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# Temporal and Spatial Variation of Terpenoids in Eastern Hemlock (*Tsuga canadensis*) in Relation to Feeding by *Adelges tsugae*

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**Abstract** The terpenoid content of eastern hemlock (*Tsuga canadensis*) foliage was measured over an annual cycle of development from bud opening, shoot elongation, shoot maturation, to bud-break at the start of the next growing season. The objective was to determine if variation in terpenoid composition is linked with spatial and temporal feeding preferences of the hemlock woolly adelgid (HWA; *Adelges tsugae*). The HWA has two periods of feeding over the course of 1 yr spanning two complete generations. There are two periods of feeding separated by a nonfeeding period where the adelgid estivates. HWA prefers to feed on mature, rather than young, expanding tissue. Feeding occurs in the leaf cushion at the base of the needle. The needle is the only tissue in hemlock with resin canals that store terpenoids. The needle and leaf cushion of both the current and previous years' growth were analyzed separately over a 1-yr period to examine the variation of terpenoid composition in space and time. Terpenoids were quantified by using headspace solid-phase microextraction/gas chromatography/mass spectrometry (SPME/GC/MS). New growth needles and leaf cushions do not resemble the previous year's growth either visually or in chemical composition until October/November, when the adelgid breaks estivation and begins feeding. Nearly all of the 23 terpenoids present exceeding 0.1% varied significantly either temporally or spatially, usually with complex interactions. Ordination and factor analysis revealed that terpenoids are less variable in mature leaf cushions than in young tissue. By entering a nonfeeding diapause during the late spring and summer, HWA avoids the unstable, variable levels of terpenoids in the immature leaf cushion and needles.

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## Introduction

The genus *Tsuga* (hemlock trees) consists of nine species, two in eastern North America, two in western North America, and five in Asia (Farjon, 1990). The Asian and western North American hemlock species are considered resistant to the hemlock woolly adelgid, *Adelges tsugae* Annand, whereas the eastern North American species are susceptible to feeding by the adelgid, resulting in a slow decline in tree health and eventual tree death (McClure et al., 2001). The hemlock woolly adelgid (HWA) is an introduced pest to North American hemlock. The predominant eastern North American species is *T. canadensis* (L.), with pockets of *T. caroliniana* Engelm. in the Southern Appalachians. We previously examined the terpenoid profiles of seven species [*T. caroliniana*, *T. canadensis*, *T. chinensis* (Franch.) E. Pritz., *T. diversifolia* (Maxim.) Mast., *T. heterophylla* (Raf.) Sarg., *T. mertensiana* (Bong.) Carriere, and *T. sieboldii* (Carriere)] by solid-phase microextraction (SPME) sampling with gas chromatography/mass spectrometry (GC/MS) analysis in the headspace of a single needle of mature foliage (Lagalante and Montgomery, 2003). Data reduction by principal component analysis (PCA) revealed a "species" component and a "resistance/susceptibility" component. Examination of factor loadings of individual terpenoids within the resistance/susceptibility component revealed 13 terpenoids with statistically significant ($P < 0.05$) factor loadings.

While our previous study provided a foundation for understanding the role of terpenoid chemistry in species level resistance to HWA, this study focused on how the patterns of terpenoids vary in time and space within the susceptible species, *T. canadensis*. Although the needle is the most convenient and consistent place to sample for comparing terpenoid variation among hemlock species, it is not the exact location where the adelgid feeds. The adelgid crawler (a newly hatched nymph that is the only mobile stage on eastern hemlock) settles at the base of the needle and inserts a stylet bundle (a feeding tube three times its body length) just below the leaf abscission layer into the leaf cushion. It technically feeds on nutrients in the stem wood and the xylem ray parenchyma tissue rather the leaf needle (Young et al., 1995). The needle possesses a single resin canal along the ventral side of the midrib; however, defined resin canals are absent in the woody tissue (Farjon, 1990). Therefore, it is important to compare the terpenoid chemistry in the needle and the leaf cushion.

The lifecycle of the HWA consists of two parthenogentic generations per year on hemlock. The spring generation (progrediens) develops between March and June, and the over-wintering generation (sistens) develops between June and March, with overlap of the two generations in the late spring. Between June and mid-July, hatched crawlers of the sistens generation settle preferentially at the base of the current year's growth needles and insert their stylet bundles into the immature leaf cushion. They do not feed during this period; instead, they enter summer diapause (estivation). In October, when the weather cools, estivation is broken and feeding commences, and the sistens' nymphs mature over the winter to produce the progredien generation the following spring (McClure, 1987). The progredien generation crawlers settle preferentially on the same age tissue on which their sistens' parents fed. Generally, both generations of the adelgid feed on foliage that is mature, but less than 14 mo old at the tip of the branch. In the late spring and early summer, the sistens crawlers

move onto the new foliage, settle, and enter diapause. Thus, there are "winter" and "spring" feeding periods on mature foliage separated by a "summer" nonfeeding period on immature foliage.

The current study examined how the terpenoids in the foliage of *Tsuga canadensis* vary: (1) temporally during three feeding periods of the HWA, (2) spatially between the new growth and the previous year's growth, and (3) spatially between the needle and leaf cushion tissues. The spatial and temporal results obtained are interpreted in relation to the settling preferences and feeding of the HWA on eastern hemlock.

## Methods and Materials

*Plant Material* Samples were collected from June 2003 to May 2004 from a hemlock stand located at Lake Scranton (41.3858°N, 75.6286°W) in Scranton, PA, USA. The area around Lake Scranton possesses hemlock stands that span the entire range of HWA population densities, from dense conspicuous infestations where branches are covered with white ovisacs to stands that are visually uninfested. A stand of healthy hemlock with a light infestation (possessing only a few visible ovisacs scattered among trees in the stand) on the eastern shore of the lake was selected as a sampling site. This particular stand was selected because the vast majority of branches were devoid of adelgid or other pests, yet a neighboring stand (approximately 0.5 km closer to the lakeshore) possessed moderate to heavy levels of adelgid where we could observe the settling behavior of the crawlers of the 1st instar nymph stage. Thus, our analytical results are based on the terpenoid present in healthy hemlock, presumably devoid of induced host-plant responses from adelgid wounding.

