

# Variation in Plant Defense against Invasive Herbivores: Evidence for a Hypersensitive Response in Eastern Hemlocks (*Tsuga canadensis*)

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**Abstract** Herbivores can trigger a wide array of morphological and chemical changes in their host plants. Feeding by some insects induces a defensive hypersensitive response, a defense mechanism consisting of elevated  $H_2O_2$  levels and tissue death at the site of herbivore feeding. The invasive hemlock woolly adelgid *Adelges tsugae* ('HWA') and elongate hemlock scale *Fiorinia externa* ('EHS') feed on eastern hemlocks; although both are sessile sap feeders, HWA causes more damage than EHS. The rapid rate of tree death following HWA infestation has led to the suggestion that feeding induces a hypersensitive response in hemlock trees. We assessed the potential for an herbivore-induced hypersensitive response in eastern hemlocks by measuring  $H_2O_2$  levels in foliage from HWA-infested, EHS-infested, and uninfested trees. Needles with settled HWA or EHS had higher  $H_2O_2$  levels than control needles, suggesting a localized hypersensitive plant response. Needles with no direct contact to settled HWA also had high  $H_2O_2$  levels, suggesting that HWA infestation may induce a systemic defense response in eastern hemlocks. There was no similar systemic defensive response in the EHS treatment. Our results showed that two herbivores in the same feeding guild had dramatically different outcomes on the health of their shared host.

**Key Words** *Adelges tsugae* · *Fiorinia externa* · *Tsuga canadensis* · Hypersensitive response

## Introduction

The rapid expansion of global trade and transport links has been accompanied by a concomitant increase in the number of invasive species and the threat they pose to native ecosystems (Mooney and Cleland, 2001). As the number of biological invasions increases, the potential for interactions among invasive species will become more likely. Since invasive species tend to have high growth rates and population densities (Sakai et al., 2001), the likelihood and importance of these interactions is great (Denno et al., 1995). Such interactions are especially likely among specialist sessile herbivores that cannot emigrate in response to changes in host quality induced by competing insect species (Karban, 1989).

Herbivores can have differing effects on their host plants if one species, but not another, induces changes in plant physiology. Infestation by one herbivore may lead to the systemic induction of plant defenses and reduce the plant's susceptibility to subsequent invaders (Karban, 1990; Alborn et al., 1996; Bezemer et al., 2003). Some herbivores also are able to directly manipulate host plant metabolism and nitrogen allocation to their benefit, changes that can lead to the induction of systemic effects (Karban and Agrawal, 2002). Herbivores may be particularly harmful to their host if they are capable of inducing a pronounced hypersensitive response (HR). The HR is a plant defense response that increases reactive oxygen species, including hydrogen peroxide ( $H_2O_2$ ), superoxide anions, and hydroxyl radical levels, and induces cell death in herbivore-damaged areas that isolates and starves invading organisms (Heath,

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2000; Liu et al., 2010). Such localized tissue death usually is a response to sessile herbivores (Karban and Baldwin, 1997) and reduces the negative effects of insects such as balsam woolly adelgids, bark beetles, woodwasps, and gall midges (Fernandes, 1990; Ollerstam and Larsson, 2003). The hypersensitive response also defends plants against gall formation by isolating the galling insects (Fernandes and Negreiros, 2001).

