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Tree-level distribution of a novel insect-pathogen complex and its potential contribution to eastern white pine dieback☆

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ABSTRACT

Bottom-up branch dieback and sapling mortality of eastern white pine (*Pinus strobus* L.) has been observed range-wide during the last two decades. Observational studies thus far implicate these symptoms to an insect, the eastern white pine bast scale (*Matsucoccus macrocicatrices* Richards), and a canker-forming, fungal pathogen, *Caliciopsis pinea* Peck. The scale insect was historically considered an innocuous herbivore of eastern white pine restricted only to New England and Canada, but is now found in high densities on symptomatic trees, in close association with Caliciopsis canker, and in almost every region where the host grows. We sampled branches and boles of eastern white pines in the southern Appalachians to better understand the distribution of the insect-pathogen complex on individual trees and among size classes. Results indicate distinct patterns, as branches of poletimber, boles of saplings, and branches lowest in the canopy harbored the greatest numbers of bast scales and had the highest proportional Caliciopsis canker area. The incidence of scales and cankers was generally highest on older tissue with high percent lichen cover, but with thinner outer bark. Tree-level distribution of the bast scale and Caliciopsis canker was non-random and in fact mirrored the observed dieback patterns reported for eastern white pine, indicating that these two organisms may be important contributors to tree dieback and mortality in the southeastern USA.

1. Introduction

Trees are spatially and temporally heterogeneous habitats for animals, with abiotic and biotic conditions varying at multiple strata (e.g., root, bole, branch, twig, cone, and foliage levels). As hosts, structural heterogeneity in trees influences herbivorous communities and populations (Lawton, 1983). Associated with varying tree size, age, and zonation are marked differences in physiology (e.g., tissue type, volume, texture, nutritional quality, and secondary defensive compounds), micro-environmental conditions (e.g., temperature and moisture), and natural enemy load (e.g., predators and parasitoids), which directly or indirectly affect the spatial colonization of herbivores (Lawton, 1983; Ulyshen, 2011; Wardhaugh, 2014). Many studies have investigated differences in herbivore communities within and across trees (e.g., Maguire et al., 2014; Plewa et al., 2017; Weiss et al., 2016), but fewer studies are available that assess such distributions of forest pest populations (e.g., Wardhaugh et al., 2006), including those associated with tree-killing pathogens. An understanding of tree-level colonization patterns may elucidate if a link exists between observed tree symptoms and the contributing pests and pathogens.

A novel dieback phenomenon is posing ecological and economic threats to eastern white pine (*Pinus strobus* L.) across its entire range in eastern North America (Asaro, 2011; Costanza et al., this issue; Lombard, 2003). The current symptoms are unrelated to known threats of eastern white pine, white pine blister rust (*Cronartium ribicola* Fisch) and white pine weevil (*Pissodes strobi* Peck), but rather include a progressive bottom-up thinning of the crown, where lower branches die steadily until the live canopy is reduced to terminal branches and the leader (Schulz et al., this issue-a, this issue-b). Accompanying this pattern of branch dieback is the presence of characteristic cankers on branches and boles of trees. Severe resin outflow from cankers is common, especially on large diameter trees, which leads to decreased

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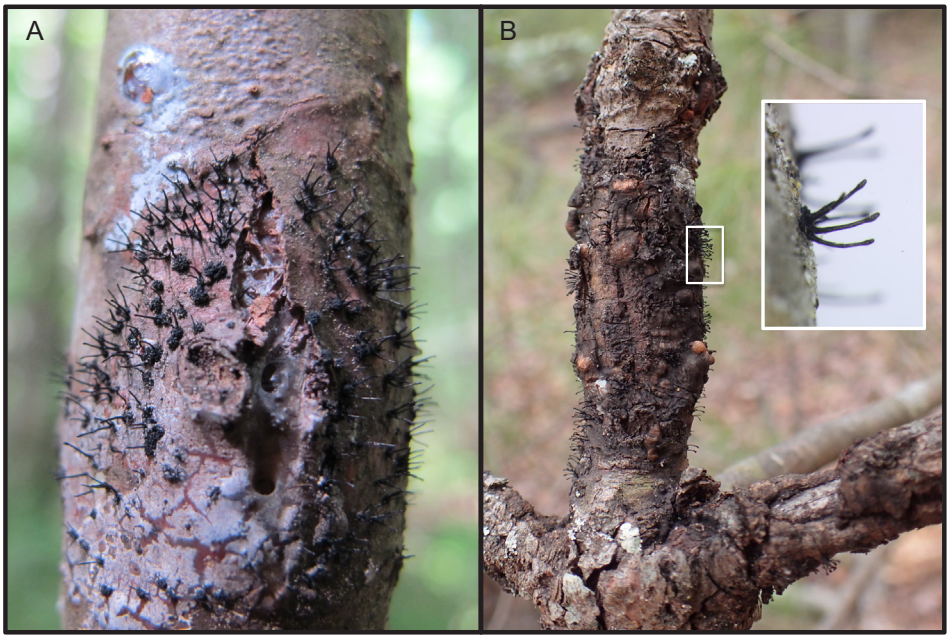

Fig. 1. (A) Early-stage Caliciopsis canker with characteristic fruiting structures, causing a fissure in the tree bark and resin flow. (B) Late-stage Caliciopsis canker girdling a young eastern white pine stem. Inset is a close-up of *Caliciopsis pinea* ascocarps, which are the characteristic sexual fruiting bodies.

wood quality (Costanza, 2017). The cankers also cause girdling of stem and branches, thus killing trees (Overholts, 1930). Seedlings and saplings are particularly vulnerable to mortality, but the phenomenon has been reported from all diameter classes, thus threatening the resilience and sustainability of eastern white pine as a canopy species in eastern forests (Asaro et al., this issue; Costanza et al., this issue).

A variety of common fungi have been isolated from cankers on symptomatic eastern white pine, with *Caliciopsis pinea* Peck as the most ubiquitous and virulent species (Cram et al., 2009; Ray, 1936; Schulz et al., this issue-b). A native pathogen primarily of eastern white pine, *C. pinea* has been known throughout the eastern United States to be associated with sapling and branch mortality (Overholts, 1930; Ray, 1936), but never to an extent that would significantly threaten the health of the host-tree species across its range. Recent studies have established that *C. pinea* is strongly associated with current eastern white pine dieback and is now considered a main contributing factor throughout the host range (Lombard, 2003; Munck et al., 2015; Schulz et al., this issue-b). The fungal hyphae of *C. pinea* establish under the tree bark, grow into the vascular cambium, and kill host tissue. This infection results in the steady expansion of necrotic lesions from which annual crops of ascocarps, the distinctive hair-like fruiting bodies, are produced on the external surface of trees (Fig. 1) (Funk, 1963; Ray, 1936). Cankers sometimes coalesce with each other and will girdle branches and young stems (e.g., Fig. 1b). The epidemiology of the disease (Caliciopsis canker, hereafter) is not fully understood, but it has long been speculated that *C. pinea* requires an infection court in the form of an old lenticel, a natural bark crack, or an insect feeding wound to first colonize a host (Funk, 1963). In 2006, forest health specialists in Virginia discovered a scale insect, known as the eastern white pine bast scale, *Matsucoccus macrocicatrices* Richards (Hemiptera: Matsucoccidae), along the edge of Caliciopsis cankers; it has been suggested that the bast scale is likely facilitating *C. pinea* in its initial infection stage (Asaro, 2011; Mech et al., 2013). Historically believed to be a benign sap-sucking insect of eastern white pine, the bast scale is now strongly associated with both the dieback symptoms and the presence of Caliciopsis canker range-wide (Schulz et al., this issue-b).

