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14   **Abstract**

15   Eastern hemlock (*Tsuga canadensis* (L.) Carriére; hemlock) is an eastern North American

16   conifer threatened by the invasive hemlock woolly adelgid (*Adelges tsugae* Annand). Changes in

17   foliar terpenes and phenolics were evaluated in new (current year growth) and mature (1-year old

18   growth) hemlock needles during the growing season and into plant dormancy. From April

19   through September, foliar concentrations of non-volatile soluble phenolics, condensed tannins,

20   lignin, mono- and sesquiterpenes α-pinene, camphene, isobornyl acetate, and diterpene resin

21   were quantified. After September, additional analyses of metabolites that continued to differ

22   significantly in new versus mature foliage were carried out. Total soluble phenolic concentration

23   and condensed tannin concentration in new foliage remained low relative to mature foliage

24   throughout the growing season and converged in December. Lignin concentration in new foliage

25   converged with that of mature foliage by July. Concentrations of α-pinene, camphene, isobornyl

26   acetate, and diterpene resin in new foliage converged with mature foliage within one month of

27   budbreak. The convergence of terpene concentrations in new and mature foliage suggests that

28   these metabolites may play a role in herbivore defense during the peak growing season.

29   Conversely, soluble phenolics, including condensed tannins, may defend foliage from herbivory

30   outside of the spring growth period.

31   **Key words**

32   Phenology, terpenes, phenolics, conifers, hemlock

33   **Introduction**

34   Phenology is the study of seasonally varying developmental events driven by

35 environmental cues. Plant phenology is characterized by temporal patterns of growth associated

36 with abiotic factors such as degree day, amount of transmitted light, and precipitation (Nault

37 2003; Rathcke and Lacey 1985; Smith 1982). In seasonal climates, leaf growth and development

38 occur largely during a specific time of year (Fenner 1998). In temperate forests of the northern

39 hemisphere, for example, plants leaf out rapidly in the spring to take full advantage of the

40 growing season. Rapid leaf expansion/development is associated with substantial physiological

41 changes during tissue maturation (Koricheva and Barton 2012; Wiggins et al. 2016). In mountain

42 birch (*Betula pubescens* subsp. *czerepanovii* Ehrhart), for example, leaf toughness increases,

43 amino acid levels decrease, and different phenolic compounds (e.g., proanthocyanidins,

44 gallotannins, flavonoids) show discrete accumulation patterns throughout the growing season

45 (Riipi et al. 2002).

46   Knowledge of plant leaf phenology is largely derived from deciduous trees (for example:

47 Fenner 1998; Mauffette and Oechel 1989; Schultz et al. 1982)(Wiggins et al. 2016). Evergreen

48 conifers, however, make up a significant proportion of global woody plant biomass (Waring and

49 Franklin 1979) and exhibit unique foliar expansion patterns. For instance, loblolly pine (*Pinus

50 taeda* Linnaeus) needles have three distinct periods of seasonal development, shifting from

51 winter dormancy to an April-September period of rapid growth and completing needle expansion

52 before the end of the year (Sampson et al. 2003). Conifer foliar chemistry also varies temporally,

53 with shifting amounts of various secondary metabolites that are central to defense against

54 herbivory as needles mature. Expanding jack pine (*Pinus banksiana* Lambert) needles, for

55 example, have high levels of a *Neodiprion* sawfly antifeedant compound that rapidly decreases in

56 concentration as the needles mature (Ikeda et al. 1977). Phenolic compounds and terpenes are the

57 two primary classes of defensive metabolites present in conifer needles (Raffa et al. 2017); their

58 concentrations generally relate to needle age (Mumm and Hilker 2006). Understanding the

59 phenology of secondary metabolites is important, since changes in these compounds can alter

60 plant resistance to abiotic (e.g., drought, excess light) and biotic (e.g., herbivore and pathogen

61 attack) stressors.

62   Terpenes function in a wide array of ecological processes vital to conifer survival. These

63 include regulating community dynamics through allelopathic inhibition of seed germination,

64 altering rates of soil nutrient cycling and nitrification, and conferring resistance to herbivores and

65 pathogens (Langenheim 1994; Michelozzi 1999; White 1994). For individual terpene compounds

66 in conifer needles, the time-concentration relationship is often nonlinear and can vary throughout

67 the growing season. In newly emerged needles of scots pine (*Pinus sylvestris* Linnaeus), for

68 instance, α-pinene accumulates during the growing season while δ-3-carene levels decrease

69 rapidly after budbreak (Thoss et al. 2007). Emerging douglas fir (*Pseudotsuga menziesii* (Mirb.)

70 Franco) needles show similarly dynamic changes in monoterpene levels, with α-pinene and β-

71 pinene reversing their abundances as young needles expand (Nealis and Nault 2005).

72   Phenolic compounds in conifers contribute to needle structural development, tissue

73 toughness, and defense against damage by pests and pathogens (Isah 2019). In response to leaf

74 damage, various phenolic compounds are polymerized and covalently bound to cell walls,

75 sealing off sites of infection or injury and strengthening leaf tissue against further damage

76 (Beckman 2000). Phenolics also play a direct role in defense (i.e., toxicity to herbivores and

77 pathogens) and can deter insect herbivore oviposition and affect larval performance (Pasquier-

78 Barre et al. 2000). Phenolics have well-known roles in abiotic stress resistance as well, such as

79 oxidative stress relief and protection from UV radiation (Appel 1993; Isah 2019). These

80 compounds also vary substantially with needle age: Hatcher (1990) found that the immature

81 needles of five conifer species had lower phenolic concentrations than mature needles on the

82 same branch.

