False Ring Formation in Eastern Hemlock (*Tsuga canadensis*) After Hemlock Woolly Adelgid (*Adelges tsugae*) and Elongate Hemlock Scale (*Fiorinia externa*) Feeding

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FALSE RING FORMATION IN EASTERN HEMLOCK (*Tsuga canadensis*)

AFTER HEMLOCK WOOLLY ADELGID (*Adelges tsugae*) AND ELONGATE

HEMLOCK SCALE (*Fiorinia externa*) FEEDING

BY

LIAHNA GONDA-KING

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ABSTRACT

Hemlock forests in the eastern United States are threatened by two sessile invasive herbivores: the elongate hemlock scale, *Fiorinia externa* Ferris (Hemiptera: Diaspididae; ‘EHS’) and the hemlock woolly adelgid *Adelges tsugae* Annand (Hemiptera: Adelgidae; ‘HWA’). EHS and HWA occupy similar feeding guilds but have enormously different effects on tree health. EHS reduces hemlock growth and causes needle discoloration and loss, but only causes tree mortality under high EHS densities (McClure 1980b). In contrast, HWA has devastated stands of hemlocks on the east coast of the United States. Although EHS reduces fitness of the tree and can kill already stressed trees (McClure 1980), HWA is known to kill hemlocks in as few as four years (McClure 1991). The mechanism by which HWA and EHS kill trees is not yet elucidated and little is known as to the physiological effects each invasive has on hemlock. For the first part of my master’s research, I focused on differences in abnormal wood production among uninfested trees, EHS-infested trees and HWA-infested trees at the branch level. Specifically, I measured false ring density, ring growth and earlywood:latewood ratios in the two most recently deposited growth rings. Branches from HWA-infested trees had 30% more false ring than branches from EHS-infested trees and 50% more than branches from uninfested trees. In contrast, growth and earlywood:latewood ratios did not differ among treatments. This result suggests that two invasive insects from similar feeding guilds have differing effects on false ring formation in eastern hemlock. These false rings may be the product of a systemic plant hypersensitive response to feeding by HWA on hemlock branches. If false rings are responsible for or symptomatic of hemlock water stress, this may
provide a potential explanation for the relatively large effect of HWA infestations on tree health.

For the second part of my master’s thesis I looked at the impact of HWA on eastern hemlock anatomy and physiology. Specifically, I looked at growth and production of new buds on terminal and side branches in hemlock infested with and without HWA. We found that trees infested with HWA have significantly less new growth and fewer new buds. Additionally, I measured water potential, photosynthesis and stomatal conductance in trees infested with and without HWA during diapause and immediately after HWA resumes feeding. HWA undergoes summer diapause while still attached to eastern hemlock and it is unknown if this ‘inactive’ period affects tree health. We found that actively feeding HWA exacerbate reductions in photosynthesis and stomatal conductance, but not water potential. The presence of HWA, irrespective of feeding activity, decreases eastern hemlock water potential, photosynthesis, and stomatal conductance. Additionally, water potential and stomatal conductance were negatively correlated with HWA density. These data indicate that HWA negatively impacts tree health even when not actively feeding and depleting carbon reserves. These results also suggest that HWA-infested trees are water stressed, shedding light on possible mechanisms behind HWA-induced death.
ACKNOWLEDGEMENTS

I would like to thank my advisor, Dr. Evan Preisser for his guidance, support and mentoring in completing the research. I would also like to thank my committee members Dr. Richard Casagrande and Dr. Alison Roberts for the expertise and advice on experimental design. Dr. Steven Alm served as my committee chair and provided invaluable assistance in managing the East Farm Site. This thesis would not have been possible without the advice and guidance from Dr. Sara Gomez and Laura Radville. A special thanks goes to the Preisser Lab members, Jamie Rafter, Mauri Hickin, Elwood Roberts, and Phyll Ford. I would also like to thank my family and friends for their support and encouragement.
PREFACE

The first chapter of this thesis is being submitted in manuscript form. Chapter one, “False ring formation in eastern hemlock branches: Impacts of hemlock woolly adelgid and elongate hemlock scale”, has been published in the journal Environmental Entomology with co-authors Laura Radville and Evan Preisser. Chapter two, “Impacts of hemlock woolly adelgid (Adelges tsugae) on eastern hemlock (Tsuga canadensis) physiology during and after diapause”, will be submitted to the journal Entomologia Experimentalis et Applicata with co-authors Sara Gomez and Evan Preisser.
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CHAPTER 1

“False ring formation in eastern hemlock branches: Impacts of hemlock woolly adelgid and elongate hemlock scale”

by

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ABSTRACT

Herbivores can alter plant physiology through the induction of abnormal wood formation. Some insect herbivores induce the formation of false rings, a band of thick-walled latewood cells within the earlywood portion of the tree ring that reduces water transport. Hemlock woolly adelgid (*Adelges tsugae*), and elongate hemlock scale (*Fiorinia externa*) are invasive insects that feed on eastern hemlock (*Tsuga canadensis*). *Adelges tsugae* has a greater effect on tree health than *F. externa*, but the mechanism underlying their differential effect is unknown. We explored their effects by assessing growth ring formation in branches of trees that had been experimentally infested for four years with *A. tsugae*, *F. externa*, or neither insect. We measured false ring density, ring growth, and earlywood:latewood ratios in the two most recently deposited growth rings. Branches from *A. tsugae*-infested trees had 30% more false rings than branches from *F. externa*-infested trees and 50% more than branches from uninfested trees. Branches from *F. externa*-infested trees and control trees did not differ in false ring formation. Radial growth and earlywood:latewood ratios did not differ among treatments. Our results show that two invasive herbivores with piercing-sucking mouth parts have differing effects on false ring formation in eastern hemlock. These false rings may be the product of a systemic plant hypersensitive response to feeding by *A. tsugae* on hemlock stems. If false rings are responsible for or symptomatic of hemlock water stress, this may provide a potential explanation for the relatively large effect of *A. tsugae* infestations on tree health.
INTRODUCTION

Herbivores can alter plant physiology directly through tissue and nutrient removal and indirectly through the induction of increased chemical (Bezemer et al. 2003, Kaplan et al. 2008) and/or morphological defenses (Levin 1973). Although such responses vary between herbivores, alterations in plant physiology are especially likely with invasive or other species that reach high densities on their host plants (Sakai et al. 2001). Although herbivore-induced changes to plant structure are most commonly thought to involve architectural shifts resulting from bud/branch mortality or altered height/radial increments (Traw and Dawson 2002, Sopow et al. 2003), herbivory may also induce changes in woody plant tissues in the stems of conifers and other woody plants (Fernandes 1990).

In conifers, false rings are thick-walled xylem cells that appear as dark bands of latewood flanked on both sides with earlywood (Copenheaver et al. 2006). False rings occur within an annual ring but, although they resemble the end of an annual ring, do not occur on a yearly or seasonal basis. Normal rings are composed of large, thin-walled cells formed early in the growing season and small, thick-walled cells formed later in the year. These true rings are characterized by an abrupt increase in cell size at the start of the new growing season, while false rings are identified by a slow increase in cell diameter and decrease in cell wall thickness adjacent to the false ring (Copenheaver et al. 2006). Like compression wood, false rings have thick-walled xylem cells that increase resistance to water flow (Bolton and Petty 1978). False rings are associated with water stress and insect infestation, and have been observed in
conifers such as *Pinus sylvestris* and *P. banksiana* (Hollingsworth and Hain 1992, Cherubini et al. 2003, Copenheaver et al. 2006). Drought may induce false rings by reducing photosynthesis and stopping cambial activity during the summer (Cherubini et al. 2003). During periods of water stress, small, thick-walled cells are formed in the wood; if conditions become more favorable, subsequent cells will be larger with thinner walls (Wimmer et al. 2000). This alternation in cell size may appear as a false ring. In support of this, Wimmer et al. (2000) found that false rings were associated with periods of alternating wet and dry months.

