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Seasonal changes in eastern hemlock (*Tsuga canadensis*) foliar chemistry

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Abstract

Eastern hemlock (*Tsuga canadensis* (L.) Carrière; hemlock) is an eastern North American conifer threatened by the invasive hemlock woolly adelgid (*Adelges tsugae* Annand). Changes in foliar terpenes and phenolics were evaluated in new (current year growth) and mature (1-year old growth) hemlock needles during the growing season and into plant dormancy. From April through September, foliar concentrations of non-volatile soluble phenolics, condensed tannins, lignin, mono- and sesquiterpenes α-pinene, camphene, isobornyl acetate, and diterpene resin were quantified. After September, additional analyses of metabolites that continued to differ significantly in new versus mature foliage were carried out. Total soluble phenolic concentration and condensed tannin concentration in new foliage remained low relative to mature foliage throughout the growing season and converged in December. Lignin concentration in new foliage converged with that of mature foliage by July. Concentrations of α-pinene, camphene, isobornyl acetate, and diterpene resin in new foliage converged with mature foliage within one month of budbreak. The convergence of terpene concentrations in new and mature foliage suggests that these metabolites may play a role in herbivore defense during the peak growing season. Conversely, soluble phenolics, including condensed tannins, may defend foliage from herbivory outside of the spring growth period.

Key words

Phenology, terpenes, phenolics, conifers, hemlock
Introduction

Phenology is the study of seasonally varying developmental events driven by environmental cues. Plant phenology is characterized by temporal patterns of growth associated with abiotic factors such as degree day, amount of transmitted light, and precipitation (Nault 2003; Rathcke and Lacey 1985; Smith 1982). In seasonal climates, leaf growth and development occur largely during a specific time of year (Fenner 1998). In temperate forests of the northern hemisphere, for example, plants leaf out rapidly in the spring to take full advantage of the growing season. Rapid leaf expansion/development is associated with substantial physiological changes during tissue maturation (Koricheva and Barton 2012; Wiggins et al. 2016). In mountain birch (Betula pubescens subsp. czerepanovii Ehrhart), for example, leaf toughness increases, amino acid levels decrease, and different phenolic compounds (e.g., proanthocyanidins, gallotannins, flavonoids) show discrete accumulation patterns throughout the growing season (Riipi et al. 2002).

Knowledge of plant leaf phenology is largely derived from deciduous trees (for example: Fenner 1998; Mauffette and Oechel 1989; Schultz et al. 1982)(Wiggins et al. 2016). Evergreen conifers, however, make up a significant proportion of global woody plant biomass (Waring and Franklin 1979) and exhibit unique foliar expansion patterns. For instance, loblolly pine (Pinus taeda Linnaeus) needles have three distinct periods of seasonal development, shifting from winter dormancy to an April-September period of rapid growth and completing needle expansion before the end of the year (Sampson et al. 2003). Conifer foliar chemistry also varies temporally, with shifting amounts of various secondary metabolites that are central to defense against herbivory as needles mature. Expanding jack pine (Pinus banksiana Lambert) needles, for example, have high levels of a Neodiprion sawfly antifeedant compound that rapidly decreases in
concentration as the needles mature (Ikeda et al. 1977). Phenolic compounds and terpenes are the
two primary classes of defensive metabolites present in conifer needles (Raffa et al. 2017); their
concentrations generally relate to needle age (Mumm and Hilker 2006). Understanding the
phenology of secondary metabolites is important, since changes in these compounds can alter
plant resistance to abiotic (e.g., drought, excess light) and biotic (e.g., herbivore and pathogen
attack) stressors.

Terpenes function in a wide array of ecological processes vital to conifer survival. These
include regulating community dynamics through allelopathic inhibition of seed germination,
altering rates of soil nutrient cycling and nitrification, and conferring resistance to herbivores and
pathogens (Langenheim 1994; Michelozzi 1999; White 1994). For individual terpene compounds
in conifer needles, the time-concentration relationship is often nonlinear and can vary throughout
the growing season. In newly emerged needles of scots pine (*Pinus sylvestris* Linnaeus), for
instance, α-pinene accumulates during the growing season while δ-3-carene levels decrease
rapidly after budbreak (Thoss et al. 2007). Emerging douglas fir (*Pseudotsuga menziesii* (Mirb.)
Franco) needles show similarly dynamic changes in monoterpene levels, with α-pinene and β-
pinene reversing their abundances as young needles expand (Nealis and Nault 2005).

