

**Costs of herbivory: Resource partitioning and biomechanics in Eastern  
hemlock (*Tsuga canadensis*) under exotic herbivore attack**

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## Abstract

Plants balance resource allocation to growth with antiherbivore defenses for survival. Sometimes, these defenses fail and insect herbivores outbreak. Because they lack a recent coevolutionary history, exotic herbivores often overcome plant defenses, becoming invasive in impacted ecosystems. I examine the effect of two invasive sap-feeding insects, hemlock woolly adelgid (HWA, *Adelges tsugae*) and elongate hemlock scale (EHS, *Fiorinia externa*) on Eastern hemlock (*Tsuga canadensis*). While extensive changes associated with hemlock stress and population decline are apparent in affected forests, I elucidate the plant-level effects of these herbivores through two studies. To understand increased litterfall in infested stands, I studied the effect of HWA on biomechanics of needles, twigs, and branches. To understand decreased growth in infested trees, I studied the effects of HWA and EHS on carbon resource allocation. I found evidence of stress in both the biomechanics and chemistry of infested trees, with implications for ecological processes.

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## **Chapter 1. Introduction - Lost in the woods: The tree-level factors in hemlock decline**

### INTRODUCTION

The classic conundrum of plant development, to balance growth against resources allocated to antiherbivore defenses, shapes plant communities globally. But these adaptive strategies often leave plants poorly defended against sudden explosions of herbivore populations. A triangle of factors contributes to outbreaks; the presence of an herbivore population, widespread susceptibility in the host, and an environment conducive to epidemic spread (Stevens, 1960). These outbreaks can dramatically alter plant species composition, habitat for native animals, and forest biogeochemistry (Lovett *et al.*, 2006).

Trees of North America evolved with forest herbivores such as the spruce budworm (*Choristoneura spp.*), mountain pine beetle (*Dendroctonus ponderosae*), and forest tent caterpillar (*Malacosoma disstria*). Due to changes in environmental conditions or asynchrony between host and insect life cycles (Foster *et al.*, 2013), each of these herbivore species periodically reach epidemic proportions. Impacts of these outbreaks are widespread, altering forest dynamics from canopy composition down to soil chemistry through their effects on leaf litter. Extreme herbivory directly changes plant inputs to the soil by altering quantity and timing of defoliation, and indirectly by inducing chemical defenses that alter nutrient content and decomposition rates of plant detritus (Gandhi and Herms, 2010).

With increasing frequency, forest herbivore outbreaks are caused by invasive species. Globally, 14 of the top 100 harmful alien species are invasive herbivores (Lowe *et al.*, 2000). The gypsy moth (*Lymantria dispar*), Asian long-horned beetle (*Anoplophora glabripennis*), European elm bark beetle (*Scolytus multistriatus*), winter moth (*Operophtera brumata*), and emerald ash borer (*Agrilus planipennis*) are just a few of at least 85 exotic species that have caused forest tree epidemics in the northeastern US (Mattson *et al.*, 1991). It is not uncommon for native plant species to be poorly defended against exotic herbivores. Successful invasive herbivores often shift from native hosts to close relatives in the new range. Since herbivore defenses are costly, often specialized to target specific herbivores, in the absence of native herbivores any defenses would likely be lost. In fact, these naïve host populations, in the absence of historical pressure from an exotic herbivore clade, may prioritize growth over defenses. In essence, these alien invasive insects benefit from widely susceptible hosts, environmental conditions conducive to spread, and the presence of an enemy-free space (Liebhold *et al.*, 1995). Ineffective antiherbivore defenses mean there is little to no bottom-up control of the invading insect. In addition, they are often freed from competition with other herbivores, and predators or parasites as forms of top-down control. As such, severe infestation by these exotic species is common (Gandhi and Herms, 2010). Further, high densities of monospecific hosts, as often occurs in mature forest dominated by shade-tolerant species, exacerbate the effect. Outbreaks of these insects often contribute to native species decline.

## STUDY SYSTEM & RESEARCH GOALS

Eastern hemlock (*Tsuga canadensis*), a dominant conifer throughout eastern North America, is a species in decline due to the spread of two invasive herbivores, hemlock woolly adelgid and elongate hemlock scale (Orwig *et al.*, 2002). Hemlocks provide major forest ecosystem services in the form of habitats for birds, insects, and mammals, and regulation of soil chemistry (Stadler *et al.*, 2005). Slow-growing and shade tolerant, they often occur in high-density stands (Stadler *et al.*, 2005). Heavy infestation of hemlock stands by these two herbivores is leading to localized population dieback. We expect dramatic shifts in forest composition following hemlock removal, as gap formation (Orwig and Foster, 1998) alters competition among plants, leading to increasing dominance of deciduous species (Stadler *et al.*, 2005; Small *et al.*, 2005). Soil nitrification and a shift in forest biogeochemistry are expected to result, as indirect consequences (Stadler *et al.*, 2005).

In the 1950s, hemlock woolly adelgid (HWA) began spreading across the eastern hemlock range, quickly becoming a major pest (Stoetzel, 2002). Resistance to HWA is very low in *T. canadensis* (McClure, 1995), and as a consequence entire stands are dying back and new hemlock recruitment in infested stands is near zero (Orwig *et al.*, 2002; Preisser *et al.*, 2011). In its native range, HWA feeds from *Tsuga* and *Picea spp.* in alternating generations. There, HWA remains at low densities and does not strongly affect health of *Tsuga* hosts (Havill *et al.*, 2006). Interestingly, HWA forms galls on *Picea*, altering carbon resource dynamics by increasing localized sink strength within the host. In the

introduced range, HWA settles at the bases of needles, but does not gall, on *Tsuga canadensis*. HWA inserts its stylet bundle to extract water and nutrients from the xylem ray parenchyma of the stem (Young *et al.*, 1995) and likely impacts nutrient transport and storage (van Bel, 1990). Attacked hemlocks suffer numerous stress symptoms including needle loss (Stadler, 2005) and reduced growth (McClure, 1991), leading to rapid mortality (Broekling *et al.*, 2003).

Elongate hemlock scale (*Fiorinia externa*) is also a widespread invasive herbivore throughout the *T. canadensis* range in New England. Though detrimental to the host as it sucks nutrients from the leaf mesophyll, its effects are milder and it does not often cause mortality in healthy trees (McClure, 1980). When elongate hemlock scale (EHS) and HWA co-occur on *T. canadensis*, many of the symptoms of HWA infestation are partially alleviated (Miller-Pierce *et al.*, 2010).

Clearly, HWA and EHS feeding can elicit stress by altering local demand or reducing plant-wide resource availability through the removal of carbon and nutrients. Feeding behavior similar to galling can further manipulate local resource balance. Additionally, HWA may cause a systemic hypersensitive response, evinced by elevated peroxide levels in needles (Radville *et al.*, 2011), and the formation of false rings in branches (Gonda-King *et al.*, 2012; Domec *et al.*, 2013), which may reduce water transport (Bolton and Petty, 1978). Questions remain as to how changes in resource distribution and in plant growth contribute to observed hemlock decline.

To connect the stand-level effects of HWA (light gaps, increased litter deposition, and changing biogeochemistry) to individual tree responses, we examined the effects of HWA on biomechanical properties of the plant. Through the hypersensitive response (Radville *et al.*, 2011; Gonda-King *et al.*, 2012) or chemical changes (Davin and Lewis, 2005; Liu *et al.*, 2007), we expected that HWA may compromise the mechanical resilience of attacked tissues, leading to premature needle abscission and increased branch and twig brittleness. Such mechanical effects would accelerate hemlock dieback in response to physical stressors, both by resource loss and meristem loss in the fractured shoots. To measure the biomechanics of hemlocks in response to HWA, we tested young, currently-infested twigs and needles, and mature branches, in mechanical loading experiments.

To clarify the differences in severity of HWA and EHS effects on hemlock growth and longevity, we examined carbon resource partitioning. Universal resource depletion, or manipulation of sink strength within plants, as occurs when HWA galls on *Picea*, may contribute to hemlock stress in response to these herbivores. To examine carbon allocation, we measured long-term stores via starch concentration, and short-term translocation of photosynthate by an isotope labeling study.

Through these studies, we elucidate the responses of hemlock trees to herbivory by HWA and EHS. We expect to see increased sink strength of tissues infested by HWA and EHS, such that infested branches provide less photosynthate to the rest of the tree, as insects consume the needles' sugar

exports. Further, we expect to find weakness and reduced flexibility in all tissues of infested trees. If we can integrate these findings and connect them to ecosystem-level processes, we may gain a better understanding of how invasive herbivory is altering the landscape of northeastern forests.

## **Chapter 2. Failure under stress: the effect of the exotic herbivore *Adelges tsugae* on biomechanics of *Tsuga canadensis***

### INTRODUCTION

Plants and insect herbivores often have a long history of coevolution. Over time, insects develop the ability to efficiently remove nutrients from plant tissues, and the plants in turn develop toxins and other deterrents to help protect against herbivore damage (Gatehouse, 2002; Xiang and Chen, 2004; Strauss, 1999; Núñez-Farfán, 2007). Relationships between specialist herbivores and their host plants can lead to complex coevolutionary arms races (Agrawal, 2000; Ruuhola, 2001). However, in the case of exotic species, this system of coadaptation is circumvented (Gandhi and Herms, 2010). An exotic herbivorous insect may encounter naïve plant species, allowing the insect to rapidly increase its population and become invasive. In such a system, the host plants may experience severe damage and population decline (reviewed in Gandhi and Herms, 2010).

Furthermore, the type of insect feeding can affect the severity of damage sustained by the plant (*sensu* Walling, 2000; also see Preisser and Bastow, 2006). Leaf-chewing herbivores can cause extensive cell damage and induce defensive responses, yet their effects on plant fitness are often limited (Karban and Baldwin, 1997; Orians *et al.*, 2011). Compared to leaf-chewing herbivores, piercing-sucking herbivores often have larger effects on plant fitness (Meyer, 1993; Zvereva, Lanta, and Kozlov 2010). These insects target phloem or parenchyma cells that are critical to nutrient transport and long-term storage (van Bel, 1990), and by avoiding large-scale rupture, prevent rapid plant defensive signaling

(Walling, 2008; Heidel and Baldwin, 2004; Kaloshian and Walling, 2005). Thus high densities of an invasive piercing-sucking specialist herbivore that targets stem tissues should therefore present a worst-case herbivory scenario, as the insects thrive even on highly stressed plants (Koricheva and Larsson, 1998).

The hemlock woolly adelgid (*Adelges tsugae*, HWA), is an exotic hemipteran that presents one example of this narrative. HWA is decimating stands of eastern hemlock (*Tsuga canadensis*) in its invaded range, the hemlock forests of the eastern United States. Resistance to HWA is very low in *T. canadensis* (McClure, 1995), and as a consequence entire stands are dying back and new hemlock recruitment in infested stands is minimal (Orwig *et al.*, 2002; Preisser *et al.*, 2011). Upon infestation, HWA rapidly decreases eastern hemlock growth (McClure, 1991), alters foliar nitrogen content (Miller-Pierce *et al.*, 2010; Gomez *et al.*, 2012), and induces a hypersensitive response (HR) (Radville *et al.*, 2011) and increased needle loss (Stadler, 2005).