Although not all herbivores induce changes in ring formation (Priya and Bhat 1997, Heijari et al. 2010), certain insects have been linked to their occurrence. Increased densities of the balsam woolly adelgid (*Adelges piceae* Ratz.) are correlated with the formation of rotholz rings, a type of abnormal wood similar to compression wood, in Fraser fir (*Abies fraseri*) (Hollingsworth and Hain 1992), and rotholz rings are only found near areas of adelgid feeding. Since these rings contain cells that do not conduct water and balsam woolly adelgid feeding is also associated with an increase in non-conducting heartwood (Arthur and Hain 1986), the resulting water stress may eventually kill the tree (Hollingsworth and Hain 1991). The formation of rotholz rings may defend against low-density adelgid infestations by forming necrotic tissue around the feeding site that isolates and starves the insects (Arthur and Hain 1985). With many points of adelgid feeding, however, so much of the stem may become non-conductive that the increased water stress actually kills the tree (Arthur and Hain 1985, McClure 1988).
The hemlock woolly adelgid (*Adelges tsugae* Annand (Hemiptera: Adelgidae)) is an invasive hemipteran herbivore that is causing high mortality of eastern hemlock (*Tsuga canadensis*) across the eastern United States (Orwig et al. 2002). *Adelges tsugae* was first reported in Virginia in the 1950’s (Souto et al. 1996) and has since spread rapidly along the east coast, now ranging from northern Georgia to Maine (McClure and Cheah 1999, USFS 2008). *Adelges tsugae* completes two generations per year in its invaded range (McClure 1989), and feeds on eastern hemlock at the base of the needle petiole by inserting its stylet bundle into xylem ray parenchyma tissue (Young et al. 1995). Feeding by *Adelges tsugae* devastates hemlock stands; mature hemlocks infested with *A. tsugae* can die within four years of infestation (McClure 1991), and seedlings appear similarly vulnerable (Preisser et al. 2011).

The elongate hemlock scale (*Fiorinia externa* Ferris (Hemiptera: Diaspididae)) is another invasive sessile herbivore that feeds on eastern hemlock (McClure 1978). *Fiorinia externa* arrived in New York in the early 1900’s but remained in the mid-Atlantic until the 1970’s, when its range and population density began rapidly increasing. It can now be found in over 14 states from northern Georgia to southern Maine (McClure 1978, Preisser and Elkinton 2008, Abell 2010). *Fiorinia externa* produces two generations per year (as it does in its native range) in southern states, but only one generation per year in New England (McClure 1978; Abell 2010). It feeds on mesophyll cells from its location on the underside of the needles and reduces hemlock growth while causing needle discoloration and loss (McClure 1980b). Although *A. tsugae* infestation is usually lethal to hemlocks, *F. externa* infestation only results in tree death with heavy, sustained infestations (McClure 1980b, 1991). Experimental
and survey work has confirmed that A. tsugae is much more harmful than F. externa to eastern hemlock (Preisser and Elkinton 2008, Preisser et al. 2008, Miller-Pierce et al. 2010).

Although little is known about why these species differ in their impact, there is evidence that A. tsugae induces an especially pronounced hypersensitive response in the tree (Radville et al. 2011). The hypersensitive response is a plant defense response that increases the levels of reactive oxygen species such as superoxide anions, hydroxyl radicals, and hydrogen peroxide (H$_2$O$_2$), thereby inducing cell death in herbivore-colonized areas in order to isolate and starve feeding organisms (Heath 2000, Liu 2010). The cue for this response is often the presence of a foreign substance indicative of herbivore feeding (reviewed in (Cornelissen et al. 2002), and the ensuing localized tissue death is a particularly effective response to sessile herbivores (Karban and Baldwin 1997). This response has been shown to reduce plant damage caused by balsam woolly adelgids, bark beetles, and a host of other herbivore species (Fernandes 1990, Ollerstam and Larsson 2003). In the case of A. tsugae, Radville et al. (2011) found that infestation caused a larger localized hypersensitive response (measured as an increase in H$_2$O$_2$ concentrations) than was present in either F. externa-infested or uninfested trees. Although both F. externa and A. tsugae produced a localized hypersensitive response, A. tsugae infestation also caused a systemic hypersensitive response not observed in the other treatments. Hypersensitivity has been linked to abnormal wood formation and water stress in other adelgid-conifer interactions (Arthur and Hain 1985), and Gómez et al. (2012a) found that A. tsugae-infested trees had increased levels of proline, an amino acid associated with drought conditions.
Recent research (Walker-Lane 2009) on mature hemlocks in the field found significantly more false rings in *A. tsugae*-infested hemlocks than in hemlocks treated with insecticide. Walker-Lane (2009) also determined that the *A. tsugae*-infested hemlocks were drought stressed, but was unable to establish whether *A. tsugae* caused this or was simply more abundant on drought-stressed trees. Because of the insects’ differing ability to induce a systemic hypersensitive response in eastern hemlock, we hypothesize that *A. tsugae* infestation will induce a greater degree of false ring formation than will infestation with *F. externa* or neither insect (control). Through this research, we hope to provide insight into why feeding by *A. tsugae* is much more damaging than feeding by *F. externa*.

**METHODS**

In April 2007, we collected uninfested *T. canadensis* saplings 0.7-1 m in height from Cadwell Forest (Pelham, MA), a forest research facility managed by the University of Massachusetts, Amherst. This site was past the northern range limit of both *A. tsugae* and *F. externa* at the time the plants were collected, and careful examination prior to collection confirmed that neither insect was present on any of the saplings or in the surrounding stand. The trees were planted at East Farm, a research facility managed by the University of Rhode Island (Kingston, R.I.), in a rectangular grid in an open field. The experiment utilized a randomized complete block design, with each row containing each treatment. The experimental grid originally contained eight rows (spaced 4 m apart) and thirteen trees per row (spaced 2 m apart); most of the trees in each row were utilized in an unrelated experiment (Miller-Pierce et al
Within each row, the trees used in our experiment were randomly assigned to one of three treatments: *A. tsugae* only, *F. externa* only, or neither herbivore (control). Trees in the herbivore treatments were inoculated using hemlock foliage infested with *A. tsugae* or *F. externa* each spring from 2007-2011, a total of five yearly infestations, using standard protocols (Butin et al. 2007). Details on the experimental design, including the source locations of the herbivore populations used in the inoculation, the precise timing of infestation, and the infestation protocols are reported elsewhere (Miller-Pierce and Preisser 2010). Insect densities in each treatment were recorded in the fall and spring of each year in order to confirm that each insect was present throughout the experiment (Fig. 1). To minimize cross-contamination between treatments, each tree was enclosed in a 1 x 1 x 2 m (length x width x height) plastic PVC pipe frame cage covered with mosquito netting (100 holes/cm² mesh size; Barre Army-Navy, Barre VT). Although the experiment began with a fully-balanced design, over the four-year study a combination of transplant shock, herbivore-induced mortality, and cross-contamination reduced the number of replicates per treatment to eight no-herbivore trees (controls), seven *F. externa*-only trees, and six *A. tsugae*-only trees, for a total of 21 experimental replicates.