Phenolic compounds in conifers contribute to needle structural development, tissue
toughness, and defense against damage by pests and pathogens (Isah 2019). In response to leaf
damage, various phenolic compounds are polymerized and covalently bound to cell walls,
sealing off sites of infection or injury and strengthening leaf tissue against further damage
(Beckman 2000). Phenolics also play a direct role in defense (i.e., toxicity to herbivores and
pathogens) and can deter insect herbivore oviposition and affect larval performance (Pasquier-
Barre et al. 2000). Phenolics have well-known roles in abiotic stress resistance as well, such as
oxidative stress relief and protection from UV radiation (Appel 1993; Isah 2019). These compounds also vary substantially with needle age: Hatcher (1990) found that the immature needles of five conifer species had lower phenolic concentrations than mature needles on the same branch.

Research exploring temporal variation in conifer foliar chemistry has focused on a few species in the pine family (Pinaceae), to the exclusion of other ecologically significant species (see: Hatcher 1990; Nault 2003; Nealis and Nault 2005; Nerg et al. 1994; Thoss et al. 2007). Eastern hemlock (Tsuga canadensis (L.) Carriére; hemlock) is one such conifer, a long-lived canopy dominant endemic to forests of the eastern U.S. (Orwig et al. 2008). Hemlock is responsible for important ecological functions, including soil moisture regulation and protecting riparian habitat from lethal temperature extremes (Ellison et al. 2005). It is currently under threat of extirpation by hemlock woolly adelgid (Adelges tsugae Annand; adelgid), an invasive stylet-feeding insect from Japan that has caused widespread mortality and decline of hemlock (see: Dharmadi et al. 2019, McClure 1987) since its introduction nearly 70 years ago (Havill et al. 2006).

Both the sistens and progrediens adelgid generations feed preferentially on mature hemlock foliage (Lagalante et al. 2006), and this is likely driven by chemical differences between new and mature needles. Adelgid resistance in western hemlock (Tsuga heterophylla (Raf.) Sargent) and Asian hemlock species, for instance, has been linked to terpene profiles that differ substantially from those of adelgid-susceptible species (Lagalante and Montgomery 2003). Adelgid-resistant eastern hemlock cultivars also have unique foliar terpene profiles (Lagalante et al. 2007). Additionally, resistance to adelgid has been documented in rare individual eastern hemlocks lingering in adelgid-devastated forests (Ingwell and Preisser 2011); chemical analyses
found higher terpene concentrations in the needles and twigs of these putatively adelgid-resistant eastern hemlocks compared to adelgid-susceptible controls (McKenzie et al. 2014).

Moreover, increasing terpenoid concentrations in expanding hemlock needles has been correlated with reduced fecundity of elongate hemlock scale (*Fiorinia externa* Ferris), another pest of eastern hemlock introduced from Japan (McClure and Hare 1984). Herbivores of other feeding guilds are also impacted by hemlock’s foliar chemical phenology. Hemlock looper (*Lambdina fiscellaria* Guenée), an important defoliator of eastern hemlock, feeds preferentially on specific needle age classes, with early instar larvae feeding on expanding needles and mature larvae shifting to old-growth foliage (Carroll 1999).

While multiple eastern hemlock studies have explored chemical defense induction in response to adelgid and other herbivores (e.g. Broeckling and Salom 2003; Rigsby et al. 2019), there has been less work addressing constitutive levels of foliar terpenes (but see: McKenzie et al. 2014). Only two studies have addressed phenological changes in hemlock terpene emission rates (Lagalante et al. 2006; McClure and Hare 1984). These studies only considered the volatile fraction of hemlock terpenes, and there has been no work addressing non-volatile terpenes in hemlock. While volatile terpene emissions are ecologically significant (e.g., antixenosis), since herbivores feed directly on plant tissue, non-volatile terpenes are more important in direct resistance to herbivore attack (i.e., antibiosis).

At least three studies have identified the foliar terpenes α-pinene, camphene, and isobornyl acetate as the most significant terpenes in hemlock’s interactions with adelgid (Lagalante et al. 2006; Lagalante and Montgomery 2003; Lagalante et al. 2007), a fact that suggests they play a role in hemlock’s defense against adelgid herbivory. We are unaware of any studies examining the role of these terpenes in eastern hemlock’s interactions with hemlock
looper, however, relationships between these terpenes and other lepidopteran folivores are well
documented. Western spruce budworm (*Choristoneura occidentalis* Freeman) resistance in
douglas fir, for example, is associated with higher foliar concentrations of camphene and
isobornyl acetate (Chen et al. 2002). Likewise, hemlock foliar phenolics have also been
explored, but only in the context of induced defenses (Rigsby et al. 2019, Rigsby et al. in
review). Increased cell-wall bound phenolic and lignin concentrations, for example, have been
documented in adelgid-infested hemlock foliage without corresponding changes in oxygenated
terpenes (Rigsby et al. 2019). Ultimately, within the context of phenology, dynamic changes in
non-volatile phenolic and terpene accumulation in eastern hemlock remains unexplored.

