2020

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

Ian G. Kinahan
University of Rhode Island

Chad M. Rigsby
University of Rhode Island

Suzanne K. Savage
University of Rhode Island

Nick L. Houseman
University of Rhode Island

Andrew S. Marsella
University of Rhode Island

Citation/Publisher Attribution

Kinahan, I. G., Rigsby, C.M., Savage, S.K., Houseman, N.L., Marsella, A.S., Oppong-Quaicoe, A., DeBoef, B.L., Orians, C.M., and E.L. Preisser. 2020. Seasonal changes in eastern hemlock (Tsuga canadensis) foliar chemistry. Canadian Journal of Forest Research in press.

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

Ian G. Kinahan[1], Chad M. Rigsby[1,2], Suzanne K. Savage[1], Nick L. Houseman[1], Andrew S. Marsella[1], Anita Oppong[3], Brenton L. DeBoef[3], Colin M. Orians[4], and Evan L. Preisser[1*]

[1]Department of Biological Sciences, The University of Rhode Island, Kingston, RI 02881 USA

[2]Current address: Bartlett Tree Research Laboratories, The Morton Arboretum, Lisle, IL 60532 USA

[3]Department of Chemistry, The University of Rhode Island, Kingston, RI 02881 USA

[4]Department of Biology, Tufts University, Medford, MA 02155 USA

*Author for correspondence

Abstract

Eastern hemlock (*Tsuga canadensis* (L.) Carriére; 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 monoterpene 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 $\geq$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 $NaHCO_3$ 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 OD_{550} 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 ($R^2 > 0.99$).

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 *difflsmeans* 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 $F_{[1,4]} >$

61.04, $P < 0.001$). 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 $P < 0.05$; Fig. 2A – 2D). 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.

α-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

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

415 *Conclusions*

416 Here, we provide a first look at eastern hemlock's chemical phenology. Broadly,
417 expanding hemlock needles had low concentrations of soluble phenolics and condensed tannins
418 throughout the growing season and into plant dormancy, becoming indistinguishable from levels
419 in mature foliage by December (Fig. 1A, 1B). Conversely, concentrations of the structural
420 metabolite, lignin, rapidly increased in new foliage and converged with levels in mature foliage
421 by July (Fig. 1C). Similarly, levels of α-pinene, camphene, isobornyl acetate, and resin in new

422 foliage converged with levels in mature foliage within one month of May budbreak (Fig. 2a – D).
423 Rapid convergence of terpene concentrations in new and mature foliage may implicate this class
424 of secondary metabolites in eastern hemlock's defense against early-season herbivores, while
425 soluble phenolics and condensed tannins may be act in hemlock's defense against herbivores that
426 feed during plant dormancy.

427 It is important to note, however, that there are limitations associated with the bulk-
428 analysis approach to measuring plant secondary metabolites, and more refined tests will be
429 needed to support ecological connections between the chemical patterns that we observed and
430 the activity of hemlock herbivores. In addition to concentration, for example, the composition of
431 terpenes and phenolics in foliage can change throughout the growing season (reviewed in: Iason
432 et al. 2012). In expanding white birch (*Betula papyrifera* Marshall) leaves, for instance,
433 hydrolysable tannins show distinct seasonal patterns, with certain individual tannins having an
434 inverse time-concentration relationship (Salminen et al. 2001). Several studies have also
435 documented this pattern for individual terpenes in conifer needles; their relative concentrations
436 are often opposed, and can vary as needles expand (see: Nealis and Nault 2005; Thoss et al.
437 2007). Broad phenologic trends in hemlock's foliar phenolic and terpene concentrations, while
438 useful, cannot elucidate the role of individual chemical species in this tree's herbivore defense
439 complex. In particular, research that builds on and extends our findings by addressing the
440 important roles of individual phenolics in mediating plant-insect interactions in this system is
441 critical.

442 Since the introduction of adelgid nearly a century ago (Havill et al. 2006), there has been
443 extensive mortality and decline of eastern hemlocks throughout eastern U.S. forests (Eschtruth et
444 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.

