Hemlock Woolly Adelgid and Elongate Hemlock Scale Induce Changes in Foliar and Twig Volatiles of Eastern Hemlock

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Abstract Eastern hemlock (*Tsuga canadensis*) is in rapid decline because of infestation by the invasive hemlock woolly adelgid (*Adelges tsugae;* 'HWA') and, to a lesser extent, the invasive elongate hemlock scale (*Fiorinia externa;* 'EHS'). For many conifers, induced oleoresin-based defenses play a central role in their response to herbivorous insects; however, it is unknown whether eastern hemlock mobilizes these inducible defenses. We conducted a study to determine if feeding by HWA or EHS induced changes in the volatile resin compounds of eastern hemlock. Young trees were experimentally infested for 3 years with HWA, EHS, or neither insect. Twig and needle resin volatiles were identified and quantified by gas chromatography/mass spectrometry. We observed a suite of changes in eastern hemlock's volatile profile markedly different from the largely terpenoid-based defense response of similar conifers. Overall, both insects produced a similar effect: most twig volatiles decreased slightly, while most needle volatiles increased slightly. Only HWA feeding led to elevated levels of methyl salicylate, a signal for systemic acquired resistance in many plants, and benzyl alcohol, a strong antimicrobial and aphid deterrent. Green leaf volatiles, often induced in wounded plants, were increased by both insects, but more strongly by EHS. The array of phytochemical changes we observed may reflect manipulation of the tree's biochemistry by HWA, or simply the absence of functional defenses against piercing-sucking insects due to the lack of evolutionary contact with these species. Our findings verify that HWA and EHS both induce changes in eastern hemlock's resin chemistry, and represent the first important step toward understanding the effects of inducible chemical defenses on hemlock susceptibility to these exotic pests.

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Introduction

Conifers in the family Pinaceae are among the largest and longest-living organisms on earth. Their striking longevity means that individual trees face an imposing array of biotic and abiotic challenges. They respond to these challenges via complex constitutive and inducible defenses that enable them to survive under highly diverse and taxing conditions and dominate vast areas of the earth's temperate and alpine forests (Dudareva et al. 2006; Trapp and Croteau 2001).

Conifers commonly produce oleoresin-based chemical defenses that combat herbivorous insects and pathogens (Zulak and Bohlmann 2010). Oleoresin, or simply 'resin,' is a complex and species-specific mixture of phytochemicals that is usually dominated by volatile monoterpenoids and non-volatile diterpenoid acids but also contains smaller

amounts of volatile organic chemicals such as sesquiterpenoids, benzenoids (including phenolics), and fatty acid derivatives. These compounds are produced in resin-cells of buds, needles, and woody tissue, and in some conifers (such as *Pinus* species) they accumulate in intercellular ducts or canals, either constitutively or in response to trauma (Keeling and Bohlmann 2006). Many conifers can respond to insect and microbial challenges via inducible increases in the biosynthesis and accumulation of resin (Hudgins et al. 2004). These defenses variously act to physically engulf and expel insects from the tree by the force of resin flow, seal off infected regions from surrounding tissue, deter herbivory or oviposition, chemically interfere with insect developmental pathways, ATP production, and nervous system functioning, and disrupt microbial cell membranes causing cell leakage and death (Eyles et al. 2010; Langenheim 1994). Herbivore attack also can induce the release of volatile resin semiochemicals that attract predators of the colonizing plant-feeder (Koepke et al. 2010; Mumm et al. 2003).

Over the last century, factors such as non-native pest introductions, forestry practices, and climate change have sharply increased the amount of conifer mortality due to pests or pathogen (Cudmore et al. 2010; Trapp and Croteau 2001). The increasing frequency and severity of such outbreaks have spurred intensive molecular and biochemical research into the factors underlying host susceptibility and pest/pathogen defense in spruce (*Picea*; Bohlmann 2008), fir (*Abies*; Hain et al. 1991; Lewinsohn et al. 1993a), and pine (*Pinus*; Sampedro et al. 2011). Defense induction in conifers by mechanical wounding (Lewinsohn et al. 1993a), experimental insect attack (Miller et al. 2005; Sampedro et al. 2011), or 'simulated' herbivory by application of chemical elicitors such as methyl jasmonate (Martin et al. 2002, 2003; Sampedro et al. 2010) leads to dramatic increases in bark and stem-wood terpenoid accumulation and volatile release from needles. An increasing number of the active genes and biosynthetic enzymes underlying defensive chemical outputs in these conifer systems have been identified, establishing strong evidence that resin-based—and primarily terpenoid-based—chemical defenses are central to the trees' evolved responses to insect or pathogen colonization (Franceschi et al. 2005; Keeling and Bohlmann 2006).

In eastern North America, the invasive twig-feeding hemlock woolly adelgid (*Adelges tsugae*; 'HWA') threatens to extirpate the native eastern hemlock (*Tsuga canadensis* Carr.; McClure and Cheah 1999). The first documented population of the adelgid in eastern North America was detected in the early 1950s, and appears to be of Japanese origin (Havill et al. 2006). The insect now has spread to the southern extent of eastern hemlock's range in northern Georgia, and is moving northward into Vermont, New Hampshire, and Maine (Forest Health Protection Program 2011; Preisser et al. 2008). The insect can take a year or two

to reach high densities, but its effect on hemlocks is needle desiccation, branch mortality, and marked suppression of new spring growth, often leading to tree death in 4 years or less (McClure 1991). As the only native shade-tolerant conifer in the eastern United States, eastern hemlock acts as a foundation species (sensu Ellison et al. 2005) that creates cool and moist microclimates in the midst of deciduous forests. The nearly complete removal of mature and seedling eastern hemlocks following HWA infestation (Preisser et al. 2011) substantially increases soil and stream temperatures, alters soil chemistry and nutrient cycling patterns, and favors fast-growing, early-successional trees—a series of changes that dramatically transforms the forest landscape (Gandhi and Herms 2010; Orwig et al. 2008).

