall, when current year's foliage has matured, the sessile sistens nymphs resume activity and complete development and egg deposition by April (McClure and Cheah 1999).

Experimental Design. In mid-April 2010, branches of 20 mature hemlocks (4–5 m minimum height) and 20 saplings (1–2 m in height) at a forested site in the Quabbin Reservation Watershed in Shutesbury, Massachusetts, were artificially infested with hemlock woolly adelgid collected from naturally infested trees in the same region. Careful inspection of the study site revealed no prior infestation by hemlock woolly adelgid. Effects of adelgid infestation and hemlock age on levels of volatile compounds in twigs were tested using a 2 by 2 fully factorial randomized block design to account for spatial heterogeneity in microclimate and soil conditions. In each of 20 blocks, there was one infested sapling, one uninfested sapling, one infested branch on a mature tree and one uninfested branch on a different tree. Trees and branches were separated by at least 3 m and blocks were separated by ≈10–50 m within the stand. The crossed treatments were hemlock woolly adelgid (infested or uninfested) and tree age (mature or sapling). Because the adelgid is wind-dispersed during its crawler phase (McClure and Cheah 1999), each treated branch was contained in a mesh bag for the duration of the inoculation process to prevent cross-contamination. Containment bags were made from PECAP LE monofilament polyester plain weave (77 threads per cm, 55 micron thread diameter, 70 micron mesh opening).

Adelgid Inoculations. Inoculations of hemlock woolly adelgid were conducted following standard procedures (see Butin et al. 2007). On 21–22 April 2010 trees in infested treatments were inoculated with progrediens generation hemlock woolly adelgids. Immediately before crawler emergence, naturally infested branches with comparable adelgid densities were collected from sites in southern New England and attached to a healthy, haphazardly selected branch of each tree in the appropriate treatment group; control trees received uninfested branches. Individual inoculum-containing branches were placed in aquapics to slow needle desiccation and decrease adelgid mortality. To verify the success of inoculations, densities of inoculum progredientes and their sistens progeny (adelgids per centimeter) were sampled in August and December 2010, respectively. In the case of sistentes, (summertime) aestivation mortality was also measured. Two 20-cm-long branch tips on each tree or branch were selected and adelgid individuals counted on them nondestructively. To detect adelgid effects on hemlock growth, proportion of branchlets with new growth and overall new growth length were quantified on the same branches.

Sampling Hemlock Foliage. Foliage samples were collected on three dates in 2010 after the hemlocks had experienced either active feeding (in June and November) or stylet bundle insertion before the commencement of aestivation (in July): 1) 22–23 June, mature previous year's foliage after progrediens generation adelgid had settled and fed for several weeks ($n = 19$–20); 2) 15 July, young current year's foliage after first-instar sistens crawlers had settled and entered aestivation ($n = 15$); and 3) 22–23 November, current year's growth after the appearance of ovisac wax ("wool"), which indicated the sistentes had finished aestivation and begun feeding ($n = 20$). An average of 10 cm of twig with foliage was obtained. In the case of infested treatments, foliage samples with adelgids were selectively chosen. Each sample was promptly placed in a polypropylene cryovial, flash-frozen in liquid nitrogen, transported to the laboratory on dry ice and stored at −80°C until extraction and analysis. Because the adelgids are quite small and adhere tightly to their twig feeding sites, complete removal of adelgids and their ovisacs from infested samples before analysis was not practical. To ensure that all volatiles subsequently detected were of hemlock origin, we obtained several samples of adelgid-infested foliage of comparable size and adelgid density to our experimental samples, collected the insects, eggs, and the wax of ovisacs into vials, and extracted and analyzed the insect material using the plant volatile protocol described herein.

Extraction of Volatiles From Twigs. Our procedures for extraction of twig volatiles were adapted from Lewinsohn et al. (1993). Reagents and reference standards were purchased from Sigma-Aldrich (St. Louis, MO). Solvents were HPLC or GC grade purity.

Needles were separated from twigs under liquid nitrogen and discarded. Twig samples of ≈10–50 mg (dry weight) were cooled with liquid nitrogen in a mortar and pestle, ground to a coarse powder, and combined with methyl tert-butyl ether (MTBE; 1.0 ml) containing a known concentration of the internal standard isobutylbenzene (2 µg ml^{-1}) in a pre-weighed 2 ml vial (glass with PTFE-coated screw cap, Sigma-Aldrich, St. Louis, MO). Ground twig samples were extracted overnight (19 h) with constant shaking at room temperature. Aqueous (NH$_4$)$_2$CO$_3$ (0.2 ml; 1 M) was added to each extract, followed by thorough mixing. The organic layer was then transferred directly to a Pasteur pipette filter packed with silica gel (0.2 g, Sigma-Aldrich, 60Å) overlaid with MgSO$_4$ (0.13 g). Oxygenated volatile compounds were subsequently eluted by washing the filter with diethyl ether (0.5 ml), and combined eluates were collected in a GC vial (PTFE-coated screw cap, Agilent, Santa Clara, CA) and stored at −20°C until analysis. All extracted tissue samples were weighed after drying for at least 48 h at 55–60°C.

Analysis of Volatile Compounds. Twig volatile extracts were analyzed on a Shimadzu GC-2010 system equipped with a QP2010-Plus mass spectrometer (EI mode, 70 eV), running GCMSolution software (Shimadzu Corp., Kyoto, Japan). For all analyses, the injection volume was 1 µl, with an injector temperature of 220°C. Volatile compounds were separated on an Agilent DB-5, 0.25 mm i.d. by 30 m, 0.25 µm coating thickness, fused silica capillary column. The He carrier gas flow was in constant linear velocity mode at 36.5 cm s^{-1}, with column flow set at 1.0 ml min^{-1} and a split ratio of 5:1. The GC oven was programmed with an initial temperature of 60°C (no hold), followed by an increase at 3°C min^{-1} to 175°C, then 30°C min^{-1} to 300°C (hold 5 min). The interface and ion source

Table 1. Estimates (± SE) of densities of hemlock woolly adelgid (*A. tsugae*) life stages, proportion sistentes surviving, and new growth on artificially infested and uninfested forest eastern hemlocks