In the present study, changes in non-volatile terpenes and phenolics were evaluated in
both newly-produced and mature eastern hemlock foliage though a growing season and into
plant dormancy. Temporal changes in the foliar concentration of some major defensive
secondary metabolites were outlined, including monoterpenic compounds, non-volatile resin,
soluble phenolics and condensed tannins, in both expanding and mature needles. Foliar
concentrations of lignin, a structural as well as defensive secondary metabolite, were also
measured. These data were used to identify when metabolite levels in expanding needles were
statistically indistinguishable from those found in mature needles. We hypothesized that (1)
relatively low levels of lignin in new foliage would be accompanied by relatively greater levels
of phenolic and terpene defensive metabolites. Our reasoning was that new foliage, being more
attractive to herbivores (Lempa et al. 2001) and of greater fitness value (Heath et al. 2014),
would rely on these non-lignin defensive metabolites while actively growing. We also
hypothesized that (2) concentrations of all metabolites would converge with levels in mature
needles by the end of the spring growth period. Studies of the chemical phenology of model
conifer species indicate that terpene and structural metabolite levels in expanding needles become indistinguishable from mature needles by the end of the growing season (Hatcher 1990; Nault 2003; Thoss et al. 2007). Specifically for eastern hemlock, volatile terpene concentrations in new foliage have been shown to converge with mature foliage by fall leaf-off (Lagalante et al. 2006). Here, we provide a first look at eastern hemlock’s chemical phenology.

Materials and Methods

Common Garden – In April 2014, 320 adelgid-free, chemically untreated hemlock saplings (0.5-0.7 m tall; two years in age) grown from seed collected in Pennsylvania, were purchased from Van Pines Nursery (West Olive, MI). These saplings were planted in five blocks of 64 plants, with ≥1.5 m between each sapling, in the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, RI). Plants were protected from both vertebrate and invertebrate herbivory with chicken-wire cages covered in mesh bags (Agribon-15, Johnny’s Selected Seeds, Waterville, ME, USA; 90% light transmission). In early spring 2018, we randomly selected 12 1.0-1.2 m tall herbivore-free saplings from four of the five blocks for this work. Between two and five plants were sampled from each block. This discrepancy in the number of plants sampled per block existed because we desired to include insect-free trees from each block, but multiple experiments were either occurring or had occurred within this common garden. Several of the trees in this garden also experienced a spruce spider mite (Oligonychus ununguis Jacobi) infestation that was avoided by our plant selection. These necessitated the selection of these specific plants.

Beginning on 26 April 2018, we removed one 20 cm terminal branch of mature (1-year old growth) foliage per plant; newly produced foliage was not sampled in April since bud break did not occur until mid-May. We returned to each sapling on 31 May 2018 and destructively
sampled two 20 cm terminal branches, one of mature foliage and another of expanding (current year growth) foliage. This protocol meant that we collected one branch per tree during the April sampling and two branches per tree during the May sampling and all subsequent sampling dates. Each terminal branch was excised with pruning shears, wrapped in aluminum foil, placed in a cooler on ice and brought back to the laboratory, where it was stored at -80°C until processed. The total sampling time (i.e., from when the first sample was clipped to the time all samples were placed at -80°C) always took < 1 hr to perform, samples were immediately immersed in ice as soon as they were sampled, and trees were sampled haphazardly (with regards to the order of sampling) to avoid treatment artifacts that were due to our sampling procedure. We chose this procedure over clipping individual needles from sampled branches in the field (thus leaving behind a completely defoliated woody stem) on the basis of time. While our chosen method always took less than one hour (first branch clip to when all tissue was flash-frozen), in a pilot experiment we found that carefully removing each individual needle from each sampled branch in the field more than tripled the time between tissue removal and flash-freezing. Branch clippings occurred monthly on the following dates: 26 April, 31 May, 28 June, 26 July, 30 August, and 27 September 2018. After this final date, we only continued to assay secondary metabolites that differed significantly in new versus old foliage. We added two additional sampling dates (19 December 2018 and 28 January 2019) in which we quantified these remaining metabolites. By the end the experiment, our cuttings had removed less than 5% of the total foliage from each sapling.