References

468 Appel, H.M. 1993. Phenolics in ecological interactions: The importance of oxidation. Journal of
469 Chemical Ecology **19**(7): 1521-1552.
470 Appel, H. M., Govenor, H. L., D'ascenzo, M., Siska, E., and Schultz, J. C. 2001. Limitations of
471 Folin assays of foliar phenolics in ecological studies. Journal of Chemical Ecology **27**(4):
472 761-778.
473 Bates, D., Maechler, M., Bolker, B., and Walker, S. 2015. Fitting linear mixed-effects models
474 using lme4. Journal of Statistical Software, **67**(1): 1-48.
475 Beckman, C.H. 2000. Phenolic-storing cells: Keys to programmed cell death and periderm
476 formation in wilt disease resistance and in general defence responses in plants?
477 Physiological and Molecular Plant Pathology **57**(3): 101-110.
478 Bhiry, N., and Filion, L. 1996. Mid-Holocene hemlock decline in eastern North America linked
479 with phytophagous insect activity. Quaternary Research **45(**3): 312-320.
480 Broeckling, C.D. and Salom, S.M. 2003. Volatile emissions of eastern hemlock, Tsuga
481 canadensis, and the influence of hemlock woolly adelgid. Phytochemistry 62(2): 175-
482 180.
483 Carroll, A.L. 1999. Physiological adaptation to temporal variation in conifer foliage by a
484 caterpillar. The Canadian Entomologist 131(5): 659-669.
485 Chen, Z., Kolb, T.E., and Clancy, K.M. 2002. The role of monoterpenes in resistance of Douglas
486 fir to Western spruce budworm defoliation. Journal of Chemical Ecology **28**(5): 897-920.
487 Dharmadi, S.N., Elliott, K.J., and Miniat, C.F. 2019. Lack of forest tree seedling recruitment and
488 enhanced tree and shrub growth characterizes post-Tsuga canadensis mortality forests in
489 the southern Appalachians. Forest Ecology and Management 440: 122-130.

490 Dobesberger, E.J. 1989. A sequential decision plan for the management of the eastern hemlock
491 looper, *Lambdina fiscellaria fiscellaria* (Lepidoptera: Geometridae), in Newfoundland.
492 Canadian Journal of Forest Research **19**(7): 911-916.
493 Eschtruth, A.K., Cleavitt, N.L., Battles, J.J., Evans, R.A., and Fahey, T.J. 2006. Vegetation
494 dynamics in declining eastern hemlock stands: 9 years of forest response to hemlock
495 woolly adelgid infestation. Canadian Journal of Forest Research **36**(6): 1435-1450.
496 Ellison, A.M., Bank, M.S., Clinton, B.D., Colburn, E.A., Elliott, K., Ford, C.R., Foster, D.R.,
497 Kloeppel, B.D., Knoepp, J.D., and Lovett, G.M. 2005. Loss of foundation species:
498 Consequences for the structure and dynamics of forested ecosystems. Frontiers in
499 Ecology and the Environment **3**(9): 479-486.
500 Fenner, M. 1998. The phenology of growth and reproduction in plants. Perspectives in Plant
501 Ecology, Evolution and Systematics **1**(1): 78-91.
502 Hadley, J.L. 2000. Understory microclimate and photosynthetic response of saplings in an old-
503 growth eastern hemlock (*Tsuga canadensis* L.) forest. Ecoscience **7**(1): 66-72.
504 Hatcher, P.E. 1990. Seasonal and age-related variation in the needle quality of five conifer
505 species. Oecologia **85**(2): 200-212.
506 Havill, N.P., Montgomery, M.E., Yu, G., Shiyake, S., and Caccone, A. 2006. Mitochondrial
507 DNA from hemlock woolly adelgid (Hemiptera: Adelgidae) suggests cryptic speciation
508 and pinpoints the source of the introduction to eastern North America. Annals of the
509 Entomological Society of America **99**(2): 195-203.
510 Heath, J.J., Kessler, A., Woebbe, E., Cipollini, D., and Stireman, J.O. 2014. Exploring plant
511 defense theory in tall goldenrod, *Solidago altissima*. New Phytologist **202**(4): 1357-1370.

