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

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Summary

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 aboveground 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 two and four years. Belowground 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 belowground 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, while prior EHS attack weakened HWA effects.

4. Our results collectively suggest that prior EHS attack dampens the impact of HWA on belowground functional traits. This highlights how the timing and sequence of herbivore arrival

can alter plant-mediated interactions between herbivores and their effects on above-belowground linkages and associated tree health.

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

Introduction

Forest pests, exotic and native, are pervasive agents of tree stress and mortality worldwide (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 aboveground functional traits (Zvereva, Lanta & Kozlov, 2010), less is known about their impact on belowground traits. Attack by individual herbivores and their associated cues have been shown to both increase biomass and resource allocation towards roots in herbaceous (Schwachtje & Baldwin 2008; Gómez et al. 2010) and woody plants (Babst et al. 2005, 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 aboveground tissues (where resources for defense 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 et al. 2011; Gómez, Orians & Preisser 2012). Understanding the effects of multiple herbivores on belowground 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 mobilize enough carbohydrates to sustain fundamental processes such as respiration, growth, and defense; left unchecked, these losses can lead to carbon starvation and death (Sala, Piper & Hoch 2010; McDowell 2011). Moreover, mobilization 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 aboveground consumers can drive plastic responses in belowground traits, we have only a limited understanding of the underlying mechanism(s) and consequences to belowground 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 et al. 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 (Schwachtje & Baldwin 2008; Gómez et al. 2010), 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), while 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 aboveground 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 belowground 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*). HWA feeding often causes rapid mortality, and hemlock loss can alter a suite of habitat characteristics important for community diversity and ecosystem function (Ford & Vose 2007; Orwig et al. 2008; Adkins & Rieske 2013). 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 (Preisser & Elkinton, 2008; Miller-Pierce & Preisser, 2012; Gómez et al. 2014).

Feeding by HWA can affect a suite of aboveground 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 et al. 2014). Moreover, it has been hypothesized that hemlock mortality following HW A 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, Radville & Preisser 2012). It remains unclear how these effects cascade to affect belowground 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 of 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 in order to maximize water and nutrient uptake while reducing carbon costs. Previous research also suggests that infested trees experience symptoms similar to those of water-stressed trees (Gómez, Orians & Preisser. 2012; Domec et al. 2013), which may induce trees to produce longer roots for maximizing water acquisition.

Second, attack by HWA induces movement of nutrients away from belowground tissues. Adelgid feeding can increase and/or alter nitrogen (N) and N-rich metabolites, including free amino acids, local to feeding sites (Stadler et al. 2006; Gómez, Orians & Preisser 2012). Such changes may suggest an ability for HWA to manipulate host resources for its benefit, like other members of the hemipteran family Adelgidae (Havill & Foottit 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 (Preisser & Elkinton 2008; Gómez et al. 2014), we expect an intermediate effect on root traits for trees inoculated with both exotics simultaneously and sequentially.

Materials and Methods

Study system

The hemlock woolly adelgid is an aphid-like exotic that was introduced to the U.S. from Japan around 1950 and currently inhabits over a dozen eastern states (Souto, Luther & Chianese 1996). Mature adelgids inserts 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.

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 one-yr. old *T. canadensis* seedlings (Van Pines Nursery, West Olive, MI, USA) were planted 1 m apart in a grid (10 x 20) using a randomized 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 (Fig. 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 et al. 2007). First, we inoculated trees in the herbivore treatments annually with HWA (A), EHS (S), or both (B) insects from 2011 to 2014 (treatments: A_4, S_4, and B_4). Additional trees were kept uninfested for the first two years of the study and then inoculated with HWA, EHS, or both insects in the third year (treatments: A_2, S_2, and B_2). 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 A_4S_2 and S_4A_2). Control trees received annual sham treatments using uninfested hemlock foliage to control for tree handling.

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.

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 randomize 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 one and three harvest groups were collected per day between May 18 - 29, 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 sub-samples 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.

Ectomycorrhizal colonisation and diversity

Root fragments were analyzed 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 (White et al. 1990; Gardes & Bruns 1993). 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.

Statistical analyses

Adelgid and scale densities over the course of the experiment were analyzed 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 (HW A 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 6903 sequences (the smallest amount of sequences for any sample). To analyze 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 visualized 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 1000 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).

