PLANT-ANIMAL INTERACTIONS - ORIGINAL RESEARCH

Exotic herbivores on a shared native host: tissue quality after individual, simultaneous, and sequential attack

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Abstract Plants in nature are often attacked by multiple enemies whose effect on the plant cannot always be predicted based on the outcome of individual attacks. We investigated how two invasive herbivores, the hemlock woolly adelgid (*Adelges tsugae*) (HWA) and the elongate hemlock scale (*Fiorinia externa*) (EHS), alter host plant quality (measured as amino acid concentration and composition) when feeding individually or jointly on eastern hemlock (*Tsuga canadensis*), an important long-lived forest tree that is in severe decline. The joint herbivore treatments included both simultaneous and sequential infestations by the two herbivores. We expected resource depletion over time, particularly in response to feeding by HWA. In contrast, HWA dramatically increased the concentration and altered the composition of individual free amino acids. Compared to control trees, HWA increased total amino acid concentration by 330% after 1 year of infestation. Conversely, EHS had a negligible effect when feeding individually. Interestingly, there was a marginally significant HWA × EHS interaction that suggests the potential for EHS presence to reduce the impact of HWA on foliage quality when the two species co-occur. We suggest indirect effects

of water stress as a possible physiological mechanism for our results. Understanding how species interactions change the physiology of a shared host is crucial to making more accurate predictions about host mortality and subsequent changes in affected communities and ecosystems, and to help design appropriate management plans.

Introduction

Herbivory can cause plants to undergo morphological, chemical, and physiological changes (Karban and Baldwin 1997). Attacks by multiple herbivore species can have additive effects on their host plant. For example, simultaneous herbivory by two weevil species on spotted knapweed reduced flowering and biomass corresponding to the combined magnitude of each insect individually (Knochel et al. 2010). However, the outcome cannot always be predicted on the basis of single-species attacks, as shown in tomato plants attacked by either aphids, beet-army worm caterpillars, or both. Plants with both herbivores exhibited biochemical and gene expression that did not correspond to those predicted by each individual herbivore (Rodriguez-Saona et al. 2010). Despite this fact, most studies investigating plant–insect interactions focus on single herbivores, an approach that provides important data but may not always be relevant to multiple-herbivore systems (Marquis 1990; Vos et al. 2001).

Herbivory by early-arriving species affects later-arriving attackers. This is most obviously important in cases where the early-arriving species causes extensive defoliation,

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outbreaking species being an extreme case. In many cases, however, tissue removal is small and has a negligible impact on resource availability (Kaplan and Denno 2007). Even a small amount of tissue damage, however, can induce both local and systemic changes in plant morphology and chemistry that can affect herbivore performance and fitness (Karban and Baldwin 1997). Such herbivore-induced changes can last days or weeks (Underwood 1998; Gómez et al. 2010a), and may even persist across seasons (Zvereva et al. 1997). Because of this, even when the initial attacker is no longer present, phenotypic changes in host plants can indirectly impact future enemies by increasing resistance (Inbar et al. 1999; Soler et al. 2007) or by increasing susceptibility to a second attacker (Thaler et al. 2002; Zarate et al. 2007). These plant-mediated changes can alter herbivore population dynamics through resource competition (Branson 2010), the attraction of natural enemies (De Boer et al. 2008), and/or induced changes in leaf chemistry (Poelman et al. 2008).

The potential unpredictability of multiple herbivore attacks is an aspect for great concern in cases where herbivores are responsible for significant ecological and economical damage, such as invasive insects (Liebhold et al. 1995). The ever-increasing number of invasive insects also increases the likelihood that multiple invasive herbivores will utilize a common host in their invaded habitat. In order to better understand the potential impact of multiple invasive insects on native hosts, it is important to study how they affect their host's physiology when feeding alone and together.

On the east coast of the United States, the invasive hemlock woolly adelgid (*Adelges tsugae* Annand; HWA) threatens native eastern hemlock (*Tsuga canadensis* Carr.). This insect can kill mature trees in as little as 4 years (McClure 1991), and the decline and death of eastern hemlock can alter light availability, soil conditions, canopy structure, stream chemistry, mycorrhizal composition/abundance, and litter decomposition. These changes can in turn affect birds, fish, insects, and other fauna associated with these unique ecosystems (Orwig and Foster 1998; Jenkins et al. 1999; Stadler et al. 2005; Cobb 2010). The invasive elongate hemlock scale (*Fiorinia externa* Ferris; EHS) also feeds on eastern hemlock in the same region, and has rapidly expanded its range in recent decades (Preisser et al. 2008). Although EHS rarely causes tree death, it can kill already stressed individuals (McClure 1980a). The contemporaneous range expansion of HWA and EHS results in their frequent co-occurrence in the same hemlock stands and host trees (Preisser et al. 2008). Host-sharing by these two sessile sap-feeders should result in high interspecific competition due to their high densities and limited ability to move to more favorable environments (Denno et al. 1995).

