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https://doi.org/10.7275/5743349 https://scholarworks.umass.edu/masters_theses_2/104

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VOLATILE PROFILES AND RESISTANCE TO HERBIVORY IN EASTERN HEMLOCK

A Thesis Presented

by

ELIZABETH ALEXA MCKENZIE

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
of the requirements for the degree of

MASTER OF SCIENCE

September 2014

Organismic and Evolutionary Biology

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VOLATILE PROFILES AND RESISTANCE TO HERBIVORY

IN EASTERN HEMLOCK

A Thesis Presented

by

ELIZABETH ALEXA MCKENZIE

Approved as to style and content by:

____
Joseph S. Elkinton, Chair

____
Rachel A. Levin, Member

____
Ruthanne H. Paradise, Member

____
Paul R. Sievert, Member

____
Elizabeth R. Dumont, Department Head,
Organismic & Evolutionary Biology

DEDICATION

I fondly dedicate this thesis to the friends and family members who supported me throughout this project and pulled me out of the lab for important life experiences – and especially to Eli Sennesh, for broadening my horizons and enriching my life in countless ways.

ACKNOWLEDGEMENTS

I owe tremendous thanks to Joshua Pezet, OEB graduate and Elkinton lab veteran, who pioneered the methods I am using and who trained me in their use. I also gratefully acknowledge the other members of the Elkinton lab for their support, especially Jeff Boettner for his constant advice and encouragement, and Liz Sussky for use of her adelgid population data and for all her efforts in maintaining the Quabbin study site. I am also grateful to my labmates Natashia Manyak, Tessa Dowling, and Courtney Hoffman for their efforts in logistical support.

My work on the resistant hemlocks would not have been possible without the prior work of University of Rhode Island investigators, especially Evan Preisser, Richard Casagrande, and their lab members. A special thanks also to Elwood Roberts, who grew the trees I sampled at URI and who climbed a cliff in the rain with me to find good control trees at the New Jersey field site.

Many thanks to my advisor and thesis committee members for their insight and generosity with their time: Joseph Elkinton, Paul Sievert, Rachel Levin, and Ruthanne Paradise.

This work is supported by the United States Department of Agriculture National Institute of Food and Agriculture grant #2010-03710 to ELP and JSE, and RI-AES Hatch Grant RI00HI-4004 to ELP and RAC. Access to the New Jersey forest sites was permitted through the New Jersey Department of Environmental Protection, Division of Fish & Wildlife and the Delaware Water Gap National Recreation Area, National Parks Service.

ABSTRACT

VOLATILE PROFILES AND RESISTANCE TO HERBIVORY

IN EASTERN HEMLOCK

SEPTEMBER 2014

ELIZABETH ALEXA MCKENZIE, B.S., UNIVERSITY OF MASSACHUSETTS AMHERST

M.S., UNIVERSITY OF MASSACHUSETTS AMHERST

Directed by: Professor Joseph S. Elkinton

Eastern hemlock hosts the hemlock woolly adelgid, an introduced sap-feeding insect that causes rapid deterioration of the host. Like most conifers, eastern hemlock produces a variety of constitutive and induced defenses, primarily terpenoids. To explore the relationship of terpenoid defenses with adelgid infestations, we artificially infested hemlocks at a forest site and a plantation site, and compared their terpenoid concentrations to those in control trees. Infested trees showed lower terpenoid concentrations than control trees, suggesting that eastern hemlock not only fails to induce production of terpenoids in response to adelgid infestation, but becomes less able to produce carbon-based defenses due to loss of carbon resources to the adelgid. Greater light intensity may account for consistently higher terpenoid concentrations at the plantation site, supporting the explanation that carbon limitation restricts terpenoid production.

Recent studies have identified a small number of individual eastern hemlock trees that demonstrate relative resistance to the hemlock woolly adelgid. We compared concentrations of terpenoids in susceptible and relatively resistant trees, both in the forest and in propagated cuttings in a common-garden setting. Terpenoid concentrations were

higher in twig tissue of resistant versus susceptible trees, across six sampling dates and at both sites. Because the common-garden cuttings were free of herbivores, the higher terpenoid concentrations are interpreted as a constitutive defense. Increased levels of monoterpenes and sesquiterpenes imply an overall increase in the input of carbon precursors to both terpenoid synthesis pathways. This result suggests either an altered growth-defense balance favoring allocation of carbon resources towards production of defenses, or overall greater carbon availability in growing twig tissue of adelgid-resistant eastern hemlock individuals.

We contribute detailed terpenoid data to the study of the eastern hemlock – hemlock woolly adelgid system. Our solvent extraction method permits us to examine needle and twig tissues separately, capture minor components at low concentrations, and focus on stored rather than volatilized terpenoids. By relating terpenoid concentrations to insect densities, we explore the relationships of tentatively defensive chemistry to insect population dynamics. The question remains which terpenoids, if any, directly affect hemlock woolly adelgid and what role phenols may play in the system.

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CHAPTER I

TERPENE RESPONSE TO ADELGID INFESTATION

Introduction

The eastern hemlock (*Tsuga canadensis*) of eastern North America is currently suffering high mortality rates from the continuing spread of hemlock woolly adelgid (*Adelges tsugae Annand*, abbreviated HWA), a sap-feeding insect introduced from Japan over five decades ago (McClure 1991, Orwig and Foster 1998, Orwig et al. 2002). HWA population density on a given host tree follows a two year boom-and-bust cycle, attributed to a pattern of tree health declines and recoveries affecting nutrient availability to each generation of HWA (McClure 1991). The cycle typically culminates in hemlock mortality after four years (McClure 1991), although at the northern extent of HWA invasion, infested trees have been observed to survive over ten years in reduced health (Orwig and Foster 1998, Paradis et al. 2008). The rapid decline of eastern hemlocks upon HWA infestation has led to recent research on the mechanism by which HWA infestation leads to host mortality in this species (Radville et al. 2011, Domec et al. 2013). Evidence of systemic defense signaling (Pezet et al. 2013) suggests that eastern hemlock attempts an induced defense response to HWA. Failure of induced defenses may contribute to tree health decline via expenditure of carbon resources (Bonello et al. 2006) and alteration of xylem structure due to defensive release of phenolic compounds (Puritch 1977, Domec et al. 2013).

Like most conifers, hemlocks deter herbivores using constitutive defenses, in the form of resin cells and polyphenolic parenchyma cells in the secondary phloem and resin canals in the needles (Hudgins et al. 2004, Lagalante et al. 2006); and induced defenses, in the form of traumatic resin ducts in the xylem and secondary resin production (Hudgins et al. 2004). Resin cells and canals produce and store oleoresin, which is primarily composed of volatile mono- and

sesquiterpenes serving as toxins or signals and viscous diterpene acids that seal wounded tissue upon exposure to air (Trapp and Croteau 2001). Polyphenolic parenchyma cells produce toxic phenolic compounds (Franceschi et al. 2005). Previous studies of terpene chemistry in eastern hemlock identified 43 terpenoids in the needle headspace, of which 28 were present as >0.10% of total volatile content (Lagalante and Montgomery 2003). HWA infestation increased the emission rate of monoterpenes, but not the stored concentration of monoterpenes (Broeckling and Salom 2003, Pezet et al. 2013). HWA infestation also increased the stored concentration of the phenolic compound benzyl alcohol by over five-fold (Pezet et al. 2013). Additionally, methyl salicylate, a key hormone in plant responses to pathogens and sessile herbivores (Vlot et al. 2009, Wu and Baldwin 2010), increased by 10- to 80-fold under HWA infestation (Pezet et al. 2013), strongly suggesting that eastern hemlock responds to HWA with systemic induced defenses.

A uniting hypothesis for the mechanism of hemlock death, proposed with variations by Gomez et al. (2012) and Domec et al. (2013), describes a xylem-altering defense response, reduced conductance of water to foliage, and hemlock mortality due to the resulting carbon deficit. In summary, HWA infestation triggers abnormal xylem development of false rings (Gonda-King et al. 2012), possibly via a systemic hypersensitive response (Radville et al. 2011) and related signaling pathways (Wu and Baldwin 2010). Similar abnormal and detrimental xylem development has been described in balsam fir (*Abies balsamea*) in response to feeding on the stem bark by balsam woolly adelgid (*Adelges piceae*), with xylem distortion triggered by an apparently defensive release of phenols in the wood (Balch et al. 1964, Puritch 1977, Domec et al. 2013). In eastern hemlock, as in balsam fir, xylem and root hydraulic conductance are reduced, leading to water stress and restricted photosynthesis (Domec et al. 2013). To compensate with increased investment in photosynthetic machinery and to mobilize nutrients out of infested tissues, hemlock moves nitrogen to the new growth of needles (Domec et al. 2013, Gomez et al. 2012). HWA's second generation each year moves forward to feed on the new growth, apparently

depleting the mobilized nutrients (Gomez et al. 2012). Domec et al. (2013) reported a decline in tree water use by over 40% and a reduction in gross primary productivity by 25% in infested hemlocks. Shortage of photosynthate accounts for the observed cessation of growth and decline of stored terpene concentrations in heavily infested hemlocks (McClure 1991, Miller-Pierce et al. 2010, Pezet et al. 2013), as respiration would be prioritized over growth or costly defenses (Domec et al. 2013, McDowell 2011, Paré and Tumlinson 1999). The resulting lack of defense could be interpreted as a case of "induced susceptibility" (Bonello et al. 2006).

In this study, we contribute detailed terpene chemistry data to the portrait of HWA-infested eastern hemlocks. Within the framework of a two-year study of HWA population dynamics (2011-2012), we measured concentrations of terpenoids stored in hemlock needles and twigs in September 2012 for eastern hemlocks in a natural forest setting and a plantation setting. We examine relationships of HWA treatment, density, and survivorship with terpenoid concentrations, while considering tree age, within-site location, and environmental setting as potential covariates. By combining detailed HWA population density records with our tissue-specific terpene concentration data, we hope to provide new insights on the immediate and multi-year effects of HWA on its host's carbon resources and defense responses.

Materials and Methods

Experimental Design

A 2x3 factorial design of tree age and infestation duration was established in mixed hemlock-hardwood forest (Quabbin Reservoir protected land, Pelham, MA), assigning 96 initially uninfested hemlocks to 16 blocks for replication. For tree age, each block contained 3 saplings (height < 2 m, whole sapling infested) and 3 mature trees (height > 3 m, one branch infested per tree). One tree of each age category was assigned to each infestation duration treatment: "control," "new," and "previous." The 2012 "control" trees were identified as HWA-free in 2011

and replaced with clean trees as needed in 2012. The 2012 "new" trees were initially clean trees, artificially infested in April 2012. The 2012 "previous" infestation trees were initially clean trees, artificially infested in April 2011, and artificially re-infested in April 2012 to imitate natural infestation patterns. In addition, each experimental tree was assigned to a treatment group for initial density of artificial infestation by varying the number of heavily infested twigs applied to the mature branch or sapling (1, 3, 10, or 30 twigs). From 2011-2012, Sussky (2013) recorded the infestation history of each tree over four generations of HWA to construct a life history table, from which the 2012 data are used here to relate terpene chemistry to infestation history.