One proposed mechanism for the severe impact of HWA on eastern hemlock is that a systemic HR induces a general stress response, leading to altered wood quality (Radville *et al.*, 2011). Evidence for phytotoxicity is partially supported by elevated peroxide levels (indicative of an HR) found in both the infested and distal needles of HWA-infested trees (Radville *et al.*, 2011). This HR appears to lead to changes in wood anatomy: branches of HWA-infested trees form more false rings [an abnormal band of thick-walled latewood cells that reduce water transport (Bolton and Petty, 1978)] than branches of uninfested trees (Gonda-King *et al.*, 2012; Domec *et al.*, 2013). Similarly, infestation of *Abies*

*balsamea* by balsam woolly adelgid (*Adelges piceae*) has been shown to cause abnormal xylem resembling compression wood (which is characterized by thickened cell walls, lignification, a decrease in water conductivity, and increased wood brittleness) (Balch *et al.*, 1964). Additionally, in low-vigor (Skene, 1972) and HWA-infested (Walker, 2012) eastern hemlock, latewood tracheid cell walls become thinner, and the cell diameter decreases, likely with biomechanical consequences such as weakened branches. It is also possible that HWA induces lignification (Davin and Lewis, 2005; Liu *et al.*, 2007), which could increase wood brittleness.

We hypothesize that HWA feeding alters the mechanical properties in stems, including enhanced lignification and the thinning of xylem cell walls. Because the damage can lead to osmotic stress, we expect this to lead to premature needle abscission and increased branch and twig brittleness. In this study, we used biomechanical measurements to test this potential mechanism. We studied the biomechanics of HWA-infested trees by measuring tissue responses to mechanical loading, under the hypothesis that HWA feeding causes weaker and less flexible responses to mechanical forces in the needles, twigs, and branches.

To quantify the effects of HWA on eastern hemlock biomechanics under a wide range of infestation histories, we used branches from trees at multiple sites. We selected a forested site and an urban site to include hemlocks grown in different abiotic environments. Additionally, to control for environmental variation and the history of infestation among trees, we sampled from a common garden experiment.

## MATERIALS AND METHODS

### *Study sites*

We chose three study locations based on infestation history, environmental conditions, and site types, allowing for greater levels of environmental heterogeneity and for extrapolation to hemlock stands in nature. Shade conditions in particular can alter plant structure by affecting the balance between resource allocation to light capture and biomechanical support (Givnish, 1995). In 2011 and 2012, we sampled from two naturally infested sites, a rural and an urban site. The rural site, located at Mt. Tom State Reservation in Holyoke, MA, is a shaded forest stand typical of hemlock habitat, with a well-established history of HWA infestation (Orwig *et al.*, 2002; Preisser *et al.*, 2008; 2011) and a high density of downed foliage in the infested regions (C. Orians personal observation). The urban site, a sunny field at Tufts University in Medford, MA, is a mix of ornamental trees. Additionally, we sampled from a common garden experiment established in 2007 (uninfested hemlock saplings from Peltham, MA) at an experimental farm at the University of Rhode Island (Kingston, RI). These seedlings were then inoculated with either HWA or control foliage following standard protocols (Butin *et al.*, 2007) over the course of four growing seasons. Trees were caged in individual PVC enclosures covered by mosquito netting to prevent cross contamination between treatments. Further details of the establishment and maintenance of the common garden are described elsewhere (Miller-Pierce *et al.*, 2010).

### *Sample collection*

We collected samples of the following three foliage categories at 1 to 1.5 meter height from each tree: branches (5 mm diameter, approximately three to four years old), and the youngest twigs (1 mm diameter, ten months old) and corresponding needles. We assessed branch age by leaf scar nodes. Table 1 summarizes the type and number of samples collected.

For all twig and needle samples, we randomly sampled one large branch per cardinal direction on each tree, and randomly selected a three-branch subset. Twig segments from these branches were sorted into the following categories: uninfested (no HWA present), low infestation (0.01 - 6 HWA per cm), and moderate infestation (6.01 - 23 HWA per cm). In order to focus on the localized HWA effect, we sampled only needles with HWA feeding at the base. As such, we allocated all moderately-infested segments to needle testing, and randomly subsampled the remaining segments for twig and needle testing. For needles, upon collection all samples were wrapped in damp paper towels in sealed plastic bags, refrigerated (4°C) and tested within two days of collection. We stored all prepared twig samples at room temperature in sealed plastic bags, and tested them within five days of initial collection.

While HWA feed actively on the youngest twig class, the insects do not feed directly on older branches. For branches from the rural and urban sites, samples were categorized as infested if any HWA were present on the youngest foliage distal to the sample branch, and as uninfested if we did not observe HWA on distal young foliage. We selected all branches for linear growth and few

secondary branches. Following sample collection, we pruned all secondary branches, and stored the branch segments in damp paper towels in sealed plastic bags at 4°C. Branches were tested within two days of sampling.

### *Mechanical testing*

We assessed strength and flexibility of all samples. Strength was assessed by measuring the stress (load applied per unit area), while flexibility was determined by measuring tissue strain (a measure of relative axial deformation). Strain describes the flexibility of a tissue. We measured stress and strain under deformation and failure, to quantify mechanical responses to HWA. Under small strains, plant tissues behave elastically, such that all deformation is reversible, and stress is proportional to strain. This stress:strain ratio (i.e. Young's modulus) describes a structural property of the material (Figure S1A). The stress required to initiate permanent deformation (i.e. yield strength) marks the point where the plant material begins to behave plastically (Figure S1B).

Mechanical failure, or breaking, of samples can occur in multiple ways. Under tensile stress, which we applied to needle and twig samples, fracture occurs due to crack initiation and propagation. Under bending, which we applied to branch samples, the sample experiences three modes of stress; tensile stress, compressive stress, and shear stress. We minimized shear stresses through our choice of sample dimensions (see *branches*), such that sample failure occurs primarily due to a combination of compressive stress on the concave side, and tensile stress on the convex side of the branch, causing bending. Eventually, flexure causes failure due to excessive plastic deformation and sample buckling of

the light hemlock wood, beyond the yield strength of the sample; a similar result is also seen in *Salix* (van Casteren *et al.*, 2011).

In cases of sample fracture, we quantified maximum stress and the corresponding strain prior to specimen failure (Figure S1B). We used an Instron universal testing machine (UTM, Model 3366) for mechanical testing, and performed data collection and processing with Bluehill software (Instron).

### *Needles*

We tested five ten-month old needles, from a single twig segment per tree, unless otherwise omitted [e.g. due to failure during loading (Table 1)], for the mechanical properties listed above. We quantified needle mechanics only at the field sites. We averaged the multiple measurements per twig segment to calculate one replicate value per tree. Needle samples were standardized by cutting 3-cm-long segments, and from these selecting five central needles oriented 40° from the stem. We secured twigs in a metal press vise along their length (Figure 1A), oriented at a 40° angle. We attached a small (1 g) binder clip to the load cell arm by a fine motor wire to facilitate loading of single needle samples (modified from Thurber *et al.*, 2010) (Figure 1A). Each tested needle was secured at its midpoint by the clip, and stressed with a 10N load cell at 0.2 mm/min. For low- and moderate-infestation sampling, we tested only needles with HWA attached at the base. We tested tension on needles to quantify the strength necessary for needle abscission (maximum stress). The strain at maximum stress (displacement at the time of abscission) is a measure of flexibility.

## *Twigs*

In order to correlate current insect feeding with mechanical properties, we quantified tension in randomly selected ten-month-old twigs. From each branch sampled (see *sample collection*), only one twig per large branch was analyzed. Twig diameter, length, and HWA density of each sample was measured. Twigs were stripped of their needles and glued with cyanoacrylate adhesive into a two-layered card frame support with 15mm span (modified from DesRochers *et al.*, 2009) (Figure 1B). The frame was loaded into the Instron UTM tension setup with pneumatic clamps, and the support was cut to allow tensile loading of the twig. Tension was applied using a 100N load cell at  $0.2 \text{ mm min}^{-1}$ . We quantified maximum stress (force required to fracture twig) as a measurement of strength, and strain at maximum stress (vertical displacement at the time of breakage) as a measure of flexibility. The nonlinear stress-strain response of twigs under tension precluded us from examining yield stress.

Following mechanical testing of twigs, we quantified lignin concentration and tissue density (oven-dry mass per fresh volume) of twig and branch samples using standard methods (Bonello and Blodgett, 2003). In small wood specimens, dry tissue density and cell wall chemistry (as lignin, cellulose, and hemicellulose content) are often better predictors of mechanical behavior than histological traits (Gibson, 2012; Winandy and Rowell, 2005). For instance, lignification of secondary cell walls increases stiffness and strength of woody tissues (Gibson, 2012).

### *Branches*

We collected 40-month-old branches previously exposed to HWA over multiple growing seasons to study the cumulative effects of insect feeding on biomechanics over time. We randomly selected two branches, one each from the east and west sides, per tree. From the urban site, we were only able to sample from the south side of each tree. All trees at the urban site were heavily HWA-infested, such that no comparison could be made between infested and uninfested branches. We measured branch diameter and length for all samples. We tested all branches on the Instron UTM 3-point flexure setup, with a 100N load cell (Figure 1C). When possible, branches were loaded topside-down to mimic downward bending in the field. We oriented them with a support span of 8.5-10cm (at least 15 times the branch diameter to minimize shear stresses), and applied the central load at 5mm/min. Branches that twisted or slipped during the run were omitted from the analyses. Branch measurements were averaged to calculate one value per tree. As the most ecologically relevant factor in large branch stresses is irreversible deformation, we focused on yield strength (stress required to cause plastic deformation, MPa) as a measurement of strength, and modulus of elasticity (initial rate of increase of stress per strain, MPa) as a measure of flexibility.

### *Statistical analysis*

We inspected all data for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) prior to analysis. To meet assumptions of normality, twig density was square-root transformed, twig strain was log transformed, and needle stress and strain were inverse square-root transformed.

To account for variation in insect infestation density, infestation level was included [i.e. none, low (0.01-6 HWA/cm), or moderate (6.01-23 HWA/cm)] as a factor in twig and needle analysis. In twigs, we analyzed the effect of tissue density and lignin concentration on stress and strain by linear regression. The effects of insect level on HWA density and lignin concentration were assessed by t-test. For each tissue type, we used a 2-way multivariate analysis of variance (MANOVA) to find mechanical response variables impacted by site and insect level. We then ran ANOVAs on any response variables that were significant under MANOVA. For significant ANOVAs with more than 2 insect levels, or a significant interaction, we ran post-hoc Fisher's LSD tests. We performed all statistical analyses in R (R development core team, 2011).

## RESULTS

### *Needles*

We measured a significant effect of HWA infestation level and site on the mechanical traits tested in needles (2-way MANOVA: Table 2A). Subsequent ANOVA analysis for maximum stress revealed that insect infestation significantly reduced the strength required to cause needle abscission (Table 2A, Figure 2A). However, the effect of HWA on needle strength was lost in post-hoc Fisher's LSD analysis (Figure 2A, B). Neither site nor insect presence significantly impacted needle flexibility (Figure 2 C,D) (ANOVA: Table 2A).

### *Twigs*

In twigs, we observed significant effects of insect infestation level and site on biomechanical properties (2-way MANOVA: Table 2B). Further analysis

indicated that infested twigs were consistently weaker (by 25% on average) under tensile stress than uninfested twigs across all three sites (2-way ANOVA: Table 2B, Figure 3 A,B,C). We observed a disordinal interaction between infestation and site for twig brittleness (2-way ANOVA: insect  $p=0.0047$ , site  $p<0.001$ , insect\*site  $p=0.0165$ , Figure 3 D,E,F). Post-hoc analysis by Fisher's LSD indicated that while infested twigs were brittle at the urban and common garden sites, HWA infestation had no effect at the rural site (Figure 3 D, E, F).

We also found that lignin content and tissue density predicted maximum tensile stress across treatments, but not tensile strain. There was a significant positive relationship between maximum stress and lignin concentration (linear regression:  $R^2 = 0.145$ ,  $p<0.001$ , Figure 4A) and tissue density (linear regression:  $R^2 = 0.207$ ,  $p=6.771E-07$ , Figure 5A) across all samples. Tensile strain appeared to be insensitive to lignin concentration (linear regression:  $R^2=0.0002$ ,  $p=0.9013$ , Figure 4B) and to tissue density (linear regression:  $R^2=0.029$ ,  $p=0.0777$ , Figure 5B). Lignin and density, however, did not differ between the two insect treatments (t-test,  $t = 0.8543$ , 96 df,  $p=0.3951$ ;  $t=-0.5664$ , 105 df,  $p=0.5723$ , respectively) and thus do not explain the effects of HWA on branch mechanics.

