

RESEARCH ARTICLE

Individual and non-additive effects of exotic sap-feeders on root functional and mycorrhizal traits of a shared conifer host

Robert N. Schaeffer^{1*}  | Claire M. Wilson^{2*} | Laura Radville^{2,3} |
Mauri Barrett^{2,4} | Elizabeth Whitney² | Sofia Roitman¹ | Esther R. Miller¹ |
Benjamin E. Wolfe¹ | Carol S. Thornber^{2,5} | Colin M. Orians¹ | Evan L. Preisser²

¹Department of Biological Sciences, Tufts University, Medford, MA, USA

²Department of Biological Sciences, University of Rhode Island, Kingston, RI, USA

³Department of Ecosystem Science and Management and Ecology Intercollegiate Graduate Degree Program, Pennsylvania State University, University Park, PA, USA

⁴USDA-APHIS, Buzzards Bay, MA, USA

⁵Department of Natural Resources Science, University of Rhode Island, Kingston, RI, USA

Correspondence

Robert N. Schaeffer
Email: schaeffer.robert@gmail.com

Funding information

National Science Foundation, Grant/Award Number: NSF-DEB 1256826 and NSF-DEB 1256769; National Institute of Food and Agriculture, Grant/Award Number: 2011-67013-30142; National Science Foundation EPSCoR Cooperative Agreement, Grant/Award Number: EPS-1004057

Handling Editor: Alison Bennett

Abstract

1. Forest pests drive tree mortality through disruption of functional traits linked to nutrient acquisition, growth and reproduction. The impacts of attack by individual or multiple above-ground herbivores on root functional traits critical to tree health have received little attention. This is especially true for exotic herbivores, organisms often found in disturbed forests.
2. We excavated whole-root systems from eastern hemlock (*Tsuga canadensis*) individuals experimentally infested with hemlock woolly adelgid (HWA: *Adelges tsugae*) and elongate hemlock scale (EHS: *Fiorina externa*) individually, or in combination, for periods of 2 and 4 years. Below-ground root biomass, functional traits and storage nutrients were measured to assess impacts of herbivory. We also quantified ectomycorrhizal fungal (EMF) colonisation of fine roots and used culture-independent methods to examine EMF diversity.
3. Trees infested with HWA had a greater root mass fraction (root to total biomass ratio), although feeding had no observable effects on root functional traits (e.g. specific root length) or on resource allocation to roots. HWA feeding did significantly reduce EMF colonisation of hemlock fine roots, though surprisingly, EMF diversity and that of other fungal associates were unaffected. In contrast to HWA, EHS (alone or in conjunction with HWA) feeding had no observable effect on below-ground traits or EMF colonisation alone; however, its presence mediated HWA effects when trees were co-infested. Simultaneous infestation within the same year yielded significant reductions in EMF colonisation, whereas prior EHS attack weakened HWA effects.
4. Our results collectively suggest that prior EHS attack dampens the impact of HWA on below-ground functional traits. This highlights how the timing and sequence of herbivore arrival can alter plant-mediated interactions between herbivores and their effects on above–below-ground linkages and associated tree health.

KEYWORDS

ectomycorrhizal fungi, elongate hemlock scale, hemlock woolly adelgid, herbivory, root functional traits, specific root length, *Tsuga canadensis*

1 | INTRODUCTION

Forest pests, exotic and native, are pervasive agents of tree stress and mortality world-wide (Anderegg, Kane, & Anderegg, 2013). The impacts of these pests are mediated through disruption of functional traits linked to nutrient acquisition, growth and reproduction. While much is known about the effects of pests on above-ground functional traits (Zvereva, Lanta, & Kozlov, 2010), less is known about their impact on below-ground traits. Attack by individual herbivores and their associated cues have been shown to both increase biomass and resource allocation towards roots in herbaceous (Gómez, Ferrieri, Schueller, & Orians, 2010; Schwachtje & Baldwin, 2008) and woody plants (Babst et al., 2005; Babst, Ferrieri, Thorpe, & Orians, 2008), as well as decrease investment, observed in several speciose genera, including *Populus* (Stevens, Kruger, & Lindroth, 2008), *Quercus* (Frost & Hunter, 2008) and *Eucalyptus* (Eyles, Pinkard, & Mohammed, 2009). Many of these species respond to defoliation by shifting biomass away from roots to above-ground tissues (where resources for defence induction are required; see Arnold & Schultz, 2002). These studies tend to focus on the impacts of single pests or stressors, which provides little insight into the synergistic, antagonistic or additive effects of multiple herbivores on plant functional traits (Blossey & Hunt-Joshi, 2003; Erb, Robert, Hibbard, & Turlings, 2011; Gómez, Orians, & Preisser, 2012). Understanding the effects of multiple herbivores on below-ground functional traits will provide important insights into how trees respond to pressures experienced in increasingly disturbed forests.

Physiological processes involving resource acquisition and transport have important consequences for tree growth, survival and reproduction (Reich et al., 2003), and attack by pests can disrupt these processes via a number of mechanisms. For instance a tree's carbon budget can be negatively impacted through defoliation by chewing insects or nutrient loss linked to phloem-feeders. Such consumption can render a tree unable to assimilate or mobilise enough carbohydrates to sustain fundamental processes such as respiration, growth and defence; left unchecked, these losses can lead to carbon starvation and death (McDowell, 2011; Sala, Piper, & Hoch, 2010). Moreover, mobilisation and distribution of resource reserves from roots and other storage organs requires a functioning hydraulic system, which may also be compromised by antagonists and feeding-induced shifts in morphology that can affect whole-plant water acquisition (Arthur & Hain, 1986).