The eastern white pine bast scale is native to North America and is a specialist on eastern white pine (Richards, 1960). The vast majority of its lifecycle is spent in its feeding stage as a sessile, 2nd instar juvenile: a black, eyeless, and legless cyst (Fig. 2a). With their relatively long piercing-sucking mouthparts (stylet) inserted into the outer phloem (bast), the insect cysts extract sugar-rich, vascular fluids for nourishment (Fig. 2b). Adult insect emergence occurs annually during the spring in the Southern Appalachians (Mech et al., 2013; Whitney pers. obs.), but occurs biennially at higher latitudes, presumably because winter is more prolonged (Watson et al., 1960). Winged males mate with quiescent females, and the females lay eggs in early summer. First instar “crawlers” hatch from eggs in summer and act as the main dispersal stage, hypothesized to utilize the wind to move to new trees, as in other scale insects (Bean and Godwin, 1955; Gullan and Kosztarab, 1997). After locating a suitable feeding site in late summer, often within bark crevices, under lichen, or along the edge of cankers, they insert their mouthparts into the tree and undergo a molt into the 2nd instar cyst (see: Costanza et al., this issue, Fig. 7 for additional lifecycle details). Mech et al. (2013) hypothesized that the feeding wounds created by the eastern white pine bast scale during its cyst stage facilitate *C. pinea* infection of its host. This proposed pathway of infection is similar to the beech bark disease complex, where two native fungi (*Neonectria faginata* Lohm. & Watson and *N. ditissima* Tul. & C. Tul.) exploit the minute feeding wounds of the introduced beech scale (*Cryptococcus fagisuga* Lindinger), leading to infection and loss of American beech (*Fagus grandifolia* Ehrh.) (Houston, 1994). No mechanistic work has yet investigated if eastern white pine bast scale feeding behavior aids and/or expedites pathogen infection, and thus it would be premature to attribute this as the cause of the dieback. However, there is correlative evidence to suggest there is an association between the two organisms and that together they contribute to extensive canker formations on branches and bole (Mech et al., 2013; Schulz et al., this issue-b).

Due to their historical reputations as negligible damaging agents, there is currently a paucity of substantive research on the eastern white pine bast scale, *C. pinea*, and their association (hereby referred to as the insect-pathogen complex). To assist with effective management or mitigation strategies, the basic biology of these organisms must be elucidated, including mechanisms of dispersal, colonization, and the relationship between *C. pinea* infection and insect feeding. Particularly urgent is the quantitative assessment of their distribution on symptomatic trees to infer where they may be preferentially colonizing. Such information may assist managers to identify particular trees within a

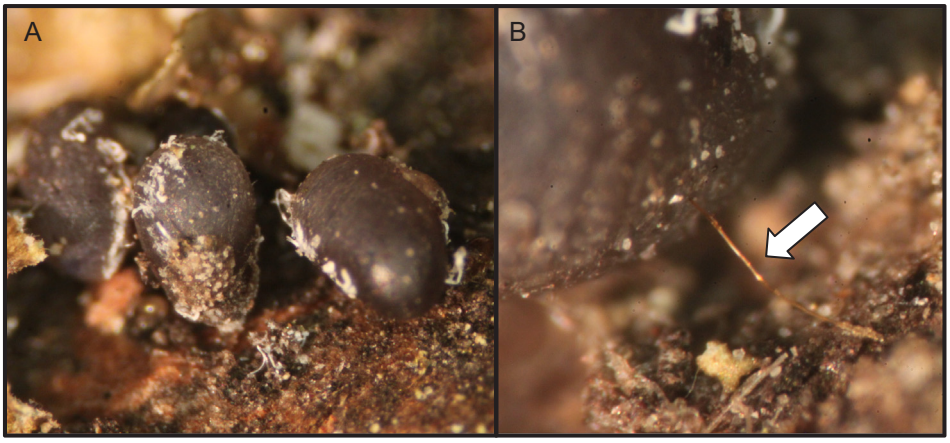

Fig. 2. (A) Second instar cysts of the eastern white pine bast scale (*Matsucoccus macrocicatrices*) congregated under lichen on an eastern white pine branch. (B) Close-up view shows the bast scale stylet used to reach the outer phloem (bast) and extract vascular fluids.

stand or specific areas on a tree that are especially susceptible to insect-pathogen colonization for specific control prescriptions.

Our goal was to determine the distribution of both focal organisms, the eastern white pine bast scale and *C. pinea*, within and across eastern white pine trees. We assessed the incidence of the insect-pathogen complex on branches and boles according to: (1) tree size class (saplings, poletimber, and sawtimber) and (2) vertical distribution within the canopy (lower, middle, and upper crown). Our field observations indicate that intermediate-sized poletimber experience the most severe branch dieback and small saplings experience the most mortality. If the insect-pathogen complex were a driving force behind these symptoms, then the incidence of both organisms would be highest on branches of poletimber trees and on boles of saplings as compared to other size classes. The pattern of branch dieback across size classes also occurs from the bottom up on individual trees. Thus, higher incidence of bast scale and Caliciopsis canker was also expected on branches in the lower, rather than the upper canopy. We also compared incidence of the insect-pathogen complex according to: (3) horizontal distribution along the branches (proximal, medial, and distal), (4) vertical distribution on boles (lower, middle-lower, middle-upper, and upper), (5) branch-section diameter (an indication of tissue age), (6) bark thickness, and (7) percent lichen cover. We evaluated these variables in addition to our main objectives to determine if bast scales and *C. pinea* may colonize branches from the outside in or inside out, if vertical bole level or bole bark thickness is more of a limiting factor, and if the presence of lichen increases the incidence of colonization.