512 Horner, J.D. 1988. Astringency of Douglas-fir foliage in relation to phenology and xylem
513 pressure potential. Journal of Chemical Ecology **14**(4): 1227-1237.
514 Iason, G.R., Dicke, M., and Hartley, S.E. 2012. The ecology of plant secondary metabolites:
515 From genes to global processes. Cambridge University Press.
516 Ikeda, T., Matsumura, F., and Benjamin, D.M. 1977. Mechanism of feeding discrimination
517 between matured and juvenile foliage by two species of pine sawflies. Journal of
518 Chemical Ecology **3**(6): 677-694.
519 Ingwell, L.L., Miller-Pierce, M., Trotter, R.T., III, and Preisser, E.L. 2012. Vegetation and
520 invertebrate community response to eastern hemlock decline in southern New England.
521 Northeastern Naturalist **19**(4): 541-558.
522 Ingwell, L.L., and Preisser, E.L. 2011. Using citizen science programs to identify host resistance
523 in pest-invaded forests. Conservation Biology **25**(1): 182-188.
524 Isah, T. 2019. Stress and defense responses in plant secondary metabolites production. Biological
525 Research **52**(1): 39
526 Koricheva, J., and Barton, K.E. 2012. Temporal changes in plant secondary metabolite
527 production. *In* The Ecology of Plant Secondary Metabolites: From Genes to Global
528 Processes. *Edited by* G.R. Iason and M. Dicke and S.E. Hartley. Cambridge University
529 Press, Cambridge. pp. 34-55.
530 Kuznetsova, A., Brockhoff, P.B., and Christensen, R.H.B. 2017. lmerTest package: Tests in
531 linear mixed effects models. Journal of Statistical Software **82**(13): 1-26.
532 Lagalante, A.F., and Montgomery, M.E. 2003. Analysis of terpenoids from hemlock (*Tsuga*)
533 species by solid-phase microextraction/gas chromatography/ion-trap mass spectrometry.
534 Journal of Agricultural and Food Chemistry **51**(8): 2115-2120.

535 Lagalante, A.F., Lewis, N., Montgomery, M.E., and Shields, K.S. 2006. Temporal and spatial
536 variation of terpenoids in eastern hemlock (*Tsuga canadensis*) in relation to feeding by
537 *Adelges tsugae*. Journal of Chemical Ecology **32**(11): 2389-2403.
538 Lagalante, A.F., Montgomery, M.E., Calvosa, F.C., and Mirzabeigi, M.N. 2007. Characterization
539 of terpenoid volatiles from cultivars of eastern hemlock (Tsuga canadensis). Journal of
540 agricultural and food chemistry **55**(26): 10850.
541 Langenheim, J.H. 1994. Higher plant terpenoids: A phytocentric overview of their ecological
542 roles. Journal of Chemical Ecology **20**(6): 1223-1280.
543 Leather, S.R., Watt, A.D., and Forrest, G.I. 1987. Insect-induced chemical changes in young
544 lodgepole pine (*Pinus contorta*): The effect of previous defoliation on oviposition,
545 growth and survival of the pine beauty moth, *Panolis flammea*. Ecological Entomology
546 **12**(3): 275-281.
547 Lempa, K., Loponen, J., Ossipova, S., Ossipov, V., and Pihlaja, K. 2001. Delayed greening of
548 mountain birch leaves: Ecological and chemical correlates. Ecoscience **8**(1): 68-75.
549 Levin, D.A. 1971. Plant phenolics: An ecological perspective. The American Naturalist
550 **105**(942): 157-181.
551 Mauffette, Y., and Oechel, W.C. 1989. Seasonal variation in leaf chemistry of the Coast live oak
552 *Quercus agrifolia* and implications for the California oak moth *Phryganidia californica*.
553 Oecologia **79**(4): 439-445.
554 McClure, M.S. 1987. Biology and control of hemlock woolly adelgid. Connecticut Agricultural
555 Experiment Station New Haven, CT.
556 McClure, M.S., and Hare, J.D. 1984. Foliar terpenoids in *Tsuga* species and the fecundity of
557 scale insects. Oecologia **63**(2): 185-193.

