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Facilitation between invasive herbivores: hemlock woolly adelgid increases gypsy moth preference for and performance on eastern hemlock

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Abstract.

1. Interactions between invertebrate herbivores with different feeding modes are common on long-lived woody plants. In cases where one herbivore facilitates the success of another, the consequences for their shared host plant may be severe. Eastern hemlock (*Tsuga canadensis*), a canopy-dominant conifer native to the eastern U.S., is currently threatened with extirpation by the invasive stylet-feeding hemlock woolly adelgid (*Adelges tsugae*). The effect of adelgid on invasive hemlock-feeding folivores remains unknown.

2. We evaluated the impact of feeding by hemlock woolly adelgid on gypsy moth (*Lymantria dispar*) larval preference for, and performance on, eastern hemlock. To assess preference, we surveyed 245 field-grown hemlocks for gypsy moth herbivory damage and conducted laboratory paired-choice bioassays. To assess performance, gypsy moth larvae were reared to pupation on adelgid-infested or uninfested hemlock foliage and pupal weight, proportional weight gain, and larval period were analyzed.

3. Adelgid-infested hemlocks experienced more gypsy moth herbivory than uninfested control trees, and laboratory tests confirmed that gypsy moth larvae preferentially feed on adelgid-infested hemlock foliage. Gypsy moth larvae reared to pupation on adelgid-infested foliage gained more weight than larvae reared on uninfested control foliage.

4. Our results suggest that the synergistic effect of adelgid and gypsy moth poses an additional threat to eastern hemlock that may increase extirpation risk and ecological impact throughout most of its range.

Key words. Facilitation, herbivores, *Adelges tsugae*, *Lymantria dispar*, invasional meltdown

38 Introduction

39 Many interactions between co-occurring insect herbivores are mediated by their impact
40 on the shared host plant (Kaplan & Denno, 2007). Feeding by one insect may cause alterations in
41 plant quality, such as the induction of toxic secondary metabolites or changes to various leaf
42 structural traits, which can affect simultaneously- or sequentially-feeding competitors (Nykänen
43 & Koricheva, 2004). Although many such changes negatively impact the other species, they can
44 also be facilitative (Kaplan & Denno, 2007; Ohgushi, 2008). Sap feeding by the aphid
45 *Brevicoryne brassicae*, for example, improves the performance of folivorous *Pieris brassicae*
46 larvae by attenuating chemical defense induction in *Brassica oleracea* (Li *et al.*, 2014).

47 Understanding herbivore-herbivore interactions is especially important in cases where
48 one or both herbivores can substantially affect plant growth and fitness. One such species is
49 hemlock woolly adelgid (*Adelges tsugae*; ‘adelgid’ hereafter), a destructive pest that has caused
50 widespread mortality and decline of an ecologically significant conifer, eastern hemlock (*Tsuga*
51 *canadensis*; ‘hemlock’ hereafter), in eastern U.S. forests. Adelgid feeds by inserting its stylet
52 bundle into the xylem ray parenchyma cells at the base of a hemlock needle (Shields *et al.*,
53 1995). This feeding reduces the production of new foliage (Gonda-King *et al.*, 2014; McClure,
54 1991), alters wood morphology (Domec *et al.*, 2013; Gonda-King *et al.*, 2012), and substantially
55 impacts plant physiology. Adelgid-infested hemlocks have elevated tissue levels of salicylic acid
56 (SA) and emissions of its methylated form, methyl salicylate (Pezet *et al.*, 2013; Pezet &
57 Elkinton, 2014). SA is a phytohormone that plays a critical role in plant response to abiotic
58 stresses and biotrophic pathogens; it has also been shown to accumulate following stylet-feeding
59 insect infestations (Walling, 2000). SA accumulation and subsequent monomerization of NPR1,
60 a transcriptional regulator that promotes the expression of SA-responsive genes, can interfere

with the biosynthesis of jasmonic acid (JA)/ethylene-dependent defenses that help protect against leaf-chewing herbivores (Walling, 2008; Zarate *et al.*, 2007). Adelgid feeding has also been shown to increase nitrogen (Gonda-King *et al.*, 2014) and total amino acid content (Gomez *et al.*, 2012) in hemlock needles. Because nitrogen is critical to insect growth (Awmack & Leather, 2002; Kerslake *et al.*, 1998), such adelgid-mediated increases may enhance host plant quality for folivorous herbivores.

