PLANT-MICROBE-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Two invasive herbivores on a shared host: patterns and consequences of phytohormone induction

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Abstract

Herbivore-induced changes in host quality mediate indirect interactions between herbivores. The nature of these indirect interactions can vary depending on the identity of herbivores involved, species-specific induction of defense-signaling pathways, and sequence of attack. However, our understanding of the role of these signaling pathways in the success of multiple exotic herbivores is less known. Eastern hemlock (*Tsuga canadensis*) is attacked by two invasive herbivores [elongate hemlock scale (EHS; *Fiorinia externa*) and hemlock woolly adelgid (HWA; *Adelges tsugae*)] throughout much of its range, but prior attack by EHS is known to deter HWA. The potential role of phytohormones in this interaction is poorly understood. We measured endogenous levels of phytohormones in eastern hemlock in response to attack by these invasive herbivores. We also used exogenous application of methyl jasmonate (MJ) and acibenzolar-S-methyl (ASM), a salicylic acid (SA) pathway elicitor, to test the hypothesis that defense-signaling phytohormones typically induced by herbivores could deter HWA. Resistance to adelgid attack was assessed using a behavioral assay. Adelgid feeding significantly elevated both abscisic acid (ABA) and SA in local tissues, while EHS feeding had no detectable effect on either phytohormone. HWA progrediens and sistens crawlers preferred to settle on ASM-treated foliage. In contrast, HWA crawlers actively avoided settlement on MJ-treated foliage. We suggest that induction of ABA- and SA-signaling pathways, in concert with defense-signaling interference, may aid HWA invasion success, and that defense-signaling interference, induced by exotic competitors, may mediate resistance of native hosts.

Keywords Abscisic acid · *Adelges tsugae* · *Fiorinia externa* · Salicylic acid · *Tsuga canadensis*

Introduction

Plants have evolved varied constitutive and inducible defenses to defend themselves against herbivores (Karban and Baldwin 2007; Wu and Baldwin 2010). Initial herbivore-induced changes in a host's chemical phenotype can facilitate or inhibit attack by subsequent herbivores, including those operating on spatial and temporal scales different from those of the initial attacker (Underwood 1998; Zvereva et al. 2010; Erb et al. 2011). Herbivore-induced defenses are mediated by plant defense-signaling pathways (Thaler et al. 2012). In addition to attack by many native herbivores, plants today are often attacked by one or more invasive herbivores (Preisser and Elkinton 2008; Gandhi and Herms 2010), which lack a shared co-evolutionary history with their native host. The success of invasive herbivores is likely to be determined by phytohormonal responses within a native host, which are likely to err in their defensive investment, as they are not attuned to recognize species-specific cues associated with feeding by invasives. However, we know very little about (1) how invasive herbivores alter the phytohormone expression of native hosts or (2) how these hormones might mediate both invasive herbivore performance and competition with other herbivores.

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The success of an induced defense response hinges on a plant's ability to respond both accurately and in rapid fashion to its attacker. Thus, surveillance systems are required that can recognize biotic attackers, while minimizing costs inherent to a mounted defense response (Kaloshian and Walling 2005; Howe and Jander 2008). Beyond preformed physical and chemical defenses, such as storage of toxic compounds in vacuoles or trichomes that are released upon tissue damage, these systems often rely upon the identification of exogenous compounds present in herbivore oral secretions. Such compounds range from fatty acid–amino acid conjugates, as observed in lepidopteran saliva (Alborn et al. 1997; Halitschke et al. 2001, 2003), to enzymes and effector molecules often injected by Hemiptera through their feeding stylets into plant tissues (Kaloshian and Walling 2005; Bos et al. 2010; Hogenhout and Bos 2011). Even among Hemipteran herbivores, there is evidence of differential host defense induction, influenced by both the identity of tissues attacked, as well as the structure of herbivore mouthparts and associated damage inflicted as they seek phloem resources or those stored in cells (Walling 2000). Natural systems commonly contain multiple herbivore attackers, whose unique effectors induce several different defensive signaling pathways. Subsequent defenses can be either strengthened or suppressed depending on the identities of co-infesting herbivores and their sequence of arrival. Despite widespread recognition that herbivore induction of different signaling pathways commonly results in negative cross talk (von Dahl and Baldwin 2007; Hillwig et al. 2016), which may influence the success of a subsequent attacker, the potential importance of such processes in interactions between exotic herbivores remains largely unexplored. In the eastern USA, attack on eastern hemlock (*Tsuga canadensis*) by two invasive piercing/sucking Hemiptera, the highly destructive hemlock woolly adelgid (HWA; *Adelges tsugae*) and the less destructive elongate hemlock scale (EHS; *Fiorinia externa*), represents an excellent system for examining the patterns and consequences of differential phytohormone induction by multiple invasive herbivores.