Tissue Preparation – Needles were removed from each branch sample and ground to a powder in liquid nitrogen using a mortar and pestle. New foliage was processed separately from mature foliage. The powder was partitioned into three tubes: 100 (± 5) mg in a 1.5 ml microtube
for the phenolic analyses (total soluble phenolics, condensed tannins, and lignin), 100 (± 5) mg
in another 1.5 ml microtube for GC-FID analysis of major mono- and sesquiterpenes, and 1 (±
0.05) g in a 15 ml Falcon tube for non-volatile resin analysis. Tubes were stored at -30° C until
analysis, and analyses were conducted within two days of tissue grinding.

**Total Soluble Phenolics** – Soluble phenolics were extracted in HPLC-grade methanol and
total soluble phenolic levels quantified similarly to Rigsby et al. (2019), via Folin assay, with
minor enhancements for optimization. Twenty-five µl extract was first diluted in 75 µl methanol,
and 500 µl water was then added, followed by 40 µl Folin-Ciocalteu reagent (Sigma-Aldrich).
Tubes were incubated at room temperature for 10 min, then 40 µl 1 M NaHCO₃ was added, and
the tubes incubated at room temperature for 1 hour. Absorbance was then quantified at 725 nm
using a SpectraMAX M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA,
USA) and Greiner UV-Star® 96 well plates (Monroe, NC, USA). In place of gallic acid,
chlorogenic acid (Sigma-Aldrich) was used to generate a standard curve, and phenolic
concentration was expressed as chlorogenic acid equivalents (mg g⁻¹ FW). Condensed tannin
concentration was also quantified as per Rigsby et al. (2019), by incubating methanol extracts
(250 µl) with 750 µl 95:5 butanol:HCl for three hours at 95° C. After cooling, absorbance at 550
nm was quantified using a Turner® SP-830 cuvette spectrophotometer and plastic cuvettes
(expressed as OD₅₅₀ g⁻¹ FW).

Although Appel et al. (2001) raised concerns regarding the use of Folin assays in
quantifying temporal or species variation in foliar phenolic levels in ecological studies, recent
work by our research group has revealed that chlorogenic acid derivatives dominate (≥ 70%) the
soluble phenolic profile of hemlock foliage (Rigsby et al. in review) throughout the year
(unpublished data). We conducted preliminary experiments showing that total soluble phenolic
content estimated with the spectrophotometric procedure described above, using chlorogenic acid as standard, is highly correlated with phenolics quantified via HPLC-UV$_{280nm}$ ($R^2 \geq 0.88$; unpublished data), regardless of sampling month or tissue age. This background work led us to conclude that this more cost- and time-effective Folin spectrophotometric assay provided reasonable estimations of foliar soluble phenolic concentrations in lieu of phenolics quantified via HPLC. We acknowledge, however, that despite this preliminary work and that fact that we could obtain a close estimation of HPLC-quantified phenolics with Folin-quantified phenolics, the Folin method could, and likely did, miss quantitative variation in the contents of phenolic compounds.

**Lignin** – Lignin levels were quantified as per Villari et al. (2012). Briefly, the leftover pellet from the soluble phenolic extraction was washed twice with methanol, allowed to air-dry overnight, then resuspended in 400 µl 1 M NaOH and incubated for 24 hours at 40° C. The homogenate was acidified with 200 µl 1.5 M formic acid, and 400 µl methanol was added. The tubes were centrifuged at 16,000 x g for 5 min and the supernatant discarded. Pellets were then washed twice with 1 ml methanol, and 1 ml 2 M HCl was added to the tubes followed by 250 µl thioglycolic acid. The tubes were incubated for 4 hours at 85° C. Once cooled to room temperature, tubes were centrifuged at 16,000 x g for 5 min and the supernatant was discarded. Pellets were then washed once with 1 ml water, and thioglycolic acid-lignin pellets were extracted overnight in 1 ml 1 M NaOH. The tubes were centrifuged at 16,000 x g for 5 min and the supernatant saved; this extraction step was repeated and supernatants combined. Extracts were acidified with 300 µl concentrated HCl and incubated at room temperature for 4 hours. Tubes were then centrifuged at 20,000 x g (5 min), supernatants discarded, and pellets allowed to dry overnight at 40° C. The following day, pellets were resuspended in 1 ml 1 M NaOH. The
absorbance of 20-fold dilutions (with 0.5 M NaOH) at 280 nm was quantified using the SpectraMAX microplate reader and Greiner UV-Star® 96 well plates against a standard curve of spruce lignin (expressed as mg g⁻¹ FW).

