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Establishment, hybridization and impact of *Laricobius* predators on insecticide-treated hemlocks: Exploring integrated management of the hemlock woolly adelgid

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ABSTRACT

An integrated management approach is needed to maintain eastern hemlock (*Tsuga canadensis* (L.) Carrière) in eastern North America and to minimize tree damage and mortality caused by the invasive hemlock woolly adelgid (*Adelges tsugae* Annand). This study examined the hypothesis that chemical control with low rates of insecticide and biological control can be combined in the same stand to impact adelgid populations, prolong crown health, and allow predator proliferation. Sixty *T. canadensis* trees in northern Georgia were individually treated via soil injection with 0%, 10%, or 25% of the label rate of imidacloprid insecticide, and the biological control predator *Laricobius nigrinus* Fender was released in the stand, two and four years later. By year seven, hemlocks treated with the 25% imidacloprid rate lost their insecticide protection, had significantly better crown health and higher adelgid densities than untreated trees, and supported as many *Laricobius* predator larvae as untreated trees. In year seven, no residues of imidacloprid were detected in *Laricobius* larvae feeding on previously-treated hemlocks. Most (77%) of the predators collected on study trees were identified as *L. nigrinus*, 12% were the native congener *Laricobius rubidus* LeConte, and 11% were hybrids between the introduced and native species. The hybridization rate remained stable over time. The density of undisturbed *A. tsugae* ovisacs was twice as high on branches protected from predators as compared with branches exposed to predators. Results suggest that chemical and biological control of *A. tsugae* can be successfully integrated to help prolong hemlock health, although additional predators may be necessary to protect hemlock trees in the southern Appalachians.
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1. Introduction

Eastern hemlock (*Tsuga canadensis* (L.) Carrière) is an ecologically important North American conifer distributed from Nova Scotia, west to Minnesota, and south through the Appalachian Mountain region into northern Georgia and Alabama (Godman and Lancaster, 1990). This long-lived, shade tolerant evergreen is considered a "foundation" species due to its robust influence on the vegetative structure, faunal species assemblages, and ecological processes in the ecosystems in which it occurs (Ellison et al., 2005; Vose et al., 2013). Due to its crown architecture, potential size, and ease of cultivation, eastern hemlock also has high aesthetic value in parks, recreational areas, and residential landscapes (Holmes et al., 2010; Havill et al., 2014). The sustainability of both eastern hemlock and the southern Appalachian species Carolina hemlock (*Tsuga caroliniana* Engelm.) are threatened by the invasive hemlock woolly adelgid (*Adelges tsugae* Annand), an insect native to Asia and western North America but which was introduced to

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the eastern U.S. from Japan by 1951 (Havill et al., 2006). Hemlock woolly adelgid has caused widespread decline and mortality in all age classes of *T. canadensis* in forests from New England to the southern Appalachians, with resulting or expected impacts on forest stand dynamics (Orwig, 2002); hydrologic processes (Ford and Vose, 2007), carbon and nutrient cycling (Knoepp et al., 2011), and vertebrate and invertebrate communities (Tingley et al., 2002; Adkins and Rieske, 2013).

In North America, *A. tsugae* produces two asexual generations per year: an overwintering "sistens" generation and a spring "progrediens" generation. First instar "crawlers" of both generations disperse either actively by crawling or passively by wind, birds or other animals. Crawlers insert a thin bundle of stylet mouthparts into the twig and feed on carbohydrates stored in the ray parenchyma cells. Shortly after settling on new shoots in early summer, sistens nymphs enter an inactive aestivation period but resume development in the fall, and secrete a waxy, wool-like substance that eventually serves as an ovisac for the adult female. Brood produced by sistens adults hatch in the spring and develop into either sessile progrediens adults or winged sexuparae. In Japan, sexuparae migrate and initiate a sexual generation on spruce (*Picea* sp.) but this phase of the life cycle is unsuccessful in North America and does not contribute to adelgid population growth (McClure, 1989, 1990; Havill et al., 2014).

A number of management strategies to minimize the impacts of *A. tsugae* on *T. canadensis* are being pursued, including chemical control (Cowles et al., 2006), biological control (Onken and Reardon, 2011), silvicultural manipulations (Fajvan, 2008), host gene conservation (Jetton et al., 2013), and enhancement of host resistance (Montgomery et al., 2009). The systemic neonicotinoid insecticide imidacloprid has been used widely to provide temporary protection to individual hemlock trees and can persist at efficacious concentrations within twigs for multiple years (Cowles et al., 2006; Coots et al., 2013). Imidacloprid is metabolized within the tree to produce at least two other compounds with insecticidal properties: olefin-imidacloprid and 5-hydroxy-imidacloprid. The olefin-imidacloprid metabolite is up to ten times more toxic than imidacloprid against some species of aphids (Nauen et al., 1998) and persists in treated hemlock trees for three or more years, whereas 5-hydroxy-imidacloprid is less toxic and less persistent (Coots et al., 2013). Although use of insecticides has been a critical short-term method for saving hemlock trees, it is not a viable stand-alone management strategy for several reasons, including: (1) treatments must be applied on an individual tree basis, (2) environmental concerns limit the location and amount of insecticide that can be applied per hectare, and (3) the time, cost, and labor associated with re-treating trees at regular intervals is prohibitive.

Biological control of *A. tsugae* has focused on several predator species from the native ranges of *A. tsugae* in Asia and western North America (Onken and Reardon, 2011). One of these predators, *Laricobius nigrinus* Fender (Coleoptera: Derodontidae) is native to the northwestern U.S. and western Canada where it feeds on *A. tsugae* on western hemlock (*Tsuga heterophylla* (Raf.) Sarg.). *L. nigrinus* adults feed on the sistens generation during the fall and winter and lay their eggs in the wool of sistens ovisacs. The larvae feed on *A. tsugae* sistens adults and progrediens eggs in the spring before dropping from the tree to pupate in the duff layer (Zilahi-Balogh et al., 2002, 2003). Since 2003, *L. nigrinus* has been released and become established at numerous locations across the range of *T. canadensis*, and in some areas has reached population densities sufficient for collection and redistribution of beetles to other locations (Mausel et al., 2010; Onken and Reardon, 2011). Significant reductions in *A. tsugae* sistens densities caused by *L. nigrinus* have been documented in controlled predator–prey enclosure studies (Lamb et al., 2006) and in young hemlock plantations (Mausel et al., 2008), but data quantifying the impact of *L. nigrinus* releases on *A. tsugae* densities in natural forests are lacking. Unexpectedly, *L. nigrinus* has subsequently hybridized with *Laricobius rubidus* LeConte, the only native congener in eastern North America and a predator of pine bark adelgid (*Pineus strobi* (Hartig)) on eastern white pine (*Pinus strobus* L.). These *Laricobius* congeners and their hybrids are morphologically similar and cannot be confidently distinguished without genetic analysis (Havill et al., 2012).

