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Authors: Formby, John P., Krishnan, Natraj, and Riggins, John J.

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## SUPERCOOLING IN THE REDBAY AMBROSIA BEETLE (COLEOPTERA: CURCULIONIDAE)

JOHN P. FORMBY\*, NATRAJ KRISHNAN AND JOHN J. RIGGINS

Department of Biochemistry, Molecular Biology, Entomology and Plant Pathology, Mississippi State University,  
Box 9775, Mississippi State, MS 39762-9775, USA

\*Corresponding Author; E-mail: jpf9@msstate.edu

### ABSTRACT

The redbay ambrosia beetle, *Xyleborus glabratus* Eichoff, (Coleoptera: Curculionidae: Scolytinae) is a severe pest of North American trees and shrubs in the family Lauraceae. Supercooling point (SCP) is an important physiological baseline for cold tolerance studies and could provide useful insights into the invasive potential of *X. glabratus* in northern latitudes of North America. The supercooling point (SCP) of *X. glabratus* was experimentally determined on field-collected and artificially cold hardened specimens. Field-collected beetles were captured in Jackson County, Mississippi using Lindgren funnel traps baited with manuka oil lures. Testing was conducted from June through August 2011. The mean SCP for field-collected *X. glabratus* was  $-21.7 \pm 0.5$  °C ( $\pm$  SE). A significant negative trend in the SCPs of field-collected beetles occurred over the summer testing period. *Xyleborus glabratus* specimens were reared from redbay (*Persea borbonia* (L.) Sprengel bolts in June 2012 and artificially cold hardened in a low temperature incubator at a thermo-photoperiod of 7 °C:2 °C (10:14 h L:D) for 31 days. Artificially cold hardened *X. glabratus* supercooled to a mean temperature of  $-23.9 \pm 0.4$  °C ( $\pm$  SE), which was significantly lower than that of field-collected beetles. Biometric indices of beetles (size, weight, and size x weight interaction) had no effect on the mean supercooling SCPs of either field-collected or artificially cold hardened beetles. Results from environmentally conditioned beetles suggest that *X. glabratus* has a high degree of thermal plasticity. Based on the artificially cold hardened mean SCP, *X. glabratus* and laurel wilt disease have the possibility to impact sassafras and northern spicebush throughout eastern North America. The data, although preliminary, suggests that a previous spatio-temporal model based on climate match data may have substantially underestimated the geographical area that may be affected by *X. glabratus*. This study will help form the basis of building and validating models to better predict the North American invasion potential of *X. glabratus*.

Key Words: cold tolerance, invasion potential, laurel wilt, supercooling point, *Xyleborus glabratus*

### RESUMEN

El escarabajo ambrosia, *Xyleborus glabratus* Eichoff, (Coleoptera: Curculionidae: Scolytinae) es una plaga severa de árboles y arbustos de la familia Lauraceae en Norteamérica. El punto del sobre-enfriamiento (PSF) es una línea de base fisiológica importante para los estudios de tolerancia al frío y puede proveer una pista relevante del potencial de invasión de *X. glabratus* en Norteamérica. Por lo tanto, se determinó el punto de sobre-enfriamiento (PSF) de *X. glabratus* experimentalmente utilizando especímenes recolectados en el campo y endurecidos por el frío artificialmente. Escarabajos recogidos en el campo fueron capturados en el condado de Jackson, Mississippi utilizando trampas de embudo Lindgren cebados con un señuelo de aceite de manuka. Se realizó la prueba entre junio y agosto del 2011. El promedio del PSF para los *X. glabratus* recolectados de campo fue de  $-21.7 \pm 0.5$  °C ( $\pm$  SE). Una tendencia negativa significativa en el PSF de los escarabajos recolectados en el campo sucedió durante en el transcurso del período de prueba el verano de 2011, lo que posiblemente indica un aumento en la tolerancia al frío. Especímenes del *Xyleborus glabratus* fueron criados sobre pernos del laurel rojo (*Persea borbonia* (L.) Sprengel en junio 2012 y artificialmente endurecidos por al frío en una incubadora de baja temperatura a un termofotoperiodo de 7 °C: 2 °C (10:14 h L:D) por 31 días. El PSF de los *X. glabratus* artificialmente endurecidos por el frío y sobre-enfriados a un promedio de temperatura de  $-23.9 \pm 0.4$  °C ( $\pm$  SE) fue significativamente más bajo que el de los escarabajos recogidos en el campo. El tamaño y el peso de los escarabajos no tuvo ningún efecto sobre el promedio del punto de sobre-enfriamiento (PSF) de los escarabajos recolectados en el campo o los endurecidos artificialmente por el frío. Los resultados sugieren que *X. glabratus* es capaz de ajustarse rápidamente a bajas temperaturas, independientemente de la exposición previa, o sea los endurecidos por el frío rápidamente. Basado sobre el promedio de PSF, *X. glabratus* y la

