2016

Long-Term Impacts of Invasive Herbivores on Tree Physiology, Growth, and Phenology: A Whole-Tree Perspective

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LONG-TERM IMPACTS OF INVASIVE HERBIVORES ON TREE
PHYSIOLOGY, GROWTH, AND PHENOLOGY: A WHOLE-TREE
PERSPECTIVE

BY

CLAIRE M. WILSON

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF
MASTER OF SCIENCE
IN
BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2016

MASTER OF SCIENCE THESIS

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2016

ABSTRACT

Insect herbivores play an essential role in structuring plant communities and species interaction therein. Plant response to herbivory, particularly to non-native insects, can be difficult to predict. A diverse array of feeding strategies, including leaf-chewing, wood-boring, and piercing-sucking, leads to varied plant responses following attack. Piercing-sucking insects are known to alter source-sink dynamics but are relatively understudied, particularly in woody plants. Two piercing-sucking invasive insects, hemlock woolly adelgid (*Adelges tsugae*; 'HWA') and the elongate hemlock scale (*Fiorinia externa*; 'EHS'), are commonly found on eastern hemlock (*Tsuga canadensis*; 'hemlock') in the eastern United States. Hemlock, a native shade-tolerant conifer, provides unique habitat for a range of biota and plays an important role in structuring ecosystems, but is threatened throughout much of its range because of HWA. HWA drives rapid decline in tree health, whereas EHS rarely kills trees. The individual and interactive impacts of HWA and EHS on resource allocation, phenology, and metabolite profiles were explored following two and four years of infestations. HWA-infested trees, regardless of EHS presence, had relatively more biomass belowground and less aboveground biomass. Consistent needle desiccation and drop indicative of HWA infestation explains these allocation changes. EHS did not drive changes in biomass allocation. HWA-infested trees broke bud on average three days later than HWA-free trees and new flush production (grams/day) in early spring was 30% less compared to HWA-free trees. Although EHS and HWA both impacted primary metabolites, the effects of HWA are more pronounced. While EHS has virtually no impact, HWA substantially alters resource acquisition and allocation

in eastern hemlock. Assessing whole-plant impacts of two invasive piercing-sucking insects on a native woody plant following long-term experimental infestations in a long-lived conifer provides a unique contribution to the literature. The lack of interaction between HWA and EHS at a whole-plant level, which conflicts with prior branch-level studies, reinforces the importance of considering long-term impacts in an ecologically relevant setting.

The accompanying appendices contain additional details about canopy closure (Appendix S1) and statistical models (Appendix S2).

ACKNOWLEDGMENTS

I would like to first thank Dr. Evan Preisser, my major professor, for his mentorship and support during my time at the University of Rhode Island. I would also like to thank my committee members, Dr. Richard Casagrande and Dr. Alison Roberts for their guidance and feedback throughout this project. I am grateful that Dr. Larry Englander agreed to serve as my committee chair. Dr. Carol Thornber also contributed to the analyses and experimental design. Many members of the Preisser Lab during my tenure here (Mauri Hickin Barrett, Katherine Harrison, Mary Mallinger, Jamie Rafter, Dr. Chad Rigsby, Elwood Roberts, Elizabeth Whitney) have provided invaluable support. Several undergraduates also lended their help throughout the course of the experiment, including Aidan Barry, Julie Bozzo, Grace Hanson, Schirley Tostes, and Monica Tynan. They have bright futures ahead of them. Dr. Colin Orians (Tufts University) and Dr. Robert Schaeffer (Tufts University and University of Rhode Island) were essential contributors to this manuscript. I am grateful for other mentors, friends, and family that encouraged, listened, and supported me during my time at URI. I would also like to thank my funding sources that helped make this opportunity possible to pursue. During the academic year, the Graduate School provided support for three semesters through teaching assistantships and NSF grant DEB-1256769 supported for two semesters and one summer.

PREFACE

The following thesis has been submitted in manuscript format following the formatting guidelines of the journal *Ecology*.

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CHAPTER 1

Long-term impacts of invasive herbivores on tree physiology, growth, and phenology:
a whole-tree perspective

Authors

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Manuscript in preparation for *Ecology*

ABSTRACT

Insect herbivores play an essential role in structuring plant communities and species interaction therein. Plant response to herbivory, particularly to non-native insects, can be difficult to predict. A diverse array of feeding strategies, including leaf-chewing, wood-boring, and piercing-sucking, leads to varied plant responses following attack. Piercing-sucking insects are known to alter source-sink dynamics but are relatively understudied, particularly in woody plants. Two piercing-sucking invasive insects, hemlock woolly adelgid (*Adelges tsugae*; ‘HWA’) and the elongate hemlock scale (*Fiorinia externa*; ‘EHS’), are commonly found on eastern hemlock (*Tsuga canadensis*; ‘hemlock’) in the eastern United States. Hemlock, a native shade-tolerant conifer, provides unique habitat for a range of biota and plays an important role in structuring ecosystems, but is threatened throughout much of its range because of HWA. HWA drives rapid decline in tree health, whereas EHS rarely kills trees. The individual and interactive impacts of HWA and EHS on resource allocation, phenology, and metabolite profiles were explored following two and four years of infestations. HWA-infested trees, regardless of EHS presence, had relatively more biomass belowground and less aboveground biomass. Consistent needle desiccation and drop indicative of HWA infestation explains these allocation changes. EHS did not drive changes in biomass allocation. HWA-infested trees broke bud on average three days later than HWA-free trees and new flush production (grams/day) in early spring was 30% less compared to HWA-free trees. Although EHS and HWA both impacted primary metabolites, the effects of HWA are more pronounced. While EHS has virtually no impact, HWA substantially alters resource acquisition and allocation

in eastern hemlock. Assessing whole-plant impacts of two invasive piercing-sucking insects on a native woody plant following long-term experimental infestations in a long-lived conifer provides a unique contribution to the literature. The lack of interaction between HWA and EHS at a whole-plant level, which conflicts with prior branch-level studies, reinforces the importance of considering long-term impacts in an ecologically relevant setting.

Keywords: amino acids, elongate hemlock scale, hemlock woolly adelgid, phenology, primary metabolism, *Tsuga canadensis*

INTRODUCTION

The ubiquity and relative immobility of autotrophs in terrestrial and aquatic communities makes them a tempting target for a diverse array of herbivorous insects (Karban and Baldwin 1997, Stam et al. 2014). The impact of these attackers on the morphology, physiology, and growth of individual plants can substantially alter community structure (Crawley 1989, Marquis 2004). Researchers have made significant strides in understanding how insects individually (Kessler and Baldwin 2002) and jointly (Stam et al. 2014) affect their host plants. As the number of herbivore invasions rise and their cumulative ecosystem impacts increase, it is increasingly important to understand the individual and combined effect of invasive herbivores on plant fitness (Lovett et al. 2006, Gandhi and Herms 2010).