Despite the successful establishment and proliferation of *L. nigrinus* in the eastern U.S., rapid decline and mortality of *T. canadensis* has occurred at a number of sites where this predator has been released (Mausel et al., 2011). Operationally, only a few hundred to a few thousand *L. nigrinus* beetles have typically been released at one time in a given forest stand, due to practical limits on the number of beetles that can be field-collected or produced in rearing laboratories. Dispersal of *L. nigrinus* populations from release points is initially slow, extending only a few hundred meters after 5 years (Davis et al., 2012). It is therefore likely to take several years before established populations of *L. nigrinus* can increase to levels capable of substantially reducing the millions of *A. tsugae* that occur annually on individual trees. By that time, however, *T. canadensis* health may deteriorate past the point of potential recovery. Without management, decline and mortality in *T. canadensis* stands can be extremely rapid, particularly in the southern Appalachians, where average crown loss and percent tree mortality have exceeded 80% as quickly as four and seven years post-infestation, respectively (Elliott and Vose, 2011; Ford et al., 2012).

These operational and biological challenges indicate a need to integrate multiple types of *A. tsugae* control strategies in the same hemlock stands. One proposed management approach is to prolong hemlock health on select trees through temporary protection with insecticide, while simultaneously establishing *L. nigrinus* on the adelgids of nearby untreated trees (Salom et al., 2011) or unprotected portions of trees treated with low rates of insecticide (Joseph et al., 2011; Eisenback et al., 2014). In the theory of this approach, by the time insecticide protection diminishes and *A. tsugae* infests the previously-treated trees, the predator population is abundant and has a better chance of protecting those trees because they are in superior health and have better potential longevity than untreated trees. Furthermore, such previously-treated trees should be a better source of prey because they have more new shoots for *A. tsugae* to infest. Such an integrated management approach has yet to be implemented widely, partly due to concerns that *L. nigrinus* might experience lethal or sublethal effects of imidacloprid exposure upon eating adelgids settled on treated trees (Eisenback et al., 2010). However, *A. tsugae* is effectively controlled on *T. canadensis* at very low foliar concentrations of imidacloprid (>120 ppb, Cowles et al., 2006) indicating that exposure to insecticidal residues on branches where adelgids are feeding may be minimal. Field studies are needed to test these assumptions and determine whether *L. nigrinus* can successfully colonize, reproduce, and impact *A. tsugae* populations on trees previously treated with imidacloprid.

In previous work at our study location, Joseph et al. (2011) demonstrated that soil injection with low rates of imidacloprid (10% and 25% of label rate) could improve hemlock crown health relative to untreated trees, while also permitting a low density of adelgids two years after treatment. In the current study we subsequently released *L. nigrinus* in the same forest plot to determine if this predator would establish and proliferate on insecticide treated trees, and also addressed the following questions:

1. *Imidacloprid effects on predator and prey densities and hemlock health:* What are the longer-term (5–7 years post treatment) effects of imidacloprid treatment rate on *Laricobius*

and *A. tsugae* densities, and what are the concurrent changes in hemlock insecticidal residues, crown health, and tree mortality over time? These questions are of interest because for chemical treatment to enhance biological control, the chemically-treated trees should, in the long run:

a. lose chemical protection, thereby supporting enough adelgid prey to maintain predator populations, and,
b. have better crown health and better survival than untreated trees.

2. *Predator hybridization:* What proportions of the established population are comprised by *L. nigrinus*, the native congener *L. rubidus*, and *L. nigrinus* × *L. rubidus* hybrids? This question is important because for *L. nigrinus* releases to be successful, their populations on hemlock must not be replaced or diluted by *L. rubidus*.
3. *Predator impact:* What is the effect of the established *Laricobius* population on *A. tsugae* sistens densities in the forest stand? This question is of interest because if *L. nigrinus* cannot reduce populations of the sistens generation in natural stands, there is little reason to continue releasing it in forest environments.

2. Materials and methods

2.1. Study site and insecticide treatments

The study was conducted on the Chattahoochee National Forest in White County, GA (34.7869°N, −83.7620°W) using the same experimental site and study trees described by Joseph et al. (2011). Sixty *T. canadensis* trees ranging 5–38 cm diameter at breast height (dbh) and 7.3–24.6 m tall (mean 15.6 m) were selected in 2006 due to their close proximity to the forest road and accessibility to an observer raised into the canopy using a hydraulic lift truck. Study trees represented understory and mid-story hemlocks in a mixed evergreen–deciduous stand dominated primarily by eastern white pine (*Pinus strobus* L.) and yellow poplar (*Liriodendron tulipifera* L.). Initial infestation of *T. canadensis* with *A. tsugae* was observed in this area beginning in 2004 (Joseph et al., 2011). Trees were arranged in 5 blocks of 12 trees, with blocks decreasing in elevation from approximately 853 m to 792 m as the road descended into the Wilks Creek drainage. Four trees in each block (20 trees per treatment) were randomly assigned to receive 0%, 10%, or 25% of the label rate (1.5 g active ingredient per 2.5 cm of dbh) of the imidacloprid insecticide Merit 75 WP (Bayer Environmental Science, Research Triangle Park, NC). Insecticide was applied on 14 November 2006 with a Kioritz soil injector (Kioritz Corp., Tokyo, Japan) using one injection point per 2.5 cm trunk dbh and dispensing approx. 30 ml solution per point (Joseph et al., 2011).

2.2. L. nigrinus releases

A total of 510 and 900 adult *L. nigrinus* were released at the study site on 19 February 2008 and 15 October 2010, respectively. In the 2008 release, *L. nigrinus* adults were obtained from the predator-rearing laboratory at the University of Georgia (Athens, GA) and 17 beetles were placed in each of 30 ventilated, 1.9 L plastic containers (Rubbermaid, Wooster, OH) with fresh *T. canadensis* terminals infested with *A. tsugae*. Beetles were kept at 12 °C and 50% RH for two days prior to release. The contents of one container were released on each of 30 *T. canadensis* trees (two trees of each insecticide treatment per block) by carefully removing the infested hemlock terminals with predators from the container and fastening them to branches in the upper crown using twist ties. Hemlock crowns were accessed using a truck equipped with a hydraulic boom lift and operator bucket (maximum working height 19.8 m). Beetles were released between 1000 and 1600 EST at temperatures <8 °C and wind speed <8 km/h.

In the 2010 release, adult *L. nigrinus* were obtained from the predator rearing facility at Virginia Polytechnic Institute and State University (Blacksburg, VA) and 30 beetles were placed in each of 30 ventilated plastic containers with fresh *T. canadensis* terminals infested with *A. tsugae*. Thirty beetles were released onto each of the same 30 trees using the method described for the 2008 release. In 2010, beetles were released between 0900 and 1600 EST at temperatures <16 °C and wind speed <8 km/h.