Eastern hemlock is a long-lived foundational tree species, but attack by HWA can rapidly kill mature trees (McClure 1991) and result in widespread mortality. This herbivore-induced mortality can have profound consequences for hemlock-associated fauna and ecosystem-level processes (Jenkins et al. 1999; Ellison et al. 2005; Sackett et al. 2011; Raymer et al. 2013). In contrast, EHS attack has minimal impact on plant growth (Miller-Pierce and Preisser 2012; Soltis et al. 2015) and appears to kill only previously stressed trees (McClure 1980). Moreover, EHS presence on co-infested trees can significantly reduce both HWA performance and its impact on hemlock (Preisser and Elkinton 2008; Miller-Pierce and Preisser 2012) and inhibit settlers

(Gómez et al. 2014), and chemical changes may explain these contrasting effects.

Infestation and feeding by HWA and EHS induce disparate chemical and physiological changes in hemlock. Adelgid feeding alters both primary and secondary metabolism through reduced growth and photosynthetic capacity (Gonda-King et al. 2014; Nelson et al. 2014), mobilization of free amino acids in foliar tissue (Gómez et al. 2012), and changes to the volatile profile of hemlock resin (Pezet et al. 2013). In contrast, EHS has minimal or no effect on new growth production (Soltis et al. 2015) or foliar amino acid concentrations (Gómez et al. 2012). In fact, Gómez et al. (2012) showed that EHS presence appears to inhibit the HWA-induced increases in amino acids. In addition, the array of HWA-induced changes in secondary metabolism contrasts sharply with those produced by EHS feeding, which induces a smaller and localized hypersensitive response and emission of green leaf volatiles (Radville et al. 2011; Pezet et al. 2013). Three lines of evidence suggest that differential phytohormone induction may mediate the interactions between the plant and these two herbivores. First, HWA infestation increases the emission of both benzyl alcohol and methyl salicylate (MeSA), a volatile methyl ester of SA which functions in plant defense against pathogens (Shulaev et al. 1997; Park et al. 2007). Second, settlement and feeding induce a localized and systemic hypersensitive response that is typical of SA-induced systemic acquired resistance (Radville et al. 2011). Given the capacity of HWA to proliferate on HWA-infested trees, these results suggest that activation of the SA pathway may enhance plant susceptibility to this insect. Third, EHS causes a greater increase in green leaf volatile (GLVs) emissions following attack than HWA (Pezet et al. 2013); GLVs play an important role in defense priming and can coordinate with the JA pathway to promote disease and herbivore resistance (Christensen et al. 2013; Naeem ul Hassan et al. 2015). Thus, elicitation of GLV emissions by EHS may prime hemlock JA-based defenses and explain the negative effects of EHS on HWA.

We measured endogenous phytohormones and employed exogenous application of plant defense elicitors to examine induced defenses in hemlock and to test for pathway-specific resistance against HWA. We hypothesized that HWA activates the SA-signaling pathway, while EHS activates the JA-signaling pathway. In addition, we hypothesized that SA signaling will facilitate settlement, that JA signaling will inhibit HWA settlement by crawlers, and that application of both signaling molecules will result in no change in settlement via negative cross talk. We quantified endogenous phytohormone levels in response to attack by both herbivores, and used methyl jasmonate (MJ) and acibenzolar-S-methyl (ASM), an SA-pathway elicitor, to examine JA- and SA-based resistance against HWA. Resistance was assessed through examination of

HWA early instar, mobile 'crawler' settlement behavior on induced hemlock foliage. Moreover, we quantified changes in total phenolics in response to these phytohormones.

Materials and methods

Study system

Eastern hemlock (*Tsuga canadensis*) is a late-successional, shade-tolerant conifer that plays an important role in shaping both the function and biodiversity of forest communities throughout the eastern USA (Ellison et al. 2005). Unlike co-occurring hardwoods, photosynthesis rates of hemlock peak in early spring and late fall, taking advantage of light in the open canopy (Hadley and Schedlbauer 2002).

