Reproduction of Walnut Twig Beetle in Black Walnut and Butternut

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Summary. **The walnut twig beetle [WTB (*Pityophthorus juglandis* Blackman)] is the primary insect vector for a pathogen that causes thousand cankers disease (TCD), a disease complex that leads to mortality in species of walnut (*Juglans* L.). We performed field and laboratory trials to determine if reproduction by WTB varies between two black walnut (*Juglans nigra* L.) parent trees of a full-sib mapping population of 323 offspring, and between black walnut and butternut (*Juglans cinerea* L.). These two tree species are native to eastern North America. In field trials, we found no significant differences in colonization density or mean number of adult offspring per female among branch sections from black walnut parent trees or among branch sections from black walnut and butternut, respectively. In laboratory trials with controlled colonization densities of WTB, we found that significantly fewer adult offspring developed in branch sections of the black walnut maternal 'Sparrow' parent than the paternal 'Schessler' parent over three summer months and one winter month. In the field, high colonization densities likely limited reproduction due to increased intraspecific competition beneath the bark. In the laboratory, where we established a lower colonization density, reproduction was likely influenced by differences in host quality. In laboratory trials, no differences were detected in the number of adult offspring emerging from black walnut and butternut accessions. This finding suggests that butternut is a suitable host for WTB. Future screening of the full-sib mapping population of 323 offspring of black walnut parent trees for WTB resistance is a warranted next step in developing alternative management strategies for TCD in black walnut.**

Thousand cankers disease affects walnuts and related species [e.g., wingnut (*Pterocarya* Kunth)] and is caused by the interaction between WTB and a phytopathogenic fungus, *Geosmithia morbida* Kolařík et al. (Kolařík et al., 2011). WTB is native to Mexico and the southwestern United States, where the greatest genetic diversity of the species has been measured (Rugman-Jones et al., 2015), but has spread and occurs in 16 U.S. states (9 western states, 7 eastern states) as of Sept. 2015 (Seybold et al., 2016). Feeding by WTB in the phloem can inoculate healthy host trees with the canker-causing fungal pathogen. Intensive phloem feeding by larvae and adults coupled with coalescence of the cankers can cause girdling of the host branches and stem, which may lead to host mortality (Seybold et al., 2013b). The extent of TCD in the United States appears to be linked to adventive plantings of highly susceptible black walnut, particularly in the western half of the country (Tisserat et al., 2011; Utley et al., 2013).

In addition to black walnut, colonization of butternut by WTB and infection by *G. morbida* were reported for the first time in Oregon in 2011 (Serdani et al., 2013). Butternut is also highly susceptible to another invasive fungal pathogen [*Ophiognomonia clavigignenti-juglandacearum* Nair, Kostichka, & Kuntz (Broders and Boland, 2011)], which has caused butternut canker followed by widespread mortality of butternut across eastern North America (Ross-Davis and Woeste, 2008). As an already threatened hardwood species, remaining butternut populations in North America could be at risk from TCD. Both black walnut and butternut are the only walnut species with native distributions in eastern North America. Resistant cultivars of black walnut and butternut are needed to lower the risk of TCD to regionally important nut and timber industries, germplasm resources, and forests in the eastern United States (Leslie et al., 2010; Newton and Fowler, 2009). The research problem that we investigated, and have described here, is the potential interaction between host selection and reproduction by the WTB and resistance of black walnut and butternut.

Generally, host selection by bark beetles [Scolytidae (sensu Bright, 2014)] follows discrete behavioral steps that are mediated by host characteristics and physiological cues (Wood, 1972). The process begins when newly emerged adults begin to search for hosts. Adults land on trees, often in response to visual and/or odor cues. Visual, tactile, olfactory, and gustatory cues may elicit boring behavior by the beetle into the outer bark and phloem. If the host is unsuitable, the adult will either reject the host before boring (Walter et al., 2010) or abandon the tree after boring into the outer bark or phloem (Elkinton and Wood, 1980). Sustained feeding in the phloem generally leads to pheromone production and aggregation (Wood, 1982). Preliminary work on the colonization dynamics of WTB on northern california black walnut (*Juglans hindsii* Jepson, R.E. Smith) led to the development of a male-produced aggregation pheromone, which can be released artificially from a sponge-stabilized bubble cap device (Seybold et al., 2013a, 2015). Experimentally, the lure can be attached to a branch section, whereby pheromone is released to attract males and females to the cut branch.

