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Factors affecting *Xyleborus glabratus* attack and host utilization in sassafras and redbay in the Carolinas

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The laurel wilt disease complex is a destructive combination of a non-native beetle vector [redbay ambrosia beetle (RAB), *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae)] and a symbiotic fungus (*Harringtonia lauricola* (Ophiostomataceae) T.C. Harr., Fraedrich & Aghayeva), which serves as a pathogen in the host trees infested by RAB. The complex originated from Asia and was first discovered in the United States near Savannah, GA in 2002, and has rapidly made its way across the southeastern US, causing mortality for redbay and other important Lauraceae species, including sassafras, giving this disease complex the potential to have far-reaching ecological effects across North America. Our goal with this study was to examine the spatial distribution of RAB attacks in redbay and sassafras trees along the leading edge of disease progression. RAB attacks were clustered in both tree species, with attacks being most concentrated on the south side of the tree in sassafras, and with RAB clustering more with other RAB attacks on redbay. When comparing bolts that produced adult RABs, the average number of RABs emerged was higher in redbay compared to sassafras. Entrance hole density, RAB emergence, and moisture content were higher near the base of the stem compared to stems sections higher on the bole of both tree species. Our results suggest that physiological differences, such as size and structure of vessels, between these tree species may drive beetle attack patterns and, therefore, affect the progression and spread of disease throughout sassafras and other Lauraceae.

Key words: laurel wilt disease, ambrosia beetles, spatial distribution

Introduction

Non-native insects represent a major forest health threat across the globe and, of these pests, one of the most successful groups of invaders are bark and ambrosia beetles (Coleoptera: Curculionidae: Scolytinae) as they are readily transported into new areas via tree and wood products due to their cryptic nature (Haack 2001, Rassati et al. 2018). Their small size allows them to be easily overlooked during inspections, while their tendency toward generalist host selection behavior provides biological advantages that facilitate invasion (Rabaglia et al. 2006).

The redbay ambrosia beetle (RAB; *Xyleborus glabratus* Eichhoff, 1877) is an invasive ambrosia beetle that was first discovered in the United States in 2002 in Port Wentworth, GA. It originates from Asia where it has been reported on hosts in the families Dipteracarpaceae, Fagaceae, Fabaceae, and Lauraceae, but is not known to be a major pest in its native range (Hulcr et al. 2017). In the United States, however, RAB is known to utilize only one host family (Lauraceae) and attacks living, apparently healthy trees, suggesting that RAB could have experienced a functional shift from a generalist decomposer beetle to a specialist primary pest (Kendra et al. 2013, Hanula and Mayfield 2014, Hulcr et al. 2017). As with many other ambrosia beetles, RAB has the ability to reproduce by parthenogenesis and sibling mating, allowing it to establish a new infestation with the introduction of a single female (Brar et al. 2013).

The combination of RAB along with its symbiotic fungus, *Harringtonia lauricola* (T.C. Harr., Fraedrich & Aghayeva) Z.W. de Beer & M. Procter (formerly *Raffaelea lauricola*), causes a vascular disease known as laurel wilt in North American Lauraceae. Within a few years after initial detection, laurel wilt had caused widespread mortality in redbay and sassafras in South Carolina, Georgia, and Florida (Fraedrich et al. 2008, Cameron et al. 2015) and has now spread throughout the entire southeastern Coastal Plain and as far north as Kentucky (Loyd et al. 2020, Mayfield et al 2022). Numerous additional lauraceous species have been identified as

suitable hosts (Mayfield et al. 2013, Kendra et al 2014, Olatinwo et al. 2021), indicating the potential for laurel wilt to spread even more widely in the United States. Furthermore, the progression of laurel wilt through these Lauraceae species has already been shown to cause detrimental cascading effects for certain insect fauna that rely on these trees (Riggins et al. 2019). Previous studies have identified the supercooling point of –22 °C for RAB, which suggests an ability to survive extreme temperatures (Formby et al. 2013); however, the lethal temperature for RAB is –10 °C, which is warmer than its supercooling point and, therefore, makes it a chill susceptible species (Formby et al. 2018). The authors also noted that only 0.1% of extant sassafras was in the range of temperatures sufficient to prevent RAB establishment, suggesting that temperature is unlikely to prevent the movement of RAB and spread of laurel wilt further north. This is further evidenced by the fact that in some states, such as South Carolina, RAB has been observed in all 12 months of the year (Hanula et al. 2008).