Although above-ground consumers can drive plastic responses in below-ground traits, we have only a limited understanding of the underlying mechanism(s) and consequences to below-ground functional traits linked to resource acquisition, including investment in mycorrhizal mutualisms. Investment in roots, for example involves trade-offs between their costs of production and the potential benefits of resource acquisition. Root traits such as specific root length ("SRL" root length/weight), tissue density and hair density can mediate plant stresses such as carbon or water limitation and greatly impact plant performance (Comas, Becker, Cruz, Byrne, & Dierig, 2013; Fort et al., 2015). Variation in, and plasticity of, these and other root traits is often tied to environmental variation and the need to obtain

soil-based resources. If herbivore cues increase biomass and resource allocation towards roots (Gómez et al., 2010; Schwachtje & Baldwin, 2008), this could increase the production of absorptive fine roots (e.g. first- and second-order; *sensu* Pregitzer et al., 2002) necessary to acquire water and nutrients essential for regrowth (Moreira, Zas, & Sampedro, 2012). Other foraging traits are also important. Increased nutrient availability can decrease SRL (Freschet, Swart, & Cornelissen, 2015), whereas plants with higher SRL are less impaired by water and nutrient stress (Fort et al., 2015). Moreover, root associations with mycorrhizal fungi are also important to the health of species that rely on this mutualism for nutrient acquisition. A meta-analysis found that the impact of above-ground pests on mycorrhizal mutualisms can range from positive to negative (Barto & Rillig, 2010). The nature of this interaction can depend on whether mycorrhizae are ectomycorrhizal or arbuscular, as each have distinct physiologies (Smith & Read, 2008) and likely respond differently to herbivore-induced shifts in plant primary metabolism and tissue loss. These disparate effects highlight the need to understand the consequences of multiple herbivores on below-ground functional traits.

The past half-century has seen a rapid and widespread decline in many tree species, often due to the effects of exotic pests (Gandhi & Herms, 2009). Eastern hemlock (*Tsuga canadensis*), a long-lived, shade-tolerant conifer that is considered a foundation species in eastern forests, has suffered a severe decline in the eastern United States (Ellison et al., 2005) and is attacked by at least two exotic herbivores. This decline has been linked to the hemlock woolly adelgid (HWA: *Adelges tsugae*), and to a lesser degree, elongate hemlock scale (EHS: *Fiorina externa*). Hemlock woolly adelgid feeding often causes rapid mortality, and hemlock loss can alter a suite of habitat characteristics important for community diversity and ecosystem function (Adkins & Rieske, 2013; Ford & Vose, 2007; Orwig, Cobb, D'Amato, Kizlinski, & Foster, 2008). In contrast, EHS infestation has a minimal effect on hemlock growth and only has a pronounced effect on already-weakened trees (McClure, 1980; Miller-Pierce, Orwig, & Preisser, 2010). However, previous studies have indicated that EHS may deter HWA and mitigate HWA-induced damage to hemlocks in areas where both co-occur (Gómez, Gonda-King, Orians, & Preisser, 2014; Miller-Pierce & Preisser, 2012; Preisser & Elkinton, 2008).

Feeding by HWA can affect a suite of above-ground functional traits important for overall tree health. For instance HWA feeding can affect hemlock's ability to assimilate carbon through reduced photosynthetic capacity (Gonda-King, Gómez, Martin, Orians, & Preisser, 2014). Moreover, it has been hypothesised that hemlock mortality following HWA attack may be due to hydraulic failure (Domec et al., 2013). Numerous lines of evidence support this hypothesis, including HWA-induced formation of abnormal wood (Gonda-King, Radville, & Preisser, 2012) and reductions in stomatal conductance (Gonda-King et al., 2014). Branches from HWA-infested trees have upwards of 50% more false rings, layers of thick-walled xylem cells that can significantly restrict water flow (Spicer & Gartner, 1998), than uninfested trees (Gonda-King et al., 2012). It remains unclear how these effects cascade to affect below-ground processes and functional traits.

Here, we explore the effects of HWA and EHS on eastern hemlock root functional traits. Using a full-factorial experimental design (Figure 1), we examined individual and combined (simultaneous vs. sequential infestation) effects of both exotics on foraging-related traits: resource concentration, root morphology and ectomycorrhizal fungal (EMF) colonisation and diversity. We tested the following hypotheses. First, trees attacked by HWA exhibit root traits that can limit their foraging ability. Given HWA reduction in photosynthesis and carbon assimilation (Gonda-King et al., 2014), we expect root production and maintenance to be limited. HWA-infested trees may produce thinner roots to maximise water and nutrient uptake while reducing carbon costs. Previous research also suggests that infested trees experience symptoms similar to those of water-stressed trees (Domec et al., 2013a; Gómez et al., 2012), which may induce trees to produce longer roots for maximising water acquisition. Second, attack by HWA induces movement of nutrients away from below-ground tissues. Adelgid feeding can increase and/or alter nitrogen (N) and N-rich metabolites, including free amino acids, local to feeding sites (Gómez et al., 2012; Stadler, Müller, & Orwig, 2006). Such changes may suggest an ability for HWA to manipulate host resources for its benefit, like other members of the hemipteran family Adelgidae (Havill & Footitt, 2007). Third, HWA-attacked trees have reduced EMF colonisation and altered root-fungal community composition. Eastern hemlock, a member of the Pinaceae family, interacts symbiotically with EMF, which form dense hyphal sheathing around its roots and heavily mediate water and nutrient uptake (Smith & Read, 2008). As with root production and maintenance, we expect that HWA-infested trees will be limited in their ability to support EMF associates through carbon provisioning due to reduced photosynthesis (Gonda-King et al., 2014). Overall, considering that EHS generally weakens rather than kills hemlock, we expect to observe only a small negative effect of EHS on root functional traits, including shifts in nutrient allocation from below- to above-ground, as well as EMF