558 McKenzie, E.A., Elkinton, J.S., Casagrande, R.A., Preisser, E.L., and Mayer, M. 2014. Terpene
559 chemistry of eastern hemlocks resistant to hemlock woolly adelgid. Journal of Chemical
560 Ecology **40**(9): 1003-1012.
561 Michelozzi, M. 1999. Defensive roles of terpenoid mixtures in conifers. Acta Botanica Gallica
562 **146**(1): 73-84.
563 Moreira, X., Sampedro, L., Zas, R., and Pearse, I.S. 2016. Defensive traits in young pine trees
564 cluster into two divergent syndromes related to early growth rate. PLOS One **11**(3):
565 E0152537.
566 Mumm, R., and Hilker, M. 2006. Direct and indirect chemical defence of pine against folivorous
567 insects. Trends in Plant Science **11**(7): 351-358.
568 Nault, J.R. 2003. Site temperatures influence seasonal changes in terpene composition in
569 Douglas-fir vegetative buds and current-year foliage. Canadian Journal of Forest
570 Research **33**(11): 2269-2273.
571 Nealis, V.G., and Nault, J.R. 2005. Seasonal changes in foliar terpenes indicate suitability of
572 Douglas-fir buds for Western spruce budworm. Journal of Chemical Ecology **31**(4): 683-
573 696.
574 Nerg, A., Kainulainen, P., Vuorinen, M., Hanso, M., Holopainen, J.K., and Kurkela, T. 1994.
575 Seasonal and geographical variation of terpenes, resin acids and total phenolics in nursery
576 grown seedlings of Scots pine (*Pinus sylvestris* L.). New Phytologist **128**(4): 703-713.
577 Orwig, D.A., Cobb, R.C., D'Amato, A.W., Kizlinski, M.L., and Foster, D.R. 2008. Multi-year
578 ecosystem response to hemlock woolly adelgid infestation in southern New England
579 forests. Canadian Journal of Forest Research **38**(4): 834-843.

580 Orwig, D.A. and Foster, D.R. 1998. Forest response to the introduced hemlock woolly adelgid in
581 southern New England, USA. Journal of the Torrey Botanical Society **125**(1): 60-73.
582 Orwig, D.A., Foster, D.R., and Mausel, D.L. 2002. Landscape patterns of hemlock decline in
583 New England due to the introduced hemlock woolly adelgid. Journal of Biogeography
584 **29**(10-11): 1475-1487.
585 Pasquier-Barre, F., Géri, C., Goussard, F., Auger-Rozenberg, M.A., and Grenier, S. 2000.
586 Oviposition preference and larval survival of *Diprion pini* on Scots pine clones in relation
587 to foliage characteristics. Agricultural and Forest Entomology **2**(3): 185-192.
588 Pezet, J., and Elkinton, J.S. 2014. Hemlock woolly adelgid (Hemiptera: Adelgidae) induces twig
589 volatiles of eastern hemlock in a forest setting. Environmental Entomology **43**(5): 1275-
590 1285.
591 Preisser, E.L., Lodge, A.G., Orwig, D.A., and Elkinton, J.S. 2008. Range expansion and
592 population dynamics of co-occurring invasive herbivores. Biological Invasions **10**(2):
593 201-213.
594 Preisser, E.L., Miller-Pierce, M.R., Vansant, J., and Orwig, D.A. 2011. Eastern hemlock (*Tsuga*
595 *canadensis*) regeneration in the presence of hemlock woolly adelgid (*Adelges tsugae*) and
596 elongate hemlock scale (*Fiorinia externa*). Canadian Journal of Forest Research **41**(12):
597 2433-2439.
598 Raffa, K.F., Mason, C.J., Bonello, P., Cook, S., Erbilgin, N., Keefover-Ring, K., Klutsch, J.G.,
599 Villari, C., and Townsend, P.A. 2017. Defence syndromes in lodgepole–whitebark pine
600 ecosystems relate to degree of historical exposure to mountain pine beetles. Plant, Cell &
601 Environment **40**(9): 1791-1806.

