Physiological responses of eastern hemlock (*Tsuga canadensis*) to light, adelgid infestation, and biological control: Implications for hemlock restoration

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ABSTRACT

The rapid loss of eastern hemlock (*Tsuga canadensis*) in the southern Appalachian Mountains due to hemlock woolly adelgid (*Adelges tsugae*, HWA) infestation has resulted in substantial changes to ecosystem structure and function. Several restoration strategies have been proposed, including silvicultural treatments that increase incident light in forest understories. We conducted a four-year manipulative field experiment on surviving midstory hemlock trees to investigate the effects of release from light limitation on HWA infestation and physiological parameters, expecting that higher light levels would improve tree carbon balance. Mixed hardwood forest sites were either previously uninfested with HWA (UN), infested with HWA (I), or infested with HWA and had a history of predatory beetle releases for biological control (I + P). At each site, we identified ten eastern hemlock trees in the mid-story and cut ~15 m radius canopy gaps around half of them while leaving the canopy intact over the other half. We compared short- and long-term indices of carbon gain and stress: leaf net photosynthesis (A_{net}); leaf fluorescence (F_v/F_m); leaf total non-structural carbohydrate concentration ([TNC]); new shoot growth; HWA density; and basal area growth. We found that trees experienced greater leaf-level stress in gaps and when HWA was actively feeding. Despite being more stressed, trees in gaps fixed 2x more carbon than those in reference conditions. High A_{net} in the spring translated into high leaf [TNC] in the spring, coinciding with when HWA was actively feeding. Although infested and uninfested trees had similar leaf [TNC] maxima, infestation prevented trees from allocating this carbon to shoot and basal area growth; this was particularly true for reference trees. Greater shoot growth in gap trees translated to greater annual basal area growth—by the end of the study, trees in gaps were growing 9x more than trees in reference conditions, and this was generally regardless of infestation status. In terms of growth and carbon balance, eastern hemlock consistently benefited from the increased light and soil moisture found in gaps; there was inconsistent and rather weak evidence that predator beetles conferred an additional advantage. Our results indicate that silvicultural treatments may improve long-term health and survival of infested trees and that integration of such treatments with existing strategies is worthy of continued exploration.

1. Introduction

In the southern Appalachians, hemlock woolly adelgid (*Adelges tsugae* Annand, HWA) infestation has had substantial ecological and social effects. Widespread infestation of the region during the 2000s led to rapid eastern hemlock (*Tsuga canadensis* (L.) Carrière) mortality; in some watersheds, 97% of the eastern hemlock population died within 10 years after initial infestation (Dharmadi et al., 2019). The rapid loss of this foundation species, which was often concentrated in riparian zones, had significant impacts on regional hydrologic processes (Ford and Vose, 2007; Brantley et al., 2013; Brantley et al., 2015) and ecosystem properties in the region (Nuckolls et al., 2009; Ford et al., 2012;

Dharmadi et al., 2019). Large-scale restoration of eastern hemlock across the southern Appalachians has been a strategic goal of land management agencies (Cerretani, 2011; NC Forest Service, 2013; USDA Forest Service, 2015; U.S. Department of the Interior, 2018). Nonetheless, there are currently no effective landscape-scale strategies for restoring eastern hemlock, and little evidence of resistance to HWA in this species to-date (Bentz et al., 2008; Montgomery et al., 2009), but see (Ingwell and Preisser, 2011; McKenzie et al., 2014).

HWA produces two generations per year that feed on eastern hemlock—one in winter (sistens) and another in spring (progrediens). Adult sistens females lay eggs in ovisacs in late winter/early spring; these hatch in March and April as mobile 'crawler' progrediens nymphs that settle at the bases of hemlock needles. The progrediens generation feeds throughout the spring and lay eggs in May and June (Havill et al., 2014). Sistens eggs will hatch in June and July, producing nymphs that settle and enter dormancy until October. They then break dormancy and begin feeding and developing throughout the winter. Typically, many more eggs and nymphs are produced in late winter/early spring than in late spring/summer (Havill et al., 2014). Peak feeding pressure on an eastern hemlock is in the spring (when both adelgid generations occur and overlap), followed by late fall and winter. Nymphs feed by inserting their long mouthparts into xylem ray parenchyma cells at the hemlock needle base (Young et al., 1995).

Xylem ray parenchyma cells play important functional roles, including storing and transporting solutes and carbohydrates, loading and unloading solutes into and from the transpiration stream, and storing water reserves (Secchi et al., 2017). Depletion of carbohydrates from xylem ray parenchyma cells by HWA should impact both carbon and water cycling in infested trees, including lowering carbon reserves and loss of ability to refill embolized xylem conduits (Secchi et al., 2017). These effects may be most apparent in trees that already have a marginal carbon balance due to unfavorable environmental conditions, such as deep shade and high competition for soil resources. Indeed, earlier studies show that smaller hemlock trees in more shaded forested conditions have higher mortality rates from HWA than larger or more dominant canopy trees (Orwig and Foster, 1998), and the greater the HWA infestation, the lower the leaf water potential (Coots et al., 2015) and stomatal conductance (Domec et al., 2013; Rubino et al., 2015).

While hemlock mortality is widespread, there are anecdotal accounts of eastern hemlock survival despite infestation, particularly when the trees are receiving full or elevated levels of sunlight. A growing body of work is beginning to confirm this pattern and the mechanisms behind it. Recent studies suggest that both high light (Mayfield and Jetton, 2013; Hickin and Preisser, 2015; Brantley et al., 2017; Lapham et al., 2018) and high temperature (Mech, 2015; Sussky and Elkinton, 2015) regimes may negatively affect HWA success. On artificially-infested seedlings across a range of shade treatments, HWA density increased with decreasing light, and was greatest in 90% shade (Brantley et al., 2017). Similarly, decreased HWA density and increased new shoot growth has been demonstrated on unshaded trees (McAvoy et al., 2017). As such, silvicultural treatments such as forest thinning that increase light exposure on surviving hemlock (henceforth silvicultural release) may help reduce HWA abundance and improve eastern hemlock carbon (C) balance and overall vigor.

Silvicultural treatments could potentially complement and integrate with existing management strategies for HWA control, which currently include chemical insecticides and biological control. Insecticide treatments are widely used in forests and other landscapes and some products (such as the neonicotinoid insecticide imidacloprid) can provide multiple years of protection with a single application (Benton et al., 2016). However, these insecticides are applied on an individual tree basis, and associated costs and environmental concerns prohibit chemical control as a long-term, stand-alone strategy (Vose et al., 2013). Classical biological control of HWA with predaceous beetles has been pursued since the 1990s, beginning with widespread release of *Sasajiscymnus tsugae* (Sasaji and McClure) from Japan (Cheah, 2011),

followed by *Laricobius nigrinus* Fender from the North American Pacific region (Mausel et al., 2010), and *Laricobius osakensis* from Japan (Toland et al., 2018), and most recently with silver flies (*Leucopis argenticollis* (Zetterstedt) and *Leucopis piniperda* (Malloch)) from the Pacific Northwest US (Motley et al., 2017). The beetle species have established in eastern forests, but the persistence of hemlock decline in areas of predator release suggests that biological control alone may be insufficient to sustain healthy eastern hemlock populations (Mayfield et al., 2015).