The introduced hemlock woolly adelgid (*Adelges tsugae*; HWA) is causing mortality of eastern hemlocks along the east coast of North America (Orwig et al., 2002). A second invasive herbivore, the elongate hemlock scale (*Fiorinia externa*; EHS), also feeds on eastern hemlocks in North America (McClure, 1977). Although both species are sap-sucking insects that are considered pests, they have different impacts on their shared host (Preisser and Elkinton, 2008). In a multi-year experiment, Miller-Pierce et al. (2010) found that infestation with HWA, but not EHS, significantly decreased tree growth. While mature trees infested with HWA can die in as few as 4 years, mortality rarely occurs as a result of EHS infestation (McClure, 1980, 1991). The disproportionate hemlock mortality observed in response to HWA feeding has led to the hypothesis that HWA might have a systemic effect on the plant (Young et al., 1995), resulting in widespread tissue necrosis and tree mortality. We tested this hypothesis by measuring foliar  $H_2O_2$ , indicative of a hypersensitive response, in eastern hemlocks that were infested with HWA, EHS, or neither insect. Since plants often respond to sessile herbivores by mounting localized defenses (Fernandes, 1990), we expected both insects to induce a localized response at the site of infestation. We hypothesized that HWA settlement would induce a stronger HR in hemlock than EHS settlement, and that the HWA-induced HR would be detectable even in undamaged tissue (a ‘systemic’ response). Since a localized HR is marked by  $H_2O_2$  exclusively surrounding the site of feeding,  $H_2O_2$  accumulation in tissues not being directly fed upon would suggest the presence of a magnified HR in HWA-infested trees.

## Methods and Materials

Our research took advantage of a pre-existing experimental manipulation intended to assess the long-term responses of hemlock trees to infestation with HWA or EHS (Miller-Pierce et al., 2010). While both projects sampled the same trees, our research question (assessment of an insect-induced hypersensitive response in hemlocks) differed substantially from those addressed in the initial research project (long-term changes in plant growth and C:N ratio in response to HWA and EHS infestation; Miller-Pierce et al., 2010).

**Plant Material** Hemlock saplings (1–1.5 m in height) were collected in April 2007 from the understory of a hemlock forest in Pelham, Massachusetts, USA. This area was outside the range of both HWA and EHS when the saplings were collected, and the saplings and surrounding trees were inspected prior to removal to ensure the absence of both insects. Saplings were transplanted into a field surrounded by a deer-proof fence at the University of Rhode Island’s East Farm (Kingston, RI, USA). They were planted in a grid pattern and randomly assigned to each of 3 experimental treatments: HWA only, EHS only, or control (neither insect). Details of the insect inoculations are contained in Miller-Pierce et al. (2010); briefly, we used standard protocols (Butin et al., 2007) to inoculate saplings in the HWA and EHS treatments with the appropriate insect in May (for HWA) or June (for EHS) of every year from 2007 through 2010. From 2007 to 2008, trees in the HWA and EHS treatments had similar densities (2007–2008 mean: 0.97 and 0.93 insects/cm for HWA and EHS, respectively; Miller-Pierce et al., 2010), but in 2009 trees in the EHS treatment had higher densities (0.3 and 2.3 insects/cm for HWA and EHS, respectively; Miller-Pierce et al., 2010). To prevent contamination between treatments, each tree was caged during the time of year in which crawlers could be dispersed by wind.

In June 2010, we sampled foliage from 7 HWA-infested trees, 6 EHS-infested trees, and 8 uninfested control trees (21 total trees). We harvested four branches from each tree; each branch consisted of a single leader of new growth emerging from a  $\geq 15$  cm section of old growth. Each branch was bagged and returned to the lab where we recorded the number of HWA or EHS and the number of needles on the terminal 4 cm of new growth. All sampled branches in the insect treatments had settled HWA or EHS on the old growth. The spring generation of HWA had emerged, but had not yet settled on old growth. Because the new growth on each branch was produced after the first generation of HWA had emerged and begun to settle on old growth and prior to the emergence of EHS, there were no settled insects on any of the new growth; a careful examination of the new growth directly adjacent to the old growth on each branch confirmed that no insects had settled in this area.