Tree age Insect treatment	Mature		Sapling	
	Infested	Uninfested	Infested	Uninfested
Adult progredientes/cm[a]	0.20 (0.04)	0	0.18 (0.03)	0
Sistens nymphs/cm[b]	6.33 (0.44)	0	5.27 (0.66)	0
Proportion surviving sistentes[b]	0.20 (0.04)	0	0.05 (0.01)	0
Proportion branchlets with new growth[c,d]	0.66 (0.05)	0.60 (0.04)	0.71 (0.04)	0.68 (0.05)
Mean new growth length (cm)[c,e]	1.80 (0.16)	1.97 (0.19)	1.98 (0.20)	2.20 (0.25)

[a] Counted in August 2010.
[b] Counted in December 2010.
[c] Measured in December 2010.
[d] Mean values compared by two-way ANOVA; life-stage: $F = 1.89$, $P = 0.17$; adelgid: $F = 0.97$, $P = 0.33$.
[e] Mean values compared by two-way ANOVA; life-stage: $F = 0.14$, $P = 0.71$; adelgid: $F = 0.09$, $P = 0.76$.

temperatures were both set at 300°C, and the MS scan range was m/z 40–400.

Identification of volatile compounds was based on comparison of experimental retention times and mass spectra with those of authentic standards; when a pure standard was unavailable, tentative identification was based on comparison with retention index and mass spectral information reported in the literature (Adams 2001) and with mass spectra in the NIST05 and NIST05s mass spectral libraries (Stein 2005). Concentrations of all compounds were determined by normalizing integrated peak areas against that of the internal standard isobutylbenzene in each chromatogram. Tissue volatile concentration values were standardized to $\mu g\,g^{-1}$ dry weight by dividing by the sample dried weights.

Statistical Analysis. Resin volatile concentrations were log transformed before statistical analysis when necessary to reduce heterogeneity of variance between treatment groups. We used two-way MANOVAs to test for effects of adelgid infestation and tree age on the overall twig volatile concentration profile, (R 2.14.0, R Development Core Team 2012). Our model included adelgid (infested or uninfested) and tree age (mature or sapling) as fixed factors, as well as their interaction. When an overall MANOVA was significant, individual two-way mixed-model ANOVAs (Proc Mixed, SAS 9.3; SAS Institute 2011) were used to detect treatment effects on concentrations of each individual volatile tested in the multivariate analyses, as well as summed total monoterpenoids, total sesquiterpenoids, total combined benzenoids (including phenolics), and total volatiles. Our univariate model included hemlock woolly adelgid (infested or uninfested) and tree age (mature or sapling) as fixed factors, their interaction, and block as a random factor. Because each of the three samples taken over the growing season represented different phases of hemlock growth, we analyzed each time period separately, rather than combining them into a single repeated measures analysis.

Finally, we conducted a post hoc analysis to compare constitutive and adelgid-induced twig volatile concentrations of wild forest grown saplings with those measured in an analogous study conducted at the University of Rhode Island's (URI) East Farm plantation (Pezet et al. 2013). Each volatile measured in twigs of forest hemlocks (sampled in June and November, respectively) was divided by the average concentration of that compound for the appropriate treatment group (adelgid infested or uninfested) and sampling date (spring or autumn) of the URI plantation hemlock study; Quabbin forest tree values of total monoterpenoids, total sesquiterpenoids, total combined benzenoids, and total combined twig volatiles were also normalized to the plantation average values in this way. These ratio values were averaged for each forest tree treatment group to provide a relative measure of the difference in accumulation of twig volatiles between the two groups of hemlocks due to the influence of site factors. The monoterpenoids sabinene, α-phellandrene, terpinolene, linalool, camphor, piperitone, and geranyl acetate, the sesquiterpenoids (*E*)-β-farnesene, γ-muurolene, and germacrene D-4-ol, as well as one unidentified volatile, were not present in the volatile arrays of both sites. These compounds were included as appropriate in total volatile and structural class total calculations, but were not subjected to individual comparisons.

Results

The results of August 2010 counts of progredientes and December 2010 counts of sistentes show similar densities of each adelgid generation were established on mature and sapling eastern hemlock trees (Table 1). Additionally, adelgid feeding did not significantly affect 2010 new growth in trees of either age class, compared with uninfested trees (Table 1). Adelgid infestation has been shown to affect new growth, but it is densities in the previous year that have this effect (McClure 1991a, Sussky and Elkinton 2014).

Thirty-six resin volatiles, including monoterpenoids, sesquiterpenoids, and benzenoids, were detected in hemlock twig tissue, and all but three were identified (Table 2, Supp. Table 1 [online only]). Ten of the 36 volatiles in June and 12 in July were not present with adequate regularity or in sufficient quantity to be unequivocally identified or quantified, and were omitted from statistical analyses for those sampling dates. Monoterpenoids were the most abundant class of compounds, both in diversity and mass proportion;

Table 2. Resin volatile concentration "relative change" (infested average/uninfested average ratio) for mature and sapling eastern hemlocks artificially infested with hemlock woolly adelgid (*A. tsugae*)