On May 31, 2011, we collected one branch (~0.6 cm diam) per cardinal direction from each of the 21 experimental trees (84 branches total). Following their removal from the tree, each branch was immediately submerged in DI water. After 24 hours, we mounted the base of the branch on a sliding microtome and cut two 60μm sections per branch. Sections were placed in a 0.1% safranin O stain solution for three minutes, rinsed for two minutes and mounted on slides. We counted the number of
false rings formed in the last two years of branch growth (2009 and 2010) using a light microscope. Our decision to assess the most recent two years of branch growth was motivated by the fact that although branches varied in age, all of them had at least two distinct growth rings. Each growth ring consisted of both earlywood and latewood; the two wood types were easily distinguishable by their distinctive colors and by the smaller, thick-walled cells characteristic of latewood. False rings were easily identifiable using standard diagnostic characteristics; i.e., a gradual change from earlywood to a band of latewood with earlywood on the opposite side (Copenheaver et al. 2006). After counting the false rings in the 2009 and 2010 growth rings, we photographed each section (Fig. 2) and used ImageJ 1.44 (Abràmoff et al. 2004) to measure the total number of annual growth rings, the branch radius, and the thickness of the earlywood and latewood in the 2009 and 2010 tree rings of each branch. Because every section was asymmetrical, we measured branch radius in three different axes and averaged them to calculate a mean branch radius. We followed the same procedure to calculate the mean thickness of the 2009 and 2010 growth rings and the mean thickness of latewood in the 2009 and 2010 tree rings of each branch. We calculated the width of each ring’s earlywood by subtracting the latewood thickness from the mean ring width, and the earlywood:latewood ratio by dividing the thickness of each ring’s earlywood by its latewood.

**Statistical analysis:** The unit of replication for our analyses was the mean response per tree per treatment (21 replicates). Data were square-root transformed when necessary to improve normality; variances were homogenous between treatments. When analyzing data on branch diameter and total annual growth rings, we
used ANOVA to test for the main effects of treatment (A. tsugae-only, F. externa-only, and control) and location within the experimental grid (included as a blocking variable), and for their two-way interaction. All other data were analyzed using repeated measures ANOVA to test for the main effects of treatment, location, time (either the 2009 or 2010 growth ring), and their interactions. We performed means separation tests, where appropriate, using Tukey’s HSD. Statistical analyses were performed using JMP 9.0.0 (SAS 2010). When initial p-values are significant, we report both the initial p-value as well as the p-value corrected for multiple comparisons at $\alpha=0.05$ using step-up FDR, a sequential Bonferroni-type procedure (Benjamini and Hochberg 1995).

**RESULTS**

*Branch size and age:* There were no treatment-level differences in either branch radius or age, measured as the number of annual growth rings (Tables 1, 2A-B). Branches averaged $0.61\pm0.036$ [SE] cm in diameter and had similar numbers of annual growth rings. There was no effect of tree location within the experimental grid (Table 2A-B), and no significant treatment*location interactions.

*False ring density:* Adelges tsugae-infested branches had significantly more false rings than either F. externa-infested or control branches (Table 3A; Fig. 3). Adelges tsugae-infested trees averaged 0.96 false rings/growth ring, significantly more than in either control or F. externa-infested trees (0.48 and 0.66, respectively; Tukey’s HSD, $P < 0.05$). In contrast, F. externa-infested trees did not differ from the uninfested controls (Fig. 3). There was a marginally significant effect of tree location
within the experimental grid (Table 3A), but no significant change in false ring density across time (Table 3A). All two- and three-way interactions were nonsignificant (Table 3A).

*Earlywood, latewood, and ring width:* There were no treatment-level differences in the width of earlywood (Tables 1, 3B), latewood (Tables 1, 3C), or the annual rings (Tables 1, 3D). There was also no effect of treatment on the earlywood:latewood ratio (Tables 1, 3E). These four variables did not change over time and were unaffected by tree location within the experimental grid (Tables 1, 3B-E). There were no significant two- or three-way interactions (Tables 1, 3B-E).

**DISCUSSION**

While both insects have piercing-sucking mouth parts, infestation by *F. externa* and *A. tsugae* had markedly different effects on wood formation. Branches from *A. tsugae*-infested trees had a greater number of false rings than branches from uninfested trees (Fig. 3). Branches infested with *A. tsugae* had 50% more false rings than control branches and 30% more false rings than *F. externa*-infested trees. In contrast, infestation by *F. externa* did not significantly increase false ring formation. Despite the difference in false ring formation, there were no between-treatment differences in annual ring width or earlywood and latewood production.

The presence of false rings in all three treatments suggests that environmental factors can influence false ring formation in eastern hemlock. Dry conditions and drought stress have been associated with the formation of false rings in several other conifer species (Wimmer et al. 2000, Copenheaver et al. 2006, Hoffer and Tardif...
Although our experiment was not set up to test this hypothesis, our results nonetheless suggest that eastern hemlock may respond similarly. The highest densities of false rings occurred in summer 2010, a period that was much warmer and drier than summer 2009 (NOAA 2011). In 2009, Kingston RI experienced its 3rd wettest summer (43.1 cm of rainfall) since 1895, with an average temperature of 20.5 °C and seven days in which the temperature exceeded 30 °C. In contrast, summer 2010 had one-third less rainfall (28.2 cm) but was the hottest summer since 1895, with an average temperature of 22.4 °C and seventeen days over 30 °C (NOAA 2011). Although it cannot be tested, the substantial climatic differences between the two summers provide a plausible explanation for the overall increase in the number of false rings in 2010.

Although environmental parameters are undoubtedly important, *A. tsugae*-infested branches still contained significantly more false rings than branches from either *F. externa* or control trees. This finding corroborates observations made on mature eastern and Carolina hemlock (Walker-Lane 2009). Walker-Lane (2009) noted an association between false ring formation and *A. tsugae* infestation, but was unable to determine whether this association was correlative or causative. By experimentally infesting trees with *A. tsugae* and *F. externa* and observing the resulting changes in wood anatomy, our work confirms that *A. tsugae* infestation is responsible for an increase in false ring formation.