In the genus *Pityophthorus* Eichhoff, most species are polygynous; a male initiates gallery construction by creating a nuptial chamber in the phloem and is joined by at least

Units

To convert U.S. to SI, multiply by	U.S. unit	SI unit	To convert SI to U.S., multiply by
0.3048	ft	m	3.2808
3.7854	gal	L	0.2642
2.54	inch(es)	cm	0.3937
6.4516	$inch^2$	cm^2	0.1550
28,350	oz	mg	3.5274×10^{-5}
(°F – 32) ÷ 1.8	°F	°C	(°C × 1.8) + 32

two females (Kirkendall, 1983). For WTB, males typically mate with two to four females (P.L. Dallara, personal communication). Females will then lay eggs individually along the walls of an egg gallery as they tunnel away from the nuptial chamber through the phloem. Thereafter, larvae emerge from eggs and continue to mine perpendicularly to the egg gallery in the phloem.

In the United States, WTB reproduce and develop in native and cultivated stands of walnut species such as northern california black walnut, southern california black walnut (*Juglans californica* S. Watson), black walnut, arizona walnut (*Juglans major* Torr., A. Heller), and butternut, and three species of wingnut (Hishinuma et al., 2016). However, little is known about comparative reproduction of WTB in different hosts. For other bark and ambrosia beetles, reproduction varies significantly among host species (Eager et al., 2004; Lee et al., 2008; Mayfield et al., 2013; Walter et al., 2010; Zeiri et al., 2015). The number of adult brood per established female (F_1) can be influenced by host resistance (Raffa and Berryman, 1983), interspecific, and intraspecific competition (i.e., for food and space); and phloem quality (Ayres et al., 2000; Haack et al., 1987). Methods for artificially infesting cut wood to measure bark beetle reproduction in hosts were developed for mountain pine beetle [*Dendroctonus ponderosae* Hopkins (Cole and Weenig, 1966)], but have not yet been developed for WTB.

An opportunity to screen two black walnut parent trees for differences in WTB colonization behavior and reproduction was available through an applied breeding program for the improvement of nut cultivars, established in 1996 at the University of Missouri, Center for Agroforestry, Columbia, MO. A total of 57 cultivars were fingerprinted by using 10 microsatellite markers and subsequently confirmed based on seven phenological descriptors for each cultivar over four seed years (Coggeshall and Woeste, 2010). These same markers and phenological descriptors were used to identify the parents, the maternal tree ('Sparrow'), and paternal tree ('Schessler'), of 323 full-sib (F_1) trees (Coggeshall, 2011; M.V. Coggeshall, unpublished data). In our experiments, we focused on comparing reproduction of WTB between parent trees in the field and laboratory. If we were successful in defining that these two "parent" trees in fact differed in their susceptibility to WTB attack (i.e., numbers of F_1 offspring produced), then the next step would be to assess the responses to WTB attack among the 323 tree mapping population. If the parents vary in their phenotype, then the offspring will also, which would then allow us to map quantitative trait loci (QTL) associated with WTB susceptibility in this species (or in fact any walnut species) for the first time.

In this study, we investigated WTB brood production in black walnut and butternut cultivars in field and laboratory trials. The objectives of our study were to 1) measure WTB reproduction in cut branch sections that were colonized in the field and 2) develop a laboratory assay for WTB reproduction by artificially infesting cut branch sections with WTB. If there were differences in WTB reproduction between black walnut parent trees or butternut cultivars resulting from field colonization densities or controlled colonization densities in the laboratory, further study might then identify resistance genes related to female fecundity and beetle development so that the heritability of those traits could be tested. Finally, we conducted the laboratory portion of this study because future host screening assays planned by our project team required a reliable method of infesting hosts with WTB in the laboratory. For these future studies, we also needed to identify the month(s) during the growing season when field-collected cut branches might yield the maximum number of WTB brood.

Materials and methods

WALNUT TWIG BEETLE REPRODUCTION IN THE FIELD. Branch sections, ≈18 inches long and 1.5–3 inches diameter, were cut from the maternal tree ['Sparrow' (n = 10)] and the paternal tree ['Schessler' (n = 10)]. If these two parents exhibited a differential host response to WTB, then further screening of the full-sib mapping population would be warranted to identify potential QTL regions associated with WTB reproductive capacity. Furthermore, there were a limited number of accessions of 'Sparrow' and 'Schessler' to sample from in the collection, so we were not able use cultivar as the unit of replication in our experiment.

