



Biology and distribution of *Agrilus macer* LeConte (Coleoptera: Buprestidae), a species associated with sugarberry (*Celtis laevigata* Willd.) mortality in the southeastern USA

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Received: 15 June 2018 / Accepted: 18 December 2018

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Abstract

• **Key message** *Agrilus macer* is attacking sugarberry trees in the southeastern USA, a region from which few specimens have been previously collected. Despite attacking at high densities, this species appears to be a secondary pest, and there is no evidence it carries harmful fungal pathogens.

• **Context** Because the genus *Agrilus* Curtis includes significant forest pests, the association of a poorly known species, *Agrilus macer* LeConte, with unexplained sugarberry (*Celtis laevigata* Willd.) mortality in the southeastern USA is a cause for alarm.

• **Aims** This study sought to investigate the distribution and biology of *A. macer* and determine whether the species is a primary cause of observed tree mortality.

• **Methods** Through a series of studies and literature searches, we documented aspects of *A. macer* biology and distribution while focusing on egg-laying behavior and searching for fungal pathogens associated with oviposition sites.

• **Results** *A. macer* appears to be widely distributed throughout the southern USA, but most records are from Texas and Louisiana. Egg mass densities up to 1.2 masses per 10 cm² (equivalent to ~1.9 eggs per cm²) were observed on trunks, branches, and exposed roots of dying *C. laevigata* trees in our study area, with an average of 16 eggs per mass. Fungi isolated from discolored sapwood around larval galleries did not cause defoliation, dieback, or mortality of sugarberry in inoculation trials.

• **Conclusion** Our findings suggest that *A. macer* is a secondary pest on sugarberry and does not transmit harmful fungal pathogens.

Keywords *Agrilus* · Eggs · Egg masses · Fungal isolation · Oviposition · Saproxylic

Handling Editors: Aurélien Sallé & Christophe Bouget

Contributions of the co-authors Michael Ulyshen conceived of the studies on the distribution and biology of *A. macer* while Rabi Olatinwo and Michelle Cram led the search for potential pathogens. Field work was performed by Michael Ulyshen, Scott Horn, Emilee Poole, Stephen Fraedrich, and Michelle Cram, while Rabi Olatinwo isolated fungi from some samples and sequenced fungal isolates. Michelle Cram and Stephen Fraedrich conducted pathogenicity tests on fungi. Emilee Poole and Michael Ulyshen analyzed the results and wrote the paper with contributions from all co-authors.

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1 Introduction

Insects and diseases are often found to play a significant role in forest health issues, and the movement of these organisms among and within continents has had disastrous consequences for a number of tree species. Some insect taxa have been repeatedly implicated in major episodes of tree mortality. Within North America, for example, a number of nonnative buprestid species belonging to the genus *Agrilus* Curtis have been responsible for major losses of *Fraxinus* (*A. planipennis* Fairmaire), *Quercus* (*A. auroguttatus* Schaeffer), and *Sapindus* (*A. prionurus* Chevrolat). Other buprestid species cause losses within their native range such as the damage seen by outbreaks of *A. bilineatus* Weber on *Quercus* and *A. anxius* Gory on *Betula* (Barter 1957; Bauer et al. 2014; Billings et al. 2014; Coleman and Seybold 2016; Haack 2006; Haack and Benjamin 1982; Muilenburg and Herms 2012). Additionally, concern over losses of *Fraxinus* from *A. planipennis* and

Quercus from *A. biguttatus*, Fab. and *A. bilineatus* is increasing in Europe with the growing awareness of the damaging abilities of *Agrilus* species (Baranchikov et al. 2008; Brown et al. 2015; Hizal and Arslangundğdu 2018; Reed et al. 2018). Although few *Agrilus* species act as primary pests, a close association between an *Agrilus* species and an emerging forest health issue should not be overlooked. The purpose of this study was to explore what role a largely unknown species of *Agrilus*, *A. macer* LeConte, is playing in the mortality of sugarberry (*Celtis laevigata* Willd., Cannabaceae) in the southeastern USA.

Trees belonging to the genus *Celtis* grow throughout North America including Mexico. Although ranges overlap considerably in Texas and other south-central states, three species (*C. lindheimeri* Engelm ex K. Koch, *C. pallida* Torrey, and *C. reticulata* Torrey) are primarily western species whereas three others (*C. laevigata* Willd., *C. occidentalis* L., and *C. tenuifolia* Nuttall) are largely concentrated in the east (www.eFloras.org, accessed Aug. 2018). In addition to these native species, species of non-native *Celtis*, such as *C. sinensis* Pers., have become naturalized in North America. Sugarberry commonly grows on floodplains and along rivers and streams, where it thrives as a dominant or codominant member of hardwood forests (Duncan and Duncan 1988; Samuelson and Hogan 2003; Timmerstein 1990). Sugarberry can dominate disturbed sites, and occasionally pure stands can be found (Ford and Van Auker 1982). In addition to numerous benefits to wildlife as a food source and nesting site, sugarberry is planted as an ornamental tree, and the wood is used for furniture, flooring, or pulp (Duncan and Duncan 1988; Timmerstein 1990).

Declining health of sugarberry trees (Fig. 1a) was reported around Columbia, South Carolina, in 2009 with symptoms including yellowing leaves and defoliation (Andy Boone, personal communication). Efforts to identify insects or diseases responsible for these symptoms began soon thereafter. In 2011, scientists from the US Forest Service, South Carolina Forestry Commission, and other entomologists and pathologists met to further evaluate reports of symptoms present in other areas of South Carolina. Early inspections reported the larvae of an unidentified species of buprestid beneath the bark of dying sugarberry (Fig. 2a–c), and a large number of light-colored egg masses were observed on declining trees in some areas (Fig. 1b, c). It was also clear from early observations that areas of sapwood discoloration, usually appearing as streaks, were typically found in association with sites injured by feeding buprestid larvae (Fig. 2d). This is not the first episode of sugarberry mortality to be observed in the southeastern USA. A previous occurrence was reported in Louisiana between 1988 and 1990 (Solomon et al. 1997). Although the presence of *Agrilus* beetles at low levels was noted in that study, it was concluded they were not the principal cause of the dieback and mortality.

Our objectives in this study were to (1) determine what species of buprestid was infesting sugarberry trees, (2) estimate the density of egg masses on declining sugarberry trees and the number of eggs per mass, (3) investigate the known geographic distribution of the species, (4) describe aspects of its biology, giving special attention to its egg-laying behavior, and (5) determine whether any pathogenic fungi were present in discolored sapwood associated with the beetle activity.

