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Brood Production by *Xyleborus glabratus* in Bolts from Trees Infected and Uninfected with the Laurel Wilt Pathogen, *Raffaelea lauricola*

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Raffaelea lauricola is the causal agent of laurel wilt and a fungal symbiont of its vector, the redbay ambrosia beetle, *Xyleborus glabratus*. Beetle populations increase rapidly where redbays have died from laurel wilt. In a series of experiments using bolts from healthy and diseased trees, *X. glabratus* reproduced better in bolts from diseased redbay; ratio of beetles emerged to entrance holes (BE/EH ratio) ranged from 4.1 to 6.0 in diseased bolts versus 0.3 to 1.9 in healthy bolts. Emergence was greater from redbay bolts when their ends were treated with paraffin wax compared to untreated bolts. Although sassafras sapwood has been considered poor brood material for *X. glabratus*, bolts from a diseased sassafras had BE/EH ratios comparable to those from diseased redbay bolts. It was hypothesized that better brood production in bolts from diseased trees was due to precolonization of the bolts by *R. lauricola*, which would allow for better establishment of the pathogen in beetle tunnels. However, the frequency of *R. lauricola* isolation and colony forming units (CFU) per beetle did not differ between beetles emerged from diseased versus healthy redbay bolts. Changes in the xylem of trees with laurel wilt appear to provide conditions favorable for brood production.

Keywords: laurel wilt, redbay ambrosia beetle, Persea borbonia, Sassafras albidum, beetle and host interaction

Laurel wilt has caused widespread mortality of redbay (*Persea borbonia* [L.] Spreng.) in the coastal plains of the southeastern United States since at least 2003 (Fraedrich et al. 2008). The disease is caused by the Asian fungus, *Raffaelea lauricola* T.C. Harrin., Fraedrich & Aghayeva (Wuest et al. 2017), a fungal symbiont of the redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae: Scolytinae), which carries the pathogen in paired, oral pouches called mycangia (Fraedrich et al. 2008; Harrington et al. 2008). The redbay ambrosia beetle was first detected near Savannah, Georgia in 2002, and reports of redbay mortality followed in nearby Hilton Head, South Carolina (Fraedrich et al. 2008). Since that time the pathogen and vector have spread to over 150 counties across nine southern states (Wuest et al. 2017). In addition to redbay, the disease has affected other species of Lauraceae indigenous to the southeastern United States, including sassafras (*Sassafras albidum* [Nuttall] Nees) (Fraedrich et al. 2008). Redbay appears to be an exceptionally good host tree for brood production by *X. glabratus* (Hanula et al. 2008), and other American Lauraceae appear to vary greatly in suitability as brood material (Mayfield and Hanula 2012; Mayfield et al. 2013).

Xyleborus glabratus is native to Asia (e.g., India, Japan, Taiwan, and China), where it is often associated with tree species in the Lauraceae, such as *Cinnamomum camphora* (L.) J. Presl. (Hulcr and Lou 2013) and *C. osmophloem* Kanehira (Harrington et al. 2011; Wuest et al.

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2017). *Raffaelea lauricola* is consistently associated with *X. glabratus* in both the United States and Asia (Harrington and Fraedrich 2010; Harrington et al. 2011; Wuest et al. 2017), and in the United States, *X. glabratus* brood production has been found exclusively in trees with laurel wilt. However, Asian species of Lauraceae appear to be more resistant to the disease, and it is not clear if the beetle in Asia reproduces only in trees that have been precolonized by *R. lauricola* (Fraedrich et al. 2015a; Harrington et al. 2011).

The vast majority of ambrosia beetle species are regarded as opportunists that live and breed only in dying and dead plants (Beaver 1989; Francke-Grossmann 1967; Wood 1982). However, the biology and behavior of *X. glabratus* seems to differ from many other ambrosia beetles in several respects. Female *X. glabratus* beetles carry as many as nine *Raffaelea* spp., though *R. lauricola* is consistently the most abundant fungus in mycangia of *X. glabratus* (Harrington and Fraedrich 2010; Harrington et al. 2010, 2011; Campbell et al. 2016). *Raffaelea lauricola* is unique among fungal symbionts of ambrosia beetles in that it is a highly virulent plant pathogen that is capable of systemic colonization of trees and causes a lethal vascular wilt. While many ambrosia beetles are generalists and are attracted to stress signals or fermentation products such as ethanol, *X. glabratus* is attracted to plant volatiles other than ethanol (Hanula and Sullivan 2008; Kendra et al. 2014; Kuhns et al. 2014). In Asia, the beetle favors aromatic tree species in the Lauraceae and a small number of other aromatic species in other plant families (Wood and Bright 1992; Hulcr and Lou 2013). In the United States, *X. glabratus* creates its brood galleries in wilted trees, but some individual females appear to make isolated attacks on healthy trees of Lauraceae native to both the United States and Asia (Fraedrich et al. 2008, 2015a). Although these initial attacks are aborted, they introduce *R. lauricola* into the xylem of healthy trees, which can later serve as brood material for *X. glabratus* after the pathogen has systemically colonized and killed the tree host. Thus, the attraction to host volatiles rather than ethanol, attacks on living host trees, and an association with an aggressive, systemic pathogen suggest that *X. glabratus* and *R. lauricola* have evolved a unique, plant pathogen-vector relationship (Harrington et al. 2011; Fraedrich et al. 2015a). This primary symbiosis persists under forest conditions in spite of the numerous *Raffaelea* spp. associated with *X. glabratus* (Harrington and Fraedrich 2010; Harrington et al. 2011; Campbell et al. 2016) and the association of other ambrosia beetle species with trees killed by laurel wilt (Fraedrich et al. 2008; Hanula, et al. 2008; Ploetz et al. 2017).

During the summer of 2010, we attempted to compare bolts from different host tree species for their potential to produce *X. glabratus* brood and for development of *R. lauricola* and other *Raffaelea* spp. The bolts were baited with manuka oil, and female *X. glabratus* bored into the bolts from healthy trees, but brood production was low in bolts of all species, including redbay. Previous studies using bolts from healthy redbay trees also found that brood production by *X. glabratus* was quite low (Mayfield and Hanula 2012; Mayfield et al. 2013). Based on our observations, experiences and previous studies, we hypothesized that redbay trees that died from laurel wilt would serve as better reproductive material for *X. glabratus* than sapwood from recently cut, healthy trees. Bolts of trees that had died from laurel wilt would be precolonized by *R. lauricola*, and the ambrosia growth in the galleries of the developing brood would be more luxuriant and dominated by its primary symbiont. Under this scenario, one might expect isolations from females emerging from bolts killed by laurel wilt to yield more *R. lauricola* than females emerging from bolts taken from healthy trees.

A primary goal of this study was to compare production of *X. glabratus* brood in bolts from healthy and diseased redbay trees. We also isolated *Raffaelea* spp. from brood emerged from bolts taken from healthy and diseased trees, expecting more *R. lauricola* from brood developing in bolts from diseased trees than healthy trees. Since some studies have questioned if sassafras was a suitable reproductive host for the redbay ambrosia beetle (Hanula et al 2008; Mayfield and Hanula 2012), we evaluated *X. glabratus* brood production in sassafras bolts from a tree killed by laurel wilt. Lastly, we evaluated brood production of *X. glabratus* in redbay and sassafras bolts with ends sealed with paraffin wax to determine if sealing in sapwood moisture would improve brood production.

Materials and Methods

Three experiments were conducted during 2011 using bolts baited with manuka oil lures (Synergy Semiochemical Corporation; Burnaby, British Columbia, Canada) on the southern portion of Jekyll Island, Georgia, where redbay trees were being attacked by *X. glabratus* and dying from laurel wilt. The forest was a live oak maritime hammock with a dominant overstory of *Quercus virginiana*, *Q. hemisphaerica,* and *Pinus elliottii*. Redbay occurred primarily in the understory and midstory. A fourth experiment to examine beetle production in sassafras bolts was conducted near Demopolis, Alabama.