2.3. Predator density

Sampling for *Laricobius* spp. larvae and adults was conducted in mid-March 2011–2013. The hydraulic lift truck was used to access five separate points spaced throughout the crown of all 60 study trees. A sample for *Laricobius* adults was collected at each point by placing a canvas beat sheet (71 × 71 cm, BioQuip Products, Rancho Dominguez, CA) beneath hemlock foliage and striking the branch 10 times with a 1.6 cm-diameter wooden stick. Adult *Laricobius* dislodged onto the beat sheet were collected with an aspirator and placed into vials of 95% ethanol. Prior to each beat sheet sample, a 20 cm-long hemlock branch tip infested with *A. tsugae* sistens ovisacs was clipped to sample for *Laricobius* larvae, placed in a plastic zipper-seal bag in a cooler with blue ice, and returned to the laboratory. The cut ends of the five branch tips per tree were inserted into a 30 ml plastic shot glass (Hill & Markes Inc., Amsterdam, NY) containing floral foam (Oasis Instant Deluxe, Smithers-Oasis Company, Kent, OH) hydrated with tap water and sealed with laboratory film (Parafilm M, Pechiney Plastic Packaging, Menasha, WI). The resulting hemlock bouquet was suspended horizontally within a square-sided 1.4 L clear plastic container (Container & Packaging Supply Inc., Eagle, ID) by tightly fitting the shot glass through a 2.5 cm hole in the lid. To provide ventilation, two 5 cm holes were cut on opposite sides of the container and covered with fine (200 μm) mesh screen (Dynamesh, West Chicago, IL) using hot melt glue. Containers of hemlock foliage were kept in a biological incubator (Model I-36LL, Percival Scientific Inc., Perry, IA) at 6°: 4 °C (day: night) until the last week of March and 10°: 8 °C thereafter; photoperiod was set to 12 h: 12 h throughout the monitoring period. Containers were checked every 1–2 days for larvae of *Laricobius* spp. that dropped from the hemlock foliage (indicating completion of feeding) onto the bottom of the container. Larvae were counted and preserved for DNA extraction in 95% ethanol.

2.4. Adelgid density, hemlock health, and tree mortality

Estimates of *A. tsugae* population level, hemlock shoot dieback, and new hemlock shoot production were made in March (2011–2013) and June (2011 and 2013), coincident with the adult *A. tsugae* sistens and progrediens generations, respectively. Ten 25 cm-long branch tips, five in the upper half of the crown and five in the lower half, were sampled non-destructively per tree using the hydraulic lift truck. An adelgid density index was calculated for each crown level using a rating method similar to that of Cowles et al. (2006). The number of *A. tsugae* ovisacs on each 25 cm branch tip was counted up to a maximum of 20 ovisacs. Adelgid ratings from the five branches in each crown level were summed to produce an index with a potential value range of 0–100. Hemlock shoot health was rated on the same branches by evaluating the outer 10 shoots of each branch tip (the terminal shoot and the nine most distal side-shoots). The number of dead shoot tips, and the number of tips that represented new growth expansion from the most recent growing season, were counted. Percent dead tips and percent new shoot growth were calculated

by dividing the number of tips in each category by the total number of shoot tips rated per crown level ($n = 50$). Whole-crown estimates of adelgid density index, percent dead tips and percent new shoot growth were calculated by averaging the upper crown and lower crown ratings. In addition, tree mortality status (live vs. dead) was evaluated on 10 September 2009, 16 March 2011, 31 January 2013, and 10 February 2014.

2.5. Insecticide and metabolite residues

Residues of imidacloprid and its 5-hydroxy-imidaclopird and olefin-imidacloprid insecticidal metabolites were analyzed in *T. canadensis* twig samples collected in March (2011–2013) and June (2011 and 2013), coincident with sampling for adelgid density and hemlock shoot health. In 2011, two to three 10 cm long shoot tips were collected from five locations distributed in different parts of the tree crown. In 2012 and 2013, the collection procedure was the same except that separate collections were made for foliage with *A. tsugae* present on the branches and foliage on which *A. tsugae* was absent. For each tree, all foliage of the same infestation status (with vs. without *A. tsugae*) was combined in one plastic zipper-seal bag, placed in a cooler with blue ice, returned to the laboratory and stored in a freezer (−20 °C) until used in analysis. Sample tissue was dried in a drying oven at 60 °C for 2 h, pulverized using a coffee grinder (KitchenAid, model BCG1000OB, Shelton, CT), and placed in amber vials to prevent photodegradation (Coots et al., 2013). In addition to hemlock tissue, in 2013 approximately one third ($n = 353$) of all the *Laricobius* larvae collected as described in Section 2.3 were processed for detection of imidacloprid and metabolite concentrations, whereas approximately two thirds ($n = 693$) of the larvae were processed for genetic species identification as described in Section 2.6. Imidacloprid and metabolite concentrations were determined using high pressure liquid chromatography coupled with tandem mass spectroscopy (HPLC/MS/MS) per methodology detailed in Coots et al. (2013). Residue concentrations were converted from nanograms per gram to parts per billion (ppb) and were calculated by using the average of peak areas of each target compound and conversions for each analyte per the formula described by Schöning and Schmuck (2003).

2.6. Predator identification

The identities of *Laricobius* larvae ($n = 1736$) and adults ($n = 152$) collected from the study trees were determined using genetic analysis. Six nuclear microsatellite loci (Klein et al., 2010; Havill et al., 2012) were amplified using the conditions described in Klein et al. (2010). Fragment analysis was conducted with a 3730xl 96-capillary genetic analyzer (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA) at the DNA Analysis Facility on Science Hill at Yale University, New Haven, CT. Genotypes were scored using the software Genemapper 4.0 (Applied Biosystems, Thermo Fisher Scientific Inc., Waltham, MA). Early generation hybrids of *L. nigrinus* and *L. rubidus* were distinguished from parent species using the software programs Structure 2.3.2 (Pritchard et al., 2000) and NewHybrids 1.1 (Anderson and Thompson, 2002) using the methods detailed in Havill et al. (2012).

2.7. Predator impact

An experiment to evaluate the effect of predator exclusion on *A. tsugae* sistens density was conducted in winter 2012–2013 using a hydraulic lift truck to access the hemlock crowns. A preliminary survey for trees suitable for the experiment (defined as those with an abundant population of live *A. tsugae* sistens nymphs settled on shoot growth of the current year throughout the crown) was conducted in October 2012. Nine of the 60 hemlock study trees (representing one, three, and five trees from the 0%, 10%, and 25% imidacloprid rate treatments in 2006, respectively) met these criteria and were used for the experiment. On 6 December 2012, five branch pairs in the upper crown of each tree were tagged and non-destructively sampled for adelgid density. One branch in each pair was randomly assigned to the cage treatment and was firmly tapped 10 times with a beat stick to dislodge *Laricobius* adults and other potential predators from the branch. The number of *A. tsugae* sistens nymphs, each evidenced by an undisturbed woolly ovisac, on the outer 20 cm of each branch was counted in the field. A fine mesh (300 μm) sleeve cage ($L \times W = 48 \times 71$ cm, MegaView Science Company Ltd., Taichung, Taiwan) was placed over the foliage and cinched tight to the branch using foam pipe insulation and plastic cable ties. The other branch in each pair was left uncaged.