marchitez del laurel tienen la posibilidad de impactar los sasafrás por todo la región este de América del Norte. Esto sugiere que un modelo espacio-temporal hecho anteriormente basado en los datos de compatibilidad del clima puede haber subestimado sustancialmente el área geográfica que puede ser afectada por *X. glabratus*. Este estudio ayudará a formar una base para construir y validar modelos para predecir mejor el potencial de invasión de *X. glabratus* en Norteamérica.

Palabras Clave: tolerancia al frío, potencial de invasión, marchitez del laurel, punto de sobre-enfriamiento, *Xyleborus glabratus*

Laurel wilt disease (LWD) is a non-native vascular wilt of North American trees and shrubs in the Lauraceae family. Laurel wilt is initiated by the wood-boring redbay ambrosia beetle, *Xyleborus glabratus* Eichoff, a beetle endemic to parts of Asia (Taiwan, Japan, Myanmar, India, and Bangladesh) (Rabaglia et al. 2006). The pathogen, *Raffaelea lauricola* T. C. Harrington, Aghayeva, and Fraedrich, is a newly described fungal symbiont of *X. glabratus* and causes lethal vascular wilt in redbay (*Persea borbonia* (L.) Sprengel), swampbay (*Persea palustris* (Rafinesque) Sargent), sassafras (*Sassafras albidum* (Nuttall) Nees), northern spicebush (*Lindera benzoin* (L.) Blume), and the commercially important avocado (*Persea americana* Miller) among other North American lauraceous species (Fraedrich et al. 2008; Mayfield et al. 2008a).

Endemic ambrosia beetles and their fungal symbionts are an essential part of hardwood forest succession, as the beetles seek out stressed or dying trees and the fungal symbionts play an important role in decomposition of wood (Wood & Bright 1992). Ambrosia beetles bore into the heartwood of trees and create brood galleries, where they inoculate species-specific symbiotic fungi into the woody vascular tissue (Batra 1963; Francke-Grosmann 1967). Ambrosia beetles do not consume the wood, but rather cultivate their symbiotic fungi (i.e. ambrosia) as food for both the adults and larvae (Batra 1967; Kühnholz et al. 2001). Usually these fungal symbionts are not the primary causal agents of tree mortality (Atkinson & Peck 1994; Mayfield & Thomas 2006; Mayfield 2007); however, *X. glabratus* carries the only known ambrosia beetle symbiont to cause a lethal vascular wilt disease (Fraedrich et al. 2008; Harrington et al. 2008). Tree mortality can follow initial fungal inoculation in as little as 4 weeks (Mayfield et al. 2008a).