Experiment One: Healthy and Laurel Wilt Affected Redbay

Two redbay trees, one healthy and the other symptomatic for laurel wilt, were located on Jekyll Island on May 11, 2011. The

Management and Policy Implications

Mutual dependence of pathogen and vector supports quarantine and other disease management strategies that focus on the vector. This study demonstrates that populations of *X. glabratus* can increase rapidly in redbay (*Persea borbonia*) trees that have died from laurel wilt. Recently felled, healthy trees may be attacked by the beetle, but those attacks may not lead to suitable brood production. In contrast to earlier studies, we found that sassafras trees that have died from laurel wilt can be a good brood host for *X. glabratus*, thus explaining the northern progress of the disease in forests where sassafras is the only known host. In some circumstances, prompt sanitation cuts in combination with cutting and leaving healthy and infected asymptomatic trees in and around areas with laurel wilt may help to maintain *X. glabratus* populations at low levels, thus reducing the probability of beetle and disease spread. However, laurel wilt moves rapidly through stands of redbay, and once the disease is established in an area, efforts to control the disease by reducing beetle populations could be futile. Perhaps, the greatest potential application for sanitation cutting and "cut and leave" management strategies are in areas where redbay trees occur around susceptible, rare, and endangered species such as pondberry, or near susceptible, high-value tree crops such as avocado. Such management strategies may also be applicable in forest types with sassafras, a species that generally occurs at lower densities than redbay in southern forests.

wilted tree had recently succumbed to laurel wilt based on the reddish discoloration of the foliage and the streaking and discoloration in the sapwood. Prior to cutting, there was no evidence of mass attack (no frass at entrance holes nor at base of tree) by *X. glabratus* or other ambrosia beetles, and bark was removed from several areas (~100 cm^2) to confirm that there were no beetle entrance holes into the sapwood. Four bolts, approximately 30 cm long were cut from each tree. The average diameters of bolts were 10.4 cm (SE = 1.48) from the diseased tree and 10.0 cm (SE = 0.39) from the healthy tree. Both ends of the bolts were sealed with paraffin wax (Gulf Wax, Royal Oak Enterprises, Roswell, Georgia) to reduce moisture loss. A metal hook was screwed into one end of each bolt, and a manuka oil lure was attached through the hook at the top of each bolt to enhance the attraction of *X. glabratus* (Hanula et al. 2008; Hanula and Sullivan 2008).

The bolts were deployed in a randomized complete block design (RCBD) at each of the four locations (i.e., blocks) that were separated from one another by at least 250 m, and one bolt of each treatment was hung at each of the four locations. Bolts within locations were separated from one another by at least 5 m and hung vertically on a rope between two trees 1.2 to 1.5 m above the ground. After 44 days, the bolts were placed in emergence cages (Mayfield and Hanula 2012) at room temperature in a building with a controlled environment. Beetles were collected in cups at the bottom of the cages, and the number of *X. glabratus* beetles and other ambrosia beetles were recorded at intervals of two to four weeks for 114 days, at which time beetle emergence from bolts had ceased or significantly slowed.

At the end of the emergence period, the bark surface area of each bolt was determined, bolts were debarked, and the number of entrance holes into the sapwood of a diameter typical for *X. glabratus* was counted (Hanula et al. 2008). The number of emerged *X. glabratus* beetles and entrance holes were expressed per 100 cm^2 of bolt surface. The ratio of the number of emerged *X. glabratus* beetles to the number of entrance holes (BE/EH ratio) was determined for each bolt.

Experiment Two: Bolts from Inoculated and Uninoculated Redbay

This experiment was a repeat of the first experiment with the exception that we inoculated a healthy redbay tree with *R. lauricola* to produce the diseased bolts. Two healthy redbay trees, one 17 cm dbh and the other 10 cm dbh, were selected on Jekyll Island on May 12, 2011. Both trees were wounded with a 6 mm drill bit to a depth of 2 to 3 cm at 1.4 m height. The larger diameter tree was inoculated with a plug of malt extract agar (MEA) colonized by *R. lauricola* for 14 days, and the other tree was inoculated with a sterile plug of MEA. The wound sites were covered with Parafilm (Pechiney Plastic Packaging, Menasha, Wisconsin) after inoculation and then wrapped with duct tape.

The inoculated and uninoculated redbay trees were felled on June 22, 2011, and cut into bolts approximately 30 cm in length. The uninoculated redbay had no symptoms of wilt disease (i.e., no wilted foliage or staining of the sapwood), and there was no evidence of beetle attacks on the stem. The inoculated redbay tree had wilted (i.e., all leaves dead or limp), and vascular discoloration characteristic of laurel wilt was noted in the xylem. There was no external evidence of ambrosia beetle frass, and no entrance holes characteristic of ambrosia beetle attacks were observed when bark was removed from several areas (~100 cm^2) along the stem. The average diameters of bolts from the tree with laurel wilt were 16.8 cm (SE = 0.063) and 9.9 cm (SE = 0.1974) for bolts from the healthy tree.

On June 23, 2011, the ends of bolts were sealed with paraffin wax, and one bolt for each of the two treatments was hung at each of the four locations on Jekyll Island in a RCBD as previously described. A manuka oil lure was placed on the top of each bolt to enhance their attractiveness to *X. glabratus*. Bolts were removed from the field after 58 days and placed in emergence cages as previously described. The number of beetles that emerged was recorded at irregular intervals of one to three weeks for 135 days, at which time beetle emergence from bolts had ceased or significantly slowed. The bark was then removed from the bolts, and the density of beetle entrance holes similar in size to those created by *X. glabratus* was determined.

Experiment Three: Redbay Bolts from Healthy and Diseased Trees with and Without Waxed Ends

The objectives of this experiment were to repeat the previous experiments comparing *X. glabratus* reproduction in bolts cut from healthy and infected redbay trees and to evaluate the effect of sealing the ends of bolts with paraffin wax. Two redbay trees, one in the initial stages of laurel wilt and the other healthy, were felled near Swainsboro, Georgia, on August 18, 2011. The dbh of the tree with laurel wilt was 11 cm, and it had foliage on many shoots in the upper crown that were drooping and had begun to turn brown. There was a light discoloration characteristic of laurel wilt in the outer sapwood. There were no frass tendrils characteristic of *X. glabratus* attack on the stem of the tree, and no beetle entrance holes were found when bark was removed from several areas (~100 cm^2) along the stem. The healthy tree was 12 cm dbh and had no evidence of ambrosia beetle attacks, wilted foliage, or xylem discoloration. Eight bolts were cut from the main stem of each tree, the ends of four bolts from each tree were sealed with paraffin wax, and the ends of the other four bolts from each tree were not sealed. The mean diameters of bolts from the tree with laurel wilt were 11.0 cm (SE = 0.30) and 12.3 cm (SE = 0.24) for bolts from the healthy tree. On August 19, 2011, one bolt from each of the four treatments was hung at each of the four locations on Jekyll Island in an RCBD, and a manuka oil lure was placed on top of each of the bolts.

The bolts were removed from the field site after 64 days, placed inside plastic bags in a cooler, transported to Athens, Georgia, and placed in emergence cages. Bolt lengths for this experiment ranged from 23 to 27 cm. The number of *X. glabratus* beetles that emerged from bolts was recorded over the next 100 days, at which time emergence had ceased or significantly slowed. The bark was then removed from the bolts, and the density of beetle entrance holes similar in size to those created by *X. glabratus* was determined.