2. Methods

2.1. Field sites

Sampling was conducted at the southernmost extent of the Appalachian Mountains and range of eastern white pine within the Chattahoochee National Forest in northern Georgia, USA (Fig. 3). Five sites were selected and were separated by at least 10 km (Table 1). In addition to eastern white pine, forest overstory consisted of American beech (*F. grandifolia*), dogwood (*Cornus florida* L.), eastern hemlock (*Tsuga canadensis* (L.) Carrière), oaks (*Quercus* spp.), red maple (*Acer rubrum* L.), sweetgum (*Liquidambar styraciflua* L.), and tulip poplar (*Liriodendron tulipifera* L.). The understory was dominated mostly by *Rhododendron* spp. and mountain laurel (*Kalmia latifolia* L.). Annual precipitation of this region ranges from 150 to 300 cm, average daily temperature ranges from 8 to 16 °C, and the dominant soil orders are Inceptisols and Ultisols with the general textural class being loamy (USDA, 2006).

2.2. Sampling of trees

From October 2015 through February 2016, we established three, 1-ha circular plots spaced > 100 m apart within each site. Wintertime sampling was necessary for the most reliable observations, because the minute bast scale cysts are largest during this time (Mech et al., 2013). A sapling- (DBH < 12.5 cm), a poletimber- (≥12.5–30 cm), and a sawtimber- (DBH > 30 cm) sized eastern white pine was felled within each plot. We selected 15 trees within each size class from across five sites for a total of 45 trees in the study. Our selection criteria prioritized size class requirements and felling feasibility, and thus dieback severity of trees ranged from light to severe (dieback rating of 2–4 out of 5, per Schulz et al., this issue-a, this issue-b).

Samples were taken from both the bole and branches of felled trees (Fig. 4). First, the vertical length of the living tree canopy was measured and divided into three equal sections for branch sampling: lower, middle, and upper crown. Two live branches were selected from each of the three sections. After removing axillary twigs, the length of each branch was measured and when > 3 m, it was partitioned into three equal parts, representing the horizontal stratification of the canopy. One-meter segments were removed from each third of each branch and were categorized as proximal (adjacent to bole), medial (middle of the branch), or distal (furthest from bole). Some branches were < 3 m in length, especially those from saplings and branches of the upper canopy from poletimber-sized trees. In these instances, branch segments were categorized only as proximal or distal. We sampled between 7 and 18 branch segments from each of the 45 trees for a total of 604 samples.

To obtain representative bole samples, the entire height of each tree was measured and then partitioned into four equal sections. A 12.5 × 12.5 cm sample (156.25 cm^2 area) was removed from the bole surface from the middle of each of the four partitions (lower, middle-lower, middle-upper, and upper), providing 180 total bole samples for the assessment of vertical bole distribution. All bole and branch samples were transported to the University of Georgia, Athens, and kept at 4 °C until scale counts and canker measurements were made to ensure that the immature bast scales remained dormant.

2.3. Assessment of Matsucoccus macrocicatrices and Caliciopsis pinea

Counts of live cysts (current generation) and exuviae, the shed exoskeletons from which adults emerged in recent years (previous generations), were made on each bole and branch sample under a microscope. Surface areas of Caliciopsis cankers were visually estimated using a 1-cm^2 gridded plastic transparency. When *C. pinea* fruiting bodies or asexual structures were present, we used a vegetable skinner to remove the outer bark and reveal the larger extent of necrotic,

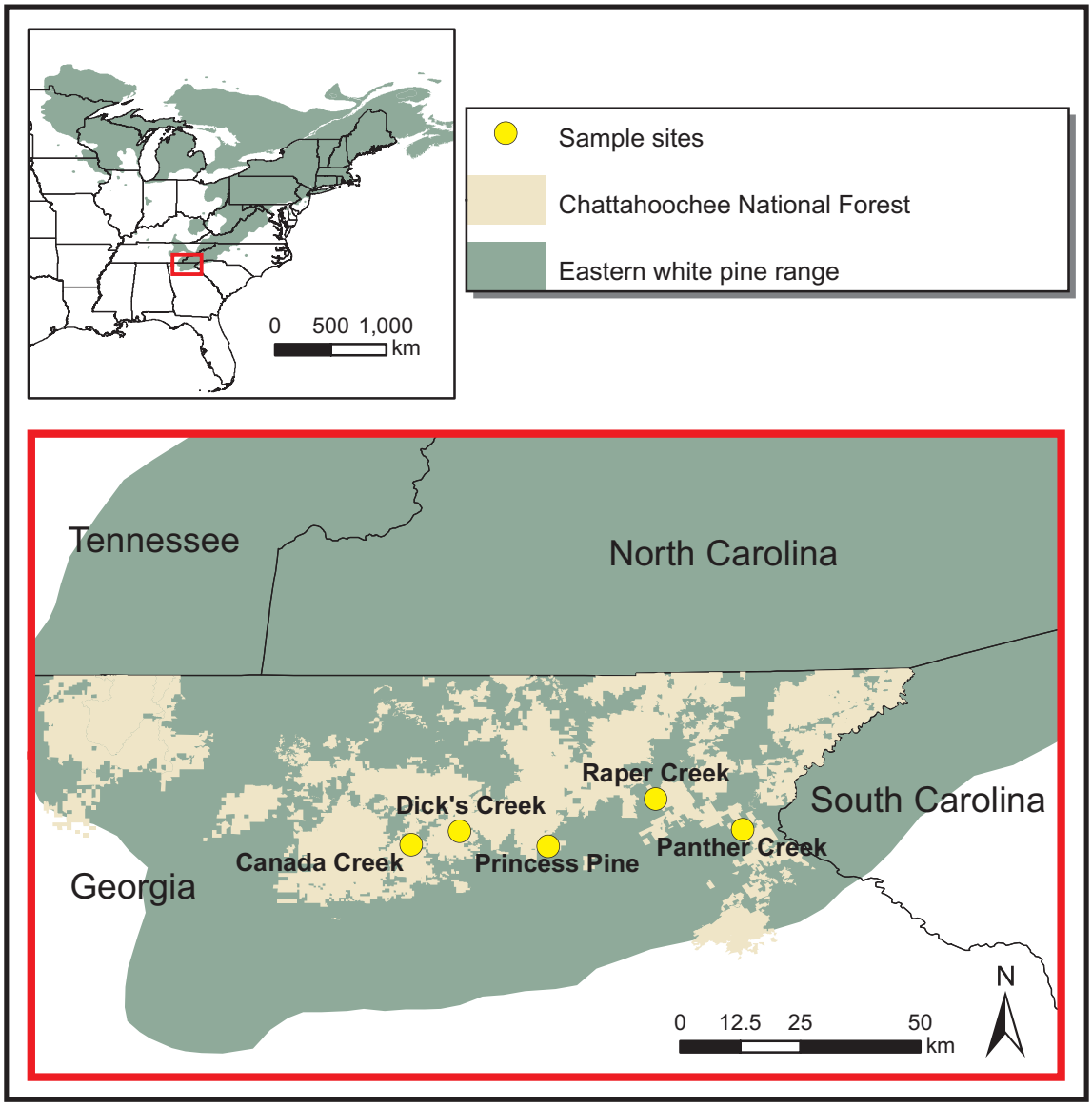

Fig. 3. Range map (Little, 1971) of eastern white pine in North America with focal inset displaying geographic locations of the five study sites within the Chattahoochee National Forest, Georgia, USA.

