CHAPTER 8.

Assessment and Etiology of Thousand Cankers Disease within the Native Range of Black Walnut (*Juglans nigra*)

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INTRODUCTION

Thousand cankers disease (TCD) is caused by a fungus (*Geosmithia morbida*) vectored by the walnut twig beetle (WTB), *Pityophthorus juglandis* (Coleoptera: Curculionidae: Scolytinae). This pest complex was first described in Colorado where it has caused the widespread death of eastern black walnut (*Juglans nigra*) along the Front Range and throughout the Western United States (Tisserat and others 2009). In August 2010, TCD was found in Knoxville, TN—the first discovery of the disease within the native range of black walnut (Grant and others 2011). Since that time, six other States in the Midwest and East have detected the beetle or pathogen on black walnut trees: Virginia, Pennsylvania, North Carolina, Maryland, Ohio, and Indiana (Juzwik and others 2015, Seybold and others 2012).

In 2012, the Ohio Department of Natural Resources captured eight adult beetles near a veneer mill in Butler County. Hundreds and/or thousands of WTB adults were captured during more intensive surveys conducted in the subsequent year. The outbreak was centered on several dead and dying black walnut trees in adjoining residential neighborhoods (Ashwood Knolls and Avalon Station) approximately 5 km from the mill. These trees exhibited advanced symptoms of TCD, including yellowing and thinning canopies and branch dieback. In Indiana, a trap tree survey conducted in 2011 detected no WTB but rather recovered *G. morbida* associated with a weevil, *Stenomimus pallidus*, at one site in the Yellowwood State Forest in Brown County, IN (Juzwik and others 2015). This was the first report of *G. morbida* from Indiana and the first report of the fungus from an insect other than WTB. Although the pathogen responsible for TCD is present at the site, the trees remain non-symptomatic at this time. From a similar survey conducted on TCD-symptomatic trees in Butler County, OH, in 2014, *G. morbida* was recovered from *Xyleborinus saxeseni*, *Xylosandrus crassiusculus*, and *S. pallidus* (Juzwik and others 2016). This association of *G. morbida* with beetles other than WTB suggests that they may be capable of transmitting the fungus in areas affected by TCD. In the Western States, eastern black walnuts are typically killed within 2 years after initial symptoms appear; however, smaller trees and those growing on poor sites decline more rapidly (Tisserat and others 2009). Recent observations in Knoxville, TN, and Richmond, VA, suggest, however, that TCD progresses more slowly within the native range of eastern black walnut, and some trees may even appear to survive the disease (Griffin 2015).

The goal of this study was to monitor the health of TCD-symptomatic trees and surrounding trees and assess the roles of *G. morbida* and other fungal pathogens in affecting tree health. This information is essential for understanding the etiology and potential threat of this disease complex within the native range of eastern black walnut. In the first objective, we monitored changes in forest health at Yellowwood State Forest, Brown County, IN, and Butler County, OH, where *G. morbida* alone and TCD, respectively, have been found.

Specifically, we assessed the change in canopy condition of trees at varying spatial scales from the epicenter of the TCD outbreak in Butler County, OH. We also compared the change in canopy condition of trees from the epicenter to other parts of Butler County and an area with *G. morbida* and no WTB (i.e., Indiana).

For the etiology objective, we evaluated the potential for *G. morbida* and other fungal pathogens of walnut to cause branch dieback. Specifically, field experiments were established in 2015 to determine (1) whether multiple inoculations with *G. morbida* in the absence of the WTB can lead to branch death over two growing seasons, and (2) the relative virulence of *G. morbida* compared to other known or putative canker pathogens of eastern black walnut within its native range. It is important to understand the role of *G. morbida* in causing branch dieback and tree death in TCD-affected trees so that effective and cost-efficient control methods can be devised. Other native, canker-causing fungi of eastern black walnut have been found to colonize TCD-symptomatic trees (McDermott-Kubeczko 2015, Montecchio and others 2015). Known canker pathogens of eastern black walnut that occur in the native range of the species include several *Fusarium* species and *Botryosphaeria dothidea* (Carlson and others 1993, Pijut 2005). Other fungi (e.g., *Diplodia seriata*, *Biscogniauxia atropunctata*) are known to cause bark diseases on other hardwood species and have been found on eastern black walnut, but their pathogenicity on walnut has not been proven.