Site Comparison

To compare site-related effects, a plantation site (South Deerfield, MA) with full sun and amended soil was included in addition to the forest site (Sussky 2013). In 2007, 130 uninfested saplings at 1-meter height were transplanted and established in a 1.5-meter spaced grid. In April 2012, 16 saplings were assigned as controls and 16 were newly infested by the above method. Light intensity was measured at both sites in July 2013, using a WatchDog data logger and light meter (Spectrum Technologies, Inc., Aurora, IL) to record intensity of photosynthetically active radiation (PAR, photons m^{-2} s^{-1}). PAR was recorded by hand between 11am-1pm on days with no cloud cover. Three measurements above the outermost foliage of 8 saplings at each site were averaged to calculate site means. Temperature was tracked in July and August 2012, using iButtons (Embedded Data Systems, Inc., San Jose, CA) to automatically record air temperature every 2 hours.

Extraction of terpenes from plant material

Hemlock tissue samples were collected in September 2012 by collecting the current-year and previous-year flushes of growth from 10-15 tips scattered over one mature branch or sapling tree. Samples were promptly flash-frozen in liquid nitrogen and transported on dry ice until storage at -80°C in the laboratory.

In each sample, current-year and previous-year growth were separated with scissors. From each growth sample, a total of 18 cm of growth was selected at random for extraction. HWA on the sample were counted (HWA/cm). Needles were separated from twigs with tweezers. Approximately 1 mL volume of needles, or ca. thirty needles, were selected at random and placed in a pre-weighed vial. Twigs were ground under liquid nitrogen and placed in a separate pre-weighed vial. Tissue dry weight was determined following extractions and two to six weeks in a 75°C drying oven. This procedure yielded four vials per sample: current-year needles, current-year twigs, previous-year needles, and previous-year twigs.

One milliliter of methyl tert-butyl ether (MTBE) was applied to each vial as an extraction solvent. Tissue was extracted for 16-19 hours with continuous agitation. Extracts were treated with 0.3 mL of 0.1 M aqueous ammonium carbonate, filtered on silica gel, activated carbon, and magnesium sulfate (3:1:2 ratio), and eluted with 0.5 mL hexanes. Filtered eluates were stored at -20°C in glass vials capped with PTFE/silicone septa.

Quantification of terpene compounds

Terpene compounds in samples were quantified by gas chromatography with flame ion detection (Hewlett-Packard Agilent 6890, running Agilent ChemStation software). Separations were performed on an Agilent HP-5 capillary column, non-polar with crosslinked 5% phenyl / 95% methyl siloxane, 0.25 µm film thickness, 0.32 mm diameter, 30 m length. The column was trimmed by 8 cm during previous use. The helium carrier gas was in constant flow mode at 2.2 mL min-1 and average velocity 36 cm sec-1; sample was injected with split ratio of 3:1, split flow of 6.5 mL min-1, and total flow of 11.1 mL min-1. The injection volume was 1 µL at inlet temperature of 250°C. The GC oven temperature was programmed to start at 60°C and rise to 158°C with holds at 64°C, 100°C, and 126°C to improve separation of compounds, followed by burn-off at 200°C. The flame-ion detector was set at 300°C, with hydrogen flow at 30 mL min^{-1},

air flow at 300 mL min^{-1}, and nitrogen makeup flow at 25 mL min^{-1}. The detector began data collection after 3 minutes of solvent cut-time.

Raw quantity (pA sec) was calculated for each terpene by software integration of peak area on the chromatogram (Hewlett-Packard ChemWare). Concentration (μg mL^{-1}) was calculated using experimentally-determined calibration curves. For terpenoids with no commercial standard, averaged calibration curves of structurally similar terpenoids were used. Tissue concentration (μg g^{-1} dry weight) of each terpene was calculated by dividing terpenoid concentration by the sample's tissue dry weight.

Compounds were identified based on previous work (Pezet et al. 2013), retention time comparison to analytical standards on the GC-FID, and comparison to chromatograms from gas chromatography-mass spectrometry on select samples (GCMS; Shimadzu GC-2010 and GCMS-QP2010 Plus with HP-5 column). References for mass spectrometry included a software library (Stein 2005), published reference (Adams 2009), and analytical standards.

While previous research has described monoterpenes, sesquiterpenes, small phenolics, and green leaf volatiles in eastern hemlock (Pezet et al. 2013; Lagalante and Montgomery 2003), the present method captured only mono- and sesquiterpenes and their derivatives. Using analytical standards, we determined that our filtration step almost completely removed the phenolics benzyl alcohol and methyl salicylate. While quantification of these compounds was desirable, the sample preparation required for the available instrumentation prevented quantification.

Statistical analyses

All statistical analyses were conducted using R (version 2.15.2, R Development Core Team 2012). The four tissue types sampled (current-year needles, previous-year needles, current-year twigs, and previous-year twigs) were analyzed separately. Prior to analysis, outliers were identified and removed. Univariate outliers were defined as being over four standard deviations

away from the mean in any one compound, and multivariate outliers were over 10x further from the centroid than 90% of the observations, based on Mahalanobis distance (McCune and Grace 2002). Compounds were removed from the analysis as insufficiently sampled if they were undetected in more than 20% of samples or if the raw quantity median was 10 pA sec or less. The resulting datasets contained twenty-two compounds, of which ten were present in twigs and twenty-one present in needles (Table 1). Two more variables were added, "total monoterpenes" and "total sesquiterpenes," representing the sum of concentrations of the fourteen monoterpenes and six sesquiterpenes in each sample, respectively. A logarithmic transformation of ($\log_e(x+1)$) was applied to all concentration data and all HWA density data to improve normality.

To test for effects of HWA treatment (control / 6 month HWA / 18 month HWA) on terpenoid concentrations in the forest site, multivariate analysis of covariance (MANCOVA) was performed with tree age and block as covariates. As the covariates were not found to be significant, analysis of variance (ANOVA) was performed to identify individual terpenoid compounds affected by HWA treatment. To test for relationships of terpenoid concentration to HWA density and tree health in the forest site, terpenoid concentrations were compared in linear regression against HWA density, rate of HWA survivorship, and rate of new growth among branch tips (Sussky 2013). For site effects, terpenoid concentrations in the control saplings at the forest site and plantation site were compared by MANOVA and subsequent ANOVA.

Results

Terpene concentrations in HWA-infested and control trees: Forest Site

A trend of lower concentrations of terpenoids in HWA-infested tissue was observed across tissue types and growth flushes (Table 1). In needles, total concentration of monoterpenes and total concentration of sesquiterpenes were lower in all HWA-infested needles than in controls (ANOVA; $p<0.05$; Figure 1). Concentrations of 10/13 monoterpenes and 5/6 sesquiterpenes were

significantly lower in needles of HWA-infested trees (ANOVA; $p<0.05$; Table 1). In twigs, a multivariate pattern of lower terpenoid concentrations in infested tissue was observed in both current-year and previous-year growth (MANOVA, $p<0.05$); however, total monoterpene and total sesquiterpene concentrations in twigs did not differ significantly between treatments (ANOVA, Figure 1). Concentrations of only 1/7 monoterpenes and 0/3 sesquiterpenes were significantly lower in twigs of HWA-infested trees (ANOVA; $p<0.05$; Table 1).

Tree age and block designation, representing location within the forest site, were not significant covariates for any individual terpenoid compound (MANCOVA; age: $0.074<p<0.881$, block: $0.058<p<0.934$).

Site-related effects on terpene concentrations: Forest Site vs. Plantation Site

Forest saplings contained significantly higher total concentrations of monoterpenes and of sesquiterpenes than their plantation counterparts, in both needles and twigs (ANOVA; $p<0.05$; Figure 2). In needles, 11/13 monoterpenes and 4/6 sesquiterpenes analyzed were present at significantly higher concentrations in forest saplings (ANOVA; $p<0.05$; Table 2). In twigs, 2/7 monoterpenes and 2/3 sesquiterpenes analyzed were present at significantly higher concentrations in forest saplings (ANOVA; $p<0.01$; Table 2); however, 3/7 monoterpenes were present at significantly lower concentrations in forest saplings (ANOVA; $p<0.01$; Table 2). Block designation within each site did not explain a significant part of variance in concentration of terpenoids (MANCOVA).

Temperature data showed lower summer temperatures at the forest than at the plantation in July and August 2012. The forest site was cooler by 2.9°C in average daily temperature, by 5.8°C in average daily maximum temperature, and by 4.7°C in absolute maximum temperature in July and August. Light availability data showed a 10-fold lower intensity of photosynthetically active radiation (PAR) for understory forest saplings than for exposed plantation saplings.

Terpene concentrations and HWA density: Forest Site

HWA density on samples from artificially infested trees ranged from 0.05 to 19.67 insects per cm for sistens nymphs on current growth and 0.11 to 3.39 insects per cm for progrediens adults on previous growth. Control trees were excluded from the density analysis. A logarithmic transformation of ($\log_e(x+1)$) was applied to all concentration data and all HWA density data to improve normality prior to linear regression.

Density of HWA nymphs exhibited a trend of non-significant negative linear relationships with terpenoid concentrations, except in previous-growth needles where the relationships are significantly positive. In twigs, a significant negative linear relationship of concentration with HWA density existed in total sesquiterpenoids in current-growth twigs with 18 months of HWA infestation (linear regression; $p<0.01$, $R^2=0.38$; Table 3). The total sesquiterpene relationship was marginally significant and negative in previous-growth twigs with HWA infestation (linear regression; Table 3). No significant linear relationships were detected in monoterpenes in twigs.

In current-growth needles, significant negative linear relationships of concentration with HWA density existed in 18-month infested needles in 2/13 monoterpenes and 2/6 sesquiterpenes (linear regression; $p<0.05$, $0.17<R^2<0.63$; Table 3). In 6-month infested needles, the relationship appeared negative but was non-significant in total monoterpenes and total sesquiterpenes (linear regression; Table 3). By contrast, in 18-month infested previous-growth needles, positive relationships with HWA density were found in 7/13 monoterpenes and 2/6 sesquiterpenes (linear regression; monoterpenes, $p<0.05$ and $0.11<R^2<0.27$; sesquiterpenes, $p<0.05$ and $0.13<R^2<0.14$; Table 3).

Terpene concentrations and HWA rate of survivorship: Forest Site

Rate of survivorship of sistens nymphs across aestivation likewise exhibited negative linear relationships with terpenoid concentrations, where significant relationships could be

detected. In needles, a significant negative linear relationship to survivorship was observed in total monoterpenes in current-year needles with 18-month HWA infestations (linear regression; $p=0.02$, $R^2=0.24$; Table 4). In addition, 5/13 monoterpenes and 4/6 sesquiterpenes in these needles exhibited significant or marginally significant negative linear relationships with survivorship (linear regression; $p<0.10$, $0.11<R^2<0.28$; Table 4). In twigs, significant negative relationships with terpenoids in current-year and previous-year growth were observed, but these were distributed across terpene compounds and growth flushes, and non-significant positive relationships were also present (linear regression; Table 4).

Terpene concentrations and hemlock rate of new growth production: Forest Site

Proportion of hemlock tips producing new growth exhibited a trend of negative linear relationships to terpenoids in twigs and positive linear relationships to terpenoids in needles. In current- and previous-growth twigs, 2/3 sesquiterpenes exhibited significant negative linear relationships to new growth production (linear regression; $p<0.05$, $0.10<R^2<0.25$; Table 5). In current-growth needles with 6 months of HWA infestation, 3/13 monoterpenes exhibited significant positive linear relationships to new growth production (linear regression; $p<0.05$, $0.10<R^2<0.15$; Table 5). Current-growth needles with 18-month infestation and previous growth needles displayed non-significant negative linear relationships to new growth production (linear regression; Table 5).