Hemlock woolly adelgid is an aphid-like invasive introduced to the eastern USA from Japan around 1950 (Souto et al. 1996). Adelgid feeds by inserting stylets into the base of needles into xylem ray parenchyma cells, from which they acquire stored nutrients (Young et al. 1995). Lacking natural predators, it has spread rapidly throughout the eastern USA and can cause rapid hemlock mortality (McClure 1991). Its lifecycle consists of two parthenogenetic stages on hemlock per year: a quick developing spring generation and an overwintering generation with a longer development period (Ward et al. 2004). The April–June progrediens generation emerges in early spring and settles on hemlock foliage produced in the previous growing season. Females in this generation can produce ~ 75 eggs per individual. Their offspring, the July–April sistens generation, settle on newly produced foliage and immediately enter estivation until late autumn. Following the end of estivation, settled individuals feed throughout the winter and are capable of producing anywhere from 70 to 200 eggs per female (Paradis 2011).

Elongate hemlock scale arrived in the early 1900s from Japan and is commonly found throughout the eastern USA, often occurring alongside HWA (Sasscer 1912; Preisser et al. 2008) and replacing it in some regions (Gómez et al. 2014). A sessile herbivore, EHS settles on the underside of needles and feeds by piercing mesophyll cells and extracting the cell contents (McClure 2002).

Plant material

For all of the following experiments, we obtained 2-year-old *T. canadensis* saplings, ~ 0.3 m in height, from Van Pines Nursery (West Olive, MI, USA). These saplings had not been previously exposed to HWA and were grown without the use of insecticides.

Effects of herbivore attack on phytohormone induction

We measured phytohormone responses in potted saplings growing in a garden plot at Tufts University (Medford, MA USA) as well as in saplings planted into the ground within an understory common garden at the Kingston Wildlife Research Station (Kingston, RI, USA). Potted saplings were randomly assigned to either a control or HWA treatment ($N = 15$ for each), with HWA trees infested using a standard protocol (Butin et al. 2007). Common garden saplings were planted in March 2014 as part of a long-term common garden study. Briefly, 320 2-year-old ~ 0.3 m saplings were planted among five 8×8 blocks ($N = 64$ trees per block) using a randomized complete block design. Saplings were then individually enclosed in mesh cages and inoculated with HWA and EHS individually, both herbivores simultaneously or in alternating sequence, or with neither insect (control). Inoculations were applied annually, with HWA inoculations occurring before EHS inoculations as per natural conditions, following standard protocols (Butin et al. 2007). Control trees in both experiments received a sham treatment consisting of uninfested hemlock foliage. For both experiments, three 15 cm branches were harvested per tree, immediately frozen in liquid nitrogen (N), and then transferred to the laboratory on dry ice for storage in a – 80 °C freezer until future analyses. Foliage for the potted experiment was collected 48 h post-inoculation with HWA, while foliage for the field-transplanted trees was collected ~ 6–8 months after the second round of treatment with HWA and EHS, respectively. With these two approaches, we could also assess potential differences in phytohormonal responses to short- and long-term feeding by HWA.

To quantify the endogenous concentrations of JA, SA, and abscisic acid (ABA), samples were sent to the Chemical Ecology Core Facility at Cornell University for analysis using a slightly modified protocol (Rasmann et al. 2012). Briefly, phytohormones were extracted from ~ 900 mg (FW: fresh weight) of needle material. Needle material for each sample was first coarsely ground in liquid N using a mortar and pestle and then partitioned into ~ 300 mg subsamples, each placed in 2.0 mL microcentrifuge tubes containing 0.9 g of zirconia/silica beads (2.3 mm diameter; Bio Spec Products Inc., Bartlesville, OK, USA) before being flash frozen in liquid nitrogen. 1 mL of extraction buffer (2:1:0.005, isopropanol:water:HCl) was added to each sample. d5-JA, d4-SA, and d6-ABA (CDN Isotopes Inc., Pointe-Claire, Quebec, Canada) were added as internal standards, and samples were homogenized in a Mini-Beadbeater-16 homogenizer (Bio Spec Products Inc.) at 6 m s^{-1} for 90 s. Samples were dissolved in 200 µL of methanol after extraction with dichloromethane and solvent evaporation, and 15 µL was analyzed using a triple-quadrupole liquid chromatography–tandem