Research examining host attractiveness and suitability has had conflicting results. Some studies have shown a low level of attractiveness of the beetle to sassafras and other non-redbay Lauraceae (Hanula et al. 2008) while others have demonstrated that sassafras is equally as attractive to RAB but that redbay is more vulnerable to RAB attacks, and thus more suitable for development than sassafras (Kendra et al. 2014). This difference indicates that there are additional drivers behind the observed disease progression than simply host distributions. Currently, we do not fully understand the mechanisms behind host selection by RAB females and, therefore, cannot make accurate predictions about future spread and impacts to non-redbay Lauraceae. To better understand potential driving mechanisms behind host selection, we examined patterns in attacks by RAB in redbay and sassafras, we compared the spatial distribution on tree stems (by height and circumference) of RAB attacks and adult emergence using both redbay and sassafras trees in South Carolina and North Carolina, along the leading edge of disease progression.

Materials and Methods

Site Selection

In fall 2019 we surveyed two sites in the Midlands region of South Carolina (Sesquicentennial State Park (SP): 34.087307, –80.907624; Leesville: 33.927670, –81.495727) for signs of laurel wilt disease in both redbay and sassafras. In fall 2020 we surveyed an additional site at Cliffs of the Neuse State Park in North Carolina (35.235687, –77.891047). Both Sesquicentennial SP and Leesville are classified as humid subtropical climates and have Pelion loamy sand with 2–6% slopes. Cliffs of the Neuse SP is classified as a humid warm temperate climate and consists mainly of Kalmia loamy sand with 15–25% slopes (Soil Survey Staff 2024). All three sites are primarily loblolly–longleaf pine forests with mixed hardwoods.

Sample Collection

We identified a total of 17 symptomatic redbay ($n = 4$) and sassafras ($n = 13$) trees in October of 2019 and 2020 for felling (Table 1). Prior to felling, the diameter (cm) at breast height (DBH; 1.4 m) was measured and the north-facing side of each tree (i.e., N line) was marked with spray paint. Each tree was cut as close to the ground as possible, and the total height (m) of each tree was measured before cutting it into 1 m bolts from the bottom-most cut to a top diameter of 7.6 cm (due to evidence that stems become less visually attractive to RAB at diameters below 10 cm; Mayfield and Brownie 2013). Each 1 m bolt was then sub-divided into three subsections for the following purposes: (1) a 65 cm sub-section was used to assess the spatial distribution of RAB attacks, (2) a 5 cm sub-section was used to quantify moisture content (MC; %), and (3) the last 30 cm subsection was used to quantify RAB emergence (Fig. 1).

Beetle Attack Spatial Distribution

To determine the distribution of RAB attacks on host stems, the bark from each 65 cm sub-section was removed, all *X. glabratus* entrance holes (EH) were identified and marked by sticking a size no. 1 paperclip with an end diameter of 0.8 mm (Hanula et al. 2008) into each beetle hole. This size corresponds to the exact dia. of RAB EH, while other commonly collected ambrosia beetles (e.g., black twig beetle, *Xylosandrus compactus*) have holes much larger in diameter. At each marked EH we measured: the circumference of the bolt (cm), height of the EH from the bottom of the bolt (cm), and absolute distance from the N line (i.e., circumference; cm) to the EH. To assess the spatial distribution of attacks, we standardized both the height (cm) and absolute distance of each EH on a scale of 0 to 1 by dividing the height and distance of each EH by the total bolt length and maximum circumference of each bolt, respectively.