**Detection of  $H_2O_2$  in Needles** Detection of  $H_2O_2$  involved a combination of chemical treatment and image analysis looking for a stain that is present if  $H_2O_2$  is present in the needle. We removed the first two pairs of needles both above and below the base of new growth on each branch, giving 4 old-growth and 4 new-growth needles per branch. A DAB (1 mg ml<sup>-1</sup> 3,3'-diaminobenzidine) solution was prepared following a protocol modified after Thordal-Christensen et al. (1997). The DAB was dissolved in DMSO (0.002% dimethyl sulfoxide) and then slowly added to water while stirring with a stir bar. Each needle was

placed in a separate 1.5 ml vial containing 1 ml of DAB-DMSO solution. We placed each vial in a bell jar and vacuum infiltrated each needle twice using Tween 20 as the surfactant (Katagiri et al., 2002). The vacuum was run to 26 in Hg and then left to run for 1 min. Following the second infiltration, the vials containing the needles were removed from the jar and allowed to sit in the DAB solution for 2 hr. To decolorize the needles and reveal the reddish-brown DAB polymer if  $H_2O_2$  were present, we placed a cap with a small puncture on each vial and placed them in boiling ethanol (95%) for 10 min once a day for 3 d. Needles were stored in 95% ethanol.

**Image Analysis** We photographed treated needles using a Pixelink PL-A662 camera attached to an Olympus SZX12 microscope. Each digital image (1280×1020 pixels) was analyzed using Photoshop CS3 (Adobe; San Jose, CA, USA). In each needle, the number of stained pixels (indicative of  $H_2O_2$  presence) was recorded and expressed as a percentage of the total needle area.

**Statistical Analysis** Response variables were needle area (in  $mm^2$ ), the proportion of needle area that stained positive for the presence of  $H_2O_2$ , and needle density in the terminal 4 cm of new growth foliage. For both needle area and proportion of needle area stained, responses for the between-treatment analyses were calculated using the mean response of old- and new-growth foliage per tree for each treatment (21 replicates per foliage type, 42 total replicates). Because needle density was measured only on new growth foliage, there were 21 replicates in total for this variable. Data were square-root transformed when necessary to meet the assumptions of normality; variances were homogenous between treatments. We used ANOVA to test for the main effects of treatment, foliage age (old vs. new growth), and their interaction. Where appropriate, we performed means separation tests using Tukey's HSD.

Responses for the within-treatment analyses were calculated using the mean response of old- and new-growth foliage per branch per tree for each treatment (4 branches per tree, 28 replicates per foliage type in the HWA treatment, 24 replicates per foliage type in the EHS treatment). We used MANOVA to test for the main effects of insect density, foliage age, and tree, as well as an insect density\*foliage age interaction. Statistical analyses were performed using JMP 6.0.2 (SAS Institute 2007).

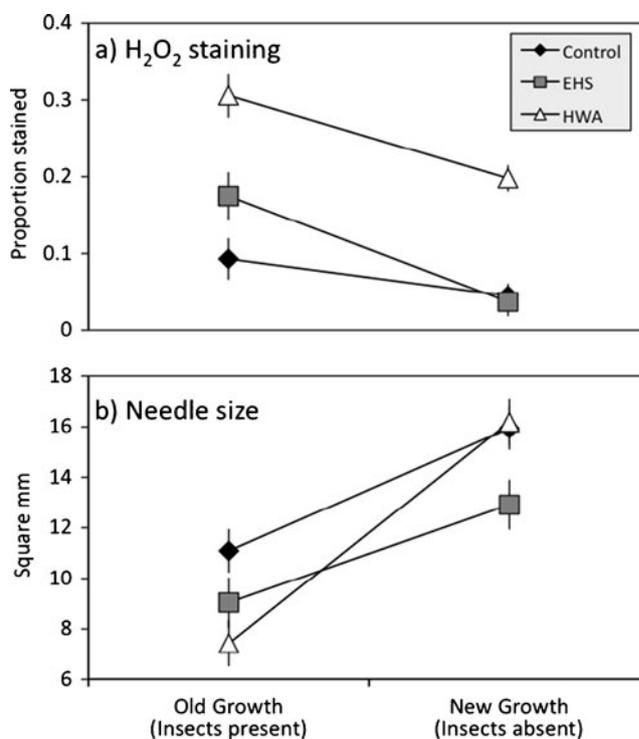
## Results

**Between-Treatment Analyses** Needles from HWA-infested trees had higher  $H_2O_2$  levels than EHS and control

treatments (Fig. 1a;  $F_{2,36}=37.9$ ,  $P<0.001$ ). In the HWA treatment,  $25.2\pm 2.30\%$  (SE) of the needle area contained  $H_2O_2$ . Mean  $H_2O_2$  levels in the EHS and control treatments were substantially lower ( $10.6\pm 2.50\%$  and  $6.9\pm 2.15\%$  of total needle area, respectively).