Table 1
Study site information. All sites were located within the Chattahoochee National Forest in northern Georgia, USA.

Site	Latitude	Longitude	Elevation (m)	Ranger District
Boggs Creek	34.70083	−83.88603	560	Blue Ridge
Canada Creek	34.67940	−84.04239	860	Blue Ridge
Panther Creek	34.69949	−83.41986	460	Chattooga
Princess Pine	34.66688	−83.78466	540	Chattooga
Raper Creek	34.74417	−83.57211	530	Chattooga

subcortical tissue, which allowed for a more precise estimate of canker area. Bark thickness was measured and percent lichen cover was estimated in 25% increments on each bole and branch sample.

2.4. Non-Caliciopsis canker survey

A subset of cankers without the distinguishing *C. pinea* fruiting bodies were collected from ten of the study trees and then plated on three different types of general media to isolate and identify other potential fungal pathogens in our study. Up to four cankers without the characteristic signs of *C. pinea* were sampled per branch for a total of 43 cankers for isolation and potential identification. The branches were stored at 4 °C until processed. For isolation, the surface bark was removed with a scalpel and the cankered tissue surface was sterilized for 10 s with 95% ethanol, followed by 4 min in 1.08% sodium hypochlorite solution (18 mL of 6% bleach in 100 mL water) (Blodgett and Stanosz, 1997). Surface sterilized tissues were then washed in sterile water for 1 min and blotted dry with sterile paper towels. Each canker sample was divided evenly into 5-mm^2 diseased tissue sections and plated on three different media types: (1) modified Nash-Snyder media (Nelson et al., 1983), (2) pine needle agar (PNA) media (Blodgett et al., 2003), and (3) potato dextrose agar with streptomycin and terigitol (PDA + S + T) media (Steiner and Watson, 1965).

Plated samples were incubated for four weeks at 20 °C, with weekly observation for identification and/or transfer of isolates to other media. Samples with unidentifiable mycelium isolates were transferred to carnation-leaf water agar (Nelson et al., 1983) or pine needle agar to induce spore production for identification. Plates from secondary transfers were visually inspected weekly for another four weeks. Frozen samples of isolates representing each genus or unknown morphological types were sent for DNA sequencing of the internal transcribed spacer

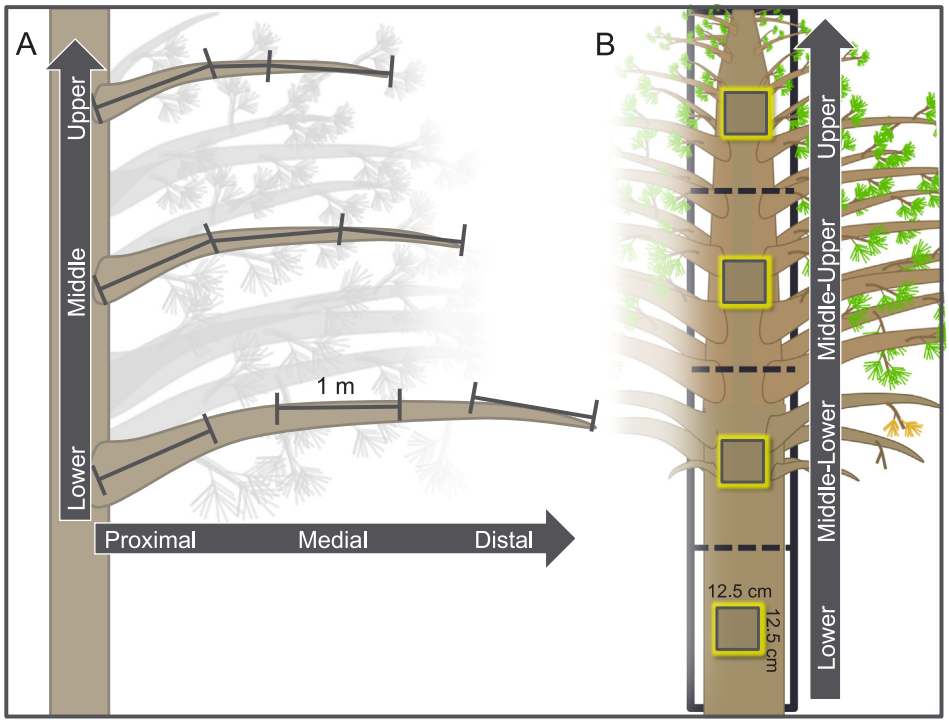

Fig. 4. Sampling strategy on each tree (N = 45) for scale insect counts and Calciciopsis canker measurements. (A) Six live branches were analyzed per tree, two from each vertical third of the canopy (lower, middle, and upper). Each branch was partitioned into horizontal thirds and a 1-m section was removed from each (proximal, medial, and distal) (N = 604). (B) Four 12.5 × 12.5 cm area bole samples were sampled per tree (N = 180), one from each vertical fourth of the total bole height (lower, lower-middle, upper-middle, and upper).

(ITS) region, which is widely used in fungal barcoding. In a few cases, there were amplification issues and the small subunit was used. We used primers ITS1F (5′-CTTGGTCATTTAGAGGAAGTAA-3′) (Gardes and Bruns, 1993) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3) (White et al., 1990) to amplify the ITS rDNA region under the following cycling conditions: 85 °C for 2 min, 95 °C for 95 s, and then 36 cycles of 58 °C for 1 min, 72 °C for 80 s, and 95 °C for 70 s, followed by a 52 °C for 1 min and 72 °C for 15 min. Sequences were compared to GenBank and other accessions using BLAST searches (NCBI). Species identifications from ITS barcodes were assigned when isolates had ≥99% matching ITS sequences (Gazis et al., 2011). In the cases where an isolate did not meet these criteria, the isolate morphology and relative sequence similarity were combined to infer the most probable genus, family, or order. Of the 43 cankers initially sampled, 36 underwent further identification. Seven samples were removed from the survey, because of mold contaminants (e.g., *Penicillium* spp.) or lichenized fungi (e.g., *Sarea* spp.).

2.5. Statistical analyses

Surface areas of branch and bole samples were used to standardize counts of bast scales and area of cankers. Surface area of each branch segment differed and required estimation using the area of a truncated cone: $A = s\pi (r_1 + r_2) + \pi {r_1}^2 + \pi {r_2}^2$, where s is the slant length (length of the branch segment) and r_1 and r_2 are the radii of each circular end. Surface area of all bole samples was ~156.25 cm^2. The adjusted bast scale counts were analyzed as number of scales per m^2 (i.e., scale density) and the adjusted Caliciopsis canker areas were analyzed as proportional canker area (i.e., the ratio of canker area to branch/bole sample surface area). Measurements of scale density only included counts of the current generation (live cysts), however we included both the current and previous generations (exuviae) of bast scales in the presence/absence data.