Discussion

The observed decrease in terpenoid concentration following both 6-month and 18-month infestations by HWA (Figure 1) suggests that eastern hemlocks not only fail to induce production of mono- and sesquiterpenoids in response to HWA infestation, but become less able to produce carbon-based defenses when infested with HWA. HWA's consumption of sugars and other carbon compounds from sap in the phloem likely contributes to this effect (Young et al. 1995),

but the HWA-induced false ring structures in the xylem may also be responsible for reduced water conductance to photosynthetic tissues (Gonda-King et al. 2012, Domec et al. 2013). If the tree's carbon is being consumed through herbivory and its ability to capture carbon through photosynthesis is compromised, then less carbon would be available for production of growth and carbon-based defenses such as terpenoids (McDowell 2011).

Terpenoid concentrations differed more between sites than between HWA infested and control trees (Figure 2), suggesting that light intensity and temperature differences between sites are highly important in determining terpenoid content. Light intensity may drive photosynthetic assimilation of carbon, resulting in the carbon availability that provides precursors for terpenoid production as well as nutrition for HWA.

Even in ideal conditions, plants are expected to balance resource investments between production of defenses and growth (Herms and Mattson 1992). This may explain the observed lower terpenoid content of plantation saplings (Figure 2). Saplings exposed to full sunlight are growing at a greater rate than shaded forest saplings; thus, they must support growth by directing carbon resources away from defense. In addition, the same raw quantity of terpenoids would become "diluted" in the greater tissue mass produced by fast-growing trees.

Under a carbon deficit, the need to balance resources results in plants ceasing growth and production of defenses altogether to reserve carbon for respiration, resulting in the case of "induced susceptibility" (Bonello et al. 2006). HWA infestation is believed to cause carbon deficit in eastern hemlock by inducing false rings that restrict water flow to foliage and reduce gross primary productivity by an estimated 25% (Gonda-King et al. 2012, Domec et al. 2013). Thus, our HWA-infested trees are expected to experience carbon deficit, and to produce a combination of reduced growth and reduced terpenoid content. This is consistent with reported reduced growth in HWA-infested eastern hemlocks (McClure 1991) and the trend of positive linear relationships between concentrations of terpenoids in needles and proportion of branch tips

producing new growth (Table 5). The opposite negative relationship in twigs may be attributed to faster growing twigs possessing greater dry weight, "diluting" the concentration of terpenoids. "Dilution" in tissue mass is not an important effect in needles of fast-growing tissue because needles do not increase in girth as twigs do in high-sunlight conditions (EA McKenzie, unpublished results comparing mass of needles and twigs in sun and shade conditions).

Density of springtime HWA adults displayed a positive linear relationship with autumn terpenoid concentrations in the previous-growth tissue that they fed on (Table 3; 18-month infested trees). Subsequently, on the same 18-month infested trees, autumn HWA nymph survivorship displayed a negative linear relationship with autumn terpenoid concentrations in the current-growth tissue that this generation of HWA fed on (Table 4). Tentatively, these findings may suggest that trees infested for 18 months do increase terpenoid concentrations in response to springtime HWA densities, serving to reduce HWA densities in the autumn generation. However, with no replication across years or seasons, and no investigation of the direct effects of terpenoid compounds on HWA, it is not possible to draw a firm conclusion.

Future research on hemlock defense chemistry should target phenols, which were not captured by the methods of this study but which are known to be produced in hemlock by polyphenolic parenchyma cells in the secondary phloem (Hudgins et al. 2004). Pezet et al. (2013) found significant increases under HWA infestation in several low-molecular-weight, volatile phenols, so other phenols may be active as well. Phenols are also of special interest because abnormal xylem development leading to water stress in balsam fir (*Abies balsamea*) is believed to be triggered by release of phenols in the wood in response to feeding by balsam woolly adelgid (*Adelges piceae*; Balch et al. 1964, Puritch 1977, Domec et al. 2013). The key question remains whether eastern hemlock possesses defense chemistry that affects HWA's ability to survive and reproduce. Our findings emphasize the importance of light availability and overall tree health in determining both terpenoid concentration and HWA population.

Tables

Table I.1: Difference in concentrations of terpenoid compounds in HWA infestation duration treatments relative to controls. Values indicate a fold change in concentration, calculated by dividing each compound's concentration in the treatment by concentration in the control. Increased concentrations are shaded in dark gray, decreased concentrations in light gray, and no difference (0.95- to 1.05-fold difference) unshaded. Significance for ANOVA of log concentration is indicated by circle and italics for 0.10 alpha level, asterisk and bold italics for 0.05 alpha level.

	Twig Terpenoids			***Needle Terpenoids***			
	Current growth		***Previous growth***	***Current growth***		***Previous growth***	
	6mo. HWA	18mo. HWA	18mo. HWA	6mo. HWA	18mo. HWA	18mo. HWA	
Monoterpenoids							***Monoterpenoids***
Tricyclene	1.05	1.56	N/A	**_0.82*_**	_0.82°_	**_0.72*_**	Tricyclene
a-Pinene	**_0.75*_**	_0.87°_	_0.54°_	**_0.80*_**	**_0.82*_**	**_0.72*_**	a-Pinene
Camphene	0.92	1.15	N/A	**_0.80*_**	_0.85°_	**_0.75*_**	Camphene
				_0.77*_	**_0.76*_**	**_0.66*_**	Sabinene
B-Pinene	0.73	0.82	N/A	**_0.77*_**	**_0.71*_**	**_0.64*_**	B-Pinene
Myrcene	0.91	0.87	0.55	_0.81°_	0.81	0.84	Myrcene
				_0.71*_	**_0.68*_**	**_0.62*_**	a-Phellandrene
				0.96	0.85	**_0.73*_**	p-Cymene
Limonene	0.98	1.00	0.85	**_0.76*_**	**_0.77*_**	**_0.74*_**	Limonene
				1.06	0.89	0.77	Eucalyptol
				0.82	0.76	0.78	Camphor
				_0.75*_	0.78	_0.78°_	Piperitone
Bornyl Acetate	0.98	1.09	0.29	**_0.83*_**	_0.85°_	**_0.79*_**	Bornyl Acetate
Total monoterp.	0.85	0.89	0.65	**_0.81*_**	**_0.83*_**	**_0.76*_**	Total monoterp.
Sesquiterpenoids							***Sesquiterpenoids***
B-Caryophyllene	0.83	1.00	0.87	**_0.86*_**	0.87	**_0.71*_**	B-Caryophyllene
a-Humulene	0.85	1.04	0.95	_0.86°_	0.88	**_0.71*_**	a-Humulene
				0.89	0.86	**_0.69*_**	y-Muurolene
Germacrene D	0.80	0.83	_0.62°_	_0.53°_	0.49	_0.37°_	Germacrene D
				0.92	0.98	**_0.79*_**	y-Cadinene
				_0.89°_	0.95	**_0.76*_**	d-Cadinene
Total sesquiterp.	0.81	0.89	0.76	**_0.83*_**	**_0.84*_**	**_0.70*_**	Total sesquiterp.
Unidentified							***Unidentified***
				0.94	0.93	**_0.82*_**	Unknown A
				1.15	0.95	0.81	Unknown B (Ac.)

Table I.2: Difference in concentrations of terpenoid compounds in forest saplings relative to plantation saplings. Values indicate a fold change in concentration, calculated by dividing each compound's concentration in the treatment by concentration in the control. Increased concentrations are shaded in dark gray, decreased concentrations in light gray, and no difference (0.95- to 1.05-fold difference) unshaded. Significance for ANOVA of log concentration is indicated by asterisk and bold italics for 0.05 alpha level.

	Twig Terpenoids	***Needle Terpenoids***	
	Current growth	***Current growth***	
Monoterpenoids			***Monoterpenoids***
Tricyclene	***0.23****	***2.68****	Tricyclene
a-Pinene	0.90	***2.42****	a-Pinene
Camphene	***0.37****	***2.87****	Camphene
		4.45*	Sabinene
B-Pinene	0.67	***1.99****	B-Pinene
Myrcene	***5.07****	***2.47****	Myrcene
		2.08*	a-Phellandrene
		1.63*	p-Cymene
Limonene	***1.33****	***2.12****	Limonene
		3.27	Eucalyptol
		0.94	Camphor
		2.17*	Piperitone
Bornyl Acetate	***0.45****	***2.83****	Bornyl Acetate
Total monoterp.	***1.44****	***2.60****	Total monoterp.
Sesquiterpenoids			***Sesquiterpenoids***
B-Caryophyllene	***2.86****	***1.34****	B-Caryophyllene
a-Humulene	***2.36****	***1.32****	a-Humulene
		1.51	y-Muurolene
Germacrene D	8.32	6.07	Germacrene D
		1.92*	y-Cadinene
		1.85*	d-Cadinene
Total sesquiterp.	***4.69****	***1.54****	Total sesquiterp.
Unidentified			***Unidentified***
		1.18*	Unknown A
		1.56*	Unknown B (Ac.)

Table I.3: Best-fit slopes from linear regression of terpenoid concentrations against HWA density on samples (Sept.). HWA were counted on the sampled tissue prior to extraction. On current growth, autumn sistens nymphs exiting aestivation were counted. On previous growth, dead spring progrediens adults were counted. Natural log of HWA per centimeter was regressed against natural log of concentration of each terpenoid. Positive slopes are shaded in dark gray, negative slopes in light gray, and zero slopes unshaded. Significance for ANOVA of log concentration is indicated by circle and italics for 0.10 alpha level, asterisk and bold italics for 0.05 alpha level. R-squared is reported for significant regressions.

	Twig Terpenoids			***Needle Terpenoids***			
	Current growth		***Previous growth***	***Current growth***		***Previous growth***	
	6mo. HWA	18mo. HWA	18mo. HWA	6mo. HWA	18mo. HWA	18mo. HWA	
Monoterpenoids							***Monoterpenoids***
Tricyclene	0.72	0.39	N/A	-0.21	-0.03	***0.23**** R^2=0.14	Tricyclene
a-Pinene	0.04	-0.51	1.07	-0.17	-0.07	***0.24**** R^2=0.17	a-Pinene
Camphene	-0.63	-0.94	N/A	-0.16	-0.03	***0.23**** R^2=0.16	Camphene
				-0.18	-0.09	***0.30**** R^2=0.15	Sabinene
B-Pinene	-0.65	-2.42	N/A	-0.18	-0.08	***0.30**** R^2=0.18	B-Pinene
Myrcene	0.20	-0.76	-0.81	-0.31	-0.10	0.09	Myrcene
				-0.13	-0.24	*0.24°* R^2=0.11	a-Phellandrene
				-0.33	***-0.32**** R^2=0.63	0.06	p-Cymene
Limonene	0.10	-0.19	0.76	-0.09	-0.19	***0.32**** R^2=0.27	Limonene
				0.90	0.47	0.18	Eucalyptol
				-0.07	0.00	0.17	Camphor
				-0.11	***-0.37**** R^2=0.21	0.08	Piperitone
Bornyl Acetate	-0.89	-0.25	1.31	-0.13	-0.06	***0.18**** R^2=0.13	Bornyl Acetate
Total monoterp.	0.11	-0.45	-0.08	-0.14	-0.07	***0.19**** R^2=0.15	Total monoterp.
Sesquiterpenoids							***Sesquiterpenoids***
B-Caryophyllene	0.11	*-1.08°* R^2=0.14	-1.24	-0.05	-0.01	***0.23**** R^2=0.13	B-Caryophyllene
a-Humulene	0.10	-0.72	-0.64	-0.06	-0.00	***0.23**** R^2=0.14	a-Humulene
				0.14	***-0.30**** R^2=0.46	0.01	y-Muurolene
Germacrene D	0.91	-3.26	-0.79	0.62	-0.68	0.17	Germacrene D
				0.07	***-0.21**** R^2=0.22	0.06	y-Cadinene
				0.08	*-0.20°* R^2=0.17	0.06	d-Cadinene
Total sesquiterp.	0.39	***-1.57**** R^2=0.38	*-0.83°* R^2=0.08	0.00	-0.07	*0.17°* R^2=0.09	Total sesquiterp.
Unidentified							***Unidentified***
				0.04	***-0.25**** R^2=0.47	0.00	Unknown A
				0.06	1.42	-1.43	Unknown B (Ac.)