Experiment Four: *X. glabratus* Reproduction in Sassafras Bolts

The objectives of this experiment were to evaluate the reproduction of *X. glabratus* in sassafras bolts from an infested tree that had died from laurel wilt and to further determine if the use of paraffin wax as an end-seal to restrict moisture loss would enhance beetle reproduction. A 10 cm diameter sassafras tree with symptoms of laurel wilt was cut down near Demopolis, Alabama, on April 25, 2012. The tree had wilting and dead foliage, sapwood discoloration

characteristic of the wilt, and beetle entrance holes and frass tendrils characteristic of *X. glabratus*. It was uncertain how long the tree had the disease, but the sapwood tissue was still moist.

Six bolts that ranged in length from 18 to 23 cm were cut from the lower portions of the stem, placed in large plastic bags, and then transported in coolers to Athens, Georgia. In the laboratory, the ends of three of the bolts were waxed with paraffin, another three were left unwaxed, and the bolts were hung in separate emergence cages in an unpaired two sample *t*-test. The number of *X. glabratus* beetles that emerged from bolts over the next 75 days was recorded. The density of *X. glabratus* entrance holes in bolts was determined as previously noted.

Raffaelea spp. Isolated from Beetles

Brood of species in the Xyleborini are dominated by females, and only females can fly and establish galleries (Wood 1982). Only females have mycangia, and fungal isolations were only attempted from emerged or excavated female adults. The number of colony forming units (CFU) of various *Raffaelea* spp. was evaluated in samples of adult female *X. glabratus* beetles and other ambrosia beetles obtained from redbay and sassafras bolts in all experiments. Emerged beetles were collected, stored under refrigeration, macerated using tissue grinders, and plated at 10X, 100X, and 1000X dilutions on cycloheximide-streptomycin malt agar (CSMA), a medium selective for *Ophiostoma* spp. and related genera such as *Raffaelea* spp. (Harrington 1981; Harrington 1992), or on SMA (CSMA without cycloheximide) media (Harrington and Fraedrich 2010). Plates were incubated at 25° C, and colonies on plates were morphologically characterized and counted. At least one culture of each morphotype was identified from each beetle using a 28S rDNA barcoding system (Harrington and Fraedrich 2010; Harrington et al. 2010, 2011; Wuest et al. 2017). Sixteen representative isolates that were identified by 28S rDNA barcoding as *R. lauricola* were also confirmed to be that species by genotyping with two species-specific microsatellite markers in a separate study (Wuest et al. 2017).

In experiment one, female *X. glabratus* adults that emerged from a bolt of a redbay tree with laurel wilt and other ambrosia beetles that emerged from bolts with laurel wilt (including *Xyleborinus saxeseni* [Ratzeburg], *Xyleborinus andrewesi* [Blandford], and *Xylosandrus crassiusculus* [Motschulsky]) were ground and dilution plated on CSMA and SMA. In experiments two and three, female *X. glabratus* adults emerged from bolts of trees that had died from laurel wilt and from healthy trees were ground and plated on CSMA. In order to confirm that the *Raffaelea* spp. were isolated from the oral mycangia and not some other part of the beetle, the heads of some of the beetles in experiment three were cut off from the rest of the body, and the two parts of the beetle were separately macerated and plated on CSMA. Before separating the head from the rest of the body, some beetles were surface sterilized as previously described (Harrington and Fraedrich 2010) to determine that the propagules were internal and not superficial contaminants of the beetle.

In addition to the emerged adults in experiment four, immature *X. glabratus* adults were excavated from the tunnels by sawing and splitting the wood of bolts from the same sassafras tree with laurel wilt near Demopolis, Alabama, in April 2012. The adults were excavated in the laboratory and classified as callow (lightly colored exoskeleton), dark, or intermediate. All beetles from the sassafras bolts were cut in half, and only the half with the head were ground and plated on CSMA at 10X and 100X dilutions.

Statistical Analyses

Data for *X. glabratus* entrance holes, beetle emergence, and BE/BH ratios from experiments one, two, and three were each analyzed as randomized complete block designs with two treatments (experiments one and two) or four treatments (experiment three) and four blocks (locations). In experiment three, differences among treatment means were evaluated with Tukey's HSD Test, and in all tests, the Type I error rate was established at a probability level of 0.05. Experiment four was analyzed as a two-sample *t*-test with two treatments (i.e., bolts with waxed and unwaxed ends), and each treatment had three replicates. Homogeneity of variances was evaluated with Levene's Test (Milliken and Johnson 1984) in experiment three. In experiments one, two, and four, the equality of the two variances was evaluated with an F-test (Steel and Torrie 1980). Data were transformed using the log base 10 transformation in most experiments to reduce heterogeneity among variances as appropriate. Statistical analyses were conducted using SYSTAT (Version 13.00.05, SYSTAT Software, Inc. San Jose, CA).

The number of CFU of *Raffaelea* spp. present in X. *glabratus* beetles from healthy and diseased redbay bolts was compared with a chi-square test. Likewise, the number of CFU of *Raffaelea* spp. obtained from *X. glabratus* beetles in different developmental stages (i.e., callow, intermediate, and mature) was compared with a chi-square test.

Results

Experiment One: Healthy and Laurel Wilt Affected Redbay

The density of *X. glabratus*-size beetle entrance holes was significantly greater in bolts from the tree with laurel wilt than in the bolts from the healthy tree (Table 1; Figure 1A). Likewise, significantly more *X. glabratus* beetles emerged from bolts from the tree with laurel wilt compared to the healthy tree (Table 1; Figure 1B). Approximately 3,000 *X. glabratus* beetles emerged from the bolts from the tree with laurel wilt, but only 165 beetles emerged from bolts taken from the healthy tree. Males emerged from bolts of both treatments (Table 2), further indicating that beetle brood had been produced in the bolts. The BE/EH ratio was significantly greater in bolts from the redbay tree with laurel wilt compared to bolts from the healthy tree (Table 1; Figure 1C).

Xylosandrus crassiusculus also bored into bolts from healthy and wilted redbay trees (1.8 and 2.8 entrance holes/100 cm^2 bolts surface, respectively). In contrast to *X. glabratus*, similar numbers of adult *X. crassiusculus* emerged from the bolts from the healthy and diseased trees, although male *X. crassiusculus* only emerged from bolts taken from the healthy tree (Table 2). Other ambrosia beetles, including 54 female Xyleborinus andrewesi and 28 female Xyleborinus saxesenii, were recovered from diseased redbay bolts, but none emerged from bolts of the healthy redbay.

Experiment Two: Bolts from Inoculated and Uninoculated Redbay

There was a greater density of beetle entrance holes in the bolts from the diseased (inoculated) redbay tree than in bolts from the healthy tree, but this difference was not statistically significant ($P = 0.0506$; Table 1; Figure 1A). A significant treatment difference

Table 1. Results of ANOVA for experiments that examined the influence of trees species, tree condition or waxing the ends of bolts on numbers of beetle entrance holes in bolts, beetle emergence, and the ratio of beetle emergence to entrance holes (BE/EH ratio).