Although HWA and EHS can have a strong impact on their host and each other, only three other studies have investigated the interaction between these two important invasive herbivores (Danoff-Burg and Bird 2002; Preisser and Elkinton 2008; Preisser et al. 2008). A landscape-level survey of 7,100 hemlock trees in 142 hemlock stands along a latitudinal transect showed a negative correlation between HWA and EHS densities (Preisser et al. 2008; but see Danoff-Burg and Bird 2002), and also showed that tree mortality was correlated with HWA (but not EHS) density. The only experimental study addressing this interaction showed that densities of HWA and EHS were both ∼30% lower when the two insects co-occurred in the same branch compared to when either species was present alone (Preisser and Elkinton 2008). The same study also showed that simultaneous HWA and EHS infestation was more harmful to plant growth than was EHS alone, but less harmful than when HWA was the only species present. These findings strongly suggest that the interaction between these two herbivores affects tree physiology in ways that may not be predictable on the basis of single-species evidence alone.

Here, we explored the impacts of HWA and EHS on eastern hemlock tissue quality in response to experimental infestation by both insects individually, simultaneously, and sequentially using a full-factorial experimental design (Table 1). Tissue quality was measured as differences in concentration and composition of individual and total free amino acids. Amino acids are nitrogen-rich compounds that are often limiting nutrients for herbivores (Mattson 1980). The quantification of total and individual free amino acids, rather than just total nitrogen, provides a more detailed overview about the plant's physiological status. Furthermore, free amino acids might be a more relevant form of nitrogen since they are readily available to herbivores without undergoing prior digestion (Cockfield 1988). We hypothesized that if resource depletion by HWA is the mechanism underlying the observed tree death, HWA feeding should substantially lower the content of free amino acids over time. In contrast, EHS should have a smaller negative effect since this species generally weakens trees rather than kills them outright. In the presence of both herbivores simultaneously and sequentially, we predicted an intermediate effect. If the effects of each herbivore were additive, the multiple-herbivore treatments should have lower amino acid concentrations compared to the individual treatments; this trend should be particularly pronounced as the length of HWA colonization in a given treatment increases.

Materials and methods

Insects

The hemlock woolly adelgid was introduced to eastern North America from Japan (Havill and Foottit 2007). Upon

Table 1 Treatments arranged in a 3 × 3 full-factorial design and insect densities (average ± SE; no. cm^{-1}; $n = 7$–10 per treatment) prior to tissue sampling

	Elongate hemlock scale		
	0 year	1 year	3 years
Hemlock woolly adelgid			
0 year	*Control*	*EHS 1 year*	*EHS 3 years*
	EHS = 0	EHS = 0.20 ± 0.1	EHS = 0.87 ± 0.2
	HWA = 0	HWA = 0	HWA = 0
1 year	*HWA 1 year*	*Both 1 year*	*EHS → HWA*
	EHS = 0	EHS = 0.13 ± 0.04	EHS = 0.84 ± 0.4
	HWA = 1.30 ± 0.4	HWA = 0.85 ± 0.2	HWA = 0.85 ± 0.2
3 years	*HWA 3 years*	*HWA → EHS*	*Both 3 years*
	EHS = 0	EHS = 0.27 ± 0.1	EHS = 0.58 ± 0.4
	HWA = 1.41 ± 0.6	HWA = 1.42 ± 0.3	HWA = 1.53 ± 0.4

EHS elongate hemlock scale, *HWA* hemlock woolly adelgid

egg hatching, first instar 'crawlers' insert their stylet bundle under the abscission layer of needles where they remain feeding on the xylem ray parenchyma cells until they complete their development (Young et al. 1995). The elongate hemlock scale was also introduced from Japan (McClure 1980a). Crawlers attach to the underside of needles, where the sessile adults feed on the mesophyll cells (McClure 2002).