Hemlock trees, approximately 10 m in height, were tagged within the sampling site, and the peripheral 10 cm of foliage was periodically clipped from branches lying approximately 2 m above ground level. Due to time constraints imposed by the analytical method, one of the six trees was sequentially sampled each day to provide a single branch clipping for analysis. The clipped branch was immediately placed in a polyethylene bag in a cooler on ice packs and taken to the laboratory. Dissection and analysis were initiated <1 hr postsampling. Although the buds started opening in mid-May 2003, needle dissections were not possible on the tiny (3 mm), pliant, bright green foliage. Thus, sampling was delayed until June when the shoot had elongated to an average of 2 cm and the needle length had reached 10 mm. Initially, about 5 samples were collected each week, with the sampling frequency decreasing in November when the 2003 foliage had visually matured (turned dark green). Sampling was terminated when bud break occurred for the 2004 year's growth in May.

*Sample Preparation* On each sampling date, 6 samples were analyzed from one hemlock twig; 2 samples were needles representing new growth (2003), 2 samples were needles representing the previous year's growth (2002), 1 sample comprised 5 combined, new growth (2003) leaf cushions, and 1 sample comprised 5 combined, previous year's growth (2002) leaf cushions. The samples were collected, prepared, and analyzed for terpenoids on the same day. This method minimized extraneous influences on comparisons between the two types of tissue and the two foliage ages.

We define the twig as a branch tip consisting of the linear length from the tip back to the start of the previous year's growth, ignoring any lateral shoots. The needle and leaf cushion (pulvinus) are separated by an abscission layer (Fig. 1). In hemlocks, as in most conifers,

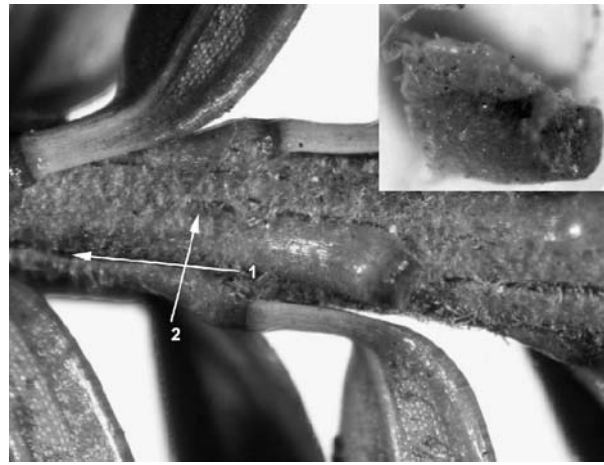


**Fig. 1** Photograph indicating the cuts performed in the leaf cushion dissection. Arrows indicate the direction of cuts and numbers are referred to in the text. A removed leaf cushion is shown in the inset picture

the leaf or needle consists of the blade and a very short petiole. During the sampling period, the abscission layer was distinct in both young and mature tissue. The leaf cushion is the thickened base below the abscission layer that remains after the leaf falls off the stem. The leaf cushion is technically part of the woody stem tissue and contains the nutrient laden ray parenchyma cells that the adelgid penetrates with its stylet bundle during feeding.

Under a stereoscope at 40×, 2 needles from the 2002 growth and 2 from the 2003 growth were broken cleanly away from the leaf cushion at the leaf abscission layer with a stainless steel scalpel. Using the same shoot from which the needles were taken, 5 needles representing the previous year's growth (2002) and 5 representing new growth (2003) were selected for leaf cushion dissection. The needles were removed at the leaf abscission layer as described above. The needle portion was discarded, and two cuts were made with a stainless-steel scalpel (Fig. 1). The first cut (arrow 1) was made along the twig from the distal to the basal end of the leaf cushion. The second (arrow 2) was made perpendicular to the first cut at the bend denoting the base of the leaf cushion. On the mature previous year's growth, the leaf cushion is distinctly darker brown than the light-tan stem, thus allowing a visual cue for the second cut. On immature needles, the distinct visual color difference does not exist, and so the placement of the cut was made at the same relative angle and position used for dissecting mature tissue. The five leaf cushions from new growth (2003) and five cushions from previous year's growth (2002) were collected in two separate 4-ml screw-top vials and capped with PTFE/silicone septa (VWR Scientific, Pittsburgh, PA, USA). Five leaf cushions were necessary to produce a measurable terpenoid profile, owing to the reduced amount of plant volatiles in the leaf cushion as compared to the needle that contains a foliar canal where the resins are concentrated.

*SPME/GC/MS Analysis* We used a headspace SPME sampling procedure for both the needle and leaf cushion analyses (Lagalante and Montgomery, 2003). The GC/MS conditions were identical except a splitless injection was used for the individual sample containing 5 leaf cushions. The same 51 compounds previously quantified by Lagalante and Montgomery (2003) were identified from a mass spectrum database search (Varian NIST MS database, 1998, and IMS terpene library, 1992), and on the basis of their measured retention indices as compared to the retention indices reported using an equivalent DB-5 column (Adams, 2001). The area under an identified peak was integrated

by using a single *m*/*z* fragment from the total ion spectrum for each compound. The *m*/*z* fragment was the most intense ion in the mass spectrum that was provided from our previously published work. Relative, as opposed to absolute, amounts (area percent) of an individual compound were calculated from the ratio of the peak area for an individual compound, relative to the total peak area for all identified compounds in a chromatogram.

On a given day, the SPME/GC/MS analyses were performed on 6 samples from a single twig as described above. Fifty-one terpenoids were measured for each of the 6 samples on each sampling day. Of the 51 terpenoids, 28 were present in amounts that averaged <0.1%, and these were not included in the statistical analysis.

*Statistical Analysis* There were 11 sampling days during the first "spring" period (June 10 to July 14, 2003), 10 sampling days during the second "summer" period (July 15 to October 14, 2003), and 15 sampling days during the third "leaf-off" period (October 15, 2003 to May 10, 2004). This division of periods is made to correspond to distinct aspects of the adelgid's life history on hemlock: spring, summer, and leaf-off. Spring is when the progrediens generation crawlers select feeding sites, generally settling on the current year's growth and immediately feeding and completing development in about 2 mo. Summer is when the sistens generation crawlers settle on the new, current year growth but enter diapause. Leaf-off is when the sistens generation feeds and develops.

We used a three-way ANOVA in the general linear model (GLM) procedure of Systat 11 (Systat Software, Richmond, CA, USA) to examine influence of the three factors: (1) *tissue* (needle or leaf cushion), (2) *age* [current year (2003) or previous year (2002) growth], and (3) *period* (spring, summer, leaf-off) and their interactions. Each twig sample within the seasonal periods was treated as a block, and, thus, twig is nested within tissue. *F*-tests were performed on the main effects and the interactions and *P* values were evaluated at the 0.05 significance level (95% confidence interval). Systat Software was also used to conduct PCA on the symmetric correlation matrix of the terpenoids in order to collapse the variability into a few components so that meaningful relationships could be identified.