Perhaps the most likely explanation for our findings is that the increased number of false rings in *A. tsugae*-infested branches is a consequence of plant hypersensitivity, a defense mechanism against sessile herbivores and pathogens
(Fernandes 1990). The hypersensitive response induces cell death by increasing the reactive oxygen species (Heath 2000), which isolates the herbivore or pathogen and prevents it from establishing a suitable nutritional site (Wong and Berryman 1977, Fernandes 1990, Bonello et al. 2006). At low herbivore densities, a successful hypersensitive response can increase plant resistance; if herbivores survive and increase to high densities, however, the response could become lethal to the host plant (Bi and Felton 1995, Bonello et al. 2006). Radville et al. (2011) demonstrated A. tsugae infestation stimulated both a localized and systemic hypersensitive response in needles adjacent to A. tsugae settlement, while F. externa infestation only induced a localized response. In North American fir trees, the formation of rotholz rings is associated with the hosts’ hypersensitive response to feeding by the balsam woolly adelgid (Arthur and Hain 1985). In such cases, the increased production of non-conductive rotholz rings may serve to isolate the wounded tissue where the balsam woolly adelgid feeds. While this is an effective defense at low densities, high adelgid densities cause the stem to become non-conductive and can kill the tree (Arthur and Hain 1985). While the preceding applies specifically to rotholz and it is unknown whether false rings are related to rotholz formation, false rings are also known to be indicative of drought stress, and recent research suggests A. tsugae-infested hemlocks have lower transpiration rates and increases in δ13C, both symptoms of drought stress (Walker-Lane 2009). In addition, Gómez et al (2012a) showed that A. tsugae-infested trees contained higher levels of proline, an amino acid indicative of water stress, than uninfested control trees. In such a scenario, feeding by A. tsugae would cause a systemic hypersensitive response that alters wood anatomy, disrupting water transport
and increasing water stress. It is also possible that *A. tsugae* infestation may directly (i.e., without the involvement of plant hypersensitivity) increase water stress in eastern hemlocks. If *A. tsugae* infestation intensifies the degree of water stress experienced by hemlocks, the resulting drought-like conditions may increase false ring formation.

The most likely explanation for the fact that *F. externa* did not induce false ring formation involves this species’ feeding location. While *A. tsugae* feeds at the base of the needle petiole on xylem ray parenchyma cells (Young et al. 1995), *F. externa* feeds directly on the underside of the needle on mesophyll cells (McClure 1980b). Although located in close physical proximity to each other, *F. externa* is thus a foliar feeder while *A. tsugae* is a stem feeder. While defensive responses to *F. externa* could be confined to foliar tissue, similar responses to *A. tsugae* feeding would be much more likely to affect cambial growth (and thus water transport). This argument is compatible with the idea that, by virtue of its role as a stem feeder, *A. tsugae* induces a systemic hypersensitive response in cambial tissue that ultimately affects false-ring formation and water transport. The decreased response of the plant to *F. externa* feeding could also be due to species-specific differences in *A. tsugae* and *F. externa* densities. *Adelges tsugae* has two generations per year in New England, while *F. externa* has only one (McClure 1978). It should be noted, however, that whole-tree *A. tsugae* densities were consistently lower than *F. externa* densities for the duration of the study (Figure 1), a fact that suggests even low *A. tsugae* densities induce a greater degree of false ring formation than higher *F. externa* densities. Although unproven, it has also been suggested that a component of *A. tsugae* saliva is ‘toxic’ (Young et al. 1995) and that it injects chemicals during feeding that adversely affect
plant health (Preisser and Elkinton 2008). If true, this toxic substance could also help explain why *A. tsugae* induces false ring formation and *F. externa* does not.

Despite finding differences in the number of false rings, we did not find differences in other variables. This fact may be due to our relatively low degree of within-treatment replication; although we began our experiment with a higher degree of replication, sapling mortality and cross-contamination over the four-year course of the experiment reduced our numbers. Another issue concerns microclimate: although all of our hemlocks were enclosed in fine-mesh mosquito netting that acted as shade cloth, it is nonetheless likely that they experienced hotter and drier conditions than hemlocks growing in the shaded forest understory. While our decision to grow the trees at East Farm was motivated by the need for deer fencing and accessibility to a water source (trees were watered in the summer of 2007 to reduce transplant-related mortality), our findings highlight the need for similar experiments under closed-canopy conditions.

The occurrence of false rings in *A. tsugae*-infested trees may provide insight into the detrimental effect of *A. tsugae* on tree health. Regardless of the mechanism behind the false ring formation, their presence restricts water flow. False rings are anatomically similar to compression wood in that they both have thick-walled xylem cells which increase resistance to the flow of water through xylem tissue (Bolton and Petty 1978). Compression wood conducts water less efficiently than does normal wood (Spicer and Gartner 1998), and there is also evidence that insect-induced false rings impede water transport. Mitchell (1967) found that trees infested by the balsam woolly adelgid absorbed and transported less dye (a proxy for water) than uninfested
subalpine and grand fir trees. Rotholz rings appeared to inhibit dye transport and infested trees had half as many conducting tree rings (Mitchell 1967). Puritch (1971) showed that balsam woolly adelgid interfered with the water conduction in grand fir, evident in the reduced permeability of sapwood in balsam woolly adelgid-infested trees. Since balsam woolly adelgid and its hosts are closely related to *A. tsugae* and eastern hemlock, it seems reasonable to assume that false rings formed in *A. tsugae*-infested eastern hemlock will correlate with changes in water transport efficiency (an idea first suggested by Walker-Lane 2009). If the false rings produced in *A. tsugae*-infested trees are indicative of water stress, this may explain why *A. tsugae* has such a severe impact on tree mortality.

**ACKNOWLEDGEMENTS**

Drs. S. Gómez, F. Hain, D. Orwig, A. Roberts, and three reviewers made very helpful comments on previous versions of this manuscript. We thank J. Backer, D. Cox, M. Miller-Pierce, B. Peixoto, and J. Rafter for assistance with fieldwork, Dr. A. Roberts for advice with staining protocols, and Drs. G. Berlyn and P. Marshall for assistance with microtoming. Funding for this work came from NSF DEB#0715504 and NIFA 2011-67013-30142 to EP.
TABLES

Table 1. Mean branch radius, number annual growth rings, earlywood width in 2009 and 2010 growth rings, latewood width in 2009 and 2010 growth rings, ring width and earlywood: latewood ratio in 2009 and 2010 growth rings (± 1 SD) of uninfested trees, *F. externa*-infested trees, or *A. tsugae*-infested trees. There were no significant differences across treatments in all variables.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th><em>F. externa</em></th>
<th><em>A. tsugae</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Branch radius (cm)</td>
<td>0.313 (0.074)</td>
<td>0.296 (0.050)</td>
<td>0.290 (0.078)</td>
</tr>
<tr>
<td>Number of annual growth rings</td>
<td>4.792 (2.340)</td>
<td>5.097 (1.777)</td>
<td>4.964 (1.666)</td>
</tr>
<tr>
<td>Earlywood width 2009 (cm)</td>
<td>0.041 (0.023)</td>
<td>0.034 (0.015)</td>
<td>0.037 (0.023)</td>
</tr>
<tr>
<td>Earlywood width 2010 (cm)</td>
<td>0.054 (0.029)</td>
<td>0.050 (0.018)</td>
<td>0.052 (0.029)</td>
</tr>
<tr>
<td>Latewood width 2009 (cm)</td>
<td>0.050 (0.022)</td>
<td>0.039 (0.020)</td>
<td>0.037 (0.017)</td>
</tr>
<tr>
<td>Latewood width 2010 (cm)</td>
<td>0.063 (0.030)</td>
<td>0.056 (0.025)</td>
<td>0.055 (0.027)</td>
</tr>
<tr>
<td>Ring width 2009 (cm)</td>
<td>0.091 (0.033)</td>
<td>0.073 (0.024)</td>
<td>0.074 (0.031)</td>
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<tr>
<td>Ring width 2010 (cm)</td>
<td>0.117 (0.035)</td>
<td>0.105 (0.025)</td>
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<tr>
<td>Earlywood: Latewood 2009</td>
<td>0.956 (0.751)</td>
<td>1.171 (0.958)</td>
<td>1.110 (0.626)</td>
</tr>
<tr>
<td>Earlywood: Latewood 2010</td>
<td>1.100 (0.859)</td>
<td>1.190 (0.852)</td>
<td>1.233 (0.864)</td>
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**Table 2**: ANOVA table for the treatment effects on branch size (Panel A) and age (Panel B).