Because herbivores differ in their phenology, their attacks on the same plant can be simultaneous or sequential. Across different spatial and temporal scales, the resulting damage can be the sum of individual herbivore effects (e.g. Knochel et al 2010); it is also possible, however, for the single-species effects to interact in a non-

additive manner (Kaplan and Denno 2007). Non-additive impacts on a shared host plant are particularly likely when early-arriving herbivores induce changes in host plant physiology and chemistry (Fournier et al. 2006, Morris et al. 2007, Pieterse and Dicke 2007, Stam et al. 2014). An early-season herbivore can increase damage by a late-arriving herbivore if early-season herbivory makes the plant more susceptible to pathogens (Wallin and Raffa 2001) or attenuates plant defenses (Soler et al. 2012). Alternately, an early-arriving herbivore may reduce the damage caused by a later-arriving herbivore through the induction of plant defense or changes in plant quality (Hunter 1987). Understanding the nuances of such herbivore interactions is especially critical in cases involving invasive species with community- or ecosystem-level impacts. The presence of several such species could lead to invasional meltdown (Simberloff 1999), for instance, or generate invasional interference (Yang et al. 2011, Rauschert and Shea 2012).

Sap-feeding herbivores are a diverse group of insects whose impact on woody plants can equal or exceed that of defoliators (Zvereva et al. 2010). Woody plants are less likely than herbaceous plants to respond to an attack with compensatory growth, instead show a reduction of photosynthate production (Schowalter 1981). Additionally, woody plants are less tolerant of sap-feeders than defoliators, yet research is skewed towards examining defoliator impacts on plant health (Zvereva et al. 2010). Studies addressing the impacts of multiple sap-feeding herbivores often utilize naturally-infested plants (e.g., Dungan et al. 2007, Grégoire et al. 2015, Karban 1980), an approach that cannot be used to experimentally assess non-additive effects (Nykänen and Koricheva 2004). As a result, few studies have examined how

simultaneous and sequential attacks by sap-feeding insects impact woody plants, especially over the multi-year timescales most appropriate to assessments of their health (Zvereva et al. 2010).

While numerous stand-alone metrics can be used to quantify plant health, the complementary and interactive nature of many plant responses highlights the appeal of a 'whole-plant' approach to herbivory studies. This approach simultaneously measures herbivore-induced alterations in factors such as growth, metabolism, and resource allocation; while logistically complex, such work is essential to identifying synergistic or compensatory responses across multiple 'compartments' (e.g., foliar versus woody biomass, or above- versus belowground tissue). Work by Moreira et al. (2015) illustrated the complex plant responses induced by two folivores feeding sequentially on lima bean (*Phaseolus lunatus*): the order of herbivore arrival influenced some metrics of plant reproduction (seed mass, germination) but not others (seed number). A whole-plant approach also makes it easier to assess how herbivore-induced change in growth or metabolism affect plant life history events, including biomass allocation and phenology. The latter is especially important for forest understory plants that rely on high photosynthetic rates in early spring before canopy leaf-out; herbivore-induced delays in bud break may put them at particular risk.

We report the results of a four-year experiment assessing the responses of a foundational tree species to the individual and combined presence of two invasive sap-feeding herbivores. We describe how chronic herbivory by the hemlock woolly adelgid (*Adelges tsugae*, 'HWA') and elongate hemlock scale (*Fiorina externa*, 'EHS') affected the growth, physiology, and phenology of eastern hemlock (*Tsuga*

canadensis, 'hemlock') saplings. Eastern hemlock is a late successional foundational tree species that grows well in high-shade forest understories (Ellison et al. 2005, Orwig et al. 2008). The fact that both herbivore species are sessile, along with previous research into this interaction (Preisser and Elkinton 2008, Gómez et al. 2012, Miller-Pierce and Preisser 2012, Domec et al. 2013) make this an ideal model system for exploring whole-plant impacts of chronic herbivory on a woody plant. Over the course of four years, hemlock saplings planted into a deciduous forest understory were individually, simultaneously, or sequentially inoculated with neither, one, or both herbivores. This design allows us to explore the multi-year impact of herbivore identity, single- versus multi-species infestations, and consumer priority effects on the growth, metabolism, and phenology of a long-lived woody plant. Our results illustrate the complex and multi-faceted impacts of chronic herbivory on a long-lived tree species, and highlight the importance of long-term experimental manipulations for exploring the interplay between herbivores and woody plants.

MATERIALS AND METHODS

Natural History

Eastern hemlock (*Tsuga canadensis*, 'hemlock') is a late-successional, shade-tolerant conifer that relies on early- and late-season carbon capture that occurs prior to spring hardwood leaf-out and following fall hardwood leaf-loss (Hadley and Schedlbauer 2002). It plays a critical role in structuring forests in the eastern United States, and has been identified as a 'foundational species' in these ecosystems (Ellison et al. 2005).

Hemlock woolly adelgid (*Adelges tsugae*, 'HWA') was introduced from Japan to the eastern United States in the 1950s (Havill et al. 2006); its invaded range now extends from Georgia northwards to Maine (Morin et al. 2009, Gómez et al. 2015). Mobile first-instar 'crawlers' settle at the base of hemlock needles and become sessile adults that extract photosynthate from xylem ray parenchyma cells (Young et al. 1995). It poses a serious threat to hemlock, with many attacked trees dying within 5-10 years of infestation (Orwig et al. 2002).

Elongate hemlock scale (*Fiorinia externa*, 'EHS') arrived in the eastern United States from Japan in the early 1900s (Sasscer 1912) and co-occurs with HWA on hemlock throughout the eastern United States (Lambdin et al. 2005, Preisser et al. 2008). First-instar EHS crawlers settle on the underside of hemlock needles, where they become sessile adults that feed upon nutrients found in leaf mesophyll cells (McClure 2002). Although it can reach extremely high densities in its invaded range (Preisser et al. 2008) and may occasionally kill already-stressed trees (McClure 1980), EHS generally appears to have a minimal impact on hemlock health (Miller-Pierce and Preisser 2012).

Experimental design

In April 2011, we planted 200 hemlock saplings (~0.3m in height; Van Pines Nursery, West Olive, MI, USA) into the understory of a mixed-hardwood forest at Kingston Wildlife Research Station (Kingston, RI). The seeds were collected in Berks County, Pennsylvania. Saplings were planted in a 10x20 grid with 1-1.5 m between trees; initial heights and basal diameters were recorded prior to planting. Each sapling was enclosed in a mesh-covered (Agribon-15, Johnny's Selected Seeds, Waterville,

ME, USA; 90% light transmission) wire cage to exclude deer browsing and prevent cross-treatment contamination. Between December and March, while both insects are immobile, we removed the mesh bags to prevent snow from damaging or destroying the cages.

Following planting, each tree was randomly assigned to a treatment (Table 1); a small number of trees were subsequently reassigned so that each row and column contained each treatment. Inter-plant dispersal of HWA and EHS is most likely prior to spring leaf-out, when both sub-canopy wind velocities and crawler densities are high (McClure 1989). Each spring, we simulated this yearly dispersal event by inoculating each tree with foliage infested with the appropriate insect; herbivore-free trees were 'inoculated' with uninfested foliage in order to control for handling. Inoculations were conducted using a standard protocol (Butin et al. 2007); because HWA emerges earlier than EHS, inoculations were conducted in May and June, respectively.