Twig volatile[a]	Previous growth (June)[b]		New growth (July)[b]		New growth (Nov.)[b]	
	Mature	Sapling	Mature	Sapling	Mature	Sapling
Monoterpenoids						
Tricyclene	0.87[c]	1.07	1.03	0.81	0.87	0.91
α-pinene	***0.72**	***0.85**	1.01	0.84	1.05	0.96
Camphene	0.82	1.03	1.04	0.82	0.89	0.88
Sabinene	0	0	1.04	0.98	0.93	1.09
β-pinene	0.81	0.81	1.00	*0.82	1.05	0.95
Myrcene	0.74	0.81	1.01	0.81	1.11	1.16
α-phellandrene	0	0	1.14	0.68	1.02	1.20
Limonene	0.81	0.73	1.02	0.79	1.02	1.09
Terpinolene	0	0	0.95	0.87	0.95	0.94
Linalool	0	0	1.33	0.88	1.05	1.12
α-campholenal	0.76	0.94	0	0	***0.54**	***0.50**
L-*trans*-pinocarveol	0.69	1.03	0	0	0.64	0.52
cis-verbenol	*0.69	*0.95	0	0	***0.40**	***0.45**
Camphor	0	0	0	0	***0.75**	***0.61**
trans-verbenol	0.81	0.92	0	0	*1.10	*0.65
Pinocarvone	0.76	0.96	0	0	***0.62**	***0.51**
Borneol	***0.62**	***0.81**	1.15	1.05	0.91	0.78
Myrtenol	0.74	0.90	0	0	***0.61**	***0.53**
Verbenone	0.78	0.91	0	0	***0.60**	***0.51**
Piperitone	0	0	1.42	1.00	1.04	1.11
Bornyl acetate	0.94	1.23	1.07	0.88	*0.78	*0.97
Geranyl acetate	0	0	0.58	0.72	0.50	1.51
Total	*0.75	*0.87	1.01	0.83	1.03	0.99
Sesquiterpenoids						
β-caryophyllene	0.75	1.27	*1.10	*1.19	0.84	1.39
α-humulene	0.77	1.17	*1.10	*1.24	0.83	1.45
(E)-β-farnesene	0	0	1.04	0.98	1.11	1.23
γ-muurolene	0	0	1.00	1.09	0.79	1.37
Germacrene D	1.40	0.50	1.12	1.27	0.98	1.46
Germacrene D-4-ol	0	0	1.11	1.16	1.07	1.20
Caryophyllene oxide	0.86	0.96	0	0	*0.71	*0.95
Total	0.96	0.92	1.11	1.25	0.92	1.43
Benzenoids/Phenolics						
p-cymene	0.84	1.04	0.92	0.86	0.77	0.92
Benzyl alcohol	***3.01**	***2.44**	***1.21**	***1.75**	***19.42**	***16.39**
Methyl salicylate	***13.67**	***5.64**	***13.36**	***181.14**	***91.97**	***59.78**
Raspberry ketone	***0.83**	***0.77**	1.12	0.97	1.06	0.99
Total	0.98	0.96	1.15	1.03	***1.58**	***1.45**
Unknown A	0.71	1.18	0	0	*0.64	*0.61
Unknown B	***1.90**	***1.39**	0	0	*0.69	*0.59
Unknown C	***0.56**	***0.53**	0	0	0.87	0.82
Total twig volatiles	*0.77	*0.88	1.03	0.88	1.02	1.07

See Supp. Table 1 (online only) for actual concentrations, F-statistics, and *P* values.

[a] Compounds are ordered first by structural class, then by ascending order of elution from a nonpolar DB-5 GC column. A summed total for each structural class of phytochemical is included.

[b] Foliage sampled in June was mature, previous year's growth infested with progrediens generation adelgid; foliage sampled in July and November was young, current year's growth infested with sistens generation adelgid.

[c] Statistically significant differences from uninfested trees (planned contrast, $P < 0.05$) are marked in bold text with asterisks. Marginally significant ($0.05 < P < 0.10$) values are marked with underlining.

notably, the monoterpenoids α-pinene and myrcene together constituted 60–70% of the total mass of all measured volatiles. Seven sesquiterpenoids were quantified; several of these were absent (i.e., germacrene D-4-ol, γ-muurolene, and (E)-β-farnesene) or at relatively low levels (i.e., germacrene-D, α-humulene, and β-caryophyllene) in June previous year's growth compared with July and November current year's growth. The remaining four twig volatiles were benzenoids, including two monophenolics.

All volatiles were at highest concentration in July, being in most cases over an order of magnitude more abundant than in June. This difference was most dramatic for sesquiterpenoids, and least dramatic for ben-zenoids, which were 40-fold and 8-fold more concentrated in July, respectively (Supp. Table 1 [online only]). By November, new growth twig terpenoids had decreased to roughly half of July levels; benzenoids, in contrast, remained at ≈90% of July levels.

Extraction and analyses of insect material showed that neither the adelgid, nor its eggs, nor ovisac wax contained volatiles at levels detectable with our methods. This verified that the changes in volatile compounds we measured in adelgid-infested twig tissue did indeed reflect changes in hemlock volatile accumulation.

Effects of Adelgid Infestation. MANOVAs for each of the three sampling dates indicated significant ef-

Table 3. Test statistics and significance values from two-way MANOVAs of effects of adelgid infestation and tree age (sapling vs mature) on volatile concentrations of mature and sapling forest eastern hemlocks artificially infested with hemlock woolly adelgid (*A. tsugae*)

Statistics	Effect of adelgid infestation (df = 1)			Effect of hemlock age (df = 1)		
	Previous growth (June)	New growth (July)	New growth (Nov.)	Previous growth (June)	New growth (July)	New growth (Nov.)
Pillai's trace	0.56	0.58	0.90	0.55	0.66	0.66
F	2.37	1.86	10.74	2.34	2.62	2.22
Numerator df	26	24	36	26	24	36
Denominator df	49	33	41	49	33	41
P	0.005	0.05	<0.001	0.005	0.005	0.007

fects of adelgid infestation on twig volatile profiles (Table 3). Subsequent mixed-model ANOVAs showed that a number of individual twig volatiles were significantly or marginally significantly ($0.05 < P < 0.10$) influenced by adelgid infestation on each sampling date (Table 2, Supp. Table 1 [online only]).

June Twig Volatile Analyses. In previous year's growth twigs sampled in June, the adelgid significantly decreased α-pinene and borneol, and decreased nonsignificantly most of the other monoterpenoids (Table 2, Supp. Table 1 [online only]). The insect increased the benzenoid benzyl alcohol nearly threefold (Fig. 1), and the monophenolic methyl salicylate (MeSA) by an order of magnitude (Fig. 2), while it decreased another benzenoid, raspberry ketone, by ≈20%. The hemlock woolly adelgid also increased one unidentified volatile by ≈60%, and decreased another by almost half.

July Twig Volatile Analyses. In twigs of current year's new growth sampled several weeks after sistentes began settling, the adelgid again increased both benzyl alcohol (Fig. 1) and MeSA (Fig. 2) the most strongly, by nearly 50% and almost 25-fold, respectively, over uninfested levels.

November Twig Volatile Analyses. In twigs of current year's new growth sampled several weeks after sistens nymphs broke aestivation, the adelgid significantly decreased six monoterpenoids, each by 30–50%; two others decreased with marginal significance (Table 2, Supp. Table 1 [online only]). Overall monoterpenoid levels, however, were not significantly different from uninfested levels. Postaestivation adelgid

feeding decreased caryophyllene oxide with marginal significance, while total sesquiterpenoids increased nonsignificantly. Again most notably, the adelgid increased benzyl alcohol levels nearly 20-fold (Fig. 1), and mean MeSA levels nearly 80-fold (Fig. 2); total combined benzenoids were increased significantly as well.