colonisation. Finally, since EHS limits HWA abundance (Gómez et al., 2014; Preisser & Elkinton, 2008), we expect an intermediate effect on root traits for trees inoculated with both exotics simultaneously and sequentially.

2 | MATERIALS AND METHODS

2.1 | Study system

The hemlock woolly adelgid is an aphid-like exotic that was introduced to the United States from Japan around 1950 and currently inhabits over a dozen eastern states (Souto, Luther, & Chianese, 1996). Mature adelgids insert their stylets at the base of needles and feed on stored sugars in xylem ray parenchyma cells (Young, Shields, & Berlyn, 1995). Elongate hemlock scale, introduced from Japan in 1908, feeds through an inserted stylet on needle mesophyll (McClure, 1980). Because both herbivores are sessile fluid-feeders, their interspecific competition and limitation of HWA performance by EHS is likely mediated through differential responses of plant functional traits to each herbivore.

2.2 | Experimental approach

In April 2011, we established a long-term common garden experiment at the Kingston Wildlife Research Station (Kingston, RI, USA) in the understory of a mixed-hardwood forest. Full details concerning the experimental site and common garden design are provided in Appendix S1. Briefly, 200 ~0.3 m 1-year-old *T. canadensis* seedlings (Van Pines Nursery, West Olive, MI, USA) were planted 1 m apart in a grid (10 × 20) using a randomised complete block design, with each seedling enclosed in a wire-mesh cage and then covered with a mesh bag (Agribon-15, Johnny's Selected Seeds, Waterville, ME, USA; 90% light transmission). These enclosures protected trees from deer browsing and prevented inadvertent cross-contamination of treatments, as insects are easily wind-dispersed (McClure, 1990).

We employed a full-factorial design, which resulted in nine treatments at the tree level (Figure 1). Treatments were randomly assigned within each row; every row contained each treatment. Treatments were applied annually, in a staged process, following a standard protocol (Butin, Preisser, & Elkinton, 2007). First, we inoculated trees in the herbivore treatments annually with HWA (A), EHS (S) or both (B) insects from 2011 to 2014 (treatments: A₄, S₄ and B₄). Additional trees were kept uninfested for the first 2 years of the study and then inoculated with HWA, EHS or both insects in the third year (treatments: A₂, S₂ and B₂). By staging our treatments, we could study the effect of herbivore feeding on tree physiology and growth over time while accounting for year-to-year differences. In the remaining two treatments, the order of infestation was taken into account. Trees inoculated with only HWA or EHS in 2011 and 2012 were inoculated with both insects in 2013 and 2014 (treatments A₄S₂ and S₄A₂). Control trees received annual sham treatments using uninfested hemlock foliage to control for tree handling.

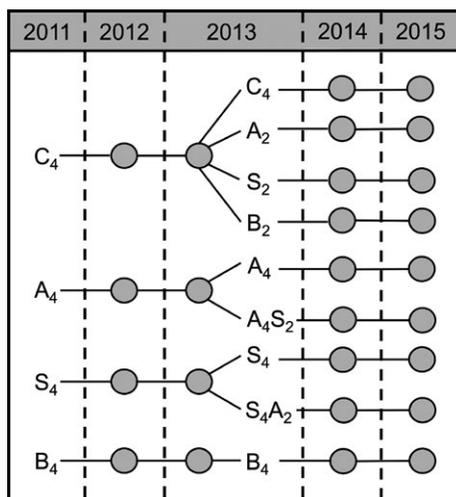


FIGURE 1 Experimental design. C, control (neither insect); A, hemlock woolly adelgid (HWA); S, elongate hemlock scale (EHS); B, both insects. Inoculation duration (years) for each insect is indicated by subscripts

2.3 | Herbivore abundance

To assess infestation levels, insect densities were monitored annually in early spring and late fall. Briefly, in each season, two 5-cm branchlets were selected per tree, and all HWA or EHS individuals encountered on a branchlet were counted.

2.4 | Plant harvest and measurement of root traits

Of the 200 trees planted in 2011, 88 were harvested in 2015. The remaining 112 trees went unharvested due to either being: (1) destroyed by several large branches from canopy trees that fell during Hurricane Sandy in 2012; (2) accidentally cross-contaminated; or (3) browsed by deer that dislodged their cages. To randomise date of harvest across treatments, the 88 trees were randomly split into 22 harvest groups (four trees/harvest group), where each treatment appeared in at least every third group. This accommodated for uneven replicates across treatments. Between 1 and 3 harvest groups were collected per day between 18 and 29 May 2015.

Full details concerning methods for tree and root excavation are provided in Appendix S1. Briefly, upon harvest, whole-root systems were fully excavated, washed and root fragments were obtained for measurements of root morphology (e.g. SRL), biomass, resource allocation (i.e. carbon, nitrogen and starch) and EMF colonisation and diversity. Root subsamples used for these measures were obtained from throughout the whole-root system, taken at approximately 45° angles to ensure the entire root system was represented.