Mono- and Sesquiterpenes – α-Pinene, camphene, and isobornyl acetate (Sigma-Aldrich) were extracted and quantified via GC-FID, similarly to Rigsby et al. (in review) with minor adjustments in the oven program to optimize rapid quantification of these three compounds. These are the three dominant terpene species of hemlock foliage and constitute ≥ 75% of all terpenes identified in eastern hemlock (Broeckling and Salom 2003; Rigsby et al. in review).

Foliar terpenes were extracted in 700 µl n-hexane containing 1 µl ml⁻¹ m-xylene as an internal standard by sonicating homogenates for 10 min in an ice bath. Tubes were then vortexed for 10 s and the 20,000 x g (5 min, 0° C) supernatant transferred to a 2 ml glass autosampler vial, capped with a PTFE-coated screw cap, and stored at -30° C until injected into the GC (within 48 hours).

The instrument, settings, gases, column, injection volume, and external standards described by Rigsby et al. (in review) were used here. Mono- and sesquiterpene identification and quantification took place using a Shimadzu GC 2010 Plus gas chromatograph equipped with an AOC-20i autosampler and a flame ionization detector (GC-FID). Nitrogen was used as the carrier gas at a flow rate of 1.0 ml min⁻¹ and an HP-5MS column (30 m x 0.25 mm internal diameter; 0.25 µm film thickness). Terpene extract (2 µl) was injected using a split flow ratio of 30:1, and the injector and detector temperatures were set to 260° C and 300° C, respectively. The adjusted oven program was: 40° C for 5 min, increased by 5° C min⁻¹ to 225° C, increased by 25° C min⁻¹ to 280° C, and held at 280° C for 5 min (total run time = 49.2 min). Peaks were matched to external standards based on retention times, and tissue amounts of terpenes (µg g⁻¹ FW) were estimated using three-point standard curves of standards (R² > 0.99).
Diterpene Resin – Non-volatile diterpene resin concentration was estimated gravimetrically using standard techniques for estimating the non-volatile resin concentrations of pine foliage, which is highly correlated with diterpene resin acid concentration measured via GC-MS (Moreira et al. 2016). Briefly, 1 g tissue powder was extracted in 3 ml n-hexane for 10 min in a sonicator, centrifuged at 4,000 x g, and the supernatant transferred to a 15 ml Falcon tube. This extraction procedure was repeated twice and the supernatants combined in a pre-weighed 15 ml Falcon tube. Uncapped tubes were then placed in a fume hood and the solvent evaporated to dryness (approximately four days) before reweighing. The before-after difference in tube weight was considered the mass of non-volatile resin (expressed as mg g⁻¹ FW). It is worth noting that hemlock diterpenes have not been characterized and we, therefore, are unable to directly relate diterpene content quantified with GC-MS with content quantified by this gravimetric method as has been shown and used in many species of pine (e.g, Moreira et al. 2016). Our n-hexane extracts would certainly have extracted other non-polar, non-volatile metabolites aside from diterpenes and these could have contributed to the change in tube weights. Our results should, therefore, be interpreted cautiously until hemlock diterpenes can be characterized.

Statistical Analysis – R software v. 3.5.0 was used for all analyses (R Development Core Team, 2018). Secondary metabolite concentrations in new and mature foliage were analyzed via linear mixed-effects models, using lme4 (Bates et al. 2015). Foliage age, month, and their interactions were treated as fixed effects, and block and tree were treated as random effects. A type III analysis of variance (ANOVA) was used to evaluate each model term for significance. Month-specific differences in metabolite concentrations in new and mature foliage were analyzed for significance using diffmeans in the lmerTest package (Kuznetsova et al. 2017),
and the Holm-Bonferroni family-wise error rate-controlling procedure was used to correct for multiple comparisons.

Results

Phenolics – Phenolic concentration differed significantly in new versus mature foliage for all measured compounds (all $F_{[1,1]} > 183.17, P < 0.05$; Fig. 1A – 1C). Phenolic levels also varied by month (all $F_{[1,7]} > 77.82, P < 0.001$), and there was a significant month × foliage age interaction (all $F_{[1,6]} > 7.47, P < 0.001$). Specifically, total soluble phenolic and condensed tannin concentration was lower in new relative to mature foliage throughout the growing season (May through September) (means separation test both $P < 0.001$; Fig. 1A, B). It took until December for concentrations of total soluble phenolics and condensed tannins in new and mature foliage to converge (means separation test both $P > 0.05$). At this point, total soluble phenolic concentrations in new and mature foliage reached 59.6 mg/g FW ± SE and 73.5 mg/g FW ± SE, respectively, and condensed tannins in new and mature foliage reached 11.7 mg/g FW ± SE and 14.4 mg/g FW ± SE, respectively. Conversely, lignin levels in new foliage rapidly increased at the start of the growing season and became indistinguishable from that of mature foliage by July, with concentrations reaching 11.6 mg/g FW ± SE in new foliage, and 14.0 mg/g FW ± SE in mature foliage (means separation test $P = 0.097$; Fig. 1C).