Effects of Tree Age (Sapling Versus Mature). MANOVAs for each of the three sampling dates also indicated a significant influence of hemlock age on the twig volatile profiles (Table 3). Subsequent mixed-model ANOVAs (Supp. Table 1 [online only]) revealed a number of modest differences between the twig volatile chemistry of mature and sapling hemlocks.

Total constitutive levels of monoterpenoids, sesquiterpenoids, and benzenoids were not significantly different between mature and sapling hemlocks at any point. But in June, mature trees had significantly higher levels of the monoterpenoids tricyclene, camphene, beta-pinene and borneol, as well as the benzenoid benzyl alcohol, than did saplings (Supp. Table 1 [online only]); total volatiles were marginally significantly higher in mature trees than saplings (≈20%). Twigs from mature hemlocks in July had significantly higher levels of the monoterpenoids tricyclene, camphene, and bornyl acetate than those from saplings. As in July, twigs from mature hemlocks in November contained significantly higher levels of the monoterpenoids tricyclene, camphene, and bornyl acetate, as well as borneol, than did saplings. Conversely, in November, several other volatiles, includ-
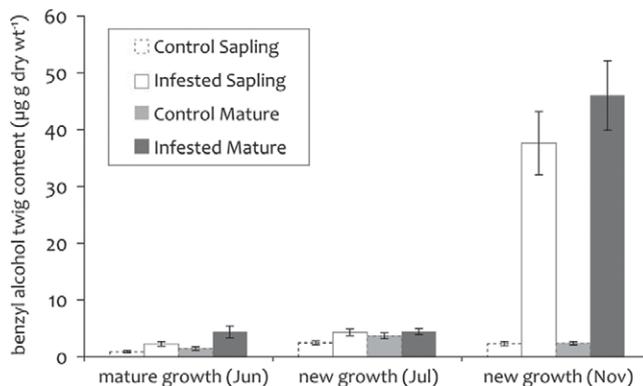

Fig. 1. Benzyl alcohol content (average ± SE) in twig tissue of mature and sapling eastern hemlock trees with and without artificial infestation with hemlock woolly adelgid (*A. tsugae*).

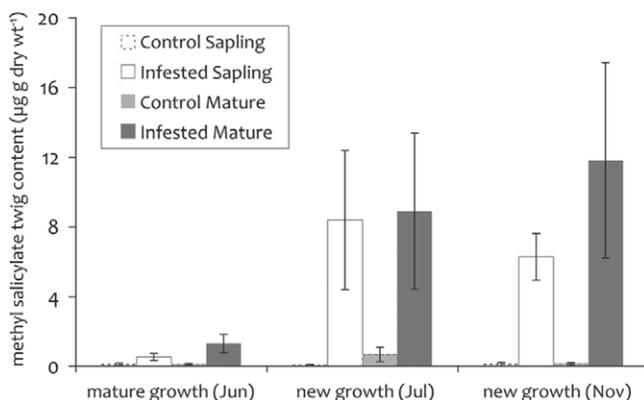**Fig. 2.** Methyl salicylate content (average ± SE) in twig tissue of mature and sapling eastern hemlock trees with and without artificial infestation with hemlock woolly adelgid (*A. tsugae*).

ing the monoterpenoids α-campholenal, L-*trans*-pinocarveol, *cis*-verbenol, pinocarvone, myrtenol, and verbenone, the sesquiterpenoid caryophyllene oxide, and one unidentified volatile, were at significantly higher levels in saplings than mature trees.

The adelgid × tree age interaction term in the MANOVAs were nonsignificant for all sampling dates (Table 3). ANOVAs of individual and structural classes of volatiles generally indicated this interaction was nonsignificant, with the exceptions of one unidentified volatile measured in June and three terpenoids in November (Table 2, Supp. Table 1 [online only]). In analyses of July volatiles, the interaction was marginally significant for the dominant volatile myrcene and total monoterpenoids; this was characteristic of the modest adelgid-induced decrease (<20%) of most sapling monoterpenoids, for which the opposite trend was observed in mature hemlocks. Similarly, analyses of volatiles from November twigs revealed a significant adelgid effect × tree age interaction for both β-caryophyllene and α-humulene. This was due to the moderate adelgid-induced increase of both compounds in saplings and concomitant decrease in mature hemlocks, and was reflective of the general trend in November for sesquiterpenoids.

Effects of Site Conditions: Plantation Versus Forest Setting. A post hoc comparison of volatile levels of forest and plantation hemlock saplings (Table 4) revealed that nearly all volatiles were considerably more abundant in twigs of forest trees. We found total monoterpenoids for all tissue ages and insect treatments were three- to fourfold more concentrated in the forest trees. Likewise, autumn current year's twigs of forest hemlocks had 13-fold (in infested trees) or 16-fold (in uninfested trees) higher concentrations of total sesquiterpenoids; the corresponding differences in June sampled twigs were fourfold or fivefold, respectively. Total benzenoids were detected at one- to twofold higher levels in autumn sampled twigs of forest trees than plantation trees, but, unlike most other volatiles, were less abundant in June sampled forest trees, by 60% (if infested) or 5% (if uninfested). Total combined twig resin volatiles in forest hemlocks were

three- to fourfold higher than plantation tree levels. The magnitude of this nearly universal effect varied dramatically among individual volatiles, from differences of less than twofold for some compounds to >15-fold for others. Most of the sesquiterpenoids, especially, were substantially higher in the forest hemlocks; notably, mean levels of germacrene D were 300-fold higher in all autumn current year's growth samples. These putatively site-driven differences did not appear to interact consistently with adelgid infestation, but were generally larger in current year's twigs.