Cut branch sections were shipped overnight from the University of Missouri Center for Agroforestry to Knoxville, TN, during the week of 22 Apr. 2013. Cut surfaces (ends and large branch stubs) were sealed with paraffin wax. Branch sections were suspended horizontally from stainless steel poles ≈1.5 m above the ground in a plantation of ≈140 stems of black walnut [Seymour, TN (lat. 35.876196°N, long. 83.762168°W, elevation 1135 m)] known to harbor WTB. The branch sections were installed in a grid (5 × 5 m spacing) interspaced between plantation trees in a completely randomized design. One lure releasing 1.2 mg·d^{-1} of WTB male-produced aggregation pheromone from a sponge-stabilized bubble cap release device (Scotts Canada, Delta, BC, Canada) was centered on the underside of each branch section and attached with push-pins. Branch sections were held outdoors for 5

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weeks, collected, and shipped to a Biosafety Level-2 quarantine laboratory in St. Paul, MN, for analysis.

Upon arrival in St. Paul, MN, entrance hole density (i.e., number of male colonization attempts per square decimeter) was determined for each branch section. Entrance holes were counted, and the surface area of each branch was determined from the length and mean diameter (diameter of each end, averaged together). Branches were held on a laboratory benchtop in 1-gal plastic jars (Uline; Pleasant Prairie, WI) with a modified micromesh [625–725 holes/inch^2 ("No-see-um" mesh; Quest Outfitters, Sarasota, FL)] top that allowed for air exchange. Broods (F_1) were allowed to develop for 12 weeks at ambient temperatures [14/10 h (light/dark), 30% to 50% relative humidity (RH), ≈21 °C] in the laboratory. The number of beetles that emerged was counted. Branches were peeled to determine the number of adults that remained under the bark. Because branches were so heavily attacked in Spring 2013, we sampled six 3×3 cm^2 areas: three on the side of the branch where the lure had been placed and three on the opposite side. To estimate the number of beetles that had not emerged, we calculated the mean number of adults per square centimeter and multiplied by the surface area of the cut branch. The numbers of adults that had and had not emerged were totaled. The number of colonizing (i.e., parent) females were estimated by multiplying the number of entrance holes by two as a conservative estimate based on the polygynous mating behavior (Langor and Raske, 1987).

For a second field trial, one branch was collected per tree on 28 Aug. 2013 from butternut (n = 9) and black walnut (n = 10) in a germplasm collection in Rosemount, MN (University of Minnesota–UMore Park, MN). Branches (1.5- to 3-inch diameter) were cut to lengths of 18 inches. As before, all cut surfaces with exposed xylem were dipped in paraffin wax. Branch sections were shipped overnight to Knoxville, TN, and installed at the same site and in the same manner as the first field trial. Branch sections were left in the field for 5 weeks to be colonized by WTB. On 7 Oct. 2013, branch sections were shipped overnight to the Biosafety Level-2 quarantine laboratory in St. Paul, MN. Upon arrival, entrance holes on each piece were counted. Brood (F_1) were allowed to develop for 12 weeks in a growth chamber [14/10 h (light/dark), 50% RH, 21 °C], after which branch sections were sampled and the number of adult offspring (F_1) per female was estimated as described for the first field trial.

Statistical analysis of field trial data. For the first field trial, we used analysis of variance (ANOVA at $\alpha = 0.05$) to examine the effect of parent tree (i.e., maternal/paternal) on colonization density (number of entrance holes per square decimeter) and on the number of adult offspring (F_1) per female. We used branch section as the unit of replication. Analytical assumptions (normality of errors, homoscedasticity of variances) were assessed by visual inspection of residual plots. For the second field trial, we also used ANOVA to examine the effect of host species (i.e., black walnut, butternut) on colonization density (number of entrance holes per square decimeter) and on the number of adult offspring (F_1) per female. In this instance, colonization densities and adult offspring per female were transformed by using a square root transformation to satisfy assumptions of normality of errors and homoscedasticity. We report means and standard errors of nontransformed values for this field trial. All analyses were conducted in R 2.15.1 (R Core Team, 2013).