Experimental design

In April 2007, eastern hemlock saplings (0.7–1 m) were collected from Cadwell Forest (Pelham, MA, USA), an area where neither HWA nor EHS was present. The saplings were transplanted into an open field (East Farm, Kingston, RI, USA). To avoid cross-contamination by dispersing crawlers, each sapling was enclosed in a mesh-covered cage from spring to fall. Cages were removed each winter, when neither insect is dispersing, to prevent snow-related damage. The cages (1 × 1 × 2 m) were made of a plastic frame and covered by a fine mesh. The ground beneath each cage was covered by a weed-block fabric (Ben Meadows, Janesville, WI, USA). Full details of the experimental set-up are described elsewhere (see Miller-Pierce et al. 2010).

A 3 × 3 full-factorial experimental design was used, resulting in nine insect combinations applied to the trees (Table 1). Trees in the herbivore treatments were inoculated annually with HWA, EHS, or both insects from 2007 to 2010 (treatments were: HWA 3 years, EHS 3 years, and both 3 years). Additional trees were kept uninfested for the first 2 years of the study and then inoculated with HWA, EHS, or both insects in the third year of the study (treatments were: EHS 1 year, HWA 1 year, and both 1 year). Having 1- and 3-year infestation treatments allowed us to study the effect of insect feeding on tree physiology over time while accounting for year-to-year differences. In the remaining two herbivory treatments, the order of

infestation was taken into account. Trees that had been inoculated with either HWA or EHS in 2007 and 2008 were also inoculated with the second insect in 2009 and 2010 (treatments EHS→HWA and HWA→EHS). Control trees remained uninfested for the duration of the study. Because all the experimental trees were originally transplanted at the same time, tree age was similar among treatments regardless of the time of inoculations. Because of transplant-related tree death or contamination, at the time of foliage sampling there were 7–10 trees per treatment.

Insect inoculation and density

The inoculations followed standard protocols to maximize insect settlement (Butin et al. 2007). Briefly, in April, immediately prior to egg hatching, inoculum branches of comparable size containing HWA were collected from nearby naturally-infested locations. EHS inoculum branches were collected prior to egg hatching in June. Promptly after collection, branches were placed on the tree inside the cages matching the appropriate treatment. Control trees received uninfested branches. The branches were inserted in plastic bags with water to avoid premature desiccation of the inoculum branch that might compromise the survival of the insects. Insect density was recorded in March 2010 to estimate the level of infestation in each treatment (Table 1). This was done by counting insects on 12 randomly selected branchlets within each tree. The counts in each tree were averaged to obtain one score per tree.

Free amino acid measurements

In May 2010, a sample of newly produced needles from each tree, collected from branches approximately 1 m above the ground, was used to measure free amino acid concentration and relative composition. Samples were

collected at this time of the year because newly produced needles are mostly still uninfested by either insect; colonization of new foliage occurs in June (EHS) and mostly July (HWA), respectively. A ∼5-cm uninfested sample of young foliage from infested branches was randomly collected from each tree, flash-frozen in liquid nitrogen, and transported in dry ice to the laboratory where they were stored at –80°C until further analysis. Samples consisted of needles attached to unlignified stem tissue. Samples were ground under liquid nitrogen using a mortar and pestle. An amount of 200 mg (fresh weight) of sample material was extracted in 1 ml of pre-cooled 80% ethanol (v:v) at room temperature for 20 min, vortexing periodically. Then, the samples were centrifuged at 7,000 rpm for 10 min and the supernatant filtered through a 0.45-μm pore size Acrodisk® Syringe filter (Pall Gelman Laboratory, Ann Arbor, MI, USA). Next, 500 μl of the filtered extract were used for free amino acid determination using the commercial EZ: Faast™ kit (Phenomenex, Torrence, CA, USA) and GC-FID. The gas chromatographic analysis was performed as recommended in the EZ: Faast kit. Briefly, 2 ml of sample were injected (15:1 split) at a temperature of 250°C on a Zebron ZB-AAA column (0.25 mm × 10 m; Phenomenex). Helium was used as carrier gas at a flow rate of 1.5 ml/min. The initial oven temperature was set to 110°C and increased at a rate of 32°C per min to a final temperature of 320°C and held for 3 min. We identified and quantified 22 amino acids and 1 dipeptide (proline-hydroxiproline); for simplicity in the text, we refer to all 23 compounds as amino acids. The EZ: Faast™ kit does not allow for the detection of arginine. Individual free amino acids were identified in the foliage samples by comparing spectra and retention time to free amino acid standard solutions (with norvaline as internal standard) provided

with the EZ: Faast kit and quantified using ChemStation software (Rev. B.04.02; Agilent Technologies, Waldbron, Germany). For each treatment we determined the concentrations of each amino acid and then determined the relative concentration of each (hereafter referred to as amino acid composition).