METHODS AND RESULTS

Assessment (Objective 1)

Data Collection—During late spring 2015, we marked and collected standard tree measurements for 61 eastern black walnuts growing at five sites in Butler County, OH. At Yellowwood State Forest (Brown County, IN), we marked 40 black walnuts growing along four transects in an unmanaged plantation where *G. morbida* has been recovered. Over the next 3 years, we evaluated these trees for (1) symptoms of TCD and (2) the percentage of live crown (to the nearest 10 percent) during the growing season (June–September). Although three trees were symptomatic for TCD at Ashwood Knolls (Butler County, OH), percent live crown at each site generally ranged from 70 to 90 percent at the beginning of the study. From 2015–2017, we visually assessed the percent live crown of each tree every other week from June–September. In 2018, visual assessments of percent live crown were conducted on a monthly basis throughout the growing season. Only the crown condition assessment nearest to July 1 each year was used in our analysis, because that time corresponded to when crowns were fullest during the growing season. To detect any WTB at each site, two pheromone-baited four-unit funnel traps were deployed and serviced at the same interval as crown ratings.

Analysis of Percent Live Crown—To determine whether there was a significant difference in the rate of crown deterioration of TCD-symptomatic trees (n = 3) compared to nearby non-symptomatic trees in the TCD epicenter (i.e., Avalon Station and Ashwood Knolls, n = 9) and the rest of Butler County, OH (n = 49), data were subsetted and compared at three levels of spatial scale (table 8.1). A fourth level of analysis was included to compare rate of change in crown condition between Butler County, OH, where TCD was present (n = 12), other sites in Butler County (n = 49), and Yellowwood State Forest (Brown County, IN), where only *G. morbida* is present (n = 40).

All data analysis was performed in R (R Foundation for Statistical Computing, Vienna). At each spatial scale, cumulative probit-link mixed model regressions were fitted with the Laplace approximation to test for significance of interaction between year and group. Individual trees were treated as a random effect, and year and group as fixed effects. A significant improvement in model fit upon inclusion of interaction between year and group in a likelihood ratio test indicated a difference in rate of change in crown condition between sites. Interannual changes in crown condition between years within groups were analyzed with a Kruskal-Wallis rank-sum test.

Results—At all four levels of analysis, the inclusion of random terms improved model fit (table 8.1). In Ashwood Knolls and adjacent Avalon Station, TCD-symptomatic trees had a marginally lower percent live crown across years in the interactive model (z = -1.82, p = 0.07) and higher rate of annual deterioration in crown condition (LR = 7.29, p <0.01). Overall rate of percent live crown loss in Ashwood Knolls and Avalon Station was not significantly different from the rest of Butler County, OH (LR = 2.44, p = 0.12). However, there was a significant decrease in crown condition overall in Butler County, OH (z = -2.67, p <0.01). When TCD-symptomatic trees in Ashwood Knolls and

Table 8.1—Spatial levels of analysis used in modelling percent of live crown in *Juglans nigra* at varying spatial scales in areas with thousand cankers disease (TCD)[a]

				Psuedo-R^{2} [b]	
Spatial level of analysis	Groups compared in the analysis			Fixed	Fixed + random
Neighborhood: by tree	TCD-symptomatic	Other trees in epicenter		0.63	0.68
County: by tree	TCD-symptomatic	Other trees in Butler County, OH		0.23	0.55
County: by neighborhood	TCD epicenter	Other trees in Butler County, OH		0.01	0.51
TCD epicenter; Butler County, OH; Brown County, IN	TCD epicenter	Other trees in Butler County, OH	Yellowwood State Forest (Brown County, IN)	0.02	0.47

[a] In Butler County, OH, both the vector and pathogen were present, whereas only the TCD pathogen was present in Brown County, IN.
[b] Calculated using Nagelkerke method (1991).

Avalon Station were compared to neighboring trees and the rest of Butler County, OH, crown loss was significantly faster in TCD-symptomatic trees (*LR* = 4.3, $p < 0.01$), and TCD-symptomatic trees had lower average crown condition across years ($z = -2.30$, $p = 0.02$). Across sites in Butler County, OH, and Brown County, IN, no parameters for main or interactive effects were significant ($p > 0.1$).

The only significant interannual changes in crown condition detected between two subsequent years within a group were a significant improvement in percent of live crown in non-symptomatic trees in Avalon Station and Ashwood Knolls from 86.7 ± 2.9 to 95.6 ± 2.4 between 2015 and 2016 (fig. 8.1A) and a decrease in overall percent live crown outside of the TCD epicenter in Ohio from 85.1 ± 1.7 to 74.9 ± 3.0 between 2017 and 2018 (fig. 8.1B).