The elongate hemlock scale (*Fiorinia externa*; 'EHS') is another exotic pest of eastern hemlock; an armored scale introduced to the Northeastern United States in the early 20th century. This insect also is now present in much of the tree's range and continues to spread northward (Preisser et al. 2008). Reports seemingly based on observational, rather than experimental, evidence suggest that although EHS is usually not lethal; high densities can cause significant needle loss and contribute to the mortality of already stressed trees (Abell and Driesche 2012; McClure 1980).

Despite the existence of several studies documenting a correlation between terpenoid levels and herbivory in eastern hemlock, there has been no direct investigation into whether either of these exotic pests elicits resin defenses in eastern hemlock. One study reported a positive correlation between volatile terpenoid levels and the fecundity of both EHS and a second armored scale pest of eastern hemlock (McClure and Hare 1984). Lagalante and Montgomery (2003) compared the constitutive volatile terpenoid profiles of HWA resistant and susceptible *Tsuga* species and suggested that several volatile terpenoids may act as deterrents or attractants ('feedants') to HWA. In a follow up study focused on eastern hemlock, Lagalante et al. (2006) measured spatial and temporal variability in resin volatiles and hypothesized that these phytochemical fluctuations drive the HWA's unusual annual patterns of settlement, aestivation, and feeding. European silver fir (*Abies alba*), a conifer of a genus related to *Tsuga*, showed increased levels of monoterpenoid accumulation in bark naturally infested with *Adelges piceae*, the balsam woolly adelgid (Hain et al. 1991). In addition, western hemlock (*Tsuga heterophylla*) responded to simulated herbivory (treatment with methyl jasmonate) in a manner typical of the conifers of Pinaceae: traumatic resin ducts formed and terpenoid concentrations increased (Hudgins et al. 2004). This evidence suggests resinosis also may occur in species of *Tsuga*. However, despite the prevalence of research into herbivore-defense responses of other conifers of Pinaceae, little is known about the inducible resin defenses of hemlocks.

There is growing evidence that HWA infestation induces changes in eastern hemlock chemistry and physiology. Evidence of a localized and systemic hypersensitive response (a common plant defense against pathogens and sessile herbivores leading to tissue necrosis at the infected site; Radville et al. 2011), substantially higher foliar free amino acid concentrations (Gomez et al. 2012), changes in woody plant anatomy (Gonda-King et al. 2012), and a reduction of both new growth and percent total foliar nitrogen (Miller-Pierce et al. 2010) have been reported in response to HWA feeding on eastern hemlocks. EHS, on the other hand, appeared to produce only a localized hypersensitive response, and did not significantly affect free amino acid concentration, percent total foliar nitrogen, woody plant anatomy, or subsequent new growth.

We investigated whether HWA or EHS infestation induced oleoresin production in eastern hemlock, an ecologically unique native U.S. conifer in rapid decline in many areas. Previous research has suggested spatial and temporal fluctuations in volatile resin compounds can influence the establishment of colonizing hemipteran herbivores (Lagalante et al. 2006; McClure and Hare 1984). To test this, we measured levels of resin volatiles in both twigs and needles of eastern hemlocks experimentally infested with HWA, EHS, or neither insect in early summer and again in mid-autumn, each time following periods of active feeding by both insects. We predicted that both insects would elicit changes in the concentrations or composition of volatile resin compounds. We hypothesized that an agent as rapidly lethal as HWA would elicit a defensive resinosis typical of many conifers of Pinaceae: pronounced increases in toxic or deterrent phytochemicals, especially terpenoids. We also predicted that the much milder effects of EHS on the host tree's physiology (Gomez et al. 2012; Gonda-king et al. 2012; Miller-Pierce et al. 2010; Radville et al. 2011) would be accompanied by an induced resin response distinct from that of HWA.

Methods and Materials

Study System

Eastern hemlock buds begin opening in May in the Northeastern United States, and the young new growth shoots, at first green and pliant, complete their elongation at approximately the end of summer. By that time, the foliage has hardened and taken on the form and appearance of the fully mature, previous year growth.

The HWA completes two clonal generations per year in eastern North America, as in its range of origin in Japan (McClure and Cheah 1999). In the Northeastern United States, first instar nymphal crawlers of the progrediens generation settle in April (before bud-break) on already mature previous year's growth just below the needle abscission layer, and feed through a stylet bundle on xylem ray parenchyma cells in the twig (Young et al. 1995). The sexuparae, a winged, sexually reproducing generation of HWA, hatch concurrently with the clonal female progredientes and, in Japan, subsequently disperse to a spruce (Picea) primary host to complete reproduction. Sexuparae in North America are unable to complete their life-cycle due to the absence of a suitable spruce host, and thus, only asexual reproduction occurs. The sessile progrediens adults complete egg laying in June, at which point the crawlers of the sistens generation emerge, settle preferentially on the new, young current year's growth, and promptly enter aestivation. In early fall, by the time the new growth has matured, the sistens nymphs resume feeding, completing development and oviposition in April (McClure and Cheah 1999).

The EHS completes two full generations per year in its natural range in Japan, but in the Northeastern United States it appears to lack a distinct and regular cycle of life stage development, and completes between one and two generations annually (Abell and Driesche 2012). First instars begin to hatch in early June and settle preferentially on the undersides of young hemlock needles. EHS is also a sessile stylet feeder, inserting a thread-like stylet bundle and sucking fluid from needle mesophyll cells (McClure 1980). Since generation times in the Northeast are irregular, life stages appear to overlap, and often two or more instars may be found developing concurrently on the same foliage (Abell and Driesche 2012).