83   Research exploring temporal variation in conifer foliar chemistry has focused on a few

84 species in the pine family (Pinaceae), to the exclusion of other ecologically significant species

85 (see: Hatcher 1990; Nault 2003; Nealis and Nault 2005; Nerg et al. 1994; Thoss et al. 2007).

86 Eastern hemlock (*Tsuga canadensis* (L.) Carriére; hemlock) is one such conifer, a long-lived

87 canopy dominant endemic to forests of the eastern U.S. (Orwig et al. 2008). Hemlock is

88 responsible for important ecological functions, including soil moisture regulation and protecting

89 riparian habitat from lethal temperature extremes (Ellison et al. 2005). It is currently under threat

90 of extirpation by hemlock woolly adelgid (*Adelges tsugae* Annand; adelgid), an invasive stylet-

91 feeding insect from Japan that has caused widespread mortality and decline of hemlock (see:

92 Dharmadi et al. 2019, McClure 1987) since its introduction nearly 70 years ago (Havill et al.

93 2006).

94   Both the sistens and progrediens adelgid generations feed preferentially on mature

95 hemlock foliage (Lagalante et al. 2006), and this is likely driven by chemical differences

96 between new and mature needles. Adelgid resistance in western hemlock (*Tsuga heterophylla*

97 (Raf.) Sargent) and Asian hemlock species, for instance, has been linked to terpene profiles that

98 differ substantially from those of adelgid-susceptible species (Lagalante and Montgomery 2003).

99 Adelgid-resistant eastern hemlock cultivars also have unique foliar terpene profiles (Lagalante et

100 al. 2007). Additionally, resistance to adelgid has been documented in rare individual eastern

101 hemlocks lingering in adelgid-devastated forests (Ingwell and Preisser 2011); chemical analyses

102 found higher terpene concentrations in the needles and twigs of these putatively adelgid-resistant

103 eastern hemlocks compared to adelgid-susceptible controls (McKenzie et al. 2014).

104   Moreover, increasing terpenoid concentrations in expanding hemlock needles has been

105 correlated with reduced fecundity of elongate hemlock scale (*Fiorinia externa* Ferris), another

106 pest of eastern hemlock introduced from Japan (McClure and Hare 1984). Herbivores of other

107 feeding guilds are also impacted by hemlock's foliar chemical phenology. Hemlock looper

108 (*Lambdina fiscellaria* Guenée), an important defoliator of eastern hemlock, feeds preferentially

109 on specific needle age classes, with early instar larvae feeding on expanding needles and mature

110 larvae shifting to old-growth foliage (Carroll 1999).

111   While multiple eastern hemlock studies have explored chemical defense induction in

112 response to adelgid and other herbivores (e.g. Broeckling and Salom 2003; Rigsby et al. 2019),

113 there has been less work addressing constitutive levels of foliar terpenes (but see: McKenzie et

114 al. 2014). Only two studies have addressed phenological changes in hemlock terpene emission

115 rates (Lagalante et al. 2006; McClure and Hare 1984). These studies only considered the volatile

116 fraction of hemlock terpenes, and there has been no work addressing non-volatile terpenes in

117 hemlock. While volatile terpene emissions are ecologically significant (e.g., antixenosis), since

118 herbivores feed directly on plant tissue, non-volatile terpenes are more important in direct

119 resistance to herbivore attack (i.e., antibiosis).

120   At least three studies have identified the foliar terpenes α-pinene, camphene, and

121 isobornyl acetate as the most significant terpenes in hemlock's interactions with adelgid

122 (Lagalante et al. 2006; Lagalante and Montgomery 2003; Lagalante et al. 2007), a fact that

123 suggests they play a role in hemlock's defense against adelgid herbivory. We are unaware of any

124 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

148   conifer species indicate that terpene and structural metabolite levels in expanding needles

149   become indistinguishable from mature needles by the end of the growing season (Hatcher 1990;

150   Nault 2003; Thoss et al. 2007). Specifically for eastern hemlock, volatile terpene concentrations

151   in new foliage have been shown to converge with mature foliage by fall leaf-off (Lagalante et al.

152   2006). Here, we provide a first look at eastern hemlock's chemical phenology.

