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

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14 **Abstract**

15 Eastern hemlock (*Tsuga canadensis* (L.) Carrière; 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.) Carrière; 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

125 looper, however, relationships between these terpenes and other lepidopteran folivores are well
126 documented. Western spruce budworm (*Choristoneura occidentalis* Freeman) resistance in
127 douglas fir, for example, is associated with higher foliar concentrations of camphene and
128 isobornyl acetate (Chen et al. 2002). Likewise, hemlock foliar phenolics have also been
129 explored, but only in the context of induced defenses (Rigsby et al. 2019, Rigsby et al. in
130 review). Increased cell-wall bound phenolic and lignin concentrations, for example, have been
131 documented in adelgid-infested hemlock foliage without corresponding changes in oxygenated
132 terpenes (Rigsby et al. 2019). Ultimately, within the context of phenology, dynamic changes in
133 non-volatile phenolic and terpene accumulation in eastern hemlock remains unexplored.

134 In the present study, changes in non-volatile terpenes and phenolics were evaluated in
135 both newly-produced and mature eastern hemlock foliage through a growing season and into
136 plant dormancy. Temporal changes in the foliar concentration of some major defensive
137 secondary metabolites were outlined, including monoterpene compounds, non-volatile resin,
138 soluble phenolics and condensed tannins, in both expanding and mature needles. Foliar
139 concentrations of lignin, a structural as well as defensive secondary metabolite, were also
140 measured. These data were used to identify when metabolite levels in expanding needles were
141 statistically indistinguishable from those found in mature needles. We hypothesized that (1)
142 relatively low levels of lignin in new foliage would be accompanied by relatively greater levels
143 of phenolic and terpene defensive metabolites. Our reasoning was that new foliage, being more
144 attractive to herbivores (Lempa et al. 2001) and of greater fitness value (Heath et al. 2014),
145 would rely on these non-lignin defensive metabolites while actively growing. We also
146 hypothesized that (2) concentrations of all metabolites would converge with levels in mature
147 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 ≥ 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: $100 (\pm 5)$ mg in a 1.5 ml microtube

194 for the phenolic analyses (total soluble phenolics, condensed tannins, and lignin), 100 (\pm 5) mg
195 in another 1.5 ml microtube for GC-FID analysis of major mono- and sesquiterpenes, and 1 (\pm
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 μ l extract was first diluted in 75 μ l methanol,
201 and 500 μ l water was then added, followed by 40 μ l Folin-Ciocalteu reagent (Sigma-Aldrich).
202 Tubes were incubated at room temperature for 10 min, then 40 μ l 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 μ l) with 750 μ l 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 $\text{OD}_{550} \text{ g}^{-1}$ 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 ($\geq 70\%$) 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 μ l 1 M NaOH and incubated for 24 hours at 40° C. The
229 homogenate was acidified with 200 μ l 1.5 M formic acid, and 400 μ l 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 μ l
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 μ l 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 $\geq 75\%$ of all
247 terpenes identified in eastern hemlock (Broeckling and Salom 2003; Rigsby et al. in review).
248 Foliar terpenes were extracted in 700 μ l *n*-hexane containing 1 μ l 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 μ m film thickness). Terpene extract (2 μ l) 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 (μ g 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 *diffsmeans* 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 \times 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 $59.6 \text{ mg/g FW} \pm \text{SE}$ and $73.5 \text{ mg/g FW} \pm \text{SE}$,
297 respectively, and condensed tannins in new and mature foliage reached $11.7 \text{ mg/g FW} \pm \text{SE}$ and
298 $14.4 \text{ mg/g FW} \pm \text{SE}$, 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 $11.6 \text{ mg/g FW} \pm \text{SE}$ in new foliage, and $14.0 \text{ mg/g FW} \pm \text{SE}$ 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 $0.71 \text{ mg/g FW} \pm \text{SE}$ and $0.75 \text{ mg/g FW} \pm \text{SE}$, respectively. Terpene levels varied by month (all
307 $F_{[1,5]} > 154.38$, $P < 0.001$), and there was a significant month \times 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 \pm SE and 56.7 mg/g FW \pm SE, respectively. Conversely, levels of isobornyl
312 acetate in new and mature foliage were 2.9 mg/g FW \pm SE and 2.5 mg/g FW \pm SE, respectively,
313 and camphene levels in new and mature foliage had reached 4.7 mg/g FW \pm SE and 3.7 mg/g
314 FW \pm 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.