2 Methods

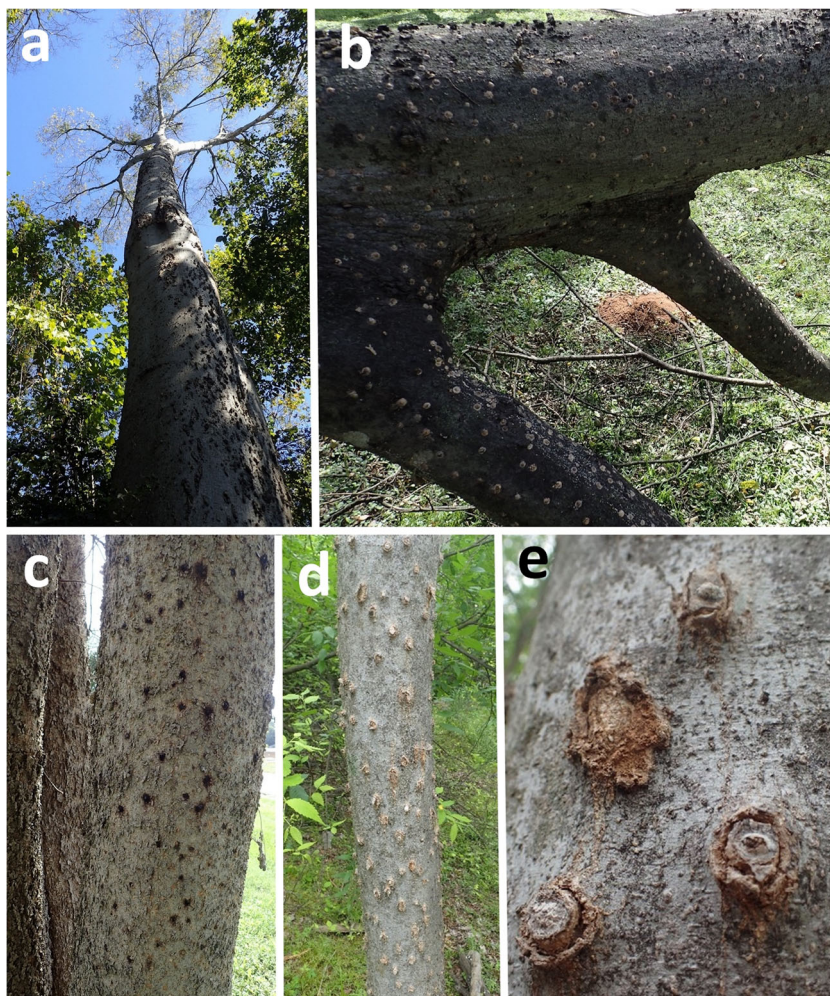
2.1 Study area

Field work was concentrated along the Savannah River in North Augusta, South Carolina (33.49, –81.98) near the North Augusta Greenway (hereafter referred to as the Greenway), a forested area with paved and unpaved recreational trails. The climate of the region is subtropical with an average annual temperature of 17.7 °C and an average annual rainfall of 1.1 m (usclimatedata.com, accessed Aug. 2018). It is estimated that 70–80% of the trees along the Greenway are sugarberry (MDU, EMP, and Roy Kibler-North Augusta Superintendent of Property Maintenance, personal observation). In 2015, when this study began, large numbers of dying sugarberry could be found on both sides of the river, but the size of the affected area was not determined. Many of the stressed and dying trees at our main study site along the Greenway were clearly experiencing high densities of buprestid attacks with large numbers of egg masses visible on the bark of trunks, branches, and exposed roots (Fig. 1). The observations and data reported in this study came from a variety of habitats along the Greenway, ranging from trees growing in mulched beds near the river as well as in adjacent woodlots, some of which were growing on slopes above the floodplain. Other tree species present include *Prunus serotina* Ehrh., *Acer negundo* L., *Pinus taeda* L., *Liquidambar styraciflua* L., *Platanus occidentalis* L., *Quercus nigra* L., and *Quercus phellos* L.

2.2 Abundance and distribution of egg masses

On 6 October 2015, five sugarberry trees of varying diameters and heights were felled in a small woodlot along the Greenway (Table 1, Fig. 1b). Beginning at a height of 0.5 m above the ground, log sections of 20 cm in length were collected every 2 m along the primary stem to the furthest-most branch until the last section exceeding 2.5 cm in diameter was collected. Depending on the height, six to nine log sections were collected from each tree (Fig. 4). The number of egg masses on each section was counted in the laboratory. The length and circumference of each log section were then used

Fig. 1 Dying sugarberry (a). Felled sugarberry covered with light-colored *Agrilus macer* egg masses (b). Dark weeping egg masses as larvae penetrate the bark (c). Tree with callus tissue formations overcoming *A. macer* attack (d). Close-up of failed colonization attempts characterized by circular tissue protruding from the bark (e). (Photos by M. Ulyshen)



to calculate the number of egg masses per square meter of bark, hereafter referred to as egg mass density (Table 1). The average egg mass density between two consecutive sections was used to estimate the number of egg masses on the intervening 2-m section, thus allowing us to calculate the total number of egg masses on the entire stem (though not including side branches). We also estimated the total number of eggs laid by multiplying the estimated number of egg masses by the average number of eggs per mass (see “[Number of eggs per mass](#)” section below). Because the number of egg masses and thus egg mass density varied considerably among the five trees, we standardized egg mass density for each section by dividing by the highest egg mass density measured from any section belonging to the same tree. The highest standardized egg mass density for any tree was therefore 1. These results were used to determine how egg mass density varied along the length of the bole. The mixed procedure of SAS was used to test whether standardized egg mass density varied with height above the ground with tree included as a random effect.

2.3 Number of eggs per mass

Individual egg masses were carefully removed from the bark to count the number of eggs present within each mass. Fresh egg masses were detached from the log sections of the five felled trees corresponding to the following heights: 0.6, 2.8, 5, 7.2, 9.4, 11.6, 13.8, 16.0, and 18.2 m. In total, 107 egg masses that still contained unhatched eggs or from which larvae were in the process of tunneling through the bark were included in these counts. The bark layers beneath the egg masses were carefully dissected under a microscope to detect any hatched larvae. The number of unhatched eggs and larvae were totaled to estimate the number of eggs initially present in each egg mass. The mixed procedure of SAS was used to test whether the number of eggs varied with height above the ground with tree included as a random effect.