153   **Materials and Methods**

154   *Common Garden* – In April 2014, 320 adelgid-free, chemically untreated hemlock

155   saplings (0.5-0.7 m tall; two years in age) grown from seed collected in Pennsylvania, were

156   purchased from Van Pines Nursery (West Olive, MI). These saplings were planted in five blocks

157   of 64 plants, with $\geq$1.5 m between each sapling, in the understory of a mixed hardwood stand at

158   the Kingston Wildlife Research Station (South Kingstown, RI). Plants were protected from both

159   vertebrate and invertebrate herbivory with chicken-wire cages covered in mesh bags (Agribon-

160   15, Johnny's Selected Seeds, Waterville, ME, USA; 90% light transmission). In early spring

161   2018, we randomly selected 12 1.0-1.2 m tall herbivore-free saplings from four of the five blocks

162   for this work. Between two and five plants were sampled from each block. This discrepancy in

163   the number of plants sampled per block existed because we desired to include insect-free trees

164   from each block, but multiple experiments were either occurring or had occurred within this

165   common garden. Several of the trees in this garden also experienced a spruce spider mite

166   (*Oligonychus ununguis* Jacobi) infestation that was avoided by our plant selection. These

167   necessitated the selection of these specific plants.

168   Beginning on 26 April 2018, we removed one 20 cm terminal branch of mature (1-year

169   old growth) foliage per plant; newly produced foliage was not sampled in April since bud break

170   did not occur until mid-May. We returned to each sapling on 31 May 2018 and destructively

171   sampled two 20 cm terminal branches, one of mature foliage and another of expanding (current

172   year growth) foliage. This protocol meant that we collected one branch per tree during the April

173   sampling and two branches per tree during the May sampling and all subsequent sampling dates.

174   Each terminal branch was excised with pruning shears, wrapped in aluminum foil, placed in a

175   cooler on ice and brought back to the laboratory, where it was stored at -80° C until processed.

176   The total sampling time (i.e., from when the first sample was clipped to the time all samples

177   were placed at -80° C) always took < 1 hr to perform, samples were immediately immersed in

178   ice as soon as they were sampled, and trees were sampled haphazardly (with regards to the order

179   of sampling) to avoid treatment artifacts that were due to our sampling procedure. We chose this

180   procedure over clipping individual needles from sampled branches in the field (thus leaving

181   behind a completely defoliated woody stem) on the basis of time. While our chosen method

182   always took less than one hour (first branch clip to when all tissue was flash-frozen), in a pilot

183   experiment we found that carefully removing each individual needle from each sampled branch

184   in the field more than tripled the time between tissue removal and flash-freezing. Branch

185   clippings occurred monthly on the following dates: 26 April, 31 May, 28 June, 26 July, 30

186   August, and 27 September 2018. After this final date, we only continued to assay secondary

187   metabolites that differed significantly in new versus old foliage. We added two additional

188   sampling dates (19 December 2018 and 28 January 2019) in which we quantified these

189   remaining metabolites. By the end the experiment, our cuttings had removed less than 5% of the

190   total foliage from each sapling.

191   *Tissue Preparation* – Needles were removed from each branch sample and ground to a

192   powder in liquid nitrogen using a mortar and pestle. New foliage was processed separately from

193   mature foliage. The powder was partitioned into three tubes: 100 (± 5) mg in a 1.5 ml microtube

194 for the phenolic analyses (total soluble phenolics, condensed tannins, and lignin), 100 (± 5) mg

195 in another 1.5 ml microtube for GC-FID analysis of major mono- and sesquiterpenes, and 1 (±

196 0.05) g in a 15 ml Falcon tube for non-volatile resin analysis. Tubes were stored at -30° C until

197 analysis, and analyses were conducted within two days of tissue grinding.

198   *Total Soluble Phenolics* – Soluble phenolics were extracted in HPLC-grade methanol and

199 total soluble phenolic levels quantified similarly to Rigsby et al. (2019), via Folin assay, with

200 minor enhancements for optimization. Twenty-five µl extract was first diluted in 75 µl methanol,

201 and 500 µl water was then added, followed by 40 µl Folin-Ciocalteu reagent (Sigma-Aldrich).

202 Tubes were incubated at room temperature for 10 min, then 40 µl 1 M $NaHCO_3$ was added, and

203 the tubes incubated at room temperature for 1 hour. Absorbance was then quantified at 725 nm

204 using a SpectraMAX M2 Multi-Mode microplate reader (Molecular Devices, Sunnyvale, CA,

205 USA) and Greiner UV-Star® 96 well plates (Monroe, NC, USA). In place of gallic acid,

206 chlorogenic acid (Sigma-Aldrich) was used to generate a standard curve, and phenolic

207 concentration was expressed as chlorogenic acid equivalents (mg g^{-1} FW). Condensed tannin

208 concentration was also quantified as per Rigsby et al. (2019), by incubating methanol extracts

209 (250 µl) with 750 µl 95:5 butanol:HCl for three hours at 95° C. After cooling, absorbance at 550

210 nm was quantified using a Turner® SP-830 cuvette spectrophotometer and plastic cuvettes

211 (expressed as OD_{550} g^{-1} FW).

212   Although Appel et al. (2001) raised concerns regarding the use of Folin assays in

213 quantifying temporal or species variation in foliar phenolic levels in ecological studies, recent

214 work by our research group has revealed that chlorogenic acid derivatives dominate (≥ 70%) the

215 soluble phenolic profile of hemlock foliage (Rigsby et al. in review) throughout the year

216 (unpublished data). We conducted preliminary experiments showing that total soluble phenolic

217 content estimated with the spectrophotometric procedure described above, using chlorogenic

218 acid as standard, is highly correlated with phenolics quantified *via* HPLC-UV$_{280nm}$ (R$^2 \geq 0.88$;

219 unpublished data), regardless of sampling month or tissue age. This background work led us to

220 conclude that this more cost- and time-effective Folin spectrophotometric assay provided

221 reasonable estimations of foliar soluble phenolic concentrations in lieu of phenolics quantified

222 *via* HPLC. We acknowledge, however, that despite this preliminary work and that fact that we

223 could obtain a close estimation of HPLC-quantified phenolics with Folin-quantified phenolics,

224 the Folin method could, and likely did, miss quantitative variation in the contents of phenolic

225 compounds.