Exp.	Response variable	Treatment effect	F value	df	*P*	Transform[a]
1	Entrance Holes	Tree condition[b]	71.6648	1, 3	0.0035	TR
	X. glabratus emerged	Tree condition	162.6554	1, 3	0.0010	TR
	BE/EH Ratio	Tree condition	150.8571	1, 3	0.0012	NT
2	Entrance Holes	Tree condition	10.0370	1, 3	0.0506	TR
	X. glabratus emerged	Tree condition	43.0022	1, 3	0.0072	TR
	BE/EH Ratio	Tree condition	27.7139	1, 3	0.0134	NT
3	Entrance Holes	Tree condition /Waxed ends	1.4447	3, 9	0.2934	TR
		Contrast "Laurel wilt versus healthy"	0.1675	1, 9	0.6910	TR
		Contrast "Waxed ends versus no wax"	4.1196	1, 9	0.0730	TR
	X. glabratus emerged	Tree condition /Waxed ends	19.5489	3, 9	0.0003	TR
		Contrast "Laurel wilt versus healthy"	48.9495	1, 9	0.0001	TR
		Contrast "Waxed ends versus no wax"	7.2121	1, 9	0.0250	TR
	BE/EH Ratio	Tree condition /Waxed ends	31.1969	3, 9	<0.0001	TR
		Contrast "Laurel wilt versus healthy"	87.8132	1, 9	<0.0001	TR
		Contrast "Waxed ends versus No wax "	1.1759	1, 9	0.3064	TR

[a] NT = nontransformed data; TR-data transformed using a $Log_{10}(x+1)$ transformation.
[b] Tree condition = diseased or healthy.

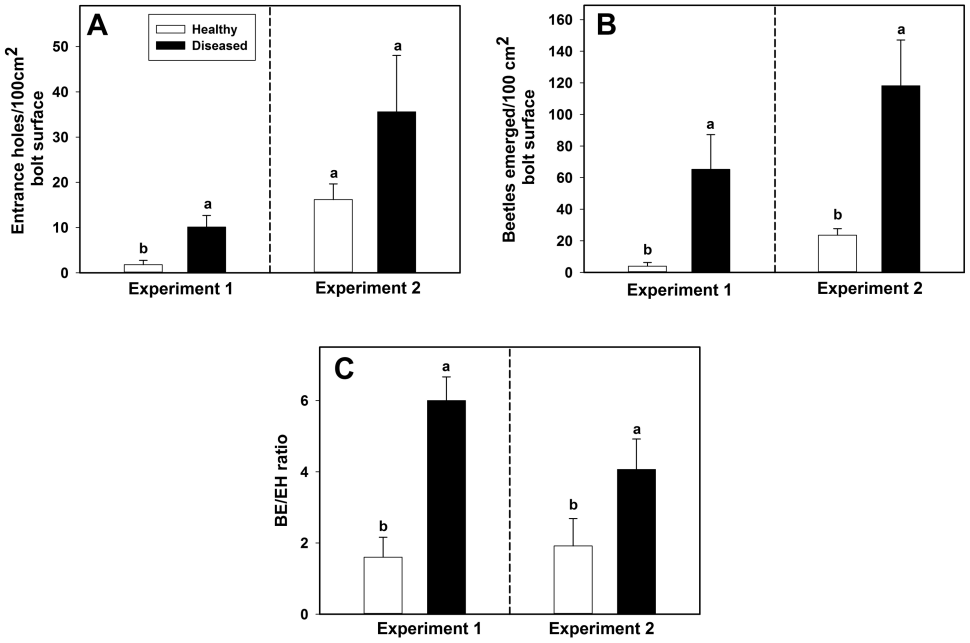

Figure 1. Density of *Xyleborus glabratus* beetle entrance holes per 100 cm² of bolt surface area (A), number of emerged *X. glabratus* beetles per 100 cm² of bolt surface area (B), and the beetle emergence/entrance hole ratio (BE/EH ratio) among bolts from healthy redbay and redbay infected with laurel wilt (C) in experiments one and two. Vertical bars and lines above bars represent means and standard errors, respectively. Means with the same letter within experiment are not significantly different ($P > 0.05$).

Table 2. Total number of female and male (and percentage males) *Xyleborus glabratus* and *Xylosandrus crassiusculus* adults that emerged from bolts taken from healthy or laurel wilt-infected redbay or sassafras trees, infested in the field and reared indoors in four experiments.[a]

				Emerged		Emerged	
	Tree	Prior Tree	Bolt end	*X. glabratus*		*X. crassiusculus*	
Exp.	species	condition	treatment	females	males (%)	females	males (%)
1	Redbay	Healthy	Waxed	153	12 (7.3)	175	9 (4.9)
	Redbay	Laurel wilt	Waxed	2944	56 (1.8)	122	0 (0.0)
2	Redbay	Healthy	Waxed	436	23 (5.0)	0	0 (0.0)
	Redbay	Laurel wilt	Waxed	4521	33 (0.7)	1049	17 (1.6)
3	Redbay	Healthy	Not waxed	44	2 (4.5)	0	0 (0.0)
	Redbay	Healthy	Waxed	368	43 (10.4)	0	0 (0.0)
	Redbay	Laurel wilt	Not waxed	881	115 (11.5)	0	0 (0.0)
	Redbay	Laurel wilt	Waxed	1367	168 (10.9)	0	0 (0.0)
4	Sassafras	Laurel wilt	Not waxed	101	5 (4.7)	0	0
	Sassafras	Laurel wilt	Waxed	271	7 (2.4)	0	0

[a]Four bolts were used for each species/treatment combination in all experiments except experiment four, for which there were only three bolts for each treatment.

was observed, however, in the number of *X. glabratus* beetles that emerged from the bolts of the two trees (Table 1). Approximately five times more beetles emerged per 100 cm^2 of surface area of diseased redbay bolts compared to the healthy redbay bolts (Figure 1B). The BE/EH ratio also varied significantly between treatments (Table 1): 4.1 for bolts from the diseased redbay tree compared to 1.9 for bolts from the healthy redbay tree (Figure 1C). Male *X. glabratus* beetles emerged from diseased and healthy redbay bolts (Table 2).

Entrance holes caused by *X. crassiusculus* were seen in bolts from the diseased redbay tree ($\bar{x}$ = 16.1 entrance holes/100cm^2), and there was good reproduction in these bolts based on relatively high numbers of emerged female beetles and the presence of *X. crassiusculus* males (Table 2). Entrance holes caused by *X. crassiusculus* were not observed in the healthy redbay bolts, and *X. crassiusculus* beetles did not emerge from these bolts.

Experiment Three: Redbay Bolts from Healthy and Diseased Trees with and Without Waxed Ends

The density of beetle entrance holes made by *X. glabratus* did not differ significantly among treatments (Table 1). An examination of the means among treatments by ANOVA suggested that the average density of entrance holes may have been greater in the waxed bolts than in the unwaxed bolts (Figure 2A), but an analysis of the data using linear contrasts indicated that these differences were not significant (Table 1).

The ANOVA indicated that emergence of *X. glabratus* from bolts varied significantly among treatments (Table 1). Beetle emergence from waxed and unwaxed bolts from the diseased tree was significantly greater than emergence from unwaxed bolts from the healthy redbay tree (Figure 2B). An analysis of the data using linear contrasts indicated that, overall, more beetles emerged from bolts from the laurel wilt tree than from the healthy tree (Table 1; $\bar{x}$ = 30.3 versus 4.5/100 cm^2 bolt surface, respectively). Furthermore, using linear contrasts, more beetles emerged from waxed bolts versus unwaxed bolts (Table 1; $\bar{x}$ = 22.0 versus 12.8/100 cm^2 bolt surface, respectively). Male *X. glabratus* beetles were obtained from all bolts except for three of the eight bolts from the healthy redbay tree (Table 2), and more males emerged from the diseased bolts than from the healthy bolts ($\bar{x}$ = 3.4 versus $\bar{x}$ = 0.4/100cm^2 bolt surface, respectively). Ambrosia beetle species other than *X. glabratus* were not obtained from bolts in this experiment.

The BE/EH ratio also varied significantly among treatments (Table 1) and was significantly greater in bolts from the tree with laurel wilt than in bolts from the healthy tree (Figure 2C). Waxing the ends of bolts had no significant effect on the BE/EH ratio (Table 1).