mass spectrometry system (Quantum Access; Thermo Scientific). Analytes were separated on a C18 reverse-phase HPLC column (3 µm, 150 × 2.00 mm; Gemini-NX; Phenomenex) using a gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 300 µL min⁻¹. The initial condition of 10% B was kept for 2 min and increased to 100% solvent B at 20 min. Phytohormones were analyzed by negative electrospray ionization (spray voltage, 3.5 kV; sheath gas, 15; auxiliary gas, 15; capillary temperature, 350 °C), collision-induced dissociation (argon gas pressure, 1.3 mTorr; CID energies 16 V for JA and SA, 13 V for ABA), and selected reaction monitoring of compound-specific parent/product ion transitions (SA, $137 \rightarrow 93$; d4-SA, $141 \rightarrow 97$; JA, $209 \rightarrow 59$; d5-JA, $214 \rightarrow 62$; ABA, $263 \rightarrow 153$; d_6-ABA, $269 \rightarrow 159$). We included the additional hormone ABA in our analyses for the following reason. Recent work on other Hemiptera has indicated a significant role for ABA induction in mediating feeding success (Studham and MacIntosh 2012; Hillwig et al. 2016). Though ABA is often induced in plants following water stress, ABA induction and resulting cross talk with defense-signaling pathways have been hypothesized to also play a role as part of an exploitative feeding strategy by some herbivores (Studham and MacIntosh 2012; Hillwig et al. 2016).

Phytohormone manipulation

Exogenous application of defensive elicitors serves as an effective tool for understanding the nature, costs, and benefits of herbivore-induced resistance in conifers (Moreira et al. 2009, 2012). Two sets of seedlings were potted in March 2014 (N = 30) and 2015 (N = 90), respectively. Following potting, seedlings were placed in a shaded (20% light transmission) garden plot on the campus of Tufts University (Medford, MA, USA) to recreate understory conditions. In 2014 and 2015, we manipulated plant defensive chemistry through exogenous application of the defensive elicitors MJ and ASM. Using a hand sprayer, the aboveground tissues (both needle and stem) of each sapling were sprayed until run-off (Moreira et al. 2009) with one of the following treatment solutions. In 2014 and 2015, trees were assigned to (control) a carrier solution comprising ethanol in deionized water (2.5% v/v), (MJ) 40 mM methyl jasmonate (95% purity; Sigma-Aldrich Corp., St. Louis, MO, USA) in carrier solution, or (ASM) 20 µM acibenzolar-S-methyl (analytical standard grade; Sigma-Aldrich) in carrier solution. In 2015, two additional treatments were used: MJ + ASM at half concentration (Both_Half) and MJ + ASM at full concentration (Both_Full). The Both_Full treatment was only tested with the sistens generation. The concentrations of both elicitors reflect those that previous studies had found to induce

a defensive, but not phytotoxic, response in other Pinaceae species (Moreira et al. 2009, 2014). Ten trees were used for each treatment. Trees in Assay 1 were sprayed on 3 July 2014 (N = 30), while separate batches of trees for Assays 2 and 3 were sprayed on 14 May (N = 40) and 10 July 2015 (N = 50), respectively.

Effects of phytohormone-induced changes to HWA

We followed the protocol outlined by Gómez et al. (2014) in conducting a series of assays to assess the effect of our elicitor treatments on HWA crawler preference. Briefly, HWA crawlers from sistens and progrediens generations were presented with individual cuttings from control and treated trees, and settlement preference was scored. Individual cuttings were placed inside 1.5 mL Eppendorf tubes with a pierced lid filled with deionized water to prevent desiccation. Paired tubes were taped to the bottom of a 9 cm diameter Petri dish. Foliage with HWA ovisacs and emergent crawlers were collected from naturally infested trees, and a small fragment with three ovisacs was positioned inside each dish so that the fragment was in contact with both cuttings. Dishes were closed and sealed with parafilm and then stored at room temperature in natural light conditions for 1 week. We inspected dishes daily for the presence of crawlers. Seven days after exposure, the infested branch was removed and the number of settled HWA crawlers per cutting was counted. This approach was utilized for three separate sets of choice tests (N = 30 replicate dishes per test). Assay 1 was conducted in July 2014 using sistens crawlers only (Experiment 1A). Assays 2 and 3 were conducted in May and July of 2015, respectively (Experiment 1B), using progrediens (Assay 2) and sistens (Assay 3) crawlers. All tests used locally obtained HWA-infested foliage fragments (Winchester, MA, USA).