We contend that if eastern hemlock trees infested with HWA incur carbon losses from feeding that ultimately contribute to morbidity and mortality, then improving the tree's carbon balance could work alone, or in concert with biological control efforts to mitigate against HWA-induced mortality, improve tree growth, and enhance stand recovery. Here we conducted a four-year manipulative field experiment on surviving mid-story hemlock trees to investigate the effects of release from light limitation on HWA infestation and physiological parameters, expecting that higher light levels would improve leaf-to-tree carbon balance. We measured the following short- and long-term indices of C gain and stress: leaf net photosynthesis (A_{net}); leaf fluorescence (F_v/F_m); leaf total non-structural carbohydrate concentration ([TNC]); HWA density; new shoot growth; and basal area growth. We expected that: release from light limitation would enhance a tree's instantaneous C gain; HWA would disrupt a tree's ability to utilize the additional C regardless of improved light; but sites with both HWA and a history of predator beetle releases would be able to translate high instantaneous C gain into improved shoot growth and higher whole-tree C gain.

2. Methods

2.1. Site and treatment descriptions

The experiment was conducted in southern Appalachian, riparian, mixed hardwood stands with live eastern hemlock present in the understory during 2014–2018. Overstory tree composition ranged from hardwood-dominated to live eastern white pine/dead hemlock dominated to live hemlock dominated, depending on site. In 2014 we located three experimental sites in NC and TN that were either uninfested or infested with HWA, and either with or without a history of predator beetle releases (*Sasajiscymnus tsugae*) (Table 1). The infested site was Dryman Fork Basin, adjacent to the Coweeta Hydrologic Lab in Otto, NC. The infested with predator beetle site was in the Blue Valley Experimental Forest. Both sites are in the Nantahala National Forest, in western North Carolina. The uninfested site was on the southwestern fringe of eastern hemlock distribution, in Bledsoe State Forest, near Pikeville, TN (Fig. 1), and had uninfested, healthy hemlock trees in the overstory and understory. In July 2016, the uninfested site had newly discovered presence of HWA.

We identified 10 trees per site ranging in diameter at breast height (DBH, 1.3 m above ground level) from 4.1 to 17.1 cm (10.0 cm average) and tagged individuals at *ca.* 1.3 m above ground height (Table S1). In each stand (uninfested, infested, and infested plus predator beetles, henceforth UN, I, I + P), we targeted half of the eastern hemlock trees to have gaps created around them, with the tree being in the center of the gap. Stands around reference trees were left intact, with a minimum buffer between treatment and reference trees of ≥30 m distance from treatment gaps. Gaps ranged from 0.073 to 0.2 ha and were either circular or oval in shape depending on how many trees were in the gap and their location to one another. Six of the seven gaps had 2–3 hemlock trees in them, and branches and crowns of these trees did not overlap. All non-hemlock trees within 15.25 m of a treatment tree were cut. Gaps were created using a chainsaw in the I site in February 2014, and in the I + P and UN sites in July–August in 2014. In March 2016, stumps in I and I + P sites were re-cut and herbicide was applied to cut stumps (see Fig. S1 for before photos of treatment gaps and trees). In September 2016 herbicide was broadcast across the competing

Table 1
Location, elevation, and climate for three sites in the southern Appalachian Mountains with varying combinations of hemlock woolly adelgid infestation and predator beetle release.

Site type	Location	Elevation (m above sea level)	Mean annual temperature (°C)*, and extreme range during study	Mean annual precipitation (mm)*, and annual range during study
I, Infested ~2004	35.032, −83.434	880	13.1, −20–34[a]	2071, 1528–2877
I+P, Infested ~2004; predator beetles released 2005, 2014, and 2015	35.006, −83.247	770	11.0, −23–29[b]	2185, 1430–3118
UN, Not infested in 2014, HWA first detected 2016	35.690, −85.254	530	17.5, −20–36[c]	1423, 1103–1870

* 30 year average, 1989–2018
[a] CS017 at Coweeta Hydrologic Lab, Otto, NC;
[b] USC00314055 in Highlands, NC;
[c] USC00407184 in Pikeville, TN

vegetation in the I and I+P sites. In March 2017, the UN site's gaps were also maintained by mechanically clearing understory woody shrubs; stump sprouting was not as prolific at this site and thus herbicides were not needed to maintain gaps.

Each year in the spring, just above the tag nail, we measured and recorded diameter (DBH). From DBH we estimated basal area increment (BAI) assuming a circular shape and estimated annual BAI growth by subtracting the current year's BAI from the previous year's BAI. If subsequent year's DBH was equal to or smaller than the previous year's DBH, we set growth values to zero.

2.2. Predator beetle releases and recovery

The I+P site had a history of predatory beetle releases (Table 1). At this site, in four locations in April/May of 2005 *ca.* 10,000 predator beetles (*Sasajiscymnus tsugae* Sasaji and McClure) were released. To augment these initial releases, an additional 520 *S. tsugae*, 100 *Laricobius nigrinus* Fender, and 2000 *S. tsugae* were released directly onto the 10 study trees at the I+P site in June 2014, December 2014, and December 2015, respectively. Post-release presence of *S. tsugae* was confirmed on May 21st 2015 and May 9th 2016 using beat sheets (70 cm × 70 cm). In 2015 only one larva was recovered from *ca.* 36 person hours of beat sheet sampling between May 6th and June 22nd on all study trees and other hemlocks within *ca.* 50 m of study trees. In 2016, nine larvae and one adult were recovered from 2 person hours of beat sheet sampling on the treatment trees in the northwest facing gap. Specimens were positively identified as *S. tsugae* at the University of Tennessee (Drs. Pat Parkman and Greg Wiggins, pers. comm). We did not sample the other sites and trees for beetle larvae or adults.

The I+P site was also adjacent to a stand of planted, mature, eastern white pine (*Pinus strobus* L.) trees. In 2015, we positively identified a native *Laricobius* species (*Laricobius rubidus* Le Conte) on eastern hemlock trees in the gaps. While *L. rubidus* feeds mainly on native pine bark adelgid, *Pineus strobi* Hartig, it can feed and develop on HWA as well as interbreed with *L. nigrinus* (Fischer et al., 2015).