2.5 | Ectomycorrhizal colonisation and diversity

Root fragments were analysed for EMF colonisation and diversity using two approaches. First, root samples were cut into 2–5 cm sections and a random sample of at least 100 root tips per tree was examined. Root tips (ends of first-order roots) were examined under a dissecting microscope and recorded as mycorrhizal or non-mycorrhizal. Root tips with a distinct and visible mantle were considered EMF, and representative EMF tips were dissected to confirm mantle presence. Ectomycorrhizal fungal colonisation was calculated as the number of EMF root tips divided by the sum of living mycorrhizal and non-mycorrhizal tips. Second, we used molecular analysis to determine the diversity and composition of root fungal communities associated with our harvested trees. Briefly, DNA was extracted and the fungal ITS1 region was amplified using primers ITS1f/ITS2 (Gardes & Bruns, 1993; White, Bruns, Lee, & Taylor, 1990). Amplicon pools were sequenced using Illumina HiSeq. Sequence processing was carried out using QIIME (v1.8) (Caporaso et al., 2010) and USEARCH (Edgar, 2010). Full details concerning library prep and bioinformatic processing are contained in Appendix S1.

2.6 | Statistical analyses

Adelgid and scale densities over the course of the experiment were analysed using repeated-measures ANOVA, with sampling date and herbivore treatment as fixed factors. We used two-way

MANOVA to test herbivore effects (HWA, EHS) and their interaction on correlated root traits and EMF colonisation. Each factor (HWA and EHS) in the full-factorial design had three levels with respect to the duration of infestation: 0 year (no insect), 2 and 4 years respectively. If an interaction was not present, the analysis was repeated without the interaction term to test for only main effects. Subsequent ANOVAs were run if the overall model was significant. Differences among treatments were determined using post hoc Tukey HSD tests.

Before analyses of sequencing data, fungal OTUs were rarefied to an even sampling depth of 6,903 sequences (the smallest amount of sequences for any sample). To analyse the diversity and composition of the total root fungal and EMF community, Chao1 and Shannon richness estimates were calculated using *phyloseq* (McMurdie & Holmes, 2013). To test for effects of herbivory on total and EMF fungal richness and diversity measures, we used one-way ANOVAs with herbivory treatment as a fixed factor and Chao1 and Shannon diversity estimates as response variables. Pair-wise dissimilarities between total and EMF fungal communities were calculated using the Bray–Curtis dissimilarity metric. Dissimilarity was then visualised via non-metric multidimensional scaling (nMDS). To estimate the explanatory power of herbivore treatments on total and EMF fungal community composition, we used permutational multivariate analysis of variance (PERMANOVA) based on 1,000 permutations in the *VEGAN* package in R (Oksanen et al., 2015). Finally, we assessed whether the abundance of individual OTUs differed between the control group and each herbivore treatment using DESeq2 with Benjamini–Hochberg corrections for multiple testing (Love, Huber, & Anders, 2014). All analyses were performed using R v. 3.2.2 (R Core Team 2015).

3 | RESULTS

3.1 | Herbivore abundance

Adelgid and EHS densities fluctuated throughout the course of the experiment, and varied significantly according to herbivore treatments (Figure S1; HWA: $F_{5,328} = 5.11$, $p < .001$; EHS: $F_{5,304} = 2.27$, $p = .05$). By the end of the experiment, trees infested individually with HWA for 2–4 years averaged densities of 2.18 (± 0.31) and 2.92 (± 0.42) insects/cm, respectively, whereas those treated with EHS for 2–4 years averaged 2.28 (± 0.52) and 2.82 (± 0.87) insects/cm respectively. In comparison, on trees that were co-infested (simultaneous and sequential treatments), HWA and EHS densities were 40% and 60% lower, respectively, than on singly infested trees.

3.2 | Herbivore effects on root biomass and morphology

Adelgid feeding affected both hemlock growth and root morphology. Trees infested with HWA had a significantly greater root mass fraction (root to total biomass ratio) (Figure 2; HWA main effect: $F_{2,83} = 5.41$, $p < .01$). However, total below-ground biomass (Figure 2; $F_{2,83} = 1.34$, $p = .27$), as well as the biomass of just 1st- and 2nd-order