Results

Herbivore abundance

Adelgid and EHS densities fluctuated throughout the course of the experiment, and varied significantly according to herbivore treatments (Fig. S1; HWA: $F_{5, 328} = 5.11$, $P < 0.001$; EHS: $F_{5, 304} = 2.27$, $P = 0.05$). By the end of the experiment, trees infested individually with HWA for two to four years averaged densities of 2.18 (± 0.31) insects/cm and 2.92 (± 0.42) insects/cm respectively, while those treated with EHS for two to four years averaged 2.28 (± 0.52) insects/cm 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.

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) (Fig. 2: HWA main effect: $F_{2, 83} = 5.41$, $P < 0.01$). However, total belowground biomass (Fig. 2 $F_{2, 83} = 1.34$, $P = 0.27$), as well as the biomass of just 1st- and 2nd -order roots ($F_{2, 82} = 1.85$, $P = 0.16$) were unaffected by HWA feeding. Biomass of ≥3rd order roots increased by ~19% following HWA attack, a marginally-significant effect ($F_{2, 82} = 2.77$, $P = 0.07$).

Adelgid effects on root morphological and functional traits were variable. Root diameter of both 1st- and 2nd and ≥3rd order roots was greater in HWA-infested trees than in control trees, although this effect was only significant for ≥3rd order roots (Fig. 2D: $F_{2, 82} = 3.37$, $P = 0.04$). For example, ≥3rd order roots of trees infested with HWA for two and four 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: $F_{2, 82} = 0.61$, $P = 0.54$; ≥3rd order: $F_{2, 82} = 0.57$, $P = 0.57$) and tissue density (1st/2nd order: $F_{2, 82} = 0.20$, $P = 0.82$; ≥3rd order: $F_{2, 82} = 0.07$, $P = 0.93$) for both root order classes were unaffected.

Changes in percent belowground biomass in multi-herbivore treatments, both simultaneous and sequential, were driven primarily by HWA. Trees infested with HWA for two and four years had 46% and 52% more of their biomass in root tissues respectively (2 yrs: $P = 0.02$; 4 yrs: $P = 0.03$). In contrast to HWA, EHS had no observable effects on patterns of allocation to root biomass, morphology, or functional traits (Supporting Information Table S1).

Herbivore effects on resource allocation and storage

HWA feeding had no observable effect on patterns of belowground resource allocation. Carbon (%), N (%), and C:N ratio were unaffected (Table S2: $P > 0.05$) by HWA feeding. Similarly, we observed no effect on starch concentration of roots ($F_{2, 82} = 1.00$, $P = 0.37$). Like HWA, EHS had no observable effect on C (%), N (%), or C:N ratio of roots ($P > 0.10$). However, EHS feeding did reduce starch concentration in roots by 20%, though this effect was only marginally significant ($F_{2, 82} = 2.80$, $P = 0.06$).

Herbivore effects on ectomycorrhizal colonisation and fungal diversity

Adelgid feeding significantly reduced EMF colonisation of hemlock fine roots (Fig. 3; $F_{2, 78} = 4.76$, $P = 0.01$). 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 ($F_{2, 78} = 0.62$, $P = 0.54$), its presence mediated HWA effects when trees were co-infested (HWA x EHS interaction: $F_{4, 78} = 2.86$, $P = 0.03$). Simultaneous infestation within the same year yielded significant reductions in colonisation, as trees treated with both herbivores in 2013 (B2) and annually thereafter had a 22% reduction in EMF colonisation compared to controls ($P < 0.01$). 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-signaling 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 (Fig. 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), while 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).

Aboveground 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: $F_{3,\,31} = 0.20$, $P = 0.89$; Shannon: $F_{3,\,31} = 0.27$, $P = 0.89$). Moreover, PERMANOVA analysis revealed no difference between the community composition of fungi from roots of control trees and those exposed to herbivore treatments (Fig. 4: $F_{3,\,31} = 0.82$, $P = 0.82$). 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: $F_{3,\,31} = 0.19$, $P = 0.91$; Shannon: $F_{3,\,31} = 1.48$, $P = 0.24$), as well as community composition ($F_{3,\,31} = 0.88$, $P = 0.69$). 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 mean = 19.87, $\log_2$ fold change = -8.51, P = 0.005).