2.3. Leaf-level physiology

Leaf-level, instantaneous, steady-state (*i.e.*, dark-adapted) chlorophyll fluorescence, and ambient net photosynthesis (A_{net}, μmol CO_2 m^{-2} leaf area s^{-1}) were measured four times per year, in the spring (April–May), summer (June–July), early fall (September–October), and late fall (November–December) (LI-6400-40, Li-Cor Biosciences, Lincoln, NE, USA). On opposite sides of each tree, fluorescence and photosynthesis measurements were taken on each of the current and previous year's needle cohorts in summer, early fall, and late fall seasons. In the spring season, only the previous year's cohort was measured because the current year's growth had not yet grown. Results were subsequently averaged across cohorts (*i.e.*, results were not presented for each cohort because we wanted to present an integrated measure for the tree's canopy). Needles were dark-adapted for ≥30 min by wrapping them in aluminum foil before fluorescence measurements were taken. Maximum quantum efficiency of Photosystem II (PSII, F_v/F_m, unitless) was calculated as $(F_m − F_o)/F_m$, where F_o is the fluorescence signal (relative units) when all reaction centers are open and F_m is the fluorescence signal (relative units) under a saturating light source that activates all reaction centers. Cuvette conditions for all measurements were as follows: leaf temperature was set to 23 °C, [CO_2] was 400 ppm, flow was 500 μmol s^{-1}, leaf fan was set to fast, and relative humidity was 50–70%. The energy balance method was used to estimate leaf temperature. Photosynthetically active photon flux density (PPFD, μmol photons m^{-2} s^{-1}) in the chamber was set to ambient conditions for the reference trees (350, 50, 50, and 200 μmol photons m^{-2} s^{-1} for the spring, summer, early fall, and late fall measurements, respectively), and set to ambient light conditions (1500 μmol photons m^{-2} s^{-1}) for the treatment trees. Gas exchange data were filtered such that
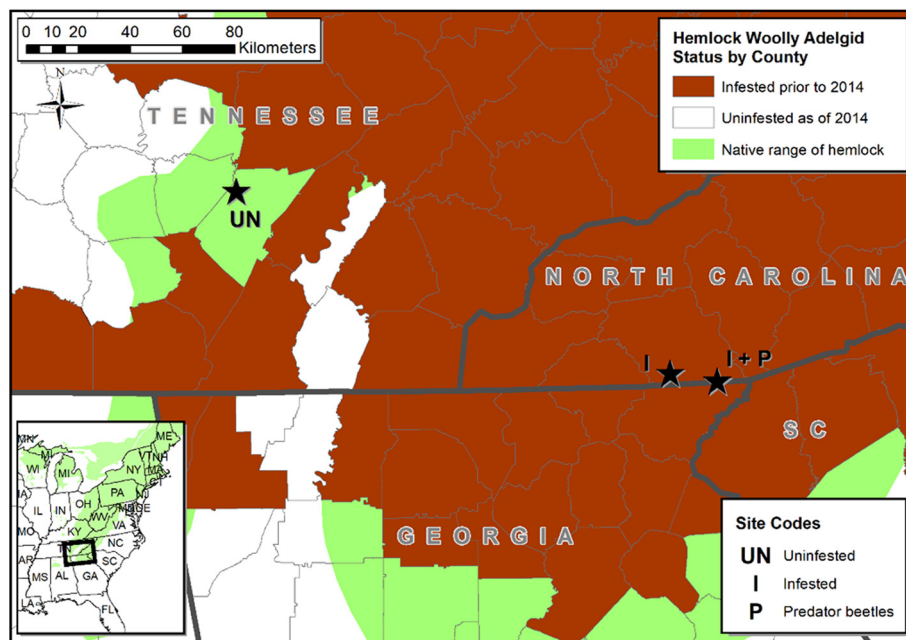
Hemlock Gap Project Sites

Fig. 1. Location of study sites in North Carolina and Tennessee that were infested (I) with hemlock woolly adelgid, had a history of predator beetle (P) releases, or were uninfested (UN) at the start of the experiment.

$0 < g_s < 0.5$, $-3 < A_{net} < 12$, $0 <$ vapor pressure deficit < 5 kPa, $0 <$ transpiration < 10 mmol H_2O m^{-2} s^{-1}, or if internal CO_2 concentration fell outside 100–500 ppm. Gas exchange measurements were accompanied by soil moisture measured 20 cm deep (Hydrosense CS620, Campbell Scientific, Inc.) at three points around the drip edge of the canopy.

Measured foliage was clipped and stored in an envelope and transported back to the lab where it was dried at 60 °C for three days and weighed (± 0.1 mg accuracy). Using a relationship between leaf area and mass (specific leaf area 81.78 cm^2 g^{-1}), we estimated leaf area in the cuvette and recalculated all gas exchange values.

2.4. Tissue carbohydrates and quality

Total soluble glucose and starch content (*i.e.*, total nonstructural carbohydrates, TNC), and nitrogen content were determined for current and previous year's leaf tissues in summer and fall, and for previous year's leaf tissue in spring seasons. TNC followed methods described by Ward and Deans (1993). Samples were collected from all trees during seasonal gas exchange measurements. Tissue samples were immediately put on ice and transferred to a freezer upon returning from the field. Samples were then dried at 100 °C for 3 h to deactivate natural enzymes before reducing the oven temperature to 70 °C for 3 days and drying to constant weight as in Brantley et al. (2017). Dried tissue was ground (8000D MixerMill) and a subsample was weighed and analyzed for C and N content (Thermo Electron Corp. Flash EA 1112 NC) following the Dumas method (USDA Forest Service, 2017) and expressed as concentrations per unit leaf dry mass. Non-structural carbohydrates were extracted on an orbital incubator and utilized water for the soluble glucose component and a separate enzymatic (amyloglucosidase, and sodium acetate) degradation of starch to soluble sugars for the stored reserves. The extracted samples were subsequently quantified colormetrically (Astoria 2 Flow Analyzer, Astoria Pacific International) and expressed as glucose concentrations on a leaf dry mass basis. Changes to the manifold described by Ward and Deans (1993) were made. These changes are described fully in Brantley et al. (2017). Results agreed well with the known starch and glucose contents of a mixed starch and

wheat standard and provided confidence to the values produced with this method of quantifying non-structural carbohydrates (certified reference samples, $n = 87$, averaged percent: actual 13.10, observed 12.94, absolute error 15.40. A two-tailed t-test for means comparing the observed with the actual yielded: $t = 0.58$, $P = 0.56$).

2.5. Shoot growth and HWA density

We examined four parameters of growth and infestation on each tree. We sampled each tree in eight locations systematically, by separating the crown into four equal quadrats in the lowest (*ca.* 1–1.5 m) and four in the highest (*ca.* 1.5–2.7 m) reachable growth of the crown. In each of the eight sections, we identified a 20 cm length of terminal shoot and its branches and on that we measured and recorded the length of all new growth (from the previous growing season), the presence of live tips without the occurrence any new growth, and the number of dead tips. On each occurrence of a tip with new growth, we measured the length of new growth and the number of HWA ovisacs (sistens generation) on that growth. All lengths were measured to the nearest 0.5 cm. Measurements were made on each tree in each site during the spring season in years 2015–2018. Measurements of new growth length and frequencies of dead tips and no new growth were summed for each 20 cm branch and averaged for each tree. HWA counts were expressed as density by dividing the number of ovisacs by the length of new growth and scaled to decimeters (dm).

2.6. Statistical analyses

We analyzed data as a repeated measures ANOVA with three fixed factors: time (year and season, up to four or ten levels respectively depending on the response variable, repeated factor, unstructured covariance), site (3 levels), and gap treatment (2 levels). Trees were the experimental unit, with five replicates, and treated as a random effect. It is noteworthy that infestation and predator beetle releases were our main factors of interest, but they could not be randomized nor replicated spatially. Thus, site was a fixed effect with the primary difference among sites being their differing management and disturbance

Table 2
Analysis of Variance (ANOVA) results by site and gap treatment and their interaction on soil moisture and light measurements. Shown is the F statistic with numerator and denominator degrees of freedom ($F_{ndf,ddf}$). Significant effects at $\alpha = 0.05$ are in bold font.

Dependent variable (units)	Site Effect	Treatment Effect	Site × Treatment Interaction
Light (PPFD, µmol m^{-2} s^{-1})	$F_{2,46} = 7.30$	$F_{1,46} = 60.39$	$F_{2,46} = 0.026$
Soil moisture (%, v/v)	$F_{2,46} = 9.17$	$F_{1,46} = 12.87$	$F_{2,46} = 0.020$

histories. Post-hoc tests on least squares means were conducted as test of simple effects (*e.g.*, not all pairwise comparisons were made), and all P values were adjusted using the Tukey option. For all analyses, we used PROC MIXED (SAS 9.4, Cary, NC), with a maximum likelihood estimation, variable covariance structures to maximize convergence, and set $\alpha = 0.05$.

During sampling times when shoot growth, HWA density, leaf level physiology, and leaf [TNC] and [N] were made, we calculated simple Pearson's correlation coefficients using PROC CORR (SAS 9.4, Cary, NC). Correlations were calculated across all trees, years and sites, and significant correlations are presented at the end of each section below.