226   *Lignin* – Lignin levels were quantified as per Villari et al. (2012). Briefly, the leftover

227 pellet from the soluble phenolic extraction was washed twice with methanol, allowed to air-dry

228 overnight, then resuspended in 400 µl 1 M NaOH and incubated for 24 hours at 40° C. The

229 homogenate was acidified with 200 µl 1.5 M formic acid, and 400 µl methanol was added. The

230 tubes were centrifuged at 16,000 x g for 5 min and the supernatant discarded. Pellets were then

231 washed twice with 1 ml methanol, and 1 ml 2 M HCl was added to the tubes followed by 250 µl

232 thioglycolic acid. The tubes were incubated for 4 hours at 85° C. Once cooled to room

233 temperature, tubes were centrifuged at 16,000 x g for 5 min and the supernatant was discarded.

234 Pellets were then washed once with 1 ml water, and thioglycolic acid-lignin pellets were

235 extracted overnight in 1 ml 1 M NaOH. The tubes were centrifuged at 16,000 x g for 5 min and

236 the supernatant saved; this extraction step was repeated and supernatants combined. Extracts

237 were acidified with 300 µl concentrated HCl and incubated at room temperature for 4 hours.

238 Tubes were then centrifuged at 20,000 x g (5 min), supernatants discarded, and pellets allowed to

239 dry overnight at 40° C. The following day, pellets were resuspended in 1 ml 1 M NaOH. The

240 absorbance of 20-fold dilutions (with 0.5 M NaOH) at 280 nm was quantified using the

241 SpectraMAX microplate reader and Greiner UV-Star® 96 well plates against a standard curve of

242 spruce lignin (expressed as mg g^{-1} FW).

243   *Mono- and Sesquiterpenes* – α-Pinene, camphene, and isobornyl acetate (Sigma-Aldrich)

244 were extracted and quantified *via* GC-FID, similarly to Rigsby et al. (in review) with minor

245 adjustments in the oven program to optimize rapid quantification of these three compounds.

246 These are the three dominant terpene species of hemlock foliage and constitute ≥ 75% of all

247 terpenes identified in eastern hemlock (Broeckling and Salom 2003; Rigsby et al. in review).

248 Foliar terpenes were extracted in 700 µl *n*-hexane containing 1 µl ml^{-1} *m*-xylene as an internal

249 standard by sonicating homogenates for 10 min in an ice bath. Tubes were then vortexed for 10 s

250 and the 20,000 x g (5 min, 0° C) supernatant transferred to a 2 ml glass autosampler vial, capped

251 with a PTFE-coated screw cap, and stored at -30° C until injected into the GC (within 48 hours).

252 The instrument, settings, gases, column, injection volume, and external standards described by

253 Rigsby et al. (in review) were used here. Mono- and sesquiterpene identification and

254 quantification took place using a Shimadzu GC 2010 Plus gas chromatograph equipped with an

255 AOC-20i autosampler and a flame ionization detector (GC-FID). Nitrogen was used as the

256 carrier gas at a flow rate of 1.0 ml min^{-1} and an HP-5MS column (30 m x 0.25 mm internal

257 diameter; 0.25 µm film thickness). Terpene extract (2 µl) was injected using a split flow ratio of

258 30:1, and the injector and detector temperatures were set to 260° C and 300° C, respectively. The

259 adjusted oven program was: 40° C for 5 min, increased by 5° C min^{-1} to 225° C, increased by

260 25° C min^{-1} to 280° C, and held at 280° C for 5 min (total run time = 49.2 min). Peaks were

261 matched to external standards based on retention times, and tissue amounts of terpenes (µg g^{-1}

262 FW) were estimated using three-point standard curves of standards (R^2 > 0.99).

263   *Diterpene Resin* – Non-volatile diterpene resin concentration was estimated

264   gravimetrically using standard techniques for estimating the non-volatile resin concentrations of

265   pine foliage, which is highly correlated with diterpene resin acid concentration measured *via* GC-

266   MS (Moreira et al. 2016). Briefly, 1 g tissue powder was extracted in 3 ml *n*-hexane for 10 min

267   in a sonicator, centrifuged at 4,000 x g, and the supernatant transferred to a 15 ml Falcon tube.

268   This extraction procedure was repeated twice and the supernatants combined in a pre-weighed 15

269   ml Falcon tube. Uncapped tubes were then placed in a fume hood and the solvent evaporated to

270   dryness (approximately four days) before reweighing. The before-after difference in tube weight

271   was considered the mass of non-volatile resin (expressed as mg g^{-1} FW). It is worth noting that

272   hemlock diterpenes have not been characterized and we, therefore, are unable to directly relate

273   diterpene content quantified with GC-MS with content quantified by this gravimetric method as

274   has been shown and used in many species of pine (e.g, Moreira et al. 2016). Our *n*-hexane

275   extracts would certainly have extracted other non-polar, non-volatile metabolites aside from

276   diterpenes and these could have contributed to the change in tube weights. Our results should,

277   therefore, be interpreted cautiously until hemlock diterpenes can be characterized.