Across all treatments, old-growth (infested) needles had higher  $H_2O_2$  levels than new-growth needles ( $18.5\pm 2.44\%$  and  $9.4\pm 1.91\%$ , respectively;  $F_{1,36}=30.9$ ,  $P<0.001$ ). There was also a significant treatment\*foliage age interaction ( $F_{2,36}=3.34$ ,  $P=0.047$ ). On old-growth foliage, both HWA and EHS showed an increase in  $H_2O_2$  levels relative to the control (Tukey's HSD at  $P=0.05$ ; Fig. 1a). On new-growth foliage, however, only trees in the HWA treatment showed increased  $H_2O_2$  levels (Tukey's HSD at  $P=0.05$ ; Fig. 1a).

Although there were no between-treatment differences in needle density ( $F_{2,18}=0.33$ ,  $P=0.72$ ), overall needle size differed between treatments (Fig. 1b;  $F_{2,36}=4.03$ ,  $P<0.026$ ). Needles in the control treatment were significantly larger than needles in the EHS treatment ( $13.52\pm 0.61 mm^2$  and  $10.98\pm 0.70 mm^2$ , respectively; Tukey's HSD at  $P=0.05$ ). Needles in the HWA treatment averaged  $11.81\pm 0.65 mm^2$  and did not differ from the control (Tukey's HSD at  $P=0.05$ ).



**Fig. 1** Response of old-growth needles (herbivores present in hemlock woolly adelgid, 'HWA', and elongate hemlock scale, 'EHS', treatments) and new-growth needles (herbivores absent in all treatments) to infestation with HWA, EHS, or neither herbivore. Top panel (A): Proportion of needle area stained with DAB polymer, an indicator of  $H_2O_2$ . Bottom panel (B): Needle area ( $mm^2$ ). Control treatment: Black diamonds, HWA present: White triangles, EHS present: Gray squares

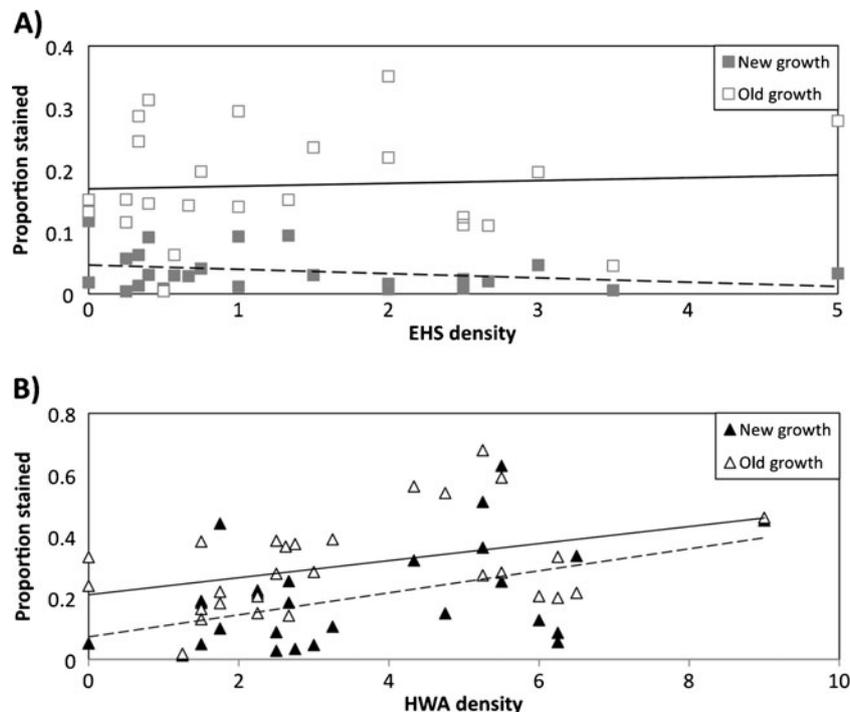
Age had an effect on needle size ( $F_{1,36}=59.4$ ,  $P<0.001$ ). Across all treatments, old-growth needles were significantly smaller than new-growth needles ( $9.28\pm 0.53$  mm<sup>2</sup> and  $15.16\pm 0.53$  mm<sup>2</sup>, respectively). There was also a significant treatment\*foliage age interaction ( $F_{2,36}=3.82$ ,  $P=0.031$ ; Fig. 1b). The proportional increase in needle size from old- to new-growth was similar in the control and EHS treatments, but not in the HWA treatment. Although old-growth needles in the HWA treatment were 33% smaller than old-growth control needles, new-growth needles in the HWA treatment were 1.5% larger than control new-growth needles (Fig. 1b).