602 Rathcke, B., and Lacey, E.P. 1985. Phenological patterns of terrestrial plants. Annual Review of
603 Ecology and Systematics **16**(1): 179-214.
604 Ricklefs, R.E., and Matthew, K.K. 1982. Chemical characteristics of the foliage of some
605 deciduous trees in southeastern Ontario. Canadian Journal of Botany **60**(10): 2037-2045.
606 Rigsby, C.M., Body, M.J.A. , May, A. Oppong, A., Kostka, A., Houseman, N., Savage, S.K.,
607 Whitney, E.R., Kinahan, I.G., DeBoef, B.L., Orians, C.M., Appel, H.M., Schultz, J.C.,
608 and Preisser, E.L. Impact of chronic stylet-feeder infestation on folivore-induced
609 signaling and defenses. **In Review**. Plant, Cell & Environment.
610 Rigsby, C.M., Shoemaker, E.E., Mallinger, M.M., Orians, C.M., and Preisser, E.L. 2019. Conifer
611 responses to a stylet-feeding invasive herbivore and induction with methyl jasmonate:
612 Impact on the expression of induced defences and a native folivore. Agricultural and
613 Forest Entomology **21**(2): 227-234.
614 Riipi, M., Ossipov, V., Lempa, K., Haukioja, E., Koricheva, J., Ossipova, S., and Pihlaja, K.
615 2002. Seasonal changes in birch leaf chemistry: Are there trade-offs between leaf growth
616 and accumulation of phenolics? Oecologia **130**(3): 380-390.
617 Salminen, J.-P., Ossipov, V., Haukioja, E., and Pihlaja, K. 2001. Seasonal variation in the
618 content of hydrolysable tannins in leaves of *Betula pubescens*. Phytochemistry **57**(1): 15-
619 22.
620 Sampson, D., Albaugh, T., Johnsen, K.H., Allen, H., and Zarnoch, S.J. 2003. Monthly leaf area
621 index estimates from point-in-time measurements and needle phenology for *Pinus taeda*.
622 Canadian Journal of Forest Research **33**(12): 2477-2490.

623 Schultz, J.C., Nothnagle, P.J., and Baldwin, I.T. 1982. Seasonal and individual variation in leaf
624 quality of two northern hardwoods tree species. American Journal of Botany **69**(5): 753-
625 759.
626 Smith, H. 1982. Light quality, photoperception, and plant strategy. Annual Review of Plant
627 Physiology **33**(1): 481-518.
628 Thoss, V., O'Reilly-Wapstra, J., and Iason, G.R. 2007. Assessment and implications of
629 intraspecific and phenological variability in monoterpenes of Scots pine (*Pinus sylvestris*)
630 foliage. Journal of Chemical Ecology **33**(3): 477-491.
631 Villari, C., Battisti, A., Chakraborty, S., Michelozzi, M., Bonello, P., and Faccoli, M. 2012.
632 Nutritional and pathogenic fungi associated with the pine engraver beetle trigger
633 comparable defenses in Scots pine. Tree Physiology **32**(7): 867-879.
634 Waring, R.H., and Franklin, J.F. 1979. Evergreen coniferous forests of the Pacific Northwest.
635 Science **204**(4400): 1380-1386.
636 Watt, A.D. 1987. The effect of shoot growth stage of *Pinus contorta* and *Pinus sylvestris* on the
637 growth and survival of *Panolis flammea* larvae. Oecologia **72**(3): 429-433.
638 White, C.S. 1994. Monoterpenes: Their effects on ecosystem nutrient cycling. Journal of
639 Chemical Ecology **20**(6): 1381-1406.
640 Wiggins, N.L., Forrister, D.L., Endara, M.-J., Coley, P.D., and Kursar, T.A. 2016. Quantitative
641 and qualitative shifts in defensive metabolites define chemical defense investment during
642 leaf development in *Inga*, a genus of tropical trees. Ecology and Evolution **6**(2): 478-492.
643 Wilson, C. M., Schaeffer, R. N., Hickin, M. L , Rigsby, C. M., Sommi, A. F., Thornber, C. S.,
644 Orians, C. M., and Preisser, E. L. 2018. Chronic impacts of invasive herbivores on a
645 foundational forest species: A whole-tree perspective. Ecology **99**(8):1783-1791.