278   *Statistical Analysis* – R software v. 3.5.0 was used for all analyses (R Development Core

279   Team, 2018). Secondary metabolite concentrations in new and mature foliage were analyzed *via*

280   linear mixed-effects models, using *lme4* (Bates et al. 2015). Foliage age, month, and their

281   interactions were treated as fixed effects, and block and tree were treated as random effects. A

282   type III analysis of variance (ANOVA) was used to evaluate each model term for significance.

283   Month-specific differences in metabolite concentrations in new and mature foliage were

284   analyzed for significance using *difflsmeans* in the *lmerTest* package (Kuznetsova et al. 2017),

285   and the Holm-Bonferroni family-wise error rate-controlling procedure was used to correct for

286   multiple comparisons.

287   **Results**

288   *Phenolics* – Phenolic concentration differed significantly in new versus mature foliage

289   for all measured compounds (all $F_{[1,1]} > 183.17$, $P < 0.05$; Fig. 1A – 1C). Phenolic levels also

290   varied by month (all $F_{[1,7]} > 77.82$, $P < 0.001$), and there was a significant month × foliage age

291   interaction (all $F_{[1,6]} > 7.47$, $P < 0.001$). Specifically, total soluble phenolic and condensed tannin

292   concentration was lower in new relative to mature foliage throughout the growing season (May

293   through September) (means separation test both $P < 0.001$; Fig. 1A, B). It took until December

294   for concentrations of total soluble phenolics and condensed tannins in new and mature foliage to

295   converge (means separation test both $P > 0.05$). At this point, total soluble phenolic

296   concentrations in new and mature foliage reached 59.6 mg/g FW ± SE and 73.5 mg/g FW ± SE,

297   respectively, and condensed tannins in new and mature foliage reached 11.7 mg/g FW ± SE and

298   14.4 mg/g FW ± SE, respectively. Conversely, lignin levels in new foliage rapidly increased at

299   the start of the growing season and became indistinguishable from that of mature foliage by July,

300   with concentrations reaching 11.6 mg/g FW ± SE in new foliage, and 14.0 mg/g FW ± SE in

301   mature foliage (means separation test $P = 0.097$; Fig. 1C).

302   *Terpenes* –In contrast to phenolics, terpene concentrations in new foliage rapidly

303   converged with those of mature foliage. Statistically similar levels of quantified terpenes in new

304   and mature foliage occurred within one month of budbreak (means separation test all $P > 0.05$;

305   Fig. 2A – 2D). By June, for instance, α-pinene concentrations in new and mature foliage were

306   0.71 mg/g FW ± SE and 0.75 mg/g FW ± SE, respectively. Terpene levels varied by month (all

307    $F_{[1,5]} > 154.38$, $P < 0.001$), and there was a significant month × foliage age interaction (all $F_{[1,4]} >$

308   61.04, *P* < 0.001). Levels of camphene, α-pinene, and isobornyl acetate were higher, and resin

309   concentration lower, in new versus mature foliage in September (separation of means test all *P* <

310   0.05; Fig. 2A – 2D). Resin concentration in new and mature foliage in September, for instance,

311   was 50.4 mg/g FW ± SE and 56.7 mg/g FW ± SE, respectively. Conversely, levels of isobornyl

312   acetate in new and mature foliage were 2.9 mg/g FW ± SE and 2.5 mg/g FW ± SE, respectively,

313   and camphene levels in new and mature foliage had reached 4.7 mg/g FW ± SE and 3.7 mg/g

314   FW ± SE, respectively.

315   **Discussion**

316   New foliage contained significantly lower concentrations of total soluble phenolics,

317   condensed tannins, and lignin compared to mature hemlock foliage (Fig. 1A – C). Phenolic

318   concentrations also varied by month, and the monthly change in the concentration of each

319   compound was different for new versus mature foliage. Total soluble phenolic and condensed

320   tannin concentration was lower in new versus mature foliage throughout the growing season

321   (May through September), rejecting our hypothesis that concentrations of all metabolites would

322   converge with levels in mature needles by the end of the spring growth period (Fig. 1A, B).

323   Moreover, concentrations of both classes of compounds in new foliage did not converge with

324   levels present in mature foliage until December (Fig. 1A, B). Concentrations of the structural

325   metabolite, lignin, in new foliage became indistinguishable from levels in mature foliage as early

326   as July (Fig. 1C). The incidence of low levels of lignin with similarly low levels of both phenolic

327   and terpene concentrations did not support our first hypothesis that relatively low levels of lignin

328   in new foliage would be accompanied by relatively greater levels of phenolic and terpene

329   defensive metabolites. Instead, concentrations of the different metabolite groups measured in

330   new foliage remained low relative to mature foliage, until the new foliage lignified.