Since initial detection of *X. glabratus* near Savannah, Georgia, the beetles have spread inland and along the coast into the Carolinas and Florida (Cameron et al. 2008). In Duval County, Florida redbay mortality increased to 92% of mature trees within 2 yr of initial beetle infestations (Mayfield et al. 2008b). As *X. glabratus* moves outside the range of redbay and swampbay, LWD has caused extensive sassafras mortality (Fraedrich et al. 2008; Smith et al. 2009; Riggins et

al. 2011; Bates et al. 2013). Disjunct populations are established in coastal counties of Mississippi and Alabama (Riggins et al. 2010; Formby et al. 2012), and in Marengo County, an interior county of Alabama (Bates et al. 2013). Marengo County, Alabama is ~200 km from the nearest infestation site and exceeds a previous temporal estimation of invasion by 12 yr (Koch & Smith 2008). Currently, Marengo County is the only documented site where no redbay occurs and sassafras is the lone lauraceous species affected by laurel wilt. In 2012, more than 20 sassafras trees were reported in various stages of wilt, up from 1 tree the previous year (Bates et al. 2013). Laurel wilt disease has not been documented infecting northern spicebush in the natural environment, but laboratory inoculation trials have shown it to be susceptible to the pathogen (Fraedrich et al. 2006). Distribution of sassafras and northern spicebush is limited to the eastern/east-central United States and southern Ontario, Canada. The susceptibility of these 2 species to LWD offers a potential avenue for the beetle and disease to spread throughout the central and northern forests of the eastern United States. Removal of sassafras and northern spicebush from the environment could have ecological consequences impacting a variety of wildlife species.

Control measures, such as insecticides or sanitation cutting, have been unable to slow the spread of the beetle and mortality from cold exposure may be the only realistic factor for limiting the expansion range of *X. glabratus*. Determination of the supercooling point (SCP) is an essential starting point for physiological investigations and limitations of cold tolerance in any insect (Salt 1961; Bale & Walters 2001; Bale 2002; Renault et al. 2002). Several other scolytine beetles have been tested for SCP in order to determine distribution limits. Ungerer et al. (1999) utilized SCP to formulate the models used for northern distribution limits of *Dendroctonus frontalis* Zimmerman and Régnière & Bentz (2007) applied the SCP of *D. ponderosae* Hopkins to model the distribution of their population as a function of daily changes in the temperature.

A variety of insects, such as scolytine bark beetles, are freeze susceptible (Ring 1977; Gehrken 1984; Miller & Werner 1987; Bentz & Mullins 1999; Lombardero et al. 2000) and initiate

behavioral and physiological processes, such as supercooling, to avoid freezing (Bale 1996). Supercooling is a form of protection against low temperatures in which insects lower the freezing points of their body fluids to avoid internal ice formation (Salt 1953; Bale 1987; Lee 1991; Lee et al. 1993; Carrillo et al. 2005). In freeze susceptible insects, the supercooling point (SCP) is defined as the temperature at which supercooling no longer assists in protection and spontaneous crystallization of the hemolymph and other body fluids occur. In some freeze susceptible insects, SCP is used as the measure of maximum cold hardiness (Lee 1989, 1991), i.e., low lethal temperature (Salt 1961; Zachariassen 1985; Hodkova & Hodek 1994; Lombardero et al. 2000; Carrillo et al. 2005; Tran et al. 2007); however, death or irreparable injury may also occur at temperatures above this point as a result of cold shock or injury (Baust & Rojas 1985; Lee & Denlinger 1985; Knight et al. 1986; Lee 1991; Sinclair 1999; Bale 2002; Renault et al. 2002). When an insect reaches the SCP, a latent spike of heat energy (exotherm) is released, which is detectable by surface thermocouple thermometry (Bale 1987). The lowest temperature reached before the sudden spike in temperature is recorded as the SCP (Lee 1989; Lee 1991; Koch et al. 2004).