Moisture Content

To measure MC, the wet (i.e., fresh) weight (g) of each 5 cm section was measured immediately upon returning samples to the Clemson Forest Health Lab (<12 h after felling) before drying in an oven (Fisher, Waltham, MA) at 102 °C for 24 h. After drying, each sample was then weighed, and MC was calculated using the following formula:

$$\text{MC} = \frac{\text{wet weight (g)} - \text{dry weight (g)}}{\text{wet weight (g)}} \times 100$$

Beetle Emergence

To collect RAB emerging from the 30 cm sub-sections, each log was placed into an individual 11.4 l (3 gal.) rearing container with a jar containing propylene glycol at the bottom to trap adults (Mayfield et al. 2014). To prevent desiccation, both ends of each 30 cm subsection were dipped into melted paraffin wax before being placed in containers. Containers were then hung from racks inside an insulated garage at the South Carolina Forestry Commission office in Columbia, SC (34.081293, –81.130828) and were checked for adult beetles every 2 wk from October 2019 to March 2020. All contents of the collection jars were poured through 190 µm mesh paint filters (Central Pneumatic, Harbor Freight Tools, Calabasas, CA) and then stored at 5 °C until sorting. Adult RAB were then

Table 1. Site descriptions. We identified a total of 17 symptomatic redbay ($n = 4$) and sassafras ($n = 13$) trees in October of 2019 (Sesquicentennial SP: 4 redbay and 4 sassafras; Leesville: 4 sassafras) and 2020 (Cliffs of the Neuse SP: 5 sassafras).

Site	Coordinates	Collection date	# redbay	# sassafras
Sesquicentennial SP	34.087117, –80.908277	14–16 October 2019	4	4
Leesville	33.927670, –81.495727	14–16 October 2019	0	4
Cliffs of the Neuse SP	35.234835, –77.889225	8–9 October 2020	0	5

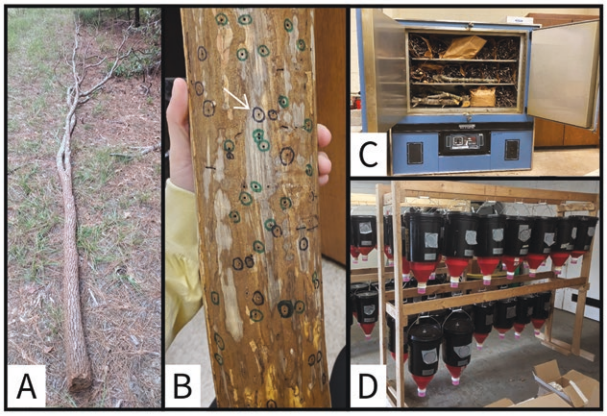

Fig. 1. Each individual redbay and sassafras tree (A) was cut into 1 m sections and each section was then cut into three smaller sections to quantify (B) ambrosia beetle attacks (white arrow pointing to black circle; 65 cm), C) moisture content (%) (5 cm), and D) adult beetle emergence (30 cm).

counted and sexed; haploid males are smaller, flightless, and have a deformed pronotum (Mann et al. 2014). Due to a lack of male emergence (n = 32; 5% emergence), however, and the fact that males are not needed for disease spread, males were not included in further analysis.

Statistical Analyses

Analyses were limited to the lowest 4 m sections of each bole because this height was common to all trees and contained nearly all visible RAB signs. The number of RAB EH and the total number of adult RAB emerged were summed across all 65 and 30 cm subsections, respectively. To test whether RAB attacks were normally distributed across bolts, we used the standardized Y (height) and X (absolute distance from N line) as described above to create a spatial point pattern along a 2D plane, which we then used to calculate $\hat{k}$ (Ripley 1976). While some spatial point pattern analyses, such as $\hat{f}$ and $\hat{g}$, can identify clustering patterns at a single scale, that is the distance between an "event" (i.e., RAB attack) and the nearest point along a grid, or the distance between two nearest "event" neighbors, Ripley's $\hat{k}$-function accounts for variation at a second scale, that is the "clustering of clusters", which is necessary to assess the large area relative to attacking RAB females and the variation in physiological and environmental gradients along that surface area (Hohl et al. 2017, Páez 2021).

We then used an Analysis of Variance (ANOVA) to test the null hypothesis that height (m) of bolt section did not significantly affect (a) RAB attack density (EH/100 cm^2), (b) RAB emergence (# beetles emerged/100 cm^2), and (c) wood MC (%) for the first 4 m sections of each tree, for each species separately. Because sassafras was found at all sites, we included both "site" and "tree" as random effects in the sassafras models, but only "tree" was considered a random effect in the redbay models because redbay was only collected at a single site. To meet normality assumptions, EH was transformed using the square root function and RAB emergence was transformed using the $\log_{10}(y + 1)$ function. Because MC was highly variable by tree (13–43%), we standardized values by dividing MC of each bolt by the mean of all bolt sections within each respective tree, essentially transforming MC to a ranked variable. We then compared means within each independent variable for significant ($\alpha = 0.05$) models using Student's t-test. To determine whether the host species differed in relation to these variables, we compared the two models using an F-test. All statistical analyses were conducted in R (R Core Team 2023).