Starting in 2011, trees in three treatments were annually inoculated with HWA only, EHS only, or both insects for four years (HWA-4, EHS-4, and Both-4, respectively). Starting in 2013, some HWA-only and some EHS-only trees were thereafter annually inoculated with both insects, creating two 'priority effect' treatments (HWA→Both, EHS→Both). In 2013 we also began annual inoculations of previously-uninfested trees with HWA-only, EHS-only, or both insects for two years (HWA-2, EHS-2, Both-2). A subset of trees remained herbivore-free throughout the experiment (Control).

Insect densities were assessed twice yearly, in early spring and late fall,

throughout the experiment. Details regarding insect density and plant growth in 2011-2014 are provided elsewhere (Hickin et al. *in preparation*); because our focus is on cumulative treatment impacts, we report November 2014 insect densities solely as an indicator of whole-tree infestation levels. In fall 2014, insect densities on newly-produced foliage were similar for HWA (2.01 ± 0.18 [SE] insects/cm) and EHS (1.99 ± 0.26 insects/cm) (Table 1). These infestation levels fall within those observed in the field and in prior studies where hemlock trees were experimentally inoculated (Miller-Pierce et al. 2010, Soltis et al. 2015). As in prior work, the densities of both HWA and EHS were higher in single-species treatments than when they co-occurred (150% higher for HWA and 50% higher for EHS).

Between 2011-2015, we lost replicates to Hurricane Sandy, cross-treatment contamination, browsing by white tailed deer (*Odocoileus virginianus*), and isolated outbreaks of secondary pests (e.g., *Oligonychus ununguis* mites and *Nuculaspis* sp. scales). There were also a few trees in the single-herbivore treatments (i.e., treatments EHS-2, EHS-4, HWA-2, and HWA-4) whose persistently-low insect densities (<0.5 insects/cm; the bottom 15% of fall 2014 insect densities) may have obscured the impact of insect damage; we excluded these trees from our final harvest. The 88 remaining trees were marked for intensive monitoring in early spring prior to the May 2015 harvest.

Early spring monitoring

In early April 2015, we haphazardly selected and marked three branches per tree for assessments of insect density, bud phenology, and post-harvest biomass and chemical analyses. Each branch was marked and all insects present on it were counted

(detailed below). This was completed prior to bud break and the emergence of HWA crawlers.

Between April 15-19th 2015, we measured gas exchange in one-year-old (2014 growth) foliage on the terminal end of each marked branch, for a total of three measurements per tree. All measurements were taken prior to bud break. We used a CIRAS-2 portable photosynthesis system (PP systems, Haverhill, MA, USA) with a 2.5 cm² cuvette and a CIRAS-2 LED light source of 1,500 $\mu\text{mol m}^{-2} \text{s}^{-1}$, a CO₂ concentration of 390 ppm, air flow rate at 350 cm³ s⁻¹, and leaf temperature of 25°C. All measurements occurred between 8:00 AM and 12:00 PM. After each measurement, the foliage inside the cuvette was photographed and ImageJ 1.44 (Abràmoff et al. 2004) was used to quantify needle area to account for blank space in the cuvette.

From April 30-May 16th, we monitored the three marked branches per tree for terminal bud break every other day. No trees broke bud prior to April 30th; any branches that had not broken bud by May 18th, the day that harvest began, were scored as broken on May 19th. We compared the timing of hemlock bud break to the bud break of co-occurring hardwoods using estimates of Normalized Vegetation Difference Index (NDVI) and Leaf Area Index (LAI) obtained from NASA MODIS (Moderate Resolution Imaging Spectroradiometer) (See Appendix S1 for details).

Harvest and biomass measurements

The 88 experimental trees were harvested between May 18-29 2015. Because of the time required for whole-tree excavation, we split the trees into 22 four-tree harvest groups, with each treatment represented in at least every third group.

Depending on the available person power, 1-3 groups were harvested daily. The growing window for each tree was estimated by subtracting the mean day of bud break for the three marked branches from its harvest date. This was used to calculate rate of new flush production.

Immediately prior to harvesting each tree, we recorded its height and trunk diameter five cm above the root ball. Following these measurements, the three previously-marked branches were each clipped at the base, placed in zip lock bags and stored on ice; to ensure we possessed sufficient plant material for chemical analyses, we also collected a fourth haphazardly-selected branch from each tree. These four branches were immediately transported to the laboratory for processing (detailed below). The trunk of each tree was clipped five cm above the root ball and the aboveground portion placed in a large paper bag and oven dried for 24 hrs at 60° C; pilot experiments revealed that this length of time was sufficient to fully dry the woody biomass. We separated dry material into three tissue classes (new flush, $\geq$ 1-yr needles, and woody) that were each weighed. After the aboveground portion of each tree had been removed, its root ball was carefully excavated, cleaned of all dirt and foreign objects, oven dried as above, and weighed. Belowground harvest and processing protocols are detailed elsewhere (Schaeffer et al., in prep.)

Branch-level insect densities and chemical analyses

Once the marked branches had been returned to the laboratory, we used dissecting tools to remove all of the insects without damaging any hemlock tissue. Each branch was separated into five tissue types (new flush, 1-yr old needles, >1-yr old needles, 1-yr old stems, and >1-yr old stems) and weighed; the wet mass of each

tissue type was converted to dry mass estimates using tissue-type-specific conversion factors we generated in a pilot experiment. Each tissue type was kept separate for each tree and stored at -20°C before being dried at -55°C for 72 hrs in a lyophilizer. Dried biomass from each tissue type was ground into a fine powder using a KLECO ball mill (Garcia Machines, Visalia, CA, USA).

For each marked branch, we combined the previously-collected data on insect numbers with the data on dry ≥ 1 -year needle biomass to calculate branch-level insect densities (insects/gram needle). We chose this metric because (A) HWA settles at the needle base and EHS settles on the needles themselves; and (B) marked branches of similar length often varied substantially in needle density. Given these facts, we felt that expressing densities on a per-gram basis provided a more ecologically-relevant density metric.

Carbon (C) and nitrogen (N) tissue content were determined by dry-combusting 2–3 mg of finely-ground material with a CHNOS analyzer (vario Micro cube, Elementar Americas, Mt. Laurel, NJ, USA). We estimated starch content using an EnzyChromTM starch assay kit (BioAssay Systems, Hayward, CA, USA) as per the manufacturer's protocol. Briefly, ten mg of finely-ground root material was boiled in one ml distilled water for five minutes, then centrifuged at 10,000g for two min; the supernatant containing soluble starch was set aside. The remaining pellet was reconstituted in 0.2 ml DMSO and boiled for five minutes to obtain more resistant starch. The supernatants were then combined and starch concentration (expressed on a dry weight basis) determined using the kit.