Statistical analysis

A two-way ANOVA was used to test for herbivore effects (HWA, EHS) and their interaction (HWA × EHS) on total amino acid concentration followed by a Student–Newman–Keuls post hoc test to test for differences among treatments (Fig. 1). Each independent variable (HWA and EHS) had three levels regarding the time post-inoculation: 0 year (no insect), 1, and 3 years, respectively, conforming to a 3 × 3 full-factorial design (Table 1). Because amino acid concentrations are related, a two-way MANOVA (Wilks' λ) was used on individual amino acid concentrations to test for overall herbivore effects and their interaction. Relative differences of each individual herbivore treatment compared to the uninfested control were calculated as ratios by dividing the concentration of each amino acid in a particular herbivore treatment by its concentration in the control group (Table 2). In order to test the specific hypotheses that each treatment differed from the control treatment (Table 2) across all dependent variables (individual amino acids), multivariate multiple regression analysis was used to account for the dependence among individual amino acids, using the individual treatments as regressor variables (SAS PROC REG, MTEST statement). Principal component analyses (Fig. 2) were performed to compare amino acid composition (percentages) profiles between different groups of treatments within the same inoculation year.
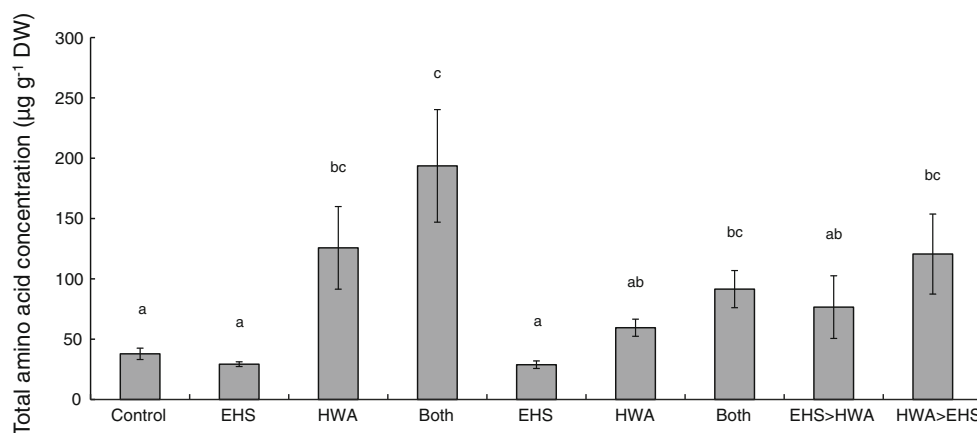
Fig. 1 Concentration of total amino acids (average ± SE) in newly produced tissue (needles and stem) of *Tsuga canadensis* subjected to different herbivore treatments. The number of replicates per treatment is indicated inside the *bars*. A Student–Newman–Keuls post hoc test was used to compare treatments. Treatments with different letters above them are significantly different among each other. HWA (*Adelges tsugae*) had a significant effect (*P* < 0.001) on amino acid concentration. EHS (*Fiorinia externa*) and the interaction between HWA and EHS only had a marginally significant effect on total amino acid concentration (*P* = 0.08 and *P* = 0.07, respectively)