Table I.4: Best-fit slopes from linear regression of terpenoid concentrations against HWA rate of survivorship (Aug. to Nov.). Survivorship was calculated as final density of live nymphs divided by initial density of live nymphs. Natural log of survivorship was regressed against concentration of each terpenoid. Positive slopes are shaded in dark gray, negative slopes in light gray, and zero slopes unshaded. Significance for ANOVA of log concentration is indicated by circle and italics for 0.10 alpha level, asterisk and bold italics for 0.05 alpha level. R-squared is reported for significant regressions.

	Twig Terpenoids			***Needle Terpenoids***			
	Current growth		***Previous growth***	***Current growth***		***Previous growth***	
	6mo. HWA	18mo. HWA	18mo. HWA	6mo. HWA	18mo. HWA	18mo. HWA	
Monoterpenoids							***Monoterpenoids***
Tricyclene	-0.45	0.59	N/A	0.05	-0.08	-0.01	Tricyclene
a-Pinene	-0.02	-0.13	***-1.15**** R^2=0.12	0.05	***-0.12**** R^2=0.21	-0.02	a-Pinene
Camphene	0.71	0.58	N/A	0.08	***-0.15**** R^2=0.28	-0.03	Camphene
				0.04	*-0.11°* R^2=0.15	-0.01	Sabinene
B-Pinene	-0.03	-0.50	N/A	0.03	*-0.14°* R^2=0.16	-0.02	B-Pinene
Myrcene	0.06	-0.15	0.06	0.11	-0.11	-0.09	Myrcene
				0.01	-0.14	0.00	a-Phellandrene
				-0.04	-0.03	0.01	p-Cymene
Limonene	0.03	***-0.12**** R^2=0.22	0.03	0.02	-0.16	-0.03	Limonene
				-0.57	0.51	0.61	Eucalyptol
				0.19	-0.12	-0.02	Camphor
				0.08	*-0.17°* R^2=0.11	-0.11	Piperitone
Bornyl Acetate	-0.07	0.07	*1.03°* R^2=0.10	0.07	-0.13	-0.04	Bornyl Acetate
Total monoterp.	0.03	-0.10	-0.30	0.07	***-0.13**** R^2=0.24	-0.03	Total monoterp.
Sesquiterpenoids							***Sesquiterpenoids***
B-Caryophyllene	-0.10	-0.21	***-1.34**** R^2=0.23	0.03	*-0.11°* R^2=0.11	0.00	B-Caryophyllene
a-Humulene	-0.05	-0.15	-0.27	0.03	*-0.12°* R^2=0.12	0.00	a-Humulene
				-0.06	0.00	0.01	y-Muurolene
Germacrene D	*-1.41°* R^2=0.07	0.47	0.43	-0.98	0.04	0.62	Germacrene D
				-0.05	***-0.12**** R^2=0.20	-0.02	y-Cadinene
				-0.07	***-0.13**** R^2=0.23	-0.02	d-Cadinene
Total sesquiterp.	*-0.26°* R^2=0.07	-0.15	-0.34	0.00	-0.10	-0.01	Total sesquiterp.
Unidentified							***Unidentified***
				0.04	-0.04	-0.02	Unknown A
				-0.28	-0.38	-0.88	Unknown B (Ac.)

Table I.5: Best-fit slopes from linear regression of terpenoid concentrations against hemlock proportion new growth (Nov.). Branch tips were counted in November 2012 and scored for presence of new growth. Proportion of tips producing new growth was regressed against concentration of each terpenoid. Positive slopes are shaded in dark gray, negative slopes in light gray, and zero slopes unshaded. Significance for ANOVA of log concentration is indicated by circle and italics for 0.10 alpha level, asterisk and bold italics for 0.05 alpha level. R-squared is reported for significant regressions.

	Twig Terpenoids			*Needle Terpenoids*			
	Current growth		*Previous growth*	*Current growth*		*Previous growth*	
	6mo. HWA	18mo. HWA	18mo. HWA	6mo. HWA	18mo. HWA	18mo. HWA	
Monoterpenoids							***Monoterpenoids***
Tricyclene	-0.31	-0.09	N/A	0.18	-0.05	-0.01	Tricyclene
a-Pinene	-0.13	-0.28	0.52	0.18	-0.04	0.01	a-Pinene
Camphene	-0.06	-0.13	N/A	0.19	-0.01	-0.01	Camphene
				0.23	-0.03	*0.23°* R^2=0.07	Sabinene
B-Pinene	*-1.08°* R^2=0.07	*-1.65°* R^2=0.07	N/A	***0.33***** R^2=0.10	0.04	0.10	B-Pinene
Myrcene	-0.04	-0.34	N/A	0.25	0.21	-0.02	Myrcene
				0.48** R^2=0.14	0.14	0.21	a-Phellandrene
				0.57	-0.12	0.08	p-Cymene
Limonene	-0.20	-0.04	0.11	***0.38***** R^2=0.15	0.13	0.13	Limonene
				0.14	0.82	-0.12	Eucalyptol
				-0.03	-0.04	-0.18	Camphor
				0.17	0.13	0.15	Piperitone
Bornyl Acetate	-1.09	-0.09	***3.08***** R^2=0.47	0.16	-0.02	-0.01	Bornyl Acetate
Total monoterp.	-0.11	-0.20	-0.22	0.19	-0.01	0.01	Total monoterp.
Sesquiterpenoids							***Sesquiterpenoids***
B-Caryophyllene	***-0.42***** R^2=0.13	***-0.61***** R^2=0.25	***-2.07***** R^2=0.22	0.01	-0.01	0.03	B-Caryophyllene
a-Humulene	***-0.31***** R^2=0.10	***-0.42***** R^2=0.20	***-0.99***** R^2=0.17	0.02	0.00	0.03	a-Humulene
				-0.12	-0.14	0.00	y-Muurolene
Germacrene D	-2.02	-0.94	-0.26	-1.41	0.23	1.09	Germacrene D
				0.04	0.00	0.04	y-Cadinene
				0.03	-0.02	0.03	d-Cadinene
Total sesquiterp.	*-0.61°* R^2=0.09	***-0.58***** R^2=0.23	***-1.16***** R^2=0.17	-0.01	-0.02	0.03	Total sesquiterp.
Unidentified							***Unidentified***
				-0.04	***-0.16***** R^2=0.27	-0.06	Unknown A
				1.45	1.52	0.28	Unknown B (Ac.)

Figures

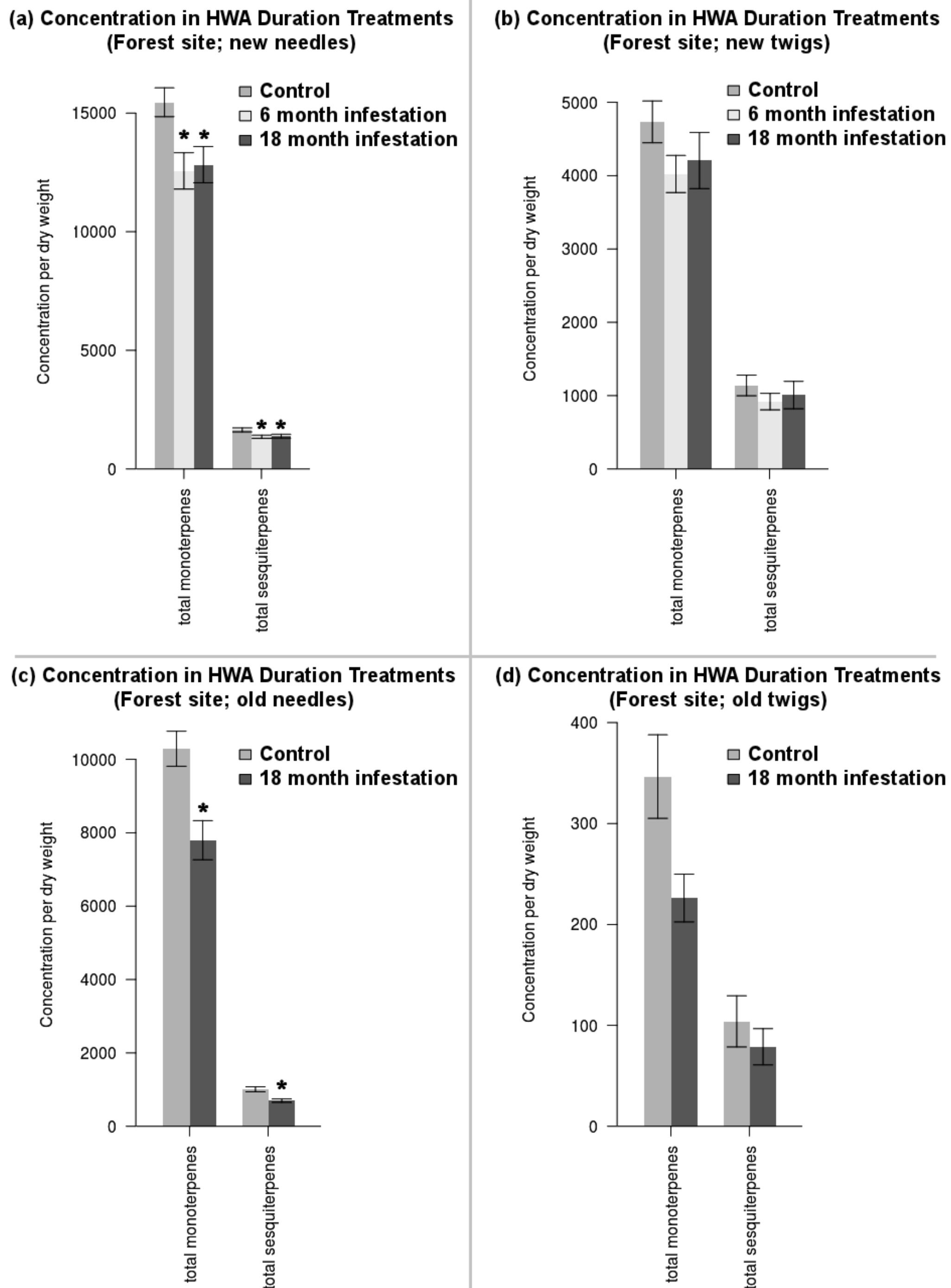

Figure I.1: Terpenoid concentrations by HWA treatment; forest site. Mean concentrations of total monoterpenes and total sesquiterpenes at the forest site (a) in current-year needles, (b) in current-year twigs, (c) in previous-year needles, and (d) in previous-year twigs. Error bars indicate standard error about the mean. Asterisk indicates significant difference from control ($p<0.05$). Concentration is reported in units of micrograms of terpenoids per gram of dry tissue.