After 3 months (12 March 2013), cages were removed and the outer 20 cm of each branch (plus an additional 5–10 cm of branch stem) was clipped and returned to the laboratory. The cut end of each branch was re-trimmed and placed in a plastic shot glass of hydrated floral foam as described in Section 2.3. A collection funnel for *Laricobius* larvae was created by forming a clear photocopier transparency ($L \times W = 22 \times 28$ cm) into a cone approx. 23 cm long with 9 cm and 6 cm diameter openings at the large and small ends, respectively. The small end of the transparent funnel was placed into the rim of a 125 ml circular, straight-sided, polypropylene jar (Nalgene, Thermo Fisher Scientific Inc., Waltham, MA) and the shot glass with hemlock branch was placed in the bottom of the jar. Funnels were kept on a table in a cool laboratory (15.5 °C) and foliage was sprayed with a mist of distilled water every 1–2 days to help prevent dehydration of foliage and insects. Funnels were checked every 1–2 days for *Laricobius* larvae dropping from foliage into the jars and larvae were counted and preserved in 95% ethanol. After three weeks branches were removed from the funnels and the number of undisturbed ovisacs on each 20 cm branch tip was counted twice by each of two independent observers. The average of the four counts was used as the final number of undisturbed ovisacs (undamaged by predation). *Laricobius* larvae and/or adults were found within cages or on caged branches of three of the 45 branch pairs (each from a different tree), indicating either failure of these cages to exclude predators or failure to remove all predators from the branch before the cage was applied. Data from these three branch pairs were excluded from statistical analyses.

2.8. Statistical analyses

2.8.1. Imidacloprid effects on predator and prey densities and hemlock variables

Repeated measures analysis of variance (ANOVA) was used to evaluate the effects of imidacloprid treatment rate (RATE) and measurement date (DATE) on the following continuous dependent variables: *Laricobius* larval density, adelgid density index (sistens and progrediens), insecticide and metabolite concentration in twigs (imidacloprid and olefin-imidacloprid), and hemlock shoot health indicators (percent new shoot growth and percent dead shoot tips) Fixed effects were RATE, DATE, and RATE × DATE and random effects were BLOCK, BLOCK × RATE, TREE(BLOCK × RATE), BLOCK × DATE, and BLOCK × RATE × DATE. A repeated measures ANOVA was also used to evaluate whether the concentration of olefin-imidacloprid in March 2012 and 2013 differed between twig samples on which *A. tsugae* sistens ovisacs were present (model term HWA = yes) and those on which ovisacs were absent (HWA = no). The model above was modified by adding HWA and its interactions with RATE and DATE as fixed effects and including random effects HWA × BLOCK (RATE) and HWA × TREE(BLOCK × RATE). Tests for fixed effects in these mixed model ANOVAs were carried out using the PROC MIXED procedure of SAS 9.4 (SAS

Institute, 2012) which pools a random effect that is estimated to be 0 with lower order random terms. Denominator degrees of freedom were obtained using the Kenward–Roger method (Kenward and Roger, 1997) and *P*-values <0.05 were considered significant. Least squares means for RATE by DATE combinations (and by HWA in the analysis of olefin-imidacloprid concentrations) were separated using the protected LSD and results summarized with the macro pdmix880.sas (Saxton, 1998).

Fisher's exact test was used to test the null hypothesis that there was no significant difference between insecticide treatment categories, within a given year, for the following dependent variables: (1) the proportion of study trees on which *Laricobius* adults or larvae were collected in 2011 and 2013, and (2) the proportion of study trees that died by February 2014. For each date, data were analyzed using the Nonparametric 2 × 2 Tables procedure in Statistica 9.1 (StatSoft, 2010). *P*-values were Bonferroni-corrected by multiplying initial *P*-values by 3 (the number of possible two-way treatment comparisons), and corrected *P*-values <0.05 were considered significant.

2.8.2. Predator hybridization

The cumulative number and proportion of *Laricobius* specimens identified as *L. nigrinus*, *L. rubidus*, and hybrids (*L. nigrinus* × *L. rubidus*) were summarized and presented graphically by STAGE (adult vs. larva), YEAR (year of collection), and RATE (source tree imidacloprid treatment rate). After preliminary analyses to eliminate higher order terms, Probit regression was used to determine the effect of the following sources of variability on the proportion of *L. rubidus*: RATE, BLOCK, YEAR, BLOCK × YEAR, STAGE, RATE × STAGE, and YEAR × STAGE. The analysis was conducted using PROC GENMOD with a probit link, and the options Pscale (to account for overdispersion) and Type 3 to generate summary likelihood ratio tests in SAS 9.4 (SAS Institute, 2012). Success was defined as a specimen being '*L. rubidus*' and failure as '*L. nigrinus* or hybrid.' Due to numerous 0s (i.e., *L. nigrinus* or hybrid specimens) in the data set, successes and failures were accumulated for each STAGE in each BLOCK, RATE and YEAR combination. *P*-values <0.05 associated with likelihood ratio *F*-tests were considered significant.

2.8.3. Predator impact

A nested ANOVA was used to evaluate the fixed effect of the predator exclusion cage treatment (CAGE) on three dependent (Y) variables: *A. tsugae* sistens density in December 2012 (pre-cage), *A. tsugae* sistens density in April 2013 (post-cage), and percent reduction in sistens density (100 × [pre-cage – post-cage]/pre-cage). Preliminary tests of imidacloprid treatment rate (RATE) and CAGE × RATE effects using the Type 3 mean square for TREE(RATE) and TREE × CAGE(RATE) as error terms, respectively, were not significant ($P > 0.10$), and so the final ANOVA included TREE, PAIR(TREE), CAGE, and TREE × CAGE as sources of variability. A test for the CAGE effect was conducted using the Type 3 mean square for TREE × CAGE as the error term. *Adelges tsugae* sistens densities (pre-cage and post-cage) were square root transformed to meet assumptions of normality and homogeneity of variance. Analyses were conducted using the GLM Procedure in SAS v9.2 (SAS Institute 2008).

3. Results

3.1. Imidacloprid effects on predator and prey densities and hemlock variables

Laricobius predators established, reproduced and increased in abundance from 2011 to 2013 on hemlocks previously treated with low rates of imidacloprid insecticide in 2006, as well as on untreated trees. Although there was no significant effect of imidacloprid treatment rate on *Laricobius* larval density, the predator population increased significantly over time (Table 1), and the largest, most sustained increase occurred in the 25% imidacloprid rate group (Fig. 1A). *Laricobius* adults and larvae were collected from only 30% of the trees in the 25% rate group in 2011, but from 90% of those trees by 2013 (Fig. 1B). The proportion of trees from which *Laricobius* adults and larvae were recovered did not differ among imidacloprid rate treatment groups in 2011 or in 2013 (Fig. 1B).