Classical multidimensional scaling (CMDS) was used to calculate distances among the four "spatial" components (two ages and two tissues) for each twig sample by using all the terpenoids listed in Table 1. The distance matrix was calculated by using a cityblock measure (Manhattan distance) and the pdist, eigval, and cmdscale commands with Matlab® Statistics Toolbox 5.2 software (MathWorks, Natick, MA, USA). This analysis was then separated into the period components to show how the "spatial" relationships change temporally. The scaling analysis was repeated by using global nonmetric multidimensional scaling (GNMDS) and Bray–Curtis coefficient distance to ensure that the obtained results were consistent with the CMDS procedure, and not dependent on the type of MDS or distance variable used. Since the results of the GNMDS agreed with those of the CMDS, they are not included here.

## Results

Visually, the new growth progressed from a bright green, pliant needle in May to a dark green, hardened needle in November. By November, the new, current year's growth of both the needle and leaf cushion were visually identical to the previous year's growth needles and leaf cushion. The twig also had matured and hardened, although it was not nearly as hard as the previous year's growth. This visual maturation coincides with the sistens generation crawler breaking estivation and commencing feeding.

**Table 1** Terpenoid percentages (Least squares means and Standard errors) and significance tests of the three factors: Age (Previous or Current year's growth), Period of the year (Spring, Summer, Leaf-off) and Tissue (Needle or Leaf cushion)

| Period | Spring | | Summer | | Leaf-off | | Spring | | Summer | | Leaf-off | | Age, $F(P)$ | Period $F(P)$ | Tissue $F(P)$ | Age × Period $F(P)$ | Age × Tissue $F(P)$ | Period × Tissue $F(P)$ | Age × Period × Tissue $F(P)$ |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Age | Current year's growth foliage | | | | | | Previous year's growth foliage | | | | | | $df$=1 | $df$=2 | $df$=2 | $df$=2 | $df$=1 | $df$=2 | $df$=2 |
| Tissue | Needle ($N$=11) | Leaf cushion ($N$=11) | Needle ($N$=10) | Leaf cushion ($N$=10) | Needle ($N$=15) | Leaf cushion ($N$=15) | Needle ($N$=11) | Leaf cushion ($N$=11) | Needle ($N$=10) | Leaf cushion ($N$=10) | Needle ($N$=15) | Leaf cushion ($N$=15) | | | | | | | |
| Tricyclene | 3.47 (0.36) | 1.56 (0.36) | 4.06 (0.38) | 1.58 (0.38) | 3.60 (0.31) | 1.57 (0.33) | 5.13 (0.36) | 3.07 (0.36) | 4.93 (0.38) | 2.50 (0.41) | 4.90 (0.33) | 1.69 (0.36) | 25.80 (0.000) | 1.33 (0.268) | 125.1 (0.000) | 1.68 (0.192) | 1.05 (0.308) | 1.70 (0.199) | 0.89 (0.415) |
| α-Pinene | 10.04 (0.98) | 13.04 (0.98) | 12.22 (1.03) | 10.90 (1.03) | 11.46 (0.84) | 6.48 (0.88) | 15.04 (0.98) | 8.45 (0.98) | 15.07 (1.03) | 6.94 (1.10) | 13.02 (0.88) | 4.10 (0.97) | 0.20 (0.654) | 11.26 (0.000) | 63.13 (0.000) | 0.16 (0.850) | 36.33 (0.000) | 7.48 (0.001) | 2.29 (0.107) |
| Camphene | 8.48 (0.50) | 2.98 (0.50) | 9.68 (0.52) | 3.15 (0.52) | 8.97 (0.43) | 3.85 (0.45) | 10.09 (0.50) | 5.68 (0.50) | 11.07 (0.52) | 4.49 (0.56) | 9.66 (0.45) | 3.43 (0.49) | 18.25 (0.000) | 1.59 (0.210) | 399.4 (0.000) | 4.68 (0.012) | 0.00 (0.979) | 2.44 (0.093) | 1.34 (0.266) |
| Sabinene | 0.61 (0.17) | 0.36 (0.17) | 0.66 (0.18) | 0.36 (0.18) | 0.40 (0.15) | 0.66 (0.16) | 0.45 (0.17) | 0.09 (0.17) | 0.36 (0.18) | 0.42 (0.19) | 0.24 (0.16) | 0.54 (0.17) | 2.65 (0.107) | 0.27 (0.767) | 1.19 (0.28) | 0.09 (0.912) | 0.24 (0.625) | 3.34 (0.040) | 0.44 (0.643) |
| β-Pinene | 0.81 (0.15) | 1.24 (0.15) | 1.35 (0.16) | 1.32 (0.16) | 1.48 (0.13) | 0.87 (0.14) | 2.47 (0.15) | 0.55 (0.15) | 2.13 (0.16) | 0.67 (0.17) | 1.69 (0.14) | 0.94 (0.15) | 8.82 (0.011) | 0.70 (0.501) | 67.03 (0.000) | 2.06 (0.133) | 55.41 (0.000) | 0.06 (0.938) | 14.67 (0.000) |
| Myrcene | 3.00 (1.40) | 21.07 (1.40) | 2.53 (1.47) | 19.81 (1.47) | 1.79 (1.20) | 2.72 (1.27) | 2.09 (1.40) | 0.48 (1.40) | 1.80 (1.47) | 2.59 (1.58) | 1.12 (1.26) | 0.61 (1.40) | 76.44 (0.000) | 19.25 (0.000) | 51.78 (0.000) | 14.01 (0.000) | 60.54 (0.000) | 12.98 (0.000) | 13.43 (0.000) |
| α-Phellandrene | 0.14 (0.15) | 0.76 (0.15) | 1.17 (0.16) | 1.06 (0.16) | 1.00 (0.13) | 0.75 (0.13) | 2.04 (0.15) | 0.43 (0.15) | 1.72 (0.16) | 0.37 (0.17) | 1.03 (0.13) | 0.57 (0.15) | 6.24 (0.014) | 3.21 (0.045) | 37.58 (0.000) | 11.13 (0.000) | 51.55 (0.000) | 1.58 (0.212) | 12.67 (0.000) |
| *o*-Cymene | 0.00 (0.011) | 0.19 (0.11) | 0.05 (0.012) | 0.19 (0.12) | 0.15 (0.10) | 0.24 (0.10) | 0.47 (0.11) | 0.76 (0.11) | 0.34 (0.12) | 0.55 (0.12) | 0.33 (0.10) | 0.09 (0.11) | 20.17 (0.000) | 2.03 (0.137) | 3.21 (0.076) | 5.85 (0.004) | 0.16 (0.690) | 2.59 (0.081) | 1.34 (0.266) |
| Limonene | 0.77 (0.17) | 0.69 (0.17) | 1.73 (0.18) | 0.64 (0.18) | 2.06 (0.14) | 1.09 (0.15) | 2.32 (0.17) | 0.44 (0.17) | 2.33 (0.18) | 0.76 (0.19) | 1.94 (0.15) | 1.34 (0.17) | 14.18 (0.000) | 11.80 (0.000) | 113.4 (0.000) | 3.28 (0.042) | 10.88 (0.001) | 2.65 (0.076) | 11.50 (0.000) |
| β-Phellandrene | 0.49 (0.11) | 0.37 (0.11) | 1.06 (0.12) | 0.36 (0.12) | 1.84 (0.10) | 0.92 (0.10) | 1.41 (0.11) | 0.25 (0.11) | 1.38 (0.12) | 0.21 (0.13) | 1.67 (0.10) | 1.07 (0.11) | 6.12 (0.015) | 55.23 (0.000) | 144.4 (0.000) | 3.78 (0.024) | 9.63 (0.003) | 1.60 (0.207) | 10.26 (0.000) |