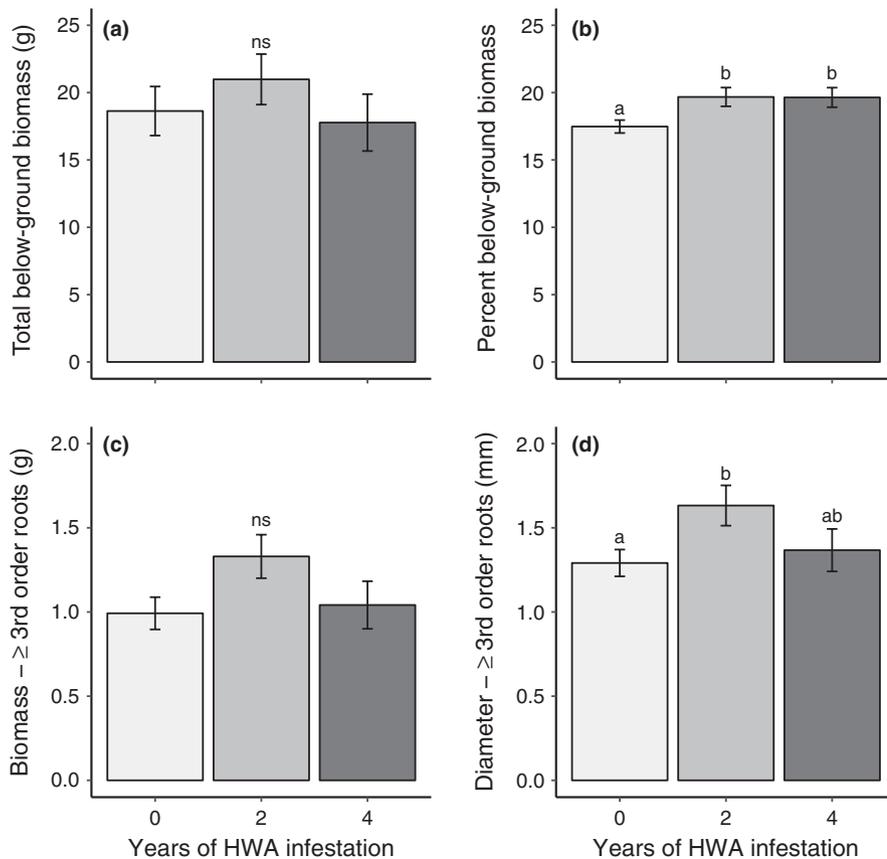


FIGURE 2 Effect of 2 and 4 years of hemlock woolly adelgid (HWA) infestation on below-ground allocation in eastern hemlock. Bars represent $M (\pm 1 \text{ SE})$ (a) below-ground biomass, (b) percent of total biomass allocated below-ground, (c) ≥ 3 rd order root biomass and (d) diameter of ≥ 3 rd order roots. Lowercase letters indicate significant differences among treatments as determined by post hoc Tukey honestly significant difference (HSD) tests ($p < .05$)

roots ($F_{2,82} = 1.85, p = .16$) were unaffected by HWA feeding. Biomass of ≥ 3 rd order roots increased by $\sim 19\%$ following HWA attack, a marginally significant effect ($F_{2,82} = 2.77, p = .07$).

Adelgid effects on root morphological and functional traits were variable. Root diameter of both 1st- and 2nd and ≥ 3 rd order roots was greater in HWA-infested trees than in control trees, although this effect was only significant for ≥ 3 rd order roots (Figure 2D; $F_{2,82} = 3.37, p = .04$). For example ≥ 3 rd order roots of trees infested with HWA for 2 and 4 years were 20% and 9% thicker, respectively, than controls. In contrast, we failed to detect significant effects of HWA on root functional traits related to foraging for water or nutrients. Specific root length (1st/2nd order: $F_{2,82} = 0.61, p = .54$; ≥ 3 rd order: $F_{2,82} = 0.57, p = .57$) and tissue density (1st/2nd order: $F_{2,82} = 0.20, p = .82$; ≥ 3 rd order: $F_{2,82} = 0.07, p = .93$) for both root order classes were unaffected.

Changes in percent below-ground biomass in multi-herbivore treatments, both simultaneous and sequential, were driven primarily by HWA. Trees infested with HWA for 2 and 4 years had 46% and 52% more of their biomass in root tissues respectively (2 years: $p = .02$; 4 years: $p = .03$). In contrast to HWA, EHS had no observable effects on patterns of allocation to root biomass, morphology or functional traits (Table S1, Supporting Information).

3.3 | Herbivore effects on resource allocation and storage

Hemlock woolly adelgid feeding had no observable effect on patterns of below-ground resource allocation. Carbon (%), N (%), and C:N

ratio were unaffected (Table S2: $p > .05$) by HWA feeding. Similarly, we observed no effect on starch concentration of roots ($F_{2,82} = 1.00, p = .37$). Like HWA, EHS had no observable effect on C (%), N (%) or C:N ratio of roots ($p > .10$). However, EHS feeding did reduce starch concentration in roots by 20%, though this effect was only marginally significant ($F_{2,82} = 2.80, p = .06$).

3.4 | Herbivore effects on ectomycorrhizal colonisation and fungal diversity

Adelgid feeding significantly reduced EMF colonisation of hemlock fine roots (Figure 3; $F_{2,78} = 4.76, p = .01$). Two and four years of continuous infestation reduced colonisation by 5% and 14% relative to controls (whose initial % colonisation remains unknown), respectively. Although EHS feeding had no observable effect on EMF colonisation ($F_{2,78} = 0.62, p = .54$), its presence mediated HWA effects when trees were co-infested (HWA \times EHS interaction: $F_{4,78} = 2.86, p = .03$). Simultaneous infestation within the same year yielded significant reductions in colonisation, as trees treated with both herbivores in 2013 (B_2) and annually thereafter had a 22% reduction in EMF colonisation compared to controls ($p < .01$). Finally, patterns of EMF colonisation in the sequential infestation treatments (A_4S_2 and S_4A_2) suggest that the timing of herbivory can affect plant signalling to and maintenance of EMF. EMF colonisation in trees first infested with HWA was 14% lower than controls, whereas EMF colonisation in trees first infested with EHS were indistinguishable from controls.