### *Branches*

Previously-infested branches did not differ significantly in any of the biomechanical traits measured between insect infestation level (2-way MANOVA: insect  $p=0.3405$ , site  $p<0.001$ , insect\*site  $p=0.9804$ , Table 2C). However, both flexural yield stress and flexibility (Young's modulus) differed by site (2-way ANOVA: for stress, insect  $p=0.1732$ , site  $p<0.001$ , insect\*site

$p=0.9096$ , Figure 6 A, B, C; for modulus, b, Figure 6 D, E, F, Table 2C). Our findings at the urban field site were consistent with the rural and common garden sites; mean yield stress and mean modulus of infested branches were 41.2 MPa and 1852 MPa, respectively.

## DISCUSSION

Our results indicate that HWA feeding alters some of the biomechanical properties of eastern hemlock, with evidence of decreased flexibility and strength in HWA-infested trees. The effects of HWA on hemlock biomechanics were strongest in currently-infested twigs, and measurable in the strength of attached needles. HWA feeding has no apparent impact on the mechanical properties of previously infested branches or on the flexibility of currently-infested needles. The changes to the twigs and needles suggest that a decrease in mechanical resistance of attacked trees could contribute to the mortality of infested trees over time (Stadler, 2005; McClure, 1991). As strength and, at some sites, flexibility, decreased in infested twigs, and abscission strength decreased in infested needles, these trees could become more sensitive to abiotic mechanical stressors such as strong winds, ice storms, or snow pack.

While the negative effects of invasive insects on native plants are well documented, the mechanisms behind these effects are often poorly understood. When herbivores cause complete defoliation, the lack of plant defense is clear. In non-defoliating invasive insects with devastating effects, the reasons can be elusive. It appears that HWA induces a hypersensitive response that changes wood properties (Gonda-King *et al.*, 2012), which may ultimately affect needle

and twig retention. This is the first study to demonstrate that feeding by an invasive herbivore alters the mechanical properties of needles and twigs. Below we discuss the effects of HWA on each tissue type and the implications of these results.

Herbivores and pathogens are known to affect needle longevity; defoliating insects increase longevity in *Pinus* due to reduced needle cast in response to slow growth (Kurkela *et al.*, 2005; Drenkhan *et al.*, 2006), while fungal infection decreases needle longevity in *Pseudotsuga* (Hansen *et al.*, 2000) and *Larix* (Krause and Raffa, 1992). We hypothesized that a decrease in needle attachment strength and in abscission zone flexibility would underlie the increased rate of needle loss observed in HWA-infested hemlocks (Orwig *et al.*, 2002; Stadler *et al.*, 2005), and as predicted needle abscission strength was reduced by HWA feeding. However, HWA had no effect on the flexibility of the needle abscission layer. Perhaps continuous HWA feeding induces localized branch drought stress (Domec *et al.*, 2013), such that decreased turgor pressure in twigs causes premature needle abscission, as seen in some *Picea* trees (Maier-Maerker and Koch, 1995). This induced abscission is likely detrimental to HWA, as needles proximate to the feeding site provide a key nutrient source to the stem-feeding HWA via xylem storage cells (Young *et al.*, 1995) laterally connected to the phloem (van Bel, 1990). Our results indicate that the increased needle loss observed in infested hemlock stands (Stadler *et al.*, 2005) may begin soon after infestation along the young, currently-infested twigs.

The changes in woody tissues were most pronounced following recent infestation. In young twigs, plausible explanations for the biomechanical changes we measured include abnormal xylem formation or a change in resource allocation priorities. Previous research indicates that HWA alters xylem anatomy by inducing false ring formation in young branches (Gonda-King *et al.*, 2012), which can result in thickened xylem walls in the false rings (Bolton and Petty, 1978). If cells in these false rings have thickened walls resembling those of adelgid-infested *A. balsamea* and of compression wood, an increase in lignin and in twig brittleness is expected (Balch *et al.*, 1964). However, in our study, twig lignification was not predictive of brittleness or impacted by HWA infestation. It has been suggested that the brittleness associated with elevated lignin is actually due to low relative cellulose content rather than lignin content *per se* (Johnson *et al.*, 2006). As such, we cannot exclude low cellulose content in the false rings as a potential cause of twig brittleness.

Since lignin did not predict mechanical responses to infestation, we suggest two additional, non-mutually exclusive, explanations. First, the loss of tissues resulting from HWA attack, along with induced changes in resource allocation, could affect the tree's ability to allocate resources to structural reinforcement in the infested twigs and to new growth. Specifically, the removal of nutrient stores from the xylem ray parenchyma by HWA feeding may decrease localized nutrient availability (Shigo, 1989 as cited in Oten, 2011; but see Gomez *et al.*, 2012). These cells store nutrients for release during periods of rapid growth, as lipids and starch granules (Pallardy, 2008; Begum *et al.*, 2010). If HWA

feeding depletes xylem ray parenchyma cells of glucose and other carbohydrates, the building blocks of cellulosic polymers will be depleted, likely leading to cell wall weakening (Pallardy, 2008), explaining the observed increase in brittleness and weakness in young HWA-infested twigs.

Second, an excess of nitrogen localized to sites of HWA feeding may decrease mechanical reinforcement. Under high-nutrient conditions, tissue density, strength, and stiffness decrease in some plant groups (Craine *et al.*, 2001; Lamberti-Raverot and Puijalón, 2012). This may be due to cell wall hydrolysis for accelerated growth and development (Labavitch, 1981), including amino acid synthesis.

Interestingly, twig brittleness under HWA attack was affected by an interaction with site, due to the weak HWA effect at the rural and common garden sites. Twigs from trees from the urban site, with intermediate infestation densities and low health (as assessed by minimal new growth foliage, dry and dull mature needles, and bare twigs, N. Soltis personal observation), were more brittle when HWA was feeding on them. It may be that recent years of HWA infestation were most severe at the urban site, and a decline in tree health has led to both increased branch brittleness and decreased HWA density. A milder infestation history at the rural site, and a briefer infestation history at the common garden, may have prevented a measurable increase in brittleness due to HWA infestation. We find it noteworthy that the common garden trees still exhibited the same pattern as urban site trees, despite the briefer infestation.

HWA feeding did not significantly affect the mechanics of three to four-year-old branches. Large branches, free of direct HWA feeding, may recover in subsequent growth seasons to structurally resemble unattacked branches. After HWA cease feeding on the storage cells within a branch, these cells are released from localized nutrient stress, and growing cells may develop normally with no subsequent reduction of strength – diminishing the effect of one layer of low-quality cells. As such, the mechanical effects of HWA herbivory are limited to the currently-attacked tissues.

### *Implications*

Our results may explain the more frequent needle (Stadler *et al.*, 2005) and twig (Nuckolls *et al.*, 2009) litterfall patterns observed in infested stands. By our measurements, young hemlock twigs break under 16 – 40 N of force. In the field, tree branches can accumulate up to 2g/cm snow and 4g/cm of ice under heavy precipitation (Cannell and Morgan, 1989), which along the length of our twig segments would amount to 0.4N snow or 0.8N ice. As such, ice buildup alone is not expected to cause breakage in hemlock twigs.

However, at wind speeds of 25 m/s (a strong gale), drag forces on tree stems can range from 20 to 80 N, while at 14 m/s (a strong breeze), drag forces reach 10 to 40 N (Butler *et al.*, 2012). Further, in softwood trees, wind loads in excess of 20 m/s cause mechanical instability (Spatz and Bruechert, 2000). While the forces experienced by small twigs will be much less, high winds or the combination of wind and ice loading may cause young branch breakage in hemlocks. Further, chronic stresses caused by recurring wind events also

contribute to mechanical failure in trees, and may be damaging to hemlock twigs (Mitchell, 2013).

In addition, the changes in branch biomechanics may have implications beyond the risk of damage in the wake of physical stressors. In addition to preventing breakage, biomechanical traits may serve to deter herbivores and protect against feeding damage. Structurally reinforced tissues, such as toughened leaves, experience less herbivore attack (Coley, 1983). Chemicals for structural reinforcement may also protect plants from feeding damage; cell walls with high cellulose or lignin content are indigestible to chewing insect herbivores (Clissold *et al.*, 2004). Hemlock woolly adelgids may avoid highly cellulosic and lignified tissues. Through cell wall fortification, plants increase mechanical barriers to prevent insect access to food sources, especially phloem-feeding aphids (Divol *et al.*, 2007). Dense tissues may also increase tissue longevity and nutrient conservation which could benefit HWA. In sum, biomechanical changes induced by HWA may increase *T. canadensis* susceptibility to further herbivory and accelerate decline.

Overall, twig breakage and needle loss due to physical factors may reduce plant fitness due to both biomass and meristem loss. Cumulative effects of structural damage, increased branch breaking, and foliage loss may alter the litter composition and nutrient cycling of infested stands, accelerating the ecological impact of HWA on eastern forests.

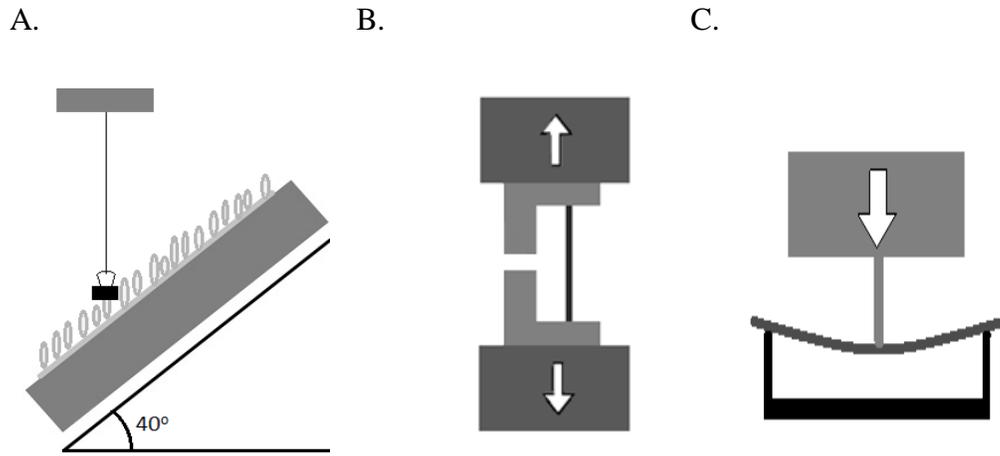


Figure 2.1: Depiction of setups for mechanical testing. A: Needle tension. B: Twig tension. C: Branch flexure.