Experiment Four: *X. glabratus* Reproduction in Sassafras Bolts

The density of *X. glabratus* entrance holes did not differ significantly between waxed and unwaxed bolts of the naturally infested sassafras tree: $\bar{x}$ (SE) = 1.8 (0.98) versus 1.4 (0.22)/100cm^2 bolt surface, respectively (P = 0.7004). Likewise, the density of emerged *X. glabratus* adults did not differ among treatments: $\bar{x}$ (SE) = 11.5 (8.2) versus 4.1 (0.17)/100 cm^2, respectively (P = 0.5609). The BE/EH ratio was $\bar{x}$ (SE) = 5.1 (1.16) for the waxed bolts and 3.1 (0.40) for the unwaxed bolts, but these means did not significantly differ (P = 0.1794). Males were recovered from both the waxed and unwaxed sassafras bolts (Table 2).

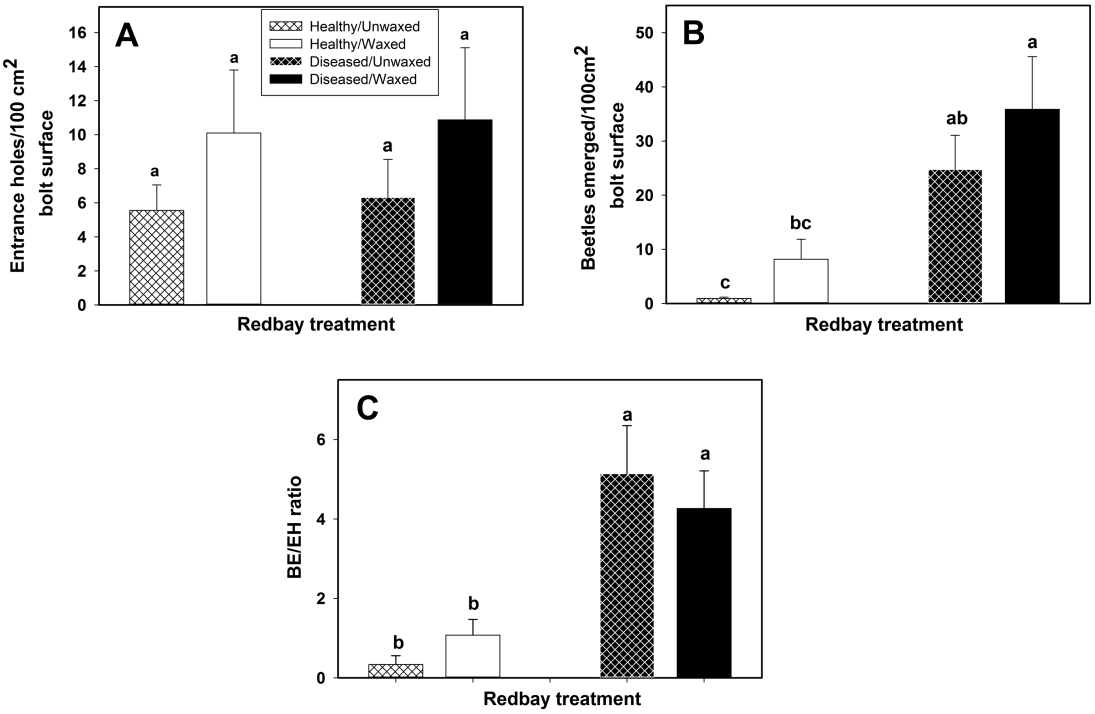

Figure 2. Density of *Xyleborus glabratus* beetle entrance holes per 100 cm^2 of bolt surface area (A), number of emerged *X. glabratus* beetles per 100 cm^2 of bolt surface area (B), and the beetle emergence/entrance hole ratio (BE/EH ratio) (C) for waxed and unwaxed bolts from healthy and diseased redbay bolts in experiment three. Vertical bars and lines above bars represent means and standard errors, respectively. Means with the same letter are not significantly different (P > 0.05).

Raffaelea spp. Isolated from Beetles Emerged from Bolts of Redbay with Laurel Wilt

Raffaelea lauricola was isolated from nine of the 10 *X. glabratus* beetles that emerged from a bolt taken from a redbay tree with laurel wilt in experiment one (Table 3). *Raffaelea subalba* was isolated from eight of the nine *X. glabratus* that also yielded *R. lauricola*; and *R. subfusca* was isolated from four of the eight beetles that yielded *R. lauricola* and *R. subalba*. Of the nine beetles yielding a *Raffaelea* sp., 133 to 6330 CFU of *R. lauricola* were recovered, for a mean of 2,081 CFU per beetle (Table 3). The number of CFU per beetle was much lower for *R. subalba* and lower still for *R. subfusca* (Table 3). *Raffaelea ellipticospora* was only isolated from a single beetle, at 1,833 CFU, and this same beetle yielded only 133 CFU of *R. lauricola*.

Isolations were attempted from females of other species of ambrosia beetles that emerged from the bolts taken from the diseased redbay tree in experiment one. *Raffaelea sulphurea*, the primary symbiont of *Xyleborinus saxeseni*, was isolated from six of the 11 *X. saxeseni* that emerged from a bolt taken from the redbay tree with laurel wilt, though the mean CFU of *R. sulphurea* was only 595 for these six beetles (Table 3). *Raffaelea lauricola* was isolated at 67 and 800 CFU per beetle, respectively, from two other *X. saxeseni* emerged from another diseased bolt. No *Raffaelea* sp. was isolated from the other three emerged *X. saxeseni* adults. Seven *X. andrewesi* and eight *X. crassiusculus* from diseased bolts were also ground and dilution plated on CSMA and SMA media, but no *Raffaelea* sp. was recovered from these beetles. Three of the eight *X. crassiusculus* yielded the mycangial symbiont *Ambrosiella roeperi* (Harrington et al. 2014) on the SMA plates (number of CFU not determined), and no filamentous fungus was recovered from *X. andrewesi* on CSMA or SMA.

Raffaelea spp. from Beetles Emerged from Bolts of Healthy and Diseased Trees

There was substantial variation among bolts in the number of *X. glabratus* adults yielding the various *Raffaelea* spp. in experiments two and three (Table 4). However, there was little difference in isolation frequencies from beetles emerging from waxed and unwaxed bolts in experiment three, and isolation results from these beetles with these two treatments were combined.

As found in earlier studies (Harrington and Fraedrich 2010), there were no significant effects of surface sterilization on the isolation frequencies of *Raffaelea* spp. or CFU per beetle. In experiment two and the first batch of emerged *X. glabratus* beetles from experiment three, whole beetles were ground and dilution plated on CSMA, and the beetles were not surface sterilized prior to grinding. In the second batch of emerged beetles in experiment three (i.e., experiment 3-2), some beetles were surface sterilized prior to dissecting and grinding. Because there was no significant effect of surface sterilization on the isolation frequencies or CFU per beetle, the data for surface-sterilized and non-surface-sterilized beetles were combined.

Isolations were compared from the head (where the mycangia are found) and pronotum versus the rest of the body in experiment 3-2. Consistent with the hypothesis that the *Raffaelea* spp. were primarily in the mycangia at the time of emergence, the CFU of *Raffaelea* spp. from the head- or pronotum-only samples (experiment 3-2), with or without surface sterilization, were comparable to the CFU from

Table 3. Isolation frequencies and CFU of *Raffaelea* spp. from *Xyleborus glabratus*, *Xyleborinus saxeseni*, *X. andrewesi* and *Xylosandrus crassiusculus* females emerged from a bolt taken from a redbay tree with laurel wilt in experiment one.