There are very limited data on the developmental stage(s) utilized by overwintering ambrosia beetles, but the overwintering stage(s) is constant and distinctive in each species (Wood 1982). For example, Weber & McPherson (1983) discovered that the ambrosia beetle, *Xylosandrus germanus* (Blandford), overwinters in the adult stage. There are reports from South Carolina and Georgia of host seeking *X. glabratus* females flying during winter months (Hanula et al. 2008), and in Georgia, Maner et al. (2013) found adult females emerging in every month of the year, suggesting overlapping generations and the presence of all life stages throughout the winter. Furthermore, Wood (1982) gives examples of scolytine adults surviving the winter and participating in production of spring brood. Many ambrosia beetles, such as *X. glabratus* are parthenogenic, thus if 1 female survives the winter to reproduce, a new area can be readily colonized (Wood 1982). Therefore, due to the empirical and observational evidence described above, this study uses adult females as a proxy for SCP determinations.

The present study is the first to investigate SCP or any aspect of cold tolerance in ambrosia beetles, probably because of the benign role most play in their native environment. However, global trade and commerce is increasing the risk for non-native ambrosia beetle introductions and the spread of their potentially pathogenic fungal symbionts. Therefore, the purpose of this study was to experimentally determine the mean SCPs of field-collected and cold hardened *X. glabratus*.

Comparing summer collected and artificially cold hardened beetles will elucidate any differences between the 2 physiological states and the degree, if any, of thermal plasticity to low temperatures by *X. glabratus*. Determining the SCP of *X. glabratus* will also help to describe its overwintering strategy, increase the understanding of cold tolerance in ambrosia beetles, and form the basis of building and validating models to better predict the invasion potential and ecological impacts of LWD in North America.

## MATERIALS AND METHODS

### Field-Collected *Xyleborus glabratus*

Female *X. glabratus* were captured using 12-unit Lindgren funnel traps (Lindgren 1983) with dry cups during the summer of 2011 in Jackson County, Mississippi. Eleven funnel traps baited with manuka oil lures (Synergy Semiochemical Corp., Burnaby, British Columbia, Canada) (Hanula & Sullivan 2008) were placed in slash pine dominant stands with a symptomatic red-bay understory. Traps were checked once daily between 09:00 am and 11:00 am from 22 Jun to 11 Aug 2011 for a total of 50 trapping sessions. Many of the daily trapping sessions resulted in zero captures (Fig. 1). Mean monthly (Jun, Jul, and Aug 2011) high/low atmospheric conditions are also available in Fig. 1 (NOAA, NCDC). No males were captured or tested because they are flightless and rarely leave the natal tree. All living and apparently healthy *X. glabratus* were transported to the laboratory immediately following daily trap checks. Due to the commute from trapping locations to the laboratory, supercooling point experiments were conducted within 2 h of trap collection. In total, 48 live *X. glabratus* female beetles were captured and transported to the laboratory for testing.

### Artificially Cold Hardened *Xyleborus glabratus*

Female *X. glabratus* were reared from symptomatic redbay bolts in Jun 2012. All symptomatic redbay bolts were obtained from the same field locations as the 2011 study. Approximately 300 beetles were collected from rearing cans on 14 Jul 2012. Beetles were placed in a low temperature incubator (VWR International, Model 2015, Radnor, Pennsylvania) for 31 days with a thermo-photoperiod of 7 °C:2 °C (10:14 h L:D). This thermo-photoperiod was chosen to correspond to a cycle found during winter months near the northerly limits of sassafras. Initially, female *X. glabratus* were introduced into a 20 °C incubator. Temperature was lowered to 7 °C at a rate of 1.0 °C/day. When 7 °C was reached (day 13), the thermo-photoperiod cycle was initiated. At 31 days, 100 beetles were removed from the