Etiology: Branch Health and Canker Development (Objective 2)

Methods—Nineteen eastern black walnut trees (18- to 60-cm diameter at breast height [d.b.h.]) either open-grown or on forest edge in three Butler County, OH, metro parks and 20 eastern black walnut trees (25- to 47-cm d.b.h.) in a plantation (established 1977) in Brown County, IN, were identified for two experiments in each location. Four branches on nine trees in Ohio and five branches on 10 trees in Indiana were selected and randomized to receive one of four/five treatments in mid-June 2015. Treatments consisted of inoculations with three or four fungal species and a water control. Inocula of locally obtained isolates of *F. solani*, *D. seriata*, and *G. morbida* were used in both States. In addition, *B. dothidea* was included in the Indiana trials. The experiments were repeated with the same treatments in mid-September 2015 but on 10 trees in each location. Aliquots (0.2 ml) of aqueous suspensions of fungal spores or of sterile distilled water were placed in 24 to 42 drilled holes (10-mm diameter and depth to outermost sapwood) of healthy (>95 percent leaves green) branches (5 to 8 cm in diameter) with number of holes dependent on branch diameter. Inoculations were designed to result in a density of one canker per 12-cm^2 bark surface area. Thus, all holes were made in a 30- to 40-cm length on each branch to achieve this density. Following inoculation, holes were covered with epoxy resin and the branches marked with brightly colored plastic flagging. The condition of each branch was rated in mid-June and early to mid-September of the two growing seasons following treatment. Branches were harvested on the final monitoring date: mid-September 2016 for mid-June 2015 inoculation trials and mid-September 2017 for mid-September 2015 inoculations. The ~40-cm-long segment of each inoculated branch area was cut, placed in two poly-bags, and stored in sealed plastic containers at 2 °C until processed. In the laboratory (Minnesota Agricultural Experiment Station Biosafety Level 2 Facility, University of Minnesota, St. Paul), bark was carefully removed to expose any cankers or dead tissue around each inoculation point and data on canker sizes recorded. Tissue from margins of each canker was plated on 1/4-strength potato