Discussion

A characterization of the adelgid-induced resin volatile chemistry of eastern hemlocks in a central New England forest site (Table 2, Supp. Table 1 [online only]) largely agrees with the results of our previous work showing adelgid effects on plantation hemlocks (Pezet et al. 2013). In both studies we found no evidence of the copious increase in terpenoid-containing resin that herbivore damage causes in the woody tissue of many other conifers of Pinaceae (Trapp and Croteau 2001). In addition, in both studies the moderate changes in twig terpenoids were overshadowed by a small number of nonterpenoid volatiles: the relatively largest and most consistent adelgid-induced effects were increases in the benzenoids benzyl alcohol and methyl salicylate. Substantial increases in mean concentration of these compounds were measured in adelgid-infested hemlocks regardless of sampling date, tree age, or tissue age, and are now shown in hemlocks growing in both plantation and forest settings. These overall phytochemical patterns are consistent with the hypothesis that the hemlock woolly adelgid, like stylet feeding hemipterans studied in numerous angiosperm systems (Moran and Thompson 2001, Martinez et al. 2003, Zhu-Salzman et al. 2004), triggers principally salicylic acid-dependent, rather than JA/ethylene-dependent, defense-related biosynthetic cascades in eastern hemlock. A detailed exploration of the significance of these findings is

Table 4. Spring and autumn relative difference in twig volatile concentration (with SE) of Quabbin Forest eastern hemlocks and University of Rhode Island plantation grown hemlocks, each with and without experimentally introduced hemlock woolly adelgid

Twig volatile[a,b]	Spring[c] (previous year's growth)		Autumn[c] (current year's growth)	
	Infested	Uninfested	Infested	Uninfested
Monoterpenoids				
Tricyclene	1.7[d] (0.2)	0.8 (0.1)	3.0 (0.4)	2.0 (0.3)
α-pinene	8.0 (1.2)	7.3 (1.4)	3.7 (0.3)	4.1 (0.6)
Camphene	2.0 (0.3)	1.0 (0.1)	3.2 (0.4)	2.5 (0.4)
β-pinene	6.9 (0.9)	6.5 (1.6)	3.0 (0.3)	3.8 (0.7)
Myrcene	26.4 (5.8)	27.5 (9.3)	18.4 (1.8)	22.3 (3.1)
Limonene	20.1 (3.6)	19.9 (8.3)	2.9 (0.5)	1.8 (0.3)
α-campholenal	1.9 (0.3)	1.2 (0.1)	0.7 (0.1)	1.0 (0.2)
L-*trans*-pinocarveol	1.7 (0.3)	1.2 (0.2)	1.1 (0.2)	1.5 (0.4)
cis-verbenol	5.7 (0.8)	5.6 (0.8)	0.6 (0.2)	1.0 (0.3)
trans-verbenol	3.5 (0.5)	2.9 (0.4)	1.4 (0.2)	1.6 (0.2)
Pinocarvone	1.5 (0.2)	1.1 (0.1)	0.8 (0.2)	1.2 (0.2)
Borneol	0.6 (0.1)	0.6 (0.1)	3.7 (0.4)	3.5 (0.7)
Myrtenol	2.0 (0.3)	1.3 (0.2)	1.0 (0.2)	1.3 (0.3)
Verbenone	1.2 (0.2)	0.8 (0.1)	0.8 (0.2)	1.2 (0.2)
Bornyl acetate	1.1 (0.2)	0.4 (0.1)	1.6 (0.3)	1.2 (0.2)
Total	4.3 (0.5)	3.0 (0.6)	4.2 (0.3)	4.1 (0.5)
Sesquiterpenoids				
β-caryophyllene	12.7 (3.7)	10.6 (2.2)	10.1 (1.1)	17.3 (2.8)
α-humulene	11.6 (2.8)	18.7 (4.1)	8.1 (0.9)	15.6 (2.7)
Germacrene D	34.8 (9.7)	72.8 (18.9)	341.4 (83.2)	273.5 (63.5)
Caryophyllene oxide	1.2 (0.2)	1.2 (0.2)	1.0 (0.2)	1.0 (0.1)
Total	3.7 (0.7)	5.1 (1.0)	13.1 (2.4)	16.4 (3.1)
Benzenoids				
p-cymene	1.5 (0.2)	1.1 (0.2)	1.0 (0.1)	1.4 (0.2)
Benzyl alcohol	0.1 (0.0)	1.2 (0.3)	3.2 (0.5)	1.9 (0.3)
Methyl salicylate	0.2 (0.1)	3.9 (2.8)	6.5 (1.4)	1.3 (1.3)
Raspberry ketone	2.2 (0.3)	2.1 (0.2)	4.2 (0.5)	2.8 (0.2)
Total	0.4 (0.0)	1.0 (0.1)	1.7 (0.1)	1.5 (0.1)
Unknown A	2.9 (0.4)	1.6 (0.2)	1.7 (0.3)	1.9 (0.3)
Unknown B	0.9 (0.1)	0.2 (0.0)	0.9 (0.2)	0.5 (0.1)
Total resin volatiles	3.0 (0.3)	2.6 (0.5)	4.6 (0.4)	4.2 (0.5)

[a] Compounds are ordered first by structural class, then by ascending order of elution from a nonpolar DB-5 GC column. A summed total for each structural class of phytochemical is included.

[b] Sabinene, α-phellandrene, terpinolene, linalool, camphor, piperitone, and geranyl acetate, and the sesquiterpenoids (E)-β-farnesene, γ-muurolene, and germacrene D-4-ol, as well as one unidentified volatile, were not present in the volatile arrays found at both sites; these compounds were included as appropriate in total volatile and structural class total calculations, but were not subjected to individual comparisons.

[c] Spring previous year's growth sampled in late June in both studies; autumn plantation study samples collected in mid-October, forest study samples in mid-November, each collection date timed to follow sistens aestivation break by several weeks.

[d] Each forest hemlock twig volatile measurement was divided by the plantation hemlock average concentration of the corresponding compound and treatment group (adelgid infested or uninfested) and sampling date (spring or autumn); forest hemlock total values for monoterpenoids, sesquiterpenoids, benzenoids, and combined twig volatiles were also normalized to the plantation average values in this way. The resulting ratio values were then averaged for each forest tree treatment group to provide a relative measure of the difference in accumulation of twig volatiles between the hemlocks at the two different sites.

presented within the manuscript in which we report the results of that companion study (Pezet et al. 2013).