2.4 Observations of *A. macer* egg-laying behavior

During the summer of 2016, the trunks of declining sugarberry trees were observed along the Greenway to determine

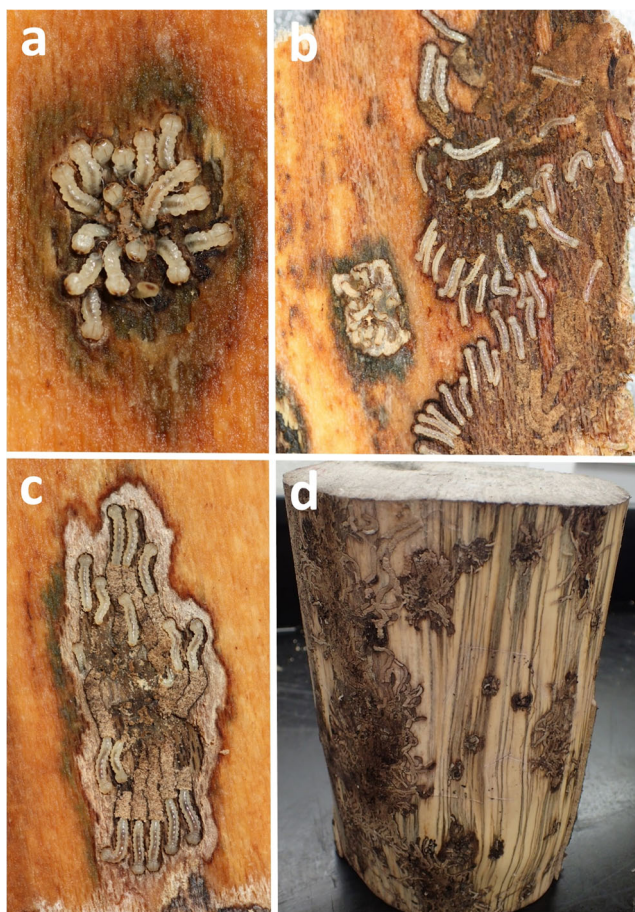


Fig. 2 *Agrilus macer* larvae from a single egg mass as seen from the underside of bark (a). Feeding *A. macer* on the underside of bark (b–c). A de-barked log showing multiple colonization points and associated xylem discoloration (d). (Photos by M. Ulyshen)

which species was responsible for the egg masses and to better understand the egg-laying behavior of the species. The observations were made opportunistically between late morning and early afternoon hours, and an effort was made to photograph the sequence of events (Fig. 3).

2.5 Adult activity period

We installed traps on 10 declining sugarberry trees in 2016, many with visible *A. macer* egg masses, along the Greenway

to determine the peak activity period for adult *A. macer*. Two brackets were installed on opposite sides of each tree. One bracket received a black Lindgren funnel trap (with eight funnels), whereas the other received a flight intercept trap. The flight intercept trap consisted of two intersecting sheets of clear plexiglass (20 × 30 cm) attached above a single Lindgren funnel. Propylene glycol and a few drops of dish soap were used as the killing and preservative agent in both traps; samples were collected every 2 weeks beginning on 16 May 2016 (after leaf expansion) and ending on 9 September 2016.

2.6 *Agrilus macer* distribution

To determine the known distribution of *A. macer*, specimen data were requested from large university and institutional arthropod collections throughout North America (see Acknowledgements). We requested label data from all specimens present in these collections, such as state, county, collection date, and any host information to assist in understanding the distribution and natural history of the species. Photographs were requested to confirm correct identifications of specimens when they came from states with few other records. *A. macer* is distinguishable in photographs due to the presence of a prominent raised ridge on each elytron. Specimen data were organized by state, county, and collection date and used to determine the known distribution of *A. macer* within the USA. Collection date information also allowed us to further examine the adult activity period of the species (Fig. 5). Because we were primarily interested in determining whether *A. macer* is new to the southeastern USA, we limited our search to primarily US records. The range of *A. macer* is not limited to the USA, however. The species occurs in the Lower Rio Grande Valley close to the USA-Mexico border (Vogt 1949) and has also been recorded from Mexico, confirming its presence south of the USA (Harpootlian and Bellamy 2014; Hespenheide et al. 2011).

2.7 Oviposition behavior of selected *Agrilus* species

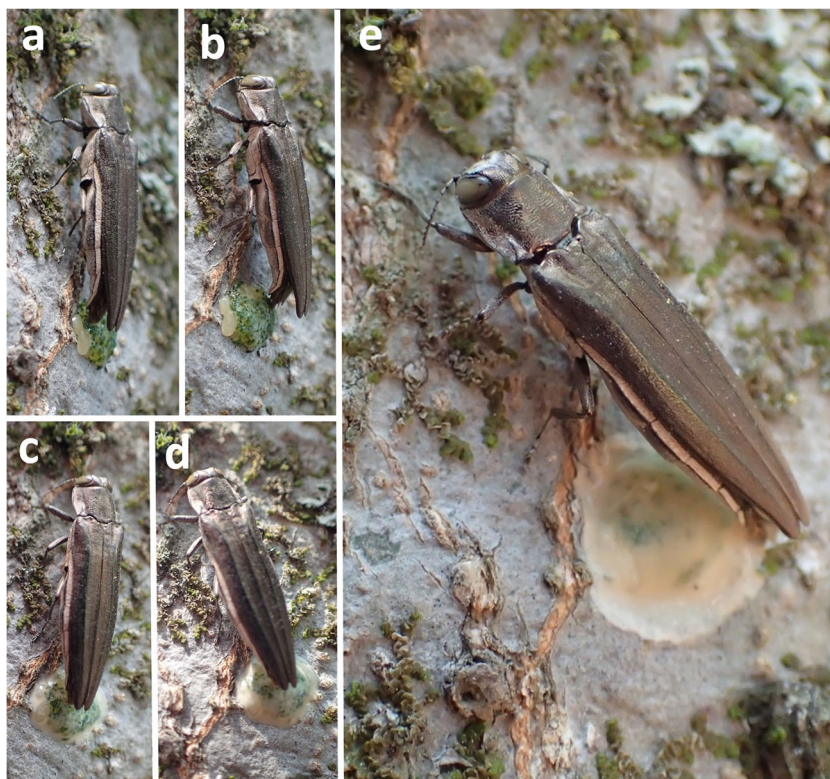
To understand how the oviposition biology of *A. macer* compared to other members of the genus, a literature search was

Table 1 Estimated number of *Agrilus macer* egg masses and eggs laid along the main trunk and the leading branch of five sampled trees

Tree	Diameter at 0.5 m (m)	Length of primary stem (m)	Bark surface area (m ²)	Estimated number of egg masses	Estimated number of eggs*
A	0.37	18.3	10.1	13,024	209,556
B	0.15	11.7	3.4	637	10,249
C	0.18	13.9	4.9	4609	74,159
D	0.19	13.9	4.5	2480	39,903
E	0.25	16.1	6.6	384	6179

*Based on an average of 16.1 eggs per mass. See text for details

Fig. 3 Oviposition sequence of *Agrilus macer*. After eggs are deposited on bark, bright green material is deposited (a) before the mass is covered with a tan secretion (b–d) that hardens into a protective covering (e). (Photos by M. Ulyshen)



conducted to investigate the habits of other *Agrilus* species. We specifically looked for information on whether eggs are laid singly or in groups, whether any protective covering is added to the eggs, and whether eggs are laid on the trunk or branches of host trees. Our survey was limited to *Agrilus* species for which information could be found on egg-laying behavior, including egg number and whether any protective coverings are added to the eggs. Supplementary notes were recorded when resources provided additional information relevant to the oviposition behavior of *A. macer*.