3. Results

3.1. Environmental variables

Gap treatments increased light and soil moisture conditions (Table 2). Light incident on the gap trees was 6-fold greater than that on reference trees, and this was consistent among the sites (treatment effect $F_{1,46} = 60.39$, $P < 0.001$; site effect $F_{2,46} = 7.30$, $P = 0.002$; no site*treatment interaction). Average (SE) ambient PPFD during gas exchange measurements over the entire study period was 328 (44) µmol m^{-2} s^{-1} in gaps compared to 63 (18) µmol m^{-2} s^{-1} in reference conditions. Mean annual precipitation during the study period (2014–2018) was near or slightly above the 30 year means for all sites (Table 1). Average (SE) soil moisture under reference trees was 26.5 (1.8)% (v/v) while soil moisture under gap trees was 34 (1.7)%; an average difference of 8%, and this was consistent across sites (treatment effect $F_{1,46} = 12.87$, $P < 0.001$; site effect $F_{2,46} = 9.17$, $P < 0.001$; no site*treatment interaction).

3.2. Leaf fluorescence, gas exchange, and nitrogen

Leaf fluorescence varied among treatments and over time, generally with trees in gaps experiencing lesser efficiency in PSII, and all trees exhibiting decreased efficiency in colder seasons (treatment effect $F_{1,24} = 56.47$, $P < 0.001$; time effect $F_{8,24} = 333.40$, $P < 0.001$; site effect $F_{2,24} = 23.64$, $P < 0.001$; time*site*treatment interaction $F_{16,24} = 6.95$, $P < 0.001$) (Fig. 2a, Table 3). Trees growing in reference conditions at the UN site were consistently above the stress zone threshold of 0.8, regardless of season. In the summer and early fall, when HWA isn't actively feeding, trees generally had the greatest PSII efficiency. In contrast, PSII efficiency was generally lower in spring and late fall when HWA is actively feeding, with trees at the I+P site generally having higher efficiency than those at the I site. This trend was particularly evident in spring 2016 (Fig. 2a).

Net photosynthesis under ambient light conditions was greater for trees in gaps and did not decline in the summer, compared to that of reference trees which was low and declined in the summer (time effect $F_{8,175} = 38.28$, $P < 0.001$; treatment effect $F_{1,24} = 763.19$, $P < 0.001$; time*treatment interaction $F_{7,175} = 27.93$, $P < 0.001$) (Fig. 2b, Table 3). Ambient light conditions for reference trees were greatest in the spring and late fall seasons when the dominant deciduous trees above them were leafless, and corresponded with peaks in A_{net} for reference trees during these seasons (Fig. 2b). Despite gap trees having lower PSII efficiency than reference trees, their A_{net} across all seasons and sites was almost 2-fold greater.

Leaf nitrogen concentration varied seasonally, with greatest concentrations in the late fall and least in the spring (time effect $F_{8,175} = 28.45$, $P < 0.001$) (Fig. 2c, Table 3). Because the spring sampling included only previous year's growth, this seasonal pattern would not be caused by including newly-developed leaves that typically have a lower [N] than older leaves. Across all sites and sampling times, leaf [N] was greater in gaps compared to reference trees (mean [SE] 1.5% [0.01] *vs.* 1.3% [0.01]) (treatment effect $F_{1,24} = 127.48$, $P < 0.001$), and was generally greater in the I+P trees in gaps compared to all other sites (time*site*treatment interaction $F_{16,175} = 3.35$, $P < 0.001$) (Fig. 2c). Increases in leaf [N] were associated with increases in A_{net} ($r_{248} = 0.38$, $P < 0.001$) and PSII efficiency ($r_{260} = 0.30$, $P < 0.001$).

3.3. Total nonstructural carbohydrates

Total nonstructural carbohydrate concentrations in the leaves varied over time, were generally greater in trees growing in gaps, and didn't always differ among sites (Fig. 3, Table 3). Leaf [TNC] was greatest in the spring averaging 30–35% of the leaf dry mass, and coincided with the timing of active HWA feeding, and minimum leaf [N] ($r_{266} = -0.49$, $P < 0.001$) and PSII efficiency ($r_{259} = -0.76$, $P < 0.001$). Starch concentrations in spring were more than twice those measured during other sampling times (time effect $F_{8,175} = 527.32$). Trees in gaps had greater leaf starch (7.9% *vs.* 5.8%) and glucose (10.8% *vs.* 9.5%) concentrations than those in reference conditions, largely driven by the summer and early fall measurements (for both treatment effect $P < 0.001$; time*treatment interaction $P < 0.001$). Site differences were most apparent in the summer and early fall of 2014. During these times, trees in gaps in all the sites had high glucose concentrations, but starch was only high in the I+P and UN gap trees (*i.e.*, trees in gaps in the I site had high glucose but not starch). These site differences largely disappeared during the remainder of the study period.

3.4. Shoot growth, lack of growth, dead tips, and HWA density

New shoot growth varied among years, and was generally, but not consistently, greater in the gap trees compared to the reference trees (time effect $F_{3,24} = 25.26$, $P < 0.001$; treatment effect $F_{1,24} = 47.545$, $P < 0.001$; time*site*treatment interaction $F_{6,24} = 6.95$, $P < 0.001$) (Fig. 4a, Table 3). In 2014 in the I site, gap trees had more new growth than reference trees, and more new growth than any trees in the other two sites (Fig. 4a). This ranking may reflect the timing of the treatments from the previous year, as the I site gaps were cut first, followed by the I+P and the UN gaps. Averaged across all years and sites, gap trees had 39 cm of new growth on a 20 cm length of shoot compared to 21 cm of new growth by trees in reference conditions, an 86% increase.

The cumulative frequency of no new growth on a 20 cm shoot was generally twice as high in the reference trees compared to the gap trees, but this was not consistent over time (treatment effect $F_{1,24} = 22.28$, $P < 0.001$; time*treatment interaction $F_{3,24} = 9.71$, $P < 0.001$) (Fig. 4b, Table 3). In the infested site with no predator beetles, by 2016 gap trees had a similar frequency of no new growth as reference trees (time*site*treatment interaction $F_{6,24} = 7.06$, $P < 0.001$).