Recent work in the hemlock system suggests that adelgid-induced phytochemical changes may influence interactions between hemlock and other herbivores (Rigsby *et al.*, 2019; Schaeffer *et al.*, 2018; Wilson *et al.*, 2018). Larvae of the native hemlock looper (*Lambdina fiscellaria*) had higher survival and enhanced larval development when reared on adelgid-infested versus uninfested hemlock foliage (Wilson *et al.*, 2016). This work led us to explore whether similar interactions might be occurring between the adelgid and more commonly-occurring folivores. We focused our attention on gypsy moth (*Lymantria dispar*), an invasive folivore that has devastated eastern U.S. forests. Since its introduction in 1890, periodic gypsy moth outbreaks have defoliated millions of acres and altered forest structure and composition (Gandhi & Herms, 2010; Lovett *et al.*, 2006). Gypsy moth can feed on eastern hemlock (Lovett *et al.*, 2006) and although it and hemlock woolly adelgid co-occur in their introduced range, their interactions have not been considered.

We report the results of work assessing the impact of adelgid infestation on gypsy moth-hemlock interactions. We surveyed hemlocks planted into a deciduous forest understory for gypsy moth herbivory and conducted two laboratory experiments to measure gypsy moth preference for, and performance on, adelgid-infested hemlock foliage. Because the adelgid inhibits hemlock anti-folivore defense pathways and increases the nutritional value of its needles,

we hypothesized that gypsy moth larvae would both prefer (consume more of) and do better (pupate at higher weights) on adelgid-infested foliage. The ‘invasional meltdown hypothesis’ suggests that much of the damage caused by introduced species may result from positive interactions between invaders that can facilitate their establishment and increase their ecological impact (Simberloff & Von Holle, 1999). Our findings illustrate the potential for such facilitation between two invasive herbivores and highlight the threat this may pose to their shared host and its associated ecosystem.

Materials and Methods

Field preference survey: Our field preference survey took advantage of a 2016 gypsy moth outbreak to assess their impacts on field-grown eastern hemlock. The trees in this survey were planted in 2014 for use in an unrelated experiment. Briefly, 1-1.2 m-tall hemlock saplings were purchased from Van Pines Nursery (West Olive, MI) in spring 2014, planted, and grown for two years in the understory of a mixed hardwood stand at the Kingston Wildlife Research Station (South Kingstown, RI). Hemlocks were planted in five 64-tree blocks, with each tree spaced 1-1.5 m apart. Trees were protected from herbivory and cross-contamination of treatments with chicken-wire cages covered by mesh bags (Agribon-15, Johnny’s Selected Seeds, Waterville, ME, USA; 90% light transmission. Sixteen trees in each block were randomly assigned one of the following two treatments: infestation with adelgid or another invasive herbivore (*Fiorinia externa*; elongate hemlock scale, ‘scale’ hereafter). The remaining 32 trees in each block were maintained as controls. Trees in the adelgid and scale infestation treatments were inoculated in the spring of 2014, 2015, 2016, and 2017 with infested foliage collected from nearby adelgid-infested and scale-infested hemlocks, respectively; trees in the control treatment had herbivore-free hemlock foliage placed on them to control for disturbance.

In spring 2016, a gypsy moth outbreak occurred at our field site. Late-instar gypsy moth larvae were regularly seen roaming on the ground, where they could crawl under the mesh bags enclosing our trees. Over a short (2-3 week) time period, we observed that many of our trees received substantial damage from gypsy moth larvae. In late June 2016, 69 trees in the adelgid-infested treatment group, 69 trees in the scale-infested treatment group, and 107 trees in the control treatment group were assessed for gypsy moth herbivory damage, for a total of 245 trees. All branches emerging from the main stem of each tree were surveyed, and each tree was given a combined damage score of 0-3 (0=0-25% foliage loss, 1=26-50%, 2=51-75%, 3=76-100%). An annual, early spring survey confirmed that trees did not experience foliage loss prior to the gypsy moth outbreak. During the survey, gypsy moth larvae were confirmed to be the only folivores present on trees.