<table>
<thead>
<tr>
<th>Test</th>
<th>Exact F</th>
<th>Numerator DF</th>
<th>Denominator DF</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Branch radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.287</td>
<td>2</td>
<td>15</td>
<td>0.755</td>
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<tr>
<td>Location</td>
<td>0.026</td>
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<td>15</td>
<td>0.874</td>
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<tr>
<td>Treatment*Location</td>
<td>0.181</td>
<td>2</td>
<td>15</td>
<td>0.836</td>
</tr>
<tr>
<td>B. Annual growth rings</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.099</td>
<td>2</td>
<td>15</td>
<td>0.906</td>
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<tr>
<td>Location</td>
<td>3.039</td>
<td>1</td>
<td>15</td>
<td>0.102</td>
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<tr>
<td>Treatment*Location</td>
<td>0.735</td>
<td>2</td>
<td>15</td>
<td>0.496</td>
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</table>
Table 3: rm-ANOVA table for the effects of treatment, location, and time on false ring density (Panel A), earlywood width (Panel B), latewood width (Panel C), radius width (Panel D), and earlywood: latewood ratio (Panel E).

<table>
<thead>
<tr>
<th>A. False Ring Density</th>
<th>Test</th>
<th>Exact F</th>
<th>NumDF</th>
<th>DenDF</th>
<th>Prob&gt;F</th>
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</thead>
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<td>15</td>
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</tr>
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<td>Location</td>
<td>3.430</td>
<td>1</td>
<td>15</td>
<td>0.084</td>
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<tr>
<td>Treatment*Location</td>
<td>0.892</td>
<td>2</td>
<td>15</td>
<td>0.431</td>
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<tr>
<td>Time</td>
<td>0.276</td>
<td>1</td>
<td>15</td>
<td>0.607</td>
<td></td>
</tr>
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<td>Time*Treatment</td>
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<td>2</td>
<td>15</td>
<td>0.880</td>
<td></td>
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<td>Time*Location</td>
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<td>1</td>
<td>15</td>
<td>0.844</td>
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<tr>
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<td>0.042</td>
<td>2</td>
<td>15</td>
<td>0.959</td>
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<table>
<thead>
<tr>
<th>B. Earlywood Width</th>
<th>Test</th>
<th>Exact F</th>
<th>NumDF</th>
<th>DenDF</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.208</td>
<td>2</td>
<td>15</td>
<td>0.815</td>
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<tr>
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<td>1.443</td>
<td>1</td>
<td>15</td>
<td>0.248</td>
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<td>Treatment*Location</td>
<td>1.391</td>
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<td>15</td>
<td>0.279</td>
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<td>Time</td>
<td>1.488</td>
<td>1</td>
<td>15</td>
<td>0.241</td>
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</tr>
<tr>
<td>Time*Treatment</td>
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<td>2</td>
<td>15</td>
<td>0.976</td>
<td></td>
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<tr>
<td>Time*Location</td>
<td>0.603</td>
<td>1</td>
<td>15</td>
<td>0.450</td>
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</tr>
<tr>
<td>Time<em>Treatment</em>Location</td>
<td>0.472</td>
<td>2</td>
<td>15</td>
<td>0.633</td>
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<table>
<thead>
<tr>
<th>C. Latewood Width</th>
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<th>NumDF</th>
<th>DenDF</th>
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<tbody>
<tr>
<td>Treatment</td>
<td>0.692</td>
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<td>15</td>
<td>0.516</td>
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<tr>
<td>Location</td>
<td>0.049</td>
<td>1</td>
<td>15</td>
<td>0.828</td>
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<tr>
<td>Treatment*Location</td>
<td>1.132</td>
<td>2</td>
<td>15</td>
<td>0.348</td>
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<tr>
<td>Time</td>
<td>2.317</td>
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<td>15</td>
<td>0.149</td>
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<td>Time*Treatment</td>
<td>0.279</td>
<td>2</td>
<td>15</td>
<td>0.760</td>
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<tr>
<td>Time*Location</td>
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<td>1</td>
<td>15</td>
<td>0.389</td>
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<tr>
<td>Time<em>Treatment</em>Location</td>
<td>1.639</td>
<td>2</td>
<td>15</td>
<td>0.227</td>
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</table>

<table>
<thead>
<tr>
<th>D. Annual Ring Width</th>
<th>Test</th>
<th>Exact F</th>
<th>NumDF</th>
<th>DenDF</th>
<th>Prob&gt;F</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.596</td>
<td>2</td>
<td>15</td>
<td>0.564</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>0.364</td>
<td>1</td>
<td>15</td>
<td>0.556</td>
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</tr>
<tr>
<td>Treatment*Location</td>
<td>0.206</td>
<td>2</td>
<td>15</td>
<td>0.816</td>
<td></td>
</tr>
<tr>
<td>Time</td>
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<td>1</td>
<td>15</td>
<td>0.189</td>
<td></td>
</tr>
<tr>
<td>Time*Treatment</td>
<td>0.151</td>
<td>2</td>
<td>15</td>
<td>0.861</td>
<td></td>
</tr>
<tr>
<td>Time*Location</td>
<td>1.286</td>
<td>1</td>
<td>15</td>
<td>0.275</td>
<td></td>
</tr>
<tr>
<td>Time<em>Treatment</em>Location</td>
<td>1.482</td>
<td>2</td>
<td>15</td>
<td>0.259</td>
<td></td>
</tr>
<tr>
<td>E. Earlywood:Latewood Ratio</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>----------------------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Treatment</td>
<td>0.125</td>
<td>2</td>
<td>15</td>
<td>0.884</td>
<td></td>
</tr>
<tr>
<td>Location</td>
<td>1.267</td>
<td>1</td>
<td>15</td>
<td>0.278</td>
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</tr>
<tr>
<td>Treatment*Location</td>
<td>2.086</td>
<td>2</td>
<td>15</td>
<td>0.159</td>
<td></td>
</tr>
<tr>
<td>Time</td>
<td>0.154</td>
<td>1</td>
<td>15</td>
<td>0.700</td>
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</tr>
<tr>
<td>Time*Treatment</td>
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<td>2</td>
<td>15</td>
<td>0.665</td>
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<tr>
<td>Time*Location</td>
<td>0.074</td>
<td>1</td>
<td>15</td>
<td>0.789</td>
<td></td>
</tr>
<tr>
<td>Time<em>Treatment</em>Location</td>
<td>0.199</td>
<td>2</td>
<td>15</td>
<td>0.822</td>
<td></td>
</tr>
</tbody>
</table>
Figure 1: Densities of the hemlock woolly adelgid A. tsugae (left axis, triangular symbols) and the elongate hemlock scale F. externa (right axis, square symbols) at the whole-tree level in the A. tsugae-only and F. externa-only treatments over the course of the experiment. March sampling was conducted prior to yearly tree inoculations and measures the number of surviving overwintered adults in each treatment. Gray bars indicate the approximate formative period for the 2009 and 2010 annual growth rings; the space between the bars corresponds to the winter period of hemlock dormancy. NOTE: These data were initially presented in online resource 1 in Gómez et al (2012a), and are reprinted here to confirm that insects were present in each treatment throughout the experiment.
Figure 2: Panel (A): Cross-section of *T. canadensis* branch. Earlywood is distinguishable by larger thin-walled earlywood cells (located in the left side of each growth ring) and latewood is distinguishable by smaller thick-walled latewood cells (located in the right side of each growth ring. Panel (B): Cross-section of *T.*
canadensis branch with false ring (indicated by arrow) located to the left of the true ring. The false ring is a diffuse band of latewood flanked by earlywood. In contrast, the true ring (located to the right of the indicated false ring) is identified by the abrupt alteration from latewood cells in the prior years’ growth to earlywood cells. Panel (C): Cross-section of T. canadensis branch with multiple false rings (indicated by arrows). All photographs were taken using a PixeLink PL-A662 camera attached to an Olympus SZX12 microscope at 400x magnification.