Culture-independent sequencing revealed a diverse root-associated fungal community (Figure S2). Post-rarefaction, quality-filtered non-chimeric reads clustered into 741 unique fungal OTUs at the 97% sequence-similarity level. Ascomycota was the dominant phylum (56.8% of total OTUs), followed by Basidiomycota (39.4%), Glomeromycota (1.9%) and Zygomycota (1.9%). Within Ascomycota, dominant orders included Pleosporales (124 OTUs) and Helotiales (98 OTUs), whereas dominant Basidiomycota orders included Russulales (133 OTUs) and Thelephorales (87 OTUs). Using the FUNGuild application, we were able to assign ~36% (264) of OTUs as ectomycorrhizal (Table S3).

Above-ground herbivory, both by HWA and EHS singly and in combination (HWA + EHS), had no observable effect on the alpha diversity of fungi associated with hemlock roots (Chao1: $F_{3,31} = 0.20$, $p = .89$; Shannon: $F_{3,31} = 0.27$, $p = .89$). Moreover, PERMANOVA analysis revealed no difference between the community composition of fungi from roots of control trees and those exposed to herbivore treatments (Figure 4: $F_{3,31} = 0.82$, $p = .82$). These patterns held when we restricted our analyses to OTUs identified as ectomycorrhizal with FUNGuild. Ectomycorrhizal fungal richness and diversity were unaffected by herbivory (Chao1: $F_{3,31} = 0.19$, $p = .91$; Shannon: $F_{3,31} = 1.48$, $p = .24$), as well as community composition ($F_{3,31} = 0.88$, $p = .69$). However, analyses of individual OTUs using DeSeq2 revealed an effect of herbivory on only one OTU (Thelephoraceae sp.: SH010158.07FU_FR852164_reps_singleton), an unidentified EMF species whose abundance was significantly depressed following herbivory by both HWA and EHS (Both treatment: base $M = 19.87$, \log_2 fold change = -8.51 , $p = .005$).

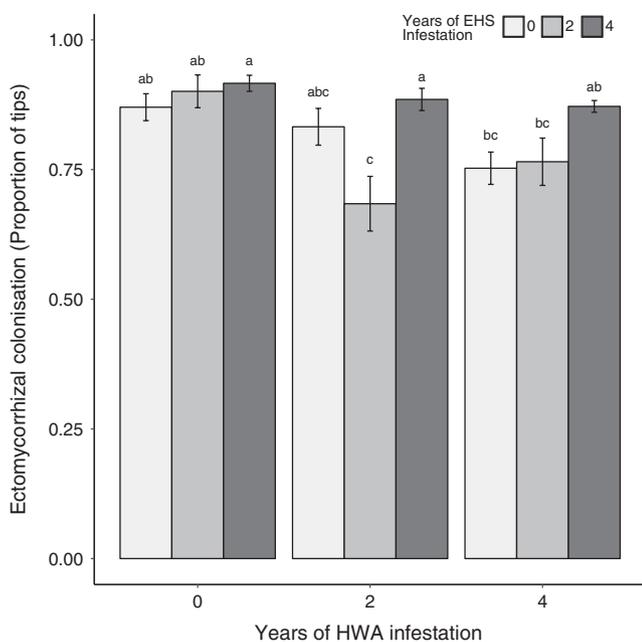


FIGURE 3 Mean ectomycorrhizal colonisation (proportion of tips colonised) of fine root tips from eastern hemlock trees infested individually or in combination (simultaneous and sequential) with hemlock woolly adelgid (HWA) and elongate hemlock scale (EHS). Bars represent $M \pm 1$ SE respectively. Bars connected by the same lowercase letter are not significantly different based on a post hoc Tukey honestly significant difference (HSD) test ($p < .05$)

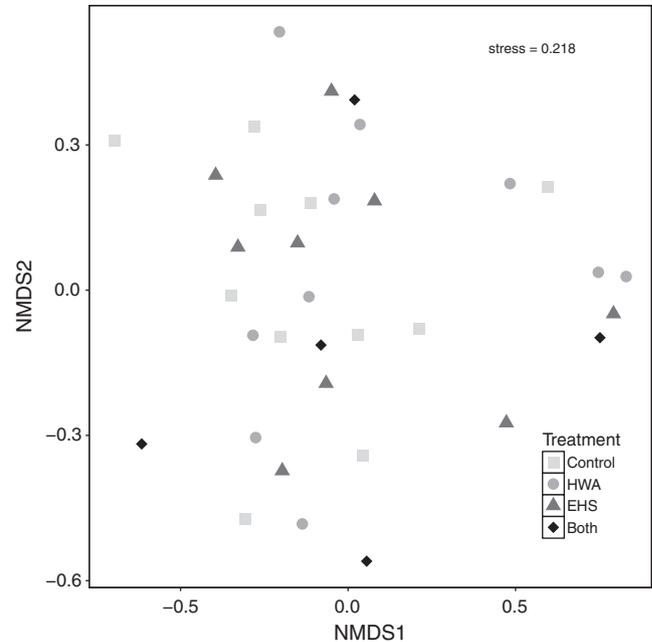


FIGURE 4 Differences in composition of fungal communities associated with roots of *Tsuga canadensis* saplings attacked singly by hemlock woolly adelgid (HWA), elongate hemlock scale (EHS) or both (HWA + EHS) for 4 years. The non-metric multidimensional scaling plot is based on Bray–Curtis measures of dissimilarity

4 | DISCUSSION

Exotic pests are increasingly common agents of tree stress and mortality in North American forests. Our understanding of the physiological processes underlying decreased performance and mortality of threatened tree species in response to exotic herbivory is largely informed by studies of above-ground functional traits (Zvereva et al., 2010), with far less attention paid to below-ground traits or processes (Zhang, Zhou, Zhou, & Ju, 2015). Here, we show that two exotic herbivores have contrasting effects on below-ground traits of a threatened foundational species of eastern US forests. Adelgid significantly affected hemlock growth, with consequences for below-ground mutualistic interactions with EMF. In sharp contrast, EHS, an herbivore within the same feeding guild, had a negligible effect on below-ground traits and extended interactions with fungal associates.