Laboratory preference assay: Hemlock foliage used in the laboratory preference assay came from 0.5-0.7m hemlock saplings purchased from Vans Pines Nursery (West Olive, MI) in spring 2016. In late spring 2016, we inoculated half of the trees with adelgid-infested foliage from nearby trees; we attached adelgid-free hemlock foliage to the other trees (the control group) to control for disturbance. All trees were covered in mesh (Agribon-15, Johnny's Selected Seeds, Waterville, ME, USA; 90% light transmission) to prevent cross-contamination between treatments and grown in 1-gallon pots outside of the greenhouse complex at the University of Rhode Island (URI; Kingston, RI). Adelgid densities on each tree were assessed in late fall 2016 and early spring 2017. Five secondary branches on each tree were randomly selected, and all adelgid present on the branches were counted. We used this data to ensure that both the trees and specific branches used in this experiment had similar adelgid densities (0.8-1 adelgid/cm).

In late spring 2017, we collected 40 gypsy moth larvae from a mixed-hardwood forest located adjacent to the URI greenhouses. We collected 4th-5th instar larvae found wandering on the ground or on tree trunks; all larvae were similarly-sized and highly active throughout the experiment. To assess gypsy moth preference for adelgid-infested hemlock, we collected 40 ~10cm terminal branches: one branch from each of 20 adelgid-infested trees, and one branch from each of 20 uninfested trees. Each branch was weighed; analysis *via* two-tailed Welch's unequal variances t-test confirmed there was no significant difference in the mean branch weight experienced by larvae in each treatment group ($t_{36} = -0.72$, $P = 0.4731$). Following weighing, the branches were inserted into individual blocks of water-saturated floral foam (Oasis brand, Kent, OH). Two pieces of foliage (one adelgid-infested and one control) were then put in a 6L polypropylene bin (Sterilite brand, Townsend, MA). The pieces of foliage were placed at the 25% and 75% marks between the left and right sides of the bin; treatment placement was alternated between left and right. After two similarly-sized gypsy moth larvae were weighed, they were both added to the center of each bin. Each bin was then covered with metal mesh held in place by a rubber band. There were a total of 20 bins in the experiment. After one day, the mesh was removed and the larvae and foliage (including any dropped needles) were weighed; the adelgid-infested and uninfested foliage were weighed separately to calculate larval consumption for each treatment.

Laboratory performance assay: Hemlock foliage used in the laboratory performance assay came from the common garden planting described in the field preference survey. In late spring 2018, 3rd-instar gypsy moth larvae were obtained from the USDA-APHIS Laboratory in Buzzards Bay, MA. These larvae originated from the New Jersey Standard Strain-APHIS substrain, a laboratory colony which has been in cultivation for >60 generations. Larvae were

reared on an artificial diet (Frontier Agricultural Sciences, USDA Hamden Formula) until they reached the fourth instar, at which point each larva was weighed and placed individually into one of 50 473 ml glass mason jars (Ball brand, Broomfield CO). Fourth-instar larvae were used in this experiment because younger stages have trouble consuming hemlock foliage, likely because their undeveloped mouthparts cannot penetrate lignified needles. By contrast, larvae in the fourth instar and above readily consume hemlock.

Half of the jars contained foliage from adelgid-infested hemlocks, while the other half of the jars contained foliage from uninfested hemlocks, for a total of 25 replicates per treatment. The foliage in each jar consisted of a single ~17cm sprig of foliage kept upright in hydrated floral foam (Oasis brand, Kent, OH); foliage was checked every day and replaced if >50% of the needles had been consumed. The top of each jar was covered with nylon mesh and all jars were kept in a growth chamber (15:9 L:D, 24° C, 60-70% RH). Larvae were checked every two days and the position of the jars rotated within the growth chamber; the date of and weight at pupation was recorded for each individual.