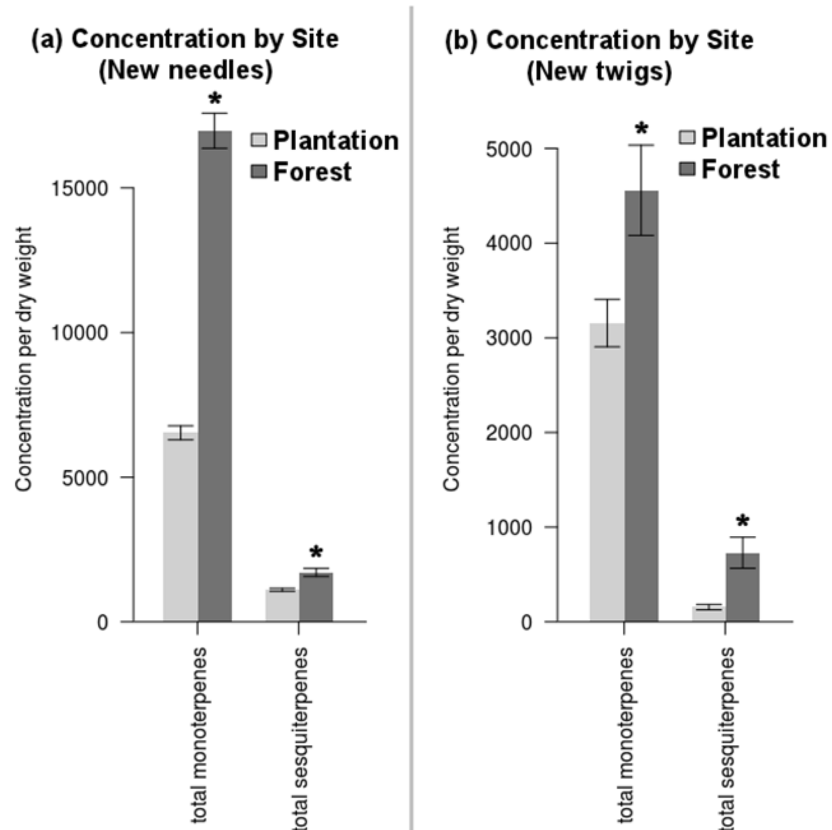

Figure I.2: Terpenoid concentrations by site. Mean concentrations of total monoterpenes and total sesquiterpenes (a) in current-growth needles of HWA-free control saplings, and (b) in current-growth twigs of HWA-free control saplings. Error bars indicate standard error about the mean. Asterisk indicates significant difference from forest site ($p<0.05$). Concentration is reported in units of micrograms of terpenoids per gram of dry tissue.

CHAPTER II

TERPENE CHEMISTRY OF EASTERN HEMLOCKS RESISTANT TO HEMLOCK WOOLLY ADELGID

Introduction

The discovery, development, or maintenance of herbivore-resistant genetic lineages can play a key role in the conservation of plant species attacked by introduced pests (Bentz et al. 2002; Burdon 2010; Ingwell and Preisser 2011; Mattson 1986; Reis et al. 2004; Schoettle et al 2012). Here we address factors that may be linked to resistance to the hemlock woolly adelgid (*Adelges tsugae* Annand, abbrev. HWA) in rare individuals of eastern hemlock (*Tsuga canadensis*). Adelges tsugae is a sessile, piercing-sucking insect introduced to the eastern United States from Japan (Havill et al. 2006; McClure 1991). While HWA causes limited damage to *Tsuga* hosts in its native range of East Asia and the American Pacific Northwest (Lagalante and Montgomery 2003; Montgomery et al. 2009; Oten et al. 2012), HWA poses a serious threat to the host species, eastern hemlock (*T. canadensis*) and Carolina hemlock (*T. caroliniana*), in its introduced range (McClure 1991; Orwig and Foster 1998; Orwig et al. 2002). Eastern and Carolina hemlock are also colonized by a second introduced piercing-sucking insect, the elongate hemlock scale ((EHS, *Fiorinia externa*), which contributes to hemlock decline, but may deter later co-infestation by HWA (Gomez et al. 2012).

HWA feeds on nutrients in the xylem ray parenchyma cells, and prefers the most recent flush of growth (McClure 1989; Young et al. 1995). Infestation by HWA has been shown to alter xylem growth and water relations in eastern hemlock (Domec et al. 2013; Gonda-King et al. 2012).This effect is associated with a hypersensitive response (Radville et al. 2011), reduced photosynthetic productivity (Domec et al. 2013), and mobilization of nitrogen to new-growth tissues (Gomez et al. 2012). In addition, infested hemlocks display a local increase in phenolic compounds (Pezet et al. 2013), and a simultaneous increase in monoterpene volatilization and

decrease in stored mono- and sesquiterpenes (Broeckling and Salom 2003; Pezet et al. 2013). A 10- to 100-fold increase in methyl salicylate, a molecule involved in the salicylic acid signaling pathway for systemic acquired resistance (Vlot et al. 2009), has also been detected in adelgid-infested eastern hemlocks (Pezet et al. 2013). In other systems, some of these responses have been linked to plant defense against herbivores or pathogens (Wu and Baldwin 2010); in eastern and Carolina hemlock, however, infestation by HWA leads to tree decline and eventual mortality (McClure 1991; Paradis 2011).

Resistance to HWA has been observed in rare individuals of eastern hemlock found growing vigorously in otherwise adelgid-devastated hemlock stands (Caswell et al. 2008; Ingwell and Preisser 2011). Heritable resistance was assessed by propagating cuttings from these trees and control (i.e., HWA-susceptible) eastern hemlocks. Once the cuttings were established, both control and resistant cuttings were inoculated with HWA and adelgid settlement and survival was assessed. Adult HWA densities were significantly lower on the resistant cuttings than on the susceptible cuttings (Ingwell and Preisser 2011), supporting the hypothesis that some rare eastern hemlock individuals possess a degree of HWA resistance, and implying that this resistance is manifest as antibiosis.

One previous study (Ingwell et al. 2009) has examined eastern hemlocks that were tentatively identified as HWA-resistant. Although potassium levels were higher in HWA-resistant versus HWA-susceptible eastern hemlocks, there were no other differences in nutritional content (Ingwell et al. 2009). Various studies have examined traits that correlate with HWA susceptibility both within and among *Tsuga* species. In Carolina hemlock, lower levels of the lipid hexacosanol may be associated with decreased HWA susceptibility (Kaur 2009). Across *Tsuga* species, thicker epicuticular wax at the point of HWA stylet insertion (Oten et al. 2012), higher levels of the terpenes alpha-pinene, alpha-humulene, beta-caryophyllene, and germacrene D, and lower levels of the terpene isobornyl acetate (Lagalante and Montgomery 2003), are associated with decreased

HWA susceptibility. A comparable degree of intraspecific variation in terpene profiles have been observed among ornamental varietals of eastern hemlock (Lagalante et al. 2007), suggesting that natural populations of eastern hemlock may also vary in their terpene profiles.

We explored one potential mechanism of HWA resistance in eastern hemlock by conducting an across-season study of terpene profiles in the identified resistant parent trees and their clonal sapling offspring, versus those of mature and sapling HWA-susceptible eastern hemlocks. Because terpenes act as toxins and semiochemicals in the complex oleoresin that serves as the primary defense of conifers against herbivory, they are likely candidates for allelochemical resistance to HWA. Phenolics, also key in conifer defense chemistry, were not addressed in this study as they appear at very low concentrations when measured by the following method of solvent extraction, filtration, and gas chromatography. Lagalante and Montgomery (2003) suggested that different terpenoids, acting either individually or in combination, may participate directly in plant resistance by serving as HWA feeding stimulants or deterrents. Alternatively, other processes in the resistant trees may influence monoterpene and sesquiterpene concentrations, so that our observed terpene chemistry would provide indirect evidence about the resistance mechanism. Our study intends to provide a thorough profile of monoterpenes and sesquiterpenes present in each season and detect both univariate and multivariate correlations to resistance status.

Materials and Methods

Resistant and control mature trees in a New Jersey forest (NJ), and resistant and control sapling trees (i.e., rooted cuttings) in a common garden at the University of Rhode Island (URI), were sampled at six intervals from May 2012 to June 2013. Solvent extraction and gas chromatography (GC) were used to measure the concentration of each identified terpene in each

tree. Terpene profiles were analyzed for concentration differences in single terpenes, as well as for multivariate differences across all terpenes present.

Study Site 1: New Jersey "Bulletproof Stand"

Previous research (Ingwell and Preisser 2011) identified eight putatively HWA-resistant eastern hemlocks growing on state-owned land adjacent to the Delaware Water Gap National Recreation Area in northern New Jersey, of which trees #1-5 have been tested for resistance by bioassay of clonal saplings (Ingwell and Preisser 2011). Trees #1-5 were sampled at all collection dates, and trees #6 and #7 were sampled beginning in October 2012 (Table 1). All of the sampled individuals are mature trees growing within a 0.25 km radius in a hemlock, white pine, and mixed hardwood forest.

Control trees were selected within a 5 km radius of the resistant trees, to control for microclimate and soil conditions, as well as genetic variation expected over longer distances. Trees in moderate to good current health, as observed by presence of current season growth, were selected to control for effects of tree health decline on terpene chemistry. To ensure that the trees were in fact HWA-susceptible, we only used trees that were infested with HWA or had evidence of needle loss due to previous infestations. We initially used five control trees for balanced replication with the five resistant trees we initially sampled. Beginning in October 2012, fifteen to twenty-one control trees were used to improve statistical power. Different control trees were used across collection dates (Table 1).

Study Site 2: University of Rhode Island Common Garden

Sapling clones of resistant trees #1-5 were established at the University of Rhode Island by propagating cuttings from the mature trees (Caswell et al. 2008; Ingwell and Preisser 2011). Cuttings of 8 cm of terminal growth were collected from the parent trees in January 2007 and rooted using a treatment of Dip-N-Grow plant hormone solution (Griffin Greenhouse Supplies, Tewksbury, Massachusetts). Saplings were established outdoors in three planting boxes, each

$3.8m^2$ and 30 cm deep, filled with a 1:1 mix of soil and compost, supplemented with soil from an established hemlock stand. Planting boxes were exposed to full sun and sheltered from wind by proximity to a low building on the south and east sides. Five saplings were successfully established from each of five parent trees, yielding twenty-five resistant saplings in total (Table 1). All saplings were free of herbivores.

For control trees, nineteen genetically individual saplings were established adjacent in the planting boxes, with equal sun exposure and no herbivores (Table 1). Ten saplings were collected from the Quabbin forest (New Salem, MA, USA) in 2009. An additional nine saplings were purchased from a Michigan nursery (Van Pine's Nursery, West Olive, MI, USA), originally grown from seed collected in Indiana County, Pennsylvania, in autumn 2009.

Extraction of Terpenes from Plant Material

Samples of hemlock tissue for chemical analysis were collected at seasonal intervals from May 2012 to June 2013 at both sites (Table 1). Each month's collection at a site was performed on a single day in the afternoon daylight hours. Each sample was collected by cutting the terminal flush of growth (current-year growth) from ten to fifteen tips scattered over two to four mature branches or circularly around one sapling tree, totaling approximately 75 cm of tissue. Samples were promptly flash-frozen in liquid nitrogen and transported on dry ice until storage at -80°C in the laboratory.