Effects of phytohormone application on total phenolics

We quantified elicitor effects on phenolic-based defenses using the Folin–Ciocalteu method (Ahmed et al. 2014). Briefly, we harvested ~ 300 mg of foliage from each tree and stored the samples on ice before returning them to the laboratory. Foliage was collected 4 days (Assay 1) and 5 days (Assays 2 and 3) post-spraying, respectively. On being returned to the laboratory, needles were removed, separated by age class, and then dried for 48 h in a lyophilizer (VirTis, SP Scientific, Warminster, PA, USA). Prior to drying, new foliage produced by trees for Assay 2 was weighed to determine the potential costs of induction. The dry needles were ground using a KLECO ball mill (Garcia Machine, Visalia, CA, USA), and 20 mg of sample was extracted in 1 mL of 80% aqueous HPLC-grade methanol

(Fisher Scientific, Pittsburgh, PA, USA). Samples were then vortexed and sonicated for 30 min at 20 °C (Quantrex 280, L&R Manufacturing Co., Kearny, NJ, USA), followed by centrifugation for 15 min at 15000 rpm (Marathon Micro A, Fisher Scientific). The resulting supernatant was transferred to new centrifuge tubes for analysis. Samples were analyzed in triplicate and absorbance values were measured at 765 nm using a Benchmark Plus microplate spectrometer (Bio-Rad Laboratories, Inc., Hercules, CA, USA); the resulting values were expressed as gallic acid equivalents (GAE) in mg g^{-1} dry plant matter. This approach provides a conservative estimate of inducibility.

Statistical analyses

All analyses were performed using R v. 3.2.2 (R Core Team 2015). To test for elicitor-induced resistance, we fit generalized linear mixed models (GLMMs) with the proportion of crawlers settling on each branch as the response variable and elicitor treatment as a fixed factor, using a binomial error distribution. Petri dish replicate IDs were included as a random effect. Models were fit with the *lme4* package (Bates et al. 2015), with separate models for each generation (progrediens or sistens) and choice assay (e.g., control vs. MJ) combination tested.

To test for herbivore induction of phytohormones, we used separate Welch's *t* tests for each phytohormone in Experiment 2A (control vs. HWA only) and separate oneway ANOVAs for Experiment 2B (control vs. HWA vs. EHS). To test for effects on phenolic-based defenses, we used linear mixed-effects models with total phenolic concentration (TPC) as a response variable and elicitor treatment and tissue type (new or old needles) as fixed factors. Tree identity was included as a random effect in models. These analyses were performed using the *nlme* package (Pinheiro et al. 2014). Separate models were run for each batch of trees used in Assays 1–3. Finally, to test for impacts on growth, we fit a linear mixed-effects model with new growth (g) as a response variable, and elicitor treatment as a fixed factor. As above, tree identity was included as a random effect in the model.

Results

Short- and long-term feeding by HWA significantly elevated the levels of ABA and SA. Both hormones increased sharply in potted hemlocks attacked by HWA (ABA: $t_{25.65} = -2.37$, $P = 0.03$; SA: $t_{17.63} = -2.59$, $P = 0.02$). These effects were also observed in field-transplanted hemlocks, which also had significantly elevated levels of ABA and SA (Fig. 1). In contrast to HWA (ABA: $F_{2,21} = 11.19$, $P < 0.001$; SA: $F_{2,21} = 17.27$, $P < 0.001$), EHS had no effect on either ABA
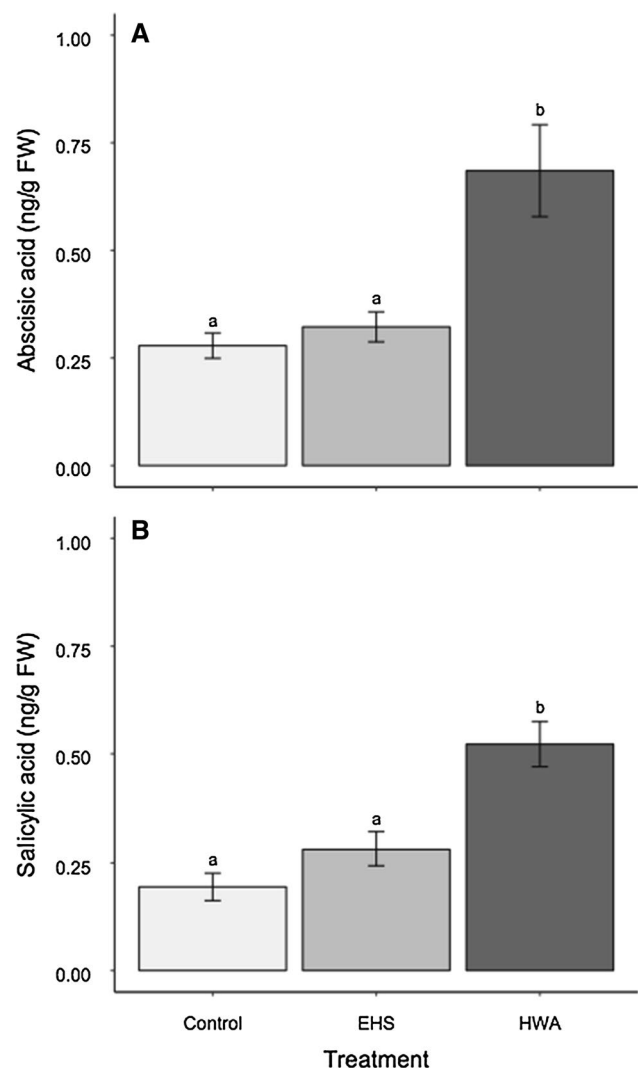