We measured free amino acid concentrations and relative composition

following the protocol of Gomez et al. (2012). For each needle tissue type, 0.2 g dry weight of sample material was extracted in one ml of pre-cooled 80% ethanol (v:v) at room temperature for 20 min. Samples were vortexed periodically and then centrifuged at 7,000 rpm for ten min; the supernatant was filtered through a 0.45- μ m pore size Acrodisk Syringe filter (Pall Gelman Laboratory, Ann Arbor, MI, USA). Following filtration, 500 μ l of extract was used for free amino acid determination using a commercial EZ: Faast™ kit (Phenomenex, Torrence, CA, USA) and GC-FID (AGILENT INFO) as per the manufacturer's protocol. Briefly, two ml of sample was injected (15:1 split) on a Zebron ZB-AAA column (0.25 mm x 10 m; Phenomenex) at 250°C. Helium was used as carrier gas at a flow rate of 1.5 ml/min. The initial oven temperature was set to 110°C, increased 32°C per minute to a final temperature of 320°C, and held for three minutes. We identified and quantified 22 amino acids; the EZ: Faast™ kit does not, however, allow for the detection of arginine. Individual amino acids were identified by comparing spectra and retention times to amino acid standard solutions (norvaline as internal standard) provided with the EZ: Faast™ kit and quantified using ChemStation software (Rev. B.04.02; Agilent Technologies, Waldbron, Germany). We used data on the absolute concentrations of each amino acid to determine the relative concentration of each compound (i.e., amino acid composition).

Statistical analyses

All analyses were performed using R v. 3.2.2 (RCoreTeam 2014). We fit linear mixed effects models and used a backward-model-selection approach to examine the individual and interactive effects of HWA and EHS on hemlock. In all models, HWA

and EHS were treated as fixed factors, each with three levels corresponding to the length of infestation (0, 2, or 4 years) and an interactive term (HWA*EHS). Full and reduced models were ranked and compared based on Bayesian Information Criterion (BIC) values, a standard criterion for model selection. Details of each model are contained in Appendix S2. The lme4 package was used to generate and compare models (Pinheiro et al. 2014).

In the full model, initial trunk diameter at planting was included as a covariate. The one exception was that initial height, not initial diameter, was used as covariate for analyses of final height. Our analyses of photosynthetic rates also included time of day that the measurement was taken as a covariate. Row position (1-20) of each tree was included as a random effect in linear mixed effects models. We used this approach to examine how HWA and EHS affected the following metrics: final height, final basal diameter, total biomass, total aboveground biomass, total belowground biomass, above-/belowground biomass ratio, needle/woody biomass ratio, new flush production, photosynthesis, and bud break.

Because tissue type and age strongly impact plant chemistry, we analyzed percent C, percent N, CN ratio, total amino acids, and total starch using a slightly different approach. For stem and needle tissue, the age of the tissue (1-year or >1-year) was included in the models. Because we did not have enough tissue to conduct full suite of chemical analyses on all tissue types, analyses including new flush are limited to percent C, percent N, and CN ratio. We ran linear mixed effects models with row/harvest date as random effects.

For amino acids (AAs), we first analyzed total AA concentrations of 1-yr and

>1-yr old needles using MANOVA (Wilk's λ), followed by separate two-way ANOVAs for each tissue class. We used a similar approach to examine treatment-level differences in individual AA concentrations: an initial MANOVA to account for the lack of independence amongst individual AAs, followed by two-way ANOVAs for individual amino acids. Among-treatment differences were assessed using Tukey *post hoc* tests. Principal component analysis was then used to compare AA composition between treatments in each tissue class.

We used linear regression to assess the relationship between branch-level insect density and the following responses of 1-year needles: photosynthesis, percent N, total AA, and total starch. Insect density was used as the predictor variable for HWA-infested trees (2, 4 years) and EHS-infested trees (2, 4 years).

RESULTS

Growth and biomass allocation

While HWA altered hemlock growth and biomass allocation, EHS did not. Because the HWA*EHS interaction was never significant, we report only the main insect effects in the text (but see Appendix S2).

Neither HWA nor EHS affected total, above- or belowground plant biomass, but HWA altered plant biomass allocation (Fig. 1; Appendix S2: Tables S1 and S2). The above-/belowground ratio was 15% less in HWA-infested trees compared to HWA-free trees ($F_{2,79}=5.44$, $P<0.01$; Fig. 1a) and the aboveground needle/woody biomass ratio was 16% lower in HWA-infested trees ($F_{2,78}=4.62$, $P=0.01$; Fig. 1c). HWA-infested trees were also 7% shorter than HWA-free trees (HWA:

$F_{2,78}=3.67$, $P=0.03$), but there was no difference in final basal diameter (Appendix S2: Table S1).

Early spring growth

There were no treatment-level effects of HWA or EHS on photosynthetic rates of 1-year-old foliage (Appendix S2: Table S3). Although there was no relationship between HWA density and photosynthetic rates (Appendix S2: Table S4), EHS density was negatively correlated with photosynthetic rates among trees infested with EHS for 2- and 4-years (Appendix S2: Table S5).

HWA feeding altered spring phenology, with HWA-infested trees breaking bud ~3 days later than HWA-free trees ($F_{2,79}=8.88$, $P<0.001$; Fig. 2); in contrast, EHS did not alter bud break (Fig. 2; Appendix S2: Table S6). New flush production in the short growing window prior to canopy leaf-out in early May (Appendix S1: Fig. S1) was ~30% lower for HWA-infested versus HWA-free trees ($F_{2,77}=36.32$, $P<0.001$; Fig. 3a) but was not reduced by EHS feeding ($F_{2,77}=1.91$, $P=0.15$; Fig. 3b).

Foliar chemistry

While HWA substantially altered multiple aspects of foliar chemistry, EHS had virtually no impact. The N content of 1-year-old needles in HWA-infested trees, for instance, was 10% higher than in HWA-free trees ($F_{2,166}=10.80$, $P<0.001$; Fig. 4a; Appendix S2: Table S7). Among trees infested with HWA for two years, HWA density was correlated with percent N; this was not, however, the case among trees infested for four years with HWA (Appendix S2: Table S4). New-flush needles on HWA-infested trees also had higher N levels ($F_{2,69}=4.22$, $P=0.02$; Fig. 4a). Although starch concentration in 1-year-old needles was similar in HWA-free trees

and trees infested with HWA for two years, it was 70% lower in needles of trees infested with HWA for four years (Fig. 4b). EHS feeding increased starch levels in 1-year needles ($F_{2,155} = 3.95$, $P=0.02$, Fig. 4b). There were no within-treatment relationships between insect density (HWA or EHS) and total starch concentration (Appendix S2: Table S4 and S5)

HWA feeding increased needle AA concentrations (Wilk's $\lambda = 0.75$, $P < 0.001$). Total AA concentrations in 1-yr and >1-yr needles were 40% and 30% higher, respectively (both $P < 0.001$), than in HWA-free trees. HWA density and total AA concentrations in 1-year needles were correlated among trees infested with HWA for two years, but not trees infested with HWA for four years (Fig. 4d; Appendix S2: Table S4). MANOVA results indicated that for both 1-yr and >1-yr needles, the presence of HWA altered concentrations of multiple amino acids, including valine, tryptophan, threonine, serine, lysine, leucine, isoleucine, glutamine and proline ($P < 0.05$ for all).