There was a significant effect of date and the interaction of imidacloprid rate × date on the mean adelgid density index for the *A. tsugae* sistens generation (Table 1). The 25% imidacloprid rate trees supported the lowest densities of adelgid prey for *Laricobius* in March 2011, but transitioned to supporting the highest prey densities by March 2013 (Fig. 1C). There were significant effects of imidacloprid rate and the interaction of rate × date on the adelgid progrediens generation as well (Table 1). Similar to the patterns in the sistens generation, the mean progrediens density index was lowest on the 25% rate trees in June 2011, but highest on the 25% and 10% rate trees by June 2013 (Fig. 1D). Adelgid densities for both generations decreased significantly on untreated trees between 2011 and 2013 (Fig. 1C, D).

The increase in adelgid densities on the 25% imidacloprid rate trees was concurrent with significant decreases in imidacloprid and olefin-imidacloprid concentrations in the twigs (Table 1, Fig. 1E, F). Mean imidacloprid residues in March 2011 (5 years post treatment) of 51 and 30 ppb for the 25% and 10% rate trees, respectively, dropped to 10 and 4 ppb respectively by June 2013 (7 years post treatment) (Fig. 1E). Similarly, mean twig residues of olefin-imidacloprid in March 2011 were 24 and 14 ppb in the 25% and 10% rate trees, respectively, but dropped to 11 and 4 ppb respectively by June 2013 (Fig. 1F). Mean (SE) olefin-imidacloprid residues were significantly lower in twigs with adelgids present [8.4 (1.9) ppb] than in those on which adelgids were absent [13.2 (1.9) ppb] ($F = 26.3$; df = 1, 8; $P < 0.001$). Concentrations of imidacloprid and olefin-imidacloprid were below the limit of detection in all untreated trees, and 5-hydroxy-imidacloprid was below the limit of detection in all treatments. Furthermore, imidacloprid, olefin-imidacloprid, and 5-hydroxy-imidaclopird were below the limit of detection in all *Laricobius* larvae collected from the foliage of the 0%, 10%, or 25% imidacloprid rate trees ($n = 113$, 103, and 137 larvae analyzed, respectively) in March 2013.

Despite the loss of insecticidal protection on treated trees, there were still significant differences in hemlock crown health (measured by new shoot growth and dead tips) by imidacloprid treatment rate and date (Table 1). New shoot production was highest in the 25% imidacloprid rate group through March 2013, and exceeded 82% through March 2012 (6 years post treatment) (Fig. 1G). By June 2013, mean new shoot production in the 25% rate group dropped to 36% and there was no significant difference between the 25% and 10% rate groups, but these groups had a significantly higher percentage of new shoots than the untreated group (15%) (Fig. 1G). Mean percent dead shoot tips was highest on untreated trees and increased to 60–70% by 2013, whereas mean percentages of dead tips in the 10% and 25% rate groups did not increase significantly during the study and did not exceed 30% (Fig. 1H). By February 2014, 25% of the trees in the untreated group died, vs. only 5% mortality in the insecticide-treated groups, but these proportions did not differ significantly (Fig. 1I).

3.2. Predator hybridization

L. nigrinus dominated the *Laricobius* larval populations that established on the hemlock study trees, representing between 69% and 89% of the specimens annually (Fig. 2A). Considering all

Table 1
Summary statistics for Type 3 tests of fixed effects (imidacloprid rate, measurement date, and interaction) on various dependent variables collected on *Tsuga canadensis* study trees in White County, GA, 2011–2013.

Dependent variable	Imidacloprid rate			Date			Imidacloprid rate × date		
	df	*F*	*p*	df	*F*	*p*	df	*F*	*p*
Laricobius density (larvae/10 cm)	2, 23	0.4	0.710	2, 7.9	8.8	0.010*	4, 22.7	1.2	0.355
Adelgid density index-sistens	2, 7.9	0.2	0.848	2, 158	9.9	<0.001*	4, 158	5.5	<0.001*
Adelgid density index-progrediens	2, 105	5.7	0.004*	1, 8.1	7.9	0.022	2, 105	10.4	<0.001*
Twig imidacloprid conc. (ppb)	1, 8	5.5	0.048*	4, 145	116.4	<0.001*	4, 145	7.0	<0.001*
Twig olefin-imid. conc. (ppb)	1, 8	5.0	0.055	4, 30.4	46.3	<0.001*	4, 30.4	2.0	0.128
New shoots (%)	2, 57.2	40.0	<0.001*	4, 17.4	34.0	<0.001*	8, 209	4.5	<0.001*
Dead shoot tips (%)	2, 55	42.1	<0.001*	4, 16.1	3.2	0.042*	8, 33.3	1.9	0.102

Note: Significant *P*-values (<0.05) are marked with an asterisk. Test statistics were generated using a mixed model, repeated measures analysis of variance and denominator degrees of freedom (df) were obtained using the Kenward–Roger method (Kenward and Roger, 1997).