Branch and bole data are discrete sets and thus their analyses were conducted separately. Both branch and bole samples are nested within trees, however, it was necessary to treat the sub-factors and whole unit factor as experimental units due to the variables of interest. Size class is a tree-level factor and all other variables we assessed (canopy level, lichen cover, etc.) are sample-level factors. Due to this and the uneven sampling of branch samples among trees, it was necessary to partition our analyses further within branch and bole data sets, explained in detail below: assessment of the whole unit factor (tree) and assessment of the subunit factor (branch or bole sample).

2.5.1. Branch sample analyses

To assess if the incidence of the insect-pathogen complex differed on branches across tree size classes, we summed the scale counts, canker areas, and total analyzed branch surface area from each tree, and standardized our response variables as described above (N = 45). This procedure was done to accommodate the uneven sub-sampling of branch segments among trees. To satisfy the assumptions of normality, scales per m^2 were log (+1) transformed, proportional canker area was log (+0.005) transformed, and then an analyses of variance (ANOVA) test was performed, blocking for site. Further, generalized linear regression was conducted with scale density as the response variable with both tree DBH and DBH^2 (to investigate a possible non-linear trend) as the explanatory variables, using the negative binomial distribution to avoid overdispersion (referred to as negative binomial regression herein). Since canker area was a proportion, the beta distribution was used for this generalized linear regression (referred to as beta regression herein) using the R package betareg (Zeileis et al., 2016). To accommodate zero values, the response variable was first transformed per the adjustment recommended in Smithson and Verkuilen (2006): $y'' = [y (N-1) + 0.5]/N$, where N is the sample size.

To determine the effect of canopy level (lower, middle, and upper) on scale density and canker area on branch segments, we conducted negative binomial regressions of scale counts (offset by branch segment area) and beta regressions of proportional canker area, using both site and tree size class as blocking factors (N = 604). Distal diameter of branch segments was also included in these tests as a proxy for age of tissue to elucidate any interactive effects between tree-level distribution and maturity of the host resource on incidence of the organisms. To determine the effect of branch segment level (proximal, medial, and distal), it was necessary to conduct separate negative binomial and beta regressions, because there was a deficiency in medial branch segments of the upper canopy, especially in saplings. Beta regressions required

the Smithson and Verkuilen (2006) adjustment to proportional canker area. We assessed whole model fit using likelihood ratio tests with the 'lrtest' function from the R package lmtest (Hothorn et al., 2017), and Wald tests assessed significance of the factor levels, using *middle* as a reference for canopy level (vertical distribution) and *medial* as a reference for segment level (horizontal distribution). We selected these references to make intuitive contrasts between the intermediate factor level (e.g. *middle*) and the extreme factor levels (e.g. *lower* and *upper*). We also conducted individual logistic regressions, blocking for site and size class, to determine whether lichen cover affected the likelihood of bast scale and Caliciopsis canker presence on branch segments.

2.5.2. Bole sample analyses

Scales and cankers were absent from a number of bole samples, and thus simple transformations failed to meet the assumption of normality. To compensate for the skewed distributions and high overdispersion, we conducted hurdle regressions for scale count data and logistic regressions for canker presence/absence. Hurdle regressions are two-part models that test zero counts and positive counts of a dataset separately. Using the 'hurdle' function from R package pscl (Jackman et al., 2017), our hurdle regressions first performed a logistic regression on the presence/absence of scales, and second performed a negative binomial regression on the scale counts (offset by bole sample surface area) using the truncated dataset of only positive values.

To determine if the incidence of bast scale and Caliciopsis canker differed on tree boles as based on DBH, scale counts (offset by sampled bole area) and canker counts were summed per tree (N = 45). We performed a hurdle model for scale insects and a logistic regression for canker presence/absence using DBH as the explanatory variable and site as a blocking factor. To determine if scale presence and density varied with vertical bole level, bark thickness, and/or percent lichen cover of individual bark samples (N = 180), we included these variables in a single negative binomial hurdle regression, blocking for site and tree size class. We similarly included bole level, bark thickness, and percent lichen cover into a logistic regression with canker presence/absence to evaluate which of these variables best predicted *C. pinea* presence on bole samples. All regressions were assessed with likelihood ratio tests to determine whole model fit and Wald tests were used to determine factor significance.

3. Results

3.1. Branch sample analysis

Scale densities (number of scales per m^2) on assessed branches per tree ranged from 0 to 3.42 per m^2 on saplings, 1.03–12.96 per m^2 on poletimber, and 0.24–3.83 per m^2 on sawtimber-sized trees. The mean density on branches of poletimber trees was 3.8 times greater than on saplings and 2.4 times greater than on sawtimber trees ($F_{2,38} = 13.58$, $P < 0.001$) (Fig. 5a). The quadratic DBH term of the generalized linear regression was also significant ($Z = -3.23$, df = 1, $P < 0.001$), indicating higher scale insect density on trees of intermediate size (poletimber). Proportional canker area (the ratio of Calciciopsis canker area to branch surface area) on branches ranged from 0 to 12% on saplings, 1–12% on poletimber, and 0.1–26% on sawtimber trees. Proportional canker area on branches was greater on poletimber trees than on saplings, but neither poletimber trees nor saplings significantly differed from sawtimber trees ($F_{2,38} = 4.81$, $P = 0.014$) (Fig. 5b). Beta regressions revealed proportional canker area was not correlated with tree DBH ($Z = 1.86$, df = 1, $P = 0.06$) or DBH^2 ($Z = -1.70$, df = 1, $P = 0.08$).

Scale insect density on branch segments differed across canopy levels ($\chi^2 = 206.43$, df = 5, $P < 0.001$), with up to 30.68, 24.18, and 9.92 per m^2 in the lower, middle, and upper canopy, respectively. With the middle canopy as reference, we found the highest and lowest scale densities were respectively, on the branches of the lower and upper canopy ($Z = 1.04$, df = 1, $P < 0.001$; $Z = -0.64$, df = 1, $P = 0.03$). There was no interaction between branch segment diameter and canopy level, but scale density increased as the diameter of branch segments also increased ($Z = 0.65$, df = 1, $P < 0.001$). Proportional canker area on branch segments also varied with canopy level ($\chi^2 = 169.05$, df = 5, $P < 0.001$), with up to 76%, 15%, and 13% in the lower, middle canopy, and upper canopy, respectively. There was an interaction between canopy level and branch segment diameter. With the middle canopy as reference, proportional canker area was greatest in the lower canopy, where branches are generally greater in diameter ($Z = 0.41$, df = 1, $P < 0.001$), and lowest in the upper canopy, where branches are generally smallest in diameter ($Z = -0.30$, df = 1, $P = 0.04$).