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|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| *cis*-Ocimene | 0.51 | 1.01 | 1.73 | 1.20 | 1.15 | 0.61 | 2.73 | 0.63 | 2.64 | 0.37 | 1.07 | 0.61 | 7.21 | 10.10 | 60.93 | 7.30 | 38.71 | 5.13 | 13.03 |
| | (0.20) | (0.20) | (0.21) | (0.21) | (0.17) | (0.18) | (0.20) | (0.20) | (0.21) | (0.22) | (0.18) | (0.20) | (0.009) | (0.000) | (0.000) | (0.001) | (0.000) | (0.008) | (0.000) |
| Terpinolene | 0.09 | 0.14 | 0.38 | 0.18 | 0.46 | 0.23 | 0.46 | 0.09 | 0.44 | 0.11 | 0.35 | 0.16 | 0.83 | 8.82 | 95.32 | 11.89 | 15.70 | 1.69 | 9.36 |
| | (0.04) | (0.04) | (0.04) | (0.04) | (0.03) | (0.03) | (0.04) | (0.04) | (0.04) | (0.04) | (0.03) | (0.04) | (0.365) | (0.000) | (0.000) | (0.000) | (0.000) | (0.019) | (0.000) |
| Borneol | 1.32 | 0.77 | 0.19 | 0.24 | 3.94 | 2.87 | 2.43 | 1.21 | 1.28 | 0.71 | 6.19 | 1.91 | 6.40 | 43.17 | 19.00 | 0.02 | 6.62 | 6.45 | 2.38 |
| | (0.51) | (0.50) | (0.53) | (0.53) | (0.43) | (0.46) | (0.51) | (0.51) | (0.53) | (0.57) | (0.45) | (0.50) | (0.013) | (0.000) | (0.000) | (0.978) | (0.012) | (0.002) | (0.098) |
| Piperitone | 0.03 | 0.92 | 1.93 | 1.33 | 4.67 | 4.92 | 4.45 | 2.62 | 5.20 | 2.14 | 5.88 | 5.48 | 66.91 | 71.07 | 10.42 | 7.28 | 15.90 | 4.52 | 1.96 |
| | (0.42) | (0.42) | (0.45) | (0.45) | (0.36) | (0.38) | (0.42) | (0.42) | (0.45) | (0.48) | (0.38) | (0.42) | (0.000) | (0.000) | (0.002) | (0.001) | (0.000) | (0.013) | (0.146) |
| Isobornylacetate | 49.28 | 21.98 | 49.53 | 29.53 | 46.65 | 48.52 | 36.60 | 53.19 | 39.43 | 50.96 | 40.87 | 55.84 | 13.48 | 11.31 | 0.07 | 3.27 | 106.9 | 10.31 | 10.78 |
| | (2.49) | (2.49) | (2.61) | (2.61) | (2.13) | (2.24) | (2.49) | (2.49) | (2.61) | (2.79) | (2.23) | (2.47) | (0.000) | (0.000) | (0.786) | (0.043) | (0.000) | (0.000) | (0.000) |
| β-Caryophyllene | 2.60 | 1.74 | 1.97 | 2.19 | 1.41 | 3.45 | 1.60 | 2.08 | 1.43 | 3.16 | 1.36 | 2.66 | 1.48 | 0.83 | 31.35 | 1.80 | 5.85 | 14.66 | 6.69 |
| | (0.25) | (0.25) | (0.27) | (0.27) | (0.22) | (0.23) | (0.25) | (0.25) | (0.27) | (0.28) | (0.23) | (0.25) | (0.226) | (0.438) | (0.000) | (0.171) | (0.018) | (0.000) | (0.002) |
| β-Gurjunene | 0.16 | 0.26 | 0.08 | 0.14 | 0.14 | 0.33 | 0.16 | 0.10 | 0.07 | 0.09 | 0.05 | 0.30 | 1.82 | 0.24 | 4.78 | 0.15 | 0.34 | 2.60 | 0.607 |
| | (0.07) | (0.07) | (0.08) | (0.07) | (0.06) | (0.07) | (0.07) | (0.07) | (0.08) | (0.08) | (0.06) | (0.07) | (0.180) | (0.096) | (0.031) | (0.860) | (0.561) | (0.080) | (0.547) |
| α-Humulene | 6.22 | 4.18 | 4.80 | 5.80 | 3.82 | 9.76 | 3.74 | 7.41 | 3.59 | 8.36 | 4.02 | 9.80 | 1.46 | 8.45 | 95.74 | 0.025 | 22.94 | 21.89 | 7.80 |
| | (0.56) | (0.56) | (0.59) | (0.59) | (0.48) | (0.51) | (0.56) | (0.56) | (0.59) | (0.64) | (0.51) | (0.56) | (0.229) | (0.000) | (0.000) | (0.780) | (0.000) | (0.000) | (0.001) |
| γ-Muurolene | 0.23 | 0.44 | 0.18 | 0.46 | 0.60 | 1.11 | 0.62 | 1.93 | 0.42 | 1.06 | 0.36 | 0.71 | 3.27 | 0.62 | 8.07 | 3.94 | 1.24 | 0.29 | 0.96 |
| | (0.34) | (0.34) | (0.35) | (0.35) | (0.29) | (0.30) | (0.34) | (0.34) | (0.35) | (0.38) | (0.30) | (0.33) | (0.074) | (0.538) | (0.006) | (0.023) | (0.268) | (0.745) | (0.386) |
| Germacrene D | 8.99 | 21.27 | 2.51 | 16.42 | 2.26 | 3.20 | 2.02 | 4.28 | 1.16 | 8.07 | 1.44 | 2.81 | 17.17 | 8.67 | 19.82 | 5.99 | 3.87 | 3.86 | 1.35 |
| | (2.44) | (2.44) | (2.56) | (2.56) | (2.09) | (2.20) | (2.44) | (2.44) | (2.56) | (2.75) | (2.19) | (2.43) | (0.000) | (0.000) | (0.000) | (0.004) | (0.052) | (0.025) | (0.264) |
| Viridiflorene | 0.04 | 0.12 | 0.08 | 0.12 | 0.05 | 0.21 | 0.14 | 0.12 | 0.15 | 0.16 | 0.09 | 0.07 | 0.84 | 0.52 | 4.68 | 3.59 | 8.73 | 0.60 | 1.36 |
| | (0.03) | (0.03) | (0.04) | (0.03) | (0.03) | (0.03) | (0.03) | (0.03) | (0.04) | (0.04) | (0.03) | (0.03) | (0.361) | (0.597) | (0.033) | (0.032) | (0.004) | (0.548) | (0.261) |
| γ-Cadinene | 0.68 | 0.82 | 0.54 | 0.88 | 0.52 | 1.89 | 0.83 | 1.62 | 0.78 | 1.78 | 0.68 | 1.67 | 12.75 | 2.07 | 66.48 | 4.23 | 2.71 | 5.45 | 3.65 |
| | (0.16) | (0.16) | (0.17) | (0.17) | (0.14) | (0.15) | (0.16) | (0.16) | (0.17) | (0.18) | (0.15) | (0.16) | (0.001) | (0.132) | (0.000) | (0.017) | (0.103) | (0.006) | (0.030) |
| δ-Cadinene | 1.24 | 1.23 | 0.80 | 1.46 | 0.68 | 2.05 | 1.27 | 2.96 | 1.21 | 3.16 | 1.10 | 2.26 | 24.09 | 0.44 | 54.60 | 2.24 | 9.09 | 0.94 | 3.91 |
| | (0.27) | (0.27) | (0.28) | (0.28) | (0.23) | (0.24) | (0.27) | (0.27) | (0.28) | (0.30) | (0.24) | (0.26) | (0.000) | (0.644) | (0.000) | (0.113) | (0.003) | (0.396) | (0.023) |