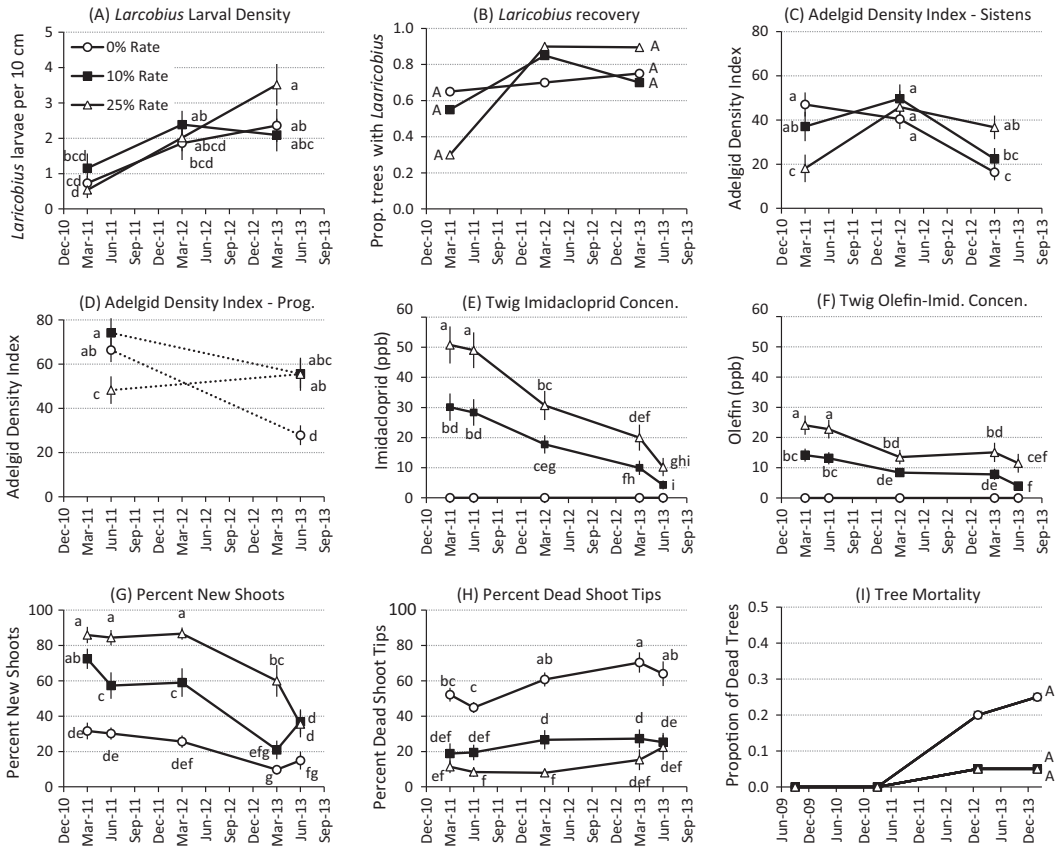

Fig. 1. Means of dependent variables collected from 2011 to 2013 within the crowns of *Tsuga canadensis* treated with 0%, 10%, and 25% of the label rate of imidacloprid insecticide in November 2006. Dependent variables are: density of *Laricobius* larvae on *A. tsugae* infested shoots (A), proportion of trees from which *Laricobius* adults or larvae were recovered (B), adelgid density indices for the *Adelges tsugae* sistens (C) and progrediens (D) generations, concentration of imidacloprid (E) and olefin-imidacloprid (F) in twig tissue, percentage of branch tips representing new shoot growth (G) and dead shoot tips (H), and proportion of dead trees (I). Vertical bars indicate standard error. Trend lines are dotted in panel D because no estimates of the progrediens generation were made in 2012. Means labeled with the same lower case letter are not significantly different, whereas proportions labeled with the same upper case letter are not significantly different within year ($\alpha = 0.05$).

years combined, percent hybridization between *L. nigrinus* and *L. rubidus* was 11% and 12.5% for the larval and adult populations, respectively, and the proportion of hybrids was relatively consistent from year to year (Fig. 2A). There were significant effects of year, life stage, and imidacloprid rate × life stage on the proportion of *L. rubidus* recovered between 2011 and 2013 (Table 2). The proportion of the *Laricobius* population comprised of *L. rubidus* was highest in 2012, when it represented nearly 60% and 20% of the larvae and adults, respectively (Fig. 2A). *L. rubidus* was present in significantly larger proportions in the adult stage than in the larval stage, representing 39% and 9% of the adult and larval populations, respectively (2011–2013 data combined) (Fig. 2A). This pattern was evident each year (Fig. 2A) and when data were grouped by tree imidacloprid rate (Fig. 2B). The proportion of *L. rubidus* adults collected was highest from trees in the 25% imidacloprid rate group, and lowest from the 0% rate group, but this pattern was not evident in the larval population, where proportions of *L. nigrinus*, *L. rubidus*, and hybrids were relatively consistent across imidacloprid treatment groups (Fig. 2B).

3.3. Predator impact

At the initiation of the predator exclusion study in December 2012 (pre-cage), the mean *A. tsugae* sistens density on caged vs.

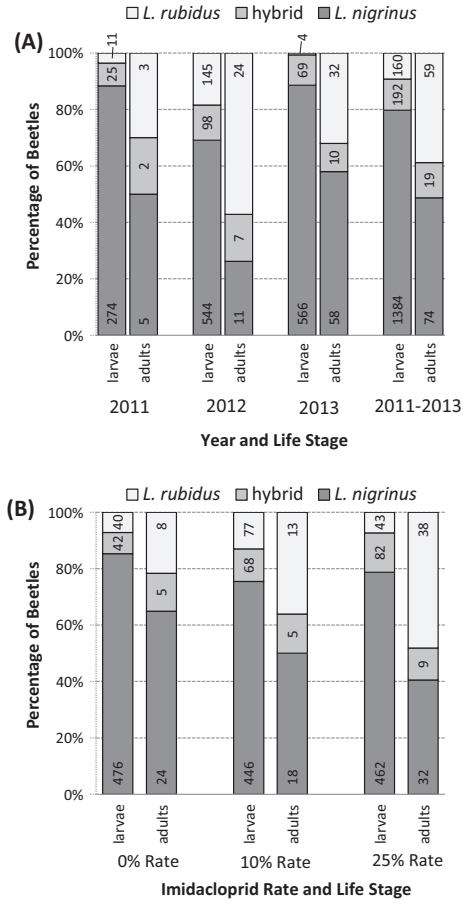

Fig. 2. Percentage (*y*-axis) and number (labeled on bars) of *Laricobius* beetles identified as *L. nigrinus*, *L. rubidus*, or hybrids using genetic analysis. Data presented by collection year and life stage (A), and by imidacloprid treatment and life stage (B).

Table 2
Summary statistics for Type 3 likelihood ratio tests of various effects on the probability of a collected *Laricobius* specimen being *L. rubidus* (vs. *L. nigrinus*, or *L. rubidus* × *L. nigrinus* hybrid), 2011–2013.

Effect	df	*F*	*p*
Imidacloprid rate	2, 51	2.57	0.087
Block	4, 51	2.2	0.082
Year	2, 51	15.29	<0.001*
Block × year	8, 51	1.82	0.096
Stage	1, 51	11.64	0.001*
Imidacloprid rate × stage	2, 51	4.39	0.017*
Year × stage	2, 51	2.62	0.083

Note: Significant *P*-values (<0.05) are marked with an asterisk. Test statistics were generated using probit regression with an adjustment for overdispersion.

uncaged branches were the same ($F = 0.91$; df = 1, 8; $P = 0.368$). At the conclusion of the experiment in April 2013 (post-cage), there was a significant effect of the cage treatment on *A. tsugae* sistens density ($F = 28.91$; df = 1, 8; $P < 0.001$) and on percent reduction in sistens density ($F = 24.90$; df = 1, 8; $P = 0.001$). Mean sistens density on caged branches from which predators were excluded (6.6 adelgids/cm) was more than twice that of branches exposed to predators (3.1 adelgids/cm) (Fig. 3). Between December and April, adelgid densities were reduced by 39% and 70% on caged and exposed branches, respectively (Fig. 3). Ovisacs on uncaged branches were commonly shredded, a characteristic of *Laricobius* larval feeding (Fig. 4). In the laboratory, *Laricobius* larvae dropped from 69% of the uncaged branches at a mean (SE) density of 5.2 (1.0) larvae per 20 cm branch tip.

4. Discussion

Joseph et al. (2011) demonstrated that low-rate treatments of imidacloprid improved crown health while allowing low densities of *A. tsugae* on *T. canadensis*. Our study subsequently demonstrated that *Laricobius* predators established and proliferated on these previously-treated trees as chemical protection diminished over time. Seven years after treatment with only 25% of the label rate of imidacloprid insecticide, hemlocks at our southern Appalachian forest site still had more new shoots and fewer dead tips than untreated trees. The greater abundance of new shoots, coupled with the loss of insecticide protection, allowed for higher *A. tsugae* densities on the previously-treated trees, providing more prey for *Laricobius* predators. Successful establishment of the introduced biological control species *L. nigrinus* (released two and four years after imidacloprid treatment) was evidenced by its proportional dominance over *L. rubidus* and hybrids in the larval population. Densities of *Laricobius* predator larvae per cm of infested branch increased over time and were as high on previously-treated trees as on untreated trees. Because *A. tsugae* density was highest on the 25% imidacloprid rate trees, these previously-treated trees likely supported higher total numbers of predators than untreated trees. The *Laricobius* population not only established and increased on *T. canadensis*, but resulted in a significant reduction in *A. tsugae* sistens ovisac density.