Table 2 Relative change in amino acid concentration

Amino acid	EHS 1 year	EHS 3 years	HWA 1 years	HWA 3 years	Both 1 year	Both 3 years	EHS > HWA	HWA > EHS
Alanine	1.11	1.06	*1.83*	1.52	**2.28**	1.35	1.33	**1.81**
Glycine	0.92	0.65	1.29	1.22	1.40	1.12	1.21	1.37
Valine	1.10	0.91	**1.95**	**1.48**	**2.80**	**1.65**	**1.46**	**2.03**
β-Aminoisobutyric acid	0.89	0.91	1.82	1.27	**2.05**	1.67	1.30	*1.63*
Leucine	0.93	0.73	**2.07**	**1.36**	**2.58**	**2.10**	1.25	**2.41**
Allo-isoleucine	1.14	0.82	**2.01**	**1.50**	**2.15**	**1.75**	1.45	**2.16**
Isoleucine	0.84	0.67	**2.80**	1.20	**3.97**	*1.81*	1.67	*2.32*
Threonine	0.97	0.85	*1.72*	1.22	**2.71**	*1.53*	**3.59**	*1.66*
Serine	0.93	0.79	1.49	1.21	1.46	1.06	1.26	**1.56**
Proline	2.12	0.63	**20**	**5.05**	**89**	**8.77**	**16**	**21**
Asparagine	0.41	0.42	*2.99*	1.26	**12.08**	2.29	2.05	**6.40**
Thioproline	0.97	0.82	*1.75*	1.21	1.77	**1.64**	1.21	**1.92**
Aspartate	0.84	0.83	2.10	**1.13**	**2.21**	**1.72**	1.03	**1.64**
Hydroxyproline	0.74	0.78	**1.67**	0.97	**1.73**	1.21	1.07	**1.52**
Glutamate	0.76	0.72	**1.86**	1.18	**1.58**	*1.43*	1.06	**1.56**
Phenylalanine	1.13	0.91	**1.46**	*1.37*	**1.50**	1.42	1.00	**1.47**
Glutamine	0.49	0.82	7.13	**2.80**	**6.18**	**5.04**	3.87	**6.09**
Ornithine	0.75	1.53	**24**	**4.59**	**42**	**19**	10.95	**27.15**
Lysine	0.68	0.96	**5.65**	*2.36*	**10**	**5.57**	3.01	**7.17**
Histidine	0.57	1.16	10.85	3.22	**14.39**	**11.38**	5.35	**12.66**
Tyrosine	0.66	0.45	0.89	0.64	*1.74*	1.44	0.77	1.68
Proline-hydroxyproline	1.22	1.00	1.67	1.39	1.93	**2.25**	1.36	2.48
Tryptophan	0.65	1.18	3.95	1.87	**4.78**	3.94	2.68	4.19

Values are calculated dividing the average concentration in each treatment by the average concentration in the control group values. The numbers of replicates per treatment ranged between 7 and 10. Values >1 represent higher and <1 lower amino acid concentration relative to controls, respectively. Significant changes resulted from a multiple regression analysis are indicated in bold

$P < 0.05$ and marginally significant changes ($P < 0.1$) are indicated in italics

Response variables were transformed as needed prior to the statistical analyses to approximate the assumptions of the general linear models used. The critical P value used in this study was $P < 0.05$. Statistical analyses were performed in SAS 9.2, JMP 9, and SPSS 19.

Results

Individual effect of HWA

Total amino acid concentration after 1 year of HWA infestation was 330% higher than in the control treatment (Fig. 1). After 3 years, amino acid concentrations tended to be lower and were not significantly different from uninfested control trees (Fig. 1). HWA infestation had an overall significant effect on individual amino acid concentrations (Wilks' $\lambda = 0.196$, $P < 0.001$). The concentration of most amino acids was higher than uninfested

trees regardless of time post-inoculation (Table 2; Online resource 1).

Amino acid composition changed dramatically in response to HWA infestation, especially after the first year (Fig. 2a). The most abundant amino acid in all treatments was glutamate, which represented 52.6% of all amino acids in uninfested trees but decreased to 32.7 and 42.1% after 1 and 3 years of HWA infestation, respectively (Online resource 2). The overall increase in individual amino acids in HWA treatments was driven by large increases in a few amino acids; the relative percentages of most amino acids tended to decrease in HWA treatments (Online resource 2). Proline increased most dramatically in response to HWA infestation: it represented 2.5% of all free amino acids in control trees, but increased to 18.2% after 1 year of HWA infestation and 5.8% after 3 years (Table 2; Online resource 2). Ornithine also increased from 0.5% in control trees to 3.2 and 1.5% after 1 and 3 years of HWA infestation, respectively (Online resource 2).