Experimental Design

In April 2007, eastern hemlock saplings (0.7–1.0 m) were removed from Cadwell Experimental Forest (Pelham, MA, USA) and planted in an open field setting (East Farm, Kingston, RI, USA) in a rectangular grid. The source forest was free of both HWA and EHS at the time of collection, and careful inspection of the sapling trees revealed no prior infestation by either insect. Artificial infestation with HWA, EHS, or neither insect was randomly applied to the saplings. Because both insects are wind-dispersed during their first-instar crawler phase, each tree (including all uninfested controls) was enclosed in a mesh cage annually from early spring to late fall to prevent cross-contamination. Each of the $1 \times 1 \times 2$ m (length by width by height) cages consisted of a plastic PVC pipe frame covered by mosquito netting (97 holes/cm^2 mesh size). Weed-inhibiting fabric (1 m^2) was placed around the base of each tree. By 2010, a combination of insect cross-contamination and tree death from transplantation-related stress reduced the level of replication to 9 trees in the HWA treatment, 7 trees in the EHS treatment, and 8 trees in the control treatment.

Insect Inoculations

Insect inoculations were conducted following standard procedures (see Butin et al. 2007). Briefly, trees were inoculated with insects each spring from 2007 to 2010 to mimic natural infestation cycles. Immediately prior to crawler emergence (May for HWA, June for EHS), naturally-infested branches with comparable insect densities were collected from sites in southern New England and attached to trees in the appropriate treatment group; control trees received uninfested branches. Individual branches were placed in aquapics to slow needle desiccation and decrease insect mortality.

Plant Material

Plant tissue samples were collected from each tree in late June 2010 (fully mature, previous year foliage segments) after the first-instar crawlers of both insects had settled and commenced feeding, and again in mid-October 2010 (young, current year growth twigs) after settled HWA had ceased aestivation and resumed feeding. An average of 10 cm of twig with foliage was clipped; in the case of the insect treatments, infested foliage samples were selected. Each sample was 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.

Extraction of Resin Volatiles

Extraction of resin volatiles was modified from a protocol developed by Lewinsohn et al. (1993b). All reagents and reference standards were obtained from Sigma-Aldrich (St. Louis, MO, USA). Solvents were HPLC or GC grade purity.

Needles were separated from twigs and ground to a homogenous powder using a mortar and pestle under liquid nitrogen. Approximately 100–200 mg (dry weight) of needle tissue were combined with methyl *tert*-butyl ether (MTBE; 1.3–1.5 ml) containing a known concentration of isobutylbenzene (40 $\mu g\ ml^{-1}$) as an internal standard in a pre-weighed 2 ml vial (glass with PTFE-coated screw cap, Sigma-Aldrich, St. Louis, MO, USA). Needle samples were extracted overnight (20 hr) with constant shaking at room temperature. Each extract was transferred to a fresh glass vial and washed with aqueous $(NH_4)_2CO_3$ (0.3 ml, 1 M) to neutralize acidic impurities. The organic layer then was filtered through a Pasteur pipette column packed with silica gel (0.3 g, Sigma-Aldrich, 60 Å) overlaid with $MgSO_4$ (0.2 g). Oxygenated volatile compounds were subsequently eluted by washing the filter with diethyl ether (1 ml), and combined eluates were collected in a GC vial (PTFE-coated screw cap, Agilent Technologies, Santa Clara, CA, USA) and stored at −20 °C until analysis.

Twig samples of approximately 10–50 mg (dry weight) were cooled with liquid nitrogen in a mortar and pestle, ground to a coarse powder, and combined with MTBE (1.0 ml) containing isobutylbenzene (2 $\mu g\ ml^{-1}$) in a 2 ml glass vial. Twigs were extracted overnight (19 hr) with constant shaking at room temperature. Aqueous $(NH_4)_2CO_3$ (0.2 ml; 1 M) was added to each extract, followed by thorough mixing. The organic layer was transferred directly to a Pasteur pipette filter packed with silica gel (0.2 g, 60 Å) overlaid with $MgSO_4$ (0.13 g). The filter was washed with diethyl ether (0.5 ml), and combined eluates were collected and stored as described above. After extraction, each sample was dried for at least 48 hr at 55–60 °C and weighed for the determination of tissue dry weight.

Analysis of Resin Volatiles

Needle volatile extracts were analyzed on a Hewlett-Packard (HP) 6890 GC equipped with a flame ionization detector (FID). For all analyses, the injection volume was 1 µl, injector temperature 220 °C. Volatile compounds were separated on an Agilent DB-5, 0.25 mm i.d. × 30 m, 0.25 µm coating thickness, fused silica capillary column. H_2 carrier gas flow was a constant 1.0 ml min^{-1}, and the split ratio was 20:1. The FID was heated to 250 °C, with H_2 flow at 40 ml min^{-1}, air flow 350 ml min^{-1}, and constant make-up flow (N_2) at 45- ml min^{-1}. The GC oven was programmed with an initial temperature of 60 °C (no hold), an increase at 3 °C min^{-1} to 156 °C, then 50 °C min^{-1} to 300 °C (hold 3 min). GC-FID generated peaks were integrated using HP ChemStation software (Agilent technologies). Datafiles for five of the October needle samples were corrupted, reducing the level of replication to 7 trees in the HWA treatment, 6 trees in the EHS treatment, and 6 trees in the control treatment.