Trees infested with HWA had a significantly greater root mass fraction. This effect was not driven by differences in total below-ground biomass or resource allocation (e.g. root starch concentration), as we observed no effect of HWA on either trait. Prior work on HWA effects on below-ground biomass and resource allocation has been limited and equivocal. Soltis, Gómez, Gonda-King, Preisser, and Oriens (2015) observed no effect of HWA on root biomass or starch content in 1-year-old trees infested for 10 weeks. With respect to coarse roots, a similar lack of an effect has been observed for mature HWA-infested trees (Nuckolls et al., 2008); however, fine root production decreased 22% following multiple years of infestation. Such equivocal results have been observed in other systems and

likely reflect important differences in herbivore identity (Zhang et al., 2015). While aphid pests of woody plants affect root traits to a lesser degree than defoliators, they have a greater impact on above-ground processes and resulting litter dynamics (Zhang et al., 2015). Shifts in the ratio of above- to below-ground biomass are more likely to have occurred via effects on above-ground tissues. Adelgid feeding can rapidly reduce new growth (Miller-Pierce et al., 2010; Preisser & Elkinton, 2008; Soltis et al., 2015) and induce premature needle abscission (Soltis et al., 2014), leading to losses in above-ground biomass.

Unlike biomass allocation, plasticity in root functional traits was not observed in response to HWA feeding. We predicted that HWA would affect root functional traits for two reasons. First, HWA infestation reduces photosynthetic rates (Gonda-King et al., 2014), which should affect a tree's carbon economy and ability to invest in production and maintenance of roots. Second, trees infested with HWA also have altered water relations and display drought-like symptoms. Adelgid feeding can reduce tree water use by >40% by altering the hydraulic properties and functioning of stomata and xylem tissue (Domec et al., 2013). Loss of hydraulic functioning is likely due to the formation of traumatic resin canals (Domec et al., 2013) and false rings (Gonda-King et al., 2012). Additional support for HWA-induced water stress is evidenced by a significant spike in proline in tissues (Gómez et al., 2012), an amino acid important for osmoregulation in plants (Delauney & Verma, 1993). Surprisingly, both SRL and tissue density were unaffected by HWA. Since high SRL increases root absorptive area (Ryser, 2006), our results indicate that hemlock does not respond to attack by increasing root foraging. The production of longer, thinner roots may come at the cost of root longevity and defences (Eissenstat, 1992; Eissenstat, Wells, Yanai, & Whitbeck, 2000; McCormack, Adams, Smithwick, & Eissenstat, 2012), which could be especially important when facing a persistent stress like HWA. The adelgid's high fecundity and limited dispersal ability means that a host often experiences attack by numerous generations until death (McClure, 1991). The increases in ≥ 3 rd order root diameter and biomass may indicate that hemlocks continue to invest in root maintenance and longevity even under constrained foraging conditions.

Exotic herbivore effects on interactions below-ground between hemlock and fungal associates were dependent on both herbivore identity and the timing of interactions. We found that HWA feeding significantly reduces EMF colonisation of fine roots; reductions reached 14% when feeding alone for 4 years. This result was expected, given that EMF maintenance can cost up to 21% of net photosynthetic production for host plants (Hobbie, 2006), and that HWA feeding reduces carbon assimilation through photosynthesis (Gonda-King et al., 2014). Our results confirm previous findings indicating a strong effect of HWA on EMF colonisation. Both Lewis et al. (2008) and Vendettuoli, Orwig, Krumins, Waterhouse, and Preisser (2015) observed drastic reductions in rates of EMF colonisation for mature hemlocks growing within HWA-infested stands. Gehring and Whitham (2002) found a similar result, that above-ground herbivory of a pine can compromise its ability to maintain EM.

While rates of EMF colonisation were affected, we failed to detect any effect of HWA on the diversity of fungi associated with hemlock roots. We highlight three possible mechanisms through which EMF diversity could be maintained in our study. First, many mycorrhizal fungi may shift their functionality in a context-dependent manner. For instance the majority of Basidiomycota identified in our study were from the Russulaceae family; taxa that remained in relatively high abundance despite prolonged HWA herbivory. Studies have suggested that *Russula* species may be able to act as saprotrophs, lessening the dependence of their carbon budget on their host (Boddy, Frankland, & van West, 2007; Štursová et al., 2014). Second, mycorrhizal responses to loss of above-ground tissue can be short term (Barto & Rillig, 2010; Nishida, Izumi, Katayama, & Ohgushi, 2009), and colonisation levels can return to normal as new growth and carbon acquisition ability is restored. HWA densities fluctuate annually. They are highly susceptible to cold winter temperatures experienced in New England, which induces high rates of winter mortality (Parker, Skinner, Gouli, Ashikaga, & Teillon, 1998) and may exhibit density-dependent dynamics (McClure, 1991). Fluctuations in densities over the course of our experiment due to these two processes may allow bouts of sapling recovery and preserve below-ground mutualisms. Finally, our study used trees that had been obtained from a nursery where they were propagated for a year prior. These trees were ectomycorrhizal upon receipt (R. Schaeffer, Pers. obser.), and thus, the lack of herbivore impact on EMF and total fungal community composition may reflect the fact that these trees were already uniformly colonised prior to the addition of our herbivore treatments. Future work using non-mycorrhizal seedlings may resolve our understanding of potential impacts of these herbivores on fungal colonisation and diversity.