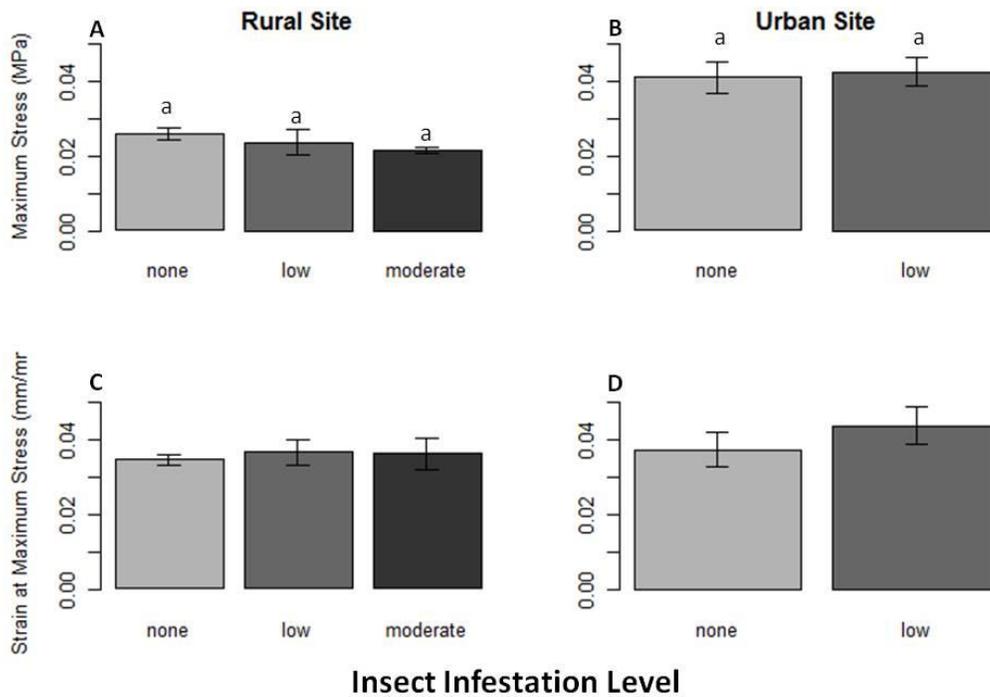


Figure 2.2: Biomechanics of needles by site and insect density, as maximum stress and as strain at maximum stress (mean  $\pm$  SE). None includes segments of 0 insects/cm, low includes 0.0-6 insects/cm, moderate includes 6.01-23 insects/cm. Different letters in figures A and B indicate significant differences between insect level within site, by post-hoc Fischer's LSD test at  $\alpha=0.05$  level.

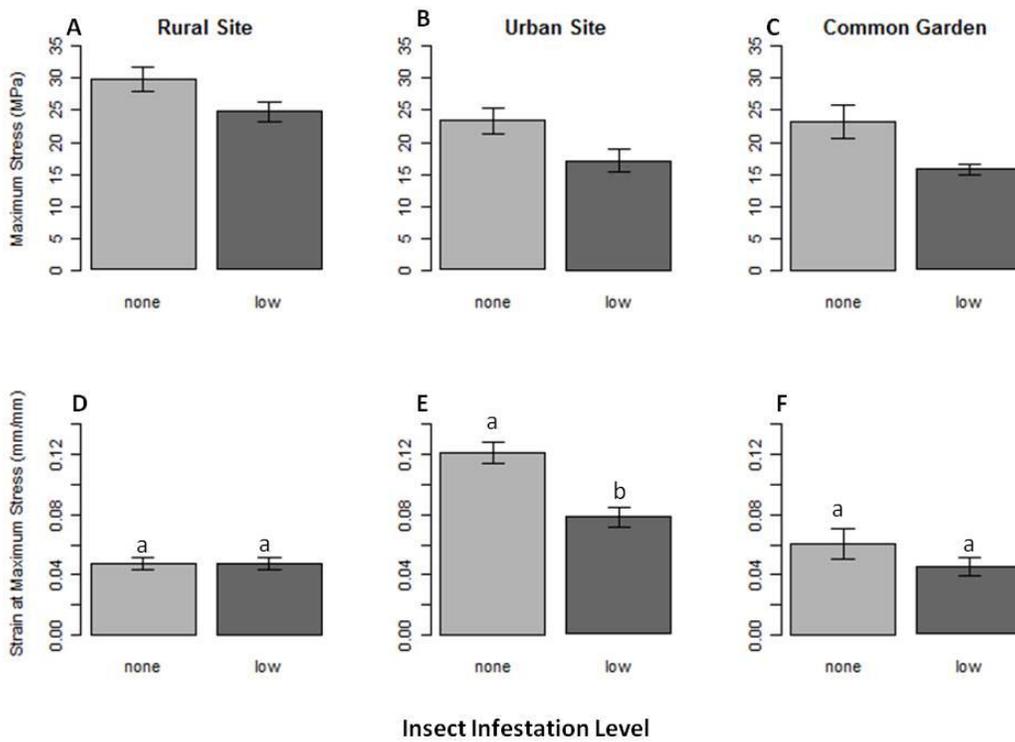


Figure 2.3: Biomechanics of 10-month-old twigs from each site, as maximum stress (A,B,C) and as strain (D,E,F) at maximum stress (mean  $\pm$  SE). None includes segments of 0 insects/cm, low includes 0.01 to 6 insects/cm. Different letters in figures D, E, and F indicate significant differences between insect level within site, by post-hoc Fischer's LSD test at  $\alpha=0.05$  level.

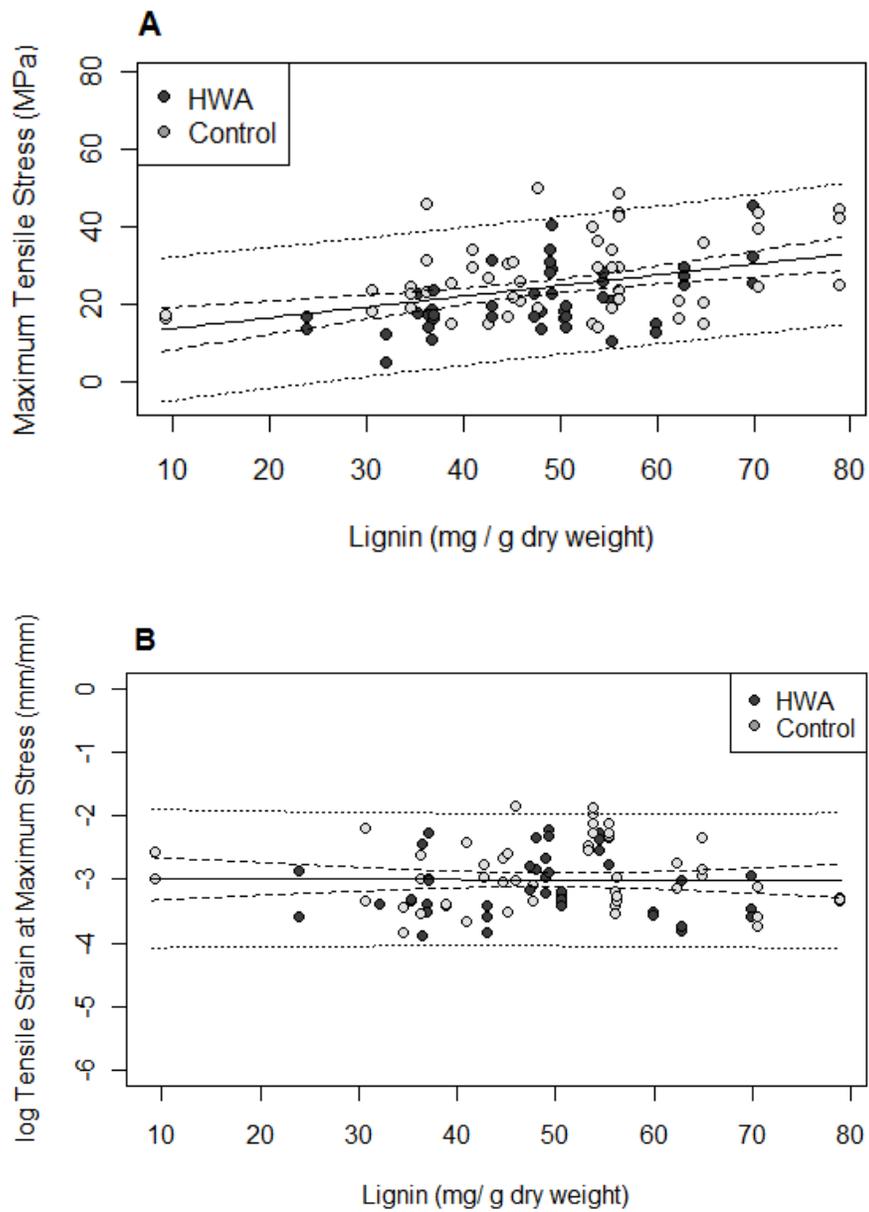


Figure 2.4. Linear regression of tensile mechanics against lignin content in twigs. Solid best fit line, dashed confidence interval, and dotted prediction interval. For stress, A,  $R^2 = 0.145$ ,  $p < 0.001$ . For log-transformed strain, B,  $R^2 = 0.0002$ ,  $p = 0.9013$ .

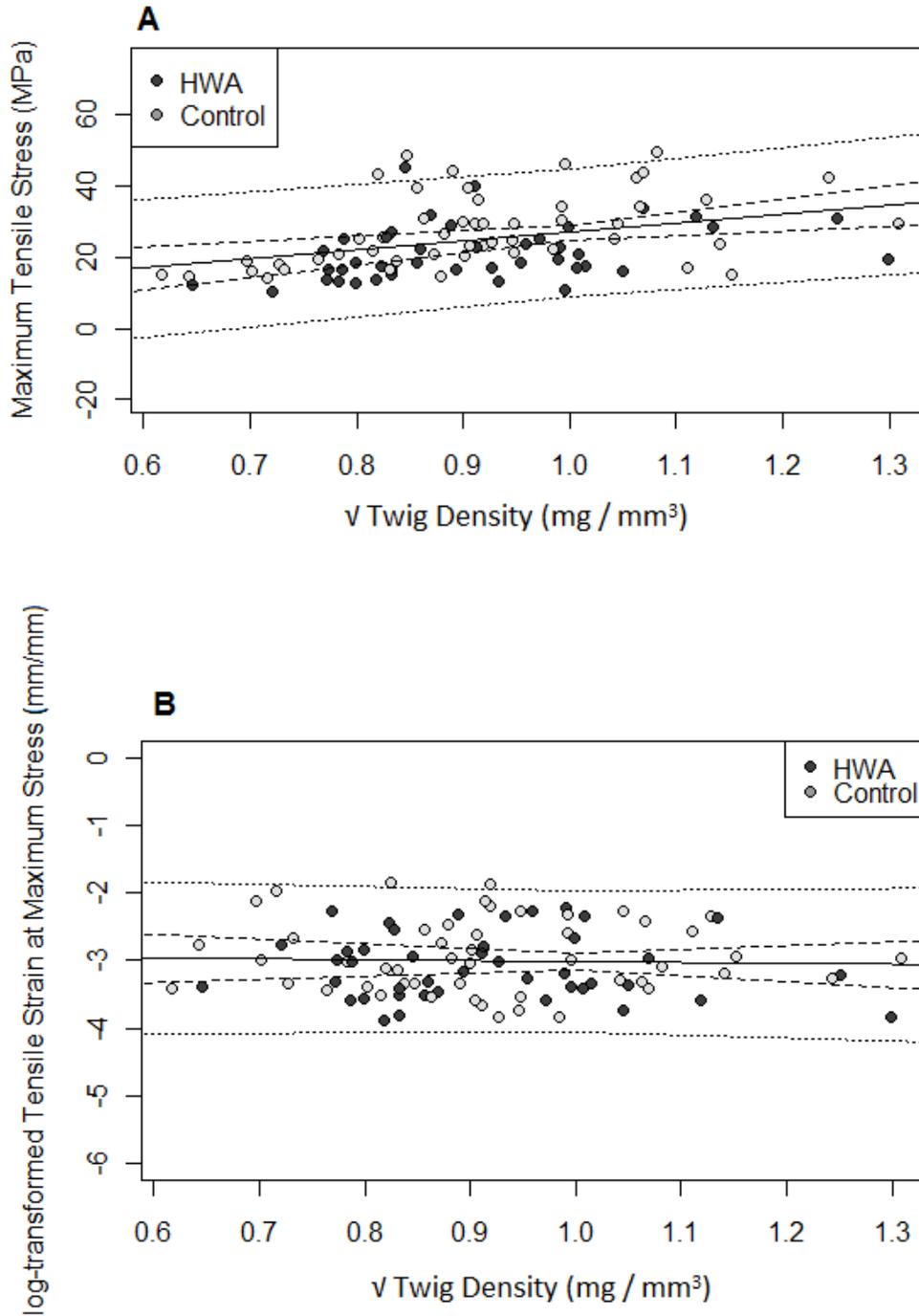


Figure 2.5. Linear regression of tensile mechanics against square-root transformed tissue density in twigs. Solid best fit line, dashed confidence interval, and dotted prediction interval. For stress, A,  $R^2 = 0.207$ ,  $p = 6.771E-07$ . For log-transformed strain, B,  $R^2 = 0.029$ ,  $p = 0.0777$ .