The low rates of imidacloprid applied to *T. canadensis* trees in 2006 provided temporary, and in some cases only partial, protection against *A. tsugae*. In the preceding analysis at this site, significant differences in *A. tsugae* density among the treatment groups were not observed until 2008–2009, when mean foliar imidacloprid concentrations were as high as 75 and 137 ppb in the 10% and 25% rate groups, respectively, and mean *A. tsugae* densities were lower (but not zero) in the treated vs. untreated groups (Joseph et al., 2011). This dose-related response of *A. tsugae* density to treatment rate persisted through March 2011 (Fig. 1C), even though twig imidacloprid concentrations in both of the treated groups (30–50 ppb) had decreased well below reported thresholds for *A. tsugae* control (e.g., >120 ppb, Cowles et al., 2006) (Fig. 1A). This suggests that the metabolite olefin-imidacloprid, which was present at mean twig concentrations above 22 ppb in the 25% rate group (Fig. 1F), was responsible for the lower *A. tsugae* densities on

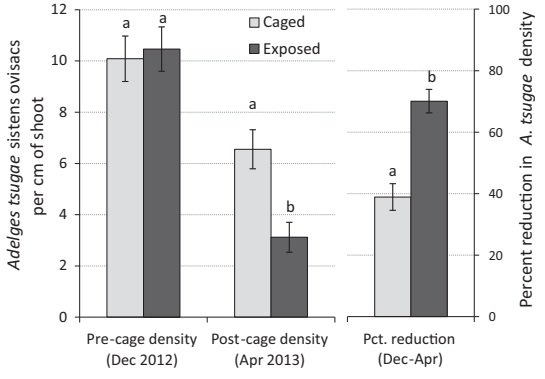

Fig. 3. Mean pre-cage (December 2012) and post-cage (April 2013) *Adelges tsugae* sistens densities, and percent reduction in sistens density on *Tsuga canadensis* branches caged to exclude *Laricobius* predators, and branches exposed to predators. Vertical bars indicate standard error. Paired means labeled with the same letter are not significantly different ($\alpha = 0.05$).

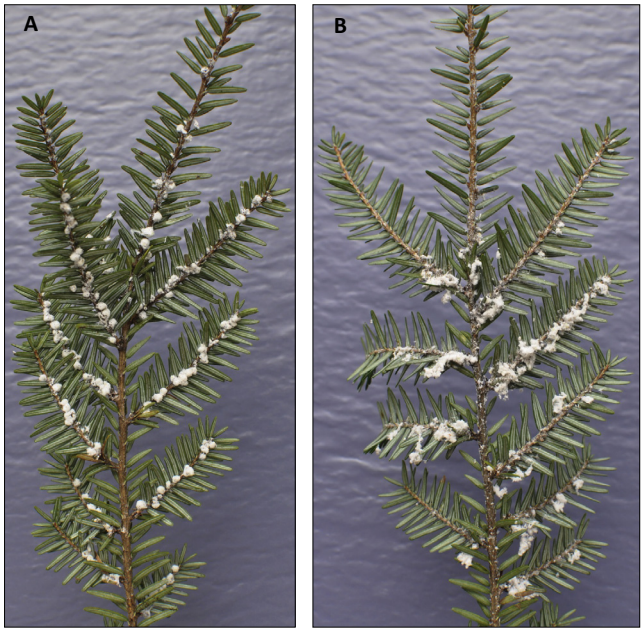

Fig. 4. A pair of branch tips collected in March 2013 from a *T. canadensis* tree treated with 25% of the label rate of imidacloprid insecticide in November 2006. The branch on the left (A) was caged to exclude winter and early-spring predation by *Laricobius* spp. and exhibits primarily globular, undisturbed ovisacs of the *A. tsugae* sistens generation. The paired branch on the right (B) remained uncaged and exhibits shredded *A. tsugae* sistens ovisacs characteristic of *Laricobius* larval feeding.

these trees in March 2011. Toxicity thresholds in *A. tsugae* are much lower for olefin-imidacloprid than for imidacloprid, and high levels (>80%) of *A. tsugae* mortality have been associated with olefin-imidacloprid concentrations in *T. canadensis* twigs as low as >7 ppb (Coots, 2012). In 2012–2013, mean olefin-imidacloprid concentrations were 9 and 14 ppb on shoots with and without *A. tsugae* ovisacs present, although concentrations in some *A. tsugae*-infested twigs were in the 20–27 ppb range for several trees. We are uncertain why *A. tsugae* ovisacs were present on twigs with olefin-imidacloprid concentrations above the mortality threshold reported by Coots (2012). Possibilities may include variability among adelgid clones in their tolerances to olefin-imidacloprid, or presence of the compound in portions of the twig tissue avoided by, or inaccessible to, the adelgid stylet mouthparts.

Our results suggest that *Laricobius* exposure to prey-mediated insecticide was minimal and not detrimental to their abundance or impact. After soaking the cut ends of adelgid-infested *T. canadensis* branchlets in various concentrations of imidacloprid solution and allowing adult predators to feed on them, Eisenback et al. (2010) observed lethal and sublethal (e.g., slower beetle flip times, intoxication symptoms) effects on *L. nigrinus* in the higher treatment groups in which mean branch imidacloprid concentrations exceeded 1000 ppb. Only the lowest treatment group, however, resulted in branch imidacloprid concentrations similar to field-treated trees (i.e., 60–200 ppb), and *L. nigrinus* mortality and flip times in that treatment did not differ from the control group (Eisenback et al., 2010). In our study, mean branch concentrations of both imidacloprid (5–50 ppb) and olefin-imidacloprid (4–24 ppb) were as low as or lower than those reported in the lowest treatment group by Eisenback et al. (2010). Also, in the study by Eisenback et al. (2010), predators were exposed to adelgids which were initially feeding on unprotected branches before consuming recently-delivered insecticide, whereas in our study, predators were released 2–4 years after initial insecticide treatments and were primarily feeding on adelgids that had returned to previously-protected trees. Furthermore, we did not detect imidacloprid or metabolite residues in any of the 240 *Laricobius* larvae collected in March 2013 from trees in the 10% and 25% treatment rate groups.