Scale insect density ($\chi^2 = 56.66$, df = 2, $P < 0.001$) and proportional canker area ($\chi^2 = 23.51$, df = 2, $P < 0.001$) also differed horizontally along each branch. With medial branch segments as a reference, lower scale density was observed on distal branch segments, furthest from the bole ($Z = -4.45$, df = 1, $P < 0.001$), whereas higher scale density was found on the proximal branch segments, adjacent to the bole ($Z = 2.31$, df = 1, $P = 0.02$). Similarly, less proportional

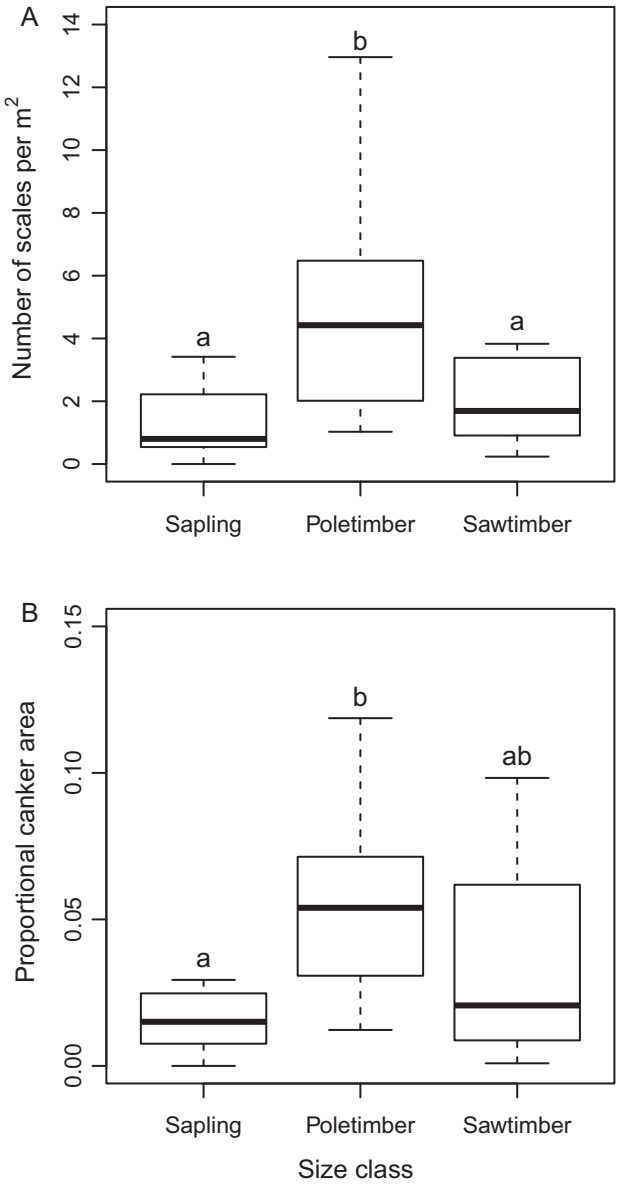

Fig. 5. Number of (A) bast scales per m^2 and (B) proportional Caliciopsis canker area found on branches analyzed according to size class: saplings (DBH < 12.5 cm), poletimber (DBH = 12.5–30 cm), and sawtimber (DBH > 30 cm) trees.

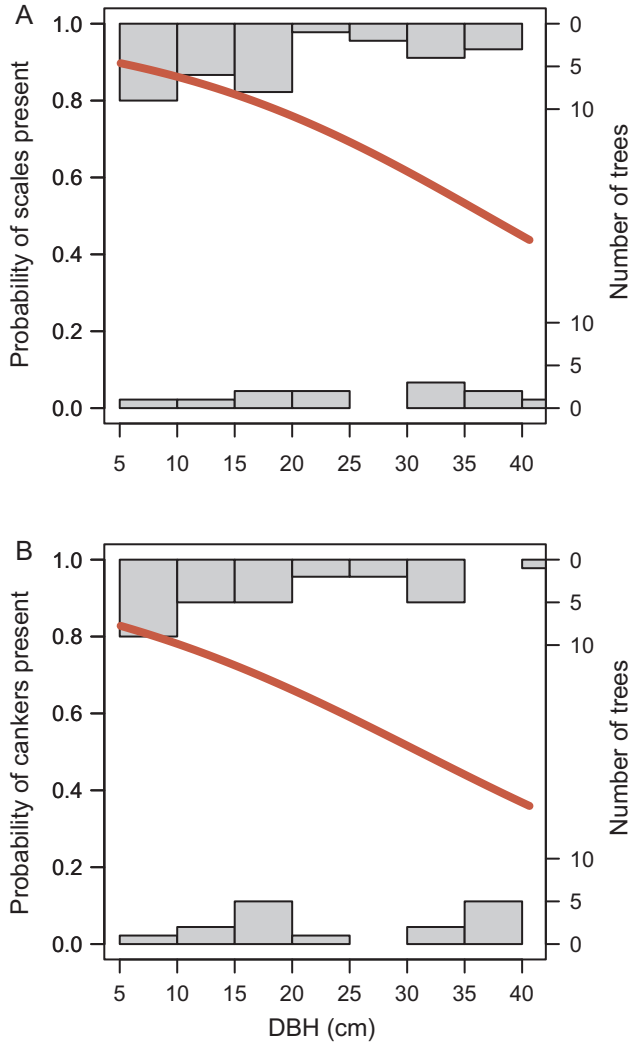

Fig. 6. Probability of (A) bast scales and (B) Caliciopsis canker presence on the boles of trees (line, left y-axis) according to tree DBH. Superimposed histogram shows the frequency of trees with and without each organism present on the bole according to DBH (bars, right y-axis).