From each sample, a total of 18 cm of growth was selected at random for extraction. HWA on the sample were counted (HWA/cm), and EHS was rated categorically for density (0 = no EHS/cm, 1 = 0-1 EHS/cm, 2 = 1-10 EHS/cm, 3 = 11-100 EHS/cm). Needles were separated from twigs with tweezers. Approximately 1 mL volume of needles, or ca. thirty needles, were selected at random and placed in a pre-weighed vial. Twigs were ground under liquid nitrogen and placed in a second pre-weighed vial. Tissue dry weight was determined following extractions and two to six weeks in a 75°C drying oven.

One milliliter of methyl tert-butyl ether (MTBE) was applied to each vial as an extraction solvent. Tissue was extracted for 16-19 hours with continuous agitation. Extracts were treated with 0.3 mL of 0.1 M aqueous ammonium carbonate, filtered on silica gel, activated carbon, and magnesium sulfate (3:1:2 ratio), and eluted with 0.5 mL hexanes. Filtered eluates were stored at -20°C in glass vials capped with PTFE/silicone septa.

Quantification of Terpenoid Compounds

Terpene compounds in samples were quantified by gas chromatography with flame ion detection (Hewlett-Packard Agilent 6890, running Agilent ChemStation software). Separations were performed on an Agilent HP-5 capillary column, non-polar with crosslinked 5% phenyl / 95% methyl siloxane, 0.25 µm film thickness, 0.32 mm diameter, 30 m length. The column was trimmed by 8 cm during previous use. The helium carrier gas was in constant flow mode at 2.2 mL min^{-1} and average velocity 36 cm sec^{-1}; sample was injected with split ratio of 3:1, split flow of 6.5 mL min^{-1}, and total flow of 11.1 mL min^{-1}. The injection volume was 1 µL at inlet temperature of 250°C. The GC oven temperature was programmed to start at 60°C and rise to 158°C with holds at 64°C, 100°C, and 126°C to improve separation of compounds, followed by burn-off at 200°C. The flame-ion detector was set at 300°C, with hydrogen flow at 30 mL min^{-1}, air flow at 300 mL min^{-1}, and nitrogen makeup flow at 25 mL min^{-1}. The detector began data collection after 3 minutes of solvent cut-time.

Raw quantity (pA sec) was calculated for each terpene by software integration of peak area on the chromatogram (Hewlett-Packard ChemWare). Concentration (µg mL^{-1}) was calculated using experimentally-determined calibration curves. For terpenoids with no commercial standard, averaged calibration curves of structurally similar terpenoids were used. Tissue concentration (µg g^{-1} dry weight) of each terpene was calculated by dividing terpenoid concentration by the sample's tissue dry weight.

Compounds were identified based on previous work (Pezet et al. 2013), retention time comparison to analytical standards on the GC-FID, and comparison to chromatograms from gas chromatography-mass spectrometry on select samples (GCMS; Shimadzu GC-2010 and GCMS-QP2010 Plus with HP-5 column). References for mass spectrometry included a software library (Stein 2005), published reference (Adams 2009), and analytical standards (Sigma-Aldrich, Saint Louis, MO).

While previous research has described monoterpenes, sesquiterpenes, small phenolics, and green leaf volatiles in eastern hemlock (Lagalante and Montgomery 2003; Pezet et al. 2013), the present method captured only mono- and sesquiterpenes and their derivatives. Using analytical standards, we determined that our filtration step almost completely removed the phenolics benzyl alcohol and methyl salicylate. While quantification of these compounds was desirable, a consistent and clean filtration method was necessary for maintenance of the GC instruments over numerous samples.

Statistical Analyses

All statistical analyses were performed in R (version 2.15.2, R Development Core Team 2012). Current-year needle and twig tissue were analyzed separately, and each site was analyzed separately. Prior to analysis, outliers were identified and removed. Univariate outliers were defined as being over four standard deviations away from the mean in any one compound, and multivariate outliers were over 10x further from the centroid than 90% of the observations, based on Mahalanobis distance (McCune and Grace 2002). Compounds were removed from the analysis as insufficiently sampled if they were undetected in more than 20% of samples or if the raw quantity median was 10 pA sec or less.

The resulting datasets contained twenty-two compounds, of which ten were present in twigs (Table 2A) and twenty-one present in needles (Table 2B). Two more variables were added, "total monoterpenes" and "total sesquiterpenes," representing the sum of concentrations of the

fourteen monoterpenes and six sesquiterpenes in each sample, respectively. A logarithmic transformation of ($\log_e (x+1)$) was applied to all concentration data and all HWA density data to improve normality.

Differences between resistant and control trees in concentrations of individual terpenoid compounds were identified using repeated measures ANOVA, followed by ANOVA of each month separately to identify seasonal patterns of resistance-correlated terpene chemistry. Multivariate differences in terpene profile were identified using MANOVA within each month. To test whether insect densities of HWA and elongate hemlock scale (EHS) confounded these results, HWA density and EHS categorical density rating were included as covariates in MANCOVA.

To further clarify the degree of confounding among explanatory variables, partitioning of variance was used to produce Venn diagrams displaying the percent of total variance explained uniquely by resistance status, HWA density, and EHS categorical density; the percent of total variance jointly explained by two or all three of the explanatory variables; and the percent of total variance not explained by the explanatory variables. Prior to partitioning of variance analysis, high collinearity among some terpenes was treated by removing terpenes from analysis if they were correlated with another terpene at Pearson's $r > 0.70$. Selection of which correlated terpene to remove was based on AIC value and previous ANOVA results. Twelve compounds were retained for partitioning of variance in needle samples and seven in twig samples.

Results

Single-Terpene Differences between Resistant and Susceptible Trees

Twigs from resistant trees tended to contain higher terpenoid concentrations, with the statistical significance of this trend differing by collection month and site (Table 2A). In the URI September and December twig collections, 9 of 10 and 10 of 10 compounds, respectively, were

found at significantly higher concentrations (ranging from 1.1- to 4.2-fold) in resistant trees versus control trees (ANOVA, P<0.05, Table 2A). Similarly, in the NJ December twig collection, 5 of 10 compounds had significantly higher concentrations (1.5- to 3.5-fold) in resistant trees (ANOVA, P<0.05, Table 2A).

Monoterpenes and sesquiterpenes in twigs appear equally likely to appear at high concentrations in resistant trees (Figure 1C, D). The twig collections noted above, URI September, URI December, and NJ December, show that the total concentration of monoterpenes and total concentration of sesquiterpenes were significantly higher (1.6 and 3.1 fold, respectively) in resistant versus control trees (ANOVA, P<0.05, Table 2A). In other twig collections, total monoterpenes and total sesquiterpenes are not significantly different between resistant and control trees (ANOVA, P>0.10), although there is a consistent trend towards higher concentrations in resistant trees (Table 2A).

In needles, no consistent trend could be identified in individual or grouped terpenoid compounds. Although monoterpenes tended to appear at higher concentrations in resistant trees (Table 2B), neither total monoterpenes nor total sesquiterpenes differed significantly between resistant and control trees (ANOVA, P>0.10, Figure 1A,B). In the URI September needles collection, a trend of lower terpenoid concentrations in needles of resistant trees appeared, with 4 of 21 compounds having a significantly lower mean in resistant trees than in control trees (ANOVA, P>0.05, Table 2B). However, the trend was not repeated in other months and sites.

Repeated measures ANOVA analysis confirmed the connection between terpene concentration and resistance status. In twigs, total sesquiterpene concentration varied significantly with resistance at both sites, and total monoterpene concentration did so at the New Jersey forest site only (Table 3A). In twigs, 6 of 10 compounds varied significantly with resistance at both sites (Table 3A). In needles, results for total concentrations were inconsistent between sites. In needles,

6 of 21 compounds varied significantly with resistance at both sites (Table 3B). Terpene concentrations also varied significantly with month, especially in needles (Table 3B).

Multivariate Terpene Profiles with Resistance Status, HWA Density, and EHS Density

Resistance status explained a significant amount of variance in twig terpenoid concentrations from URI September and December collections (MANOVA, $P<0.001$ in both cases). When HWA and EHS densities were included as cofactors in the analysis, resistance status remained a significant explanatory variable in these collections. In collections of twigs and needles from other months, resistance status only explained a significant part of variance of terpenoid concentrations when HWA and EHS densities were included as cofactors; we believe this is due to interactions between HWA, EHS, and resistance status. Notably, in most months, forest site resistant trees have zero or very low density infestations of HWA and EHS, while forest site control trees have a range of infestation densities; this creates a statistical interaction among HWA, EHS, and resistance status.

The partitioning of variance analysis separately displayed the unique and confounded explanatory power of these three variables in each collection (see Online Supplement). Resistance uniquely explained between 1% to 16% of the total variance in each month's collection, with HWA uniquely explaining 2% to 44% and EHS uniquely explaining 0.5% to 34% of the total variance. Confounded variance was greatest between resistance and HWA, ranging from 0% to 23% of the total variance in each month's collection. Interactions, displayed as negative percentages, were observed between resistance and HWA in 6 of 15 collections, and between resistance and EHS in 7 of 15 collections. Residual, unexplained variance was greater than 50% of total variance in all collections except needles of NJ June 2012. Results of partitioning of variance were not notably different between needle and twig collections.

Discussion

Higher terpene levels in the twigs of HWA-resistant eastern hemlocks provide insight into possible mechanisms for observed resistance to adelgid infestation, although no evidence exists yet that the terpenes are the cause of resistance. The trend of 1.1- to 4-fold higher terpenoid concentrations in twigs of resistant trees, across all terpenoids and all seasons (Table 3A), suggests that the resistance mechanism does not rely on any changes or up-regulation within the separate biosynthesis pathways for monoterpenoids or sesquiterpenoids (Bernard-Dagan 1982), but rather in the availability of their shared precursors, dimethylallyldiphosphate (DMADP) and isopentenyldiphosphate (IDP), or total availability of carbon. Although lack of herbivory on the resistant trees could cause greater carbon availability, the fact that the herbivore-free saplings in the URI common garden display an even stronger resistant-control difference suggests that herbivory is not the cause of the observed difference. The observation of increased terpenoid concentrations in twigs but not in needles of resistant hemlock is interesting because HWA feeds on nutrients in storage and transportation cells in the twig, the xylem ray parenchyma (Franceschi et al. 2005; Young et al. 1995). Previous studies have focused on terpene chemistry and nutrient content of the needles; following Pezet et al. (2013), this study included the twigs as HWA's direct feeding site.

There are two possible and non-mutually exclusive explanations for the higher terpene concentrations in the resistant trees. First, the growth-defense balance (Herms and Mattson 1992) in the resistant hemlocks may be altered, leading these trees to allocate more carbon toward constitutive defenses. Second, the resistant hemlocks may maintain greater or more available total carbon resources than susceptible hemlocks, resulting in more carbon available for both growth and defense, leading to the observed elevated concentrations of terpenoids in resistant trees.

The URI common garden is key to this study because it provides a controlled environment that is both herbivore-free and consistent in environmental variables. We initially

considered the possibility that, in the forest site, greater herbivory on the control trees is manifesting as overall reduced carbon resources in the control trees and, correspondingly, greater growth and defense in the low-herbivory resistant trees. In addition, environmental variables such as sunlight may influence terpene levels even between branches on a single forest tree (EA McKenzie and JS Elkinton, unpublished data), likely explaining some of the noise found by partitioning of variance analysis (Online Supplement). The consistency between the NJ forest and the URI common garden in rmANOVA results (Table 3A) and concentration trend (Table 2A) strongly support the correlation of increased terpenoid concentration in twigs with HWA-resistant status.