Although our experiment failed to detect effects of HWA on fungal diversity, it likely underestimates the full impact HWA could exert. We focused on effects of HWA on young understory saplings; in other contexts, HWA can affect entire forests. Following invasion, HWA feeding induces significant needle loss (Orwig & Foster, 1998; Stadler et al., 2006), shifting microclimatic conditions (Jenkins, Aber, & Canham, 1999; Orwig et al., 2008). These changes in microclimatic conditions, coupled with HWA-induced increases in foliar N (Domec et al., 2013; Stadler, Müller, Orwig, & Cobb, 2005), lead to increased N availability in soils, through altered rates of decomposition, N cycling and rates of nutrient uptake by affected hosts (Finzi, Raymer, Giasson, & Orwig, 2014; Rubino, Charles, Sirulnik, Tuininga, & Lewis, 2015; Stadler et al., 2006). Such changes are often associated with shifts in EMF richness and community composition (Avis, McLaughlin, Dentinger, & Reich, 2003; Lilleskov, Fahey, Horton, & Lovett, 2002; Peter, Ayer, & Egli, 2001). Given the degree to which HWA can affect soil N inputs, characterisation of microbial communities in naturally infested stands is warranted.

In contrast to HWA, EHS had negligible effects on root biomass, functional traits and interactions with EMF when feeding alone, matching prior work (Miller-Pierce et al., 2010; Soltis et al., 2015). Infestation with both herbivores generated non-additive effects on EMF colonisation. When both herbivores were introduced in 2013

and applied annually thereafter, feeding reduced EMF colonisation by 22%. In contrast, when introduced alternately across years (sequential treatments), effects were largely driven by the first arriving herbivore. Mechanisms underlying these patterns remain unclear, but we predict that species-specific plant defence responses are likely at play, which could affect plant signalling to EMF and other root associates. Adelgid feeding has been hypothesised to activate the salicylic acid (SA) defence pathway and two lines of evidence support this notion (Pezet et al., 2013). First, infestation increases emission of methyl salicylate (MeSA), a volatile methyl ester of salicylic acid which functions in plant defence against pathogens (Shulaev, Silverman, & Raskin, 1997). Second, feeding induces a localised and systemic hypersensitive response, characterised by production of hydrogen peroxide near and far from feeding sites (Radville, Chaves, & Preisser, 2011). Induction of SA defences, however, can present ecological costs, as beneficial microbial interactions involving rhizobia and arbuscular mycorrhizae can be negatively affected following induction (de Román et al., 2011; Faessel, Nassr, Lebeau, & Walter, 2009). Prior infestation with EHS has been shown to negatively affect HWA performance (Preisser & Elkinton, 2008), ameliorating its effect on hemlock growth. The ability of hemlock to maintain new growth and assimilate carbon through EHS-induced priority effects likely allows for continued provisioning of carbon and maintenance of beneficial EMF. Thus, despite being an invasive, EHS presence may ameliorate effects of HWA when introduced first, preserving EMF diversity which will be essential for future reforestation efforts.

In conclusion, we have demonstrated that two exotic herbivores can have disparate effects on below-ground functional and ectomycorrhizal traits of a threatened conifer. Our results suggest that HWA-induced loss of above-ground biomass and functioning, coupled with loss of below-ground foraging through interactions with EMF, might accelerate eastern hemlock stress and mortality through nutrient limitation. The presence of EHS either accentuates or ameliorates these effects, the precise nature of these non-additive effects being dependent on the timing of herbivore arrival. Our results thus demonstrate the importance of a community-wide approach for insights into how trees respond to pressures experienced in increasingly disturbed forests.

ACKNOWLEDGEMENTS

We thank P. Bravo, P. Candelas, L. Green, K. Pieper, E. Scott, A. Sommi, S. Tostes and M. Tynan for field and laboratory assistance and two anonymous reviewers for comments that improved the quality of this manuscript. This project was funded by National Science Foundation grants NSF-DEB 1256826 to C.O. and NSF-DEB 1256769 to E.P. and C.T., and National Institute of Food and Agriculture grant no. 2011-67013-30142 to E.P. This material is based upon work supported in part by the National Science Foundation EPSCoR Cooperative Agreement #EPS-1004057. 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 funding agencies.

AUTHORS' CONTRIBUTIONS

R.S., C.W., L.R., C.T., C.O. and E.P. conceived the idea for the study; S.R., E.M. and B.W. designed methodology for and collected EMF colonisation and diversity data, with all authors contributing to the rest of data collection; R.S. analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

DATA ACCESSIBILITY

Data on root functional traits, resource allocation and biomass are archived at the Dryad Digital Repository <https://doi.org/10.5061/dryad.9d2n3> (Schaeffer et al., 2017). Fungal community sequence data are archived in the MG-RAST public database (Project ID: 4745987.3).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

How to cite this article: Schaeffer RN, Wilson CM, Radville L, et al. Individual and non-additive effects of exotic sap-feeders on root functional and mycorrhizal traits of a shared conifer host. *Funct Ecol*. 2017;31:2024–2033. <https://doi.org/10.1111/1365-2435.12910>