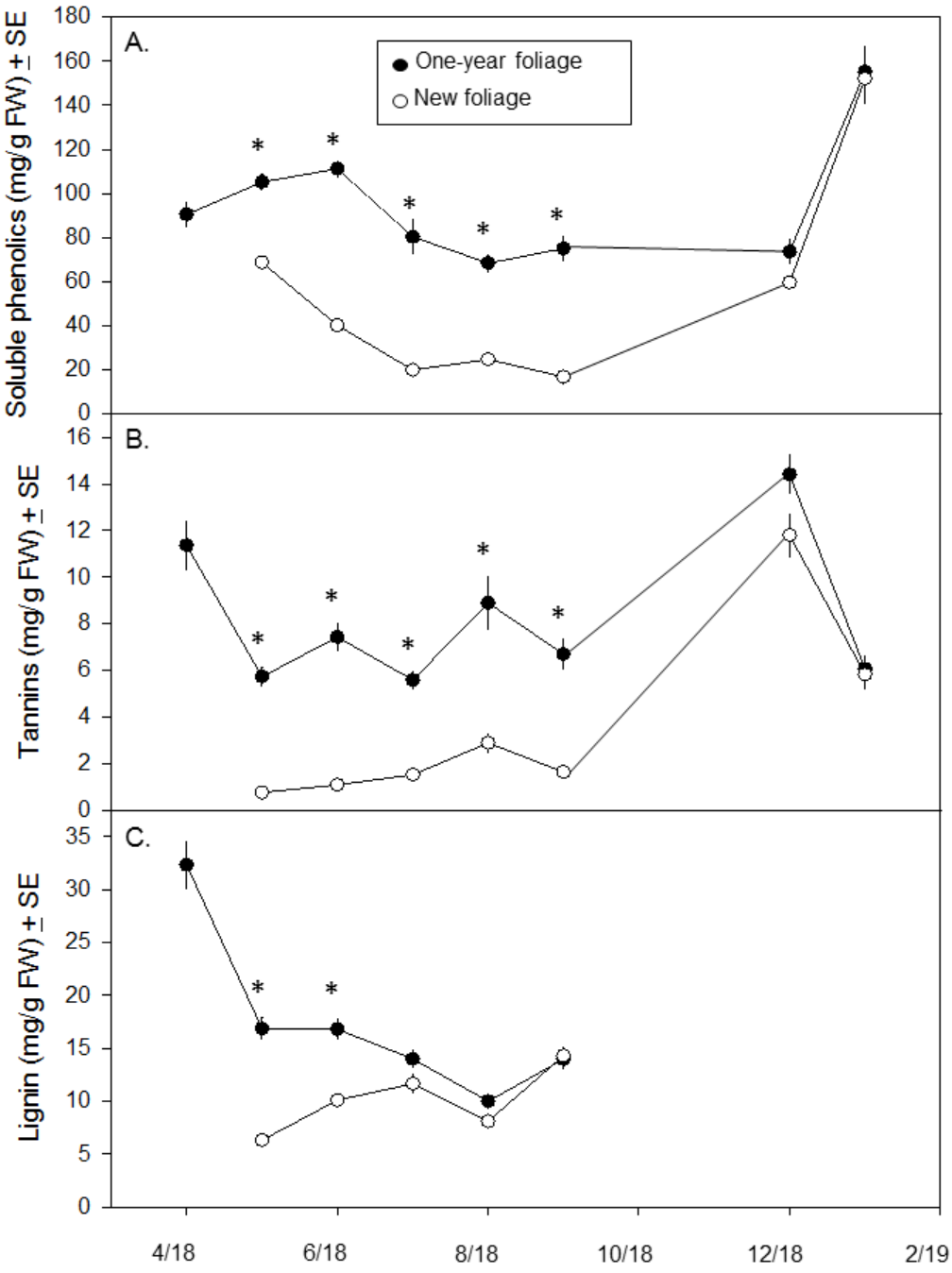
646 Zou, J., and Cates, R.G. 1997. Effects of terpenes and phenolic and flavonoid glycosides from
647 Douglas fir on Western spruce budworm larval growth, pupal weight, and adult weight.
648 Journal of Chemical Ecology **23**(10): 2313-2326.