Principal component analyses revealed that proline was primarily responsible for the between-treatment shifts in amino acid profiles. While HWA feeding altered the concentrations of several amino acids relative to the control, proline accounted for much of the change in total concentration (PC1=23% in one year needles, PC1=30% in greater than one year needles) (Fig. 5). While EHS did not significantly affect total amino acid concentrations, infestation caused significant changes in alanine concentration within both tissue types.

DISCUSSION

Although invasive species can play a central role in shaping temperate ecosystems and many exotic sap-feeding herbivores pose major economic and ecologic threats, we know relatively little about the long-term impact of multiple exotic herbivores. We found that chronic herbivory by two invasive sap-sucking herbivores had diverging impacts on their common host. Multiple years of HWA herbivory altered hemlock biomass allocation (above-/belowground and needle/woody), phenology, and metabolites. In contrast, EHS had minimal impacts and did not interact with HWA: dually-infested trees showed changes in allocation, phenology, and metabolites typical of HWA-only treatments (Miller-Pierce et al. 2010, Radville et al. 2011, Gómez et al. 2012).

Changes in plant biomass allocation may manifest differently in distinct tissue types. Several months of HWA infestation on hemlock saplings, for instance, altered needle/woody ratios but not aboveground/belowground ratios (Soltis et al. 2015). The needle desiccation associated with HWA feeding (Soltis et al. 2014) likely caused the needle loss among HWA-infested trees (Soltis et al. 2015). Plants often respond to aboveground herbivory by shifting resources away from herbivore-attacked areas, often leading to an increase in belowground biomass (Babst et al. 2005, Babst et al. 2008). Given that total belowground biomass did not increase following HWA infestation, our results support previous findings (Soltis et al. 2015) that premature needle abscission drives changes in the above-/belowground ratio rather than mobilization of resources belowground.

Since the bulk of biomass allocation and growth in conifers occurs in early

spring, open-canopy and high-light conditions early in the growing season are critical for eastern hemlock. In north-central Massachusetts, eastern hemlock forests stored the most carbon in April and May, whereas peak carbon gain in the neighboring red oak forest occurred in July and August (Hadley and Schedlbauer 2002, Hadley et al. 2008). Forest-atmosphere C exchange rates during the summer months in red oak forest were twice that of eastern hemlock forest (Hadley et al. 2008). The presence of HWA delayed the first day of bud break by an average of three days (Fig. 2), a period during which the overstory canopy was rapidly closing (Appendix S1: Fig. S1). This impact of this several-day delay on plant growth is exacerbated by HWA-induced reductions in new flush production (Fig. 3). Although early-April photosynthetic rates of 1-year old needles were unaffected by the presence of HWA (Fig. 4), the impact of HWA on new flush production is evident. A previous study (Gonda-King et al. 2014) showed negative impacts of HWA on photosynthesis but were completed in the fall on new foliage and thus the effect of HWA on photosynthesis likely varies seasonally.

Herbivore-attacked plants often protect themselves via induced changes in primary and secondary metabolism (Stam et al. 2014, Zhou et al. 2015). Although herbivory can alter both primary (essential functions) and secondary (in part, defense functions) metabolites, research to date has primarily addressed secondary metabolites. While changes in inducible defenses are well documented following an herbivory event, the impacts on primary metabolism are often less studied (Zhou et al. 2015). HWA and EHS feeding induced disparate effects on N metabolism, as evidenced through measures of bulk N and free amino acids.

Consistent with previous findings (Gómez et al. 2012), HWA feeding

significantly elevated concentrations of N and the amino acid proline local to feeding sites. Proline accumulation is a common plant response to herbivory and drought (Delauney and Verma 1993); this and other HWA-induced changes in hemlock physiology (Radville et al. 2011, Domec et al. 2013) suggest that HWA may induce drought-like stress. In contrast, EHS feeding had no effect on either N or total amino acid concentration, regardless of tissue type or age. Interestingly, however, the responses of a suite of individual amino acids following herbivory suggests different defensive responses by hemlock to each herbivore. More specifically, HWA-infested plants had significantly lower levels of isoleucine and tryptophan. A similar pattern has been observed in *Arabidopsis* plants following aphid feeding, where it is associated with aphid-induced increases in the hormone abscisic acid (ABA) (Hillwig et al. 2016). Although ABA induction is often associated with water stress (Lee and Luan 2012), its induction may also benefit sap feeders via its antagonistic interactions with jasmonic acid (JA) signaling (Erb et al. 2009, Vos et al. 2013), a key pathway for anti-herbivore defense. In contrast, EHS feeding elevates alanine and phenylalanine, aromatic amino acids that are precursors for defensive plant volatiles. Phenylalanine is also an important precursor for defensive phenolic compounds (Shah 2003). The role of these defenses and signaling pathways in mediating interactions between HWA and EHS warrant further investigation.

Starch is another key primary metabolite which plays an essential role in storage. Following herbivory, stored carbohydrates are frequently remobilized. Attacked *Arabidopsis* plants, for instance, increased the concentrations of enzymes employed to break down stored carbohydrates (Appel et al. 2014). The mobilization of

organic compounds following attack can benefit the insect or the plant (Gómez et al. 2012, Zhou et al. 2015). Following 4-years of HWA infestation, starch in 1-year needles is reduced by approximately 30%, whereas it remains unchanged in >1-year needles (Fig. 4). Increasing EHS densities led to increases in starch in 1-year needles following 2-years of infestation, but this pattern dissipated after 4-years (Appendix S2: Table S8).

There are several explanations for the lack of interaction between HWA and EHS in this experiment. Gómez et al. (2012) noted that EHS feeding reduced amino acid concentrations in hemlock foliage. Since high N levels appear crucial to HWA survival (Pontius et al. 2006), the EHS-induced reductions in amino acids noted by Gomez et al. (2012) may help explain HWA's reduced performance on foliage previously infested with EHS in this study. In a forested understory, unlike the open-field conditions of the Gómez et al. (2012) study, EHS feeding did not significantly impact N levels. As hemlock thrives in low light conditions, our experimental design more accurately represents growing conditions. Although a number of possible explanations could account for prior arrival by EHS not altering the impact reported by HWA, the most likely explanation is moderately low scale densities during the first year of the experiment (Hickin et al. unpublished data). If these densities were not high enough to drive changes in primary and secondary metabolites, it is unlikely EHS would alter the impact of HWA.

Despite branch-level and landscape-level examination of these two invasive insects, no rigorous assessment of tree-level impacts has been explored prior to this experiment. Previous work (Miller-Pierce et al. 2010, Radville et al. 2011, Gómez et

al. 2012) was carried out in an open field. Given that hemlocks typically occur in shady and resource-limited areas, the site described in this experiment is more realistic set of abiotic conditions and provides a full-tree perspective of the impacts of two invasive insects. Two invasive herbivores from the same feeding guild have disparate effects on biomass allocation, early spring growth, and metabolites.