The cumulative frequency of dead tips on a 20 cm shoot varied over time and was consistently more than 3x greater in the reference trees
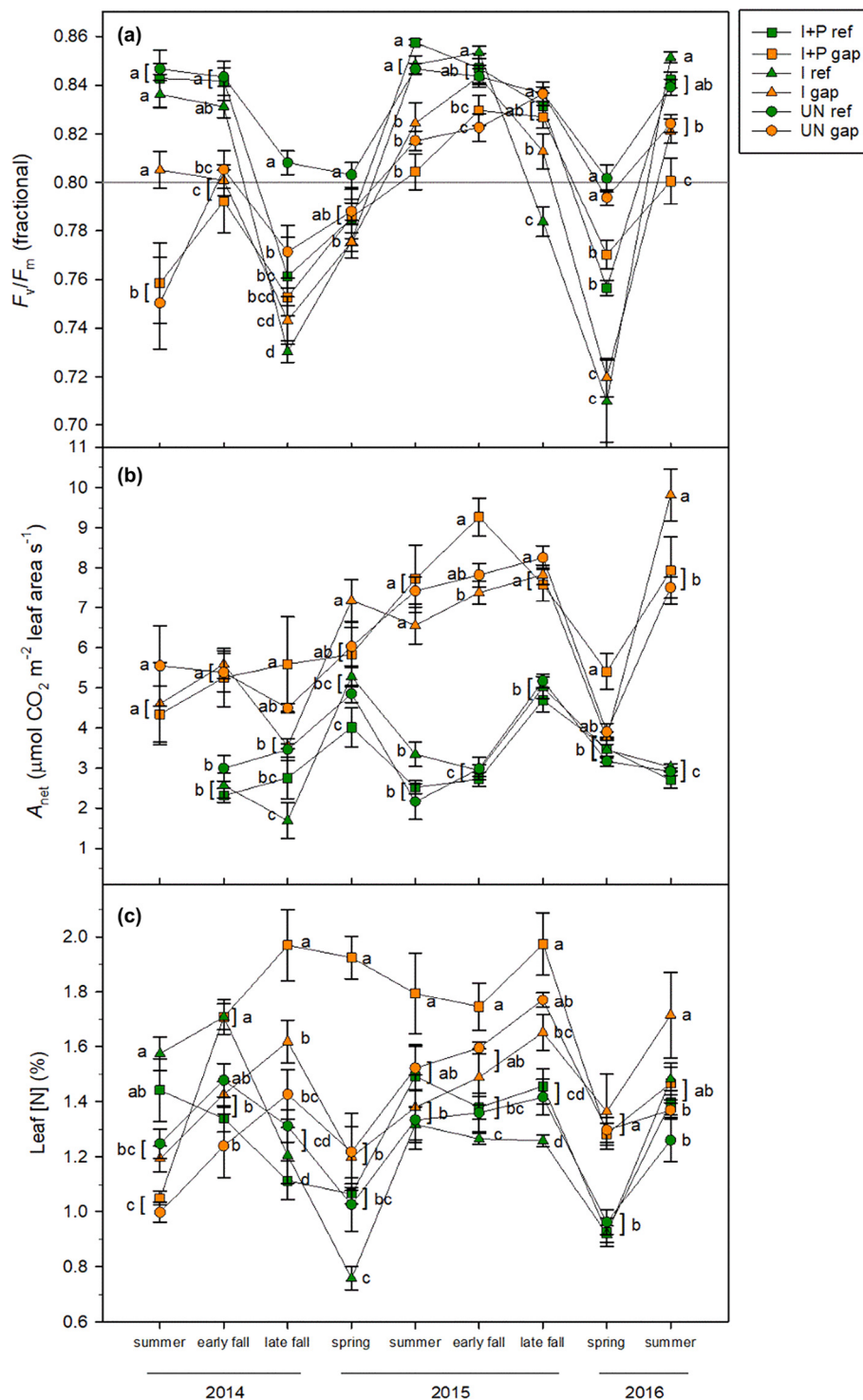
Fig. 2. Variable to maximum leaf fluorescence (F_v/F_m) (a), net photosynthesis under ambient light conditions (b), and leaf nitrogen (N) concentration (c) for trees growing in gaps (orange) or in reference forest conditions (green). Symbols sharing same lowercase letters within sampling times are not significantly different at $\alpha = 0.05$. Grey reference line in panel (a) denotes threshold, below which indicates leaf stress. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

than the gap trees (2.3 vs. 0.7) regardless of site (treatment effect $F_{1,24} = 4.25$, $P = 0.05$; time effect $F_{3,24} = 4.57$, $P = 0.01$; site effect NS; interactions NS) (data not shown, Table 3). Trees had a lower frequency of dead tips in 2014 than in 2015, with further, but non-significant, increases in 2016 and 2017.

Contrary to expectations, HWA density did not differ between the trees growing in gaps and reference conditions at any time in the I+P

site, or any site. HWA sistens ovisac density varied among years and sites, but in inconsistent ways (time effect $F_{3,24} = 26.82$, $P < 0.001$; site effect $F_{2,24} = 23.64$, $P < 0.001$; time*site*treatment interaction $F_{6,24} = 3.19$, $P = 0.02$) (Fig. 4c, Table 3). HWA density increased during the first three years in the I and I+P sites, and then declined in the final year, with a lower density at the I+P site than the I site. By the end of the study HWA density of the gap trees in the I+P site was no

Table 3
Analysis of Variance (ANOVA) results by site and gap treatment and time, and their interactions on measured dependent variables. Shown is the F statistic with numerator and denominator degrees of freedom ($F_{ndf,ddf}$). Significant effects at $\alpha = 0.05$ are in bold font.

Dependent variable (units)	Site Effect	Treatment Effect	Site × Treatment Interaction	Time Effect	Site × Time Interaction	Treatment × Time Interaction	Site × Treatment × Time Interaction
Leaf fluorescence (F_v/F_m, unitless)	**$F_{2,24} = 12.60$**	**$F_{1,24} = 56.47$**	**$F_{2,46} = 3.88$**	**$F_{8,24} = 333.40$**	**$F_{16,24} = 30.35$**	**$F_{8,24} = 29.34$**	**$F_{16,24} = 6.95$**
Net photosynthesis (A_{net}, μmol m^{-2} s^{-1})	$F_{2,24} = 0.11$	**$F_{1,24} = 763.19$**	**$F_{2,24} = 4.31$**	**$F_{8,175} = 38.28$**	**$F_{16,175} = 3.65$**	**$F_{7,175} = 27.93$**	$F_{14,175} = 1.94$
Leaf nitrogen concentration ([N], %)	**$F_{2,24} = 21.47$**	**$F_{1,24} = 127.48$**	**$F_{2,24} = 15.52$**	**$F_{8,175} = 28.45$**	**$F_{16,175} = 6.09$**	**$F_{8,175} = 21.47$**	**$F_{16,175} = 3.35$**
Leaf total nonstructural carbohydrate concentration ([TNC], fractional)	$F_{2,24} = 0.60$	**$F_{1,24} = 146.68$**	**$F_{2,24} = 5.24$**	**$F_{8,155} = 567.60$**	**$F_{16,175} = 8.12$**	**$F_{8,175} = 39.00$**	**$F_{16,175} = 2.99$**
Leaf starch concentration (fractional)	$F_{2,24} = 2.43$	**$F_{1,24} = 57.90$**	**$F_{2,24} = 5.05$**	**$F_{8,175} = 527.32$**	**$F_{16,175} = 11.05$**	**$F_{8,175} = 32.66$**	**$F_{16,175} = 3.36$**
Leaf glucose concentration (fractional)	**$F_{2,24} = 10.61$**	**$F_{1,24} = 111.27$**	$F_{2,24} = 2.19$	**$F_{8,175} = 228.33$**	**$F_{16,175} = 7.16$**	**$F_{8,175} = 5.68$**	$F_{16,175} = 1.08$
New shoot growth (cm)	$F_{2,24} = 1.16$	**$F_{1,24} = 47.54$**	$F_{2,24} = 3.37$	**$F_{3,24} = 25.26$**	**$F_{6,24} = 13.20$**	**$F_{3,24} = 17.34$**	**$F_{6,24} = 6.95$**
Cumulative frequency of no new growth (counts)	$F_{2,24} = 0.01$	**$F_{1,24} = 22.28$**	**$F_{2,24} = 5.39$**	**$F_{3,24} = 22.03$**	**$F_{6,24} = 15.73$**	**$F_{3,24} = 9.71$**	**$F_{6,24} = 7.06$**
Cumulative frequency of dead tips (counts)	$F_{2,24} = 1.33$	**$F_{1,24} = 4.25$**	$F_{2,24} = 3.25$	**$F_{3,24} = 4.57$**	$F_{6,24} = 1.40$	$F_{3,24} = 1.34$	$F_{6,24} = 2.42$
HWA ovisac density (counts dm^{-1})	**$F_{2,24} = 23.64$**	$F_{1,24} = 1.68$	**$F_{2,24} = 9.34$**	**$F_{3,24} = 26.82$**	**$F_{6,24} = 10.80$**	$F_{3,24} = 2.74$	**$F_{6,24} = 3.19$**
Basal area increment (cm^2 yr^{-1})	$F_{2,24} = 0.83$	**$F_{1,24} = 57.44$**	$F_{2,24} = 1.10$	**$F_{3,24} = 39.21$**	$F_{6,24} = 2.12$	**$F_{3,24} = 25.08$**	**$F_{6,24} = 2.76$**

different than the UN gap trees. Trees in the UN site became infested in 2016. HWA density increased with increasing frequency of no new growth ($r_{120} = 0.22$, $P = 0.01$), possibly reflecting increased density on the few new shoots that were available. The cumulative number of ovisacs per sample increased with increasing length of new growth ($r_{120} = 0.18$, $P = 0.05$), but HWA density increased with decreasing length of new growth ($r_{120} = -0.23$, $P = 0.01$), again possibly reflecting lack of available settling sites.