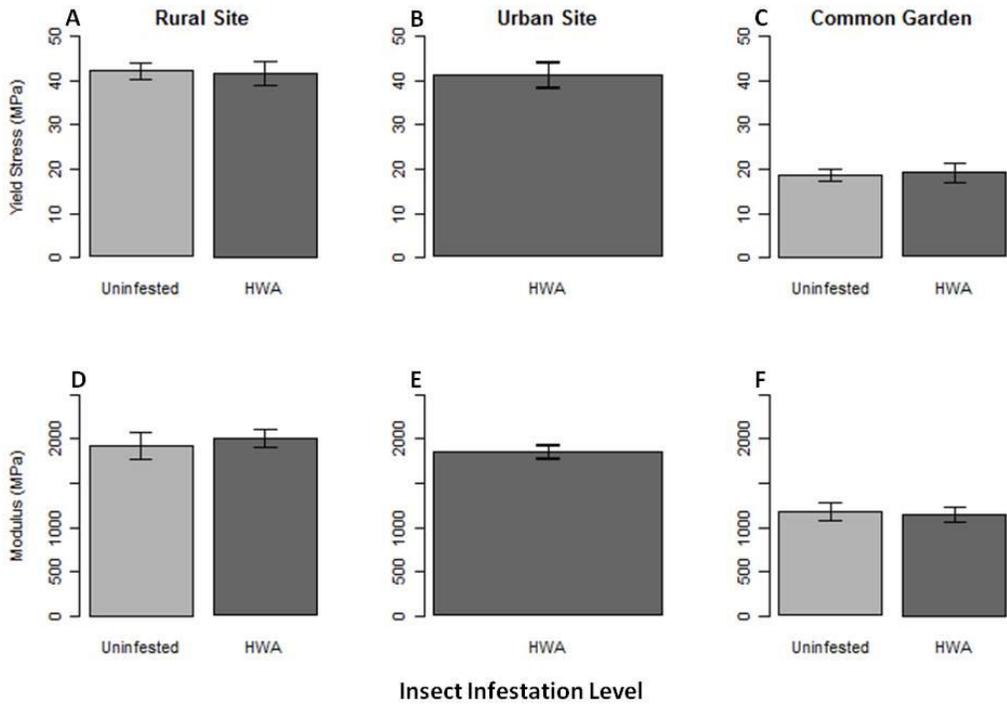


Figure 2.6: Biomechanics of large branches at each site, as yield stress and modulus. Values are means  $\pm$  SE.

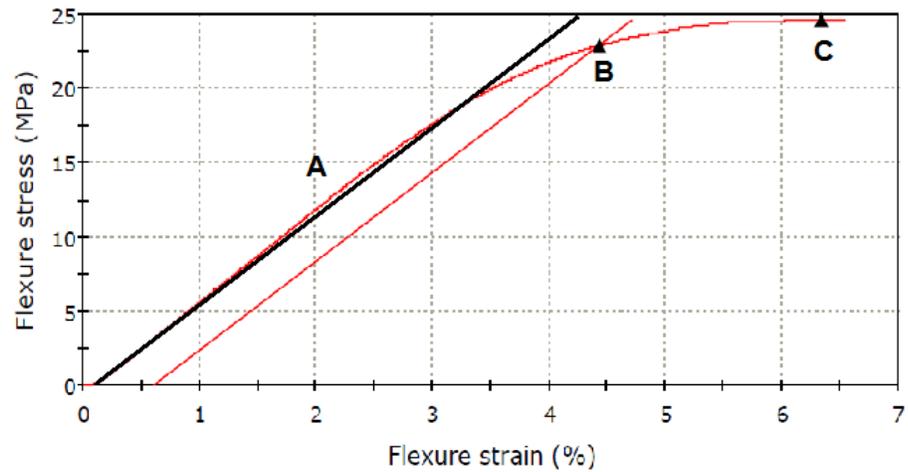


Figure 2.7. Sample output of biomechanical test depicting the stress-strain curve. A: initial slope of the stress-strain curve, the elastic modulus (Young's modulus), a structural property of the material. B: Yield strength, the stress required to initiate permanent deformation and the point at which the plant material begins to behave plastically. C: Point of maximum stress and maximum strain, immediately prior to specimen failure.

Table 2.1: Collection methods for all samples by measurement and site. ‘A’ signifies HWA-infested, ‘U’ signifies uninfested. ‘n’ signifies number of observations per site, infestation category, and tissue type. ‘Samples per tree’ and ‘n’ are equivalent for uninfested and HWA-infested samples unless noted.

Measurement	Site	Number of trees	Samples per tree	n	Unit of replication	Tree age
Needle tension	Rural	10	5 A, 5 U	10	branch	mature
	Urban	3	4 A, 4 U	3		mature
	Common Garden	n/a				
Twig tension	Rural	10	3 A, 3 U	29 A, 30 U	branch	mature
	Urban	6	2- 3 A, 2 - 3 U	13 A, 17 U		mature
	Common Garden	6 A, 8 U	3 A, 3 U	12 A, 16 U		sapling
Branch flexure	Rural	9	2 A, 2 U	9	tree	mature
	Urban	14	1	14		mature
	Common Garden	6 A, 8 U	2	6 A, 8 U		sapling

Table 2.2: Results of biomechanics analysis by tissue, site, and insect infestation category.

A. Needles					
Model	Source	df	F-ratio	P-value	Signif.
MANOVA	Insect	2	3.197	0.0174	*
	Site	1	8.241	0.0011	**
	Insect x Site	1	0.756	0.4766	
	Residual	39			
ANOVA: stress	Insect	2	6.297	0.0043	**
	Site	1	16.667	0.0002	***
	Insect x Site	1	1.132	0.2939	
	Residual	39			
ANOVA: transformed strain	Insect	2	0.525	0.5254	
	Site	1	0.982	0.9822	
	Insect x Site	1	0.606	0.6061	
	Residual	39			
B. Twigs					
Model	Source	df	F-ratio	P-value	Signif.
MANOVA	Insect	2	9.854	1.0000E-04	***
	Site	4	18.217	5.6920E-13	***
	Insect x Site	4	2.114	0.0799	.
	Residual	112			
ANOVA: stress	Insect	1	13.369	3.0000E-04	***
	Site	2	11.591	2.6600E-05	***
	Insect x Site	2	0.279	0.7573	
	Residual	112			
ANOVA: log strain	Insect	1	8.338	4.7000E-03	**
	Site	2	29.509	5.0810E-11	***
	Insect x Site	2	4.254	0.0165	*
	Residual	112			

Table 2.2: Results of biomechanics analysis by tissue, site, and insect infestation category.

C. Branches

Model	Source	df	F-ratio	P-value	Signif.
MANOVA	Insect	1	1.100	0.3405	
	Site	1	73.639	6.7620E-16	***
	Insect x Site	1	0.020	0.9804	
	Residual	53			
ANOVA: stress	Insect	1	1.906	0.1732	
	Site	1	104.260	4.0370E-14	***
	Insect x Site	1	0.013	0.9096	
	Residual	53			
ANOVA: modulus	Insect	1	0.937	0.3375	
	Site	1	90.792	4.4300E-13	***
	Insect x Site	1	0.015	0.9024	
	Residual	53			

Signif. codes: 0 '\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1

### **Chapter 3. Carbon trade deficit: The role of invasive herbivores as a resource drain on hemlock trees**

#### INTRODUCTION

Herbivores alter patterns of resource distribution that affect plant growth and development (Strauss and Agrawal, 1999; Karban and Baldwin, 1997; Orians *et al.*, 2011). In the absence of herbivory, plants exercise tight control over resource distribution to optimize their fitness. Most carbon (C) flux within plants occurs via sugars in the phloem, such that control of carbohydrate allocation is regulated by a balance between tissue demand and labile C availability. Tissue sinks compete for carbohydrates, and sink strength is dependent upon both demand and proximity to sources (Grossman and DeJong, 1994). Importantly, storage of carbohydrates as starch in persistent tissues can buffer against periods of resource stress, with the reserves re-mobilized to labile carbohydrates when needed. In long-lived trees, balanced resource allocation is particularly critical for surviving acute stresses.

Herbivory results in a loss of tissue and therefore metabolites, and affects C partitioning by increasing localized or plant-wide demand. Typically, plants respond to tissue damage by downregulating primary metabolism in favor of allocation to defense (Schwachtje and Baldwin, 2008). Insects like gallers, however, upregulate primary metabolism by manipulating sink strength of the attacked tissues (Larson and Whitham, 1991). In other cases, plants respond to herbivore cues by moving nutrients away from the site of damage to sites inaccessible to the herbivore (Orians *et al.*, 2011). In conifers such as *T.*

*canadensis*, however, trees store resources locally in non-dehiscent needles or leaves over multiple seasons, and loss of needles can have devastating impacts on plant survival (Webb, 1981).

Invasive herbivores are expected to cause major changes in resource distribution. Naïve host populations are often more susceptible to severe infestation by exotic species (Gandhi and Herms, 2010). As such, these herbivores often reach outbreak densities (Liebhold *et al.*, 1995), and cause severe damage (Lovett *et al.*, 2006). Subsequent growth in evergreen species is particularly sensitive to herbivory (Nykänen and Koricheva, 2004), likely due to their dependence on foliar resource storage. Mortality following herbivore outbreaks is common (Lovett *et al.*, 2006). When a plant is severely resource stressed, prioritizing allocation to growth and storage is critical for survival.

Eastern hemlock (*Tsuga canadensis*) is an ideal system for the study of C resource dynamics in a conifer under severe herbivory. Two invasive hemipteran species, the hemlock woolly adelgid (*Adelges tsugae*, HWA) and elongate hemlock scale (*Fiorinia externa*, EHS), are common pests of hemlock. While HWA causes extensive mortality, EHS does not. The two species differ markedly in feeding site, phenology, and severity of damage to the host, likely resulting from a range of resource allocation effects.

HWA settles at the bases of *T. canadensis* needles, and inserts its stylet bundle to extract water and nutrients from the xylem ray parenchyma of the stem (Young *et al.*, 1995), likely impacting nutrient transport and storage (van Bel, 1990). Upon infestation with HWA the long-lived *T. canadensis* suffers reduced

photosynthesis (Gonda-King, 2013), reduced growth, increased needle and branch debris fall (Stadler *et al.*, 2005; Nuckolls *et al.*, 2009), and other stress symptoms leading to rapid mortality (Broekling *et al.*, 2003). In its native range HWA forms galls on its alternate host, spruce, suggesting that it can manipulate the physiology of its host plant.

Elongate hemlock scale (*Fiorinia externa*), is also widespread throughout the *T. canadensis* range. Though detrimental to the host as it sucks from the leaf mesophyll, it does not cause mortality in healthy trees. When elongate hemlock scale (EHS) and HWA co-occur on *T. canadensis*, many of the symptoms expected under HWA infestation are alleviated (Miller-Pierce *et al.*, 2010).