For all compound identifications, as well as all twig volatile quantification, analyses were performed on a Shimadzu GC-2010 system equipped with a QP2010-Plus mass spectrometer (EI mode, 70 eV), running GCMSolution software (Shimadzu Corporation, Kyoto, Japan). Separations were performed on the same column as described above for GC-FID. The injection volume was 1 µl and injector temperature 220 °C. Helium carrier gas flow was in constant linear velocity mode at 36.5- cm sec^{-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), 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 temperatures were both set at 300 °C, and the MS scan range was *m/z* 40–400.

Identification of each volatile compound was, wherever possible, based on comparison of the experimental retention time and mass spectrum with those of an authentic standard (indicated in Table 1); 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

Table 1 Resin volatile concentration relative change (treatment average/control average ratio) for eastern hemlock saplings treated with 3-year artificial infestation with hemlock woolly adelgid (HWA) or elongate hemlock scale (EHS)

Twig Volatiles[a]	June[b] HWA	June[b] EHS	October[b] HWA	October[b] EHS	June HWA	June EHS	October HWA	October EHS	Needle Volatiles
Monoterpenoids[c]									**Monoterpenoids**
Tricyclene	*0.49[d]	0.80	*0.62	0.95	1.07	1.02	1.12	1.09	Tricyclene
α-Pinene[e]	0.78	0.93	1.08	0.71	1.08	1.05	1.15	1.09	α-Pinene[e]
Camphene[e]	*0.49	0.76	*0.68	0.91	1.04	1.05	1.08	1.13	Camphene[e]
β-Pinene[e]	0.76	1.07	1.22	0.75	1.14	0.98	1.19	0.91	Sabinene
Myrcene[e]	0.84	1.30	1.41	1.63	1.09	1.00	1.15	1.00	β-Pinene[e]
Limonene[e]	0.72	0.95	0.69	*1.47	1.05	1.02	1.08	1.08	Myrcene[e]
L-trans-Pinocarveol	0.74	0.87	0.71	*0.58	1.18	1.12	*1.34	1.09	α-Phellandrene[e]
cis-Verbenol	0.93	1.15	0.73	*0.46	1.19	1.15	1.12	1.07	Limonene[e]
trans-Verbenol	0.77	0.93	0.72	*0.58	0.88	0.83	1.14	0.92	γ-Terpinene[e]
Borneol[e]	0.73	0.87	*0.72	1.24	1.03	1.14	1.27	1.28	Terpinolene[e]
trans-Carveol[e]	0.73	0.88	0.72	*0.63	1.05	1.77	0.73	1.40	Camphor[e]
Myrtenol[e]	*0.61	*0.62	0.70	*0.56	0.05	0.10	2.30	1.68	Borneol[e]
α-Campholenal	*0.59	0.75	0.68	*0.61	0.83	0.82	0.93	0.91	4-Carvomenthenol[e]
Pinocarvone	*0.68	0.94	0.77	*0.56	0.84	0.74	1.15	1.08	p-Menth-1-en-9-ol
Verbenone[e]	*0.60	0.70	0.75	0.65	1.19	1.14	0.49	0.78	α-Terpineol[e]
Bornyl Acetate[e]	*0.45	*0.60	*0.71	0.82	0.70	*0.59	1.24	1.18	trans-Piperitol
total	*0.61	0.79	0.95	0.80	0.83	0.87	1.06	1.28	Piperitone[e]
Sesquiterpenoids					1.01	1.04	1.08	1.11	Bornyl Acetate[e]
β-Caryophyllene[e]	1.05	*0.00	2.38	1.25	1.04	1.03	1.11	1.10	total
α-Humulene[d]	1.89	*3.72	2.81	1.46					**Sesquiterpenoids**
Germacrene-D	1.05	1.34	1.17	0.55	1.01	1.07	1.24	1.13	β-Caryophyllene[e]
α-Amorphene	3.83	1.04	3.24	1.15	1.05	1.12	1.24	1.13	α-Humulene[e]
Caryophyllene Oxide[e]	0.95	0.85	0.88	*0.50	1.26	0.71	1.76	0.69	Germacrene-D
total	1.26	1.03	1.78	0.87	0.91	1.00	1.15	1.09	α-Amorphene
Benzenoids					0.97	1.02	1.24	1.13	δ-Cadinene
p-Cymene[e,f]	0.75	0.72	1.25	0.73	1.05	1.03	1.30	1.08	total
Benzyl Alcohol[e]	*31.43	0.53	*9.68	0.86					**Benzenoids**
p-Cymen-8-ol[e,f]	0.93	0.50	0.92	0.71	0.33	0.25	0.74	0.63	p-Cymene[e,f]
Methyl Salicylate[e]	*123.61	0.00	*12.05	1.10					**Green leaf volatiles**
3,4-Dimethoxyphenol	0.88	0.99	1.13	0.86	1.08	1.41	1.28	1.55	n-Hexanal[e]
Raspberry Ketone[e]	0.73	0.72	*0.66	*0.64	1.30	1.09	1.82	*2.55	trans-2-Hexenal[e]
total	*2.41	0.71	1.21	0.72	1.04	1.37	1.31	*2.55	cis-3-Hexenal
Unknown A	0.86	0.83	0.79	*0.50	1.22	1.19	*1.41	*1.87	total
Unknown B	*0.30	0.62	*0.67	0.59	1.03	1.03	1.13	1.12	**Total Needle Volatiles**
Unknown C	*0.30	0.80	*0.29	0.48					
Total Twig Volatiles	*0.77	0.78	0.98	0.79					

[a] Since mass detector response factors are likely to vary among compounds, quantification of the twig volatiles should be regarded as relative and reliable for quantitative comparison of levels of individual compounds between trees (or treatments), but not for comparison of levels of different compounds