Beetle species	No. of Beetles Sampled	No. Yielding *Raffaelea* spp.	Number of Beetles Yielding Fungus (mean ± SE CFU per fungus-yielding beetle)[a]				
			R. lauricola	*R. subalba*	*R. subfusca*	*R. ellipticospora*	*R. sulphurea*
Xyleborus glabratus	10	9	9 (2081 ± 750)	8 (771 ± 86)	4 (308 ± 96)	1 (1833)	0
Xyleborinus saxeseni	11	8	2 (433)	0	0	0	6 (595 ± 145)
Xyleborinus andrewesi	7	0	0	0	0	0	0
Xylosandrus crassiusculus	8	0	0	0	0	0	0

[a]Mean ± standard error of the CFU for beetles yielding that *Raffaelea* sp. only (beetles from which the fungus was not isolated were not included in calculating CFU).

Table 4. Isolation frequencies and CFU of *Raffaelea* spp. from *Xyleborus glabratus* females emerged from bolts of healthy redbay trees, redbay trees inoculated with *Raffaelea lauricola* in experiment two, or redbay trees naturally infected with laurel wilt in experiment three.

Exp.	Bolts from Diseased or Healthy Trees	No. of Bolts Sampled	No. of Beetles Sampled	Number of Beetles Yielding *Raffaelea* sp.	Number of Beetles Yielding that Fungus (mean ± SE CFU per fungus-yielding beetle)[a]		
					R. lauricola	*R. subalba*	*R. subfusca*
2	Healthy	3	12	5	4 (183 ± 97)	2 (183)	0
	Diseased	4	20	17	15* (913 ± 188)	12* (611 ± 197)	3 (167 ± 100)
3-1[b]	Healthy	5	9	7	6 (1756 ± 600)	3 (533 ± 58)	4* (92 ± 25)
	Diseased	6	22	16	16 (1194 ± 856)	13 (369 ± 104)	1 (233)
3-2[b]	Healthy	2	6	6	6 (1263 ± 350)	5 (307 ± 114)	3 (100 ± 39)
	Diseased	2	5	4	3 (658 ± 373)	3 (35 ± 19)	1 (3)
Total	Healthy	10	27	18	16 (1178 ± 293)	10 (350 ± 71)	7 (95 ± 20)
	Diseased	12	47	37	34 (1023 ± 134)	28 (503 ± 103)	5 (147 ± 67)

[a]Mean ± standard error of the CFU for beetles yielding that *Raffaelea* sp. only (beetles from which the fungus was not isolated were not included in calculations of CFU).
[b]3-1 and 3-2 indicate different sample times during experiment three. In experiment 3-1, whole beetles were ground and sampled. In experiment 3-2, only the head and pronotum were sampled, and two of the beetles from the healthy bolt and of the beetles from the diseased bolt were surface sterilized prior to grinding and dilution plating.
*indicates that the proportion of beetles yielding the fungus was significantly different between beetles emerged from bolts taken from an inoculated or diseased tree versus from bolts taken from a healthy tree according to chi-square analyses ($P \leq 0.05$).

whole-beetle samples without surface sterilization (i.e., experiments 2 and 3-1) (Table 4). No *Raffaelea* sp. was isolated from the main body of the beetles (mesothorax/metathorax/abdomen), but yeasts, *Penicillium* spp., and other molds were isolated in comparable numbers from the head or pronotum versus the other body segments in experiment 3-2.

When combining all isolation protocols (with and without surface sterilization and whole-beetle versus head or pronotum only) for the three samplings in experiments two and three (Table 4), 55 of the 74 sampled beetles yielded a *Raffaelea* sp., with up to three species isolated from an individual beetle. *Raffaelea lauricola* was the most frequently isolated fungus from the emerged *X. glabratus* (50 of 74 beetles sampled). *Raffaelea subalba* was recovered from 38 of the 74 beetles, which was significantly lower ($P = 0.047$) than the isolation frequency for *R. lauricola*, and the mean number of CFU per beetle was substantially lower for *R. subalba* than for *R. lauricola* (Table 4). *Raffaelea subfusca* was only isolated from 12 beetles (less than the frequency of *R. subalba*, $P < 0.001$) and at low CFU per beetle (Table 4).

In experiment two, *Raffaelea* spp. were isolated more frequently and in higher CFU per beetle from beetles emerging from bolts taken from the redbay tree with laurel wilt than from beetles emerging from bolts from the healthy tree (Table 4). Beetles from diseased bolts in experiment three yielded the *Raffaelea* spp. in similar frequencies and CFU as in the beetles emerged from diseased bolts in experiment two. However, in experiment three, there was no significant difference in the frequency of isolation of either *R. lauricola* or *R. subalba* between the beetles emerged from diseased versus healthy bolts.

Combining data from experiments two and three, *R. lauricola* was isolated from 34 of 47 beetles from diseased bolts and from 16 of 27 beetles from healthy bolts, which was not different at the $P = 0.05$ level based on chi-square analysis. Similarly, there was no significant difference in the isolation frequency of *R. subalba* from *X. glabratus* that emerged from diseased bolts (28 of 47 beetles) versus healthy bolts (10 of 27 beetles). Nor was there a significant difference in isolation frequency of *R. subfusca* from beetles emerged from diseased bolts (five beetles) versus healthy bolts (seven beetles). There were no apparent differences in CFU per beetle from diseased bolts versus healthy bolts for any of the *Raffaelea* spp. (Table 4). Thus, contrary to expectations, the microflora of the beetles emerging from bolts of diseased trees was not quantitatively nor qualitatively different from that of beetles emerging from bolts of healthy trees.

Raffaelea spp. from Excavated and Emerged *X. glabratus* from Sassafras

In experiment four, *R. lauricola* was recovered from 19 of the 21 immature adults of *X. glabratus* excavated from a naturally infested sassafras tree with laurel wilt, but *R. lauricola* was isolated at relatively low CFU (Table 5). The seven excavated adult beetles that appeared to be the most mature (darkest) yielded higher CFU per beetle ($\bar{x}$ = 652) than did the callow ($\bar{x}$ = 126 CFU) and intermediate ($\bar{x}$ = 11 CFU) beetles. *Raffaelea subalba* was isolated at 103 CFU from only one of the dark, excavated adults, and this beetle also yielded *R. lauricola* at 3433 CFU.

Of the 13 sampled *X. glabratus* that emerged from the sassafras bolts (both waxed and unwaxed), 10 yielded *R. lauricola*, and four of those beetles also yielded *R. subalba* (Table 5). The CFU of *R. lauricola* and *R. subalba* per emerged beetle were substantially greater than the CFU per excavated beetle and comparable to the CFU from *X. glabratus* beetles that emerged from bolts taken from diseased redbay trees in previous experiments (Table 4).

Discussion

The results of these studies further illustrate the unique relationship between the redbay ambrosia beetle and its plant pathogenic partner, *R. lauricola*, which provides wilted trees and ambrosia growth for its vector. Most ambrosia beetles attack and reproduce in dying and dead trees, although there are notable exceptions, such as *Corthylus columbianus* (Kabir and Giese 1966; Nord 1972), *Xyleborus vochysiae* (Kirkendall 2006), and *Xylosandrus compactus* (Ngoan et al. 1976). *Platypus quercivora* and some *Euwallacea* spp. may attack healthy trees when beetle populations are high, and weakly pathogenic mycangial symbionts may aid in killing trees or sapwood tissues around attack points (Eskalen et al. 2013; Kusumoto et al. 2015). In the case of *X. glabratus*, the beetle is attracted to and makes boring attempts in healthy trees, but these initial attacks are aborted and fail to lead to brood production (Fraedrich et al. 2008; Fraedrich et al. 2015a; Hughes et al. 2015a). In the United States, *X. glabratus* attacks trees for brood production only after they have been systemically colonized by the pathogen (Fraedrich et al. 2008). This study demonstrates that systemic colonization by *R. lauricola* enhances brood production by *X. glabratus* as compared to bolt sections taken from healthy trees.