2.8 Fungal isolation

Fungal isolations were attempted from areas of discoloration in the sapwood of sugarberry trees that had been attacked by *A. macer*. The discoloration typically occurred as dark streaks above and below points where *A. macer* had deposited egg masses and developing larvae were mining areas of the phloem, cambium, and outer sapwood (Fig. 2d). Samples of discolored sapwood were obtained in September 2015 from three infested trees located at Lake Olmstead Park (Augusta, GA) and from eight trees in February 2016 at the Greeneway. Seven additional trees were sampled at the Greeneway in July 2016. A minimum of two areas of discoloration around larval galleries were evaluated for each tree. Fungal isolations were typically attempted within 1 to 10 cm above and below the larval galleries. The samples were obtained using a hand-saw and chisel, debarked, and surface sterilized with either

1.1% sodium hypochlorite (NaOCl) solution dip (Lake Olmstead Park) or 95% EtOH dip for 1 min (Greeneway samples), then rinsed several times in sterile deionized water and blotted dry with sterile paper towels, which were sterilized with an autoclave at 250 °C at 103.4 kPa for 15 min. Sapwood chips from the Greeneway samples were then plated on malt extract agar (MEA; MP Biomedicals, LLC, Santa Ana, CA, USA), while thinly sliced stem sections from the Lake Olmstead Park trees were placed on sterile moist filter paper in petri dishes. Plates with samples were incubated at 25 °C, and fungi emerging from symptomatic tissue were evaluated. Isolates of potentially pathogenic fungi, as well as fungi routinely observed in the symptomatic samples, were subsequently single-spored and grown on either MEA or potato dextrose agar (PDA; Difco Laboratories Inc., Detroit, MI, USA). Fungal isolates were initially identified by morphological characteristics, and identities were subsequently confirmed by molecular techniques. Isolates were evaluated by PCR amplification and analysis of the internal transcribed spacer (ITS) region from genomic DNA samples extracted from cultures described earlier. Extractions were conducted using the QIAGEN DNeasy Plant Tissue Mini extraction kit (QIAGEN, Valencia, CA) following the manufacturer's instructions, and the DNA extracts were stored at –20 °C and used as templates in the PCR amplifications.

PCR amplifications were performed in a 10 µl reagent mixture containing 5 µl *TopTaq*™ PCR Master Mix (Qiagen), 1.5 µl of a 5 µM solution of the forward primer

ITS1F (5'CTTGGTCATTTAGAGGAAGTAA'3) (Gardes and Bruns 1993) and reverse primer ITS4R (5'TCCTCCGC TTATTGATATGC'3) (White et al. 1990), 1 µl of 10× CoralLoad, and 1 µl of the DNA template, and amplifications were performed using an *Eppendorf Mastercycler® Pro* PCR machine. The amplification protocol consisted of initial denaturation at 95 °C for 3 min, followed by 35 cycles of 35-s denaturation at 95 °C, 55-s annealing at 58 °C and 1-min extension at 72 °C, and a final extension at 72 °C for 10 min. Gel electrophoresis was performed to examine amplified products by loading 5 µl PCR products on 1% agarose gels. The agarose was stained with ethidium bromide after 20 min. of electrophoresis, and the resulting bands were visualized under UV illumination. PCR products were purified and sequenced at GENEWIZ Inc. (South Plainfield, NJ, USA; <http://www.genewiz.com>).

2.9 Pathogenicity tests

Pathogenicity tests were performed on *C. laevigata* saplings (approximately 3–5 years old) grown in Georgia at a local nursery (Nearly Native Nursery, Fayetteville, GA, USA). The saplings were approximately 2 m in height and 18 mm in diameter at the soil level and were grown in 25-cm pots (11 L). Isolates from three fungal species, commonly isolated from areas of discolored xylem around *A. macer* attacks, were tested to determine their effects on sugarberry. On May 3, 2016, saplings were inoculated with one of two fungal treatments, *Fusarium neocosmosporiellum* O'Donnell and Geiser or *Phialemonium dimorphosporum* Gams and Cooke, or a sterile PDA plug (control). A second test was established on August 31, 2016, using an isolate of *Clonostachys rosea* (Link: Fries) Schroers, Samuels, Seifert and W. Gams (syn. *Gliocladium roseum*; teleomorph, *Bionectria ochroleuca*) and a sterile PDA control. All isolates were grown in petri dishes with PDA for 3 weeks prior to inoculations. One treatment was applied to the stem of a sapling at 23–25 cm above the soil line. A 3.5-mm-diameter cork borer was used to remove the bark/phloem, and in order to simulate insect damage, a 1-mm drill bit and drill were used to scrap away the outermost sapwood. A 3.5-mm-diameter plug of inoculum treatment was placed with the mycelial surface down on the damaged sapwood and wrapped with parafilm. There were four replications (four saplings) for each treatment in each test. Inoculated saplings were placed outside in a partially shaded courtyard. Saplings were watered as needed and observed for any outward symptoms of disease until final data measurements were taken on July 27, 2017. The bark, phloem, and cambium were removed from around the inoculation point, and the length of the discolored sapwood above and below the inoculation point was measured. Reisolation of the fungi inoculated in stems was attempted by surface sterilizing (1.1 NaOH dip) sections of discolored sapwood from around

inoculation points and placing them on Nash-Snyder media (Nelson et al. 1983) and PDA amended with 1% tergitol. Possible differences in the mean lengths of discolored sapwood among the three treatments in test 1 were analyzed using Dunnett's 3T test for unequal variances. In test 2, data was evaluated with a two-sample *t* test to determine if means were different (SYSTAT 13, Systat Software, Inc., Chicago, IL).