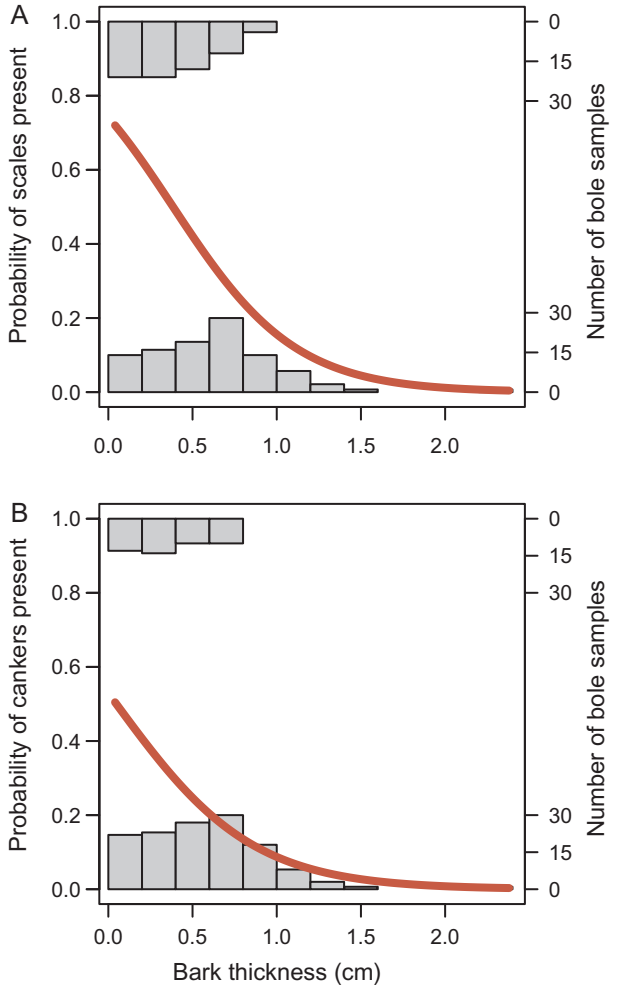

Fig. 7. Probability of (A) bast scales (B) Caliciopsis canker present on bole samples (line, left y-axis) according to bark thickness. Superimposed histogram shows the frequency of bole samples with and without each organism present according to bark thickness (bars, right y-axis).

canker area was found on distal branch segments ($Z = -3.24$, $df = 1$, $P < 0.001$), but there were no differences between the proximal and medial segments ($Z = 0.41$, $df = 1$, $P = 0.68$). Percent lichen cover significantly influenced the probability of scale ($\chi^2 = 117.39$, $df = 1$, $P < 0.001$) and canker ($\chi^2 = 182.88$, $df = 1$, $P < 0.001$) presence on all branch segments. Both the scale insect ($Z = 7.11$, $df = 1$, $P < 0.001$) and Caliciopsis canker ($Z = 8.57$, $df = 1$, $P < 0.001$) were more likely to be present on branch segments with greater percent lichen cover.

3.2. Bole sample analysis

On the bole, tree DBH was a significant predictor of bast scale presence according to the hurdle model ($\chi^2 = 6.29$, $df = 2$, $P = 0.04$) and Caliciopsis canker presence according to the logistic regression ($\chi^2 = 5.30$, $df = 1$, $P = 0.02$). Scale insect presence on the bole increased as tree DBH decreased ($Z = -2.03$, $df = 1$, $P = 0.04$) (Fig. 6a), but DBH had no effect on scale density ($Z = -1.32$, $df = 1$, $P = 0.19$). Caliciopsis canker presence on boles also increased as DBH decreased ($Z = -2.12$, $df = 1$, $P = 0.03$) (Fig. 6b).

The hurdle model that tested vertical bole level, bark thickness, lichen cover, and their interactive effects on the presence and density of scale insects fit the data better than the null model ($\chi^2 = 33.03$, $df = 16$, $P = 0.007$). The logistic regression revealed no interactive effects, and bark thickness was the only significant variable in the model, revealing that scales were more often found on thinner bark ($Z = -2.16$, $df = 1$, $P = 0.03$) (Fig. 7a). The binomial regression on the truncated dataset revealed no significant variable for predicting scale insect densities on bole samples. The logistic regression that tested vertical bole level, bark thickness, lichen cover, and their interactive effects on Caliciopsis canker presence did not perform better than the null model ($\chi^2 = 11.24$, $df = 8$, $P = 0.19$). When modeled individually, the logistic regression including vertical bole position ($\chi^2 = 8.31$, $df = 3$, $P = 0.04$) and the logistic regression including bark thickness ($\chi^2 = 5.44$, $df = 1$, $P = 0.02$) outperformed the null model (Fig. 7b). Overall, cankers were most often found on the upper-middle sections of boles ($Z = 2.83$, $df = 1$, $P = 0.005$) and on the thinnest bark ($Z = -2.30$, $df = 1$, $P = 0.02$). No scales or cankers were found on bole samples with bark thicker than 1 cm and 0.8 cm, respectively.

3.3. Fungal isolations

Of the 36 isolates from cankers without *C. pinea* fruiting structures, two were assigned to the genus *Caliciopsis*. One was identified as *C. pinea* based on the stromata fruiting structures that formed in culture on the canker tissue. The other could not be confidently given a species-level identification based on the ITS region (DQ471039), so it was instead denoted only as a member of the genus *Caliciopsis*. Seventeen

isolates belonged to one of two species from the genus *Pestalotiopsis* based on 99% ITS region matches: *P. maculans* (Corda) (KX610327) and *P. chamaeropis* Maharachch (KM199326). Eleven isolates belonged to the genus *Phaeomoniella*, based on spore morphology and 98% ITS region matches to a species within Eurotiomycetes (KM519288) (Damm et al., 2010). Two isolates were identified as *Coniochaeta velutina* (Funkel) (GQ154624) and one isolate was identified as *Clonostahys rosea* (Link) (LT576164). Three isolates without confident ITS region matches were unidentifiable.

4. Discussion

Since European settlement in the 18th century, eastern white pine has experienced a significant increase in many abiotic (e.g., logging and burning) and biotic (exotic and native herbivores and pathogens) stressors (Abrams, 2001; Wendel and Smith, 1990). The current dieback and mortality of eastern white pine is unprecedented and is associated with a previously unknown insect-pathogen complex that has been reported throughout its North American range with varying severity. Field observations indicate that poletimber-sized trees are experiencing the most severe branch dieback, while saplings have the highest levels of mortality (Asaro et al., this issue; Costanza et al., this issue; Schulz et al., this issue-a). Hence, we expected to observe greater scale density and canker area on the branches of poletimber and the boles of saplings. In evidence, we found that the distribution of both the scale insects and cankers were consistent with our expectations and hypotheses. These data suggest a link between the insect-pathogen complex and the observed tree dieback and mortality patterns relating to tree size class. Such distributional variation of insects and pathogens based on tree size has also been reported, such as for the scale insect, *Ultracoelostoma assimile* Maskell, on New Zealand beeches (*Nothofagus* spp.) (Wardhaugh et al., 2006) and for stem cankers on a Panamanian canopy tree (*Ocotea whitei* Woodsen) (Gilbert et al., 1994). It is noteworthy that although sawtimber-sized trees currently appear resilient, they probably require a higher pest load and/or longer period of time for the insect-pathogen complex to cause girdling.

The bast scales and Calciciopsis cankers were also distributed in a bottom-up gradient within trees, consistent with the observed pattern of branch dieback. The lower branches of symptomatic eastern white pine are commonly the first to die, and subsequent branch deaths occur in an upward progression (Constanza et al., this issue; Schulz et al., this issue-b). We expected and found that the incidence of the insect-pathogen complex reflected this pattern, as there were higher scale densities and proportional canker area in the lower canopy. Such bottom-up canopy distribution patterns have also been observed in two other pine bast scales, both of which are important pests. McClure (1976) found that 81% of red pine scales, *M. resinosae* [now *M. matsumurae* (Kuwana)], inhabited the lower canopy of Connecticut red pine (*Pinus resinosae* Aiton), whereas only 7% inhabited the upper canopy. Additionally, Jactel et al. (1996) found the maritime pine scale, *M. feytaudi* Ducasse, was completely absent from the upper third of maritime pine (*Pinus pinaster* Aiton) canopies in southern France, with maximum densities inhabiting intermediate tree heights. Other pathogens can also infect trees from the bottom up, such as the non-native fungus, *Cronartium ribicola* J.C. Fisch. (white pine blister rust), which tends to form cankers in the lower canopy of North American five-needle pines (Schwandt et al., 2013).