Results

A total of 699 RAB EH were marked across all 17 symptomatic trees, with 46% (n = 1,168) of these occurring on the 16 redbay bolts (μ = 73/bolt) and 54% (n = 1,378) occurring on the 96 sassafras bolts (μ = 14/bolt). Non-RAB holes accounted for an additional 2,767 attacks, with 56% (n = 1,559; μ = 97/bolt) occurring on redbay and 44% (n = 1,208; μ = 13/bolt) on sassafras. A total of 681 adult RAB (649 females, 95%; 32 males, 5%) were collected from emergence containers, with a similar number of adults emerging from 16 redbay bolts (n = 338; 49.6%) and 79 sassafras bolts (n = 343; 50.4%). A total of 157 non-RABs were collected from emergence containers with *X. crassiusculus* being the most abundant (n = 91), followed by *X. saxesenii* (n = 64), and two single individuals of *X. ferrugineus* and *X. affinis*.

EH on both redbay and sassafras were significantly clustered, as evidenced by the observed $K(r)$ line for both redbay and sassafras occurring above the envelope (i.e., the 95% upper and lower CI threshold for a random distribution) (Fig. 2). On sassafras trees, EH were clustered toward the S side of the tree. On redbay trees, EH were clustered but not near any specific side of the tree, rather they were instead clustered around other RAB attacks (Fig. 3).

There was a significant effect of bole height (m) section (for the first 4 m) on mean RAB attack density for both redbay (F = 9.34; df = 3,9; P = 0.004) and sassafras (F = 5.03; df = 3,43; P = 0.005). In redbay, mean attack density was inversely related to height on the bole, with the highest attack density on the 1 m section (2.8 EH/100 cm^2), and the lowest attack density on the 4 m section (Fig. 4A). Mean attack density on the 2 m height section was not significantly different from the 1 and 3 m sections, whereas attack density on the 4 m height section was significantly lower than all other sections (Fig. 4A). In sassafras, means of RAB attack density were much lower (< 0.5EH/100 cm^2) compared to redbay, and were lowest on the 4 m section with the first 3 sm sections having similar attack densities (Fig. 4A).

There was a significant effect of bole height (m) section on mean RAB emergence for sassafras (F = 5.49; df = 3,43; P = 0.003). In sassafras, mean RAB emergence from the 1 m section was higher than from the 3 and 4 m sections (Fig. 4B), but not the 2 m section, which did not differ significantly from any other section (Fig. 4B). Mean RAB emergence per bolt did not differ significantly across height in redbay (F = 3.23; df = 3,9; P = 0.075), however, trends of RAB emergence (Fig.4B) were similar to those for mean attack density (Fig. 4A) on redbay.

There was a significant effect of bole height section on mean normalized bolt MC in both redbay (F = 9.19; df = 3,9; P = 0.006) and sassafras (F = 2.94; df = 3,43; P = 0.044); in redbay, the 1 m bolt had the highest MC relative to all other sections (Fig. 4C), while the 1 m sassafras bolts had higher MC relative to the 3 and 4 m sections but not the 2 m sections (Fig. 4C).

Discussion

Our goal was to examine patterns of RAB attack and emergence between two laurel wilt host species, redbay and sassafras, along the leading edge of disease progression in the Carolinas. We collected similar numbers of adult RAB from both redbay and sassafras trees; even though sassafras was represented 4× more in field-collected samples [i.e., redbay accounted for only 14% (16 of 112 logs)], nearly half of the RAB attacks and half of the emerging adult RAB were from redbay logs. However, average RAB emergence per cm^2 did not differ significantly between the two species suggesting that, while beetles may be more *attracted* to redbay, both species are