In this study, we examined the short-term effects of HWA and EHS infestation on C partitioning in hemlock seedlings. The rapid, dramatic mortality of hemlock following HWA infestation may indicate resource depletion. We expect that HWA disrupt the ability of hemlocks to capture and invest carbohydrates to growth and storage. More specifically, we hypothesized that HWA, like hemipteran gallers (HWA are gallers on their alternate spruce host in their native range), act as a C sink, resulting in less C allocation to new growth and storage structures. EHS, too, are a likely C sink on hemlock trees, as they are also from a family including galling species (Miller and Howard, 1981). However, as symptoms of stress are much milder in trees attacked by EHS, we expect a weaker pattern of C reallocation in EHS infested trees.

## METHODS

### *Growth & Harvest*

To assess whole-tree effects of herbivory, we selected young seedlings for study. Thirteen 1-year-old seedlings from Evergreen Nursery, WI were randomly assigned to each treatment: HWA-infested, HWA uninfested controls, EHS-infested, EHS uninfested controls. Two trees from each control group were randomly designated unlabeled controls to determine the natural abundance of  $^{13}\text{C}$ . Due to failure of  $^{13}\text{C}$  labeling of one control tree in the EHS experiment, it was added *a posteriori* to the natural abundance subset for analysis. We measured tree height and the length of the longest primary branches upon assigning treatments, and again two days prior to C-labeling.

Each tree received inoculants according to standard protocols (see Miller-Pierce *et al.*, 2010). HWA & corresponding control foliage were collected from Medford, MA in 2012. Foliage for EHS treatment was collected from Stafford, CT in 2012. Due to the differences in insect phenology, we timed our analyses to focus on the effects of each insect. This resulted in inoculation of HWA plants in late April and EHS plants in late June. Infestation was successful, with HWA plants reaching  $1.88 \pm 0.24$  insects/cm and EHS plants reaching  $0.98 \pm 0.18$  insects/cm (mean  $\pm$  SE) at time of harvest, within abundance ranges naturally occurring in the field.

Throughout the growth season, we watered the soil to saturation every third day. Additionally, we applied a  $\text{NH}_4\text{NO}_3$  fertilizer in a 50ml 50ppm water

solution every third day for 8 weeks prior to HWA harvest, and 19 weeks prior to EHS harvest.

Ten weeks after successful inoculation, we applied a stable isotope of C ( $^{13}\text{CO}_2$ ) to each tree on a sunny morning. We exposed one primary branch in the upper portion of each tree to  $^{13}\text{CO}_2$ , by placing the branch into a sealed plastic sleeve and injecting 20% lactic acid solution into a 20mg vial of 99%  $^{13}\text{C}$  sodium bicarbonate (as in Bledsoe and Orians, 2006). Six days after labeling, we harvested control and infested plants (HWA in early July and EHS in late September) to assess growth, translocation of photosynthate, and seedling chemistry as C:N ratios and starch content.

One day prior to harvest, we measured 1) branch growth, as length of terminal current-year growth on 3 randomly-selected branches per seedling, and 2) insect infestation density. We washed soil from the roots of each plant immediately following harvest, then separated the tree into seven tissue categories: root, main stem, new growth (branch and needle separated), previous years' growth (branch and needle separated), and, separately, the branch exposed to  $^{13}\text{CO}_2$  label. We flash-froze a 5g subsample from each tissue category in liquid nitrogen (N) for chemical analysis. The remaining tissue was oven-dried (75°C) to constant mass, and weighed in its entirety. The flash-frozen subsamples were lyophilized to constant mass, then frozen until further processing.

#### *Stable isotopes and C:N*

Each tissue was ground to a fine powder using a ball mill (Kleco, Inc). 1-5mg of sample was then sent to the University of California-Davis Stable Isotope

Facility for  $^{13}\text{C}$  and  $^{12}\text{C}$  isotope ratio mass spectrometry as well as total C and N concentration. The isotopic composition of each sample was reported in  $\delta$  notation as permil (parts per thousand), relative to standards of known composition (peach leaves, nylon, USGS-41 glutamic acid).

### *Carbohydrates*

$10 \pm 0.2$  mg of each tissue type was extracted in 80% ethanol to remove ethanol soluble compounds, and the resultant pellets were analyzed for starch concentration, an important storage compound in conifers (Webb, 1981). Briefly, the pellets were digested overnight in an amyloglucosidase and sodium acetate solution at  $55^\circ\text{C}$  (Haissig and Dickson, 1979), and the resulting free sugars were analyzed. We measured absorbance for each experiment by the phenol-sulfuric acid method (Dubois *et al.*, 1956) at 487 nm using a microtiter plate reader (Bio-Rad, Hercules, CA, USA).

### *Statistical analysis*

Prior to statistical analysis, we converted starch absorbance values to mg/g dry tissue mass by use of glucose standard curve. Additionally, we converted absolute  $\delta^{13}\text{C}$  measurements to net values in excess of natural abundance levels.

Due to our nested sample design, we developed a linear mixed model for all analysis using the SAS mixed procedure (SAS Institute, 2011). Except where noted otherwise, each dependent variable ( $^{13}\text{C}$ , C:N, starch for both HWA and EHS experiments) was analyzed by pairwise comparisons under the model of insect treatment nested within tissue category, and tissue nested within plant. We included interactions between factors in the model. Due to small sample size, we

did not include covariates in our analysis. All p-values are bonferroni-corrected for multiple comparisons.

## RESULTS

Our artificial infestation resulted in  $1.88 \pm 0.24$  insects/cm (mean $\pm$ SE) for HWA-treated plants, and  $0.98 \pm 0.18$  insects/cm for EHS-treated plants, at respective time of harvest. These densities are relatively mild for HWA and moderate for EHS infestation; in a previous study using artificial infestation, branches reached 6-8 HWA/cm and 1-2 EHS/cm (Preisser and Elkinton, 2008).

### *Growth*

After only 10 weeks of infestation, HWA already reduced average branch elongation, and, although not significant after the multiple testing correction, reduced height growth as well (Table 3.1, Figure 3.1). Additionally, HWA-infested seedlings had lower new needle mass (t-test;  $t=4.57$ ,  $p < 0.001$ ) and new branch mass (t-test;  $t=5.01$ ,  $p < 0.0001$ ) (Figure 3.2). In contrast, 10 weeks of EHS infestation did not result in any change in branch elongation, height growth, or percent new branch or needle masses (Table 3.2, Figure 3.1, 3.2).

### *Stable isotopes and C:N*

In the HWA experiment, there was no effect of HWA on  $^{13}\text{C}$  uptake (t-test,  $t=0.17$ ,  $p=0.86$ , Figure 3.3A), although we observed some tissue specific effects. There was a significant increase in translocation to the main stem (Table 3.2), a marginally significant increase in translocation to the root (Table 3.2), but no effect of HWA on translocation to other tissues (Table 3.2, Figure 3.3A).

In the EHS experiment, we measured no effect of EHS on  $^{13}\text{C}$  uptake (t-test,  $t=0.57$ ,  $p=0.58$ , Figure 3.3B), and no effect of EHS on translocation to other tissues (Table 3.3, Figure 3.3B).

C:N ratio significantly decreased in HWA-attacked new branches, and also decreased with marginal significance in HWA-attacked old branches, driven by both an increase in N (Gomez, unpublished data) and a decrease in C (Table 3.2). In other tissues, C:N ratio was insensitive to HWA feeding (Table 3.2, Figure 3.4). C:N ratio was insensitive to EHS treatment (Table 3.3).

### *Carbohydrates*

Starch concentrations did not significantly differ by HWA treatment (Table 3, Figure 3.5A) or by EHS treatment (Table 4, Figure 3.5B) for any of the tissues assayed.

## DISCUSSION

HWA infestation, but not EHS infestation, affected plant growth and resource distribution within ten weeks of infestation. Considering that infestation levels were considered mild for HWA (Preisser and Elkinton, 2008), the rapidity of this effect is especially remarkable. HWA reduced branch elongation, and decreased the percent mass of new needles and new branches. Simulated herbivory in conifers often inhibits shoot growth (Moreira, Zas and Sampedro, 2012; Ayres *et al.*, 2004). While our results are consistent with the observation that HWA reduces tree growth (McClure, 1991), premature needle abscission (N. Soltis, unpublished data) may also contribute to the reduction in young needle

mass. Stadler *et al.* (2005) showed that needle litterfall is greater beneath infested hemlocks.

C:N ratio declined significantly in the new branches of HWA seedlings, and decreased with marginal significance in old branches in the HWA experiment. Simulated herbivory also reduces C:N ratio of woody tissues in *Abies* (Ayres *et al.*, 2004). The decreased C:N ratio of old branches may be a direct result of local HWA feeding; in order to consume sufficient N, herbivores remove large quantities of C-rich compounds and tissues as well. Further, low C:N in new branches may indicate an induced improvement in resource quality for HWA feeding.

In spite of differences in summer growth in the HWA experiment,  $^{13}\text{C}$  uptake was equivalent across control and HWA treatments. This may indicate that photosynthesis was nearly equivalent between control and infested trees, or that the supply of labeled C did not exceed the uptake potential of the exposed branches.

After a six-day chase period, translocation of photoassimilate in HWA plants to the main stem increased significantly, and increased to the roots with marginal significance. In conifers, C allocation shifts toward the roots and latewood stem following the completion of needle elongation in summer (Gordon and Larson, 1968; Smith and Paul, 1988). As summer progresses, the translocation of assimilates from needles to stem may be localized to starch-storing cells as in *Picea* (Langenfeld-Heyser, 1987), or allocated to the production of thick-walled latewood xylem, which correlates with current-season needle

maturation as in *Pinus* (Gordon and Larson, 1968). Thus, we suggest that the increased C translocated to the main stem may be partitioned to storage as starch, or to latewood xylem for cell growth. We did not, however, observe a change in stem starch content. Since HWA induces false rings (abnormal cells resembling latewood xylem) in branches of infested trees (Gonda-King *et al.*, 2012; Domec *et al.*, 2013), and this thickening of early and mid-season cell walls may constitute a stronger C sink in HWA-attacked seedlings, we suggest this may have been the primary sink for this labeled photoassimilate.

Alternatively,  $^{13}\text{C}$  detected in the stem at the time of sampling may have been on a path toward the root, to alleviate nutrient stresses due to herbivory. Gómez *et al.* (2012) previously found evidence of water stress and N stress in HWA-attacked hemlocks. N-deficient trees may respond by increasing C allocation to fine roots (Dyckmans, 2001). This basipetal translocation can aid in the herbivore response by increasing capacity for water and nutrient uptake (Erb *et al.*, 2009), often by growth of mycorrhizal roots, a strong belowground C sink in conifers (Smith and Paul, 1988). Simulated herbivory induces mycorrhizal colonization in *Picea* (Regvar, Gogala and Znidarsic, 1997), and herbivory may encourage mycorrhizal associations in hemlock as well. In the field, *T. canadensis* associates with ectomycorrhizae and arbuscular mycorrhizae (Godbold, Berntson and Bazzaz, 1997). Additionally, prioritizing carbohydrate storage, as indicated by the lack of HWA effect on starch concentrations, can aid in maintaining labile N pools and in producing root exudates to improve nutrient cycling (Sala, Woodruff and Meinzer, 2012). Further study of root activity differentiating large

and fine root growth, and measuring root exudation and mycorrhizal colonization, would clarify the implications of the observed photosynthate export to roots.

As indicated above, we did not find evidence for changes to starch reserves following infestation by either insect. The ability to feed without immediately depleting starch reserves is important for HWA survival. As HWA are sessile following stylet insertion for feeding (Young *et al.*, 1995), individual survival depends upon the persistence of localized nutritive tissues, such as starch within the hemlock ray parenchyma cells (Wiedenhoft and Miller, 2012). Further, EHS are sessile when feeding from the needle parenchyma cells (McClure, 1979) and similarly rely on needle tissue quality and persistence.