3 Results

3.1 General observations

When we first visited the Greenway in 2015, there were numerous sugarberry trees at all stages of decline, including many dead trees. Dying trees were observed throughout the area that included park-like settings adjacent to the river and neighboring woodlots. A total of 46 dead stems ranging from 5.1 to 84 cm dbh were visually inspected for *A. macer* activity without felling the trees. Old egg masses and D-shaped exit holes, which are typical for *Agrilus* species, were observed on 82.6% of these trees. Among living trees, only those exhibiting symptoms of decline (e.g., thin or yellow crowns) had tan-colored *A. macer* egg masses, as also assessed from the ground. Trees under attack by *A. macer* (in which larvae are tunneling through the bark) were found weeping black liquid around the egg masses, as visible externally (Fig. 1c), but weeping was not observed in trees at later stages of attack. We also observed evidence of trees overcoming attacks by *A. macer*. This consisted of the formation of callus tissue beneath each mass, resulting in a raised bump in the bark (Fig. 1d, e).

3.2 Abundance and distribution of egg masses

The estimated number of egg masses present on the main stems (i.e., not including other branches, etc.) of the five trees ranged from 384 to 13,024 (Table 1). On the tree with highest density of egg masses, tree A (Table 1), there were about 1.2 masses for every 10 cm² of bark, which is equivalent to about two eggs per square centimeter. Standardized egg mass density varied significantly with height along the bole ($F_{8,24} = 3.59$, $P < 0.01$), being significantly higher at mid-bole positions (2.7–2.9 m, 4.5–5.1 m, 7.1–7.3 m, 9.3–9.5 m) than at the base or the upper reaches of the trees (Fig. 4).

3.3 Number of eggs per mass

The number of eggs or larvae counted per mass ranged from 5 to 28, with an average of 16.1 ± 0.4 eggs per mass. The number of eggs per mass did not vary significantly with height above the ground ($F_{1,102} = 1.67$, $P = 0.2$).

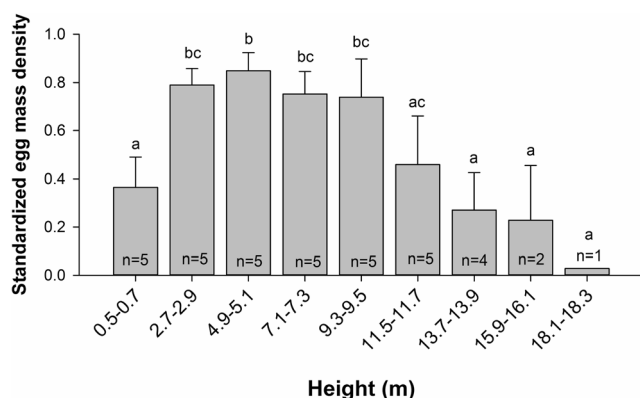


Fig. 4 Mean \pm SE standardized *Agrilus macer* egg mass density by height as measured from five trees. Bars with different letters above them are statistically significant based on differences of least square means

3.4 Observations of *A. macer* egg-laying behavior

Almost the entire sequence of egg-laying behavior was observed for three *A. macer* females in June 2016. The beetles lay their eggs on the smooth areas of bark of a declining tree, avoiding the natural warty bumps commonly found on sugarberry bark. A bright green substance, which is presumably leaf material since adults were observed feeding on sugarberry leaves, is defecated on top of the egg mass before the female begins applying a tan liquid as a final layer. This latter material is added with quick circular movements of the abdomen and quickly dries into the protective cap characteristic of the egg mass (Fig. 3).

3.5 Adult activity period

In our 2016 trapping effort, a total of 69 *A. macer* specimens were collected over the 16-week sampling period, with 40 and 29 being captured in the intercept and Lindgren funnel traps, respectively. The first specimen was not captured until June, and the largest number of specimens was collected between 15 and 28 June, which is a common activity time for other *Agrilus* species in the southeastern USA (Burke 1917; Klingeman et al. 2015; Nord et al. 1965). A single specimen was collected during our last sample period, 23 August to 9 September (Fig. 5).

3.6 *Agrilus macer* distribution

A total of 2032 specimens with applicable records and data were obtained from the collections listed in Acknowledgements. Although we received records of *A. macer* from nine southern US states, ranging from California in the west to South Carolina in the east (Fig. 6), almost all specimens (98%) were collected in Louisiana (1126) or Texas (868) (Fig. 7). Texas had the highest number of county records (30), followed by Louisiana (3 parishes).

South Carolina had the next most records, although all but a single specimen from 1934 are recent specimens from our study area. There were five specimens each from Florida and Mississippi, whereas the other five states were each represented by just a single specimen. The specimen from Georgia was a recent record from near our study area.

3.7 Oviposition behavior of selected *Agrilus* species

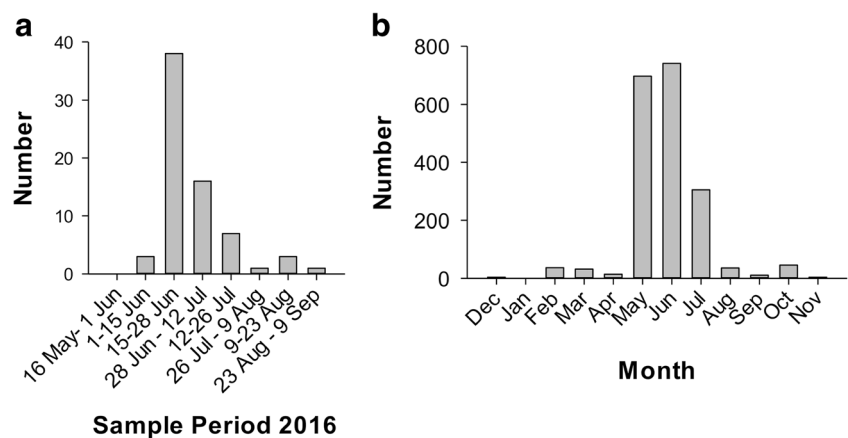
Of the 25 *Agrilus* species for which egg-laying biology was found, 12 species lay eggs singly while the remaining species are known to sometimes or always lay clusters of eggs (Table 2). *Agrilus macer* appears to lay the largest number of eggs per cluster on average, but other species, such as *A. biguttatus* Fabricius and *A. politus* Say, are also known to lay large clusters of eggs. Six (30%) of the 25 *Agrilus* species apply a secretion to the eggs, in addition to any secretion used to attach the eggs to the oviposition site (Table 2). While these are transparent secretions in most cases, the brown covering prepared by *A. politus* females is very similar to that produced by *A. macer*. Although *A. macer* is the only species known to deposit a layer of green substance over the eggs before smearing the final protective covering on the eggs, Dutt (1969) reported that female *A. acutus* Thunberg frequently deposit a “greenish-black elliptical fecal pellet” on the egg along with a fluid discharge.