331   α-Pinene and isobornyl acetate levels were significantly lower in new foliage compared

332   to mature foliage immediately post-budbreak in May, increased to convergence by June, and

333   exceeded that of mature foliage in September (Fig. 2A, C). Resin concentration in new versus

334   mature foliage was also initially low, but became indistinguishable from levels in mature foliage

335   by June (Fig. 2D). Camphene concentrations in new and mature foliage were not significantly

336   different immediately post-budbreak, and in September, camphene levels were higher in new

337   foliage than in mature foliage (Fig. 2B).

338   This broad pattern of early-season convergence of terpene concentrations in new and

339   mature foliage suggests that this class of secondary metabolites may play a significant role in

340   eastern hemlock's defense against herbivores that are active at the beginning of, and during, the

341   peak growing season. Conversely, soluble phenolics, including condensed tannins, may be

342   responsible for defending hemlock foliage from herbivory that occurs outside of the spring

343   growth period. It is important to note, however, that the phenolic content of most plants varies

344   not only in association with tissue age and/or herbivore activity, but according to other stressors,

345   such as growing conditions (reviewed in: Levin 1971). For example, latitudinal variation in

346   temperature, growing season duration, and sun exposure has a strong effect on the total phenolic

347   concentration of scots pine needles (Nerg et al. 1994). Thus, while the apparent seasonal tradeoff

348   between terpene and phenolic concentrations observed in this study may be an element of

349   hemlock's anti-herbivore defense complex, it may also be affected by seasonal variation in

350   certain biophysical factors. Future studies should evaluate the extent to which seasonal

351   herbivores, biophysical factors, and their interactions drive these patterns.

352   *Phenolics*

353   Total soluble phenolic and condensed tannin concentration in new foliage did not

354   converge with levels in mature foliage until December (Fig. 1A, B). Total soluble phenolic

355   concentration in new foliage was relatively high immediately after budbreak but declined

356   throughout the growing season (Fig. 1A). Like terpenes, phenolics are a class of secondary

357   metabolites that play a significant role in conifer defense against insect herbivory (Mumm and

358   Hilker 2006). Because hemlock retains its needles throughout the year, it resumes photosynthesis

359   well before new foliage emerges (Hadley 2000). As a result, hemlock buds are nutrient rich

360   (Wilson et al. 2018), and elevated levels of certain soluble phenolics may be necessary to protect

361   these tissues from early spring folivores. Elevated early-season phenolic concentrations is

362   consistent with work by Hatcher (1990) documenting high phenolic concentrations in western

363   hemlock needles at budbreak, followed by decreasing concentrations as needles expanded during

364   the growing season. The subsequently low levels of total soluble phenolics and condensed

365   tannins observed in expanding hemlock needles between June and September may be necessary

366   for hemlock to avoid autotoxicity. At least one study suggests that compartmentalization

367   problems prevent the accumulation of high amounts of tannins in new conifer foliage (Horner

368   1988). Evidence of low tannin concentration in expanding needles of both scots pine (Watt 1987)

369   and douglas fir (Horner 1988) during peak growth, followed by post-growing season

370   convergence with levels in mature needles for both trees, is also consistent with this hypothesis.

371   Condensed tannin concentration and total soluble phenolic concentration in new foliage

372   became indistinguishable from mature foliage in December (Fig. 1A, B). This late-season

373   convergence indicates that these metabolites may play a role in hemlock's defense against later-

374   arriving herbivores. In addition to phenolics directly deterring phytophagous insects, these

375   compounds have also been shown to affect oviposition preference in certain pine-defoliating

376 insects (Leather et al. 1987; Pasquier-Barre et al. 2000). Oviposition on plants unsuitable for

377 larval development reduces insect fitness, and elevated foliar tannins have been shown to reduce

378 oviposition rates (Leather et al. 1987). One destructive pest of eastern hemlock, the hemlock

379 looper, initiates oviposition in late September and into October (Dobesberger 1989), when our

380 data suggests that newly produced hemlock needles begin to accumulate condensed tannins and

381 other soluble phenolics. Given the shared evolutionary history of eastern hemlock and hemlock

382 looper (Bhiry and Filion 1996), the fall/winter increase in foliar condensed tannin and total

383 soluble phenolic concentration may have some significance for this interaction. Adelgid also

384 feeds on hemlock tissue from October through April. While the two species have not co-evolved,

385 adelgid feeding has been shown to dramatically increase both the condensed tannin (Rigsby et al.

386 2019) and soluble phenolic concentration (Pezet and Elkinton 2014; Rigsby et al. 2019) of

387 hemlock foliage. This further suggests that condensed tannins and other soluble phenolics may

388 be an important aspect of eastern hemlock defense against late-season herbivores.

389   Concentrations of the structural metabolite lignin in new foliage reached levels present in

390 mature foliage after terpenes in July (Fig. 1C). Contrary to our first hypothesis, lignin

391 concentration in new foliage was not exceeded by any individual defensive metabolite before it

392 reached levels found in mature foliage. Furthermore, condensed tannins and total soluble

393 phenolics in new foliage did not reach levels found in mature foliage until well after the growing

394 season had ended. This did not support our second hypothesis that concentrations of all

395 metabolites would converge with levels in mature needles by the end of the spring growth

396 period, and suggests that the latter two groups of metabolites may be important against

397 herbivores active outside of the spring growth period.