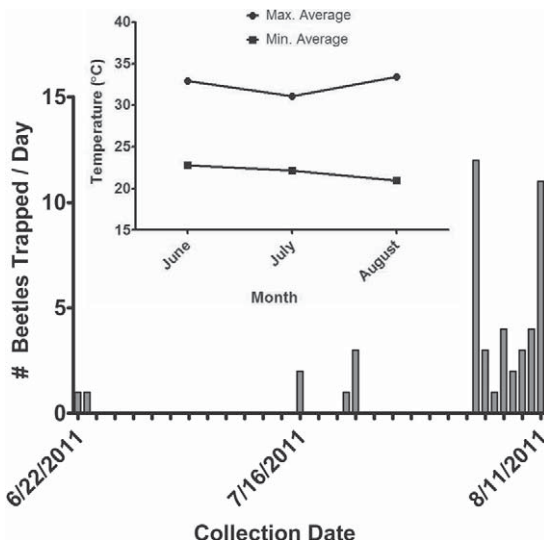


Fig. 1. Frequency of field-collected *Xyleborus glabratus* females collected during the summer of 2011 in Jackson County, Mississippi. The inset graph depicts maximum and minimum average air temperatures encountered by host seeking *Xyleborus glabratus* females during the summer of 2011 study (Jun-Aug). There were a total of 50 trapping sessions with a majority of the sessions resulting in zero healthy *X. glabratus* captures. Female *X. glabratus* were captured using Lindgren funnel traps in symptomatic redbay stands. Beetles were transported to the lab and tested within 2 h of capture.

incubator, allowed to warm to ambient temperatures ( $\sim 21^\circ\text{C}$ ) for 2 h to monitor for survival status (dead, limited locomotion, or vigorous). Only beetles that were deemed vigorous were used for SCP determination. In all, 56 vigorous and apparently healthy *X. glabratus* were artificially cold hardened and used for SCP determination.

#### Determination of Supercooling Point

Supercooling point determination of both field-collected ( $n = 48$ ) and artificially cold hardened ( $n = 56$ ) beetles was conducted in the biology laboratory of Grand Bay National Estuarine Research Reserve in Jackson County, Mississippi (N  $30.4297^\circ$  W  $-88.4279^\circ$ ; 2 m asl). Laboratory SCP testing consisted of inserting an individual *X. glabratus* into a microcentrifuge tube and a thermocouple was placed against the beetle cuticle to monitor for the latent heat release. The beetle and thermocouple were held in place by a foam stopper. The microcentrifuge tube was inserted into a microcentrifuge float and placed in a PolyScience® programmable liquid bath containing Dynalene® HC 50 fluid (Andreadis et al. 2005; Ansart et al. 2007). Thermocouples were connected to a Pico Technology® USB TC-08 data

logger, which allowed 8 replicates to run simultaneously. Real-time temperature measurements were recorded at 1 s intervals via software generated spreadsheet and graph (Pico Technology®, PicoLog). The lowest temperature reached before the exothermic reaction was recorded as the SCP (Lee 1989; Kořtal & Šimek 1996). Following SCP determinations, beetles were placed into individual microcentrifuge tubes and monitored for survival for 24 h at  $\sim 21^\circ\text{C}$ .

Tropical beetle species can exhibit extremely low SCPs (many as low as  $-30.0^\circ\text{C}$ ) (Fields 1992; Nedved & Windsor 1994). With this information and the lack of biological data for *X. glabratus* and other ambrosia beetle species, a target low temperature of  $-30.0^\circ\text{C}$  was selected. Preliminary bath temperature was held at a steady ambient room temperature ( $\sim 21.0^\circ\text{C}$ ). Salt (1966) and Hahn et al. (2008) reported that rate of temperature adjustment has little effect on SCP ( $1.0$ – $2.0^\circ\text{C}$ ), but urged the use of a consistent rate. One of the most commonly used rates observed in the literature is  $1.0^\circ\text{C}/\text{min}$  (Salt 1966; Carrillo et al. 2005; andreadis et al. 2005; Hiiesaar et al. 2011). Based on these precedents, bath temperature was lowered from ambient room temperature down to  $-30.0^\circ\text{C}$  at a rate of  $1.0^\circ\text{C}/\text{min}$ .