3.8 Fungal isolations and pathogenicity testing

Three fungi isolated from the discolored areas around *A. macer* attack points and subsequently used in pathogenicity tests were identified as *Clonostachys rosea*, *Fusarium neocosmosporiellum*, and *Phialemonium dimorphosporum*. *Clonostachys rosea* was isolated from all sampled stem sections at the Lake Olmstead Park. This fungus was also isolated and identified from various stem and root samples from seven additional symptomatic trees along the Greenway. *Fusarium neocosmosporiellum* and *P. dimorphosporum* were isolated from eight trees heavily attacked by the *A. macer* along the Greenway. *F. neocosmosporiellum* was also confirmed from one of the Lake Olmstead Park trees.

Clonostachys rosea, *F. neocosmosporiellum*, and *P. dimorphosporum* were 99%, 98%, and 99% similar to GenBank accession numbers EU552110.1 (*C. rosea*), KM231803.1 (*F. neocosmosporiellum*), and KX881590.1 (*P. dimorphosporum*), respectively. No dieback or leaf yellowing was observed in saplings of any inoculation treatment. Zones of discoloration were observed above and below the point of inoculation for fungal treatments and in the control treatment in both inoculation tests. The discolored area appeared as a fine black streak, which emanated from the inoculation point and was never more than a few millimeters in width. In test 1, the length of the discolored sapwood in

Fig. 5 Total number of *Agrilus macer* collected by sampling period in North Augusta (a) and by month based on museum records (b)



saplings inoculated with *F. neocosmosporiellum* (18.2 ± 1.2 mm) was significantly greater than saplings in the control treatment (8.97 ± 1.1 mm; $P = 0.0038$); however, the length of the discoloration in saplings inoculated with *P. dimorphosporum* (19.5 ± 4.7 mm) did not differ from control saplings ($P = 0.2406$). In test 2, the length of the discoloration in saplings inoculated with *C. rosea* (19.3 ± 3.1 mm) did not differ significantly from the discoloration length in the control saplings (12.3 ± 0.77 mm; $P = 0.1093$).

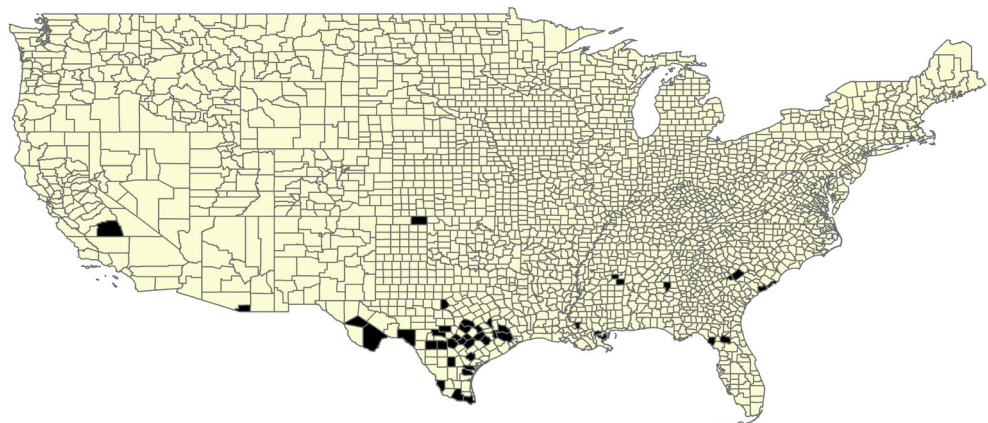
4 Discussion

To our knowledge, the density of eggs laid by *A. macer* on the trunks and stems of dying sugarberry exceed that reported for any other species of *Agrilus*. At the highest densities observed in this study, there were about 1.2 egg masses for every 10 cm² of bark, which is equivalent to about two eggs per square centimeter. These numbers are especially surprising considering there was only a single record of *A. macer* from South Carolina from 1934, and there were no records of the species being in neighboring Georgia before the current episode of sugarberry mortality. From the information provided by collection specimens, *A. macer* appears to be

widely distributed throughout the southern USA but has been most often collected in Texas and Louisiana. Specimens collected outside of sugarberry's native range were most likely found as a result of the presence of other *Celtis* species, as larvae have been previously recorded in *C. occidentalis* and *C. tenuifolia* (Harpootlian and Bellamy 2014). The abundance of stressed and dying host trees has clearly resulted in an unusual population increase for our study area.

Compared to most other members of the genus that have been studied, the egg masses laid by *A. macer* are also unusual in terms of the number of eggs laid per mass. Although there are approximately 3000 species of *Agrilus* worldwide, descriptive observations of the oviposition behavior and egg-laying techniques are not often readily available for many species (Jendek 2016). Based on the available literature, about half of *Agrilus* species lay single eggs, whereas the rest are known to lay clusters of 2–30 eggs with typically few eggs per cluster. *A. macer* has the largest known average number of eggs per cluster with 16 eggs per mass. Most *Agrilus* species prefer to lay eggs within crevices on the host plant, but *A. macer* will attach egg clusters to smooth surfaces of bark. The eggs are then concealed with a tan secretion that hardens into a covering. The substance used to create this covering

Fig. 6 Known distribution of *Agrilus macer* based on museum specimens



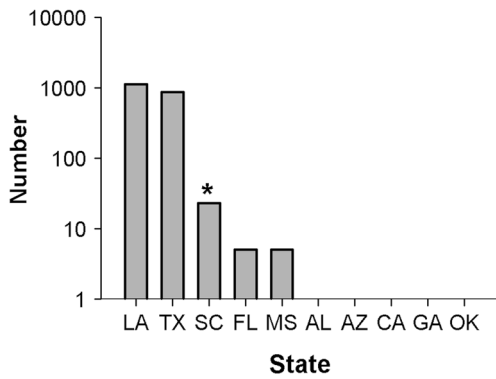


Fig. 7 Total number of *Agrilus macer* collected by state based on museum specimens. At least one specimen was collected from each state listed. Only one specimen was collected from South Carolina prior to this study

possibly originates from the accessory (colleterial) gland, considering that secretions from this gland are commonly used by insects to aid in egg adhesion (Hilker and Meiners 2002; Klowden 2013; Li et al. 2008). The cap presumably provides protection from environmental factors and natural enemies (Desurmont and Weston 2011), and we noticed ants readily attack masses with damaged caps.