Table 1 summarizes the mean values (with standard error) of the percentages and the statistical tests associated with the variables measured for the terpenoids. Additional terpenoids not listed in Table 1 were present at levels below 0.1% and could not be reliably quantified. There were significant main effects and interactions for most of the measurable terpenoids. The data are complex and patterns are not readily visualized. For instance, isobornyl acetate, the most abundant terpenoid, is at higher levels in the new growth needles and at lower levels in the new growth leaf cushions during the spring and summer periods. During the leaf-off period when the foliage has visually matured, the percentages of isobornyl acetate are similar in both the needles and cushions in both the current and previous year's growth. The statistical tests indicate that the needle and leaf cushion tissues are not different generally, but do differ from each other within age and period. Hence, the isobornyl acetate content in the needles and leaf cushions depends on the age of the foliage and the time of the year. Although inferences can be draw from Table 1 for other terpenoids by visual inspection, ordination analysis provided a more objective and powerful method to discern the terpenoid inter- and intrarelationships.

CMDS was used to assess the relationships among the two tissues and two age growth classes in each of the three time periods, based on the composition of all 23 terpenoids (Fig. 2). This approach demonstrates three key patterns relating host chemistry and adelgid feeding. First, the ordination shows that the chemical composition of both old and new needles is similar, and that the variation in chemical composition within and between both groups is relatively small. In other words, needles vary little spatially or temporally. This is not the case for leaf cushions. For the leaf cushion, there are two patterns. The leaf cushions are widely scattered and in separate groups, and this changes with the time period. The widely scattered points for the leaf cushions in both the spring and summer periods indicate that the adelgid crawlers of both generations encounter a highly unpredictable chemical mixture. In the last period, by leaf-off, the scatter for the leaf cushions is greatly reduced and data points coincide with those for the needles.

PCA was used to identify which individual terpenoids are varying in similar patterns and which may be phytochemically important. The correlation matrix was analyzed by using the 23 terpenoids in the 138 tissue and age samples as variables for the PCA. Next, dummy variables for the age, period, and tissue factors were introduced in order to more easily identify which factors were influencing the component loadings. Introducing the dummy variables had little influence on the loading of the terpenoids. The initial run produced six latent roots (eigenvalues) greater than 1.0 and explained 73% of the total variance. It was difficult to find meaning in so many components, so the components were limited to three (Table 2). First, second, and third components accounted for 30.3%, 14.3%, and 9.7% of the total variance, respectively (54.7% total).

The first component (Table 2) seems to be most associated with the tissues. There are nine terpenoids with high, positive loadings (>0.5). Table 1 indicates that these are generally higher in the needles than in the leaf cushions. There are four terpenoids with negative loading (<−0.5), and these have higher levels in the leaf cushions than in the needles. This pattern among these fourteen terpenoids with positive or negative high loadings is often reversed in the current growth tissues in the spring period. This was identified in the second component, which is interpreted as the interaction between the age and period factors. There are high, positive loadings for isobornyl acetate and piperitone, and high, negative loadings for myrcene and germacrene D. The third component represents a weak relationship that may be represented by the interaction among all three factors in the statistical model (age, period, tissue). The high positive loading terpenoids (*o*-cymene, viridiflorene, δ-cadinene, and γ-cadinene) have higher percentages in leaf