Statistical analysis: All data were inspected for normality (Shapiro-Wilk test) and homoscedasticity (Bartlett's test) prior to analysis; data were log-transformed where necessary to meet assumptions. Damage scores were tabulated by treatment group and analyzed *via* Pearson's chi-squared test. Data from the laboratory preference assay were analyzed *via* two-tailed Welch's unequal variances t-test. Percent weight gain, pupal weight and larval period were analyzed separately *via* three-way analysis of covariance (ANCOVA), with foliage type and sex as the predictors, initial larval weight as a covariate, and all two-way interactions. We classified larvae as male or female because the sexes differ substantially in their time to and weight at pupation (Myers et al., 1998); this allowed us to analyze percent weight gain, pupal weight, and larval

period of the two sexes separately for both foliage treatment groups. Tukey's test was used to separate the mean response of the two sexes in either foliage treatment group. Figures were created using *ggplot2* (Wickham, 2016). R software v. 3.5.0 was used for all statistical analyses (R Development Core Team, 2018).

Results

Field preference survey: Adelgid-infested hemlocks experienced significantly more gypsy moth herbivory damage than scale-infested or control trees ($X^2 = 48.96$, $P < 0.0001$; Fig. 1). Nearly 40% of adelgid-infested trees lost more than half of their foliage to gypsy moth herbivory, while fewer than 10% of scale-infested trees and 5% of control trees experienced similar levels of damage. Conversely, 84% of both control and scale-infested trees experienced minimal (0-25% foliage loss) herbivory.

Laboratory preference assay: When allowed to choose between adelgid-infested and control foliage, larvae consumed an average of 37% more adelgid-infested foliage than control foliage (0.36 g $\pm$ 0.054 SE and 0.22 g $\pm$ 0.034 SE, respectively; $t_{31} = -2.17$, $P = 0.0380$).

Laboratory performance assay: Larvae reared to pupation on adelgid-infested hemlock foliage gained more weight, and pupated at a higher weight, than larvae reared on uninfested foliage (both $P < 0.05$; Fig. 2 A, B). Female larvae gained more weight, pupated at a higher weight, and took longer to pupate than male larvae (all $P < 0.05$; Fig. 2 A, B, C). Initial larval weight affected larval weight gain and weight at pupation, but not larval period.

Female larvae reared on adelgid-infested foliage gained 256% of their initial weight, while those fed control foliage gained 120% of their initial weight ($P < 0.001$; Fig. 2 A). Male

larvae reared on adelgid-infested and uninfested foliage gained 115% and 67% of their initial weight, respectively ($P < 0.001$; Fig. 2 A).

Female larvae reared on adelgid-infested foliage pupated at weights 25% greater than those reared on uninfested foliage ($F_{1,36} = 12.5$, $P = 0.0011$; Fig. 2 B). Conversely, male larvae reared on adelgid-infested and control foliage pupated at similar weights ($P = 0.88$; Fig. 2 B).

Larval period was not affected by treatment or initial weight (both $P > 0.4$; Fig. 2 C), although female larvae reared on adelgid-infested hemlock foliage had a larval period five days longer than that of male larvae reared on uninfested foliage ($P = 0.0249$; Fig. 2 C).

Discussion

Here we present evidence that one destructive forest pest, hemlock woolly adelgid, facilitates the development of the invasive gypsy moth. We found that gypsy moth larvae prefer hemlock foliage infested with hemlock woolly adelgid (Fig. 1), and that feeding on this infested foliage facilitates gypsy moth larval development. Female larvae reared on adelgid-infested hemlock foliage gained more than twice as much of their initial weight (Fig. 2 A) and pupated at 25% higher weights (Fig. 2 B) than larvae reared on uninfested foliage. Male larvae reared to pupation on adelgid-infested foliage also gained 48% more weight than those fed uninfested foliage (Fig. 2 A) but pupated similar weights (Fig. 2 B). Additionally, gypsy moth larvae exhibited a preference for adelgid-infested foliage over uninfested foliage, and in a natural setting, adelgid-infested hemlocks experienced substantially more gypsy moth herbivory than uninfested trees. Our results are consistent with findings from previous studies documenting a facilitative effect of hemlock woolly adelgid on other leaf-chewing herbivores.