[b] Foliage sampled in June was mature, previous year growth infested with EHS or progrediens-generation HWA; foliage sampled in sampled in October was young, current year growth infested with EHS or sistens-generation HWA

[c] Compounds are ordered first by structural class, then by ascending order of elution from a non-polar DB-5 GC column

[d] Values >1 and <1 indicate an increase (dark gray shading) and decrease (light gray shading), respectively, from the untreated control trees. 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 in italics with asterisks

[e] Compound identification based on comparison of GC retention time and mass spectrum with those of authentic standard

[f] p-Cymene and its derivatives may also be classified on biosynthetic grounds with the monoterpenoids

spectral libraries (Stein et al. 2011). Concentrations of all compounds were determined by normalizing integrated peak areas against that of the internal standard isobutylbenzene in each chromatogram. Each tissue volatile concentration value was standardized to 'ug g^{-1} dry weight' by dividing by the sample dried weight. Since mass detector response factors are likely to vary among compounds, quantification of the twig volatiles should be regarded as relative and reliable for quantitative comparison of levels of individual compounds between trees (or treatments) but not for comparison of levels of different compounds.

Since both the HWA and the EHS are quite small and adhere tightly to their twig or needle feeding sites, complete removal of insects and their ovisacs from infested samples prior to analysis was not practical. To test whether detected volatiles could potentially be of insect, rather than hemlock, origin, we obtained several samples of HWA-infested foliage of comparable size and insect 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.

Statistical Analysis

Resin volatile concentrations were log transformed prior to statistical analysis to reduce heterogeneity of variance. Two-way mixed-model ANOVAs (Proc Mixed, SAS 9.3; SAS Institute 2011) were used to test twigs and needles separately for treatment-level differences in the concentration of individual volatiles, total monoterpenoids, total sesquiterpenoids, total green leaf volatiles (needles only), and total combined benzenoids (including phenolics; twigs only) with month (June vs. October) and treatment (HWA, EHS, and control) as fixed factors and tree as a random factor. Mixed-model analyses were appropriate because we sampled from the same trees in both months. We also used ANOVA planned contrasts to separately test HWA and EHS treatment means against the control mean, using treatment as the fixed factor (R 2.14.0; R Development Core Team 2010).

Familywise error rate for the mixed model analyses was evaluated using a false discovery rate (FDR) estimation method ('fdrtool' software package; R 2.14.0; Strimmer 2008). False discovery rate techniques are now used widely with multiple simultaneous hypothesis testing to estimate the proportion of tests with incorrectly rejected null hypotheses among tests with statistically significant findings. This is in contrast to traditional familywise error rate correction methods (e.g., the sequential Bonferroni) that estimate the probability of a false rejection among all tests conducted and, arguably, unnecessarily sacrifice statistical power.

As an additional measure of the overall strength of evidence for our mixed-model hypothesis test findings, we used the following binomial equation (sensu Moran 2003) to calculate the overall probability of obtaining K tests with P-values smaller than our specified α-level:

$$P_B = \left[N! \middle/ (N-K)!K! \right] \times \alpha^K (1-\alpha)^{N-K}$$

where N=number of tests. This procedure allowed us to estimate the probability that so many statistically significant treatment effects could arise by chance (i.e., could be 'false positives').

Results

The overall effects of infestation with HWA and EHS were similar in both June 2010 (mature previous year's growth) and October 2010 (young current year's growth) samples (Table 1, Online resource 1). Both insects produced a trend of decreases of most individual twig volatiles, though only 16 out of 90 of these decreases were statistically significant ($P<0.05$). Conversely, both insects produced a largely nonsignificant trend of increases of most needle volatiles (Table 1). Across all treatments, total volatile levels were five- to eight-fold higher in twigs sampled in October than those in June (Table 2A, B), and two-fold higher in needles sampled in October than those in June.