Several growth and infestation variables were associated with physiological measures. Increases in A_{net} were associated with increases in the amount of new growth measured ($r_{70} = 0.37$, $P = 0.001$), decreases in the frequency of no new growth ($r_{70} = -0.37$, $P = 0.001$) and dead tips ($r_{70} = -0.25$, $P = 0.03$), and increases in HWA density ($r_{70} = 0.34$, $P = 0.004$). Further, in spring when HWA ovisacs were counted, increasing HWA density was associated with increasing leaf [N] ($r_{56} = 0.31$, $P = 0.02$).

3.5. Tree basal area increment growth

Trees increased growth over time and grew more in gaps compared to reference conditions, but site differences were not apparent (time effect $F_{3,24} = 39.21$, $P < 0.001$; treatment effect $F_{1,24} = 57.44$, $P < 0.001$; time*site*treatment interaction $F_{6,24} = 2.76$, $P = 0.03$) (Fig. 5, Table 3). The first year, 2014, was a low growth year for the gap trees. This could reflect the gaps being created during the first year of the experiment, with trees not experiencing the treatment conditions for the growing season. Contrary to expectations, trees in the I + P site did not grow more than the trees in the I site for any year. In fact, during 2016 tree growth for the gap trees in the I + P site was not different than the reference trees.

4. Discussion

4.1. Leaf-to-tree carbon balance in response to gaps and HWA infestation

The creation of gaps had both positive and negative effects on eastern hemlock physiology. Trees experienced greater leaf-level stress (indicated by leaf fluorescence, henceforth stress) in gaps and during cold months, when HWA was actively feeding, and when leaf [N] was lowest. Early stress in gap trees may have been associated with gap treatment implementation and their lack of being acclimated to higher light conditions. This seems particularly likely given that leaf [N] was also lower than reference trees during the first two sampling periods, but thereafter seemed to be consistently higher. Low leaf [N] has been shown to decrease the quantum yield of PSII electron transport and thus PSII efficiency (Khamis et al., 1990; Lima et al., 1999; DaMatta et al., 2002; Huang et al., 2004). The timing of HWA feeding also coincided with increased stress, and this was seen in both the gap and reference trees. HWA has been documented to induce a hypersensitive plant defense response in eastern hemlock, which increases reactive oxygen species (ROS) both locally and systemically (Radville et al., 2011). Increases in ROS and oxidative stress influence leaf chemistry, such that chlorophyll concentration decreases (Garris et al., 2019) and thus efficiency of PSII decreases.

Despite being more stressed, trees in gaps fixed 2x more carbon than those in reference conditions, and fixed more carbon when leaf [N] was high. Trees growing in reference conditions had seasonal peaks in photosynthesis and TNC in spring. Net photosynthesis for reference trees was within the range reported for hemlock seedlings growing under a range of light conditions (Ford and Vose, 2007; Brantley et al., 2017); however, rates for trees growing in gaps and greater light conditions were considerably higher. In contrast to previous studies, we did not find consistently lower A_{net} in infested trees compared to uninfested trees within any treatment (Nelson et al., 2014). We did observe a noticeable drop in A_{net} in gaps when HWA density was greatest (spring 2016), possibly reflecting HWA respiration, lower potential A_{net} given
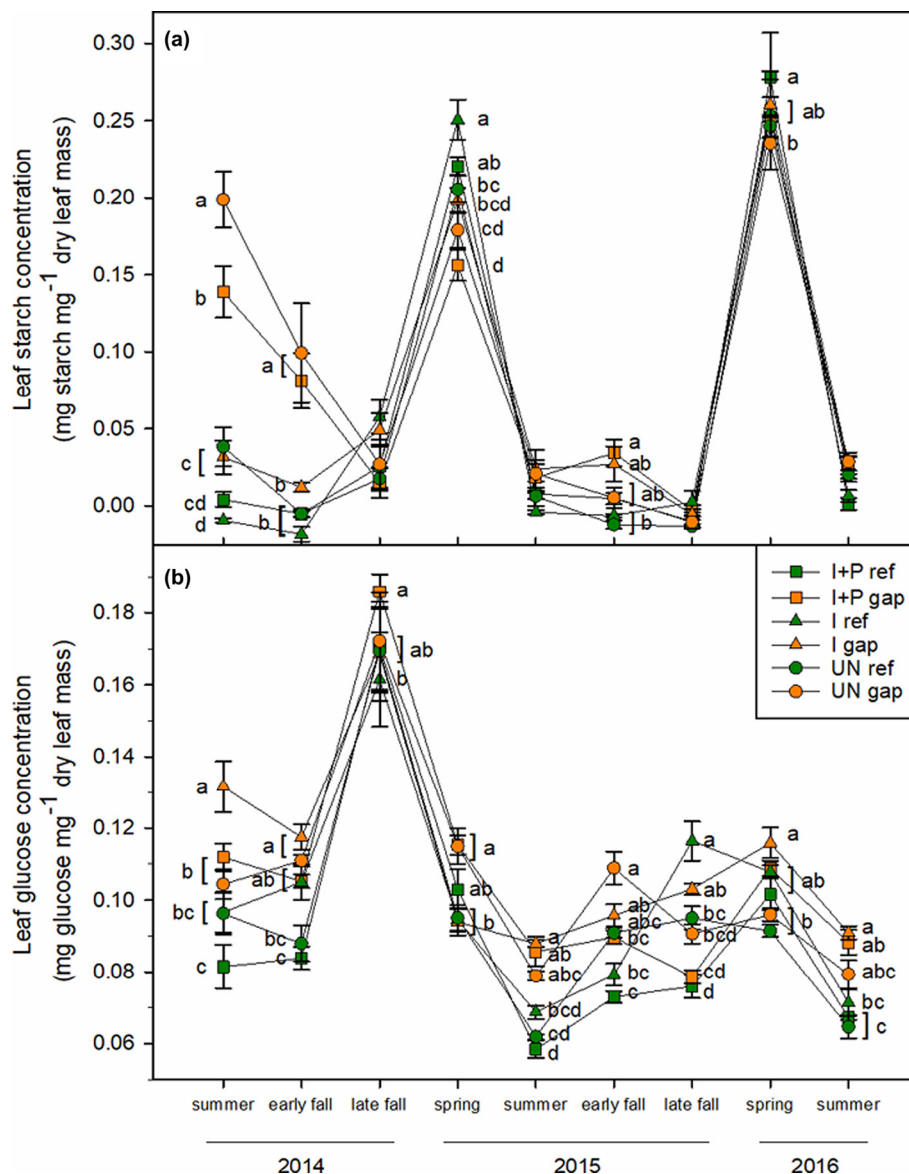
Fig. 3. Leaf starch (a) and glucose (b) concentrations for trees growing in gaps (orange) or in reference forest conditions (green). Symbols sharing same lowercase letters within sampling times are not significantly different at α = 0.05. If no lowercase letters are shown within a sampling time, no significant differences were detected. Note differences in y-axis scale. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

that leaf [N] was also lowest during this time, and or lower potential A_{net} of relatively older foliage compared to other sampling times that included newly developed leaves.