Walnut twig beetle reproduction in the laboratory. Ten branch sections were cut in Missouri from each of the same trees as in the first field trial, on 30 May, 9 July, and 6 Aug. 2013, and were shipped overnight to St. Paul, MN. Branch sections were cut to 10-inch lengths, and cut surfaces with exposed xylem were dipped in paraffin wax upon arrival. About four holes, spaced on opposite sides of the branch section (density of two entrance holes per square decimeter), were predrilled to insert WTB. We selected a low colonization density in the laboratory trial for several reasons. First, the higher colonization density that we observed in our field trial was the result of parent colonization that was under the influence of the synthetic aggregation pheromone. Work in California (S.J. Seybold, unpublished data) suggests that colonization densities of WTB on host branch sections or branches on live trees under natural conditions (i.e., in the absence of synthetic pheromone) are generally relatively low. This would especially be the case when WTB first begins to invade an area. Thus, the densities we selected have ecological relevance. Second, at the lower density, we observed that larval galleries would remain relatively distinct. A higher density would have resulted in several overlapping larval galleries (i.e., competition). Thus, increasing the colonization density would compromise our ability to accurately track parent beetles and data collected from individual galleries. Parent beetles were collected from beneath the bark of naturally infested hybrid black walnut branch sections [*J. hindsii* × (*J. nigra* × *J. hindsii*/*J. californica*)] from a commercial seed orchard in Sutter County, CA (lat. 39°03.681′N, long. 121°36.818′W, 63-ft elevation). We used this source of beetles because the population density was high at this location and WTB were readily available to us at the site for most of the calendar year. We also used WTB from this site for other laboratory studies involving cold tolerance, so for consistency, we continued to collect from this population. There is no evidence that using WTB reared from species other than black walnut has an effect on successful colonization and reproduction on other host species. Under natural conditions, switching from its putative native host (arizona walnut) to walnut species and hybrids in the western and later, eastern United States, did not appear to hinder WTB colonization or reproduction. Infested branches were shipped overnight on 4 June, 2 July, and 7 Aug. 2013, to the Biosafety Level-2 facility in St. Paul, MN, to provide a continuous supply of beetles.

Eighty adult WTB (≈40 males and 40 females) were collected by removing the outer bark from infested branch sections from California. Males were held in sealed petri dishes with moist tissues (Kimwipes; Kimberly Clark, Roswell, GA) for 2 d to induce feeding when introduced to a new host. To ensure that beetles were healthy, individuals were allowed to walk on a walnut bark surface. If a test beetle could walk normally for 10–15 s on the bark, that individual was used in the breeding trial. If the test beetle did not walk, it

was discarded. One male was placed by using a fine paint brush in each drilled hole, and the hole was covered with modeling clay (CraftsMart, Irving, TX) to prevent escape. Males were checked daily until signs of feeding (i.e., production of frass) were visible, and males were replaced if they were dead or inactive. Up to three males were inserted into each drilled hole if males continued to die. After males showed signs of establishment (i.e., frass extrusion, space for a nuptial chamber), one female was introduced by using a fine paint brush to transfer and guide her to the entrance hole. Females also underwent a walking test before they were selected. Holes were resealed with modeling clay until signs of feeding were evident. The time from placement of a male or female beetle into a drilled hole to signs of establishment varied from 1 to 5 d. Branch sections were held for 12 weeks inside a growth chamber [14/10 h (light/dark), 50% RH, 21 °C] in 1-gal plastic jars with modified lids (as described above). After this incubation period, branch sections were peeled completely and immature and adult life stages were counted. Adults that had emerged naturally also were counted.

A second laboratory experiment began in Winter 2014. Twenty branch sections were collected in Nov. 2013 from the U.S. Department of Agriculture (USDA), Agricultural Research Service (ARS) National Clonal Germplasm Repository (NCGR) facility in Corvallis, OR, from four accessions of butternut ['Herrick' = NCGR Accessions JUG 9.002 and JUG 9.001 (Iowa), and 'Craxezy' = NCGR Accessions JUG 5.001 and JUG 5.002 (Michigan); USDA, 2012]. Based on available data in the USDA-ARS database, these accessions were considered at the time to be "pure" butternut with limited or no introgression from heartnut (*Juglans ailantifolia* Carrière) (USDA, 2012). Branch sections were shipped to St. Paul, MN, on 12 Nov. 2013 and held at –20 °C until Jan. 2014 to kill any associated insects. Ten branch sections of black walnut parent trees (five from each parent) also were acquired at this time from the same Missouri source as described above. The purpose of the latter was to serve as experimental controls. Beetles used to infest the branch sections in this trial were from the same California source and involved the same method of artificial infestation as described above. Branches were held for 12 weeks in plastic jars inside a growth chamber [14/10 h (light/dark), 50% RH, 21 °C], after which branches were peeled completely and the number of immature and adult life stages were counted. In both trials, the number of parents that were initially inserted in a cut branch section was subtracted from the number of adults found to determine the number of adult progeny.

STATISTICAL ANALYSIS OF LABORATORY TRIAL DATA. For the first laboratory trial, we tested the effects of month, parent tree, and their interaction on the number of adults (F_1) per female with a two-way ANOVA. We examined how the number of adults produced varied by month or black walnut parent tree using a Tukey's means comparison procedure. The numbers of adult offspring (F_1) per female were transformed by using a square root transformation to satisfy assumptions of normality of errors and homoscedasticity. For the second laboratory trial, we tested the effect of butternut cultivar and black walnut parent tree on the number of adults (F_1) per female using one-way ANOVA. Results from two trees of 'Herrick' and two trees of 'Craxezy' were combined, as accession (tree) did not affect mean offspring per female (see Results and discussion). Variations among the numbers of adults produced by butternut cultivar and black walnut parent tree were also examined with the Tukey's means comparison procedure. Because the errors had a normal distribution and the variances were equal, no data transformation was necessary for the data set from the second laboratory trial. We report means and standard errors of nontransformed values for results of both laboratory trials. All analyses were limited to adults because immature stage counts were low in field and laboratory trials.