In our previous plantation experiment, hemlock woolly adelgid decreased nearly all individual monoterpenoids in both spring and autumn hemlock twig samples; we found the same trend in volatile levels measured in the Quabbin forest trees in June, but a more complex picture emerged in the data from sub-

sequent sampling dates. In the forest hemlock study we added a third (July) sampling date to coincide with the early aestivation period of newly settled sistens crawlers, as hemlocks in midsummer were shown to produce a distinct, and more variable, twig volatile profile than during the spring and autumn periods (Lagalante et al. 2006). Sampling in November after sistentes had broken aestivation showed the insects had significantly decreased a half-dozen volatile terpenoids; in contrast to the plantation experiment (Pezet et al. 2013), overall volatile levels were not decreased significantly by hemlock woolly adelgid.

Patterns of adelgid-induced and constitutive volatile secondary metabolism in the mature trees we studied were quite similar to those in saplings, with some modest differences (Table 2). Tree age influenced twig monoterpenoid composition, rather than abundance, on all three dates. Differences in resin composition between trees of different levels of maturity have been linked to susceptibility to adelgid damage. The balsam woolly adelgid (*Adelges piceae*), a European introduction and close relative of the hemlock woolly adelgid that caused widespread mortality of the North American fir species, is unable to reproduce on younger trees, especially seedlings, of Fraser fir (*Abies fraseri*) because of heightened levels of sesquiterpenoid defenses in the immature trees (Hain et al. 1991). Although the hemlock woolly adelgid does feed and reproduce on sapling eastern hemlocks, to our knowledge no one has yet conducted a study of the influence of tree age on adelgid-induced mortality of hemlocks. In light of our findings of higher levels of some sesquiterpenoids in immature eastern hemlocks than their mature counterparts, this would be a pertinent investigation.

Although we did not sample previous and current year's growth simultaneously, we did observe preliminary evidence suggesting that tissue age influences the composition of eastern hemlock's twig resin volatile array. Sesquiterpenoids, in particular, varied between the previous year's growth sampled in June and the new growth sampled in July and November in a number of ways (Table 2, Supp. Table 1 [online only]). There were almost twice as many sesquiterpenoids expressed in the younger twigs than in those from previous year's growth. In addition, the three most abundant sesquiterpenoids (germacrene D, α-humulene, and β-caryophyllene) were present in greater proportions (relative to total quantified volatiles) in both July and November current year's growth than in older June sampled tissue. Notably, germacrene D was detected at modest proportions in June samples but became a dominant volatile in all newer twig tissues. This compound was identified as a possible feeding deterrent to hemlock woolly adelgid (Lagalante and Montgomery 2003), and its more substantial presence in immature plant tissue may be an indicator of more heavily defended younger growth tissue, a phenomenon observed in species of pine (Persson et al. 1993) and spruce (Ikeda et al. 1977). Meanwhile, mature hemlocks had higher levels of the monoterpenoid bornyl acetate, identified as a possible

phytochemical attractant to hemlock woolly adelgid (Lagalante and Montgomery 2003), only in July and November current year's growth. This finding is consistent with the known tendency of sistens crawlers to settle on current year's growth tissue (Lagalante et al. 2006) preferentially over previous year's growth in the early summer when they have the choice.

Because eastern hemlock is both ecologically important and one of the most commonly planted landscape trees in the eastern United States (Lagalante et al. 2007), we were interested to compare the volatile accumulation in hemlock populations representative of forest and ornamental trees, respectively (Table 4). As this was not the original aim of our study and we have only one replicate per type of site, these findings are only preliminary and will need to be followed up in subsequent studies. We were able to make this comparison between our two studies because we used solvent tissue extraction, which yielded estimates of absolute accumulated volatile concentration, in contrast to many recent studies of hemlock volatile chemistry that reported relative volatile abundance obtained with static or dynamic headspace volatile extraction methods.

Our comparison revealed striking differences between the two sites. Nearly all resin volatiles were substantially more concentrated in the twigs of forest trees. Although it is possible that differences in genotype may explain this disparity, a regional chemosystematic analysis of foliar terpenoid concentrations of western hemlock (*Tsuga heterophylla*) revealed that interpopulation variation of average individual volatile levels was at most 10%, and generally much less (Von Rudloff 1975a). This indicates genotypic variation in terpenoid expression in species of *Tsuga* may be fairly small. In any case, the source population for the URI plantation trees was a wild hemlock population several miles from the Quabbin forest site of the current study. Although this close proximity does not rule out differences in genotype between the two groups of trees, it does suggest major differences in phenotype are likely related to site influences.

There are many site factors that could contribute to the differences in volatile accumulation we observed—disparities in light intensity, levels of soil nutrients, and the availability of water principal among them. Each of these in turn has been shown to affect the secondary metabolism in other conifers (Lamontagne et al. 2000, Lombardero et al. 2000, Sampedro et al. 2010). In addition, an investigation of the effects of soil nutrient levels on sapling eastern hemlock susceptibility to adelgid revealed that the survival and fecundity of the adelgid, and its depression of hemlock health and new growth, were dramatically enhanced at higher nitrogen levels (McClure 1991b). This finding, paired with our discovery that twigs of plantation hemlocks had much lower accumulations of volatiles than forest trees, suggests that environmental constraints affect eastern hemlock's volatile defenses.

Two hypotheses that have received mixed support in the literature—the carbon–nutrient balance and growth differentiation balance hypotheses—propose a competitive trade-off between growth and defense in woody plants, where an increase in some environmental or developmental constraint that limits growth (e.g., nitrogen, water, or even temperature) decreases the plant's carbon/nitrogen (C/N) ratio, which in turn spurs a shift in the allocation of photosynthate away from carbon-based secondary metabolism toward more vigorous growth (Loomis 1932, Bryant et al. 1983, Herms and Mattson 1992, Massad et al. 2012). Decreasing light availability, and hence carbon resources, in the presence of growth-limiting nutrient conditions is predicted to have the same effect on the C/N ratio. If, as is reasonable to expect, the URI plantation soil was on average more adequately supplied than the Quabbin forest soil with growth-limiting nutrients (e.g., nitrogen), then we might expect a depression of carbon-based volatile levels. Likewise, although we did not measure these site factors, water availability and temperature were both likely to be higher at the plantation site. Light intensity, however, was conspicuously higher at the plantation site, and this may be expected to increase the C/N ratio, and in turn shift the allocation of photosynthate back to secondary allelochemicals. Testing the various influences of these landscape features will prove useful in extending existing knowledge of resource and herbivore defense constraints on herbivore resistance to eastern hemlock and its most serious insect pest. In summary, we have shown that overall patterns of inducible resin volatile accumulation previously characterized in twigs of plantation saplings are also present in both mature and immature forest hemlocks; the absolute abundance of the volatiles in the forest trees, however, was much greater. In addition, tree age and tissue age both affected the composition of eastern hemlock's resin volatile array. Hemlocks that are resistant to hemlock woolly adelgid have been identified and cloned by our collaborators at the University of Rhode Island (Ingwell et al. 2009, Ingwell and Preisser 2011). The patterns of hemlock volatile induction we have revealed highlight the possibility that salicylic acid-dependent biosynthetic pathways are operative in this gymnosperm system, and represent a new path toward understanding the mechanisms of resistance of putatively adelgid-resistant biotypes of eastern hemlock.