![Mean false rings per treatment in each of the 2009 and 2010 annual growth rings.](image)

**Figure 3:** Mean ± 1 SE false rings per treatment in each of the 2009 and 2010 annual growth rings. Branches from trees in the A. tsugae-only treatment had significantly more false rings than branches from trees in either the F. externa-only or control treatments.
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CHAPTER 2

“Impacts of hemlock woolly adelgid (Adelges tsugae) on eastern hemlock (Tsuga canadensis) physiology during and after diapause”

by

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In preparation for Entomologia Experimentalis et Applicata

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ABSTRACT

Sap-feeding insects have negative impacts on tree growth and physiology but little is known about impacts of sap-feeding insects on tree performance when these insects are in diapause. Some insects diapause directly on a host plant and the effect of inactive insect presence is unknown. *Adelges tsugae* (Annand Hemiptera: Adelgidae), the hemlock woolly adelgid (HWA), is a sap-feeding insect that undergoes diapause during the summer attached to hemlock. HWA is an invasive insect rapidly killing *Tsuga canadensis* (L. Carrière), eastern hemlock trees in eastern North America. Our study is the first to look at the effect of HWA on eastern hemlock growth and physiology during diapause and immediately after HWA resumes feeding. We found that actively feeding HWA exacerbate reductions in photosynthesis and stomatal conductance, but not water potential. Interestingly, the presence of HWA, irrespective of feeding activity, decreases eastern hemlock growth, water potential, photosynthesis, and stomatal conductance. Additionally, water potential and stomatal conductance were negatively correlated with HWA density. These data indicate that HWA negatively impacts tree health even when not actively feeding and suggests that HWA-infested trees are water stressed, shedding light on possible mechanisms behind HWA-induced death.

INTRODUCTION

Many sap-feeding insects have long lasting physiological impacts on their host plant. These physiological changes are driven by both changes in plant nutrients (McClure 1980a, Masters and Brown 1992) and the production of secondary
chemicals (Karban and Myers 1989, Haukioja et al. 1990). By removing nutrients from the plants’ xylem or phloem, sap-feeding insect herbivores reduce plant growth, decrease photosynthesis rates and decrease plant reproduction (Candolfi et al. 1993, Meyer 1993). Sap-feeding insects are detrimental to trees (Vranjic and Gullan 1990, Smith and Schowalter 2001), yet there is minimal literature that quantifies the impact of sap-feeding herbivores on woody species (reviewed in Zvereva et al. 2010). Conifers may be especially susceptible to sap-feeders because unlike deciduous trees that store resources in their roots, stems, and other tissues inaccessible to sap feeders, evergreens allocate more storage to foliage (Chapin et al. 1990, Krause and Raffa 1996, Hester et al. 2004). The lack of such stored resources makes conifers vulnerable to herbivore attacks and in some cases, intense sap-feeding events can even result in tree death (Fernandes 1990, Furuta and Aloo 1994, Paine 2000).

The invasive non-native hemlock woolly adelgid (Adelges tsugae Annand Hemiptera: Adelgidae) is a specialist sap-feeding insect currently decimating eastern hemlock (Tsuga canadensis (L.) Carrière), a foundation species in eastern North American forests. Feeding occurs through the insertion of the stylet bundle at the base of a needle into the ray parenchyma tissue (Young et al. 1995). Once HWA selects a feeding place, it remains sessile throughout its entire life cycle. HWA can kill mature hemlocks within four years of infestation (McClure 1991). As eastern hemlock stands disappear, they are replaced by deciduous hardwood species such as birch (Orwig and Foster 1998, Catovsky and Bazzaz 2000). This disappearance has major impacts on ecosystem processes that can result in the regional homogenization of forest structure.
(Ellison et al. 2005) and declines of bird (Tingley et al. 2002) and invertebrate (Snyder et al. 2002) biodiversity (but see Ingwell et al. 2012).

HWA has a spring and a summer generation in its invaded range and each generation passes through four larval instars before becoming adults. The spring generation completes its life cycle between April – June and lays eggs that become the summer generation. The summer generation hatches in July and remains on hemlock until the following April when the cycle starts again (McClure 1989). While the spring generation feeds continuously throughout their shorter life cycle, the summer generation enters diapause in July immediately after hatching. Summer diapause in HWA is primarily induced by temperature (Salom 2001). The summer generation stays dormant until October, when HWA break diapause and resume feeding throughout the winter until April.

Diapausing insects undergo a period of arrested development characterized by metabolic depression (Triplehorn and Johnson 2005). Although diapause is often associated with winter, summer diapause also occurs in a wide range of insect taxa (reviewed in Masaki 1980). Insects in the Adelgidae experience diapause (Havill and Footit 2007), but only two species (Adelges tsugae and Adelges piceae) are known to go through summer diapause (Amman 1962, McClure 1989). Summer diapause is induced by a range of environmental factors that include photoperiod and temperature. In addition to abiotic influences, biotic factors such as host plant quality also affect the induction and length of insect diapause (Dalin and Nylin 2012). For example, leaf toughness influences the likelihood of diapause in the swallowtail butterfly Byasa alcinous (Takagi and Miyashita 2008). Hunter and McNeal (1997) found that host
plant species and the nutritional content of the diet influences the induction of, and mortality during, diapause in the lepidopteran herbivore *Choristoneura rosaceana*. While many studies focus on the effect of the plant host on the insect in diapause, the question remains whether the presence of a diapausing insect impacts host-plant physiology and performance.

The hemlock-HWA interaction provides an ideal system to explore the impact of sap-feeding herbivores on trees during and after diapause on their host plant. This is because HWA experiences summer diapause with its stylet imbedded at the base of hemlock needles. The aim of this study was to determine how HWA impacts hemlock performance during diapause and active feeding. Specifically, we assessed hemlock growth during the tree growing season, when HWA from the spring are feeding. Once the summer generation emerged and entered diapause (which coincides with the end of the host plant’s growing season) we measured physiological plant responses such as water potential, photosynthesis, and stomatal conductance. These responses were measured again immediately after HWA resumed feeding.

**METHODS**

*Study Site:* In April 2011, one-year hemlock trees were purchased from Van Pines (West Olive, MI, USA) and planted in a grid in a randomized complete block design at the Kingston Wildlife Research Station (Kingston, RI). There were two treatments: HWA-infested (n=12-15) and control (no insect; n=12-15). Subsequently, in April 2011 and 2012, experimental trees were inoculated with hemlock branches infested with HWA. Control trees received uninfested branches following a standard
protocol (Butin et al. 2007). Treatments were randomly assigned within each row of the grid and each row contained every treatment. Each sapling was enclosed in a cylindrical wire cage (0.3-m diameter, 0.9-m height) covered by a mesh bag (Agribon-15, Johnny’s Selected Seeds, Waterville, ME, USA; 90% light transmission) to prevent cross contamination.