Results and discussion

Walnut twig beetle reproduction in the field

COLONIZATION DENSITY. In the first field trial, mean colonization density (± SE) was 27.5 ± 2.7 WTB entrance holes/100 cm^2 for the maternal 'Sparrow' tree and 28.1 ± 2.6 WTB entrance holes/100 cm^2 for the paternal 'Schessler' tree. The colonization density among branch sections from parent trees did not differ [$F_{1,18}$ = 0.03, P = 0.85 (Fig. 1A)]. In the second field trial, colonization densities also did not differ between butternut and black walnut [$F_{1,18}$ = 0.05, P = 0.83 (Fig. 1B)] but were much lower for both species than in trial 1 (i.e., <1.0 ± 0.2 WTB entrance holes/100 cm^2). The second field trial, completed in fall, occurred during several days of rain, which likely lowered beetle flight activity when compared with the first field trial, completed in spring. Elevated spring and fall flights are typical in northern California (Chen and Seybold, 2014), and this pattern also likely occurs in Tennessee (S.J. Seybold, personal observation).

Though the flight activity of WTB may have varied seasonally, when compared between hosts tested in spring and fall, we observed that beetle colonization activity was consistent in the field. This suggests that the lure made hosts equally attractive to WTB. Based only on our reproduction results, it appears that both black walnut parent trees, as well as black walnut and butternut, are at equal risk of WTB colonization, and ultimately TCD. Because of our destructive sampling of the branch sections to estimate WTB population density, we were not able to compare development of cankers caused by *G. morbida* at each entrance hole. However, Utley et al. (2013) showed that both black walnut and butternut maternal half-sib families produced medium to large cankers in controlled inoculation greenhouse studies of 1-to 2-year-old trees. Future work on beetle landing rates in the absence of synthetic aggregation pheromone (Wood, 1982) would provide more information on whether attraction varies among these host species and cultivars in the field.

ADULT OFFSPRING. In the first field trial, the mean number of adult offspring (F_1) per female did not differ among branch sections from black walnut parent trees [$F_{1,18}$ = 0.35, P = 0.56 (Fig. 2A)]. Similarly, in the second field trial, the mean number of adult offspring (F_1) per female did not differ among branch sections from butternut and black walnut [$F_{1,17}$ = 1.30, P = 0.27 (Fig. 2B)]. Brood production of many scolytids is influenced by several

host phloem characteristics, such as nitrogen content, available carbohydrates, inner bark thickness, and moisture (Amman, 1972; Ayres et al., 2000; Webb and Franklin, 1978). Although phloem quality was not directly measured in our assays, the two black walnut parent trees as well as butternut appear to provide sufficient nutrition for development and reproduction of WTB in the field. In general, the high incidence of TCD in urban and rural landscapes in the western United States suggests that black