Fig. 1 Impacts of *Fiorinia externa* (EHS) and *Adelges tsugae* (HWA) on **a** abscisic acid (ABA) and **b** salicylic acid (SA) concentrations (ng/g) in needles [fresh weight (FW)] of field-transplanted *Tsuga canadensis* seedlings. Bars represent mean ± SE. Letters indicate significant differences among treatments based on a post hoc Tukey HSD test

or SA induction in the field-transplanted trees (ABA: post hoc Tukey test $P = 0.72$; SA: post hoc Tukey test $P = 0.09$). Finally, while the phytohormone JA was detected in a small fraction of our samples (8 of 54), it often appeared to be below our threshold limit of detection and as such we were unable to make any meaningful comparisons among herbivore treatments (data not shown).

As hypothesized, across all choice tests, both progrediens and sistens generation HWA crawlers avoided settling on MJ-treated foliage (Fig. 2). In Assays 1 (sistens), 2 (progrediens), and 3 (sistens), 63.4% ($Z = -3.69$, $P < 0.001$), 64.4% ($Z = -3.69$, $P < 0.001$), and 70.4% ($Z = -4.68$, $P < 0.0001$) of crawlers across all replicates settled on
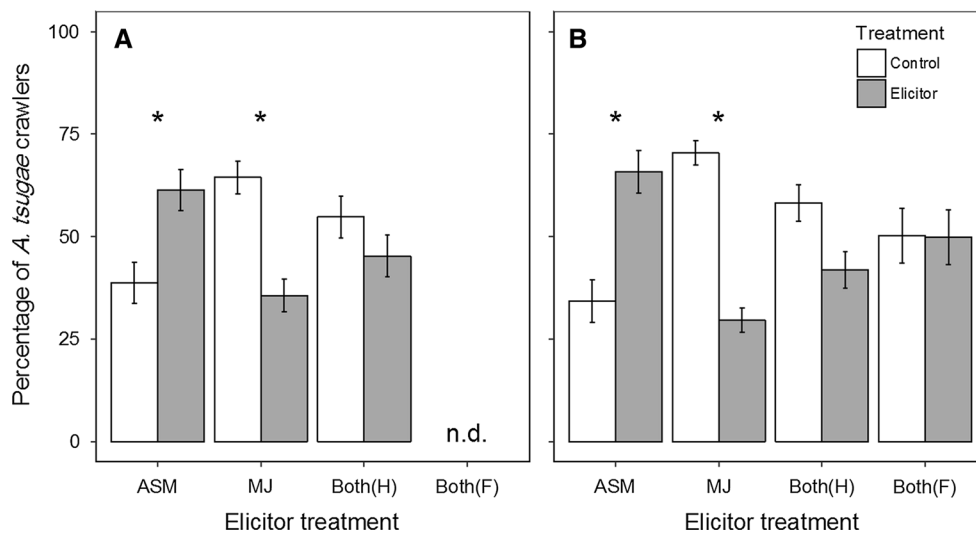
Fig. 2 Percentage of *Adelges tsugae* **a** progrediens and **b** sistens crawlers settling on control (light gray) or elicitor-treated (dark gray) foliage. Results from Assays 2 and 3 are presented. Assay 1 was qualitatively and quantitatively similar to Assay 3 results. Elicitors tested include methyl jasmonate (MJ), acibenzolar-*S*-methyl (ASM), and a combination of the two at half [Both (H)] and full concentration [Both (F)] (Assays 2 and 3 only). Asterisks indicate a significant preference ($P < 0.05$) for either control or elicitor-treated foliage. No preference test for Both_Full with progrediens was performed (n.d.)