In twig tissue, 16 monoterpenoids, five sesquiterpenoids, and six benzenoid or phenolic compounds were present in quantities sufficient for identification and quantification (Fig. 1a); in needle tissue, the corresponding numbers were 18 monoterpenoids, five sesquiterpenoids, one benzenoid, and three fatty acid derivatives (i.e., green leaf volatiles or 'GLVs'; Fig. 1b). Qualitatively, needle and twig volatile profiles were overlapping but different (Table 1). Monoterpenoids dominated in terms of both diversity and mass contribution, and had the greatest effect on the induced changes of total volatiles. Sesquiterpenoids, present at somewhat lower abundance, generally increased in both twigs and needles. GLVs were detected only in needle tissue, and were consistently increased by both insects, especially by EHS. Additionally, across all treatments, levels of these compounds increased four- to six-fold from June to October; Fig. 2.
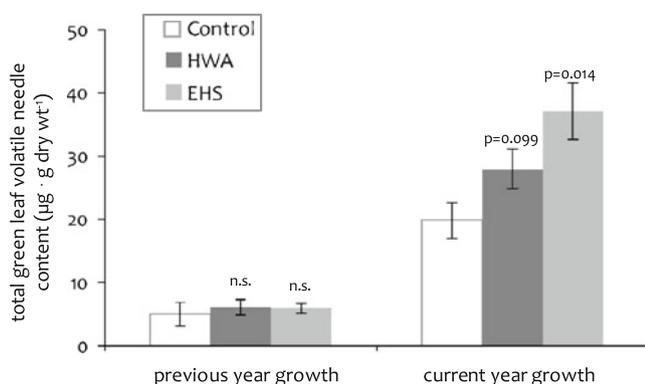
The effects of insect feeding on volatile concentration were larger in twigs than in needles (Table 1, Online Resource 2). In twigs, the results of mixed-model ANOVAs (Online Resource 2A) show that HWA feeding significantly ($P<0.05$) or marginally significantly ($0.05<P<0.10$) decreased five of 17 individual monoterpenoids and two unidentified volatiles; additionally, there were significant increases in the benzenoid benzyl alcohol (more than 30-fold in June and about 10-fold in October; Fig. 3) and the monophenolic phytohormone methyl salicylate ('MeSA'; two orders of magnitude in June and more than 10-fold in October; Fig. 4). EHS feeding
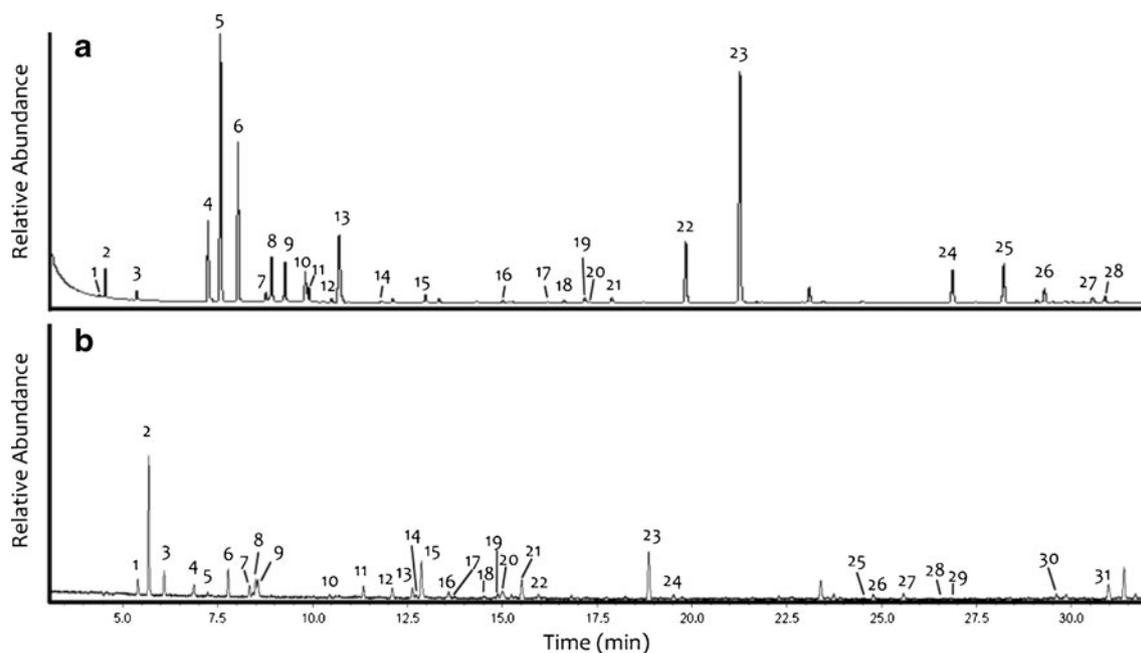
Fig. 1 **a** GC-FID total ion chromatogram showing volatiles tentatively identified in HWA-infested eastern hemlock needles: **1**, *cis*-3-hexenal; **2**, *n*-hexanal; **3**, *trans*-2-hexenal; **4**, tricyclene; **5**, α-pinene; **6**, camphene; **7**, sabinene; **8**, β-pinene; **9**, myrcene; **10**, α-phellandrene; **11**, isobutylbenzene (internal standard); **12**, *p*-cymene, **13**, D-limonene; **14**, γ-terpinene, **15**, terpinolene; **16**, camphor; **17**, borneol; **18**, 4-carvomenthenol; **19**, *p*-menth-1-en-9-ol; **20**, α-terpineol; **21**, *trans*-piperitol; **22**, piperitone; **23**, bornyl acetate; **24**, β-caryophyllene; **25**, α-humulene; **26**, germacrene-D; **27**, α-amorphene; **28**, δ-cadinene. **b** GC-MS total ion chromatogram showing volatiles in HWA-infested twigs: **1**, tricyclene; **2**, α-pinene; **3**, camphene; **4**, β-pinene; **5**, myrcene; **6**, isobutylbenzene (internal standard); **7**, *p*-cymene; **8**, D-limonene; **9**, benzyl alcohol; **10**, unknown; **11**, unknown; **12**, α-campholenal; **13**, L-*trans*-pinocarveol; **14**, *cis*-verbenol; **15**, *trans*-verbenol; **16**, pinocarvone; **17**, borneol; **18**, *p*-cymen-8-ol; **19**, methyl salicylate; **20**, myrtenol; **21**, verbenone; **22**, *cis*-carveol; **23**, bornyl acetate; **24**, unknown; **25**, β-caryophyllene; **26**, 3,4-dimethoxyphenol; **27**, α-humulene; **28**, germacrene-D; **29**, α-amorphene; **30**, raspberry ketone; **31**, caryophyllene dioxide

decreased two monoterpenoids significantly. Both insects decreased the monophenolic raspberry ketone and several other monoterpen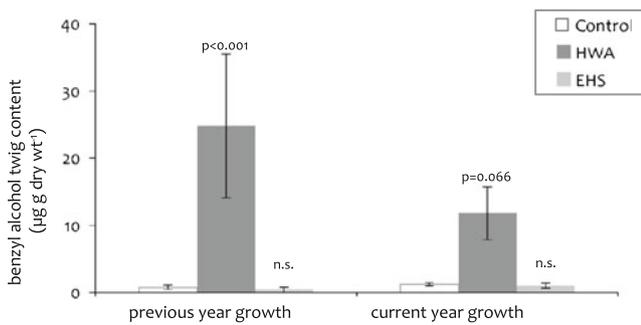oids with marginal significance. HWA feeding significantly decreased total monoterpenoids, while EHS feeding decreased both total monoterpenoids and total volatiles with marginal significance. HWA feeding marginally increased total benzenoids, while EHS feeding marginally decreased these compounds (Online Resource 2A).