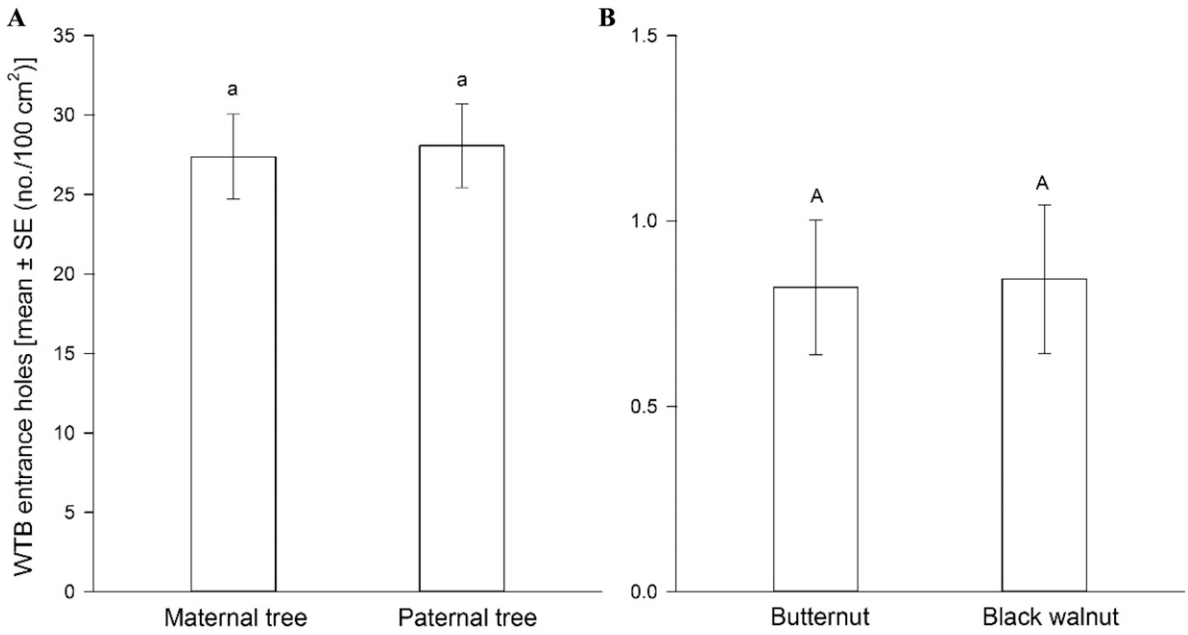

Fig. 1. Walnut twig beetle (WTB) colonization densities (population from Seymour, TN) among branch sections from (A) two black walnut parent trees (n = 10 branches each), and (B) butternut (n = 9 trees) and black walnut (n = 10 trees). Note difference in scale for y-axis between panels A and B, representing (A) spring and (B) fall flights of WTB. Bars with the same letter (lower case in panel A and upper case in panel B) are not significantly different by analysis of variance (α = 0.05); 1 entrance hole/100 cm² = 9.2903 entrance holes/ft².

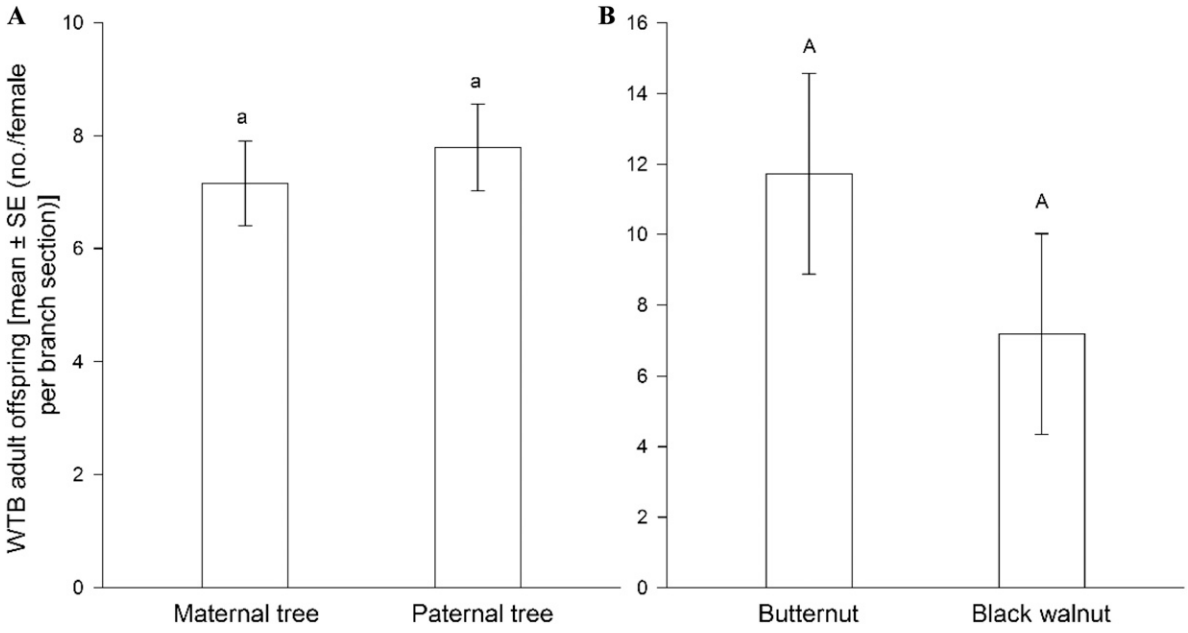

Fig. 2. Number of walnut twig beetle (WTB) adult offspring (F_1) per female (population from Seymour, TN) in branch sections from two field trials among (A) black walnut parent trees (n = 10 branches each), and (B) butternut (n = 9 trees) and black walnut (n = 10 trees). Note difference in scale for y-axis between panels A and B, representing (A) spring and (B) fall flights of WTB parents. Bars with the same letter (lower case in panel A and upper case in panel B) are not significantly different by analysis of variance (α = 0.05).

walnut is a very susceptible host (Tisserat et al., 2011).