Although not common among members of the genus, other species are also known to produce similar capped masses of eggs. The species with egg-laying behavior most similar to *A. macer* appears to be *A. politus*, which attacks willow and maple. Like *A. macer*, *A. politus* lays groups of eggs covered with a substance that hardens and creates a brown cap on the smooth bark of trunks and branches. While the advantage of producing such egg masses remains unknown, one possibility is attacking en masse helps to locally overcome host plant defenses. Gregarious larval feeding is not a common *Agrilus* mechanism but is noted in some *Dendroctonus* species as a behavior that increases the larval growth rate (Storer et al. 1997). Duan et al. (2010) showed host tree defenses, especially the formation of callus tissue, were the most important mortality factors for *A. planipennis* larvae. Furthermore, similar callus tissue has been observed as a response against *A. biguttatus* and *A. auroguttatus* egg masses and larval feeding on oak (Brown et al. 2015; Coleman and Seybold 2008). It is clear from our observations that trees are sometimes able to overcome colonization attempts, and attacks from *A. macer* do not guarantee the tree will soon die. The formation of callus tissue seems to be an important mechanism by which trees overcome attacks by *A. macer* (Fig. 1d, e), and this may have selected for the production of large egg masses by the species. To our knowledge, the green material we observed female *A. macer* adding on top of eggs has never been reported before, although the observation of *A. acutus* depositing a fecal pellet on top of individual

eggs is similar (Dutt 1969). This material presumably consists of leaf material ingested by the female, but it remains unclear how this addition benefits the eggs or first instar larvae. It is possible that this material is simply voided before the tan secretion is expelled, as it is common for *Agrilus* species to feed on foliage of the host plant (Burke 1917; Petrice et al. 2009).

Based on our trapping effort in North Augusta, SC, the adult activity of *A. macer* was highest the week of 15–28 June in North Augusta. Similar to our observations, museum specimen labels indicate the species can be captured in low numbers in the warmer months of the year but is most abundant in May, June, and July.

The necrotic zones surrounding *A. macer* oviposition sites and associated streaks of discoloration in the xylem could be related to *F. neocosmosporiellum*, which is the only fungus to provide a significant increase in xylem discoloration following wounding and inoculation. *Fusarium neocosmosporiellum* is a pathogen in agricultural crops (Cheng and Schenck 1978; Smith 1899); however, our test is the first indication that *F. neocosmosporiellum* could be weakly pathogenic to a tree species. Other fungi such as *P. dimorphosporium* and *C. rosea* were also associated with the discolored sapwood following *A. macer* oviposition, and these fungi may also elicit a host response in sugarberry. Although the mean sapwood discoloration lengths in saplings inoculated with *P. dimorphosporium* and *C. rosea* did not differ from controls, the variation in the lengths following inoculation with these fungi was considerably greater than that observed in controls. Furthermore, *P. dimorphosporium* was reisolated from only two of four inoculated stems, and bacteria and yeasts predominated in stems from which *P. dimorphosporium* was not successfully reisolated. The lengths of discoloration in stems from which *P. dimorphosporium* was reisolated were greater than 24 mm, thus suggesting that colonization by other organisms could have limited the ability of *P. dimorphosporium* to colonize host tissue in some inoculated saplings. *Phialemonium dimorphosporium* has been previously associated with bark beetle galleries and sapwood discoloration in conifer logs (Hutchison and Reid 1988) and is capable of causing soft rot in wood (Zabel et al. 1991). *Clonostachys rosea* is a cosmopolitan fungus frequently found in soil and on plant debris (Farr et al. 1990) and has been thought to be important as a biocontrol agent of plant diseases (Papavizas 1985), a mycoparasite (Jensen et al. 2000; Jensen et al. 2004), and a plant pathogen (Afshari and Hemmati 2017; Bienapfl et al. 2012). Nonetheless, none of the fungi that we isolated from discolored areas of affected sugarberries and subsequently tested in controlled inoculations had a significant effect on tree growth and health, and a possible role of these fungi in the current decline of sugarberry is doubtful.

Table 2 Oviposition biology of selected *Agrilus* species

	Host(s)	Number of eggs per group	Egg distribution	Generation per year	Protective egg covering	Covering description	Source
<i>A. acutus</i> (Thunberg, 1787)	<i>Hibiscus</i>	1	Stems near a leaf scar	2	Yes, although, it is not permanent, the covering typically falls off after several days	Dark green to black fecal pellet on the egg and an additional fluid is deposited	Dutt (1969)
<i>A. angelicus</i> Horn, 1891	<i>Quercus</i>	1	Rough bark	0.5	Yes	Eggs on bark are difficult to see due to the varnish-like covering that catches dust	Solomon (1995)
<i>A. anxius</i> Gory, 1841	<i>Betula</i>	1 or in clusters up to 14	In bark crevices beneath loose bark	0.5 to 1	No		Nord et al. (1965), Solomon (1995), Muilenburg and Herms (2012)
<i>A. biguttatus</i> (Fabricius, 1776)	<i>Quercus</i>	1 to 30	Crevices of bark	0.5–1	Yes	Egg firmly glued to smooth bark; transparent secretion covers eggs and resemble scale insects	Brown et al. (2015), Reed et al. (2018)
<i>A. bilineatus</i> (Weber, 1801)	<i>Castanea, Quercus</i>	1 to 10	In rough bark crevices	0.5 to 1 from	No		Cote and Allen (1980), Haack and Benjamin (1982), Chapman (1915), Solomon (1995)
<i>A. burkei</i> Fisher, 1917	<i>Alnus</i>	3 to 10	Bark of branches and trunks of moderately to severely stressed trees	1	Yes	Similar to that of <i>A. politus</i>	Solomon (1995), Nelson and Hespenheide (1998)
<i>A. auroguttatus</i> Schaeffer, 1905	<i>Quercus</i>	1 or in clusters	Crevices of bark on large branches and main trunk	0.5	No		Coleman and Seybold (2008), Coleman and Seybold (2016)
<i>A. cuprescens</i> (Ménétries, 1832)	<i>Rosa, Rubus</i>	1	Bark of canes near the ground	0.5 to 1	No		Solomon (1995)
<i>A. difficilis</i> Gory, 1841	<i>Gleditsia</i>	1 to 8	Bark of stressed trees	1	No		Solomon (1995)
<i>A. fleischeri</i> Obenberger, 1925	<i>Populus, Salix</i>	1 or in clusters	Bark crevices and beneath bark pieces	1	No		Zang et al. (2017)
<i>A. granulatus</i> liragus Barter and Brown 1949	<i>Populus, Salix</i>	5 to 8	Bark crevices	0.5 to 1	No		Burke (1917), Solomon (1995)
<i>A. horni</i> Kerremans, 1900	<i>Populus</i>	1 or in small clusters	Base of host plant on smooth bark surface of small stems near the ground	0.5	No		Nord et al. (1965), Solomon (1995)
<i>A. hyperici</i> (Creutzer, 1799)	<i>Hypericum</i>	1	In stems near the ground	1	No		Paynter (2012)
<i>A. liragus</i> Barter and Brown, 1949	<i>Populus</i>	Clusters of 5–8, but up to 18	Bark crevices	0.5–1	No		Barter (1965)