398   *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

445   adapted to grow in cool, understory microclimates (Hadley 2000); the loss of most canopy-

446   dominant hemlocks in this region has inhibited seedling recruitment (Ingwell et al. 2012; Orwig

447   and Foster 1998; Orwig et al. 2002), and virtually eliminated hemlock sapling regeneration

448   (Preisser et al. 2011). Hemlock-associated forests are now characterized by lower overstory

449   deciduous tree densities, novel understory vegetation communities (Ingwell et al. 2012), and

450   significantly reduced soil moisture and C:N ratios (Orwig et al. 2008). As hemlock continues to

451   be threatened with extirpation by adelgid, it will be important to understand the potency of

452   individual secondary metabolites in hemlock's interactions with herbivores, in addition to

453   understanding broad seasonal trends, to effectively conserve this species. Putatively adelgid-

454   resistant eastern hemlocks, for instance, sustain lower adelgid densities (Ingwell et al. 2011), and

455   this is likely due to their unique terpene chemistry (McKenzie et al. 2014). However, the

456   individual terpene(s) species responsible for inhibiting adelgid population growth remains

457   unknown. Such a chemical marker could provide the means to identify candidate eastern

458   hemlocks for adelgid-resistance breeding programs and reforestation efforts, aimed at not only

459   restoring eastern hemlocks, but also maintaining the vital, broad-scale ecosystem functions that

460   this tree provides.

461   **Acknowledgments**

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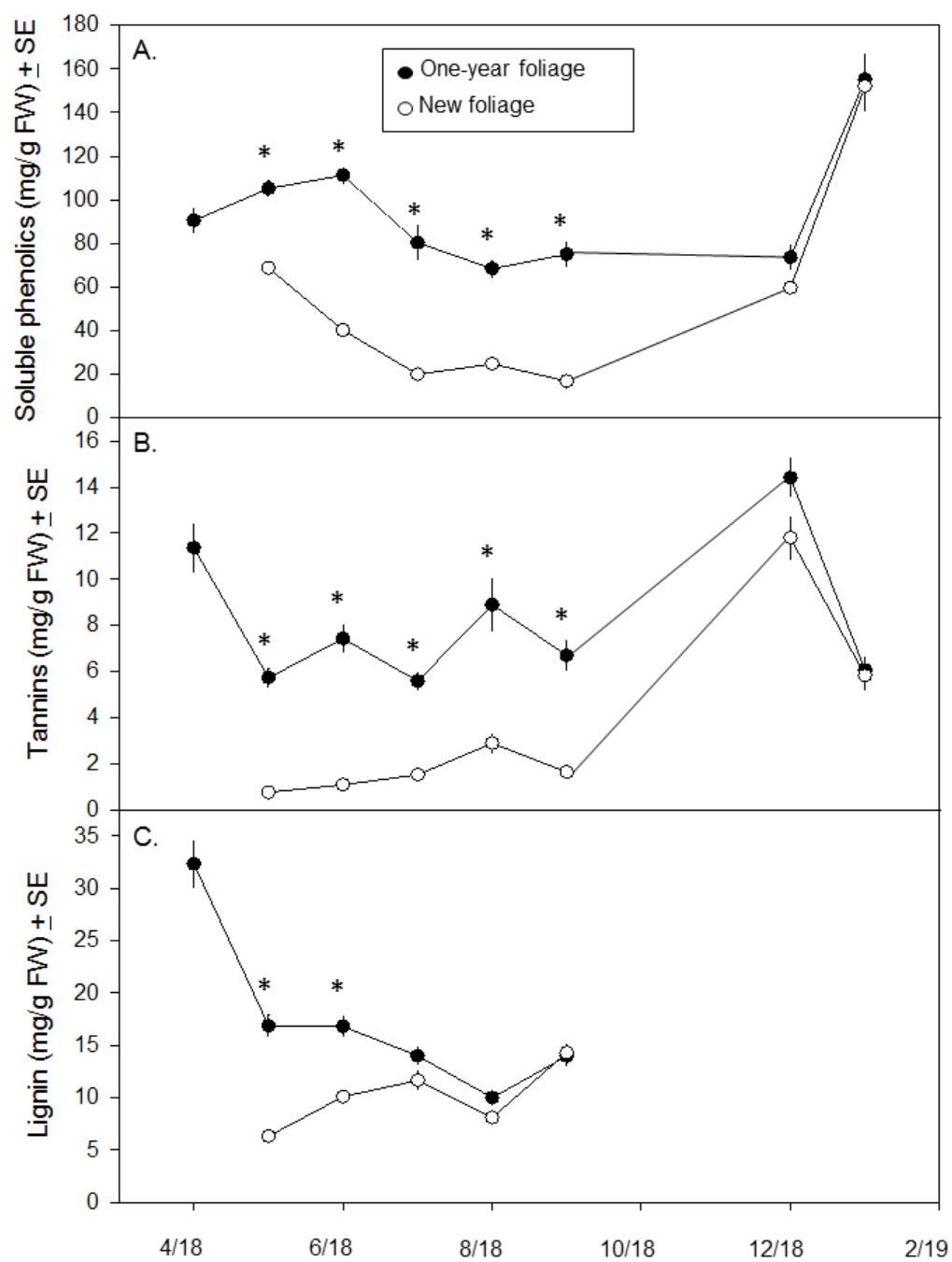645   foundational forest species: A whole-tree perspective. Ecology **99**(8):1783-1791.