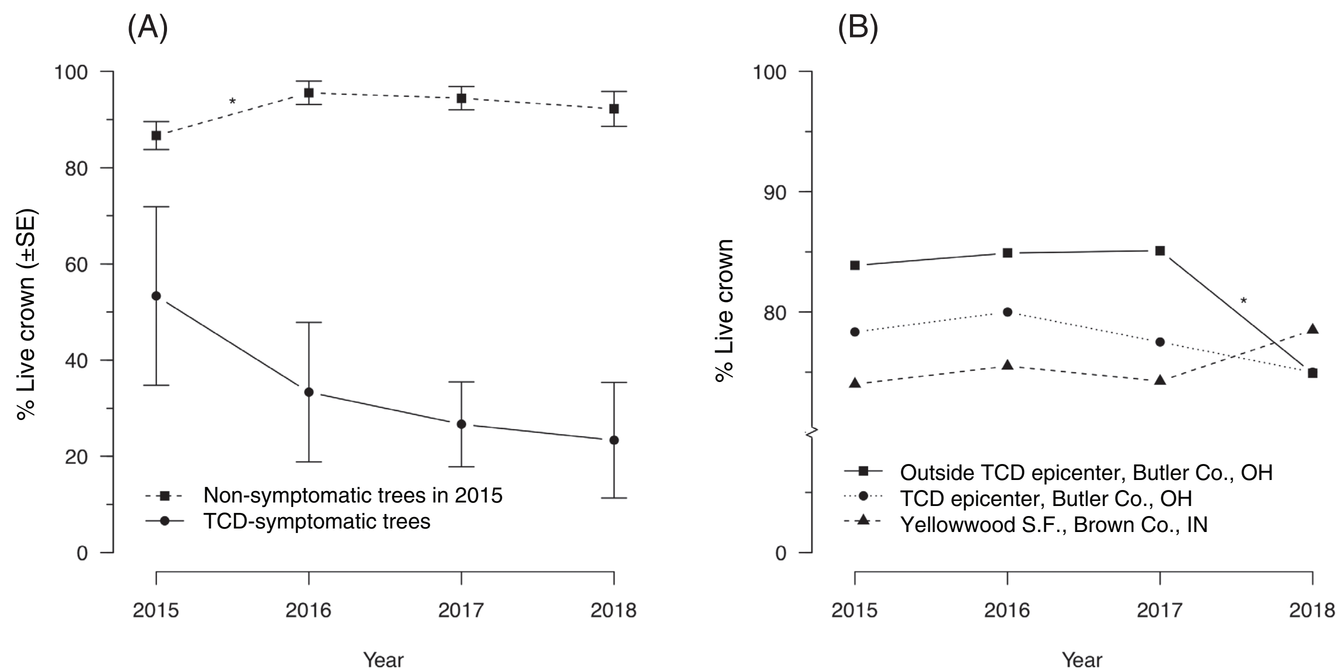

Figure 8.1—Average percent live crown of Juglans nigra. *Asterisk indicates significant change between years (Kruskal-Wallis rank sum test, p <0.05). (A) Thousand cankers disease (TCD)-symptomatic and non-symptomatic trees in the TCD epicenter (Ashwood Knolls and Avalon Station) in Butler County, OH. (B) TCD epicenter compared to other sites in Butler County, OH, and sites in Brown County, IN.*

dextrose agar amended with chloramphenicol (100 mg/L) and streptomycin sulfate (100 mg/L) in attempts to recover the fungi originally applied. Fungi isolated were identified using cultural morphology, microscopic characteristics, and polymerase chain reaction (PCR) and DNA sequencing. Branch condition data and bark canker frequencies were subjected to log-linear modeling analyses using R (R Foundation for Statistical Computing, Vienna). Canker area data were also analyzed in R using mixed-effects models.

Results of June 2015 inoculation trials— Several branches died in each location over the two growing seasons; however, none had been inoculated with *G. morbida*. No differences (p = 0.797) were found for branch condition ratings over 15 months among treatments for both locations combined (data not shown). No differences were found in proportions of inoculation points with cankers or general necrosis (>0.25-cm^2 diameter) on branches across all treatments within each location (data not shown). Mean canker sizes in Indiana were similar across treatments (fig. 8.2). In Ohio, the smallest mean canker sizes were found for control and *D. seriata* branches compared to those associated with *F. solani* isolates (*F. solani* species complex [FSSC] phylogenetic species 6 and 25) and *G. morbida* (p <0.001). No evidence of canker coalescence was observed for the latter treatment. The various fungi were recovered from associated, resultant cankers over a wide range of frequencies (data not shown).

Results of September 2015 inoculation trials—No branches died in response to inoculation in Ohio; however, one or two branches died in response to inoculation with three of four fungal treatments and the control, respectively, in Indiana. In general, the condition ratings for inoculated branches were similar to each other as they varied over 24 months within each location (data not shown), although the preponderance of ratings shifted from ≤5 percent to 10 to 20 percent for September compared to June inoculations. Although no differences were found in proportions of inoculation points with cankers or general necrosis across all treatments within each location, the proportions for September inoculation points were higher than those for June (p <0.0001). Mean canker sizes were lowest for water-inoculated branches (p = 0.0019) and similar (p = 0.992) for *D. seriata* and *B. dothidea* in Indiana (fig. 8.2). Furthermore, mean canker sizes were largest (average = 3.09 cm^2) on *G. morbida*-inoculated branches compared to *F. solani* branches and the other three treatments (p <0.001). In Ohio, mean canker sizes were lowest for water-inoculated branches (p <0.001) and similar for *D. seriata* and *F. solani* (p = 0.98). *G. morbida* inoculation also resulted in the largest mean canker sizes compared to those associated with all other treatments (p <0.001). Coalescing of *G. morbida* cankers was observed along the longitudinal axis of inoculated branch segments on 44 percent of branches from Indiana and Ohio. Bark splitting extending for most of the branch segment length was observed for 39 percent of the *G. morbida* branches in both States and appeared to be caused by strong callus formation response of the host. However, *G. morbida* was infrequently recovered from the margin of cankers found on inoculated branches: 8.5 percent of assayed cankers per branch in Indiana and 1 percent of assayed cankers per branch in Ohio. In contrast, *F. solani* was commonly recovered, i.e., 84 percent of assayed cankers per branch in both States.