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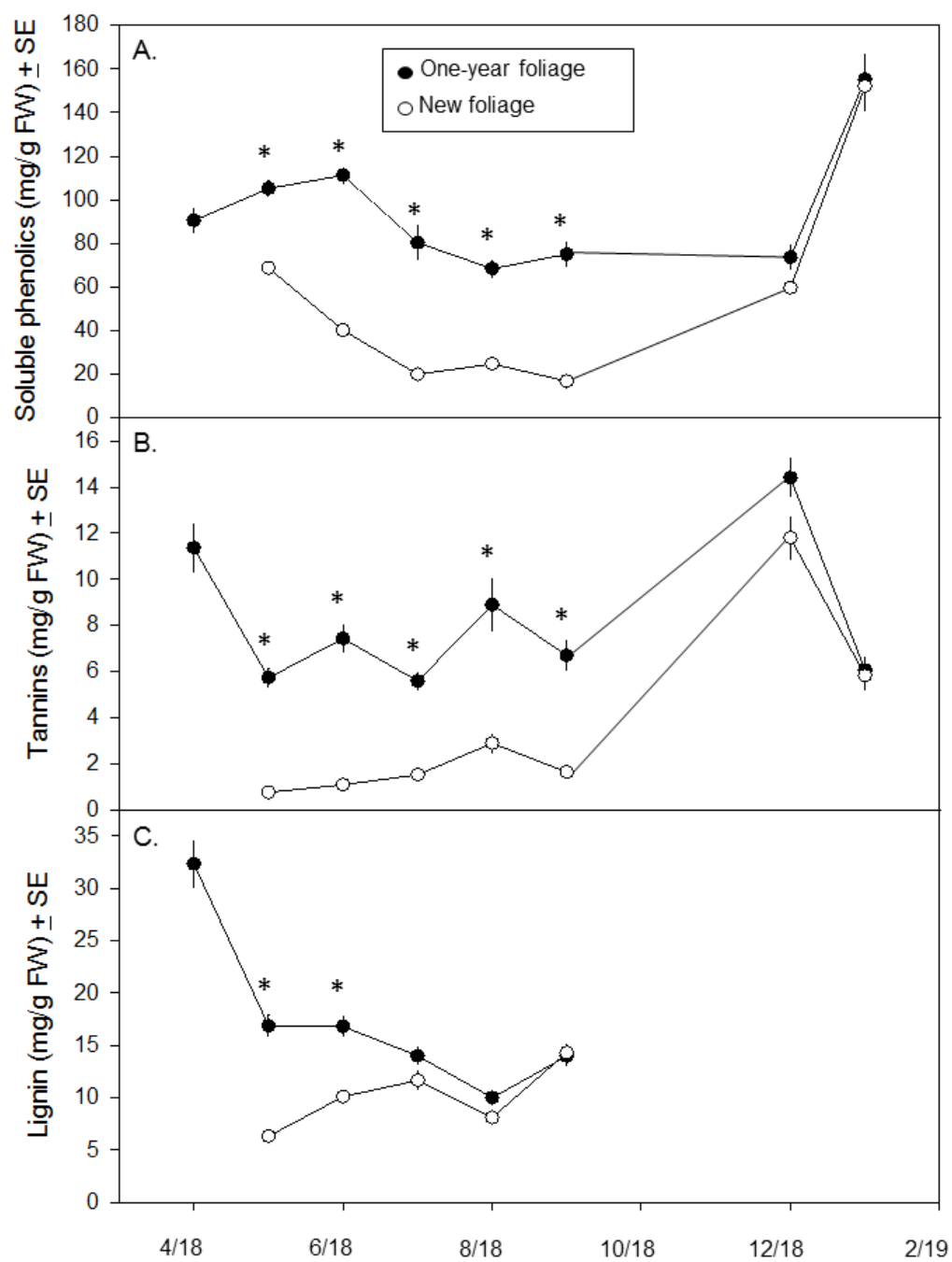
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649 **Figure Legends**