a 1 year post-inoculation

b 3 years post-inoculation

c 3 years post-inoculation
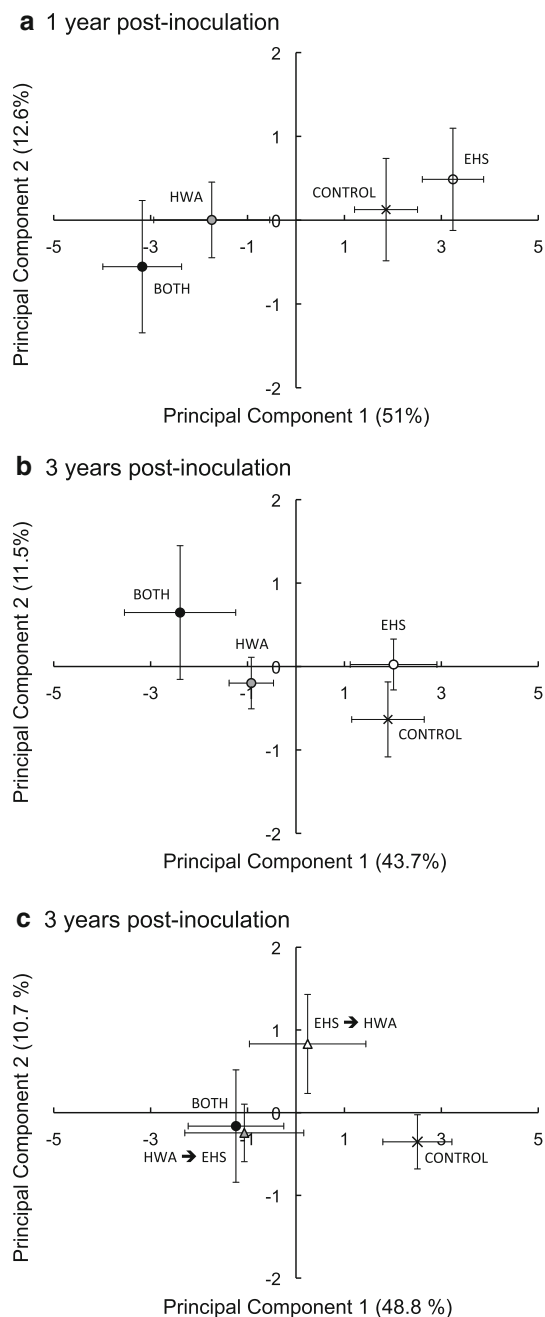
Fig. 2 Principal component scores (average ± SE; $n = 7$–10) of amino acid percentages in young needles and stems of *Tsuga canadensis* comparing herbivore treatments 1 year (**a**) and 3 years (**b**, **c**) post-inoculation. Treatments: uninfested controls (*cross*); elongate hemlock scale, EHS (*white circle*); hemlock woolly adelgid, HWA (*gray circle*); both simultaneously (*black circle*); HWA followed by EHS, HWA→EHS (*gray triangle*); and HWA followed by EHS, EHS→HWA (*white triangle*). *Numbers in parentheses* explain the amount of variation explained by each principal component

Individual effect of EHS

Infestation with EHS consistently decreased the total concentration of amino acids, but this effect was only marginally

significant ($P = 0.08$; Fig. 1). When analyzed individually, even though most amino acids tended to decrease in concentration, the overall decrease was not statistically significant (Wilks' $\lambda = 0.423$, $P = 0.52$).

In terms of amino acid composition, the profiles of EHS-infested plants did not differ from uninfested control treatments, but were different from HWA-infested plants consistently over time (Fig. 2a, b).

Multiple infestations: simultaneous versus equential infestation

In multiple-herbivore treatments inoculated during the same year, the overall response in amino acid concentration and composition was driven by the presence of HWA. In other words, the simultaneous herbivory treatments were more similar to HWA treatments than EHS treatments (Figs. 1, 2a, b). The amino acid composition profile in the simultaneous infestation treatment after 1 year post-inoculation (both 1 year) was largely the same as HWA 1 year (Fig. 2a). After 3 years, there was some separation among the HWA and the simultaneous treatments, but there was still clear separation between those treatments and the ones without HWA (Fig. 2b). The order of colonization also affected amino acid composition. The amino acid composition profile of simultaneous herbivory (3 years post-inoculation; both 3 years) was closely clustered with the sequential treatment where HWA was introduced first as opposed to the treatment were EHS was introduced first, which only partially overlapped the other two treatments (Fig. 2c). Principal component 1 (which explained 47.7% of the variation) clearly separated the control group and the treatments with the other three treatments containing both herbivores (Fig. 2c).

Total amino acid concentration in the simultaneous herbivory treatment was over five times higher than uninfested trees after 1 year of infestation (Fig. 1) and over two times higher after 3 years (Fig. 1). Although those values were higher than would be expected from adding the individual effect of both insects (Fig. 1), the HWA × EHS interaction term was only marginally significant ($P = 0.07$). In the sequential treatments, total amino acid concentration was only significantly higher than the control group when HWA was introduced first in year 1, followed by EHS in year 3 (treatment HWA→EHS; Fig. 1). When EHS was introduced first (treatment EHS→HWA), the subsequent addition of HWA in year 3 did not lead to a significant difference in total amino acid concentration compared to control trees (Fig. 1).