DISCUSSION

Walnut twig beetle and/or *G. morbida* have been detected in isolated locations across the native range of eastern black walnut, but establishment of WTB and accompanying mortality have been less pronounced than in the Western United States. Our investigation of tree decline at varying spatial scales from a TCD epicenter revealed that the disease did not spread to neighboring trees and there was no new TCD development. Thousand cankers disease-symptomatic trees had a lower percent live crown rating and higher rate of deterioration in crown condition compared to neighboring trees

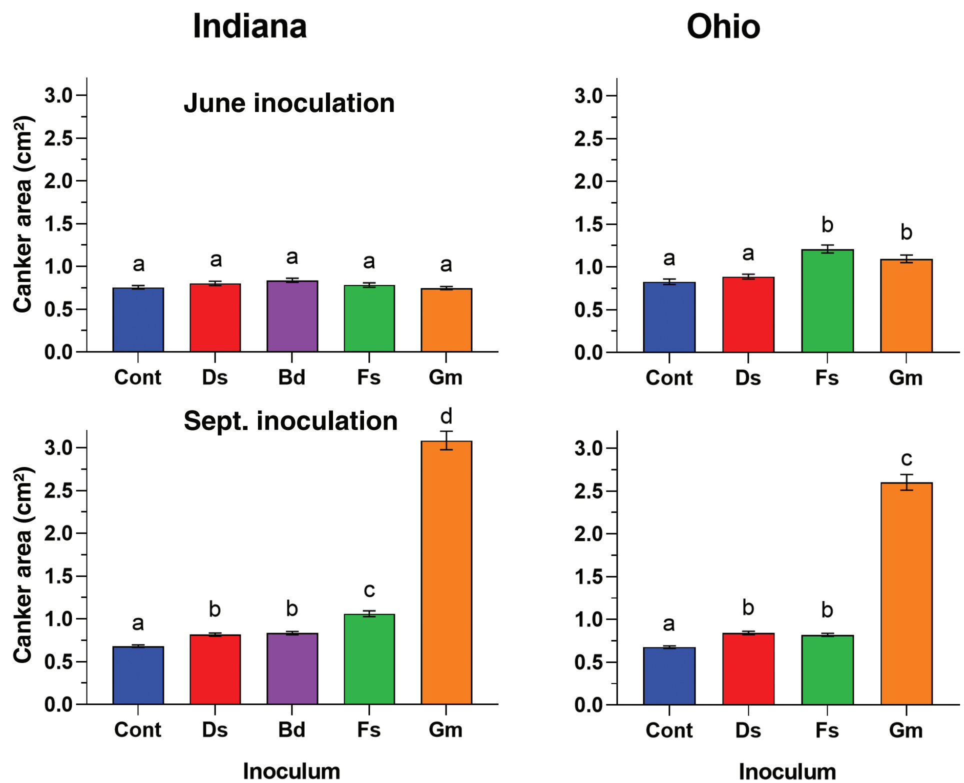

Figure 8.2—Mean size (cm^2*) of cankers of field-grown* Juglans nigra *in two Eastern States 15 (June) and 24 (September) months after multiple inoculations with known or putative canker-causing fungi. Inoculum used: CON = water control; DS =* Diplodia seriata*; BD =* Botryosphaeria dothidea*; FS =* Fusarium solani*; GM =* Geosmithia morbida*. Error bars are standard error. Bars with the same letter are not significantly different according to the Tukey least significant difference test,* $p < 0.05$*.*

in the epicenter and the rest of Butler County, OH, suggesting that the disease was localized to those individual trees. In fact, disease symptoms appeared on no new trees throughout our study, and the rate of crown decline in trees at the epicenter as a whole did not differ from that of other study sites in Butler County, OH. Focal trees at our site in Brown County, IN, were in a generally poorer condition than those in Ohio, which can likely be attributed to the suppressed or codominant position of these trees and the presence of woody invasive plants within the 30-year-old unmanaged plantation.

Ecological factors, including environmental conditions or biotic interactions, likely play a role in the etiology of TCD. Based on a case study of two locations in the Eastern United States, Griffin (2015) attributed the rapid progression of TCD in the Western United States and resilience of eastern black walnut in the Eastern United States to abiotic factors, namely rainfall. Such climatic and ecological factors may have contributed to the improvement in crown condition in Avalon Station and Ashwood Knolls in 2015 and 2016 and the overall decline of crown condition in Ohio. Climate regimes in the Western and Eastern United States differ significantly with respect to total amount and the degree of seasonality in temperature, precipitation, and humidity. Climatic differences between the expanded and native ranges of eastern black walnut could affect growth and dispersal of *G. morbida* by modifying suitability for infection and canker development under the bark and influencing its competitive interactions with other fungi.