In needles, (Online Resource 2B) EHS feeding increased *cis*-3-hexenal and total GLVs significantly, and increased *trans*-2-hexenal and the benzenoid *p*-cymene with marginal significance. There were no significant effects of HWA feeding on needle volatile levels.

Results of planned contrast ANOVA comparisons of average control vs. treatment volatile concentrations were similar to those we obtained using the mixed model analyses. Significance values from these simpler analyses are indicated in Table 1.

The binomial probability that the 60 twig volatile mixed model tests we ran would generate P-values smaller than the ones we observed was $P_B = 0.00014$ if calculated at the $\alpha = 0.05$ level, or 4.7×10^{-9} if calculated at the $\alpha = 0.10$ level. For the 54 needle volatile tests, the overall probability of no 'real' effect was greater: $P_B = 0.18$, and 0.12, respectively.

Estimated false discovery rate for twig volatile hypothesis tests is reported as q-value alongside each test's nominal P-value (Online Resource 2A). The q-value is the minimum FDR level that would be needed to reject that hypothesis.

Fig. 2 Green leaf volatile ('GLV') content (average ± SE) in needle tissue of control and insect-infested eastern hemlocks. 'HWA' or 'EHS' represent 3-year artificial infestation with hemlock woolly adelgid (*Adelges tsugae*) or elongate hemlock scale (*Fiorinia externa*). Data represents the average concentration (μg·g dry wt^{-1}) of total GLVs in mature previous year growth (sampled 28 June) and young current year growth (sampled 19 October), calculated from 6 to 9 trees per treatment group. P-values are shown when the difference between the treatment and control trees was significant ($P<0.05$), or marginally significant ($0.05<P<0.10$; planned contrast)
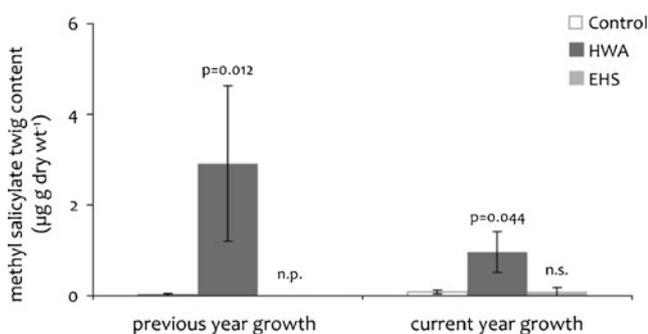
Fig. 3 Benzyl alcohol content (average ± SE) in twig tissue of control and insect-infested eastern hemlock trees. 'HWA' or 'EHS' represents 3-year artificial infestation with hemlock woolly adelgid (*Adelges tsugae*) or elongate hemlock scale (*Fiorinia externa*). Data represent the average concentration (µg·g dry wt^{-1}) of benzyl alcohol in mature previous year growth (sampled 28 June) and young current year growth (sampled 19 October), calculated from 7 to 9 trees per treatment group. *P*-values are shown when the difference between the treatment and control trees was significant (*P*<0.05), or marginally significant (0.05<*P*<0.10; planned contrast)

Selection of an appropriate FDR level, in turn, depends on the proportion of false rejections considered tolerable. We did not report FDR for needle volatile hypothesis tests (Online Resource 2B). Since the method estimates the proportion of false rejections among only tests with significant findings—and there was just one out of 54 needle volatile hypothesis tests that was statistically significant—in that case an estimate of FDR was superfluous.

Discussion

We found evidence of an induced response in eastern hemlock during infestation by both HWA and EHS, encompassing a

Fig. 4 Methyl salicylate content (average ± SE) in twig tissue of control and insect-infested eastern hemlock trees. 'HWA' or 'EHS' represents 3-year artificial infestation with hemlock woolly adelgid (*Adelges tsugae*) or elongate hemlock scale (*Fiorinia externa*). Data represent the average concentration (µg·g dry wt^{-1}) of methyl salicylate in mature previous year growth (sampled 28 June) and young current year growth (sampled 19 October) calculated from 7 to 9 trees per treatment group. *P*-values are shown when the difference between the treatment and control trees was significant (*P*<0.05), or marginally significant (0.05<*P*<0.10; planned contrast)

number of feeding-elicited changes in the tree's resin volatile profile. However, the modest induction (mostly decreases) of resin metabolites in twig tissue and the non-significant trend of modest increases in needle tissue produced by both insects, was conspicuously different from the profuse resinosis observed in insect-infested pines, spruces, and firs (Trapp and Croteau 2001). In light of the considerable evidence that HWA induces more extensive changes in eastern hemlock physiology than does EHS (Gomez et al. 2012; Gonda-King et al. 2012; Miller-Pierce et al. 2010; Radville et al. 2011), the observation that HWA and EHS produced similar overall changes in the tree's volatiles was intriguing and ran counter to our predictions.

In contrast to the modest changes in terpenoid levels, a number of the non-terpenoids were sharply increased by HWA feeding, in what may reflect a hemlock defense response (Table 1). Benzyl alcohol was induced in HWA-infested trees; this compound is a common plant volatile (Dudareva et al. 2006) previously detected in the stem-wood of mountain hemlock (*T. mertensiana*; Shepherd et al. 2007) and in volatiles released from mite-infested spruce foliage (Kannaste 2008; Fig. 3). In screening studies, benzyl alcohol deterred feeding by the greenbug aphid *Schizaphis graminum*, reducing fecundity and causing substantial mortality (Formusoh et al. 1997). MeSA, which was also induced by HWA (Fig. 4), has been found in the volatile mix released after aphid feeding and identified as a deterrent to aphid settling and fecundity in a number of plant-insect systems (Hardie et al. 1994; Quiroz et al. 1998).