#### Determination of Body Size and Mass

Following SCP determination, pronotal width of the female field-collected ( $n = 48$ ) and cold hardened ( $n = 56$ ) *X. glabratus* were measured to the nearest  $0.001\text{ mm}$  using a Leica® microscope connected to a computer with Leica Suites® software (Leica® Camera Inc.). Mean dry biomass of the beetles was determined by placing individual specimens into microcentrifuge tubes and drying them in an oven at  $70^\circ\text{C}$  for 24 h (Riggins et al. 2009). Field-collected ( $n = 48$ ) and cold hardened ( $n = 56$ ) beetles were then weighed to the nearest  $0.001\text{ mg}$  in a covered Mettler UMT2 Micro-Balance® analytical scale (Mettler-Toledo International, Inc.). Size and weight of each specimen was used to measure correlations between these variables and the SCPs.

#### Statistical Analyses

Due to the non-parametric distributions of the field-collected and artificially cold hardened SCPs, a Mann-Whitney U-test was performed to determine if significant differences were observed between the treatments. A linear regression was performed on field-collected beetles to determine if a trend existed between SCP and the test dates (22 Jun to 11 Aug 2011). Linear regressions were also performed on field-collected and cold hardened beetles to determine if beetle size (pronotal width) or weight (dry biomass) influenced the SCPs. A multivariate regression was imple-

mented, using dry weight and pronotal width as independent variables and SCP as the response, to determine if pronotal width  $\times$  dry weight interaction significantly influenced *X. glabratus* SCPs. All statistical analyses were performed using the statistical and graphing software packages GraphPad Prism® (GraphPad® Software Inc. Version 5.0) and GraphPad InStat® (GraphPad® Software Inc. Version 3.06).

#### Temperature Map

A temperature map was created in ArcGIS® (ESRI® Version 10.1) using a North American mean 50 yr (1950-2000) minimum winter temperature dataset (ArcGIS® Online, ESRI®). The isotherm layer was generated through interpolation (nearest neighbor) of climate data from local weather stations throughout North and Central America. Variables included in the layer are monthly total precipitation, minimum monthly winter temperatures, and 19 other derived bioclimatic variables (data not shown). Isotherms were converted from a raster to a shapefile for analysis (conversion tool). Isotherms (shapefile) were then used to delineate regions (dissolve function) where minimum winter temperatures are cold enough to reach the SCP of both field-collected and artificially cold hardened *X. glabratus*. A sassafras distribution dataset was obtained from the USDA Forest Service Northern Research Station spatial database (Little's Range and FIA Importance Value Distribution Maps) as a shapefile and overlaid onto the isotherm dataset. The final data layers were assembled and collated into a map.

#### RESULTS AND DISCUSSION

Supercooling resulted in 100% mortality of tested beetles ( $N = 104$ ). Supercooling points of the field-collected beetles significantly decreased throughout the summer trapping season (slope =  $-0.19 \pm 0.04$  ( $\pm$  SE);  $P < 0.0001$ ) (Fig. 2). This decreasing trend in SCPs could be related to the limited amount of data points in June, thereby having a significant influence on the slope. The limited amount of catches in late June was expected and closely corresponds to the results of Hanula et al. (2008). More importantly, test 1 ( $-10.3^\circ\text{C}$ , 22 June 2011) occurred well above the other replicates. This beetle was tested very early in the year considering peak flight (early September) for *X. glabratus* (Hanula et al. 2008). This higher SCP may be the result of one or more factors suggested by Lee (1991), e.g. developmental stage, nutritional status, thermal history, and genetic potential. Moreover,  $-10.3^\circ\text{C}$  is greater than 3 standard deviations away from the mean and could simply be an outlier. To determine if test 1 was an influential leverage point or an outlier with little influence, the sample was re-