control foliage. In contrast, when presented with foliage from ASM-induced trees, crawler preferences were more variable, but as hypothesized, tended to settle on ASM-treated foliage. Across all assays, a majority of crawlers from both generations tended to settle on ASM-treated foliage, and significantly so in Assays 2 ($Z = 2.53$, $P = 0.011$) and 3 ($Z = 3.51$, $P < 0.001$) for the progrediens and sistens generations, respectively. Interestingly, no significant adelgid preference was observed for trees treated with both elicitors simultaneously half (Both_Half) or full (Both_Full) concentration vs. control foliage ($P > 0.05$ for all GLMMs fit; Fig. 2).

Elicitor treatment effects on needle total phenolic concentration (TPC) were variable across assays. In Assay 1 conducted in 2014, treatment of foliage with MJ and ASM, respectively, yielded significant differences in needle TPC ($F_{2,40} = 17.33$, $P < 0.001$). MJ-treated foliage on average (0.22 ± 0.02 GAE) was twice as high in TPC than control foliage (0.10 ± 0.02 GAE; post hoc Tukey test: $P < 0.001$) and 1.5 times more so than ASM-treated foliage (0.15 ± 0.02 GAE; post hoc Tukey test: $P = 0.003$). These effects were not tissue dependent (treatment × tissue interaction: $F_{2,40} = 2.67$, $P = 0.08$), although older needles had significantly higher TPC than younger needles overall ($F_{1,40} = 39.32$, $P < 0.001$). In Assays 2 and 3, elicitors again differed in the magnitude of their effects on TPC; however, the direction of these effects was qualitatively similar. Methyl jasmonate application again increased TPC of needles; however, this effect was not significant (post hoc Tukey test: $P = 0.06$). With respect to ASM, in contrast to the previous assay, we observed significant effects of application

on TPC of new needles (post hoc Tukey test: $P < 0.001$). Moreover, trees treated with both elicitors had elevated TPCs in both new and 1-year-old needles (post hoc Tukey tests: $P < 0.05$), and the Both_Half and Both_Full treatments did not differ in the strength of their effect on either tissue (post hoc Tukey tests: $P > 0.05$). Induction, however, came at the cost of new growth. Elicitors altered new flush production ($F_{3,101} = 7.22$, $P < 0.001$): all elicitor-treated trees in Assay 2 had less new flush than controls. The JA and SA elicitors did not, however, differ in their effects on new flush, either when sprayed alone or in combination in the Both treatments (post hoc Tukey tests: $P > 0.05$).

Discussion

These two exotic piercing/sucking insect herbivores had very different effects on phytohormone induction. Moreover, we found that defense pathway elicitors differentially mediated eastern hemlock resistance to HWA. Feeding site selection by crawlers from both the progrediens and sistens generations was strongly dependent on which defense pathway was elicited. Specifically, crawlers from both generations avoided MJ-treated foliage. These findings highlight the potential for phytohormone-induced resistance as a mechanism for limiting HWA feeding behavior and performance.

The two herbivores had very different effects on phytohormone induction. HWA induced ABA and SA, while EHS had no effect. Prior to our study, numerous lines of evidence suggested that HWA attack and feeding elicit a pathogen

defense response characteristic of SA induction observed in other systems (Radville et al. 2011; Pezet et al. 2013). Our detection of elevated SA concentrations in needles following HWA infestation confirms this hypothesis. There are several potential (and non-exclusive) explanations for the activation and ineffectiveness of SA-based defense in hemlock to HWA. First, HWA is a close relative of aphids (hemipterans) that also feeds via a stylet and can activate SA-dependent defenses associated with pathogen defense (Moran and Thompson 2001; Smith and Boyko 2007). HWA differs, however, in that it feeds on the contents of xylem ray parenchyma cells rather than the phloem like aphids. Second, the pathogen defense response observed in hemlock may not be a reaction to HWA, but rather another agent associated with it. Like aphids and other Hemiptera, HWA has obligate endosymbionts that are essential for its survival (von Dohlen et al. 2013). These endosymbionts are present in HWA salivary glands and may be excreted via saliva into plant tissues as HWA feeds (Young et al. 1995; von Dohlen et al. 2013; Pezet et al. 2013). The activation of SA-dependent defenses by saliva-borne endosymbionts may mask HWA and protect it from more effective herbivore defenses. For example, symbiotic bacteria orally secreted into plant feeding sites can shield the Colorado potato beetle from jasmonate-based defenses (Chung et al. 2013).