All multiple-herbivore treatments showed an increase in the concentrations of some amino acids (Table 2). The greatest number of significant or marginally significant changes in individual amino acid concentrations occurred

in the sequential treatment where HWA was introduced first (20 of 23 amino acids; Table 2). Surprisingly, in the counterpart treatment where EHS was introduced first, only three amino acids significantly differed from their concentrations in the control trees (Table 2). In simultaneous treatments, 19/23 and 16/23 amino acids marginally or significantly changed after 1 and 3 years, respectively (Table 2). While most amino acids showed between a one- and two-fold increase, there were greater increases in several other amino acids. For example, proline increased dramatically in response to multiple infestations, especially when both insects were introduced simultaneously, resulting in a 89- and 9-fold increase compared to uninfested trees after 1 and 3 years of infestation, respectively (Table 2). Thus, 1 year post-inoculation proline represented 28.9% of all amino acids as compared to 2.5% in uninfested trees (Online resource 2). In the sequential treatments, proline was 16- and 21-fold higher than the control treatment in the EHS→HWA and HWA→EHS treatments, respectively. Another consistent change in treatments where HWA was present was a large decrease of glutamate, which represented 52.6% in control trees but was reduced to 23.1% in the simultaneous treatment 1 year post-infestation (Online resource 2).

Insect density

Herbivore densities recorded immediately before needle flushing showed that the inoculation protocol was successful in all treatments (Table 1). In individual herbivore treatments, EHS densities on foliage were low in trees inoculated for 1 year, but were over four times higher in trees infested for 3 years. HWA densities were moderate in both individual HWA treatments. In simultaneous infestations, the density of EHS was consistently lower than in individual treatments. This pattern was followed by the HWA but only after 1 year of infestation. In the sequential treatments, insect densities were mostly consistent with densities of each insect in the corresponding individual treatment.

Discussion

Individual attacks

Infestation by EHS gradually decreased total free amino acid concentration over the course of the infestation. After 3 years, EHS-infested foliage had a 24.2% lower total amino acid content than foliage from uninfested trees. Although this trend was only marginally significant, it was consistent with previous studies documenting that increases in EHS density were accompanied by decreases in nitrogen

(McClure 1980b). In contrast, HWA-infested trees had much higher free amino acid concentrations than did uninfested trees. There was a large initial systemic increase in total free amino acids after 1 year of infestation, with values more than three times higher than uninfested trees, decreasing to values 1.6 times higher than controls after 3 years of HWA infestation. The decreasing concentration over time after the initial spike fits the hypothesis of resource depletion as a possible cause for hemlock mortality. The higher free amino acid concentration in HWA infested trees agrees with Stadler et al. (2005), who showed that stands naturally infested by HWA had much higher foliar % N than reference hemlocks in uninfested sites. In contrast, Miller-Pierce et al. (2010) showed that HWA-infested tissue had an initial, but transient, decrease in % N compared to uninfested trees and no overall difference after 2 years of infestation. This indicates that there can be differences between local and systemic tissue quality, but most importantly that these harmful insects can have an impact at the whole-plant level despite their patchy distribution within a tree. This systemic impact was also shown by an increased hypersensitive response in uninfested hemlock foliage in response to HWA but not EHS distal feeding (Radville et al. 2011).

Multiple attackers and priority effects

Feeding by EHS tended to reduce amino acid concentration, while HWA infestation induced large increases. If these herbivores' effects were additive, simultaneous attack by both should have resulted in a moderate increase in total amino acids. In contrast, our experimental results showed that simultaneous attack by HWA and EHS increased total amino acid concentration above the levels reached in the HWA-only treatment. In the sequential treatments, total amino acid concentration was higher than in uninfested trees if HWA was introduced first but not if EHS was introduced first. Although the individual impact of EHS seemed negligible, our results suggest that EHS could increase the total impact when both insects are introduced simultaneously, but may decrease the total impact when it is the initial colonizer. This 'benign' effect of EHS might be caused by induced physiological changes triggered prior to HWA arrival (e.g., induction of defenses, nitrogen depletion), which may alter HWA performance or its effects on the plant. In particular, nitrogen has been shown to be an important factor for HWA success (Pontius et al. 2006) and prior colonization by EHS might reduce nitrogen levels under a suboptimal threshold for HWA. The effects of such changes in amino acids on plant health and herbivore density remains to be investigated. Our results support previous research in other systems showing that both the order of colonization as well as herbivore identity can profoundly

affect host quality as well as subsequent herbivore and host fitness (Poelman et al. 2008; Erb et al. 2011).