Walnut twig beetle reproduction in the laboratory

In the first laboratory trial, we found that branch sections of the paternal 'Schessler' tree produced twice as many adults (F_1) per female than those from the maternal 'Sparrow' tree [$F_{1,62}$ = 63.61, P < 0.001 (Fig. 3)]. Adult (F_1) counts indicate that the paternal tree is a more suitable host than the maternal tree. In the laboratory trials, we used a colonization density that would minimize effects of intraspecific competition on reproduction or development. In our field trials, female parents and developing offspring were competing for space and nutrients in host phloem. For example, in the first field trial, WTB colonization density was relatively high (Fig. 1A) and reproduction was low (Fig. 2A), whereas in the second field trial, WTB colonization density was relatively low (Fig. 1B) and reproduction was high (Fig. 2B). The differences in intraspecific competition resulting from the initial differences in colonization density in the two field studies may have been responsible for the higher level of normalized reproduction in the second trial. Furthermore, competition likely limited WTB reproduction overall in the paternal tree branch sections in field trials when compared with laboratory trials because fewer offspring per female were found in the field than in the laboratory trials. It is possible that the Tennessee-based field population of WTB was behaviorally or reproductively different from the California population used in the laboratory trials, and these differences may have played a role in the differences in WTB reproduction that we observed in the trials. However, populations of WTB from northern California and from several locations in Tennessee did not appear to differ greatly when their mitochondrial cytochrome oxidase I (COI) gene sequences were analyzed (Rugman-Jones et al., 2015), which does not support potential behavioral or physiological differences between our test populations.

Herbivore reproduction also may be limited by juglone, a defensive phenolic compound produced by walnut species, which varies among cultivars of english walnut (*Juglans regia* L.) and is present in the bark and phloem of black walnut and butternut (Gupta et al., 1972; Moore et al., 2015; Solar et al., 2006). Further investigation of resistance traits in the maternal tree or 'Sparrow' should be considered, as juglone or other constitutive defense compounds may differ among cultivars.

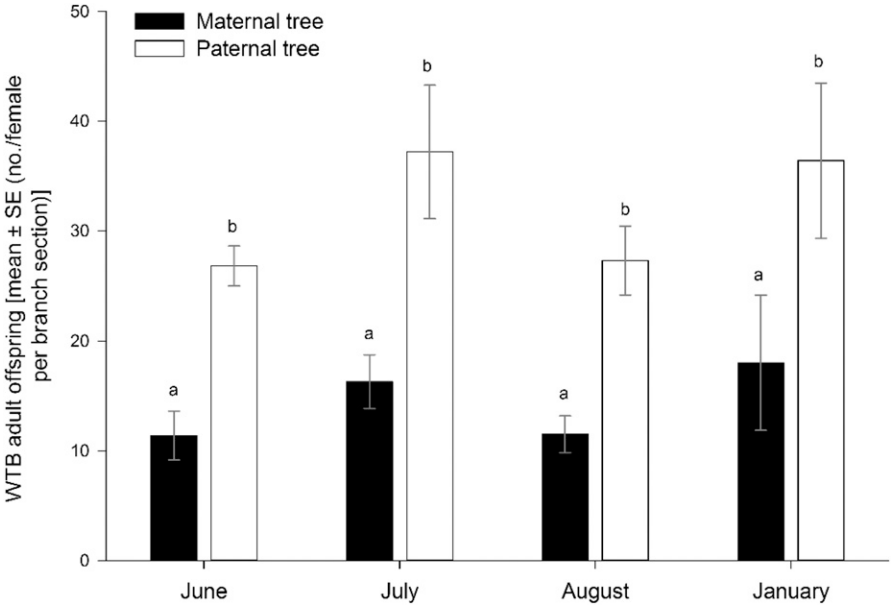

Fig. 3. Number of walnut twig beetle (WTB) adult offspring (F_1) per female per branch section in black walnut parent trees (n = 10 branches in summer months, n = 5 branches in January) across all months in laboratory experiments. Bars with the same letter are not significantly different by Tukey's test (α = 0.05).

We found some evidence (α = 0.1) that the mean number of adult offspring (F_1) per female was affected by the month of colonization [$F_{3,62}$ = 9.09, P = 0.05 (Fig. 3)]. There was no interaction between month and parent tree ($F_{3,62}$ = 0.09, P = 0.99). Water, nitrogen, and carbohydrate concentrations in the phloem can fluctuate seasonally in deciduous trees and juglone levels can fluctuate seasonally in butternut phloem (Moore et al., 2015; Pallardy, 2008; Redmer et al., 2001), but we have limited information on how seasonal changes in phloem chemistry affect WTB. To further understand seasonal changes in host phloem quality, future studies could measure the interactions among *G. morbida*, host tissues, and offspring development over time, as symbiotic fungi have also been shown to alter host nutrition in other bark beetle systems (Ayres et al., 2000; Bentz and Six, 2006; Goodsman et al., 2012).