Acknowledgements

We thank P. Bravo, P. Candelas, L. Green, K. Pieper, E. Miller, S. Roitman, E. Scott, S. Tostes, and M. Tynan for field and lab assistance. This project was funded by National Science Foundation grants NSF-DEB 1256826 to C. Orians and NSF-DEB 1256769 to E. Preisser and C. Thornber, and National Institute of Food and Agriculture grant no. 2011-67013-30142 to E. Preisser. Any opinions, findings, and conclusions or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.\

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TABLES AND FIGURES

Table 1. Experimental design. Treatments are arranged in a 3 x 3 full-factorial design, with years of infestation by both hemlock woolly adelgid (HWA) and elongate hemlock scale (EHS) indicated. Numbers in parentheses indicate the number of replicates for each treatment. Insect densities (insects/cm) were taken in November 2014 using standard protocols (Hickin et al., in prep.).

<i>Insect infested</i>	→	HWA		
↓	<i># of years infested</i>	<i>0 years</i>	<i>2 years</i>	<i>4 years</i>
EHS	<i>0 years</i>	(12) Control EHS = 0 HWA = 0	(10) HWA-2 EHS = 0 HWA = 2.36 ± 0.31	(13) HWA-4 EHS = 0 HWA = 1.74 ± 0.20
	<i>2 years</i>	(9) EHS-2 EHS = 1.79 ± 0.37 HWA = 0	(7) Both-2 EHS = 1.64 ± 0.42 HWA = 1.11 ± 0.22	(9) HWA → Both EHS = 0.83 ± 0.26 HWA = 1.29 ± 0.27
	<i>4 years</i>	(9) EHS-4 EHS = 2.19 ± 0.37 HWA = 0	(12) EHS → Both EHS = 2.10 ± 0.72 HWA = 1.37 ± 0.3334	(6) Both-4 EHS = 1.11 ± 0.18 HWA = 1.21 ± 0.24

Figure 1. Ratio of (A, B) above- to below-ground biomass and (C, D) needle to wood biomass in response to attack by HWA (A, C) and EHS (B, D) following 0, 2, or 4 years of infestation. Bars represent means $\pm$ SE. Letters indicate a significant difference between groups based on a post-hoc Tukey HSD test.

Figure 2. Percentage of trees that broke bud from May 1 – May 18 with 0, 2, and 4 years of infestation by (A) HWA and (B) EHS. Individual points represent average day of bud break for a tree and are fitted with a best-fit line (gray shading = 95% confidence interval). The vertical dashed lines indicate the mean day of bud break depending on years of HWA or EHS infestation: 0 years (light orange/blue), 2 years (medium orange/blue), and 4 years (dark orange/blue).

Figure 3. Mean $\pm$ SE rate of new foliage production (grams/day) in early spring following 0, 2, and 4 years of infestation by (A) HWA and (B) EHS. Letters indicate a significant difference between groups based on a Tukey post-hoc test.

Figure 4. Mean $\pm$ SE of (A) photosynthetic rate, (B) percent nitrogen, (C) total starch concentration, and (D) total amino acids across different tissue types (new flush, 1-yr needles, >1-yr needles). Length of HWA infestation spans 0 years (light orange), 2 years (medium orange), and 4 years (dark orange). Nd indicates no data were available. Tukey post-hoc tests were completed in each tissue type grouping.

Figure 5. Principal component scores of amino acid composition in (A) 1-yr needles and (B) >1-yr needles of trees infested with HWA for 0 years (light orange), 2 years of infestation (medium orange), and 4 years of infestation (dark orange). Numbers in parentheses explain amount of variation explained by each principal component.

APPENDICES

APPENDIX S1

Canopy monitoring

We used remotely-sensed Normalized Vegetation Difference Index (NDVI) and Leaf Area Index (LAI) values obtained from NASA MODIS (Moderate Resolution Imaging Spectroradiometer) to assess the timing and rate of forest canopy closure for the study plot in 2015. NDVI measures the difference between infrared and red wavelengths reflected off of the earth's surface, and indicates 'greenness'. LAI for broadleaf canopies is the ratio of one-sided green leaf area to ground area; it is used to indicate canopy closure. Although the measures are correlated, they measure different aspects of phenology; we looked at both to be thorough.

NDVI and LAI values for a pixel containing the study site (US-Ha1, DBF, 41.47722, 71.51166) were retrieved from the online Application for Extracting and Exploring Analysis Ready Samples (AppEEARS), courtesy of the NASA EOSDIS Land Processes Distributed Active Archive Center (LP DAAC), USGS/Earth Resources Observation and Science (EROS) Center, Sioux Falls, South Dakota, <https://lpdaacsvc.cr.usgs.gov/appeears/>.

NDVI data were recorded at 16-day intervals for a 250 m pixel, and LAI values were calculated at 4-day intervals for a 500 m pixel. NDVI data were used if the index was produced without clouds (VI Quality MODLAND = 00 or 01) and pixel usefulness was 'highest' or 'lower' (VI usefulness = 0000 or 0001). LAI data were used if the pixel was 'good quality' (i.e. MODLAND = 0) and the cloud state was 'clear' or 'mixed' (i.e. CloudState $\neq$ 01).

Figure S1. Results of models assessing canopy closure at the field site in early spring. Vertical lines show the averaged bud break day among un-infested (pink), EHS-infested (lime green), and HWA-infested (blue) trees.

APPENDIX S2

Table S1. Results of linear mixed effect models (†) assessing the impacts of HWA and EHS on full tree metrics. When linear mixed model (denoted with †) were used, row was used as the random effect. In all cases we report Type II error.

	Final Height (†)			Final Basal Diameter (†)			Total Biomass (†)			Total Aboveground Biomass (†)			Total Belowground Biomass (†)		
	F	Df	P	F	Df	P	F	Df	P	F	Df	P	F	Df	P
HWA	3.67	2,78	0.03*	1.11	2,78	0.34	1.02	2,78	0.37	1.51	2,78	0.22	0.30	2,78	0.74
EHS	0.30	2,78	0.74	0.86	2,78	0.43	1.41	2,78	0.25	1.42	2,78	0.24	0.80	2,78	0.45
HWA*EHS	0.80	4,78	0.53	0.69	4,78	0.60	0.97	4,78	0.43	1.00	4,78	0.41	1.34	4,78	0.26
Initial diam/height	38.33	1,78	<0.001***	10.53	1,78	<0.01**	26.48	1,78	<0.001***	17.93	1,78	<0.001***	26.94	1,78	<0.001***
Random effect	-			Row			Row			Row			Row		
Model Used	Linear Mixed Effect			Linear Mixed Effect			Linear Mixed Effect			Linear Mixed Effect			Linear Mixed Effect		
Error type	Type II			Type II			Type II			Type II			Type II		

Table S2. Results of ANOVAs (¢) assessing the impacts of HWA and EHS on biomass ratios. In all cases we report Type II error.

	Aboveground Biomass/ Belowground Biomass Ratio (¢)			Needle Biomass/ Woody Biomass Ratio (¢)		
	F	Df	P	F	Df	P
HWA	5.44	2,79	0.01**	4.62	2,78	0.01**
EHS	0.67	2,79	0.52	0.22	2,78	0.80
HWA*EHS	0.24	4,79	0.91	0.98	4,78	0.42
Initial diam/height	-	-	-	7.10	1,78	0.01**
Random effect	-			-		
Model Used	ANOVA			ANOVA		
Error type	Type II			Type II		

Table S3. Results of a linear mixed effects model (†) assessing the impacts of HWA and EHS on photosynthesis rates on 1-yr old (2014 growth) foliage in mid-April prior to bud break. Row as used as the random effect in the model.