In three independent experiments, brood production was much better in bolts from redbay trees naturally infected or inoculated with *R. lauricola* compared to bolts from healthy trees. Also, brood production in sassafras bolts taken from trees with laurel wilt was comparable to that in bolts taken from diseased redbay. The average BE/EH ratio ranged from 4.1 to 6.0 in bolts from redbay trees with laurel wilt, versus 0.3 to 1.9 in bolts from healthy redbay trees. The average emergence rates of four to six beetle brood per entrance hole in bolts from diseased redbay and sassafras trees in our studies

Table 5. Isolation frequencies and CFU of *Raffaelea* spp. from *Xyleborus glabratus* females excavated from or emerged from bolts of sassafras trees with laurel wilt in experiment four.

Excavated or Emerged from Bolts	Beetle Maturity	No. of Beetles Sampled	Number of Beetles Yielding *Raffaelea* sp.	Number of Beetles Yielding that Fungus (mean ± se CFU per fungus-yielding beetle)[a]	
				R. lauricola	*R. subalba*
Excavated	Callow	7	5	5 (126 ± 67)	0
	Intermediate	7	7	7 (11 ± 4)	0
	Dark	7	7	7 (652 ± 479)	1 (103)
Emerged	All	13	10	10 (1775 ± 570)	4* (342 ± 141)[b]

[a]Mean ± standard error of the CFU for beetles yielding that *Raffaelea* sp. only (beetles from which the fungus was not isolated were not included in calculating CFU).
[b]An * indicates that the proportion of beetles yielding the fungus is greater for beetles emerged from bolts (n = 13) than those excavated from bolts (n = 21) according to chi-square analyses ($P \leq 0.05$).

are comparable to the rates found by Maner et al. (2013) in naturally wilted redbay trees. The comparatively low reproduction rates of *X. glabratus* in bolts from healthy redbay trees is consistent with earlier reports, in which beetle emergence from bolts taken from healthy trees of redbay, sassafras, and other hosts has been consistently very low (Mayfield and Hanula 2012; Mayfield et al. 2013,) or absent (Pena 2012).

Many factors may be interacting to affect the colonization of bolts by *X. glabratus* and the subsequent production of brood by the beetle. *Xyleborus glabratus* has been found to respond to visual cues, and attacks on trees tend to increase in response to increasing tree diameter (Maner et al. 2013; Maner et al. 2014; Mayfield and Brownie 2013). In experiment two, the mean diameter of bolts from the tree with laurel wilt was greater than that of bolts from the healthy tree (16.8 cm versus 9.9 cm, respectively), and this may have contributed to the greater number of beetle holes in the bolts from the diseased tree. However, bolt diameters were similar for the treatments in two other experiments, and the number of beetle attacks was greater in bolts from the diseased tree in one of the experiments, while in the other experiment, the number of beetle attacks on bolts were similar for the two treatments. However, based on the BE/BH ratio, beetle galleries established in bolts from laurel wilt trees were consistently more productive than galleries in bolts from healthy trees, regardless of bolt diameter.

It is possible that altered xylem chemistry provides a localized attraction or a specific cue from the diseased tissue that stimulates gallery initiation and construction. In our bolt experiments, the use of manuka oil as bait may have overwhelmed any subtle effect of healthy versus diseased bolts for beetle attraction or stimulation, but in one of the three experiments, there were many more *X. glabratus* entrance holes in bolts taken from redbay with laurel wilt than in bolts from the healthy tree. However, Martini et al. (2017) did not detect differences in plant volatiles or *X. glabratus* preferences between healthy and diseased sapwood of swamp bay. Fungal-produced volatiles in diseased sapwood may be locally attractive or stimulate gallery construction, but *X. glabratus* only shows a weak attraction to *R. lauricola* under laboratory conditions (Hulcr et al. 2011). Under field conditions, the attraction of *X. glabratus* to host-tree volatiles is increased only slightly with the addition of volatiles from *R. lauricola* (Kuhns et al. 2014). The most consistent and dramatic difference between healthy and diseased sapwood appears to be in the efficiency of brood production (BE/BH) rather than initiation of tunnels.

The lack of a host response, alteration in moisture content, or other environmental and chemical modifications in trees with laurel wilt may make diseased sapwood tissue superior to healthy sapwood tissue for brood production. Trees with laurel wilt have discolored areas in the xylem tissues due to host responses. This discolored tissue is nonfunctional, and most or all of the parenchyma cells have died. In contrast, the sapwood of healthy trees contains parenchyma cells that can remain alive and functioning in bolts for a month or more after trees are felled (Feist et al. 1971; Ruetze and Liese 1985). The presence of live tissues in bolts from healthy trees may allow for a limited host response to wounding, and this response may inhibit tunneling by *X. glabratus* females, similar to that observed with aborted attacks in healthy trees (Fraedrich et al. 2008; Fraedrich et al. 2015a). Host responses could also inhibit egg laying, larval hatching, or ambrosial growth.

Moisture content of sapwood is thought to be critical for successful ambrosia growth and brood production by many ambrosia beetles (Francke-Grosmann 1967; McLean and Borden 1977). Sapwood of trees with laurel wilt may have greater moisture content and retain moisture longer than healthy sapwood because of tyloses and gums formed during the host response (Inch et al. 2012). In general, drying of sapwood could be a significant factor limiting beetle attacks and reproduction in bolt studies. In bolts from healthy and diseased trees, there were generally more beetle entrance holes and greater emergence numbers in redbay and sassafras bolts with ends that had been sealed with paraffin wax, but the differences were not great. These results are in contrast to those of Mayfield and Hanula (2012), who found that sealing the ends of bolts could decrease the number of attacks. However, the end seal they used was a wax and water emulsion, not a paraffin wax, and it is not clear if there could be possible interactions between end-seal types and *X. glabratus* activity. Other factors, such as bolt size, temperature, and humidity, likely affect the rate of moisture loss from bolts and beetle reproduction.

We had hypothesized that precolonization of the sapwood by *R. lauricola* would provide for a more luxuriant ambrosia growth and that *R. lauricola* would better compete with other *Raffaelea* spp. in both the fungal gardens and in the mycangia of callow females (Harrington and Fraedrich 2010). However, *R. lauricola* dominated the mycangia of *X. glabratus* that emerged from both healthy and diseased bolts. Whether the beetles were surface sterilized or not, *R. lauricola* was the most common species isolated from whole, emerged *X. glabratus* females or from the heads only. And *R. subalba*, *R. subfusca*, and *R. ellipticospora* were less commonly isolated, as reported in previous studies (Harrington and Fraedrich 2010; Harrington et al. 2010, 2011; Campbell et al. 2016). The other *Raffaelea* spp. are strictly nutritional symbionts of *X. glabratus*, competing with *R. lauricola* in the galleries and in mycangia—and not plant pathogens (Harrington and Fraedrich 2010; Dreaden et al. 2017). Based on isolations from individual emerged females, the abundance of *R. lauricola* and the relative abundance of *Raffaelea* spp. in the successful brood galleries were similar in bolts from diseased trees and healthy trees. Thus contrary to expectations, precolonization of the sapwood by *R. lauricola* does not appear to give a competitive advantage to *R. lauricola,* nor does it increase the overall abundance of *Raffaelea* spp., at least in successful fungal gardens.