The enhanced performance of gypsy moth larvae reared on adelgid-infested foliage may result from adelgid-induced changes to hemlock defenses. Adelgid infestation of hemlock increases foliar emissions of methyl salicylic acid (Pezet *et al.*, 2013; Pezet & Elkinton, 2014) and triggers salicylic acid (SA) accumulation in needles (Schaeffer *et al.*, 2018; Rigsby *et al.*, 2019), activating SA-linked stress responses in hemlock. The accumulation of SA, and subsequent monomerization of NPR1, has been shown to inhibit jasmonic acid (JA) biosynthesis and JA-responsive gene expression (Zarate *et al.*, 2007). Plant defense against leaf-chewing herbivores is primarily mediated by JA (Gilbert & Liebhold, 2010; Kroes *et al.*, 2014), and blocking the induction of JA-related defenses may make the foliage of adelgid-infested hemlocks more digestible and/or easily converted into body mass by gypsy moth larvae. This interpretation is supported by work on other systems where negative cross-talk between these pathways has been shown to improve the performance of a later-arriving herbivore (reviewed in Stam *et al.*, 2014).

The improved performance of gypsy moth may also be driven by enhanced foliar nutritional quality in adelgid-infested hemlocks. Hemlock needles on adelgid-infested stems are higher in nitrogen, suggesting that hemlock woolly adelgid may turn needles into nitrogen-rich sinks. For instance, amino acid content in adelgid-infested hemlock foliage has been measured at levels 3.3-fold greater than uninfested foliage (Gomez *et al.*, 2012). Nitrogen plays a key role in the development and fecundity of herbivorous insects (Awmack & Leather, 2002; Kerslake *et al.*, 1998). High concentrations of dietary nitrogen have been shown to increase gypsy moth larval survival and pupal weights (Lindroth *et al.*, 1997), and gypsy moth fecundity has been positively correlated with host plant foliar nitrogen content (Hough & Pimentel, 1978). This is consistent with prior work in this system by Wilson *et al.* (2016) that found hemlock looper

larvae reared on adelgid-infested foliage had higher early-instar survival and attained higher pupal weights than larvae reared on uninfested foliage.

Adelgid-infested hemlocks in our common garden planting experienced significantly higher rates of defoliation compared to both control (herbivore-free) and scale-infested trees (Fig. 1). Laboratory choice-assays confirmed that gypsy moth larvae preferentially feed on adelgid-infested hemlock foliage. In addition to documenting increased overall nitrogen and amino acid concentrations in adelgid-infested hemlocks, Gomez *et al.* (2012) reported substantial increases in levels of the amino acid proline. Proline can act as an indicator of plant stress (Mattson & Haack, 1987), and is an important source of stored energy for insects (Gäde & Auerswald, 2002). In this case, elevated proline content in adelgid-infested hemlocks may act as a phagostimulatory signal of vulnerability and elevated nutrient content. This pattern has been documented in other plant-insect systems, particularly for various Hemiptera, Lepidoptera, Orthoptera and mite species (Mattson & Haack, 1987).

The fact that adelgid feeding enhances gypsy moth preference for, and performance on, eastern hemlock, makes it likely that their co-occurrence on hemlock can additively stress and further threaten this important conifer. In southern New England, adelgid infestation has caused extensive mortality of overstory hemlocks (Eschtruth *et al.*, 2006; Orwig *et al.*, 2002; Preisser *et al.*, 2008), altering understory conditions that put hemlock seedlings at a competitive disadvantage (Orwig *et al.*, 2013; Orwig *et al.*, 2008). Hemlocks are adapted to cool microclimates and low light levels (Hadley, 2000), and increased light exposure due to crown thinning and mortality of mature trees inhibits recruitment of hemlock seedlings and favors establishment of black birch (*Betula lenta*) and other deciduous trees (Ingwell *et al.*, 2012; Orwig & Foster, 1998; Orwig *et al.*, 2002). Preferential feeding by gypsy moth larvae on adelgid-

infested overstory hemlocks may exacerbate this effect, reducing the likelihood of new hemlock recruits eventually repopulating devastated hemlock forests. The damage inflicted by gypsy moths on adelgid-infested hemlock saplings may further compromise regeneration. Over a four-year period, hemlock regeneration in adelgid-infested forests declined by 46% (Preisser *et al.*, 2011). Feeding by both species may accelerate this decline, if inhibited seedling recruitment is coupled with significant mortality of juvenile hemlock saplings.