Herbivore-induced changes in amino acids: an indication of water stress

Our results suggest that HWA feeding causes water stress in eastern hemlock. This is indicated by (1) the dramatic increase in proline, a common occurrence in water-stressed plants (Delauney and Verma 1993), (2) changes in the glutamine:glutamate ratio that suggest an increase in nitrogen recycling and remobilization (Terce-Laforgue et al. 2004), and (3) the overall increase in free amino acids in uninfested tissues.

Proline accumulation

Proline consistently increased in both absolute and relative concentration in response to HWA feeding, representing almost 30% of all amino acids as compared to 2.5% in uninfested trees. The accumulation of proline is a common response to herbivory and drought (Hare and Cress 1997 and references therein). For example, in *Spartina alterniflora*, proline made up 40% of the total free amino acids after planthopper feeding (Bacheller and Romeo 1992). In *Petunia hybrida* plants transformed to accumulate free proline, the increased proline served as an osmoprotectant that increased the drought resistance of the transformed plants (Yamada et al. 2005). However, besides its role in plant protection against different kinds of stress, proline has also been shown to act as a feeding stimulant (Behmer and Joern 1994; Meyer et al. 2006) and to be an efficient way to store energy for insects (Gade and Auerswald 2002). Whether proline accumulation in eastern hemlock is a mechanism to cope with HWA-induced water stress, or whether HWA benefits from it, is currently unknown.

Remobilization of nitrogen to uninfested tissues

HWA-infested plants showed a relative decrease in glutamate and a corresponding relative increase in glutamine. This is consistent with studies showing an increase in glutamine synthetase activity after aphid attack in different plant species (Giordanengo et al. 2010 and references therein). Glutamine synthetase is thought to be involved in the re-assimilation of ammonia during protein catabolism in senescent leaves by converting ammonia and glutamate into glutamine (Terce-Laforgue et al. 2004). Our findings thus suggest that HWA might be inducing a senescence-like process in older needles that results in an increase of recycled nitrogen in young needles.

Changes in amino acid composition can be an important factor in plant resistance/susceptibility to herbivores

(Febvay et al. 1988; Chen et al. 1997). In potato plants, aphids performed better in glutamine-rich pre-tuber-filling plants than in tuber-filling plants primarily composed of glutamate and aspartate (Karley et al. 2002). Our results showed a striking change in amino acid composition in response to HWA but not EHS. In our study, glutamate was the dominant amino acid in uninfested and EHS-infested trees, representing about 50% of all amino acids. This percentage was decreased to 23% in some HWA treatments, perhaps suggesting manipulation of the host chemistry by HWA, as shown for other members of the Adelgidae family (Havill and Foottit 2007), to increase tissue quality.

Increased nitrogen

HWA feeding increased total amino acid concentration in young uninfested needles. Localized increases in nitrogen have also been shown in other systems in response to sap-feeders (Sandstrom et al. 2000; Eleftherianos et al. 2006). Water stress is known to increase soluble nitrogen in plants through a senescence-like process that increases protein breakdown in stressed tissues (reviewed in White 1984). Remobilization of nitrogen away from stressed tissues is thought to benefit plants by optimizing resource allocation to structures with a lower risk of attack (Gómez et al. 2010b). However, free amino acids are also physiologically more accessible to insects than other forms of nitrogen. Huberty and Denno (2004) suggested that sap-feeders might benefit from water stress-induced nitrogen accumulation, but only if the water stress is not continuous. The seasonal feeding dynamics of HWA (they enter diapause in the summer and resume feeding in the fall) might be causing intermittent chronic stress resulting in temporarily elevated nitrogen. The elevated total amino acid concentration observed in our study could increase the adelgid's performance.

The HWA-induced water stress symptoms observed in our study may stem from an increased formation of abnormal wood in HWA-infested trees that hinder water transport (Walker-Lane 2009). HWA-infested trees have also been shown to shed more needles than healthy trees (Stadler et al. 2005), suggesting a greater degree of desiccation. Whether water stress occurs as a by-product of HWA feeding or whether HWA manipulates its host to improve tissue quality needs further study.

Conclusions

Our study demonstrates that two herbivores from the same feeding guild had contrasting and likely non-additive effects on foliage quality. This provides additional evidence

that the effects of multiple attackers on plant chemistry may not be predictable using single-species evidence. Hemlocks responded to herbivore identity and colonization sequence by producing different resource allocation patterns. This suggests that herbivore identity and priority effects could have long-lasting effects that shape herbivore communities and alter the herbivore-induced selective pressure on their host. Given the fact that plants generally interact with more than one herbivore species, our findings suggest that research accounting for the dynamics of multiple insect attacks will enhance our ability to accurately predict the interaction and impacts of invasive insects that share a native host.

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