Additionally, in order to optimize long-term survival, storage-limited plants downregulate growth and structural reinforcement in favor of maintaining starch and soluble sugar accumulation (Sala, Woodruff and Meinzer, 2012; Kobe, 1997), which has a relatively low metabolic cost and improves survival. Evidence for this tradeoff appears in the low needle and new branch biomass in infested seedlings, and the low biomechanical resistance in HWA-infested branches (N. Soltis, unpublished data).

A second possible explanation of starch storage maintenance in response to herbivory is the problem of low accessibility. A portion of starch stored in xylem may be anatomically or chemically sequestered, such that it is unavailable for remobilization and use (Sala, Woodruff and Meinzer, 2012). As such, seedlings may not immediately exhaust carbohydrate reserves even under intense herbivory or resource stress.

### *Synthesis & future directions*

In summary, our results suggest resource stress in branches and needles through decreased growth and induced allocation of carbohydrates to main stems and roots in HWA-attacked seedlings. We did not detect effects of EHS herbivory on any the measured traits. This difference in herbivore effects may be explained by relatively low EHS densities, feeding habit, or differences in phenology.

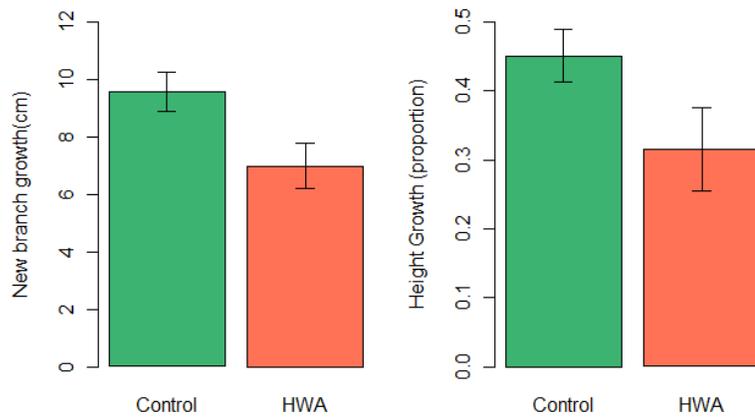
Overall, the lack of evidence for an EHS effect on any aspect of C partitioning suggests that this single season of moderate herbivory was not highly stressful to our experimental seedlings. In the field, EHS may exhibit negative density-dependence, such that populations remain at intermediate densities on their hosts (McClure, 1979). This can optimize EHS fitness by maintaining host quality and longevity, as occurs in hemlocks within the native range of EHS (McClure, 1986). Within our experiment, EHS remained below the densities at which negative population growth may occur (McClure, 1979).

The timing of EHS feeding, after the completion of shoot growth and needle expansion, may prevent stress in seedlings during the first season of feeding. Additionally, in late summer, new shoots often retain the vast majority of the C they receive, with less translocation to other tissues than in early summer (Webb, 1977; Smith and Paul, 1988), potentially explaining the lack of measurable translocation effect. A similar resource allocation experiment with multiple harvests would clarify the observed differences in herbivore effects, and

may suggest whether the difference is due to seasonality, or a difference in response rate or magnitude.

Our results can contribute broadly to our understanding of the ecological effects of HWA infestation. We observed a decrease in the C:N ratio of branches, and a decrease in needle growth potentially coupled with an increase in needle loss. Ultimately, elevated N concentrations in litterfall may contribute to changes to soil nitrification and the elevated N levels beneath infested hemlocks (Stadler *et al.*, 2005), and alter rates of decomposition (Berg, 1986). If greater C allocation to roots enhances root growth and mycorrhizal communities, this could further shift stand biogeochemistry. These changes likely accelerate the forest-level changes in nutrient cycling in response to hemlock decline and shifting community composition.

A.



B.

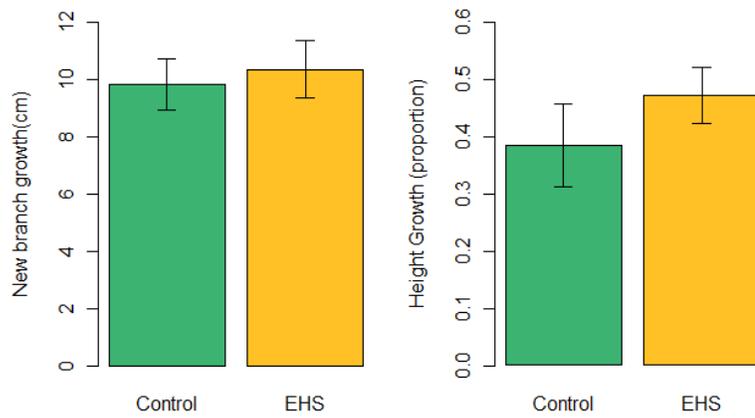
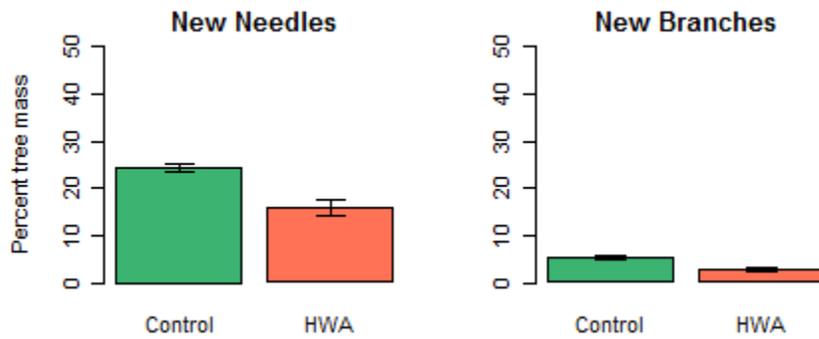


Figure 3.1: Elongation growth data for both insect experiments, A) HWA and B) EHS. New branch growth is new stem at elongating tip, height growth is proportion compared to initial height.

A.



B.

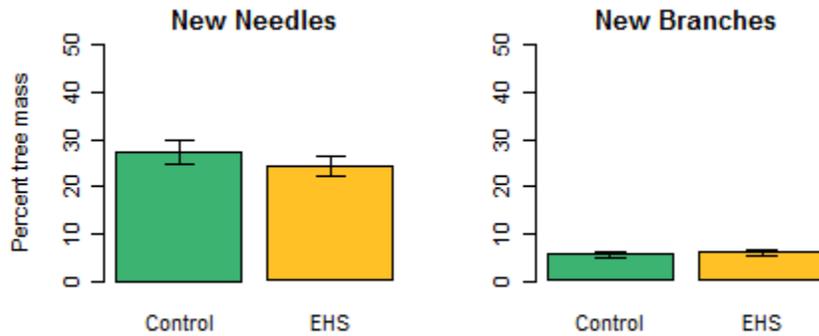
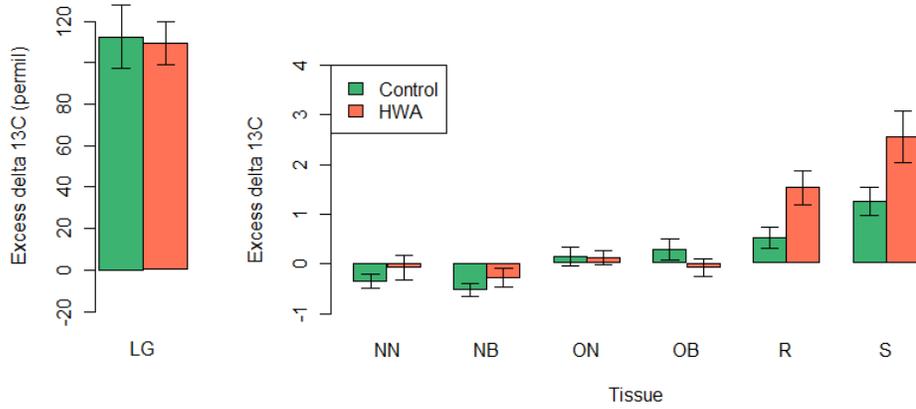


Figure 3.2: Mass growth data for both insect experiments, A) HWA and B) EHS.

Oven-dry needle and branch masses are displayed as percent total oven-dry tree mass. Results of bonferroni-adjusted t-tests as follows: A. HWA experiment: new needles,  $t=4.57$ ,  $p<0.001$ ; new branches,  $t=5.01$ ,  $p<0.001$ . B. EHS experiment: new needles,  $t=0.97$ ,  $p=0.69$ ; new branches,  $t=-0.40$ ,  $p=1.00$ .

A.



B.

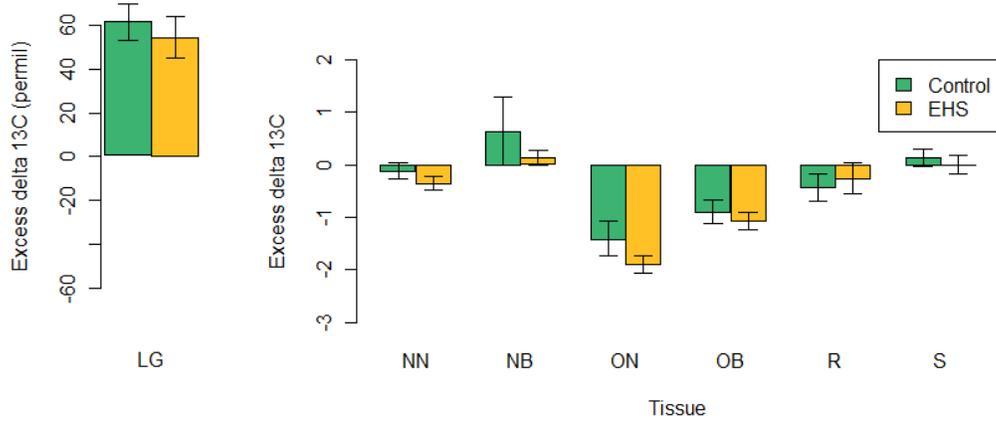
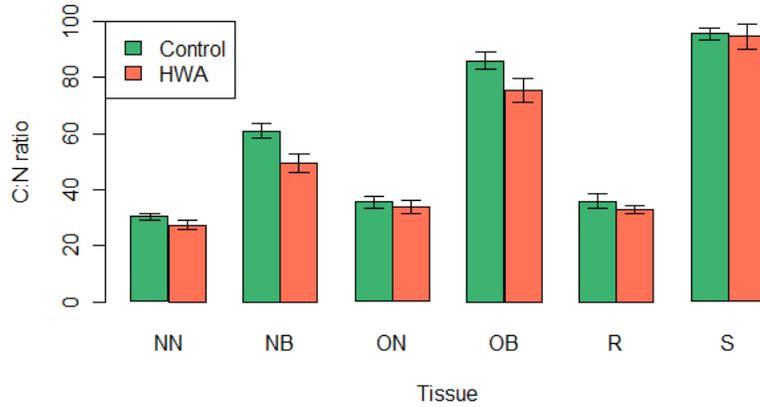


Figure 3.3:  $\delta^{13}\text{C}$  in excess of natural background for both insect experiments.

Natural background values come from 2 unexposed control seedlings for HWA experiment (A), and 3 control seedlings for EHS experiment (B). NN is new needles, NB is new branches, ON is old needles, OB is old branches, R is pooled root, S is main stem. Error bars are mean  $\pm$  SE.

A.



B.