Terpenes – In contrast to phenolics, terpene concentrations in new foliage rapidly converged with those of mature foliage. Statistically similar levels of quantified terpenes in new and mature foliage occurred within one month of budbreak (means separation test all $P > 0.05$; Fig. 2A – 2D). By June, for instance, α-pinene concentrations in new and mature foliage were 0.71 mg/g FW ± SE and 0.75 mg/g FW ± SE, respectively. Terpene levels varied by month (all $F_{[1,5]} > 154.38, P < 0.001$), and there was a significant month × foliage age interaction (all $F_{[1,4]} >
Levels of camphene, α-pinene, and isobornyl acetate were higher, and resin concentration lower, in new versus mature foliage in September (separation of means test all \( P < 0.001 \)). Resin concentration in new and mature foliage in September, for instance, was 50.4 mg/g FW ± SE and 56.7 mg/g FW ± SE, respectively. Conversely, levels of isobornyl acetate in new and mature foliage were 2.9 mg/g FW ± SE and 2.5 mg/g FW ± SE, respectively, and camphene levels in new and mature foliage had reached 4.7 mg/g FW ± SE and 3.7 mg/g FW ± SE, respectively.

**Discussion**

New foliage contained significantly lower concentrations of total soluble phenolics, condensed tannins, and lignin compared to mature hemlock foliage (Fig. 1A – C). Phenolic concentrations also varied by month, and the monthly change in the concentration of each compound was different for new versus mature foliage. Total soluble phenolic and condensed tannin concentration was lower in new versus mature foliage throughout the growing season (May through September), rejecting our hypothesis that concentrations of all metabolites would converge with levels in mature needles by the end of the spring growth period (Fig. 1A, B). Moreover, concentrations of both classes of compounds in new foliage did not converge with levels present in mature foliage until December (Fig. 1A, B). Concentrations of the structural metabolite, lignin, in new foliage became indistinguishable from levels in mature foliage as early as July (Fig. 1C). The incidence of low levels of lignin with similarly low levels of both phenolic and terpene concentrations did not support our first hypothesis that relatively low levels of lignin in new foliage would be accompanied by relatively greater levels of phenolic and terpene defensive metabolites. Instead, concentrations of the different metabolite groups measured in new foliage remained low relative to mature foliage, until the new foliage lignified.
$\alpha$-Pinene and isobornyl acetate levels were significantly lower in new foliage compared to mature foliage immediately post-budbreak in May, increased to convergence by June, and exceeded that of mature foliage in September (Fig. 2A, C). Resin concentration in new versus mature foliage was also initially low, but became indistinguishable from levels in mature foliage by June (Fig. 2D). Camphene concentrations in new and mature foliage were not significantly different immediately post-budbreak, and in September, camphene levels were higher in new foliage than in mature foliage (Fig. 2B).

This broad pattern of early-season convergence of terpene concentrations in new and mature foliage suggests that this class of secondary metabolites may play a significant role in eastern hemlock’s defense against herbivores that are active at the beginning of, and during, the peak growing season. Conversely, soluble phenolics, including condensed tannins, may be responsible for defending hemlock foliage from herbivory that occurs outside of the spring growth period. It is important to note, however, that the phenolic content of most plants varies not only in association with tissue age and/or herbivore activity, but according to other stressors, such as growing conditions (reviewed in: Levin 1971). For example, latitudinal variation in temperature, growing season duration, and sun exposure has a strong effect on the total phenolic concentration of scots pine needles (Nerg et al. 1994). Thus, while the apparent seasonal tradeoff between terpene and phenolic concentrations observed in this study may be an element of hemlock’s anti-herbivore defense complex, it may also be affected by seasonal variation in certain biophysical factors. Future studies should evaluate the extent to which seasonal herbivores, biophysical factors, and their interactions drive these patterns.