The sharp increase of these two compounds in HWA-infested trees (Table 1) is notable in light of the growing body of evidence that some plants respond to piercing-sucking hemiptera by activating biosynthetic pathways similar or identical to those used in pathogen defense (Kaloshian and Walling 2005). Benzyl alcohol is a strong antimicrobial agent against diverse microorganisms (Shenep et al. 2011), while MeSA, the volatile methyl ester of salicylic acid (SA), activates a SA-dependent biosynthetic cascade in numerous plants that leads to systemic acquired resistance (SAR) against pathogen infection (Durrant and Dong 2004). For aphids, close relatives of adelgids, feeding has been shown in many studies to activate the SA-dependent biosynthetic pathways normally associated with pathogen defense (Martinez de Ilarduya et al. 2003; Moran and Thompson 2001; Zhu-Salzman et al. 2004) or to induce pathogen-resistance outright in their host plant (Russo et al. 1997). The elevated levels of these two compounds in HWA-induced hemlock tissue is a sign that a SA-driven insect defense syndrome may be active in HWA-infested trees.

It is also possible that increased production of these volatiles reflects the tree's detection of a microbial associate of HWA rather than the insect itself. An endosymbiont has been found throughout the body of the HWA and appears essential to the insect's survival (Shields and Hirth 2005). It is

possible that the hemlocks may be responding to this bacterium, if it is introduced into the vascular tissue during HWA feeding, by mobilizing a pathogen defense response.

Our results may help elucidate why HWA causes more extensive damage to eastern hemlock than EHS. Radville et al. (2011) detected evidence of a local hypersensitive response (elevated hydrogen peroxide levels) in both EHS- and HWA-infested trees, and showed that this hypersensitive response occurs systemically in response to HWA-infestation. The hypersensitive response usually precedes the development of SAR (Durrant and Dong 2004; Kaloshian and Walling 2005). Research on tobacco has revealed that in pathogen-infected plant tissue, SA is enzymatically converted to the volatile MeSA, which acts as a mobile agent that is taken up by receptors on distant, uninfected tissue. There, the MeSA is demethylated and transformed back to SA, which in turn activates an induced resistance response to the invading organism (i.e., SAR; Park et al. 2007; Shulaev et al. 1997). Our discovery that MeSA levels were elevated in only the HWA-infested trees suggests this compound could be a mobile signal that propagates the 'pathogen-like' effects of the adelgid on uninfested foliage, extending the insect's effects and intensifying the overall damage to the tree. The observation that HWA elicited such a response, but EHS did not, may reflect the species-specific nature of the hemlock defense elicitors carried in the insects' salivary secretions, as has been observed in at least one other hemipteran-plant interaction (Ven et al. 2000).

The HWA-driven increases we observed in levels of benzyl alcohol and MeSA also may help explain previously noted changes in the primary chemistry of the hemlock saplings of the present study (Gomez et al. 2012). Although much of the biosynthetic pathway for the benzenoids has yet to be determined, radio-labeling experiments show they are derived from L-phenylalanine (Dudareva et al. 2006). As with benzyl alcohol and MeSA, a marked increase in L-phenylalanine and many other free amino acids occurred in trees infested with HWA, but not EHS (Gomez et al. 2012). Thus, the increased amino acid levels in HWA-infested trees may constitute an adaptive mobilization of precursors of defense-related volatile compounds.

Alternatively, adelgid manipulation of host-plant biochemistry could explain a number of the insect-induced changes in resin chemistry that we have shown. HWA, like many adelgids, forms extensive galls on the buds of its primary spruce host in its original range in Asia (Havill and Foottit 2007). Galling insects are adept at manipulating host plant physiology to create a more nutritious and less defended environment (Tooker and De Moraes 2009). We have demonstrated a substantial decrease in monoterpenoids, often compounds of direct defense against herbivory (Eyles et al. 2010; Schiestl 2010), in the tissue where the adelgid feed. Our results also show a less pronounced elicitation of GLVs (typical wounding response volatiles; Fig. 2; Shiojiri

et al. 2006) in response to the feeding of HWA, relative to EHS, despite the adelgid's much greater impacts on tree physiology (Gonda-King et al. 2012; Miller-Pierce et al. 2010; Radville et al. 2011). These observations, considered together with the noted increase in free amino acids only in HWA-infested trees (Gomez et al. 2012), may constitute evidence that the host-manipulating capacity conserved in adelgid biology may be an underlying mechanism in this system.

Lagalante et al. (2006) suggested that the lack of a co-evolutionary history between eastern hemlock and sessile piercing-sucking insects resulted in the absence of biosynthetic pathways with which eastern hemlock can defend against insects like HWA and EHS. This hypothesis is consistent with the finding of little or no output of anatomical of chemical resin defenses. However, we did observe a resin chemical response to HWA, and to the co-occurring EHS, though perhaps of a subtler nature than that often seen in other conifers. It is possible that the resistance traits HWA elicits in eastern hemlock are simply not well matched to the actual challenge of this introduced insect and do not confer resistance. A comparison of the induced response of susceptible eastern hemlocks to those of HWA-resistant *Tsuga* species and strains of eastern hemlock believed resistant to HWA, as well as to those conifers with putative resistance to EHS (McClure and Fergione 1977), will test these hypotheses. Nonetheless, our findings establish that HWA and EHS both induce changes in the resin chemistry of eastern hemlock, and constitute the first critical step toward understanding the role inducible chemical defenses play in determining hemlock susceptibility to these exotic hemipteran pests.

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