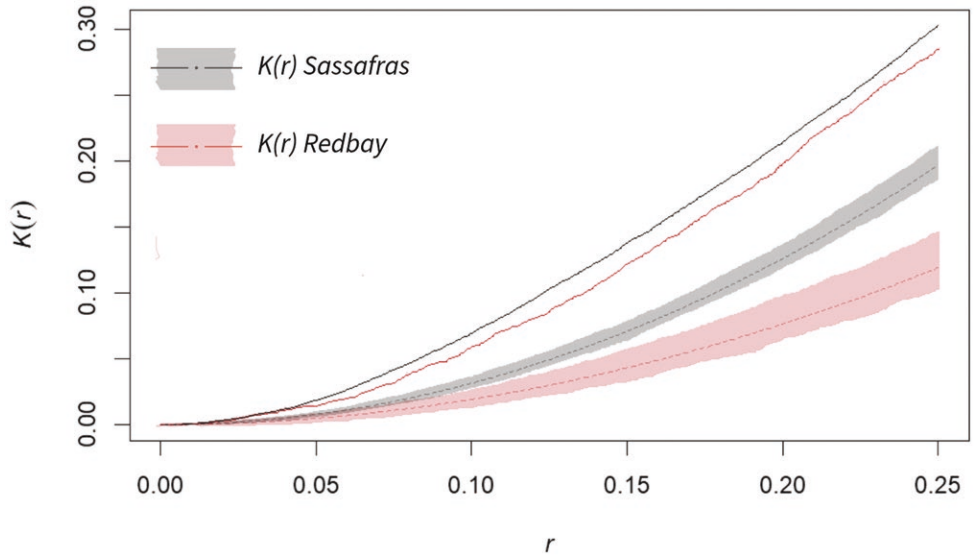

Fig. 2. Ripley's *K* test of sassafras (gray) and redbay (red) sampled trees. Observed Euclidean distance values, represented as solid lines of black (sassafras) and red (redbay) trees lie above the theoretically derived expected values, represented by the shaded envelopes of the same respective color. Observed values lying inside the envelope indicate a random distribution of points while observed values lying above the envelope indicate a significantly clustered distribution of attacks.