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 aboveground functional traits (Zvereva, Lanta & Kozlov 2010), with far less attention paid to belowground traits or processes (Zhang et al. 2015). Here, we show that two exotic herbivores have contrasting effects on belowground traits of a threatened foundational species of eastern US forests. Adelgid significantly affected hemlock growth, with consequences for belowground mutualistic interactions with EMF. In sharp contrast, EHS, an herbivore within the same feeding guild, had a negligible effect on belowground 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 belowground biomass or resource allocation (i.e., root starch concentration), as we observed no effect of HWA on either trait. Prior work on HWA effects on belowground biomass and resource allocation has been limited and equivocal. Soltis and colleagues (Soltis et al. 2015) observed no effect of HWA on root biomass or starch content in one-yr. old trees infested for ten 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 aboveground 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 aboveground tissues. Adelgid feeding can rapidly reduce new growth (Preisser & Elkinton 2008; Miller- Pierce, Orwig & Preisser 2010; Soltis et al. 2015) and induce premature needle abscission (Soltis et al. 2014), leading to losses in aboveground 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, Radville & Preisser. 2012). Additional support for HWA-induced water stress is evidenced by a significant spike in proline in tissues (Gómez, Orians & Preisser 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 defenses (Eissenstat 1992; Eissenstat et al. 2000; McCormack et al. 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 ≥3rd 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 belowground 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 four 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 et al (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 aboveground 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 & West 2007; Štursová et al. 2014). Second, mycorrhizal responses to loss of aboveground tissue can be short term (Nishida et al. 2008; Barto & Rillig 2010), 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 et al. 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 belowground 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, personal observation), and thus, the lack of herbivore impact on EMF and total fungal community composition may reflect the fact that these trees were already uniformly colonized 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, Müller & Orwig 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 (Stadler et al. 2005; Domec et al. 2013), lead to increased N availability in soils, through altered rates of decomposition, N cycling, and rates of nutrient uptake by affected hosts (Stadler et al. 2006; Finzi et al. 2014; Rubino et al. 2015). Such changes are often associated with shifts in EMF richness and community composition (Peter, Ayer & Egli 2001; Lilleskov et al. 2002; Avis et al.

2003). Given the degree to which HWA can affect soil N inputs, characterization 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, but only when introduced in the same year. 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 defense responses are likely at play, which could affect plant signaling to EMF and other root associates. Adelgid feeding has been hypothesized to activate the salicylic acid (SA) defense 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 defense against pathogens (Shulaev, Silverman & Raskin 1997). Second, feeding induces a localized and systemic hypersensitive response, characterized by production of hydrogen peroxide near and far from feeding sites (Radville, Chaves & Preisser 2011). Induction of SA defenses however can present ecological costs, as beneficial microbial interactions involving rhizobia and arbuscular mycorrhizae can be negatively affected following induction (Faessel et al. 2009; de Román et al. 2011). 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 belowground functional and ectomycorrhizal traits of a threatened conifer. Our results suggest that HWA-induced loss of aboveground biomass and functioning, coupled with loss of belowground 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.

Author Contributions

RS, CW, LR, CT, CO, and EP conceived the idea for the study; SR, EM, and BW designed methodology for and collected EMF colonisation and diversity data, with all authors contributing to the rest of data collection; RS analysed the data and led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

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Data accessibility

Data on root functional traits, resource allocation, and biomass are archived at the Dryad Digital Repository http://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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Figure legends

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.

Figure 2. Effect of 2 and 4 years of hemlock woolly adelgid (HWA) infestation on belowground allocation in eastern hemlock. Bars represent mean (± 1 SE) (A) belowground biomass, (B) percent of total biomass allocated belowground, (C) ≥3rd order root biomass, and (D) diameter of ≥3rd order roots. Lowercase letters indicate significant differences among treatments as determined by post-hoc Tukey honestly significant difference (HSD) tests ($P < 0.05$).

Figure 3. Mean ectomycorrhizal colonisation (proportion of tips colonized) 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 means ± 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 ($P < 0.05$).

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 four years. The non-metric multidimensional scaling plot is Bray-Curtis measures of dissimilarity.

Figure 1.