In the second laboratory trial, we found no difference in mean adult offspring (F_1) per female [$F_{3,26}$ = 2.35, P = 0.09 (Fig. 4)] among black walnut and butternut cultivars tested in January. Though high variability obscured significant differences between parent trees, we note that on average branch sections from the paternal 'Schessler' tree produced twice as many adults (F_1) per female than those from the maternal 'Sparrow' tree. This result is consistent with the results collected in summer months (Fig. 3). Results from two trees each of butternut cultivars, Herrick and Craxezy, were combined, as the model term for accession had no effect on adult offspring per female ($F_{1,25}$ = 0.005, P = 0.94). It appears that black walnut and butternut in winter months are equally suitable for WTB reproduction. These results support our findings from the second field trial. Future work to determine the risk of TCD to butternut, a hardwood species that is already threatened by another pathogen, should examine WTB host colonization, attraction, and establishment rates on additional butternut cultivars and naturally occurring hybrids between butternut and heartnut/japanese walnut. Genetic analyses were conducted on the specific NCGR butternut accessions used in our experiments after the research was completed, and the analyses revealed that both 'Herrick' and 'Craxezy' showed hybridization

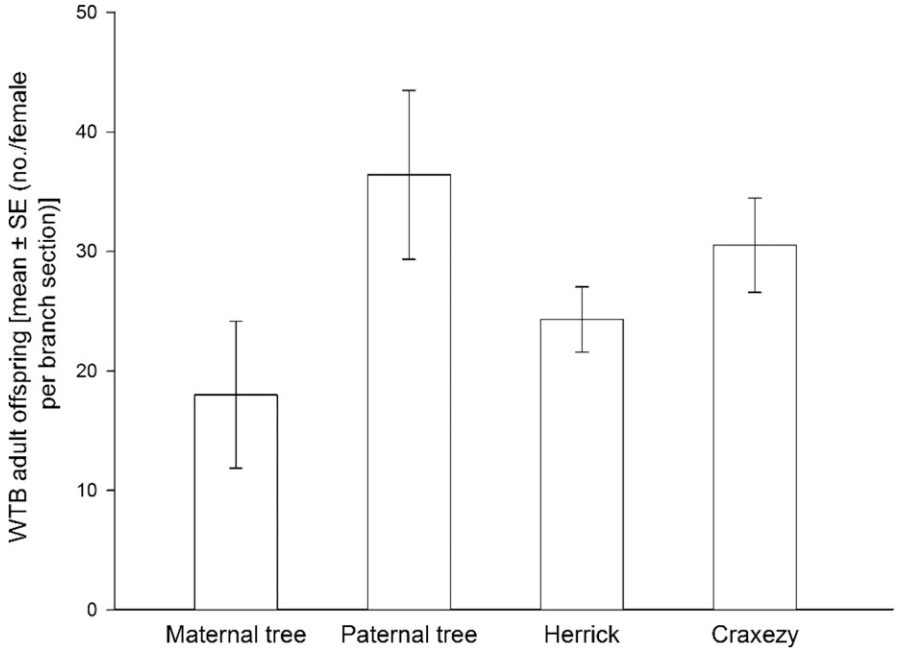

Fig. 4. Number of walnut twig beetle (WTB) adult offspring (F_1) per female per branch section from black walnut parent trees (n = 5 branches each) and two trees each of butternut 'Herrick' and 'Craxezy' (n = 10 branches each) in January laboratory experiment. Bars are not significantly different by analysis of variance ($\alpha = 0.05$).

with heartnut (J. Romero-Severson, personal correspondence).

Conclusion

We focused field and laboratory studies on the black walnut parents of 323 full-sibs. WTB reproduction differed substantially between 'Sparrow' and 'Schessler,' and this response was consistent in all months tested. Our field screenings suggested that at high colonization densities, competition may limit WTB reproduction in black walnut and butternut. Our laboratory assays suggested that at low colonization densities, host resistance may limit WTB reproduction in 'Sparrow,' but the underlying mechanisms remain to be determined. Further screening of the full-sib collection in Missouri is justified to determine the source(s) of resistance. Although our results also indicate that black walnut and butternut are equally suitable for WTB reproduction, additional screenings of other walnut species should be completed to identify other potential sources of resistance.

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