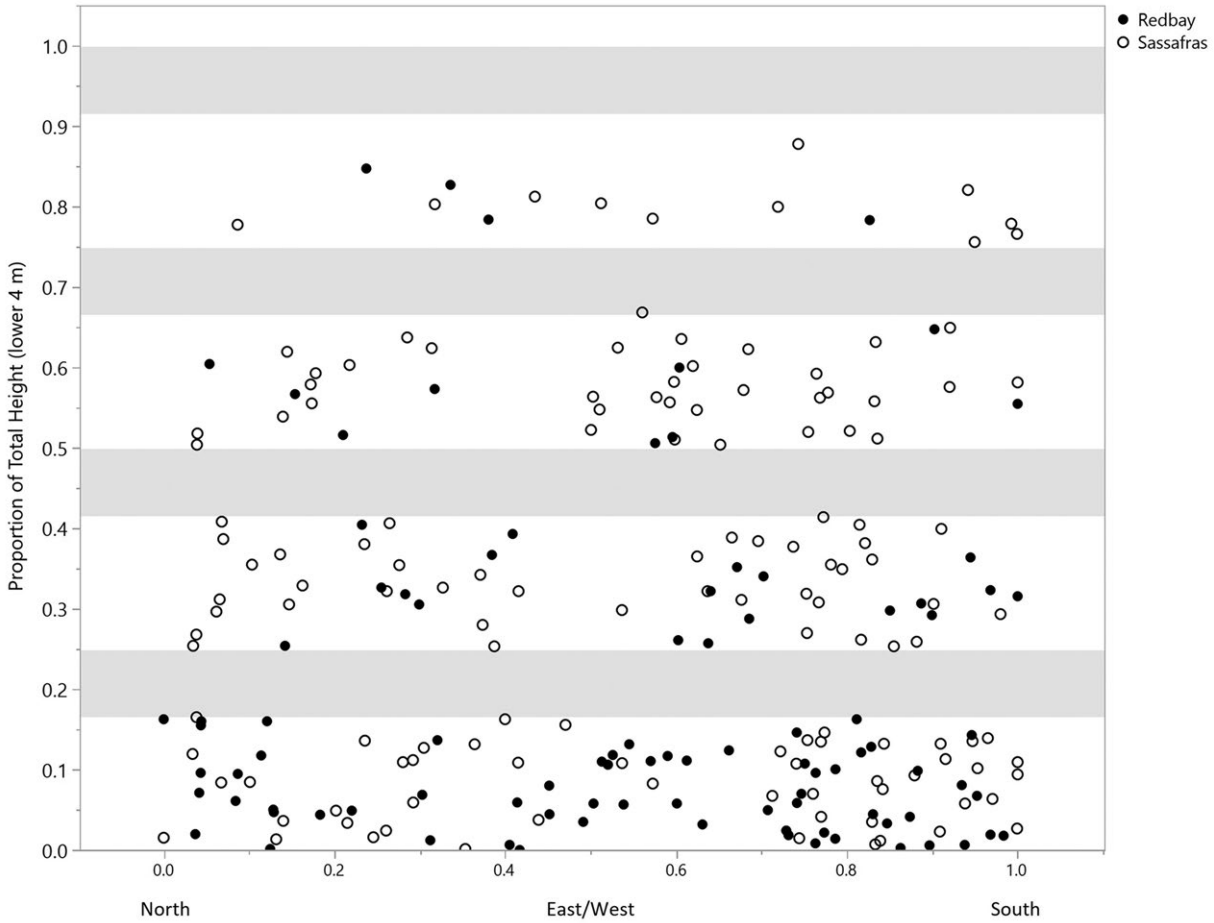

Fig. 3. Scatter plot of female RAB (*Xyleborus glabratus*) attacks along a 2D representation of whole trees (o = 13 sassafras; ●= 4 redbay trees), with gray bands indicating sections of each tree that were used for other objectives (i.e., beetle emergence and moisture content) and were not examined for EH. The *x*-axis represents the distance of each EH from the north line (0°) and the *y*-axis represents the height (m) of each attack, proportionate to the total height of each respective tree.