Multiple inoculations of eastern black walnut branches (5- to 8-cm diameter) in Indiana and Ohio with *G. morbida* in the absence of WTB pressure (survey data from P. Marshall, Indiana Department of Natural Resources, and D. Kenny, Ohio Department of Agriculture) did not result in any branch death in Ohio and one dead branch in Indiana. The inoculation density used was based on numbers of WTB galleries and/or *G. morbida* cankers counted on peeled branches of TCD-symptomatic eastern black walnut in a Butler County, OH, site in late summer 2014 and 2015. Walnut twig beetle populations had dropped to zero detectable levels in 2014 and 2015, and a single beetle was captured in Ohio each year from 2016–2018.[1] The canker densities documented ranged from a mean of 4 to 20 (with or without WTB galleries) per 100 cm^2 of bark surface area. Thus, the target density of this study (8.5 cankers/100 cm^2) was reasonable and a valid test for the first objective. To our knowledge, no other field tree inoculations with *G. morbida* have used more than three points per branch or stem.

A clear difference in the size of cankers resulting from *G. morbida* inoculations was found for late summer versus late spring or early summer inoculation dates. The range of canker areas (for measurable cankers) for the latter inoculation time ranged from 0.26 to 2.22 cm^2 in Indiana and 0.09 to 3.73 cm^2

[1] Personal communication. 2019. Daniel Kenny, Plant Health Administrator, Ohio Department of Agriculture, 8995 E. Main Street, Reynoldsburg, OH 43068.

in Ohio compared to that of canker areas for mid-September inoculations (0.17 to 12.18 cm^2 for Indiana and 0.34 to 8.07 cm^2 for Ohio). Previous studies of canker development by other pathogens have demonstrated effects of season on tree susceptibility to infection and/or canker development (e.g., *Ophiognomonia clavigignenti-juglanacearum*) (Ostry and Moore 2008). The mean canker areas for our June 2015 trials fall within the range of those reported by Sitz and others (2017) for *G. morbida* field inoculations of eastern black walnut saplings in Colorado. In the latter case, eight genetically distinct isolates of *G. morbida* were used to inoculate three wounds made along a 20-cm length of a branch above a stem crotch on each tree and resulting cankers measured 9 weeks later. The maximum sizes of *G. morbida* cankers found in our September 2015 inoculation trials were difficult to measure because coalescence with adjacent cankers occurred (along longitudinal axis of branches).

Of the two or three other pathogens compared to *G. morbida*, only the *F. solani* isolates (FSSC phylogenetic species 6 and 25) used in Ohio for the June 2015 inoculation trial caused cankers of significantly larger size than controls and *D. seriata*. The relative size of *F. solani* cankers found 15 months later were 10 percent larger than cankers caused by *G. morbida*, but differences were not significant. In a Colorado trial comparing relative *F. solani* canker sizes to those of *G. morbida* 9 weeks after June inoculation, the *F. solani* cankers were 47 and 37 percent smaller than *G. morbida* cankers in August 2014 and August 2015, respectively (Sitz and others 2017). The phylogenetic species used in the Colorado and in our trials are known to co-occur with *G. morbida*, and their natural occurrence may contribute to size of canker development on eastern black walnut (Montecchio and others 2015). Results of coinoculation of eastern black walnut with *F. solani* FSSC 6 in Colorado did not yield a synergistic response in terms of canker size (Sitz and others 2017).

CONCLUSIONS

Our assessment study revealed no new symptomatic trees, and no adult WTB were captured throughout the course of study, suggesting that TCD does not progress in the absence of the primary vector. In fact, very few WTB have been captured in Ohio since the mass emergence in 2013. It appears that if mass attack is not followed by similar attacks in subsequent years that host responses may indeed prevent further development of cankers in absence of the WTB. Although *G. morbida* was isolated from ambrosia beetles and weevils at the TCD epicenter in Ohio and from weevils at the site in Indiana (Juzwik and others 2015, 2016), our findings support the hypothesis that this assemblage of alternate vectors has little impact on disease severity and spread.

Based on the results of our etiology experiments, we speculate that branch dieback could occur following multiple years of mass attack of eastern black walnut branches by WTB, particularly in late summer and early fall. However, if 1 year of mass attack is not followed by similar attacks in subsequent years, we speculate that host responses may indeed prevent further development of cankers in absence of the WTB as previously mentioned. This speculation is supported in part by the published field observations of arrested TCD symptom development in Tennessee and Virginia attributed to changes in precipitation patterns (Griffin 2015).

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