Annual predator collections on *T. canadensis* at our study site from 2011 to 2013 yielded the introduced species *L. nigrinus*, the native species *L. rubidus*, and *L. nigrinus* × *L. rubidus* hybrids. The recovery of all three groups was expected, given the abundance of *Pinus strobus* (the host tree for *L. rubidus*' principal native prey, *Pineus strobi*) throughout the study site, and the documented hybridization of *L. nigrinus* with *L. rubidus* on *T. canadensis* at numerous other *L. nigrinus* release sites in the eastern U.S. (Havill et al., 2012; Fischer, 2013). After tracking hybridization patterns on *T. canadensis* for six years at several *L. nigrinus* release sites, Fischer (2013) found that: (a) the proportion of *L. nigrinus* larvae initially increased post-release and then dominated the populations, (b) the proportion of hybrids varied between 10% and 25% but remained relatively stable over time, and (c) the proportion of *L. rubidus* larvae decreased over time. Likewise, we also observed dominance of *L. nigrinus* in the larval population over time and a temporally stable population of hybrid larvae in proportions similar to those reported by Fischer (2013). Although we did not observe a clear decrease in the proportion of *L. rubidus* over time (proportions of this species pulsed in 2012 relative to 2011 and 2013), the proportion of *L. rubidus* larvae was very low (<1%) in our final year of observation.

Interestingly, in our field collections, *L. rubidus* consistently comprised a larger proportion of the adult *Laricobius* population (collected via beat sheets) than the larval population (reared from collected branch samples). Possible explanations for this pattern are that, relative to *L. nigrinus*, a greater proportion of *L. rubidus*: (a) fed on *A. tsugae* without ovipositing, (b) failed to develop into fully mature larvae on *A. tsugae*, or (c) both. Initial efforts have been made to compare development of *L. rubidus* on *A. tsugae* vs. *P. strobi* (Fischer, 2013), but very little is known about oviposition preferences or reproductive success of *L. rubidus* on its various prey, and further study would be necessary to elucidate a mechanism behind this pattern.

In the predator exclusion experiment, it was not possible to isolate the effect of specific predatory species on *A. tsugae* density reduction. However, *Laricobius* spp. were likely responsible for the majority of the observed predator activity for the following reasons. First, native or naturalized winter-active predators of adelgids in the southeastern U.S. are rare, and a previous study of three southern Appalachian sites found no significant impact of several potential spring predator species on *A. tsugae* densities (Wallace and Hain, 2000). Second, nearly 70% of the uncaged branches harbored *Laricobius* larvae by the end of our experiment, and these branches exhibited a pattern of shredded ovisacs characteristic of *Laricobius* larval feeding (Mausel et al., 2011). Predation was not the only mortality factor affecting overwintering *A. tsugae* populations in this study, however, as evidenced by a 39% reduction in sistens ovisac density on the caged branches from which predators were excluded (Fig. 3). Extreme minimum winter temperatures are known to cause *A. tsugae* mortality (Parker et al., 1999), although this phenomenon is not as common in the warmer southern Appalachians compared to sites at more northern latitudes (Paradis et al., 2007). Adelgid mortality via intraspecific competition (McClure, 1991; Paradis and Elkinton, 2008) may also have occurred due to high mean densities of sistens nymphs at the start of the experiment in December, and the inability of all these nymphs to complete development to the adult stage due to resource limitation. In addition, some reduction in the number of undisturbed ovisacs may have been due to physical abrasion of branches against the cage material or other branches as they moved in the wind.

Despite the possibility of other mortality factors as discussed above, the exclusion of *Laricobius* predators from caged branches resulted in mean *A. tsugae* sistens densities that were twice as high as densities observed on branches exposed to predators (Fig. 3). This represents a conservative estimate of impact of *Laricobius* on the sistens generation, because cages were not deployed until early December, and therefore effects of adult *Laricobius* feeding in October–November were not measured. Neither did this experiment distinguish between the effects of adult vs. larval predation by *Laricobius*. When comparing branches onto which *L. nigrinus* adults had been caged vs. branches without *L. nigrinus*, Lamb et al. (2005) found a greater predation effect from February to April (when larvae were hatching and feeding) than from November to January (when only adults were active). The predation impacts observed in our study are consistent with those of Mausel et al. (2008), who also observed significantly higher *A. tsugae* sistens densities and lower percentages of ovisac disturbance on caged branches from which *L. nigrinus* and *L. rubidus* were excluded. Our predator exclusion study differed from that of Mausel et al. (2008) in a number of ways, including: (a) *L. nigrinus* was released in greater numbers on fewer trees, (b) more years had passed since initial *L. nigrinus* release (4 vs. 2 years), (c) *A. tsugae* densities on caged branches were notably higher (>6 vs.<2 adelgids per cm), and (d) *T. canadensis* trees were much larger and located in a mature forest (vs. seedlings recently planted in an open field). To our knowledge, ours is the first published study to quantitatively document predatory impact on *A. tsugae* by *Laricobius* predators established on *T. canadensis* in a natural forest setting, and to examine predation throughout the canopies of mature trees.

While it is encouraging that hemlock crown health was prolonged through a one-time, low-rate imidacloprid treatment, and that the established *Laricobius* population caused substantial reductions in *A. tsugae* sistens densities on previously-treated trees, this may not be enough to save *T. canadensis* at this study site. By June 2013, the *T. canadensis* trees with the healthiest crowns and the most predators (i.e., the 25% imidacloprid treatment rate group) nonetheless exhibited a decreasing trend of new shoot production, likely due to the recently high adelgid densities on these trees (Fig. 1). Although *T. canadensis* mortality seemed to be progressing most rapidly in the 0% imidacloprid rate group, at least one tree in each of the imidacloprid treatment groups had died due to excessive crown loss by six years post-treatment. *Laricobius* predators do not feed on nymphs or adults of the subsequent progrediens generation, which is active in late spring and early summer. When simulating population dynamics of the entire *A. tsugae* life cycle, Elkinton et al. (2011) suggested that *Laricobius*-caused reductions in sistens density could be offset by reduced interspecific competition and improved fecundity in the subsequent progrediens generation, resulting in no net reduction in overall *A. tsugae* density by the following year. Furthermore, our study site is near the southern limit of *T. canadensis* in North America, where winter temperatures cold enough to compliment *Laricobius* predation via additional reduction of the sistens generation rarely occur. Thus, sustained protection via integrated management of *T. canadensis* in this southern portion of its range may require the addition of an effective predator on the progrediens generation.

5. Conclusions

The integration of chemical and biological control in the same stands shows promise for reducing the impact of *A. tsugae* on *T. canadensis*. Our study demonstrated that *L. nigrinus* predators (in concert with *L. rubidus* and *Laricobius* hybrids when *P. strobus* is also present) can successfully establish, increase in number, and reduce *A. tsugae* sistens densities on forest trees previously treated with imidacloprid insecticide. The insecticide treatment affords these trees prolonged crown health and, through the production of more new growth, the ability to eventually support more prey for *Laricobius* relative to untreated trees. Another multi-year study testing this integrated concept at a number of other sites in the southern Appalachians is in progress (Salom et al., 2011). Long-term efficacy of this approach will likely be enhanced by incorporation of one or more additional predator species (especially those active against the progrediens generation) or by its implementation at higher elevations and latitudes where winter temperatures may help regulate *A. tsugae* populations.

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