Table 2 (continued)

Host(s)	Number of eggs per group	Egg distribution	Generation per year	Protective egg covering	Covering description	Source
<i>A. macer</i> LeConte, 1858	<i>Celtis</i>	Cluster average 16, up to 28	1	Yes	Green substance is secreted over the egg clusters then a tan substance is smoothed over the eggs as a cap.	This study, Harpootlian and Bellamy (2014)
<i>A. pensus</i> Hom, 1891	<i>Alnus, Betula</i>	1	0.5	No		Solomon (1995)
<i>A. planipennis</i> Fairmaire, 1888	<i>Fraxinus</i>	1 or in small clusters	0.5 to 1	No		Bauer et al. (2014)
<i>A. politus</i> (Say, 1825)	<i>Salix, Acer</i>	1 to 12	0.5 to 1	Yes	Eggs covered with a secretion that hardens into a protective brown capsule. The visible covered eggs appear scale-like on branches	Burke (1917), Solomon (1995)
<i>A. quadriguttatus niveiventris</i> Hom, 1891	<i>Populus, Salix</i>	1		No		Burke (1917)
<i>A. ruficollis</i> (Fabricius, 1787)	<i>Rubus</i>	1	1	No		Solomon (1995)
<i>A. sayi</i> Saunders, 1870	<i>Myrica, Comptonia, Quercus, Populus</i>	1	0.5	No		Solomon (1995)
<i>A. sinuatus</i> (Olivier, 1790)	<i>Pyrus, Amelanchier, Crataegus, Cotoneaster, Quince</i>	1	0.5	No		Solomon (1995)
<i>A. subcinctus</i> Gory, 1841	<i>Fraxinus</i>	1	0.5	No		Petrice et al. (2009)
<i>A. sulcicollis</i> Lacordaire, 1835	<i>Quercus</i>	1	0.5 to 1	No		Haack et al. (2009)
<i>A. viridis</i> Linnaeus, 1758	<i>Rosa, Fagus, Alnus, Betula, Quercus</i>	1	0.5	No		Hellmut (1956)

* Data from literature sources provided

5 Conclusion

Although largely concentrated in Texas and Louisiana, *A. macer* appears to be widespread throughout the southern USA from California in the west to South Carolina in the east. Before the current episode of sugarberry mortality, *A. macer* was unknown from Georgia and had not been collected in South Carolina since 1934. This is in striking contrast to the incredible density of *A. macer* egg masses observed on weakened sugarberry in the current study. The rarity of *A. macer* in collections is not too surprising considering that methods for collecting buprestids are poorly developed. Indeed, it is clear from observations of buprestids captured by foraging *Cerceris* (Crabronidae) wasps that many buprestid species active in an area often go undetected by human collectors (Swink et al. 2013). It is therefore probable that *A. macer* is considerably more abundant and widespread than existing collection records indicate. Support for this comes from a recent *Cerceris*-based survey in Louisiana which found *A. macer* to be the most abundant buprestid species collected by the wasps (Johnson et al. 2015).

Because new and old egg masses, D-shaped exit holes, weeping wounds, and callused areas from previous attacks by *A. macer* are not obvious on all dying sugarberry in our study area (MDU and EMP, personal observations), there is no reason to believe that *A. macer* is the primary cause of mortality. This conclusion is supported by the fact relatively healthy trees are able to overcome attacks from the beetle and are generally avoided, as well as there being no evidence the beetles transmit a highly pathogenic fungus. The sapwood discoloration associated with beetle galleries is probably caused by the introduction of a weakly pathogenic fungus that causes a very limited host response, but these fungi do not appear to directly affect tree health.

It remains unknown what is responsible for the dying sugarberry in Georgia and South Carolina, and research into this question continues. While we do not believe *A. macer* plays a primary role in this mortality, the species does appear to act as an important contributing factor, and Chittenden (1900) reported the species to be “very injurious” to *Celtis* in Texas. The observation that some trees are able to overcome attacks by *A. macer* and can then live for years thereafter suggests that attacks by this beetle are not limited to extremely weakened trees (MDU personal observation). Moreover, by attacking at such high densities, *A. macer* may hasten the death of many trees, possibly preventing recovery in some cases.

Acknowledgements We thank Roy Kibler for permission to work along the Greenway in North Augusta. We are also grateful to the following individuals for responding to our request for specimen information: Lee Herman (American Museum of Natural History), Melissa Callahan (Auburn University Natural History Museum), Jacqueline Airoso (California State Collection of Arthropods), Patrice Bouchard and Anthony Davies (Canadian National Collection of Insects, Arachnids,

and Nematodes), John Rawlings and Robert Androw (Carnegie Museum of Natural History Invertebrate Collection), John Morse and Mike Ferro (Clemson University Arthropod Collection), Christopher Grinter (Entomology Collection of the California Academy of Sciences), Jim Louderman and Crystal Maier (Field Museum of Natural History Collection of Insects, Arachnids, and Myriapods and InvertEbase, NSF Award EF 14-02667), Rick Hoebeke (Georgia Natural History Museum), Philip Perkins (Harvard Museum of Comparative Zoology), Tommy McElrath (Illinois Natural History Survey Insect Collection), Louisiana State Arthropod Museum, Gary Parsons and Anthony Cognato (Albert J. Cook Arthropod Research Collection at Michigan State), Terence Lee Schiefer (Mississippi Entomological Museum), Weiping Xie and Brian Brown (Natural History Museum of Los Angeles County), Hellen Vessels (New Mexico State University Arthropod Collection), Bob Blinn (North Carolina State University Insect Collection), Luciana Musetti (Ohio State Triplehorn Insect Collection), Phil Mulder and Jana Slaughter (Oklahoma State-K.C. Emerson Entomology Museum), Robert Androw Private Collection, Charyn J. Micheli and Eugenio H. Nearn (Smithsonian National Museum of Natural History), Karen Wright (Texas A&M Insect Collection), Texas Tech Insect Collection, Wendy Moore and Wesley E. Hall (University of Arizona), Michael S. Engel (University of Kansas Natural History Museum), and Stylianos Chatzimanolis (University of Tennessee Natural History Museum). Finally, we thank three anonymous reviewers for comments that greatly improved the manuscript.

Funding This research was funded by the USDA Forest Service, Southern Research Station including a grant from Forest Health Protection’s Evaluation Monitoring program (SO-EM-17-04).

Data availability Datasets analyzed during the current study are available from the corresponding author upon request.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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