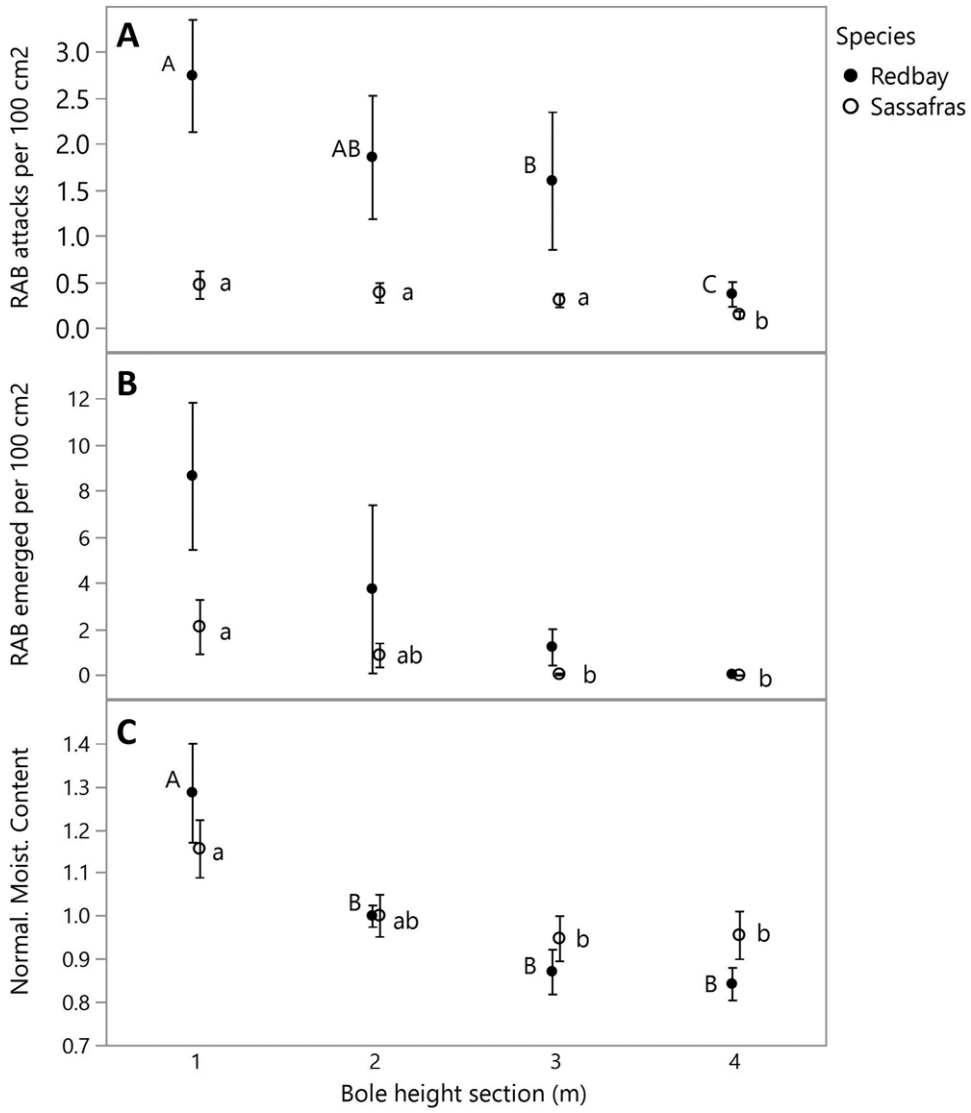

Fig. 4. Mean RAB (*Xyleborus glabratus*) attack density (attacks/m^2; A), RAB emergence (RAB/100 cm^2; B), and normalized bolt moisture content (%; C) by height (m) of the bole from which the bolt was sampled, for sassafras (o = 13 trees) and redbay (●= 4 trees). While each species was analyzed separately and transformed to meet normality assumptions [√(attack density); ($\log_{10}$(emergence + 1))], raw means are presented here. Bars indicate standard error of the mean. Means labeled with the same letter of the same case (uppercase = redbay; lowercase = sassafras) are not significantly different ($\alpha = 0.05$).

susceptible to RAB attack and mortality from laurel wilt disease. However, due to the destructive nature of our sampling, we cannot draw direct relationships between emergence from the 30 cm bolts and attack numbers on the 65 cm bolts.

Although there was a significantly clustered pattern to RAB EH/cm^2 on both redbay and sassafras, the patterns themselves differed in their respective distributions. Specifically, RAB attacks on sassafras were clustered toward the south side of host stems. This is a common pattern seen in wood boring beetles (e.g., Hartshorn et al. 2019), typically attributed to the warmer temperatures experienced on that side of the tree which receives more consistent sun exposure. However, some research suggests that, rather than keeping the under-bark environment consistently warmer compared to the ambient air temperature, the difference in temperature is not constant in direction or magnitude (Vermunt et al. 2012), suggesting that some other buffering property may be present or necessary to provide adequate protection for developing beetles. Conversely, RAB attacks on redbay were clustered specifically in relation to each other more so than to any one side of the host tree (Fig. 3). Previous studies have demonstrated the tendency of RAB females to have increased brood production in previously and actively infested trees, compared to healthy trees (Fraedrich et al. 2018), suggesting that adult female RAB may preferentially attack areas of the tree which already contain other RAB attacks. This pattern has been documented in other wood-boring beetles (e.g., Vansteenkiste et al. 2004, Hýsek et al. 2021), likely due to the loss of function in the xylem cells following physical damage from larval feeding, which then allows symbiotic, as well as competitive, fungi to enter the tree, further degrading the structure and mechanical properties of the wood, allowing developing larvae to more easily move throughout the tree.

These results combined suggest that, while adult female RAB are highly attracted to both sassafras and redbay, they encounter, and must overcome different physiological barriers in the two species. For example, while MC and surface area (cm^2) per bolt was not significantly different between the two host species, MC appeared to be an important factor in the number of RAB attacks per cm^2 of bolts,

with more attacks on bolts with lower MC. Sapwood moisture content is important for the success of ambrosia beetles both in brood production and the growth of its symbiotic fungi (i.e., ambrosia) (Francke-Grosmann 1967). It is thought that the host response to the laurel wilt pathogen increases moisture content and moisture retention due to the formation of tyloses (Inch et al. 2012). In this case, it is possible that the slightly higher average MC of sassafras (32.00 ± 0.94%) compared to redbay (25.13 ± 2.62%) means that sassafras does not require clustering of RAB to reduce functionality and MC of sassafras xylem. We expect that sassafras would have higher brood success owing to this higher MC, however, we found that most sassafras bolts were unproductive, and beetles emerged over 23 bolts, with 14 of those bolts producing fewer than 10 adult RAB. This is further evidenced by the facst that only four bolts each of sassafras and redbay produced 63.3% and 91.7% of the successful RAB brood, respectively.