	Photosynthesis Rate (†)		
	F	Df	P
HWA	1.74	2,74	0.18
EHS	2.39	2,74	0.10
HWA*EHS	0.15	4,74	0.96
Time of day	69.00	1,74	<0.001***
Random effect?	Row		
Model Used	Linear Mixed Model		
Error type	Type II		

Table S4. HWA density (insects/gram) against photosynthetic rate, Percent N, total starch, and total AA in 1-year needles. All statistics were completed with linear models. Models are split by length of HWA infestation (2-years of HWA and 4-years of HWA). These groups include dually infested trees. A dash indicates that the metric was not used in the final model.

		2-years HWA			4-years HWA		
		intercept	HWA density (insects/gram)	Time measured	intercept	HWA density (insects/gram)	Time measured
Photosynthesis rate ($\mu \text{ moles}^{-1}\text{m}^{-2}\text{s}^{-1}$)	estimate	-9.38	-0.09	26.2	-13.08	-0.07	34.46
	std. error	5.36	0.12	11.7	2.58	0.07	5.73
	t	-1.75	-0.77	2.24	-5.07	-0.97	6.01
	P	0.09	0.45	0.03*	<0.001***	0.35	<0.001***
Percent Nitrogen	estimate	0.77	0.02	-	0.81	0.0003	-
	std. error	0.03	0.01	-	0.03	0.005	-
	t	24.89	2.938	-	32.162	0.06	-
	P	<0.001***	0.01**	-	<0.001***	0.949	-
Total Starch (mg/g DW)	estimate	0.07	-0.002	-	0.03	0.002	-
	std. error	0.01	0.002	-	0.01	0.001	-
	t	7.73	-1.30	-	4.55	1.51	-
	P	<0.001***	0.21	-	<0.001***	0.14	-
Total Amino Acids (μg DW)	estimate	1096.62	82.01	-	1174.58	10.04	-
	std. error	122.82	23.73	-	90.36	16.17	-
	t	8.93	3.46	-	13	0.62	-
	P	<0.001***	<0.01**	-	<0.001***	0.54	-

Table S5. EHS density (insects/gram) against photosynthetic rate, Percent N, total starch, and total AA in 1-year needles. All statistics were completed with linear models. Models are split by length of EHS infestation (2-years EHS and 4-years EHS). These groups include dually infested trees. A dash indicates that the metric was not used in the final model.

		2-years EHS			4-years EHS		
		intercept	EHS density (insects/gram)	Time measured	intercept	EHS density (insects/gram)	Time measured
Photosynthesis rate ($\mu \text{ moles}^{-1} \text{m}^{-2} \text{s}^{-1}$)	estimate	-17.51	-0.07	44.35	-11.13	-0.02	31.43
	std. error	3.57	0.12	7.73	3.72	0.05	9.00
	t	-4.90	-0.58	5.74	-3.00	-0.40	3.49
	P	<0.001***	0.57	<0.001***	0.01**	0.70	<0.01**
Percent Nitrogen	estimate	0.81	-0.002	-	0.85	-0.01	-
	std. error	0.05	0.01	-	0.03	0.002	-
	t	17.74	-0.23	-	29.50	-2.67	-
	P	<0.001***	0.82	-	<0.001***	0.01**	-
Total Starch (mg/g DW)	estimate	0.04	0.003	-	0.04	0.0004	-
	std. error	0.01	0.002	-	0.01**	0.0005	-
	t	3.33	1.23	-	7.14	0.74	-
	P	<0.01**	<0.01**	-	<0.001***	0.47	-
Total Amino Acids (μg DW)	estimate	1308.18	-18.82	-	1294.75	-15.05	-
	std. error	170.29	32.09	-	130.60	13.90	-
	t	7.68	-0.59	-	9.91	-1.08	-
	P	<0.001***	0.56	-	<0.001***	0.29	-

Table S6. Results of a linear mixed effects model (†) and ANOVA (¢) assessing the impacts of HWA and EHS on bud break and daily new flush production in mid-May.

	Bud Break (†)			Daily New Flush (¢)		
	F	Df	P	F	Df	P
HWA	8.88	2,79	<0.001***	36.32	2,77	<0.001***
EHS	0.45	2,79	0.64	1.91	2,77	0.15
HWA*EHS	0.60	4,79	0.66	0.90	4,77	0.47
Initial diameter	-	-	-	7.70	1,77	<0.01**
Random effect?	Row			-		
Model Used	Linear Mixed Effect			Mixed Linear Model		
Error type	Type II			Type II		

Table S7. Results from Linear Mixed Effects Models assessing the impact of HWA, EHS, and foliage age on percent N, percent C, and CN ratio.

		Percent Nitrogen			Percent Carbon			Carbon:Nitrogen Ratio		
		F	Df	P	F	Df	P	F	Df	P
New Flush	HWA	4.22	2,69	0.02*	5.64	2,69	<0.01**	11.80	2,69	<0.001***
	EHS	0.25	2,69	0.78	0.45	2,69	0.64	0.41	2,69	0.66
	HWA*EHS	0.32	4,69	0.87	3.87	4,69	<0.01**	0.75	4,69	0.56
	Random effect	-			Row			Row		
	Model Used	ANOVA			Linear Mixed Effect			Linear Mixed Effect		
	Error type	Type III			Type II			Type III		
Needles	HWA	10.80	2,166	<0.001**	0.02	2,167	0.98	12.32	2,166	<0.001**
	EHS	0.03	2,166	0.97	0.03	2,167	0.97	0.17	2,166	0.85
	Age	18.45	2,166	<0.001**	-	-	-	31.32	2,166	<0.001**
	HWA*EHS	1.19	4,166	0.32	0.17	4,167	0.95	0.96	4,166	0.43
	HWA*Age	-	-	-	-	-	-	-	-	-
	EHS*Age	-	-	-	-	-	-	-	-	-
	HWA*EHS*Age	-	-	-	-	-	-	-	-	-
	Random effect	-			Row			Row		
	Model Used	Linear Mixed Effect			Linear Mixed Effect			Linear Mixed Effect		
	Error type	Type II			Type II			Type II		
Stems	HWA	0.90	2,158	0.41	0.91	2,157	0.41	3.75	2	0.14
	EHS	0.13	2,158	0.88	0.68	2,157	0.51	2.67	2	0.26
	Age	1.54	1,158	0.22	-	-	-	2.95	1	0.09
	HWA*EHS	2.10	4,158	0.08	0.89	4,157	0.47	6.82	4	0.15
	HWA*Age	1.52	2,158	0.22	-	-	-	0.82	2	0.66
	EHS*Age	82.45	2,158	<0.001***	-	-	-	55.56	2	<0.001***
	HWA*EHS*Age	34.88	4,158	<0.001***	-	-	-	48.23	4	<0.001***
	Random effect	-			Harvest Date			Row		
	Model Used	Linear Mixed Effect			Linear Mixed Effect			Linear Mixed Effect		
	Error type	Type III			Type II			Type III		

Table S8. Results from Linear Mixed Effects Models assessing the impact of HWA, EHS, and foliage age on total AA and total starch among needles and stems tissue.