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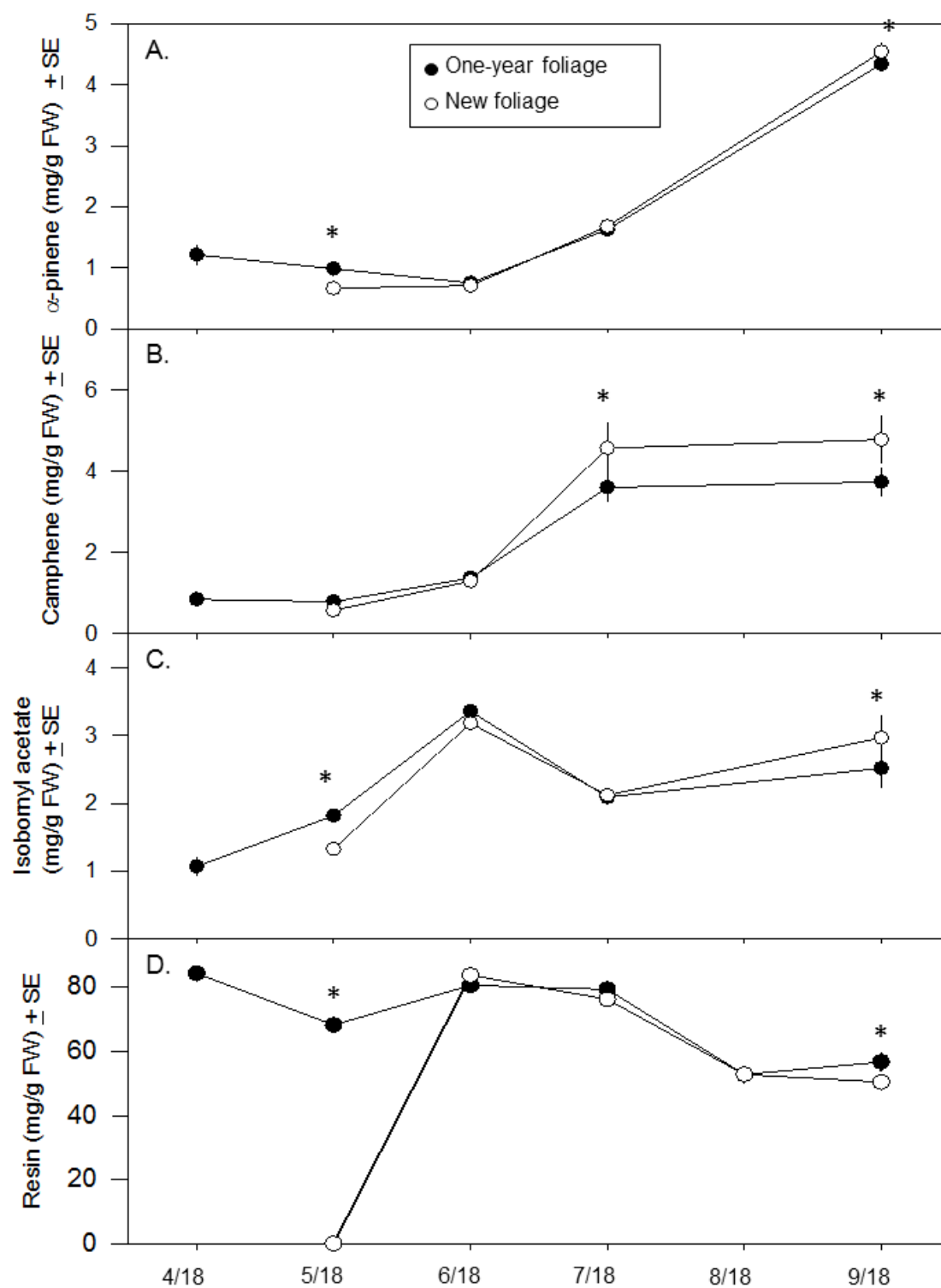
649   **Figure Legends**

650   Figure 1. Mean total soluble phenolic (A), condensed tannin (B), and lignin

651   concentrations (C) in new (current-year growth) and mature (1-year old growth) eastern hemlock

652   foliage throughout the growing season. New foliage was not produced until after the April

653   sampling date. Asterisks represent significant differences in metabolite concentrations in new

654   versus mature foliage; lines represent means ± 1 SE. Additional samples were analyzed in

655   December and January to identify when phenolic and condensed tannin levels in new foliage

656   converged with levels in mature foliage.

657   Figure 2. Mean α-pinene (A), camphene (B), isobornyl acetate (C), and resin (D)

658   concentrations in new and mature eastern hemlock foliage throughout the growing season. New

659   foliage was not produced until after the April sampling date. Asterisks represent significant

660   differences in metabolite concentrations in new versus mature foliage; lines represent means ± 1

661   SE.

662   **Figure 1.**

663

664

665 **Figure 2.**

666