*Phenolics*
Total soluble phenolic and condensed tannin concentration in new foliage did not converge with levels in mature foliage until December (Fig. 1A, B). Total soluble phenolic concentration in new foliage was relatively high immediately after budbreak but declined throughout the growing season (Fig. 1A). Like terpenes, phenolics are a class of secondary metabolites that play a significant role in conifer defense against insect herbivory (Mumm and Hilker 2006). Because hemlock retains its needles throughout the year, it resumes photosynthesis well before new foliage emerges (Hadley 2000). As a result, hemlock buds are nutrient rich (Wilson et al. 2018), and elevated levels of certain soluble phenolics may be necessary to protect these tissues from early spring folivores. Elevated early-season phenolic concentrations is consistent with work by Hatcher (1990) documenting high phenolic concentrations in western hemlock needles at budbreak, followed by decreasing concentrations as needles expanded during the growing season. The subsequently low levels of total soluble phenolics and condensed tannins observed in expanding hemlock needles between June and September may be necessary for hemlock to avoid autotoxicity. At least one study suggests that compartmentalization problems prevent the accumulation of high amounts of tannins in new conifer foliage (Horner 1988). Evidence of low tannin concentration in expanding needles of both scots pine (Watt 1987) and douglas fir (Horner 1988) during peak growth, followed by post-growing season convergence with levels in mature needles for both trees, is also consistent with this hypothesis.

Condensed tannin concentration and total soluble phenolic concentration in new foliage became indistinguishable from mature foliage in December (Fig. 1A, B). This late-season convergence indicates that these metabolites may play a role in hemlock’s defense against later-arriving herbivores. In addition to phenolics directly deterring phytophagous insects, these compounds have also been shown to affect oviposition preference in certain pine-defoliating
insects (Leather et al. 1987; Pasquier-Barre et al. 2000). Oviposition on plants unsuitable for larval development reduces insect fitness, and elevated foliar tannins have been shown to reduce oviposition rates (Leather et al. 1987). One destructive pest of eastern hemlock, the hemlock looper, initiates oviposition in late September and into October (Dobesberger 1989), when our data suggests that newly produced hemlock needles begin to accumulate condensed tannins and other soluble phenolics. Given the shared evolutionary history of eastern hemlock and hemlock looper (Bhiry and Filion 1996), the fall/winter increase in foliar condensed tannin and total soluble phenolic concentration may have some significance for this interaction. Adelgid also feeds on hemlock tissue from October through April. While the two species have not co-evolved, adelgid feeding has been shown to dramatically increase both the condensed tannin (Rigsby et al. 2019) and soluble phenolic concentration (Pezet and Elkinton 2014; Rigsby et al. 2019) of hemlock foliage. This further suggests that condensed tannins and other soluble phenolics may be an important aspect of eastern hemlock defense against late-season herbivores.

Concentrations of the structural metabolite lignin in new foliage reached levels present in mature foliage after terpenes in July (Fig. 1C). Contrary to our first hypothesis, lignin concentration in new foliage was not exceeded by any individual defensive metabolite before it reached levels found in mature foliage. Furthermore, condensed tannins and total soluble phenolics in new foliage did not reach levels found in mature foliage until well after the growing season had ended. This did not support our second hypothesis that concentrations of all metabolites would converge with levels in mature needles by the end of the spring growth period, and suggests that the latter two groups of metabolites may be important against herbivores active outside of the spring growth period.

Terpenes
Terpene concentration in expanding needles converged with levels present in mature needles within one month of May budbreak (Fig. 2A – 2D). We found that α-pinene and isobornyl acetate concentration was significantly lower in new foliage than mature foliage in May, increased to convergence by June, and was higher in new foliage versus mature foliage in September (Fig. 2A, C). Similarly, resin concentration in new foliage was initially lower but reached that of mature foliage by June. Camphene concentration in new foliage was indistinguishable from mature foliage immediately post-budbreak; in September, it was higher in new foliage than in mature foliage (Fig. 2B). The fact that terpene concentration in expanding needles rapidly converged with levels in mature needles suggests that terpenes play an important role in defense against early-season herbivores (Mumm and Hilker 2006). Elevated levels of camphene and isobornyl acetate in douglas fir needles, for example, have been linked to western spruce budworm resistance (Chen et al. 2002), and both terpenes are highly toxic to the insect (Zou and Cates 1997). Newly-produced and expanding conifer needles are softer than mature needles (for example: Hatcher 1990); conifers compensate for lower structural defense with toxic secondary metabolites (Mumm and Hilker 2006). Since new foliage on hemlock does not fully lignify until at least July (Fig. 1C), it appears likely that terpenes fill this role.