High net photosynthesis in the spring resulted in high leaf glucose and starch concentrations in the spring; this coincided with the times HWA was actively feeding. To our knowledge, this is the first study to report the seasonal dynamics of carbohydrate concentrations in leaves over multiple years in infested and uninfested eastern hemlock trees. Previous studies of hemlock leaf [TNC] were conducted only during summer, and examined dynamics between current and previous years' foliage (Schwartzberg and Montgomery, 2010; Soltis et al., 2015). Peak HWA feeding and leaf [TNC] also coincided with the lowest leaf N concentrations, and the lowest PSII efficiency. This seasonal pattern is remarkably consistent with HWA physiology, as Jones et al. (2015) found that biomass, [C], [N], [carbohydrate] and [amino acid] in HWA peaked in spring and were lowest seasonally in the winter.

Although seasonally leaf [N] was lowest when HWA was feeding, trees with high leaf [N] generally had greater HWA ovisac density in spring. It is difficult to determine if HWA was first attracted to foliage

with high [N], as is common with piercing and sucking insects (Coley, 1980; Crawley, 1983; Raupp and Denno, 1983), or if HWA had no preference and subsequently caused foliage [N] to increase as a result of feeding, as has also been reported (Gómez et al., 2012; Gonda-King et al., 2014; Williams et al., 2016; Huggett et al., 2018; Lapham et al., 2018). Increases in N-containing amino acid concentrations that result from HWA feeding do not necessarily confer greater rates of photosynthesis, as chlorophyll content decreases with infestation (Garris et al., 2019). In the present study, we found that leaf [N] was greater in gaps compared to reference trees (1.5% *vs.* 1.3%), particularly after the first two sampling periods, and this was indeed associated with greater rates of carbon gain. Higher leaf [N] in gap trees could be because we removed the canopy, thus reducing competition for uptake of this often-limiting resource. Soils were also wetter and had greater incident light, both of which could have increased N mineralization and thus availability. This suggests that thinning or gap release treatments, if implemented on a management scale in this region, could likely increase foliar quality, which is at odds with a recent study in the northeast U.S. that shows no change in foliar [N] in eastern hemlock following
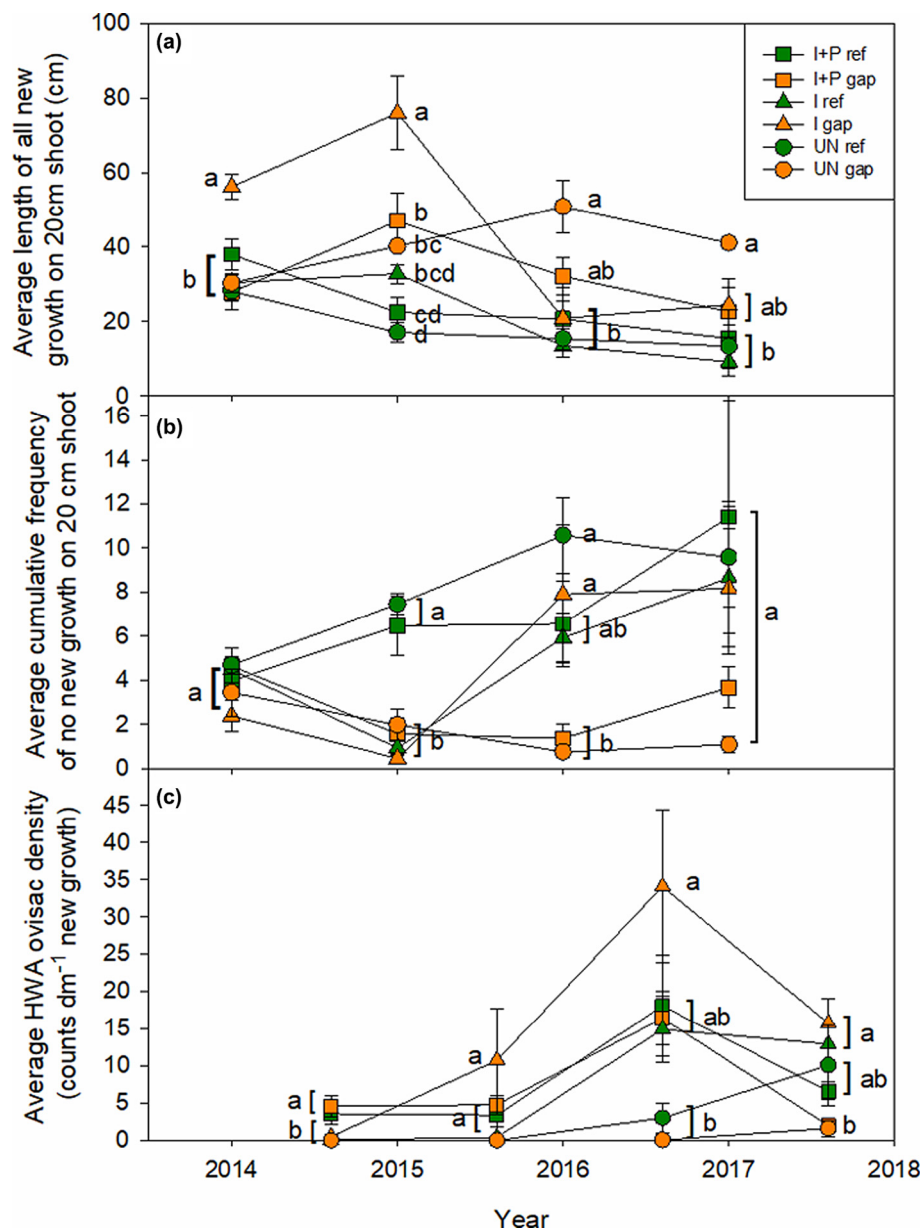
Fig. 4. Mean (SE) length of new growth (a) and cumulative frequency of no new growth on a 20 cm length of shoot; and mean hemlock woolly adelgid (HWA) ovisac density on new growth over time (c). Measurements were taken in the spring for trees growing in gaps (orange) or in reference forest conditions (green). Symbols sharing same lowercase letters within years are not significantly different at α = 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

thinning treatments (Piatek et al., 2016).

Although infested and uninfested trees had similar leaf [TNC] maxima, infestation prevented trees from allocating this carbon to shoot and basal area growth, particularly in reference trees. Infestation did impact the amount of new growth, however. Trees with greater HWA density had less new growth, and greater frequencies of no new growth and dead tips. This is consistent with previous studies reporting that HWA infested trees put on less new growth than uninfested trees (Miller-Pierce et al., 2010; Jones et al., 2015; Garris et al., 2019).

We found that gaps were highly beneficial to tree growth. Specifically, compared to reference trees, trees in gaps had: more resources (light and moisture); twice the frequency of new growth, and 86% more growth regardless of infestation status; and 3-times lower frequency of dead tips. Across all years and sites, reference trees put on *ca.* 1 cm of new shoot growth compared to *ca.* 2 cm of growth for gap trees (adjusting for length of shoot sampled). Likely the ability of gap

trees to fix more carbon than the reference trees during the time when HWA aren't feeding in the summer and early fall, translated into greater leaf [TNC] for these trees, which was allocated to new shoot and basal area growth.