Enhanced performance of gypsy moth larvae on adelgid-infested hemlock may also have a cascading effect on other plant taxa that grow with hemlock in forests of the eastern U.S. Oaks (*Quercus* spp.) are a preferred host of gypsy moth (Barbosa *et al.*, 1979; Hough & Pimentel, 1978), and feeding by gypsy moth larvae has caused extensive mortality and decline of overstory oaks throughout this region (Gandhi & Herms, 2010). Total basal area of overstory oaks has decreased due to gypsy moth herbivory, and mortality of white oak (*Quercus alba*), northern red oak (*Quercus rubra*), and chestnut oak (*Quercus montana*) specifically has increased by 40% (Fajvan & Wood, 1976). Gypsy moth herbivory in southern New England forests has increased oak mortality and reduced the growth of surviving canopy trees by as much as 65% (Gottschalk *et al.*, 1990). It is plausible that the enhanced growth of female gypsy moth larvae on adelgid-infested hemlock may translate to greater fecundity, which could increase gypsy moth population densities in southern New England forests. Since tree mortality increases as the intensity and frequency of gypsy moth defoliation increases (Davidson *et al.*, 1999), larger gypsy moth populations here could speed oak decline.

It is important to realize that ecological traits of the gypsy moth larvae used in the laboratory performance assay may not be comparable with those of wild gypsy moth larvae. Larvae used in the laboratory performance assay were part of the New Jersey Standard Strain-

APHIS substrain, a mass-reared colony of gypsy moth larvae that has been in cultivation for >60 generations. Because this colony is intended for research, certain selective regimes and control measures have been enacted upon it to maximize the survival and fecundity of the gypsy moths. These include laboratory selection for higher survival and fecundity, and an artificial diet (Frontier Agricultural Sciences, USDA Hamden Formula), which may incidentally select for genotypes that show reduced performance on a natural diet (Grayson *et al.*, 2015). However, a comparison of development between gypsy moth larvae from the New Jersey Standard Strain-APHIS substrain, -FS substrain, and three wild populations all reared on a natural diet, found no population-level differences in male and female pupal weights (Grayson *et al.*, 2015). Additionally, our observation of substantial wild gypsy moth larval herbivory damage to adelgid-infested field-grown eastern hemlocks, as well as a confirmed wild gypsy moth larval preference for adelgid-infested hemlocks, mirror results from the laboratory performance assay and further support their ecological relevance.

Adelgid-induced hemlock mortality has severely affected ecosystem dynamics in eastern U.S. forests. Hemlock supports critical habitat for unique vertebrate and invertebrate communities (Ellison *et al.*, 2010), and dramatic shifts in understory vegetation, soil nutrient cycling and hydrological regimes may have long-lasting changes that compromise these areas (Orwig *et al.*, 2008). Future work should evaluate the extent to which adelgid and gypsy moth act synergistically to speed the decline of eastern hemlock and other canopy-dominant species, and the impact this could have on hemlock-associated ecosystems.

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Contribution of Authors

IGK, ELP, CMR, and CMO were responsible for project design. IGK, AKB, ERW, SKS, EES, and ELP were responsible for data collection and analysis. IGK, ELP, AKB, CMR, and CMO were responsible for paper writing.

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457

Figure Legends

Figure 1. Gypsy moth larval herbivory damage to eastern hemlocks in adelgid (*Adelges tsugae*)-infested, uninfested control, and scale (*Fiorinia externa*)-infested treatment groups. Damage was quantified on a scale of 0-3, representing % foliage loss of trees in each treatment group (0=0-25% foliage loss, 1=26-50%, 2=51-75%, 3=76-100%).

Figure 2. Percent weight gain (A), pupal weight (B), and larval period (C) of gypsy moth larvae reared on either adelgid-infested or uninfested control hemlock foliage. Bars represent means $\pm$ 1 SE; F = female larvae, M = male larvae. Capital letters denote significant treatment-level differences ($P < 0.05$).

478

Figure 1.

479

480 **Figure 2.**