Conclusions

Here, we provide a first look at eastern hemlock’s chemical phenology. Broadly, expanding hemlock needles had low concentrations of soluble phenolics and condensed tannins throughout the growing season and into plant dormancy, becoming indistinguishable from levels in mature foliage by December (Fig. 1A, 1B). Conversely, concentrations of the structural metabolite, lignin, rapidly increased in new foliage and converged with levels in mature foliage by July (Fig. 1C). Similarly, levels of α-pinene, camphene, isobornyl acetate, and resin in new
foliage converged with levels in mature foliage within one month of May budbreak (Fig. 2a–D). Rapid convergence of terpene concentrations in new and mature foliage may implicate this class of secondary metabolites in eastern hemlock’s defense against early-season herbivores, while soluble phenolics and condensed tannins may be act in hemlock’s defense against herbivores that feed during plant dormancy.

It is important to note, however, that there are limitations associated with the bulk-analysis approach to measuring plant secondary metabolites, and more refined tests will be needed to support ecological connections between the chemical patterns that we observed and the activity of hemlock herbivores. In addition to concentration, for example, the composition of terpenes and phenolics in foliage can change throughout the growing season (reviewed in: Iason et al. 2012). In expanding white birch (Betula papyrifera Marshall) leaves, for instance, hydrolysable tannins show distinct seasonal patterns, with certain individual tannins having an inverse time-concentration relationship (Salminen et al. 2001). Several studies have also documented this pattern for individual terpenes in conifer needles; their relative concentrations are often opposed, and can vary as needles expand (see: Nealis and Nault 2005; Thoss et al. 2007). Broad phenologic trends in hemlock’s foliar phenolic and terpene concentrations, while useful, cannot elucidate the role of individual chemical species in this tree’s herbivore defense complex. In particular, research that builds on and extends our findings by addressing the important roles of individual phenolics in mediating plant-insect interactions in this system is critical.

Since the introduction of adelgid nearly a century ago (Havill et al. 2006), there has been extensive mortality and decline of eastern hemlocks throughout eastern U.S. forests (Eschtruth et al. 2006; Orwig et al. 2002; Preisser et al. 2008). Hemlock is a late-successional species that has
adapted to grow in cool, understory microclimates (Hadley 2000); the loss of most canopy-

dominant hemlocks in this region has inhibited seedling recruitment (Ingwell et al. 2012; Orwig
and Foster 1998; Orwig et al. 2002), and virtually eliminated hemlock sapling regeneration
(Preisser et al. 2011). Hemlock-associated forests are now characterized by lower overstory
deciduous tree densities, novel understory vegetation communities (Ingwell et al. 2012), and
significantly reduced soil moisture and C:N ratios (Orwig et al. 2008). As hemlock continues to
be threatened with extirpation by adelgid, it will be important to understand the potency of
individual secondary metabolites in hemlock’s interactions with herbivores, in addition to
understanding broad seasonal trends, to effectively conserve this species. Putatively adelgid-
resistant eastern hemlocks, for instance, sustain lower adelgid densities (Ingwell et al. 2011), and
this is likely due to their unique terpene chemistry (McKenzie et al. 2014). However, the
individual terpene(s) species responsible for inhibiting adelgid population growth remains
unknown. Such a chemical marker could provide the means to identify candidate eastern
hemlocks for adelgid-resistance breeding programs and reforestation efforts, aimed at not only
restoring eastern hemlocks, but also maintaining the vital, broad-scale ecosystem functions that
this tree provides.

Acknowledgments

This research was supported by PA Department of Conservation and Natural Resources
DCNR 2016-001-HWA-URI, DCNR 2018-001-HWA-URI, COLCOM Foundation #20015270,
and McIntire-Stennis RI0017-MS979 from the USDA National Institute of Food and
Agriculture. We would like to thank three anonymous reviewers for comments that substantially
improved the manuscript.

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Figure Legends

Figure 1. Mean total soluble phenolic (A), condensed tannin (B), and lignin concentrations (C) in new (current-year growth) and mature (1-year old growth) eastern hemlock foliage throughout the growing season. New foliage was not produced until after the April sampling date. Asterisks represent significant differences in metabolite concentrations in new versus mature foliage; lines represent means ± 1 SE. Additional samples were analyzed in December and January to identify when phenolic and condensed tannin levels in new foliage converged with levels in mature foliage.

Figure 2. Mean α-pinene (A), camphene (B), isobornyl acetate (C), and resin (D) concentrations in new and mature eastern hemlock foliage throughout the growing season. New foliage was not produced until after the April sampling date. Asterisks represent significant differences in metabolite concentrations in new versus mature foliage; lines represent means ± 1 SE.
Figure 1.
Figure 2.

A. 

B. 

C. 

D. 

- One-year foliage
- New foliage