Previous studies have reported both that sassafras is an inferior host for RAB (e.g., Hanula et al. 2008, Mayfield and Hanula 2012) and conversely, that sassafras is comparable to both redbay and swampbay in terms of attractiveness as well as brood productivity (e.g., Mayfield et al. 2013, Kendra et al. 2014, Fraedrich et al. 2018). It is important to note, however, that these studies were conducted in areas where laurel wilt had already caused massive declines of these species. While the destructive nature of our sampling makes it impossible to directly compare attacks with emergence from the same tree or bolt, we did find lower average RAB emergence per bolt from sassafras bolts that were productive compared to redbay bolts that were productive. Combined, our results suggest that while sassafras may be comparable in terms of attractiveness and productivity of RAB, the mechanisms behind that attractiveness and subsequent development may differ. Second, we found that while the species of Lauraceae presented does significantly impact the attack density and patterns of RAB in host trees, some of these differences are mediated by moisture within the tree. A possible explanation for this is that, as the beetles move further away from the coast, redbay trees become less abundant than sassafras and the beetles are simply using up the smaller proportion of their preferred host. Another possible explanation is that the field-collected redbay trees in this study may have been infested with RAB for a longer period of time than the adjacent sassafras trees, possibly resulting in more favorable conditions for attacking beetles and developing larvae. A third potential explanation is that there is a confounding aspect of each tree species' physiology which is mediating the response of the tree to the beetle and vice versa. For example, Castillo-Argaez et al. (2021) suggest that the susceptibility of tree species to laurel wilt was partially dictated by the anatomy of the xylem vessels, with wilting and discoloration of xylem being more pronounced in species with smaller vessel diameters.

Analyses of RAB attack density, emergence, and normalized MC for both tree species separately revealed important effects of stem height through the first 4 m. In both species, attack density and normalized bolt MC tended to decrease with increased height on the stem (Fig. 4). Emergence of RAB also followed this same pattern in sassafras, and though it was not statistically significant in redbay (due to low sample size of trees and high variability between bolts) the relative position of the means was consistent with the pattern (Fig. 4B). These attack density results are consistent with the findings that RAB have greater visual attraction for larger diameter silhouettes (Mayfield and Brownie 2013). It is also consistent with the results of Hanula et al. (2011) who found substantially higher trap catches of RAB in traps placed at 1.5 m above ground than traps placed at greater heights. Our emergence data are consistent with Maner et al. (2013) who showed that brood productivity of RAB in redbay trees increased with diameter and decreased with height. The higher normalized MC associated with lower bole sections, demonstrated here for both redbay and sassafras (Fig. 4C), may help contribute to better environmental conditions for cultivating RAB symbiont fungi in their galleries.

While our study adds to the current understanding of laurel wilt movement through North American Lauraceae, additional questions remain. Future work on this disease complex should focus on identifying those other physiological and anatomical features of host species that affect RAB attraction to, and successful development in, different trees in the expected future range of the beetle. Finally, critical information is needed on the role of native ambrosia beetles in this complex, as well as other non-native ambrosia beetles that are already known to be present and damaging. Answering these questions may be helpful to developing a successful management program for laurel wilt in the United States.

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Author contributions

Katy Crout (Data curation [equal], Formal analysis [equal], Investigation [equal], Writing—original draft [equal]), Albert Mayfield (Conceptualization [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Writing—original draft [equal], Writing—review & editing [equal]), Julia Kerrigan (Investigation [equal], Methodology [equal], Writing—review & editing [equal]), and Jess Hartshorn (Conceptualization [equal], Data curation [equal], Formal analysis [equal], Funding acquisition [equal], Investigation [equal], Methodology [equal], Project administration [equal], Supervision [equal], Writing—original draft [equal], Writing—review & editing [equal])

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