Greater shoot growth in gap trees coincided with greater annual basal area growth. By the end of the study, basal area growth in gap trees was 9x that of reference trees, and this was largely regardless of infestation status. Across the four years of our study, the only observance of infested trees putting on less basal area growth than uninfested trees was in 2015 when I+P trees in gaps grew less than UN gap trees. The tapering off of the UN site's tree growth in gaps while the I +P and I site's tree growth in gaps kept increasing may reflect the few healthy hemlock trees left in the canopy at the UN site. Increasing basal area growth could be important for long-term survival of these trees, both because eastern hemlock trees with lower radial growth are more susceptible to severe infestation (Davis et al., 2007) and once infested,
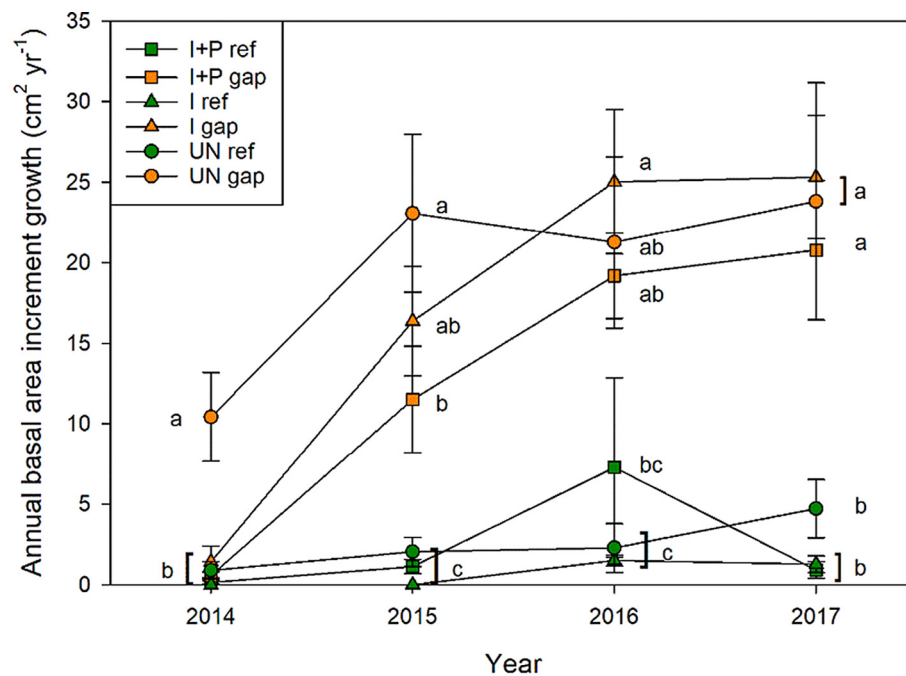
Fig. 5. Mean (SE) annual basal area increment growth over time for trees growing in gaps (orange) or in reference forest conditions (green). Symbols sharing same lowercase letters within a year are not significantly different at α = 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

trees are more likely to see consecutive years of below average radial growth (Rentch et al., 2009). This pattern in the literature of decreasing radial growth with infestation is consistent with the fact that evergreen trees primarily rely on current photosynthate to construct earlywood xylem in the spring, rather than stored photosynthate due to the latter being largely consumed by winter respiratory losses (Kozlowski, 1992). Indeed, infested trees that do put on radial growth have a lesser percentage of that growth as earlywood than uninfested trees (Domec et al., 2013). Because leaf [TNC] in spring was likely consumed by HWA feeding, trees were not able to allocate this current photosynthate to radial growth, but trees in gaps that were able to continue fixing carbon could allocate this fixed carbon to shoot and radial growth.

4.2. Did predators confer any additional advantage to infested trees?

Three lines of evidence suggest that predator beetles may have conferred an additional advantage to infested trees. First, at the infested site where predators were not released (I) the frequency of no new growth was consistently similar between gap and reference trees; whereas at the infested site where predators were released (I + P), trees in gaps had higher frequency of branches with new growth in some, but not all, years. Second, between the two infested sites, stress was less pronounced at the site with predator beetle releases at some, but not all, times when HWA was actively feeding. Lastly, in the final year of the study, gap trees at the predator release site had lower HWA density than gap trees at the non-release site.

Nonetheless, it is difficult to conclusively link these beneficial patterns to predation on HWA by the introduced beetle populations. Relatively few individuals (10 larvae, 1 adult) of *S. tsugae* were recovered at the I + P site after multiple person-hours of sampling in 2014 and 2015. The successful recovery of predator life stages indicates *S. tsugae* was established at the site, but the low numbers are consistent with other sampling efforts for this predator in the southern Appalachian Mountains that suggest such low *S. tsugae* densities are not sufficient to exert substantial control on HWA (Hakeem et al., 2010). Furthermore, the release of 100 *L. nigrinus* adults at the site in 2014 was recent relative to the study timeline, and there was no systematic effort to sample for this predator during the winter and early spring months when it is active on the trees. Future studies seeking to assess the impact of biological control on hemlock physiology would likely require

greater experimental control over the number of predators per tree and a more thorough sampling assessment.

4.3. Management implications

It is clear from our study that eastern hemlock in the under- and mid-story benefits from silvicultural release, by increasing growth, leaf [N] and [TNC] despite being initially stressed from the treatments, and being seasonally stressed when HWA is feeding. We suggest that silvicultural release could complement chemical control by improving hemlock resilience and increasing insecticide re-treatment intervals, thereby reducing both cost and environmental impact. Silvicultural release could also complement biological control by 1) slowing the rate of tree decline and thereby allowing more time for predator populations to increase in a stand, and 2) by ensuring a more consistent prey population through the maintenance of new hemlock shoots on which adelgids settle. As such, silvicultural treatments show promise as a tool in the integrated pest management approach for hemlock woolly adelgid and are worthy of continued evaluation, particularly given that these treatments may alter biodiversity and other ecosystem services of these typically deeply-shaded stands.

Because of the short-term nature of this study, we cannot know the long-term survival of these trees or the predators. Thus, we caution implementing this at a management scale before we fully understand 1) if the interaction between predators and light on HWA is overall beneficial, or if implementing silvicultural treatments would just hasten eastern hemlock's eventual mortality, and 2) if our results would apply outside the range of the inference space of this study or if they are site-specific. To address the former, controlled greenhouse studies investigating a full factorial of HWA infestation, predators, and light is warranted. While a full-scale, long-term, randomized experiment with gaps, predator beetles and HWA infestation treatments replicated across the range of eastern hemlock would address the latter, the success of such an experiment would depend on navigating several logistical and environmental challenges. These challenges include: post-release predatory beetle spread; extreme weather events that could eliminate predators and HWA where they are part of an experimental design; site heterogeneity across the range of eastern hemlock; and finally, the rapid spread of HWA could infest control stands, as happened in the present study. All these challenges make studies, such as the present

one, valuable in providing information that will help eventual restoration strategies. Because eastern hemlock's role in eastern U.S. forests is so regionally-dependent, there is also value in repeating the present study in different regions with greater variation in pre-treatment forest structure, and/or with different degrees of thinning.

CRediT authorship contribution statement

Chelcy Ford Miniat: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Supervision, Project administration, Funding acquisition. **David R. Zietlow:** Software, Validation, Formal analysis, Investigation, Data curation, Writing - review & editing. **Steven T. Brantley:** Conceptualization, Methodology, Investigation, Writing - review & editing, Supervision, Funding acquisition. **Cindi L. Brown:** Formal analysis, Investigation, Data curation, Writing - review & editing. **Albert E. Mayfield III:** Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision, Project administration. **Robert M. Jetton:** Methodology, Formal analysis, Investigation, Writing - review & editing, Supervision, Project administration. **James R. Rhea:** Conceptualization, Writing - review & editing, Funding acquisition. **Paul Arnold:** Resources, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foreco.2020.117903.

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