30142 subcontracted to J.S.E. from the University of Rhode Island.

References Cited

Adams, R. P. 2001. Identification of Essential Oil Components by Gas Chromatography/Quadrupole Mass Spectroscopy. Allured Press, Carol Stream, IL.

Bauce, E., M. Crepin, and N. Carisey. 1994. Spruce budworm growth, development and food utilization on young and old balsam fir trees. Oecologia 97: 499–507.

Broeckling, C. D., and S. M. Salom. 2003. Volatile emissions of eastern hemlock, Tsuga canadensis, and the influence of hemlock woolly adelgid. Phytochemistry 62: 175–180.

Bryant, J. P., F. S. Chapin III, and D. R. Klein. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. Oikos 40: 357–368.

Butin, E., E. Preisser, and J. Elkinton. 2007. Factors affecting settlement rate of the hemlock woolly adelgid, *Adelges tsugae*, on eastern hemlock, *Tsuga canadensis.* Agric. For. Entomol. 9: 215–219.

Ellison, A. M., M. S. Bank, B. D. Clinton, E. A. Colburn, K. Elliott, C. R. Ford, D. R. Foster, B. D. Kloeppel, J. D. Knoepp, and G. M. Lovett. 2005. Loss of foundation species: consequences for the structure and dynamics of forested ecosystems. Front. Ecol. Environ. 3: 479–486.

Forest Health Protection Program. 2011. Counties with established HWA populations 2010. U.S. Dep. Agric. Forest Service, Morgantown, WV.

Gandhi, K.J.K., and D. A. Herms. 2010. Direct and indirect effects of alien insect herbivores on ecological processes and interactions in forests of eastern North America. Biol. Invasions 12: 389–405.

Hain, F. P., R. G. Hollingsworth, F. H. Arthur, F. Sanchez, and R. K. Ross. 1991. Adelgid host interactions with special reference to the balsam woolly adelgid in North America, pp. 271–287. *In* Y. N. Baranchikov, W. J. Mattson, F. P. Hain, and T. L. Payne (eds.), Forest Insect Guilds: Patterns of Interaction with Host Trees. U.S. Dep. Agric. Forest Service General Technical Report NE-153.

Havill, N. P., M. E. Montgomery, G. Yu, S. Shitake, and A. Caccone. 2006. Mitochondrial DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation and pinpoints the source of the introductions to eastern North America. Ann. Entomol. Soc. Am. 99: 195–203.

Herms, D. A., and W. J. Mattson. 1992. The dilemma of plants: to grow or defend. Q. Rev. Biol. 67: 283–335.

Hudgins, J. W., and V. R. Franceschi. 2004. Methyl jasmonate-induced ethylene production is responsible for conifer phloem defense responses and reprogramming of stem cambial zone for traumatic resin duct formation. Plant Physiol. 135: 2134–2149.

Hudgins, J. W., E. Christriansen, and V. R. Franceschi. 2004. Induction of anatomically based defense responses in stems of diverse conifers by methyl jasmonate: a phylogenetic perspective. Tree Phys. 24: 251–264.

Ikeda, T., F. Matsumura, and D. M. Benjamin. 1977. Mechanism of feeding discrimination between matured and juvenile foliage by two species of pine sawflies. J. Chem. Ecol. 3: 677–694.

Ingwell, L. L., J. Brady, M. Fitzpatrick, B. Maynard, R. Casagrande, and E. L. Preisser. 2009. Intraspecific variation in *Tsuga canadensis* foliar chemistry. Northeastern Nat. 16: 585–594.

Ingwell, L. L., and E. L. Preisser. 2011. Using citizen science programs to identify host resistance in pest-invaded forests. Conserv. Biol. 25: 182–188.

Kaloshian, I., and L. L. Walling. 2005. Hemipterans as plant pathogens. Annu. Rev. Phytopathol. 43: 491–521.

Karban, R. 1990. Herbivore outbreaks on only young trees: testing hypotheses about aging and induced resistance. Oikos 59: 27–32.

Keeling, C. I., and J. Bohlmann. 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. New Phytol. 170: 657–675.

Lagalante, A. F., and K. E. Montgomery. 2003. Analysis of terpenoids from hemlock (*Tsuga*) species by solid-phase microextraction/gas chromatography/ion-trap mass spectrometry. J. Agric. Food Chem. 51: 2115–2120.

Lagalante, A. F., N. Lewis, M. E. Montgomery, and K. S. Shields. 2006. Temporal and spatial variation of terpenoids in eastern hemlock (*Tsuga canadensis*) in relation to feeding by Adelges tsugae. J. Chem. Ecol. 32: 2389–2403.

Lagalante, A. F., M. E. Montgomery, F. C. Calvosa, and M. N. Mirzabeigi. 2007. Characterization of terpenoid volatiles from cultivars of eastern hemlock (*Tsuga canadensis*). J. Agric. Food Chem. 55: 10850–10856.

Lamontagne, M., H. A. Margolis, and E. Bauce. 2000. Testing the ecophysiological basis for the control of monoterpene concentrations along canopy profiles in thinned and unthinned balsam fir stands. Oecologia 124: 318–331.

Lewinsohn, E., J. Thomas, M. Gijzen, and R. Croteau. 1993. Simultaneous analysis of monoterpenes and diterpenoids of conifer oleoresin. Phytochem. Anal. 4: 220–225.

Lombardero, M. J., M. P. Ayres, P. L. Lorio, and J. J. Ruel. 2000. Environmental effects on constitutive and inducible resin defences of *Pinus taeda.* Ecol. Lett. 3: 329–339.