The difference between resistant and susceptible hemlock individuals appears to be a pattern across many terpenoids in twig tissue, rather than relying on one or several specific terpenoids. In comparing resistant individuals to susceptible individuals, an overall 1.1- to 4-fold higher mean concentration of both monoterpenoid and sesquiterpenoid compounds in twig tissue was observed consistently across seasons, in twig tissue only. This suggests that resistant individuals in this study had greater carbon availability, or favored allocation of carbon broadly toward defense rather than toward growth. Future research could distinguish these two options by comparing growth rate, water usage, and carbon assimilation between resistant and susceptible eastern hemlocks, and by determining whether phenolic defenses are also increased in the resistant trees.

Without evidence of terpenoids directly affecting HWA health, we cannot conclude that the observed constitutive increase in twig terpenoid concentrations in twigs represents the resistance mechanism directly. Future research might determine the effect of specific terpenes on HWA, perhaps through induction of terpene production in susceptible eastern hemlocks or addition of terpenes to an artificial diet for HWA. The correlation of terpene concentrations with resistance status may assist individuals in developing cultivars of eastern hemlock resistant to

HWA by providing a quicker and less expensive assay for resistance than inoculation trials with the insect.

Tables

Table II.1: Collection dates and trees sampled. (a) At the New Jersey forest site (mature trees) and (b) at the University of Rhode Island common garden site (sapling trees).

(a) New Jersey forest site

Collection Date	Resistant trees	Control trees
May 2012	#1-5	5 total; three locations 1-5 km away
June 2012	#1-5	6 total; three locations 1-5 km away
October 2012	#1-7	21 total; 4 within the resistant stand, 5 on the slope, 12 at two locations 5 km away
December 2012	#1-7	16 total; 3 within the resistant stand, 6 on the slope, 7 at 5 km away
April 2013	#1-7	17 total; 3 within the resistant stand, 7 on the slope, 7 at 5 km away
June 2013	#1-7	15 total; 3 within the resistant stand, 6 on the slope, 6 at 5 km away, 8 at 6.5 km away, 7 at 1 km away

(b) University of Rhode Island common garden site

Collection Date	Resistant trees	Control trees
July 2012	5 clones each for resistant #1-5	10 Massachusetts saplings, 9 Pennsylvania saplings
September 2012	5 clones each for resistant #1-5	10 Massachusetts saplings, 9 Pennsylvania saplings
December 2012	5 clones each for resistant #1-5	10 Massachusetts saplings, 9 Pennsylvania saplings

Table II.2: Relative terpenoid concentration in (a) twigs and (b) needles. Number indicates ratio of Resistant trees' to Control trees' average concentrations. Dark gray shading indicates higher concentration in Resistant, light gray indicates lower concentration in Resistant, and white indicates no difference +/- 0.05 fold. Marginal significance ($P<0.10$) by ANOVA is indicated by italics and circle, full significance ($P<0.05$) by bold italics and asterisk. NJ = New Jersey forest mature trees, URI = University of Rhode Island common-garden saplings.

(a) Twigs: ratios for Resistant trees to Control trees

TWIGS	**NJ June 2012**	**NJ Oct 2012**	**NJ Dec 2012**	**NJ April 2013**	**NJ June 2013**	**URI Sept 2012**	**URI Dec 2012**
Monoterpenes							
Tricyclene	*0.75 °*	1.28	2.00	1.19	1.92	***1.70****	***1.09 ****
α-Pinene	1.06	1.20	***1.49 ****	1.49	1.07	***1.92 ****	***1.49 ****
Camphene	0.78	1.45	1.94	1.46	*1.58 °*	***2.80 ****	***2.26 ****
β-Pinene	1.14	*1.41 °*	*1.67 °*	2.40	1.18	***2.55 ****	***2.24 ****
Myrcene	0.96	1.16	***2.18 ****	2.28	1.13	***3.79 ****	***1.95 ****
Limonene	1.12	***1.49 ****	*1.25 °*	***1.28 ****	*1.15 °*	***1.65 ****	1.23
Bornyl Acetate	*0.77 °*	1.07	1.04	*0.82 °*	*1.51 °*	***2.04 ****	***1.84 ****
Sesquiterpenes							
β-Caryophyllene	0.96	2.00	***3.50 ****	1.86	0.97	***2.20 ****	***2.63 ****
α -Humulene	0.93	*1.83 °*	***2.63 ****	1.65	0.98	***2.08 ****	***2.34 ****
Germacrene D	n/a	*3.18 °*	***3.22 ****	1.93	1.06	***1.65 ****	***4.18 ****
Totals							
Total Monoterpenes	0.94	1.22	***1.57 ****	1.49	1.13	***2.25 ****	***1.59 ****
Total Sesquiterpenes	0.94	*2.45 °*	***3.11 ****	1.82	1.02	***1.98 ****	***2.91 ****

(b) Needles: ratios for Resistant trees to Control trees

NEEDLES	**NJ May 2012**	**NJ June 2012**	**NJ Oct 2012**	**NJ Dec 2012**	**NJ April 2013**	**NJ June 2013**	**URI July 2012**	**URI Sept 2012**	**URI Dec 2012**
Monoterpenes									
Tricyclene	1.08	1.02	1.09	0.96	1.06	1.14	1.17	0.98	1.12
α-Pinene	1.23	1.17	***1.17 ****	1.03	1.11	1.09	1.13	0.95	1.07
Camphene	1.04	1.13	1.11	0.99	1.05	1.14	1.15	0.96	1.07
Sabinene	1.16	1.04	*1.21 °*	1.15	1.37	1.10	1.15	0.87	0.93
β-Pinene	1.20	0.98	1.18	1.13	1.22	1.12	1.10	0.96	1.11
Myrcene	1.17	1.08	1.10	0.98	0.82	1.07	0.97	*0.74 °*	1.02
α-Phellandrene	1.36	1.23	*1.30 °*	*1.18 °*	1.30	1.78	***1.25 ****	0.89	1.19
p-Cymene	1.48	0.94	***1.18 ****	1.09	1.07	1.35	1.10	0.93	0.95
Limonene	1.04	0.89	1.12	1.05	1.10	1.08	0.89	0.88	1.02
Eucalyptol	0.90	***0.32 ****	1.04	1.21	*1.71 °*	0.38	0.64	0.73	0.75
Camphor	1.27	***0.57 ****	0.84	0.90	0.96	***4.08 ****	1.09	0.85	1.04
Piperitone	0.91	***0.57 ****	1.16	1.12	0.87	n/a	0.74	0.73	0.99
Bornyl Acetate	1.00	1.12	1.10	1.00	1.06	1.11	1.12	0.94	1.04
Unknown A (suspected monoterpene)	n/a	n/a	0.93	0.82	***0.76 ****	0.94	n/a	***0.76 ****	***0.89 ****
Unknown B (suspected monoterpene acetate)	n/a	n/a	*0.44 °*	0.79	0.60	0.37	*0.50 °*	***0.37 ****	0.57
Sesquiterpenes									
β-Caryophyllene	1.24	1.07	1.06	1.03	1.03	1.07	1.03	0.84	0.94
α -Humulene	1.23	1.05	1.04	1.03	1.02	1.06	1.03	0.84	0.94
γ-Muurolene	n/a	n/a	*1.18 °*	1.13	1.06	0.97	0.90	0.89	1.04
Germacrene D	n/a	n/a	*1.39 °*	***3.36 ****	***2.44 ****	0.90	1.21	0.92	1.11
γ-Cadinene	n/a	n/a	*1.09 °*	0.97	0.93	0.93	0.85	***0.79 ****	0.92
δ-Cadinene	n/a	n/a	*1.10 °*	1.03	1.01	0.93	1.06	***0.81 ****	0.96
Totals									
Total Monoterpenes	1.05	1.01	1.12	1.02	1.06	1.11	1.08	0.93	1.05
Total Sesquiterpenes	1.24	1.05	*1.07 °*	1.06	1.04	1.03	1.05	*0.84 °*	0.95

Table II.3: Repeated measures ANOVA of terpenoid concentrations against resistance status in (a) twigs and (b) needles. P-values are reported from type II ANOVA's with sample month as an interacting factor and tree identity within month as the repeated measure factor. Marginal significance ($p<0.10$) by ANOVA is indicated by italics and circle, full significance ($p<0.05$) by bold italics and asterisk. NJ = New Jersey forest mature trees, URI = University of Rhode Island common-garden saplings.

(a) Twigs: p-values for Resistant trees to Control trees

TWIGS	**NJ: resistance**	**NJ: month**	**NJ: interaction resistance* month**	**URI: resistance**	**URI: month**	**URI: interaction resistance* month**
Monoterpenes						
Tricyclene	0.104	***0.037****	*0.070°*	0.227	0.279	0.808
α-Pinene	***0.001****	***0.014****	0.677	***0.044****	***< 0.001****	0.638
Camphene	0.673	0.899	***0.049****	***< 0.001****	0.663	0.887
β-Pinene	*0.079°*	0.647	0.985	***0.003****	***< 0.001****	0.387
Myrcene	***0.034****	0.900	0.618	***0.001****	***0.020****	***0.005****
Limonene	***< 0.001****	***< 0.001****	0.790	0.109	***< 0.001****	0.718
Bornyl Acetate	***0.007****	*0.084°*	0.142	***< 0.001****	0.568	0.677
Sesquiterpenes						
β-Caryophyllene	*0.096°*	0.788	0.794	***< 0.001****	***0.005****	***0.004****
α -Humulene	***0.009****	0.500	0.575	***0.001****	***< 0.001****	0.141
Germacrene D	0.220	***0.009****	*0.084°*	0.945	*0.059°*	***0.032****
Totals						
Total Monoterpenes	***0.001****	0.187	0.355	0.114	***< 0.001****	0.569
Total Sesquiterpenes	***0.028****	0.342	0.864	***0.003****	***0.001****	*0.089°*

(b) Needles: p-values for Resistant trees to Control trees

NEEDLES	NJ: resistance	NJ: month	NJ: interaction resistance* month	URI: resistance	URI: month	URI: interaction resistance* month
Monoterpenes						
Tricyclene	0.708	< 0.001*	0.029*	< 0.001*	< 0.001*	0.423
α-Pinene	0.840	< 0.001*	< 0.001*	0.007*	< 0.001*	0.410
Camphene	0.585	< 0.001*	< 0.001*	0.002*	< 0.001*	0.226
Sabinene	0.029*	0.449	0.455	< 0.001*	< 0.001*	0.122
β-Pinene	0.001*	0.013*	< 0.001*	0.003*	0.002*	0.729
Myrcene	0.980	< 0.001*	< 0.001*	0.714	< 0.001*	0.838
α-Phellandrene	< 0.001*	0.022*	0.562	0.013*	0.028*	0.798
p-Cymene	< 0.001*	0.018*	0.022*	0.020*	0.378	0.307
Limonene	< 0.001*	0.021*	0.010*	0.040*	0.002*	0.653
Eucalyptol	0.019*	0.303	0.488	0.487	0.558	0.843
Camphor	< 0.001*	0.667	0.010*	0.066°	0.023*	0.285
Piperitone	< 0.001*	< 0.001*	0.880	0.199	0.765	0.167
Bornyl Acetate	0.798	< 0.001*	< 0.001*	< 0.001*	< 0.001*	0.165
Unknown A (suspected monoterpene)	0.285	< 0.001*	< 0.001*	0.449	< 0.001*	0.827
Unknown B (suspected monoterpene acetate)	0.412	0.001*	< 0.001*	< 0.001*	0.558	0.378
Sesquiterpenes						
β-Caryophyllene	0.004*	< 0.001*	< 0.001*	0.521	< 0.001*	0.085°
α -Humulene	0.006*	0.001*	0.047*	0.545	< 0.001*	0.086°
γ-Muurolene	0.323	< 0.001*	< 0.001*	0.285	< 0.001*	0.622
Germacrene D	0.103	< 0.001*	0.483	0.092°	< 0.001*	0.669
γ-Cadinene	0.217	< 0.001*	< 0.001*	0.028*	< 0.001*	0.938
δ-Cadinene	0.450	< 0.001*	< 0.001*	0.985	0.014*	0.248
Totals						
Total Monoterpenes	0.488	0.987	0.001*	0.002*	< 0.001*	0.317
Total Sesquiterpenes	0.003*	< 0.001*	< 0.001*	0.501	< 0.001*	0.094°

Table II.4: Partitioning of variance across terpenoid compounds. Total variance across (a) seven terpenoid compounds in twigs, or (b) twelve compounds in needles, is attributed to explanatory variables: resistance status of sample, HWA density on sample tissue, EHS density on sample tissue.