_Growth Measurements:_ We marked two terminal branches and two side branches of each HWA-infested tree (n=15) and control tree (n=15). For each marked branch, we measured length of new growth starting at the first sight of bud break (April 28, 2012) and counted number of secondary buds (starting on June 15). We measured growth every two weeks from the start date and ended on July 30, 2012 (when hemlocks ceased to put on new growth).

_Water Potential:_ On September 8 and October 27, 2012 we measured predawn shoot water potential on 12 randomly chosen trees per treatment in the HWA-infested and control treatments. We chose to take physiological measurement (water potential and gas exchange) in the autumn for the following reasons: (1) eastern hemlock photosynthesize year round (Hadley 2000); (2) it took until the late summer/early fall for foliage to fully develop; and (3) HWA aestivate during the summer months, and we wanted to compare impacts of HWA feeding vs. HWA presence alone (no feeding). Between 4:00-5:30 am on each date, we collected one ~5 cm cutting from a terminal branch from each tree, placed in a bag with a wet paper towel, and immediately took it back to the lab in a cooler. Prior to sampling we conducted HWA density counts on sampled branches. To take water potential readings, each branch was placed in the pressure chamber of a Scholander pressure
bomb (3005 Plant Water Status Console, Soil Moisture Equipment, Santa Barbara, CA, USA) and we recorded the pressure at which xylem appeared visible at the tip of the branch under a magnifying glass.

Gas Exchange: We measured gas exchange on a terminal branch (2012 growth) on each tree used to quantify water potential. Measurements were conducted between 9:00-11:00 am on September 9 and October 26, 2012. In branches from HWA-infested trees, we counted the number of HWA present/ per cm on the sampled foliage. After each measurement, foliage inside the leaf chamber was excised, placed on a white piece of paper, and photographed; we quantified total leaf area using imageJ 1.44 software (Abràmoff et al. 2004). To determine gas exchange rates we used a CIRAS-2 portable photosynthesis system (PP systems, Haverhill, MA, USA) with a 2.5 cm$^2$ leaf chamber and a CIRAS-2 LED light source of 1500 µmolm$^{-2}$s$^{-1}$, a CO$_2$ concentration of 390 ppm, air flow rate at 350 cm$^3$s$^{-1}$ and leaf temperature of 25°C.

Statistical Analyses: All statistical analyses were performed using JMP 10.0 with each data point being the mean response variable per tree per sampling date. We used repeated-measures ANOVA with treatment and branch type (terminal or side) for branch growth and number of secondary buds. We used repeated-measures ANOVA to analyze the main effects of treatment and time, and the treatment*time interaction, on the following variables: water potential, net photosynthesis, stomatal conductance, and evaporation. We used linear regression to assess the correlation between HWA density and water potential, photosynthesis, and stomatal conductance for both time points. We checked all data for normality, sphericity and homogeneity of variance and
log transformed water potential data in order to meet ANOVA assumptions. For analyses that did not meet the assumptions of sphericity, we report univariate Greenhouse-Geisser corrected p-value is reported. The critical $P$ value used in this study was $P < 0.05$.

**RESULTS**

*Growth:* By the end of the growing season, terminal branches on control trees were 41% longer than HWA-infested terminal branches (Figure 1A). In contrast, HWA infestation significantly affected side branch growth: side branches on control trees were 57% longer than on HWA-infested trees (Figure 1B). Insect treatment had a significant effect on growth ($F_{1,52}=7.16$, $P=0.010$), as did tissue type ($F_{1,52}=16.49$, $P<0.001$). The significant time*treatment interaction ($F_{6,312}=136.77$, $P=0.0078$) suggests that HWA presence was more harmful later in the growing season. There was also a significant time* tissue type interaction ($F_{6,312}=12.67$, $P<0.001$), but no time*treatment*tissue interaction. Control trees also had more new buds on both terminal and side growth ($F_{1,52}=12.34$, $P=0.0009$; Figures 1C,D, respectively). Additionally, terminal branches put on more secondary buds than side branches ($F_{1,52}=23.78$, $P<0.001$). The number of buds increased over time ($F_{3,156}=2.52$, $P<0.001$) and there were no time*treatment, time*treatment*tissue interactions. There was a time*tissue interaction ($F_{3,156}=5.22$, $P=0.003$) suggesting that terminal growth put on more secondary buds later in the growing season.

*Water Potential:* Water potential was significantly lower in HWA-infested branches ($F_{1,20}=11.36$, $P=0.003$; Figure 2). Water potential changed over time ($F_{1,20}=7.93$, $P=0.011$), but there was no time*treatment interaction ($F_{1,20}=0.02$, $P>0.05$).
On both dates, there was a significant negative correlation between HWA density and water potential (September: $F_{1,35}=7.38$, $P=0.010$; October: $F_{1,21}=9.55$, $P=0.006$).

*Gas Exchange:* Net photosynthetic rates and stomatal conductance were lower in HWA-infested trees ($F_{1,22}=7.70$ and $14.75$, $P=0.011$ and $<0.001$ respectively; Figures 3A,B) compared to control trees. Photosynthesis and stomatal conductance were greater in October ($F_{1,22}=37.89$ and $111.04$, respectively, both $P<0.001$). There was a significant time*treatment interaction for both gas exchange variables ($F_{1,22}=9.43$ and $11.35$, $P=0.006$ and $0.003$, respectively). There was no relationship between HWA density and photosynthesis for either September ($F_{1,21}=1.06$, $P=0.314$) or October ($F_{1,22}=4.02$, $P=0.057$). There was a negative correlation between HWA density and stomatal conductance for both September ($F_{1,21}=6.38$, $P=0.020$) and October ($F_{1,22}=7.18$, $P=0.013$).

**DISCUSSION**

Diapause, a period of arrested growth and metabolic depression (Hahn and Denlinger 2011), allows insects to survive in otherwise-unsuitable environments (Andrewartha 1952). Insect diapause can occur at any stage in insect development and in locations that include soil, leaf litter, and on a host plant. Although insects that diapause on their host plant should have little or no effect on plant performance when in dormancy, we are unaware of any literature exploring the impact of ‘inactive’ insect presence on plant health. Our study investigates effects of an insect’s active period (feeding) and inactive period (diapause) on plant performance. Our results showed that active feeding by HWA, an invasive sap-feeding herbivore, had a predictably
detrimental impact on hemlock growth and physiology. The fact that HWA decreased water potential, photosynthesis and stomatal conductance further suggests that it induces symptoms of water stress in eastern hemlock. These symptoms are magnified when HWA is actively feeding versus in diapause (inactive period).