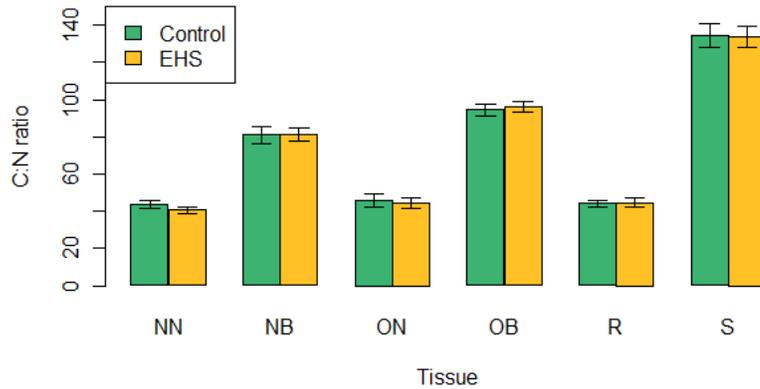
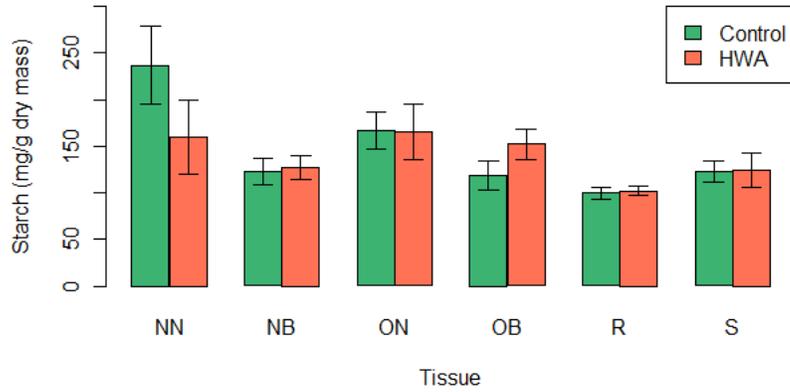


Figure 3.4: C:N ratio data in both insect experiments, A) HWA and B) EHS. EHS figure includes only infested new needles and infested new branches from EHS treatment. NN is new needles, NB is new branches, ON is old needles, OB is old branches, R is pooled root, S is main stem. Error bars are mean  $\pm$  SE.

A.



B.

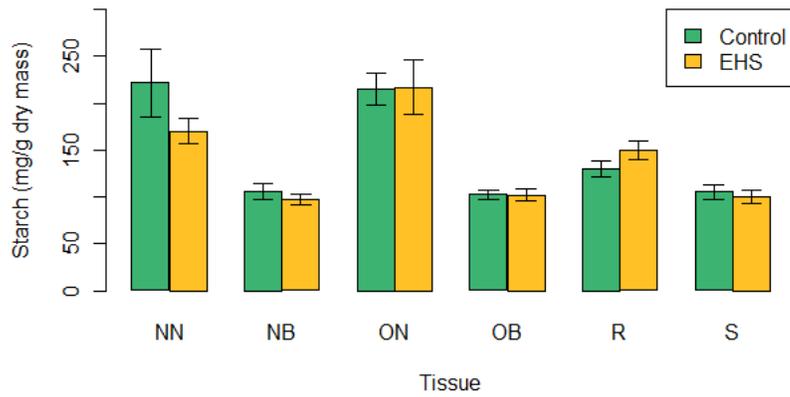


Figure 3.5: Concentration of starch by treatment and tissue in both insect experiments, A) HWA and B) EHS. NN is new needles, NB is new branches, ON is old needles, OB is old branches, R is pooled root, S is main stem. Error bars are mean  $\pm$  SE.

Table 3.1. Results of t-tests on elongation growth in each experiment. T-statistics are reported, and each insect experiment is separately bonferroni-corrected. Significance is indicated by asterisks, for p-values as follows: 0 ‘\*\*\*\*’ 0.001 ‘\*\*\*’ 0.01 ‘\*\*’ 0.05 ‘.’ 0.1 ‘ ’ 1.

Experiment	Measure	Units	Mean $\pm$ SE		Test Statistic	p-value
			Control	Infested		
HWA	Branch tip					
	elongation	cm	9.6 $\pm$ 0.7	7.0 $\pm$ 0.8	2.45	<b>0.045</b>
	Height growth	%	45 $\pm$ 4	32 $\pm$ 6	1.89	0.15
EHS	Branch tip					
	elongation	cm	9.8 $\pm$ 0.9	10.3 $\pm$ 1.0	0.96	0.69
	Height growth	%	38 $\pm$ 7	47 $\pm$ 5	0.39	1.00

Table 3.2: Significance of differences between HWA and Control trees for multiple traits. All inference under mixed model, with t-statistic results of pairwise comparisons reported. Significance is indicated by asterisks, for p-values as follows: 0 '\*\*\*\*' 0.001 '\*\*' 0.01 '\*' 0.05 '.' 0.1 ' ' 1.

		New	New	Old	Old		Main
	df	Needles	Branches	Needles	Branches	Roots	Stem
$\delta^{13}\text{C}$	85.33	0.7	0.64	-0.06	-0.92	2.62	3.37 *
Starch	135.3	-2.41	0.08	-0.04	1.05	0.08	0.05 .
C:N	75.78	-0.76	-2.9 *	-0.45	-2.64 .	-0.72	-0.24
C	110	-0.18	-1.7	-25.42***	-13.21***	118.01***	64.39***

Table 3.3: Significance of differences between EHS and Control trees for multiple traits. All inference under mixed model, with t-statistic results of pairwise comparisons reported. Significance is indicated by asterisks, for p-values as follows: 0 ‘\*\*\*\*’ 0.001 ‘\*\*\*’ 0.01 ‘\*\*’ 0.05 ‘.’ 0.1 ‘ ’ 1. New needles and new branches compare Control to EHS-infested tissues only.

		New	New	Old	Old		Main
	df	Needles	Branches	Needles	Branches	Roots	Stem
$\delta^{13}\text{C}$	113	-0.64	-1.32	-1.16	-0.35	0.48	-0.36
Starch	132	-2.22	-0.39	0.05	-0.03	0.82	-0.23
C:N	60	-0.56	-0.01	-0.27	0.31	0.08	-0.12
C	112	0.83	-8.88****	-4.1****	50.39****	27.81****	-3.59**

#### **Chapter 4. Conclusion - Sapped of strength: The costs of herbivory to *Tsuga canadensis***

Our research into carbon partitioning and biomechanics furthers knowledge of mechanisms of Eastern hemlock (*Tsuga canadensis*) decline in response to hemlock woolly adelgid (*Adelges tsugae*, HWA). Specifically, HWA reduces the strength and flexibility of young foliage in field and common garden trees. This study is the first to measure altered mechanical properties of needles and twigs in response to an invasive herbivore. In addition, HWA, in as little as 10 weeks, alters resource allocation and reduces growth in experimental seedlings. These findings may explain some of the dieback and stress responses observed in infested hemlock in the field (Stadler *et al.*, 2005). The elongate hemlock scale (*Fiorinia externa*, EHS) in contrast had no observable effect on resource allocation 10 weeks after colonization. Given that this insect is much less destructive, this is not surprising. Longer-term studies may be required to see any observable effect. Below I highlight key findings in more detail.

By studying biomechanical properties of hemlock tissues under tension, we found evidence for HWA-induced changes in eastern hemlock, particularly to twigs and to needle strength. HWA reduced twig strength and increased twig brittleness under tension, decreased twig tissue density, and decreased needle abscission strength. Further, in our study of carbon partitioning, HWA-infested seedlings showed signs of stress, as branch elongation and current-season needle and branch masses declined. HWA increased the allocation of photoassimilate

into the main stem and roots of seedlings, and decreased the C:N ratio of branches.

Though we did not study the mechanisms of the observed mechanical changes, we have several hypotheses, integrated with our findings in carbon partitioning.

The allocation of photoassimilate to main stem in the summer, as seen in HWA-treated seedlings, likely contributes to xylem cell growth. As HWA-infested branches often have abnormal xylem resembling latewood (Gonda-King *et al.*, 2012), this may explain the excess carbon allocation. This abnormal xylem likely has increased wall thickness (Bolton and Petty, 1978) and increased lignin content (Balch *et al.*, 1964) relative to cellulose. Increased wall formation in these tissues likely constitutes a stronger carbon sink. These anatomical & chemical changes are also expected to affect flexibility (Johnson *et al.*, 2006), and may cause the mechanical effects in our infested twig samples.

Though we did not see evidence of starch depletion following short-term infestation, after longer infestation or following depletion of other soluble compounds, resource stress may later occur. Nutrient availability in infested twigs is likely compromised by HWA feeding from the xylem ray parenchyma, which can release nutrients from cellular stores for growth (Pallardy, 2008; Begum *et al.*, 2010). If HWA depletes localized carbohydrate availability, accumulation of cellulose, a primary structural component of the cell wall, will decrease. Cellulose depletion due to herbivory can contribute to cell wall weakness (Pallardy, 2008), and likely to the mechanical effects we measured.

As evidenced by increased C:N ratio in our attacked twigs, HWA may increase partitioning of nitrogen to the herbivore-attacked twigs, in order to improve nutrient content of their food source. If excess nitrogen is allocated to these tissues, it may impact mechanical reinforcement as well, as tissue density and mechanical properties decrease (Craine *et al.*, 2001; Lamberti-Raverot and Puijalon, 2012), partially due to cell wall hydrolysis, which can release carbon resources for rapid growth and development (Labavitch, 1981). In order to supply this additional nitrogen, fine root growth can be supported by basipetal photoassimilate transport (Dyckmans, 2001), and increase nitrogen uptake. Further, fine root growth and mycorrhizal associations will facilitate nutrient uptake (Erb *et al.*, 2009), to alleviate resource stress.

Our findings indicate low mechanical resistance under HWA attack, which can increase breakage in response to commonly encountered physical stressors such as wind, snow, and ice. Increased twig breakage and needle loss, as seen in the field (Orwig *et al.*, 2002; Nuckolls *et al.*, 2009) likely accelerate the mortality (Stadler, 2005; McClure, 1991) and gap formation (Orwig and Foster, 1998) observed in infested hemlock stands.

Further implications of these biomechanical and chemical changes include potential effects on subsequent herbivory. Dense and structurally reinforced tissues may deter herbivores (Coley, 1983), protect storage tissues from feeding insects (Divol *et al.*, 2007), and inhibit digestion once consumed (Clissold *et al.*, 2004). Further, herbivores prefer to feed on high-nitrogen tissues. Since HWA-infested twigs had more N in our study, the structurally compromised hemlock

tissues may be more vulnerable to additional attack by other herbivores including browsing generalists.

Further, HWA may induce localized drought stress as it feeds, and low turgor pressure within attacked twigs could induce needle abscission (Maier-Maerker and Koch, 1995). Trees may compensate for drought stress by allocating carbon to fine root growth (Erb *et al.*, 2009), a response suggested by the increased carbon translocation to the roots of HWA-infested seedlings.

Considering our results, further study into resource dynamics, particularly belowground, may clarify the impact of HWA on hemlock growth and longevity. What occurs belowground following basipetal translocation of photoassimilates in infested seedlings? Measures of growth between storage and fine roots could suggest either a nutrient uptake or storage function of carbon allocation. If nutrient uptake is modulated in response to herbivory, quantifying root exudates and classifying microbial communities would be of interest. Evidence of mycorrhizal colonization would indicate that the allocation of carbon belowground in infested plants may be a strategy to mitigate nutrient stress.

Our study into resource allocation found evidence of an HWA, but not EHS, effect at a single timepoint following recent infestation. Timing may greatly affect the severity of infestation responses in hemlocks. Shifts in resource allocation occur seasonally in healthy hemlocks, between growth of new photosynthetic tissues, to growth reinforcing woody tissues, and storage. The impact of herbivory likely shifts with season as well.

In conclusion, there are multiple pathways by which biomechanical and resource stresses within these infested trees likely contribute to stand-level dieback. Increased breaking and abscission of young foliage will alter the phenology and chemistry of litterfall. The longevity of infested trees is likely impacted by decreased growth, lost biomass, and the loss of meristem with young twigs. Together, these effects may alter the biogeochemistry of infested stands, as litterfall accelerates and carbon uptake for growth declines.

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