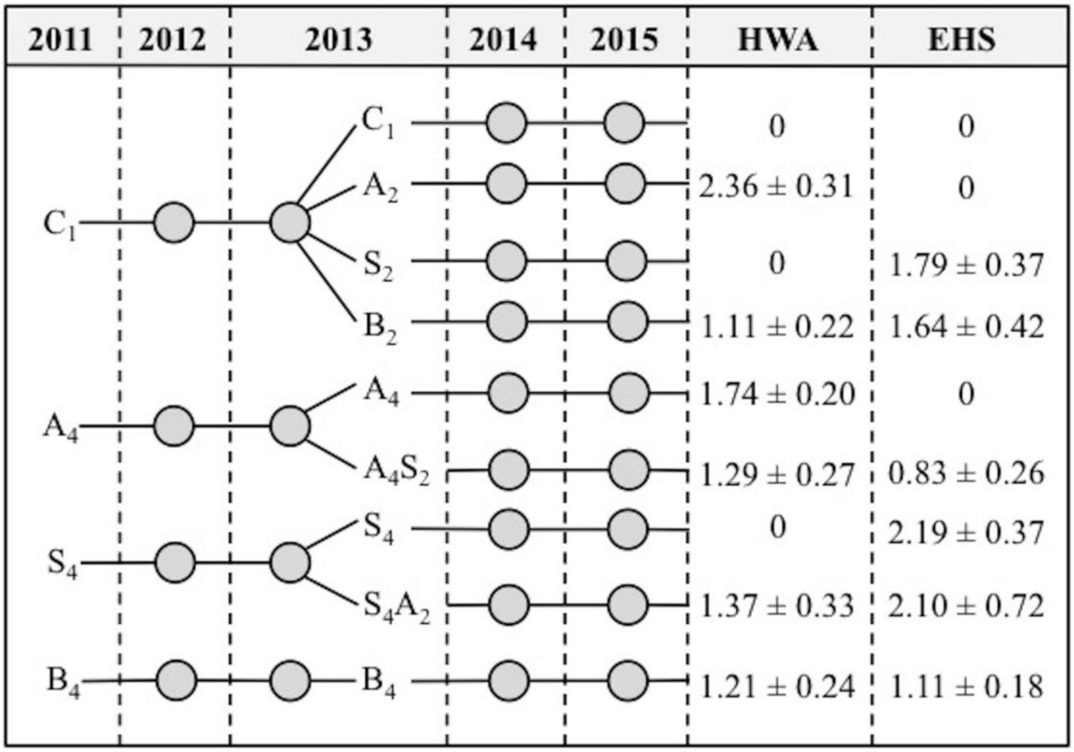


Figure 2.

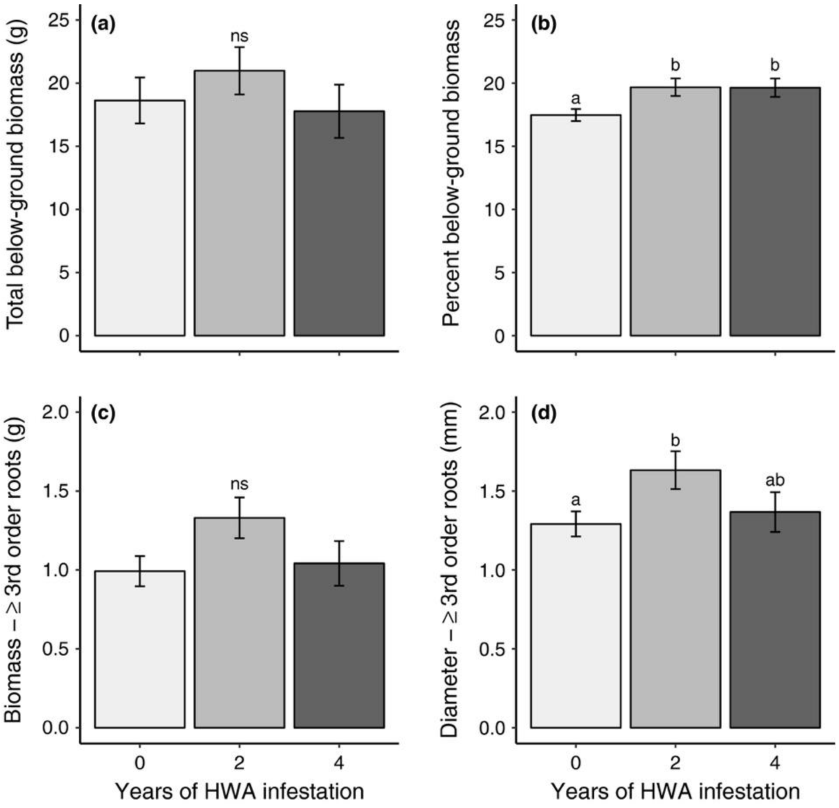
(a)
Total below-ground biomass (g)
0
5
10
15
20
25
ns
(b)
Percent below-ground biomass
0
5
10
15
20
25
a
b
b
(c)
Biomass – ≥ 3rd order roots (g)
0.0
0.5
1.0
1.5
2.0
ns
(d)
Diameter – ≥ 3rd order roots (mm)
0.0
0.5
1.0
1.5
2.0
a
b
ab
0
2
4
Years of HWA infestation
0
2
4
Years of HWA infestation

Figure 3.