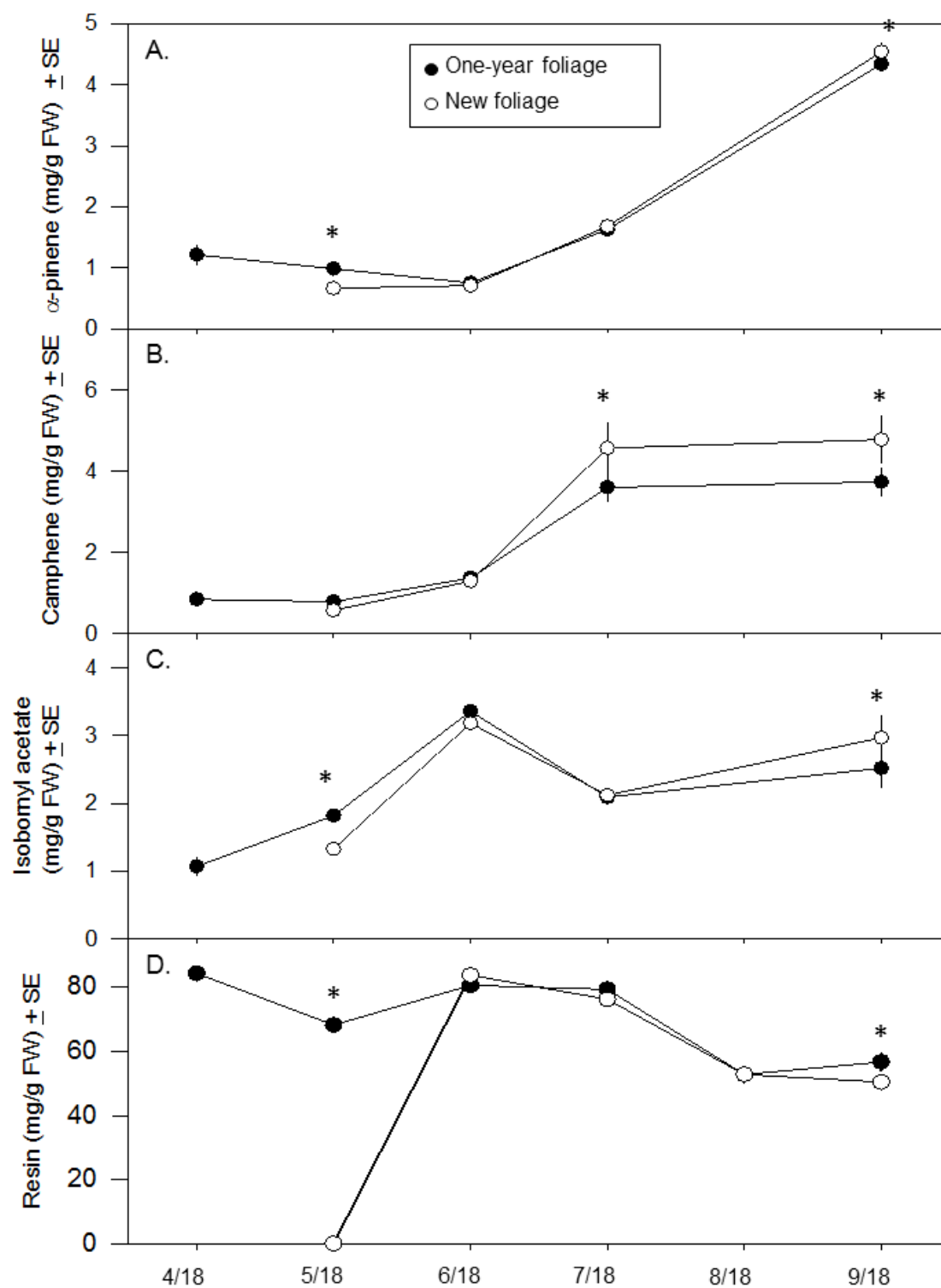
650 Figure 1. Mean total soluble phenolic (A), condensed tannin (B), and lignin
651 concentrations (C) in new (current-year growth) and mature (1-year old growth) eastern hemlock
652 foliage throughout the growing season. New foliage was not produced until after the April
653 sampling date. Asterisks represent significant differences in metabolite concentrations in new
654 versus mature foliage; lines represent means \pm 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 \pm 1
661 SE.

662 **Figure 1.**

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665 **Figure 2.**

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