		Total AA ($\mu\text{g/g DW}$)			Total Starch (mg/g DW)		
		F	Df	P	F	Df	P
Needles	HWA	19.90	2,155	<0.001***	2.19	2,155	0.11
	EHS	0.67	2,155	0.51	3.95	2,155	0.02*
	Age	38.08	1,155	<0.001***	97.17	1,155	<0.001***
	HWA*EHS	0.32	4,155	0.86	0.80	4,155	0.52
	HWA*Age	1.58	2,155	0.21	1.77	2,155	0.17
	EHS*Age	0.04	2,155	0.96	0.31	2,155	0.73
	HWA*EHS*Age	0.65	4,155	0.62	0.50	4,155	0.73
	Random effect	Harvest Date			Harvest Date		
	Model Used	Linear Mixed Effect			Linear Mixed Effect		
	Error type	Type II			Type II		
Stems	Intercept	-	-	-	0.02	2,166	0.97
	HWA	-	-	-	0.65	2,166	0.49
	EHS	-	-	-	109.01	1,166	<0.001***
	Age	-	-	-	1.76	4,166	0.13
	HWA*EHS	-	-	-	-	-	-
	HWA*Age	-	-	-	-	-	-
	EHS*Age	-	-	-	-	-	-
	HWA*EHS*Age	-	-	-	-	-	-
	Random effect	Row			Row		
	Model Used	Linear Mixed Effect			Linear Mixed Effect		
	Error type	Type II			Type II		

Table S9. Amino acids (ug/g dry tissue) from 1 year needles by treatment.

Amino acid	Control	EHS-2	EHS-4	HWA-2	HWA-4	Both-2	Both-4	EHS>Both	HWA>Both
α -Aminoadipic acid	0.069	0.053	0.025	0.039	0.048	0.037	0.075	0.021	0.073
α -Aminobutyric acid	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.002
Allo-isoleucine	0.005	0.004	0.002	0.003	0.004	0.002	0.004	0.004	0.005
Alanine	0.058	0.070	0.090	0.048	0.056	0.043	0.052	0.061	0.051
Asparagine	0.008	0.009	0.009	0.007	0.010	0.008	0.006	0.011	0.008
Aspartic acid	0.126	0.115	0.120	0.087	0.092	0.090	0.078	0.104	0.097
β -Aminoisobutyric acid	0.001	0.000	0.000	0.000	0.001	0.001	0.000	0.002	0.000
Cystine	0.002	0.002	0.001	0.001	0.002	0.001	0.003	0.004	0.003
Glutamine	0.042	0.037	0.044	0.047	0.046	0.044	0.042	0.051	0.043
Glycine	0.001	0.002	0.002	0.002	0.001	0.001	0.001	0.002	0.001
Histidine	0.016	0.013	0.018	0.011	0.011	0.011	0.007	0.013	0.011
Hydroxyproline	0.009	0.007	0.008	0.006	0.007	0.005	0.005	0.007	0.006
Isoleucine	0.014	0.015	0.013	0.008	0.005	0.004	0.004	0.009	0.004
Leucine	0.006	0.006	0.007	0.005	0.003	0.004	0.003	0.006	0.003
Lysine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Methionine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Ornithine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Phenylalanine	0.122	0.099	0.089	0.082	0.092	0.077	0.081	0.093	0.097
Proline	0.274	0.256	0.193	0.457	0.449	0.490	0.482	0.394	0.430
Sarcosine	0.000	0.000	0.000	0.002	0.000	0.000	0.000	0.000	0.007
Serine	0.151	0.225	0.283	0.143	0.121	0.138	0.119	0.154	0.114
Threonine	0.017	0.016	0.018	0.011	0.009	0.009	0.008	0.013	0.009
Tryptophan	0.034	0.030	0.035	0.017	0.019	0.016	0.013	0.021	0.016
Tyrosine	0.018	0.015	0.020	0.011	0.011	0.009	0.010	0.013	0.011
Valine	0.027	0.026	0.025	0.014	0.010	0.008	0.007	0.016	0.009

Table S10. Amino acids (ug/g dry tissue) from >1 year needles by treatment.

Amino acid	Control	EHS-2	EHS-4	HWA-2	HWA-4	Both-2	Both-4	EHS>Both	HWA>Both
α -Aminoadipic acid	0.043	0.061	0.028	0.025	0.081	0.042	0.062	0.029	0.055
α -Aminobutyric acid	0.000	0.002	0.001	0.000	0.000	0.002	0.000	0.002	0.000
Allo-isoleucine	0.003	0.003	0.003	0.003	0.003	0.004	0.002	0.003	0.005
Alanine	0.071	0.078	0.083	0.063	0.064	0.047	0.061	0.061	0.069
Asparagine	0.009	0.010	0.011	0.010	0.009	0.009	0.007	0.010	0.009
Aspartic acid	0.109	0.108	0.118	0.111	0.101	0.059	0.097	0.108	0.124
β -Aminoisobutyric acid	0.001	0.002	0.001	0.002	0.002	0.001	0.000	0.001	0.002
Cystine	0.003	0.001	0.002	0.001	0.001	0.001	0.001	0.001	0.001
Glutamine	0.038	0.040	0.037	0.025	0.039	0.029	0.028	0.043	0.033
Glycine	0.001	0.000	0.000	0.000	0.000	0.004	0.001	0.004	0.001
Histidine	0.016	0.017	0.019	0.016	0.012	0.012	0.011	0.014	0.015
Hydroxyproline	0.014	0.012	0.012	0.009	0.010	0.008	0.018	0.016	0.014
Isoleucine	0.015	0.024	0.013	0.009	0.006	0.006	0.005	0.009	0.006
Leucine	0.005	0.005	0.004	0.004	0.002	0.000	0.003	0.004	0.002
Lysine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Methionine	0.000	0.000	0.000	0.000	0.000	0.001	0.001	0.000	0.000
Ornithine	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Phenylalanine	0.139	0.131	0.118	0.098	0.103	0.101	0.134	0.118	0.112
Proline	0.294	0.245	0.229	0.446	0.385	0.437	0.387	0.370	0.402
Sarcosine	0.000	0.000	0.005	0.000	0.001	0.006	0.003	0.006	0.000
Serine	0.157	0.189	0.232	0.125	0.142	0.187	0.142	0.143	0.103
Threonine	0.016	0.014	0.018	0.012	0.008	0.008	0.006	0.011	0.010
Tryptophan	0.028	0.022	0.029	0.017	0.016	0.016	0.014	0.020	0.019
Tyrosine	0.009	0.008	0.012	0.006	0.005	0.009	0.006	0.009	0.008
Valine	0.028	0.028	0.024	0.017	0.010	0.011	0.012	0.017	0.012