**Within-Treatment Analyses** There was a positive correlation between HWA density and H<sub>2</sub>O<sub>2</sub> levels ( $F_{1,46}=16.8$ ,  $P<0.001$ ; Fig. 2a). In contrast, there was no correlation between EHS density and H<sub>2</sub>O<sub>2</sub> levels ( $F_{1,39}=0.56$ ,  $P=0.46$ ; Fig. 2b).

Although there was no correlation between HWA density and needle size ( $F_{1,46}=0.11$ ,  $P=0.74$ ), there was a positive correlation between EHS density and needle size ( $F_{1,39}=9.52$ ,  $P=0.004$ ). There was no relationship between insect density and needle density in either the HWA or EHS treatments (HWA:  $F_{1,25}=1.06$ ,  $P=0.31$ ; EHS:  $F_{1,21}=1.22$ ,  $P=0.28$ ).

## Discussion

Our research showed that invasive herbivores with similar feeding modes can have very different effects on their shared host plant. Infestation by both hemlock woolly adelgid (HWA) and elongate hemlock scale (EHS) increased H<sub>2</sub>O<sub>2</sub> levels relative to control hemlock needles, suggesting the presence of a localized hypersensitive response ('HR'; Fig. 1a). Although both insects induced a localized HR, needles with settled HWA at the base responded more strongly to insect settlement. After a year of HWA feeding, these old growth needles contained more H<sub>2</sub>O<sub>2</sub> and were smaller than needles in the EHS or control treatments (Fig. 1b). The presence of HWA, but not EHS, also induced H<sub>2</sub>O<sub>2</sub> accumulation in new growth needles that had no direct contact to feeding insects. Furthermore, HWA density on old growth needles was positively correlated with H<sub>2</sub>O<sub>2</sub> accumulation in new growth needles (Fig. 2a), while EHS feeding was not (Fig. 2b). Since the HR is associated with cell death, the induction of a systemic hypersensitive response by HWA may partially explain why HWA causes more damage than EHS to eastern hemlocks.



**Fig. 2** Within-treatment correlation between settled insect density (either hemlock woolly adelgid, 'HWA', or elongate hemlock scale, 'EHS') on old-growth foliage and proportion of needle stained with DAB polymer. Top panel (A): Correlation between EHS densities on old-growth foliage and proportion of needle stained with DAB polymer. Old-growth needles: Open gray squares, New-growth needles: Closed gray squares. Old-growth correlation: Solid line