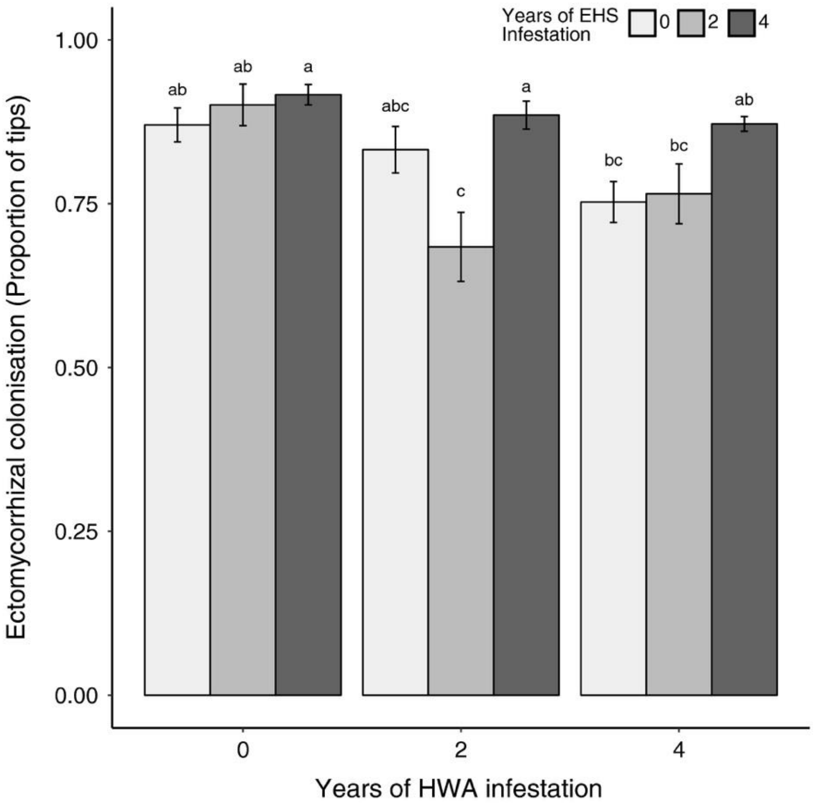
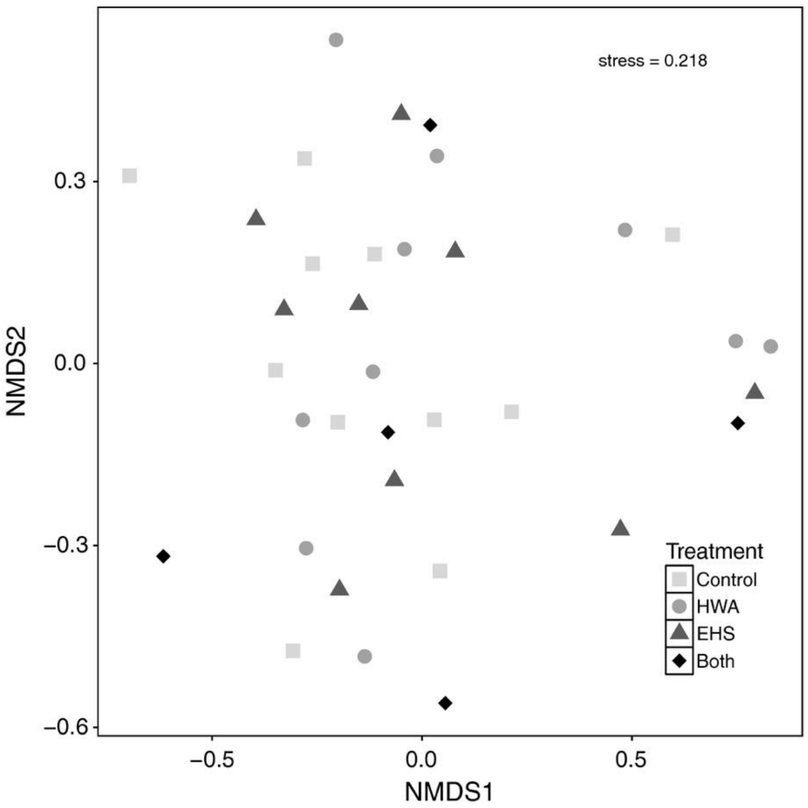

Years of EHS Infestation
0
2
4
1.00
0.75
0.50
0.25
0.00
Ectomycorrhizal colonisation (Proportion of tips)
ab
ab
a
abc
c
a
bc
bc
ab
0
2
4
Years of HWA infestation

Figure 4.


stress = 0.218
NMDS2
NMDS1
0.3
0.0
−0.3
−0.6
−0.5
0.0
0.5
Treatment
Control
HWA
EHS
Both