Other species of ambrosia beetles are potential vectors of *R. lauricola* in avocado orchards, where *X. glabratus* is uncommon (Carrillo et al. 2013; Ploetz et al. 2017), but *X. glabratus* appears to be the only important vector in natural forests (Harrington and Fraedrich 2010). In this study, other species of ambrosia beetles did not colonize the bolts to the same extent as *X. glabratus*, with the exception of *X. crassiusculus*, which commonly breeds in dead and dying redbay trees (Hanula et al. 2008). *Raffaelea lauricola* was not isolated from *X. crassiusculus* in the present study, but it has been isolated infrequently from *X. crassiusculus* adults collected from trees with laurel wilt (Carrillo et al. 2013; Ploetz et al. 2017). This beetle is not associated with *Raffaelea* symbionts and instead has *Ambrosiella roeperi* as its mycangial symbiont (Harrington et al. 2014). *Xyleborinus saxeseni*, which has *R. sulphurea* as its main symbiont (Harrington et al. 2010), was also recovered from bolts of redbay trees with laurel wilt. *Raffaelea lauricola* was isolated from a low percentage of emerged *X. saxeseni* but at low numbers of

CFU, suggesting that *R. lauricola* was a superficial contaminant. The pathogen also has been isolated from *X. saxeseni* in earlier work with other species of Lauraceae (Fraedrich et al 2011; Carrillo et al. 2013), but it attacks only recently cut, injured, or dying trees—not healthy trees (Wood 1982). *Xyleborinus andrewesi* also was obtained from bolts of a redbay tree with laurel wilt, but no *Raffaelea* sp. was isolated. This Asian and African ambrosia beetle (Wood and Bright 1992) has been reported from Florida (Okins and Thomas 2010) in sugar apple (*Annona squamosa* L.), and *R. lauricola* was isolated from two individuals trapped in flight (Ploetz et al. 2017). The recovery of *X. andrewesi* from redbay on Jekyll Island is a new Georgia state record and a new host record.

Sassafras, like redbay, is highly susceptible to laurel wilt (Fraedrich et al. 2008). However, the attraction of *X. glabratus* to sassafras has been unclear, and sassafras was generally thought to be a poor reproductive host for this beetle (Hanula et al. 2008; Mayfield and Hanula 2012). Reproductive success of *X. glabratus* in bolts from healthy sassafras was limited in earlier studies (Mayfield and Hanula 2012; Mayfield et al. 2013) and in our preliminary study. The sassafras bolts we took from a naturally infested tree with laurel wilt in Alabama had relatively low densities of entrance holes, but the BE/EH ratio averaged 4.1, which was comparable to ratios obtained with redbay bolts from trees with laurel wilt. Furthermore, *X. glabratus* males and callow adults were obtained from the bolts, showing that the beetle was reproducing. *Raffaelea lauricola* was growing in the mycangium of *X. glabratus* in sassafras bolts, as indicated by the much higher CFU of *R. lauricola* from emerged beetles, compared to the CFU from callow adults excavated from the bolts. Laurel wilt is now established in sassafras populations in areas where redbay is absent, and *X. glabratus* is apparently breeding in diseased sassafras trees at widely scattered locations (Bates et al. 2013; Fraedrich et al. 2015b; Olatinwo et al. 2016). Thus, sassafras is both highly susceptible to laurel wilt and a good reproductive host for *X. glabratus*. Unlike redbay, which primarily occurs in the southeastern coastal plains, sassafras is widely distributed in various forest types across the eastern United States. Cold tolerance studies for *X. glabratus* and climate models suggest that the beetle can survive temperature regimes found throughout much of the range of sassafras, including northern portions where sassafras is abundant (Formby et al. 2017).

Pest management practices to control *Xyleborus glabratus* and laurel wilt are currently limited. Fungicide injections can temporarily protect redbay trees from the wilt (Mayfield et al. 2008), but because of costs, this practice is feasible only for high-value landscape trees. Chipping trees that have died from laurel wilt can greatly reduce the survival of *X. glabratus* (Spence et al. 2013), although this control method would have limited application in natural forests. Populations of *X. glabratus* can increase rapidly in wilted trees after the disease becomes established in new areas (Maner et al. 2014), but combinations of sanitation cuts and "cutting and leaving" healthy and infected asymptomatic trees in areas where laurel wilt is recently established may help slow the growth of *X. glabratus* populations. Likewise, cutting and leaving healthy redbay trees in areas threatened by laurel wilt and favoring regeneration through stump sprouts may help to limit beetle populations and slow disease spread because small diameter sprouts would not be good brood material for *X. glabratus* (Cameron et al. 2015; Fraedrich et al. 2008; Maner et al. 2014). The practice of "cut and leave" may be most beneficial when leaving tree crowns intact, which would reduce stem moisture content through transpiration (McMinn, 1986) and could reduce the suitability of the felled trees for ambrosia beetle production (Johnson and Zingg, 1969). However, laurel wilt moves rapidly through stands of redbay, and once the disease is established in an area, efforts to control the disease by reducing beetle populations could be futile. A sanitation cut was attempted shortly after laurel wilt was first observed on a Georgia barrier island, but this effort failed to eradicate *X. glabratus*, and the disease ultimately spread throughout the island (Hughes et al. 2015b). Reducing the number of redbay trees of a suitable size for brood production may be most beneficial in areas where redbay occurs with rare or endangered species such as pondberry (Fraedrich, et al 2011), or in areas around high-value, susceptible trees such as avocado, which is not a good host for *X. glabratus* (Ploetz et al, 2017). Such management practices may also be applicable in forest types with sassafras, a species that generally occurs at much lower densities than redbay in southern forests (Fraedrich et al. 2015b; Formby et al. 2017).

The laurel wilt epidemic in the southeastern United States is the result of native hosts being challenged by a pathogen with which they have not evolved and to which they have limited or no apparent resistance. Further, the vector reproduces well in sassafras and exceptionally well in diseased redbay (Maner et al. 2013), and *X. glabratus* populations increase rapidly where there are redbay of suitable size (Maner et al. 2014). In Asia, *X. glabratus* is known to be associated with members of the Lauraceae and species of aromatic plants in several other families, and though *R. lauricola* is the dominant fungal symbiont, there is little indication that this beetle is a serious pest on native hosts in Asia (Wood and Bright 1992; Harrington et al. 2011; Wuest et al. 2017). Nonetheless, the attraction to volatiles from healthy hosts, the aborted attacks in healthy trees, the systemic movement of the pathogen, and the efficient brood production in diseased Lauraceae suggest a unique and highly evolved symbiosis that includes plant pathogenicity by a primary mycangial symbiont. The fungus can move systemically in Asian tree species (Fraedrich et al. 2015a), and repeated attacks by the beetle and multiple inoculations with *R. lauricola* may result in branch dieback of Asian species, which may provide some host material for brood production (Fraedrich et al. 2015a, Wuest et al. 2017). However, more thorough studies are needed with Lauraceae and other hosts in Asian forests.

Conclusions

Brood production of the redbay ambrosia beetle, *X. glabratus*, is greater in bolts from redbay trees that have died from laurel wilt compared with bolts from healthy redbay trees. It was hypothesized that precolonization of the wilted trees by *R. lauricola* would lead to better fungal growth in the ambrosia beetle galleries and help it to compete with other *Raffaelea* spp. in the galleries. However, the mycoflora of beetles emerging from bolts taken from healthy and wilted trees was similar. Instead, the physical properties of sapwood of wilted trees appear to provide a better environment for brood production compared with the sapwood of healthy trees. The sapwood of recently cut, healthy trees contains living parenchyma that may respond and inhibit beetle tunneling or brood development, and bolts taken from healthy trees may dry out faster than bolts from wilted trees. Sassafras, a species that has been questioned regarding its suitability as a brood host for *X. glabratus* based on

earlier bolt studies, was also found to be a good brood host after succumbing to laurel wilt. Thus, it is expected that *X. glabratus* and laurel wilt will continue to expand their geographic range into forest types where sassafras is the only suitable reproductive host for the vector.

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