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.

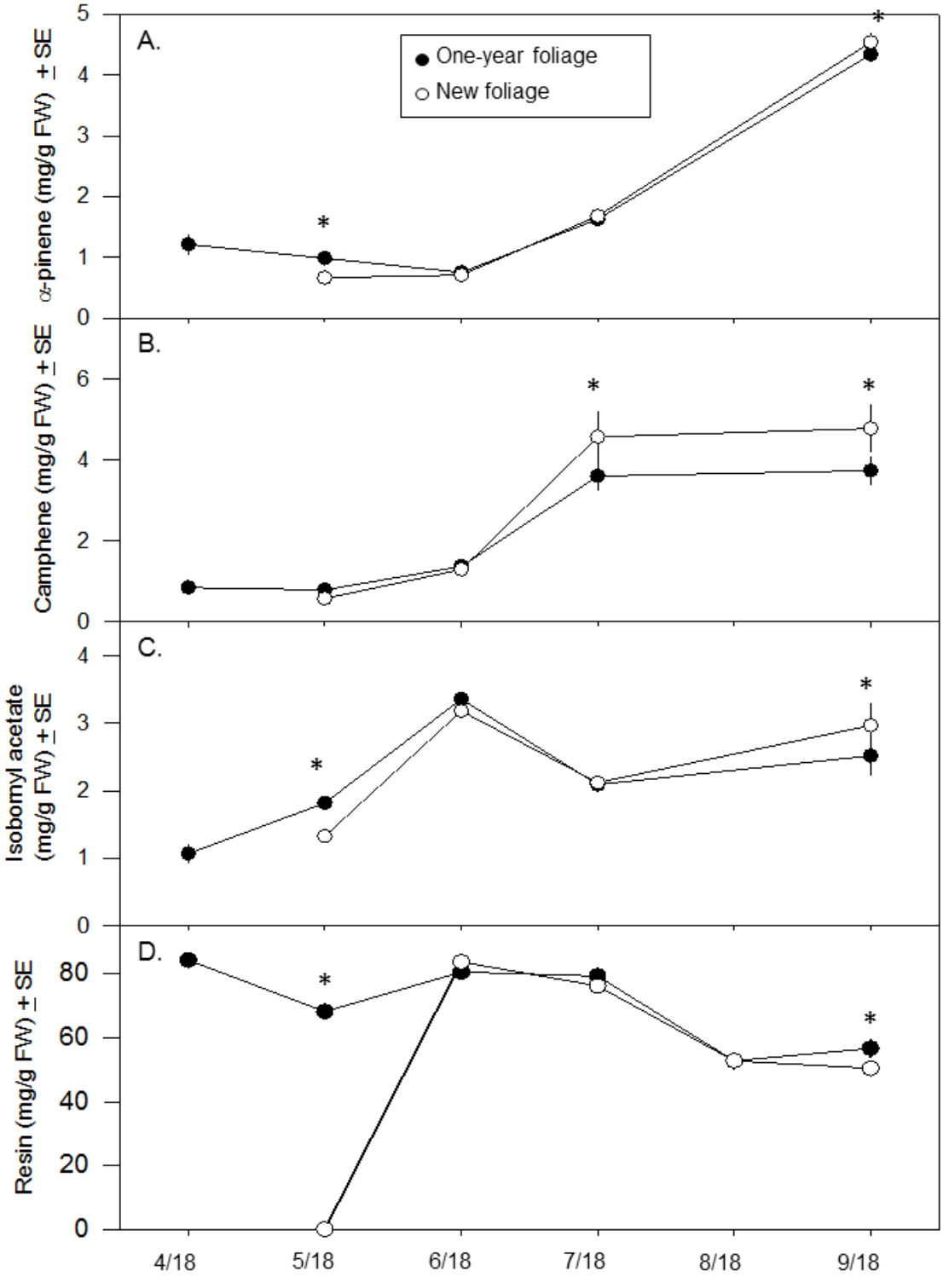
662 **Figure 1.**



663

664

665 **Figure 2.**



666