Heterogeneity in within- and across-tree distribution may be the result of factors that facilitate insects and pathogens to more easily develop in certain size classes and canopy levels, such as mode of dispersal (e.g., Brown et al., 1997) and/or climatic conditions (e.g., Rowe and Potter, 1996). For example, wind-dispersed bast scale crawlers are most likely to land on the ground and may opt to settle sites within a short crawling distance on lower branches and smaller tree boles. Cooler and moist conditions at low strata under a closed canopy can also be optimal for pathogen establishment. In evidence, Munck et al. (2016) reported a greater incidence of *C. pinea* cankers with increasing stand density, indicating that the amount of host material and specific, micro-habitat conditions may be significant predictors of the insect-pathogen complex occurrence and contribution to dieback symptoms.

Our study suggests that tree bark thickness influences the distribution and patchiness of the bast scale and Calciopsis canker formations. We found that bast scales and cankers were more frequent on thinner than thicker bark, and they were both completely absent on bole samples with bark thicknesses greater than one centimeter. Bark thickness was less variable on branches as the bark on approximately 90% of the branch samples was less than 0.1 cm thick. We posit that the bark of eastern white pine becomes an effective barrier to herbivory when it nears and exceeds this 1-cm threshold. The bark is likely too thick at this point for a bast scale's stylet to effectively penetrate and reach the outer phloem cells. Similarly, Wardhaugh et al. (2006) reported that *U. assimile* colonization is a function of host bark thickness, and Jactel et al. (1996) found that *M. feytaudi* was also unable to settle on portions of host trees with the thickest bark. If eastern white pine bast scales are absent and there are no feeding wounds on these thicker-barked surfaces of the tree, then we speculate that *C. pinea* may also lack the infection court needed to establish and form cankers.

Despite having thin bark, new growth also seemed to be unsuitable for bast scale settlement, as significantly fewer scale insects were found on younger, upper canopy branches and distal branch segments (which include axillary meristems) when compared to older, lower canopy branches and proximal branch segments. This was further supported by the finding that scale density increased with branch segment diameter (a reflection of the branch tissue's age). Bark texture, rather than bark thickness, may better explain this trend, as it is an important determinant of an insect's ability to colonize host trees (Ferrenberg and Mitton, 2014). Eastern white pine bast scales typically embed themselves in bark crevices, along canker edges, and under lichen (Mech et al., 2013; Michigan DNR, 2015; Schulz et al., this issue-b), but the smooth, young bark on new growth generally lacks these ideal settlement sites. We found that the presence of scale insects and canker formations were positively correlated with percent lichen cover on branch segments. No such pattern was found on bole samples, however, because percent lichen cover covaried with bark thickness. Bast scale settlement and *C. pinea* infection appears to occur most often on intermediate bark surfaces: old enough to provide appropriate refugia, but thin enough to provide access to nutritive fluids in the outer phloem (Wardhaugh, 2014).

We assessed the current distribution of the bast scale and Caliciopsis canker on individual trees. In previous years, scale insects may have settled on thinner-barked parts of trees that are now too thick for re-colonization. If *C. pinea* infected and produced cankers on these areas following prior scale infestation, they may have gone undetected in our study, given that we only examined surface cankers. Internal necrosis of the xylem by *C. pinea* resulting from the bark callusing over surface cankers is also common, especially in stems of larger-diameter trees (Costanza et al., this issue). Although internal cankers decrease wood quality of sawtimber trees (Costanza, 2017), surface cankers likely pose the greater threat of girdling limbs and small stems. The vast majority of the cankers we observed appeared to have been caused by *C. pinea*. By identifying the characteristic fruiting structures of *C. pinea*, we were confidently able to distinguish between Caliciopsis and non-Caliciopsis surface cankers. From our isolation work of fungi present in cankers without *C. pinea* fruiting structures, the incidence of *Caliciopsis* spp. was very low (5.6%). The other 94.4% of fungi isolated from these cankers are likely endophytic opportunists, which are common on trees but are not considered highly virulent (Damm et al., 2010; Sieber, 1989).

In summary, this is the first quantitative study to elucidate the distribution of the eastern white pine bast scale and Caliciopsis canker within individual trees and across tree size classes. The organisms in this insect-pathogen complex seem to favor thin-barked, but mature, eastern white pine surfaces, including poletimber branches, sapling

boles, and the lower canopy branches across tree all examined size classes. The incidence of the bast scale and Caliciopsis canker is consistent with, and we suggest is strongly associated with, observed dieback patterns in eastern white pine, which include: (1) highest severity of branch dieback occurring in poletimber-sized trees, (2) highest mortality occurring in saplings via stem girdling, and (3) a pattern of crown thinning occurring from the bottom up in all sized trees (Asaro et al., this issue; Costanza et al., this issue; Schulz et al., this issue-a, this issue-b). Although this research was conducted exclusively at the southernmost extent of the host-trees' range in Georgia, the dieback symptoms in eastern white pine do increase in severity with increasing latitude (Schulz et al., this issue-a). Thus, it is reasonable to suspect that the insect-pathogen complex would have a similar tree-level distribution elsewhere in their respective ranges.

There are a few management implications from our study. To reduce pest populations, managers may consider the use of registered insecticidal sprays on lower branches in the spring to coincide with adult emergence. Pruning of lower branches in the fall season, once *M. macrocicatrices* cysts have settled on trees, may have similar effects. This practice is suggested for white pine blister rust (Schwandt et al., 2013), and may also have the potential to slow the rate of infection by *C. pinea*. Tree density has been found to be an important factor in dieback and mortality patterns as open-grown trees tend to be more resilient (Munck et al., 2015; Schulz et al., this issue-a), and so selective thinning of infected trees may slow the spread of the insect-pathogen complex. Although sawtimber dieback appears less severe than the dieback and mortality in smaller diameter trees, especially in the Southern Appalachians, they should continue to be monitored for symptom advancement. Mortality in younger trees is a significant and immediate concern, but if the loss of reproductive trees occurs, this dieback phenomenon will invariably result in the loss of seed-source and regeneration, threatening its sustainability (Asaro et al., this issue). Overall, a greater focus on the management and conservation efforts for eastern white pine may result in alleviating the ecological and economic impacts of this native insect-pathogen complex in eastern North America.

Data reference

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.foreco.2018.02.002.

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