(a) Twigs: partitioning of variance across seven terpenoid compounds.

TWIGS	**Resistance:** variance attributed to Res only	**HWA:** variance attributed to HWA only	**EHS:** variance attributed to EHS only	**Confounded:** Resistance and HWA joint variance	**Confounded:** Resistance and EHS joint variance	**Noise:** unexplained variance
NJ June 2012	7%	5%	34%	*interaction*	*interaction*	58%
NJ Oct 2012	7%	6%	3%	*interaction*	*interaction*	85%
NJ Dec 2012	16%	3%	10%	2%	*interaction*	72%
NJ April 2013	7%	7%	8%	0%	*interaction*	64%
NJ June 2013	2%	7%	*n/a*	*interaction*	*n/a*	92%
URI Sept 2012	14%	3%	4%	*interaction*	*interaction*	82%
URI Dec 2012	12%	*n/a*	1%	*n/a*	1%	86%

(b) Needles: partitioning of variance across twelve terpenoid compounds.

NEEDLES	**Resistance:** variance attributed to Res only	**HWA:** variance attributed to HWA only	**EHS:** variance attributed to EHS only	**Confounded:** Resistance and HWA joint variance	**Confounded:** Resistance and EHS joint variance	**Noise:** unexplained variance
NJ May 2012	1%	*n/a*	2%	*n/a*	1%	96%
NJ June 2012	12%	44%	3%	23%	0%	19%
NJ Oct 2012	11%	8%	1%	*interaction*	0%	83%
NJ Dec 2012	6%	6%	3%	1%	3%	82%
NJ April 2013	9%	10%	2%	3%	0%	61%
NJ June 2012	4%	3%	*n/a*	0%	*n/a*	94%
URI Sept 2012	5%	2%	1%	*interaction*	*interaction*	93%
URI Dec 2012	2%	*n/a*	2%	*n/a*	*interaction*	96%

Figures

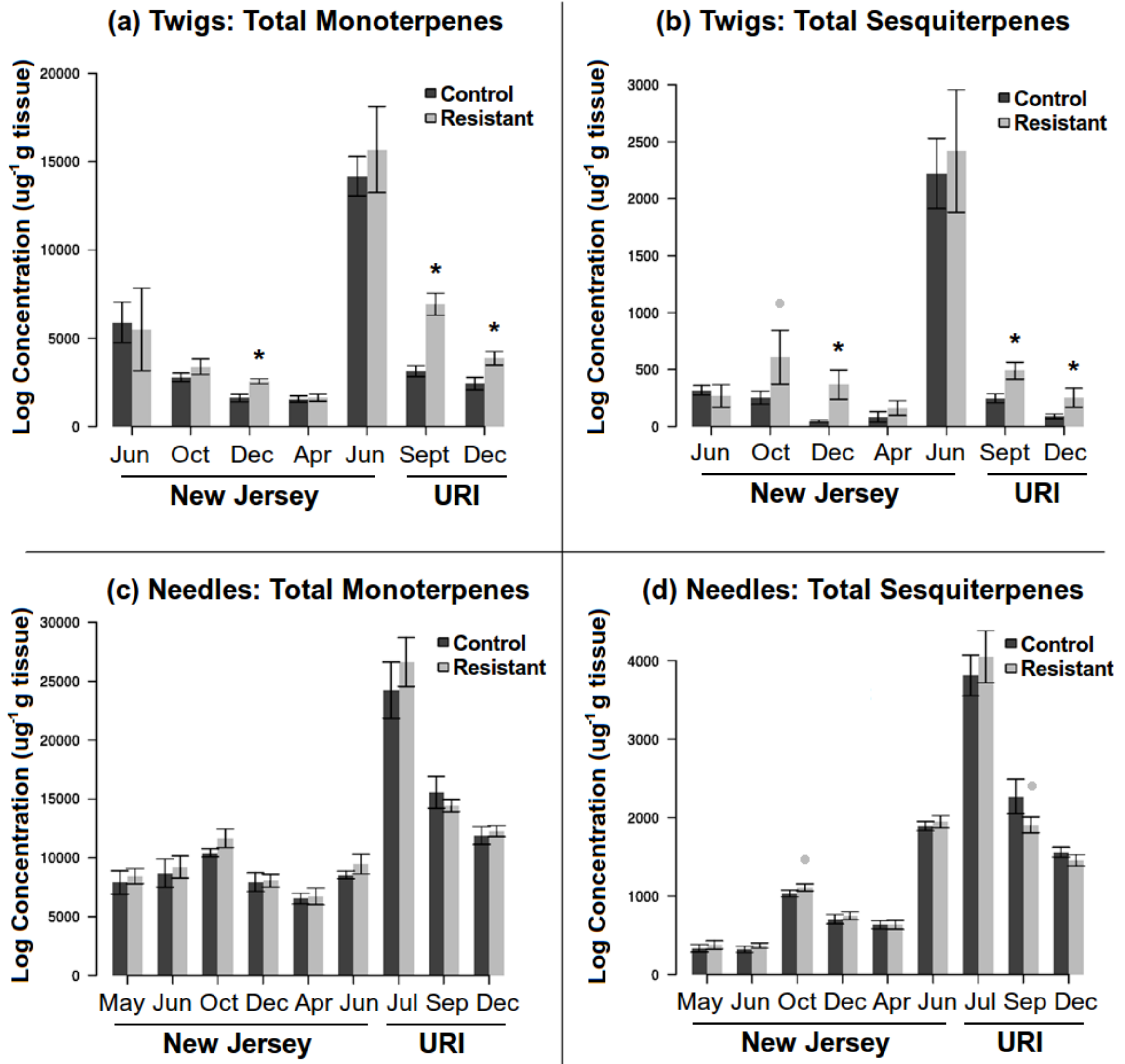

Figure II.1: Relative total concentrations of all monoterpenes, or all sesquiterpenes. (a & b) Monoterpenes and sesquiterpenes in needles; (c & d) in twigs. Bar displays average concentration in Control (dark) or Resistant (light) trees, in units of natural log of concentration. Error bars display standard error. Marginal significance ($p<0.10$) by ANOVA is marked by a gray circle, full significance ($p<0.05$) by a black asterisk.

APPENDIX

METHOD DETAILS

Filter composition and procedure

For needle samples, filters contained 0.3 g of 60Å silica gel, 0.10 g activated carbon, and 0.2 g magnesium sulfate packed in a 6-inch Pasteur pipet; for twig samples, filters contained 0.2 g silica gel, 0.07 g activated carbon, and 0.13 g magnesium sulfate. Filters were conditioned with 1 mL methanol and 1 mL MTBE, drained using a pressure bulb, and wetted with 0.2 mL MTBE immediately prior to filtration. The organic layer of each sample was transferred to the filter and allowed to drain through. Terpenes were eluted from filters with 0.5 mL hexanes and a pressure bulb was used to complete drainage.

Calibration curves

The calibration curve for a given terpene compound is an estimated linear function relating initial concentration to resulting detected quantity of that terpene compound. With an adequate range of concentrations and replication on filters, calibration curves thus account for losses in filtration and differences in detectability among compounds.

Calibration curves were determined experimentally for all compounds for which analytical standards were available. When a standard was not available, calibration curve was estimated based on neighboring compounds of similar molecular structure. Standards solutions of 25 compounds at identical concentration in MTBE were passed through the filtration procedure and quantified on the GC-FID. For solutions at 7 concentration levels, twenty-four 1-mL aliquots of each standards solution were filtered on 12 needle filters and on 12 twig filters. For solutions at 7 additional concentration levels, four 1-mL aliquots of each standards solution were filtered on 2 needle filters and on 2 twig filters.

After quantification on the GC-FID, calibration curves were created for each terpene compound by plotting initial concentration on the x-axis and detected area of that terpene on the

y-axis and fitting a linear function with 95% confidence bands. Calculations were performed in R version 2.15.2.

GC-FID instrument settings

Terpene compounds in samples were quantified by gas chromatography with flame ion detection (Hewlett-Packard Agilent 6890, running Agilent ChemStation software). Separations were performed on an Agilent HP-5 capillary column, non-polar with crosslinked 5% phenyl / 95% methyl siloxane, 0.25 µm film thickness, 0.32 mm diameter, 30 m length. The column was trimmed by 8 cm during previous use. The helium carrier gas was in constant flow mode at 2.2 mL min^{-1} and average velocity 36 cm sec^{-1}; sample was injected with split ratio of 3:1, split flow of 6.5 mL min^{-1}, and total flow of 11.1 mL min^{-1}. The injection volume was 1 µL at inlet temperature of 250°C. The GC oven temperature was programmed to start at 60°C and rise to 158°C with holds at 64°C, 100°C, and 126°C to improve separation of compounds, followed by burn-off at 200°C. The flame-ion detector was set at 300°C, with hydrogen flow at 30 mL min^{-1}, air flow at 300 mL min^{-1}, and nitrogen makeup flow at 25 mL min^{-1}. The detector began data collection after 3 minutes of solvent cut-time.

GCMS instrument settings

GCMS separations for initial compound identifications were performed on a Shimadzu SHRXI-5MS capillary column, non-polar with crosslinked 5% diphenyl / 95% dimethyl polysiloxane, 0.25 um film thickness, 0.25 mm diameter, 30 m length. The helium carrier gas was in constant linear velocity mode at 1.2 mL min^{-1} flow and 40 cm sec^{-1} velocity; sample was injected with split ratio of 3:1 and total flow of 5.8 mL min^{-1}. The injection volume was 1 µL at inlet temperature of 250°C. The GC oven temperature ramp was identical to that used on the GC-FID, running 60°C to 158°C with holds (Appendix Table 1). The MS ion source and interface temperatures were set at 200°C, and the detector scanning range was 40-400 m/z. The MS detector began data collection after 3 minutes of solvent cut-time.

Appendix Table 1: Programmed heat ramp for GC-FID oven, used in separating terpenoid compounds by volatility.

Time (min.)	Rate (°C/min.)	Target (°C)	Hold (min.)
–	–	60	0
0	2	64	3
5	2	68	0
7	20	100	3
11.6	3	126	5
25.3	3	158	0
35.9	80	200	5
40.9	–	–	–

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