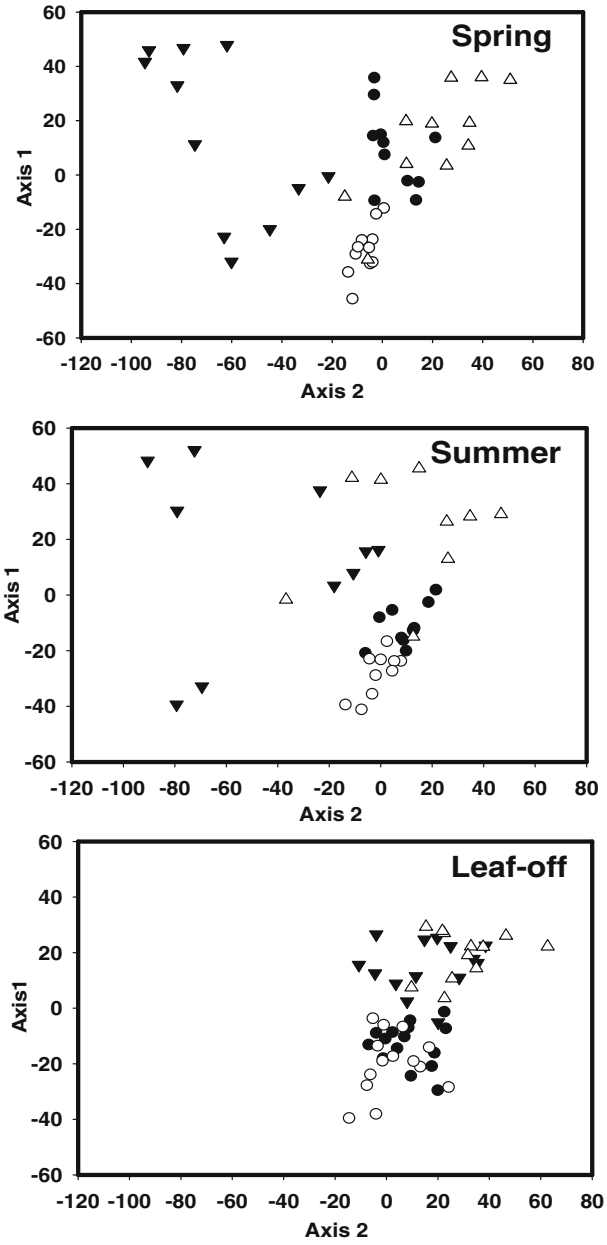


**Fig. 2** Classical multidimensional scaling ordination as a function of the terpenoid composition of each sample: 2002 leaf cushion (△), 2003 leaf cushion (▼), 2002 needle (○), and 2003 needle (●). The positions were simultaneously calculated for all samples and then separated into time periods of June 6–July 14 (spring), July 15–October 14 (summer), and October 15–May 10 (leaf-off)

cushions than needles except during the leaf-off period. This pattern is reversed for the high negative loading terpenoids isobornyl acetate and sabinine. Except for isobornyl acetate, the terpenoids with the higher loadings in this component are generally present in the samples at <1.0%. The importance of these low-level terpenoids is uncertain because in the PCA analysis the mean is subtracted across the data dimensions; hence, the PCA analysis does not consider the overall level of the terpenoid in the eastern hemlock.

**Table 2** Values of component loadings for the three factors and 23 terpenoids on the three components of the PCA

| | Components | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| *Age* | 0.126 | 0.444 | 0.373 |
| *Period* | 0.060 | 0.624 | −0.231 |
| *Tissue* | −0.783 | −0.076 | 0.268 |
| Tricyclene | 0.697 | 0.009 | 0.015 |
| α-Pinene | 0.745 | −0.449 | 0.133 |
| Camphene | 0.809 | 0.098 | −0.022 |
| Sabinene | 0.024 | 0.013 | −0.415 |
| β-Pinene | 0.839 | −0.056 | 0.222 |
| Myrcene | −0.064 | −0.725 | 0.088 |
| α-Phellandrene | 0.705 | 0.037 | 0.362 |
| o-Cymene | 0.038 | 0.033 | 0.579 |
| Limonene | 0.824 | 0.335 | 0.120 |
| β-Phellandrene | 0.713 | 0.427 | −0.021 |
| *cis*-Ocimene | 0.677 | −0.068 | 0.307 |
| Terpinolene | 0.784 | 0.265 | 0.130 |
| Borneol | 0.127 | 0.342 | −0.104 |
| Piperitone | 0.327 | 0.737 | 0.111 |
| Isobornyl acetate | −0.264 | 0.592 | −0.510 |
| β-Caryophyllene | −0.583 | 0.314 | 0.031 |
| β-Gurjunene | −0.312 | 0.024 | 0.342 |
| α-Humulene | −0.731 | 0.434 | −0.067 |
| γ-Muurolene | −0.301 | 0.069 | 0.396 |
| Germacrene D | −0.357 | −0.520 | 0.268 |
| Viridiflorene | −0.166 | 0.198 | 0.492 |
| γ-Cadinene | −0.612 | 0.405 | 0.468 |
| δ-Cadinene | −0.607 | 0.299 | 0.524 |

We identified six terpenoids considering components 1 and 2 together, and plotted their means in Fig. 3. Here, the focus was on comparison of the tissues on which the adelgid feeds (denoted with an ×) with the other tissues. Although the adelgid does not feed on needles, we hypothesized that we could use them as a means to probe host–insect chemical interactions, as the terpenoid levels in needles are more readily analyzed. Although there was not an exact match between terpenoid percentages between the two tissues, levels were similar. Furthermore, all plots in Fig. 3 show that during adelgid feeding periods in the leaf cushion, the tissue that is being fed upon has a stable percentage of the terpenoid.

Myrcene levels in the leaf cushion, but not the needles, were elevated in the immature foliage shortly after shoot extension. Myrcene dramatically increased to become a dominant terpenoid in the leaf cushion during the period over the summer months and decreased by leaf-off to endogenous levels present in previous year's growth tissues. The other dominant terpenoids in the leaf cushion were isobornyl acetate and germacrene D. In November, the needle took on a dark green color and a dramatic hardening of the cushion was evident. Thus, the visual physical maturation of new growth coincided with the chemical maturation of the new growth as well as with the conclusion of estivation and the beginning of the sistens feeding period. Throughout this time period, myrcene levels in the needles remained equivalent to the current and previous year's growth needles.