HWA had significant impacts on hemlock growth. The spring progrediens generation settles and begins to feed during the start of the hemlocks’ growing season. By the end of the growing season, terminal branches on control trees were 41% longer and had 56% more new buds than HWA-infested trees. The effect of HWA was even more pronounced on side branches. Side branches on uninfested trees grew 56% more and had 120% more new buds than HWA-infested trees. These results suggest that HWA-infested trees have significantly less lateral branching than uninfested trees. This finding matters because lateral branching can be an effective herbivore deterrent: the increasingly complex structure and lateral spread of branches can make it difficult for herbivores to navigate (Vesey-FitzGerald 1973, Archibald and Bond 2003). Increased tree architecture can also promote tolerance to herbivory by increasing sectored subunits within a plant and augmenting resource capture (reviewed in Stowe et al. 2000). In addition to HWA, white-tail deer also feed on eastern hemlock (Eschtruth and Battles 2008). If HWA infested hemlocks have less complex branching structure, the already infested trees may be more susceptible to ungulate herbivory due to easier access. Our study is the first to compare growth of terminal and side branches and these data confirm findings by McClure (McClure 1991) and Miller-Pierce et al. (2010) who both found that trees infested with HWA accrue less new growth.
Some sap-feeding insects impose water stress on plants (Wise and Abrahamson 2005) by consuming large amounts of water from the plant during the removal of photosynthates. For example, feeding by the spittlebug *Philaenus spumarius* extracts water as well as nitrogen compounds from its host plant; this may explain why leaf relative water content decreases (Meyer 1993). Because severe water stress can cause tree mortality (Adams et al. 2009), chronic herbivory-induced water stress may be similarly detrimental to plant health. The fact that HWA has a density-dependent impact on water potential provides additional support for previous research showing that water stress may play a role in HWA’s impact on hemlock health. Specifically, our results are consistent with previous research showing that HWA-infested trees had increased levels of proline, altered glutamine:glutamate ratios, an increase in overall free amino acids; Gómez et al. (2012b), all indicators of water stress.

HWA-induced changes in hemlock anatomy and physiology likely accentuate the impact of HWA-induced water stress. Changes in water status alter gas exchange rates. For instance low water potential in plants is coupled with decreases in photosynthetic activity and stomatal conductance (Farquhar and Sharkey 1982, Epron and Dreyer 1993, Dang et al. 1997) and water stress in conifers such as *Abies* spp. greatly reduces net photosynthesis (Puritch 1973). Congruently, increased photosynthetic rates in galled leaves is suggested to be explained by improved water relations (Fay et al. 1993). We showed a reduction in water potential and concomitant reductions in photosynthesis and stomatal conductance in HWA-infested trees. Sap-feeding insects generally tend to decrease photosynthesis (reviewed in Zvereva et al.
2010), and we found a similar result, HWA-infested trees had lower photosynthetic rates; a difference that was magnified when HWA were actively feeding.

The HWA-induced changes in hemlock physiology we observed may result from the formation of a higher number of false rings on HWA-infested branches (Gonda-King et al. 2012). False rings are bands of abnormal wood within an annual ring that consist of thick-walled xylem cells and which may hinder water transport efficiency (Mitchell 1967). While our data showed that actively-feeding HWA had a greater impact on hemlock physiology and growth, HWA’s impact on physiology was visible even during diapause, as shown by a 10% and 41% reduction in photosynthetic rates and stomatal conductance, respectively, in HWA-infested trees. Once HWA emerged from diapause and began feeding, photosynthesis and stomatal conductance were 56% and 70% lower in infested trees. HWA’s impact on photosynthesis and stomatal conductance during diapause might be explained by lasting prior changes in nutrient allocation from when HWA was actively feeding. Sap-feeding insects often act as resource sinks (Inbar et al. 1995, Kaplan et al. 2011) that can compete with natural plant sinks (i.e. actively growing tissues). HWA feeding alters local and systemic foliar nitrogen content (Stadler et al. 2005, Miller-Pierce 2010, Gómez et al. 2012b). Nitrogen sink competition between feeding herbivores and new foliage alters leaf nutrient status, resulting in decreased photosynthesis (Larson 1998). Since HWA feed on photosynthate from xylem ray parenchyma cells (Young et al. 1995) that transfer and store nutrients it is likely HWA induce greater sinks when feeding as opposed to in diapause. This is consistent with the idea that competition between plant and herbivore sinks reduces net photosynthesis. The act of HWA feeding does not
magnify reductions in water potential. If changes in water potential are driven by false rings formation, this would have a permanent impact on water relations as changes to wood anatomy are not transient.

Our results illustrate that HWA presence, whether in diapause or actively feeding, negatively impacts hemlock health. The during-diapause impact of HWA may be caused by physical injury or chemical cues associated with the initial stylet insertion by HWA. Stylet insertion by other sucking insects can cause long-lasting anatomical and physiological changes (Ladd and Rawlins 1965, Ecale and Backus 1995, Shackel et al. 2005), and this may be the case for HWA as well. HWA secretes a salivary sheath when feeding at the base of hemlock needles that Young et al. (1995) suggests may be ‘toxic’ and responsible for the disproportional negative impact on hemlock growth caused by HWA feeding. In support of the ‘toxic’ saliva hypothesis, Radville et al. (2011) found that HWA elicits a systemic hypersensitive response in hemlock.

Despite finding that HWA continues to impact hemlock performance during diapause, we cannot determine if this is due to the presence of inactive HWA on the plant or if these effects are residual long-term impacts from prior HWA infestations. Unfortunately, there is no true ‘control’ to compare impact of HWA diapause versus HWA feeding because the HWA life cycle always has a feeding period before diapause. A short-term feeding event always occurs prior to HWA entering diapause and short-term feeding events by herbivores can have lasting impacts on host plant health. For example, *Cinara pseudotsugae*, a sap sucking aphid, reduced root and shoot growth on its host tree and physiological impacts were still evident one year
after herbivory (Smith and Schowalter 2001). It is possible that prior HWA feeding events have had long-lasting impacts on hemlock physiology and this is reflected during the diapause period. Regardless of the mechanism driving decreased hemlock performance when HWA are in diapause, these data show that HWA has a lasting impact on hemlock performance.

While the detrimental impact of HWA on hemlock has long been recognized, the effect of HWA on hemlock physiology has received less attention. Even less recognition has been given to the impact of HWA during diapause. Our results suggest that HWA-infested trees are water stressed due to decreased hemlock growth, water potential, photosynthesis and stomatal conductance. Reductions in photosynthesis and stomatal conductance are expectedly magnified during periods of HWA feeding. These physiological changes in HWA-infested trees may shed light on possible mechanisms behind HWA-induced death. We suggest taking long-term measurements on HWA-infested trees, from infestation to mortality, to better clarify the mechanism of HWA-induced death. Our study is the first to address the impact of HWA in diapause versus actively feeding and our results suggest that HWA has a lasting physiological impact on hemlock regardless of feeding activity.

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FIGURES

Figure 1: Plant growth (top panels) and number of new buds (bottom panels) in hemlocks infested with HWA or uninfested in terminal (left panels) and side (right panels) branches
Figure 2: Water potential in HWA-infested and control trees when HWA are in diapause (September) and when HWA are actively feeding (October)
Figure 3: Impact of HWA on photosynthesis (Panel A) and stomatal conductance (Panel B) when in diapause and actively feeding.
Figure 4: Correlation between HWA and water potential. HWA density is negatively correlated with water potential in September ($R^2=0.123$), when in diapause, and in October ($R^2=0.313$) when actively feeding.
Figure 5: Correlation between HWA density and gas exchange parameters for September and October. Panel A shows no correlation between HWA density and photosynthesis. Panel B shows a negative correlation between HWA density and stomatal conductance for both September ($R^2=0.233$) and October ($R^2=0.238$).
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