These hormones also alter HWA settlement. SA elicitors increased HWA settlement, by the same pathway that is induced by HWA feeding. More specifically, progrediens and sistens crawlers preferred ASM-induced foliage across all assays. Application of MJ inhibited settlement. We also observed negative cross talk between the JA- and SA-signaling pathways. In our behavioral assays, foliage treated with both elicitors simultaneously was less resistant to HWA than MJ alone. In addition to HWA, hemlock faces pressure from other exotic and native pests, including EHS and the hemlock looper *Lambdina fiscellaria* (Bhiry and Filion 1996; Wilson et al. 2016), respectively. Previous laboratory and field research has shown that HWA crawlers avoid settling on EHS-colonized foliage (Gómez et al. 2014), a response driven through plant-mediated responses to EHS rather than the physical presence of this insect (Gómez et al. 2014). We hypothesized that this effect was mediated through EHS induction of the JA pathway and resulting cross talk. Because we failed to detect JA in our assays, however, we cannot evaluate our hypothesis at this time, although EHS-mediated induction of JA and/or another signaling pathway may still increase resistance toward HWA. First, EHS uses piercing–sucking mouthparts to feed on the contents of mesophyll cells. Such a feeding mode is likely more destructive than that employed by HWA and has been shown to induce JA in other systems (De Vos et al. 2005; Kawazu et al. 2012). Second, JA responses to herbivore feeding are often rapid and can occur over a period of hours or days (Creelman

et al. 1992; Conconi et al. 1996); our decision to measure JA 3 months after EHS settlement may have prevented us from detecting this transient phytohormone despite sustained feeding by this sessile herbivore.

Though MJ application deterred HWA, the proximate cues driving changes in behavior remain unknown. Methyl jasmonate application can induce formation of traumatic resin ducts, enhance resin flow, and stimulate increased production and emission of mono-, sesqui-, and di-terpene resin acids in conifers such as Norway spruce (Martin et al. 2003; Zeneli et al. 2006), Sitka spruce (Miller et al. 2005), and *Picea* spp. (Heijari et al. 2005; Moreira et al. 2014). Methyl jasmonate-induced resistance to HWA may be due to enhanced oleoresin defenses observed in these systems and others to herbivores (Heijari et al. 2005; Erbilgin et al. 2006; Sampedro et al. 2011) and pathogens (Zeneli et al. 2006; Krokene et al. 2008). Future studies should try to determine the mechanisms for this effect.

Adelgid success on hemlock may also be facilitated via induction of ABA. In our experiments using both potted and field-transplanted hemlocks, we found that HWA feeding elevated this phytohormone. Experimental work with *Arabidopsis thaliana* found that aphid feeding can induce ABA, that aphids prefer and perform better on wild-type versus ABA-deficient plants, and that ABA signaling may interfere with effective anti-herbivore defenses mediated by other defense pathways (Hillwig et al. 2016). The authors hypothesize that this may be an evolved strategy; however, in this novel interaction between HWA and eastern hemlock, similar mechanisms may aid its success. It is also possible, however, that elevated ABA levels are a consequence of drought-like symptoms induced by HWA feeding. Water usage by trees infested with HWA can be reduced by > 40%, occurring through loss of hydraulic functioning due to formation of traumatic resin canals (Domec et al. 2013) and false rings (Gonda-King et al. 2012). Feeding also increases the concentrations of proline in hemlock tissues (Gómez et al. 2012), an important compound for osmoregulation in plants (Delauney and Verma 1993).

In conclusion, our work suggests that successful HWA invasion may occur via exploitation of ABA and SA signaling and resulting interference with anti-herbivore defenses. Activation of these defenses, particularly through JA signaling, however, can effectively deter HWA. Characterization of these defenses, as well as their potential role in mediating competition between HWA and other herbivores such as EHS, may be key to identifying resistance mechanisms that can be leveraged to protect this threatened species. As forests become increasingly invaded, the success or failure of invasions by exotic herbivores, as well as outcomes of novel interactions with competing herbivores, native and exotic alike, may often be mediated by interference between phytohormone signaling pathways.

Author contribution statement RNS, CST, ELP, and CMO conceived and designed the experiments, RNS and ZW performed the experiments, and RNS analyzed data and wrote the first draft of the manuscript. All authors contributed to revisions.

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