The period behavior of the sesquiterpene germacrene D in leaf cushions was similar to that of myrcene in leaf cushions. However, unlike myrcene, slightly elevated levels of

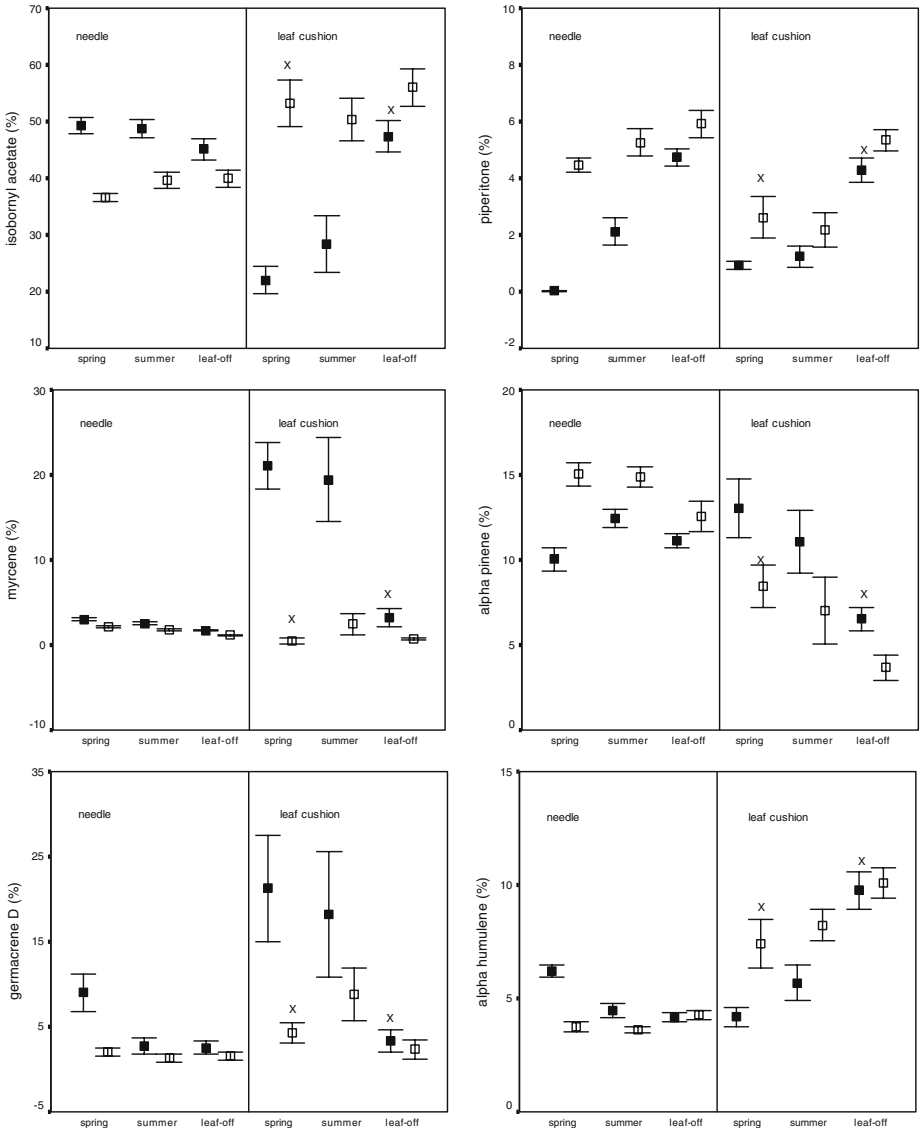


**Fig. 3** Select terpenoid percentages (mean±standard deviation) as a function of the period factor grouped according to tissue and clustered by age: current year's (2003) growth (■), previous year's (2002) growth (□). The symbol × indicates a period and tissue fed upon by the hemlock woolly adelgid

germacrene D were also found in needles during the spring period. Once again, by approximately November 2003, the levels of germacrene D decreased to levels that are indistinguishable from the new and previous year's growth in both areas of tissue. The monoterpene, α-pinene behaved similarly to germacrene D, as indicated by its 2nd component PCA loading. As indicated by a positive 1st component loading, α-pinene is slightly more abundant in needles than leaf cushions.

Isobornyl acetate is the predominant volatile chemical constituent of both the needle (40–45%) and leaf cushion (50–55%) in mature tissue. In spring, new growth needle tissue, isobornyl acetate levels are increased relative to old growth needle tissue while the leaf cushion tissue exhibits a depletion in isobornyl acetate compared to previous year leaf cushions. Although at lower relative percentages, α-humulene, and piperitone display depletion in the leaf cushion similar to isobornyl acetate as indicated by their 2nd component PCA loading.

## Discussion

The objective of this study was to determine if temporal and spatial variations in percentages of volatile terpenoids in eastern hemlock are linked to the biological life cycle of the HWA. The HWA positions itself distally to the leaf abscission layer but the stylet bundle is inserted proximally to the leaf abscission layer. Electron micrographs reveal that the 1.04- to 1.27-mm stylet bundle penetrates in the proximal direction of the twig and not in the distal direction of the needle tip, terminating in the ray parenchyma cells located within the leaf cushion (Young et al., 1995). A statistically detectable temporal and spatial variation of germacrene D and isobornyl acetate exists within the leaf cushion. In our previous work, PCA identified germacrene D as a compound with a high factor loading in the resistance/susceptibility component of an interspecies comparison among worldwide *Tsuga* (Lagalante and Montgomery, 2003). Comparison of germacrene D levels in the needles among seven species of *Tsuga* revealed that elevated percentages are found in species that are considered resistant to mortality from the HWA as compared to susceptible species (21.65% in *T. mertensiana* and 10.58% in *T. sieboldii*). Also, isobornyl acetate levels in these *Tsuga* species are elevated in the susceptible species. Unlike germacrene D, isobornyl acetate may function as a potential attractant to the HWA.

If these terpenoids have the ability to attract or deter HWA, then settling preferences may be distinguishable between the sistens and progrediens generations of the adelgid. Generally speaking, terpenoids are often toxic or repellant to insects due in part to their ability to inhibit acetylcholinesterase in the neuromuscular junction (Harrewijn et al., 2001). Although monoterpenes do not act on the acetylcholine receptor directly, they can function as competitive inhibitors through binding with acetylcholine. Additionally, HWA, like many herbivorous insects, harbors symbiotic microorganisms that are essential to its survival (Shields and Hirth, 2005). Such symbionts may be susceptible to antibiotic effects of terpenoids.