Loomis, W. E. 1932. Growth-differentiation balance vs. carbohydrate-nitrogen ratio. Proc. Am. Soc. Hortic. Sci. 29: 240–245.

Martinez, D. E., O. M. Ilarduya, Q. Xie, and I. Kaloshian. 2003. Aphid-induced defense responses in Mi-1 mediated compatible and incompatible tomato interactions. Mol. Plant Microbe Interact. 16: 699–708.

Massad, T. J., L. A. Dyer, and C. G. Vega. 2012. Costs of defense and a test of the carbon-nutrient balance and growth-differentiation balance hypotheses for two co-occurring classes of plant defense. PLoS ONE 7: e47554. doi:10.1371/journal.pone.0047554.

McClure, M. S. 1991a. Density-dependent feedback and population-cycles in *Adelges tsugae* (Homoptera: Adelgidae) on *Tsuga canadensis.* Environ. Entomol. 20: 258–264.

McClure, M. S. 1991b. Nitrogen fertilization of hemlock increases susceptibility to hemlock woolly adelgid. J. Arboric. 17: 227–230.

McClure, M. S., and J. D. Hare. 1984. Foliar terpenoids in *Tsuga* species and the fecundity of scale insects. Oecologia 63: 185–193.

McClure, M. S., and C.A.S. Cheah. 1999. Reshaping the ecology of invading populations of hemlock woolly adelgid, *Adelges tsugae* (Homoptera: Adelgidae), in eastern North America. Biol. Invasions 1: 247–254.

McKinnon, M. L., D. T. Quiring, and E. Bauce. 1999. Influence of tree growth rate, shoot size and foliar chemistry on the abundance and performance of a galling adelgid. Funct. Ecol. 13: 859–867.

Moran, P. J., and G. A. Thompson. 2001. Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Plant Physiol. 125: 1074–1085.

Orwig, D. A., and D. R. Foster. 1998. Forest response to the introduced hemlock woolly adelgid in southern New England, USA. J. Torrey Bot. Soc. 125: 60–73.

Orwig, D. A., R. C. Cobb, A. W. D'Amato, M. L. Kizlinski, and D. R. Foster. 2008. Multi-year ecosystem response to hemlock woolly adelgid infestation in southern New England forests. Can. J. For. Res. 38: 834–843.

Persson, M., A. Borg-Karlson, and T. Norin. 1993. Enantiomeric composition of six chiral monoterpene hydrocarbons in different tissues of *Picea abies*. Phytochemistry 33: 303–307.

Pezet, J., J. Elkinton, S. Gomez, E. A. McKenzie, M. Lavine, and E. Preisser. 2013. Hemlock woolly adelgid and elongate hemlock scale induce changes in foliar and twig volatiles of eastern hemlock. J. Chem. Ecol. 39: 1090–1100.

Preisser, E.L.A.G. Lodge, D. A. Orwig, and J. S. Elkinton. 2008. Range expansion and population dynamics of co-occurring invasive herbivores. Biol. Invasions 10: 201–213.

Preisser, E. L., M. R. Miller-Pierce, J. L. Vansant, and D. A. Orwig. 2011. Eastern hemlock (*Tsuga canadensis*) regeneration in the presence of hemlock woolly adelgid (Adelges tsugae) and elongate hemlock scale (*Fiorinia externa*). Can. J. For. Res. 41: 2433–2439.

R Devolopment Core Team. 2012. R 2.15.2: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. (http://www.R-project.org).

Sampedro, L., X. Moreira, J. Llusia, J. Penuelas, and R. Zas. 2010. Genetics, phosphorus availability, and herbivore-derived induction as sources of phenotypic variation of leaf volatile terpenes in a pine species. J. Exp. Biol. 61: 4437–4447.

Sampedro, L., X. Moreira, and R. Zas. 2011. Resistance and response of *Pinus pinaster* seedlings to Hylobius abietis after induction with methyl jasmonate. Plant Ecol. 212: 397–401.

SAS Institute. 2011. The SAS system for Windows, version 9.3. SAS Institute, Cary, NC.

Stein, S. E. 2005. NIST Standard Reference Database 1a. NIST/EPA/NIH Mass Spectral Database (NIST05) and NIST Mass Spectral Search Program, NIST05s, version 2.0g. U.S. Department of Commerce, Gaithersburg, MD.

Sussky, E. M., and J. S. Elkinton. 2014. Density-dependent survival and fecundity of hemlock woolly adelgid (Hemiptera: Adelgidae). Environ. Entomol. 43: 1157–1167.

Trapp, S., and R. Croteau. 2001. Defensive resin biosynthesis in conifers. Annu. Rev. Plant Phys. 52: 689–724.

Von Rudloff, E. 1975a. Chemosystematic studies in the genus *Tsuga*. Leaf and twig oil analysis of western hemlock. Can. J. Bot. 53: 933–939.

Von Rudloff, E. 1975b. Volatile leaf oil analysis in chemosystematic studies of North American conifers. Biochem. Syst. Ecol. 2: 131–167.

Wainhouse, D., J. T. Staley, R. Jinks, and G. Morgan. 2009. Growth and defence in young pine and spruce and the expression of resistance to a stem-feeding weevil. Oecologia 158: 641–650.

Young, R., K. Shields, and G. Berlyn. 1995. Hemlock woolly adelgid (Homoptera:Adelgidae): Stylet bundle insertion and feeding sites. Ann. Entomol. Soc. Am. 88: 827–835.

Zagory, D., and W. J. Libby. 1985. Maturation-related resistance of *Pinus radiata* to western gall rust. Phytopathology 75: 1443–1447.

Zhu-Salzman, K., R. A. Salzman, J. Ahn, and H. Koiwa. 2004. Transcriptional regulation of sorghum defense determinants against a phloem-feeding aphid. Plant Physiol. 134: 420–431.

Zulak, K. G., and J. Bohlmann. 2010. Terpenoid biosynthesis and specialized vascular cells of conifer defense. J. Integr. Plant Biol. 52: 86–97.

Zvereva, E. L., V. Lanta, and M. V. Kozlov. 2010. Effects of sap-feeding insect herbivores on growth and reproduction of woody plants: a meta-analysis of experimental studies. Oecologia 163: 949–960.