( $F_{1,22}=0.097$ ,  $P=0.76$ ), New-growth correlation: Dotted line ( $F_{1,22}=1.8$ ,  $P=0.19$ ). Bottom panel (B): Correlation between HWA densities on old-growth foliage and proportion stained with DAB polymer. Old-growth needles: Open black triangles, New-growth needles: Closed black triangles. Old-growth correlation: Solid line ( $F_{1,26}=4.83$ ,  $P=0.037$ ), New-growth correlation: Dotted line ( $F_{1,26}=7.91$ ,  $P=0.009$ )

There are several possible explanations for the systemic effect of HWA settlement. Since measurements were made in old and new growth on the same branch, one possibility is that the  $H_2O_2$  was produced in high quantities locally and spilled over into new growth foliage. The overproduction of defensive compounds could have been elicited by the mechanical damage caused by HWA stylet bundle insertion. This may be due to the slightly different feeding modes of the two insects. HWA settles on the petiole of the needle and feeds upon the xylem ray parenchyma cells, which function in nutrient storage (Young et al., 1995). In contrast, EHS settles on the underside of the needle and feeds upon mesophyll cells (McClure, 1980). Another possibility is that the defense compounds were elicited by some other factor related to HWA feeding (Young et al., 1995), as has been seen in several aphid species (Miles, 1999). Certain constituents in HWA saliva may be toxic at high levels of infestation, causing the hemlocks to initiate a defensive HR and produce large quantities of  $H_2O_2$  that are carried into new growth tissues. Continued feeding by subsequent generations of HWA is common, as HWA has two generations while EHS has only one, and persistently high levels of  $H_2O_2$  may lead to the suppression of bud formation after several years of HWA feeding (McClure, 1991).

The HWA-induced  $H_2O_2$  also could have been produced in new growth needles *de novo* as a result of a magnified HR that extended even to tissues without settled herbivores. In order to be effective, a systemic response should provide systemic induced resistance to further herbivore attack. For example, Moore et al. (2003) found that increases in cell-wall peroxidase in *Rumex obtusifolius* reduce the amount of future herbivore damage by strengthening cell walls. A systemic hypersensitive response in response to pathogen infection and wounding has been found in several other plants (Alvarez et al., 1998; Orozco-Cardenas and Ryan, 1999), and Alvarez et al. (1998) suggested that the  $H_2O_2$  was generated systemically and may function in induced immunity. Hemlocks mounting a successful systemic response could provide new growth needles with more protection from sessile herbivores. The widespread mortality of eastern hemlocks suggests, however, that this response does not provide adequate protection from HWA. Bonello et al. (2006) proposed that if an invader can persist despite local defenses, the plant will become increasingly stressed by the cost of defense and will become susceptible to future attack. Since high HWA densities induce a magnified HR in infested tissues, a defensive response that would otherwise be beneficial could become costly and lethal to the host plant (Bi and Felton, 1995).

Data on needle size also demonstrate that these herbivores also have different effects on plant morphology. Although old growth HWA needles were the smallest, new-growth needles on HWA-infested branches were as large as

controls (Fig. 1b). EHS and control needle size increased at the same rate from old to new growth, while HWA needle size increased at a much greater rate. The increase in needle size could be a result of mobilization of plant nutrients away from stressed older needles and into new growth. A review by Trumble et al. (1993) outlines similar results in other plants, in which leaves remaining after herbivore defoliation become larger in length and weight. This hypothesis is supported by research exploring the amino acid profile of HWA- or EHS-infested vs. uninfested hemlocks that found higher amino acid levels in new-growth needles on HWA-infested trees relative to new-growth needles on EHS-infested trees (Gomez et al. unpublished data). Remobilization of nutrients away from herbivore feeding and the site of damage may be a strategy to tolerate insect infestation (Gómez et al., 2010). Because the summer generation of HWA feeds almost exclusively on new growth tissue, HWA may benefit from the production of these nutrient-rich new-growth needles. While additional research is necessary to determine the precise mechanism(s) underlying our findings, at a broader level the work illustrates that species-specific differences in herbivore feeding can substantially affect plant responses to biological invasions.

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