The crawler phase of the 1st instar of both the sistens and progrediens generations is the only stage of adelgid on hemlock that is capable of selecting feeding sites. Thus, variation in terpenoid levels during each generation's crawler stage may influence settling preferences and survival. For the sistens generation, depending on local climate and elevation, crawler settling generally occurs by mid-July, at the end of the "spring" period. If the sistens crawler settles on new growth, it is likely to encounter a different terpenoid profile in needle and leaf cushion tissue. Depending on the health of the tree, sistens crawlers will settle preferentially on new growth in July (McClure, 1991). However, they do not feed during the summer but instead enter estivation until cooler weather in October when estivation is broken. The adelgid then feeds over the colder winter months and matures to produce up to 300 eggs by April. Following initial high colonization on new growth foliage, the production of the next year's new growth will be severely inhibited, forcing colonization on previous years growth and a subsequent population crash (McClure, 1991).

Most aphids and adelgids avoid mature tissues and feed preferentially on new growth, possibly because older foliage has lower levels of nutrients and higher levels of allelochemicals (Miles, 1990). The primary content of the parenchyma cells of softwoods are steryl esters, fats, and waxes, whereas the canal resin is primarily composed of terpenes and terpenoids (Back, 2002). The salivary sheaths of aphid and adelgid stylet bundles may be able to seal off cell ruptures along the feeding path to avoid plant chemical defenses and wounding responses by absorbing allelochemicals before the undamaged, surrounding cells can be signaled (Miles, 1987, 1990). Since the function of the parenchyma cells is to store metabolites that are mobilized for new growth the following spring, the parenchyma cells of the new growth tissue should not possess high nutrient levels until mid-fall. Thus, the sistens crawlers could not detect high nutrient levels to select an optimal feeding site during stylet probing in July.

Given the general unsuitability of older growth foliage, one might conjecture that a seasonal chemical signal may serve as an indicator of tissue suitability during the period when the adelgid probes leaf cushion tissue with its stylet bundle. The spring emergence of western spruce budworm (*Choristoneura occidentalis* Freeman) larvae has been associated with the annual cycle of select foliar monoterpenes in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) as an indicator, not the cause, of foliage suitability (Nealis and Nault, 2005). Similarly, elevated relative levels of either myrcene or germacrene D detected while probing leaf cushions may be indicators to settle on new growth tissue that will be nutrient-rich during fall. Although myrcene and germacrene D were identified as potential deterrents to HWA in our previous study, the adelgid estivates with its stylet inserted, but does not actually feed during summer when these chemicals are elevated; thus, any possible toxic effects are avoided. By the time feeding commences in mid-October, terpenoid levels in new growth tissues have been reduced to background endogenous levels.

Studies linking terpenoid composition to HWA infestation levels are limited to a single reported study in which correlations were made between the volatile emission from eastern hemlock and adelgid population levels between August 20 and August 28, 2001, in a nursery plot in Blacksburg, VA, USA (Broeckling and Salom, 2003). These researchers reported that in respect to the relative percentage of individual terpenoids, there was a stronger correlation with adelgid population levels than with foliage age, although the two variables were not independent of one another. The hemlock trees selected in our study were healthy, displaying no outward signs of even moderate adelgid infestation. Thus, we would not have observed this density-dependent feedback with volatile emissions from the previous year's growth tissues.

Unlike the sistens generation, the progrediens generation 1st instar crawler does not have a choice of settling on new or previous year's growth because all the foliage is mature in March. From April to June, the progrediens generation crawlers will feed in the previous year's growth tissue that has a more stable composition of terpenoids. Interestingly, if the population of overwintering sistens is high on the shoot, the progrediens crawlers appear to survive better on the older growth, more proximal on the shoot. The progrediens crawlers mature to produce approximately 50 eggs that will become the sistens generation. Late hatching progrediens crawlers also have the possibility of settling on new foliage. Unfortunately, we could not sample this new growth (immediately post bud-break in May) due to difficulties in dissecting the pliant, young plant tissue. Therefore, the temporal and spatial variations in terpenoid levels in this study are more open to interpretation for the sistens generation.

Mortality of progrediens crawlers on new growth foliage is in agreement with our previous PCA assertion that identified myrcene and germacrene D as potential chemical deterrents (Lagalante and Montgomery, 2003). Unlike the sistens crawler that settles on

new growth and estivates, a progrediens crawler that settles on new growth immediately begins feeding on the immature leaf cushion and may ingest elevated levels of the potentially toxic myrcene and germacrene D. Although we did not separate enantiomers on a chiral GC column, germacrene D isomers are reported in volatile chemical communications in the aphids *Euceraphis punctipennis* (Zetterstedt) (Francis et al., 2005) and *Therioaphis maculate* (Buckton) (Bowers et al., 1977). (−)-Germacrene D elicited a strong repellant response in *Sitobion avenae* (F.) although (+)-germacrene D isomer elicited no response (Bruce et al., 2005). High concentrations of limonene and myrcene in Douglas-fir deter *A. cooleyi* (Gillette) (Stephan, 1987), and high concentrations of myrcene and piperitone in Sitka spruce [*Picea sitchensis* (Bong.) Carr.] deter several species of aphids (Jackson et al., 1996). Thus, it is reasonable to hypothesize that elevated relative levels of select terpenoids in new growth of eastern hemlock leaf cushion tissue would promote mortality in the progrediens generation of *Adelges tsugae* that settles on new foliage. Likewise, if isobornyl acetate is a chemical attractant for HWA, decreased relative levels in new growth leaf cushions may promote stylet insertion in cushions from the previous year's growth where levels of isobornyl acetate are elevated (Fig. 3). This chemical cue would promote survival of the progrediens generation crawler on previous year's growth, as levels of the all terpenoids are already reduced to endogenous levels during the progrediens crawler stage. The crawler stage of *Adelges piceae* (Ratzeburg) on *Abies balsamea* (L.) continually touch the bark surface with their antennae until settling, suggesting that the crawler is possibly seeking a stimulus from parenchyma cells (Bryant, 1974).

A detailed study of HWA fecundity and population levels as related to settling preferences may further elucidate the role of temporal and spatial chemical variation in levels of terpenoids and their role in resistance/susceptibility to adelgid infestation. Visual maturation of the eastern hemlock foliage was coincident with the levels of all volatile terpenoids reaching the endogenous levels found in the previous year's tissues. We conclude that deviations from endogenous levels for isobornyl acetate, myrcene, and germacrene D have biological implications in relation to the feeding periods of the hemlock woolly adelgid on eastern hemlocks.

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