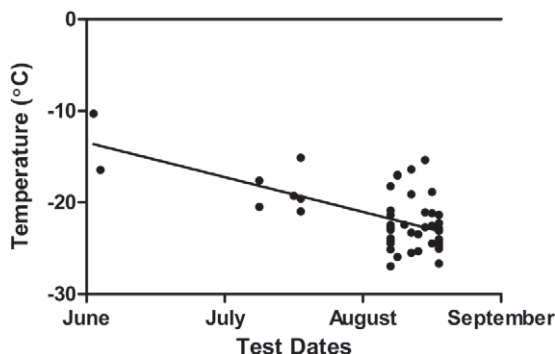


Fig. 2. Scatter plot and linear regression of *Xyleborus glabratus* supercooling points and test dates during the summer of 2011 in Jackson County, Mississippi ( $n = 48$ ). Note the significant ( $P < 0.0001$ ) negative trend (slope =  $-0.19 \pm 0.04$  ( $\pm$  SE),  $R^2 = 0.37$ ) as summer progressed.

moved from the data, and the linear regression was reanalyzed. Removing test 1 changed the slope and correlation estimates ( $-0.15 \pm 0.04$  ( $\pm$  SE);  $P < 0.0009$ ), but the slope of the new model was within the confidence interval of the original slope. Consequently, it cannot be implied that test 1 biased the model.

As summer progressed it is possible a decrease in body water concentrations of *X. glabratus* could have influenced the SCPs. Insects who live in environments where they receive little protection and are exposed to direct sunlight, wind, and solar radiation may become more desiccated during summer months. However, apart from a few hours once each generation, during the dispersal flight (usually a few meters), ambrosia beetles live entirely within the host tree, where internal woody tissues offer protection and the micro-climate stays extremely constant (Wood 1982). Due to the protected nature of the micro-environment and the consistent, daily trap checks, it is highly probable that water content in female *X. glabratus* was stable and had little to no effect on the SCPs.

The mean SCP of the 48 field-collected *X. glabratus* sampled was  $-21.7 \pm 0.5^\circ\text{C}$  ( $\pm$  SE). The mean SCP of the 56 cold hardened beetles was  $-23.9 \pm 0.4^\circ\text{C}$  ( $\pm$  SE) (Fig. 3). Artificially cold hardening *X. glabratus* significantly decreased the SCPs with respect to the SCPs of field-collected beetles (Mann-Whitney  $U = 701.0$ ;  $P < 0.0001$ ). The differences between mean SCPs in field-collected and cold hardened beetles could be attributed to amount of gut bacteria (Lee & Denlinger 1985; Cannon & Block 1988; Rosales et al. 1994; Kim & Kim 1997). In a related coleopteran, *Hippodamia convergens* Guérin-Ménéville (Coccinellidae), ice-nucleating active bacteria (i.e. *Pseudomonas syringae*, *Erwinia herbicola*)

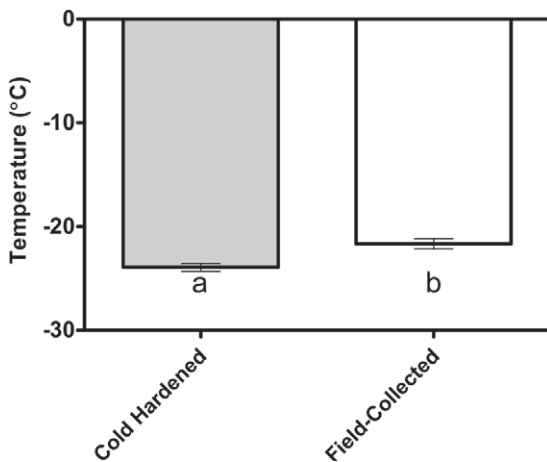


Fig. 3. Mean supercooling points from artificially cold hardened ( $-23.9 \pm 0.4^{\circ}\text{C}$  ( $\pm\text{SE}$ );  $n=56$ ) and field-collected ( $-21.7 \pm 0.5^{\circ}\text{C}$  ( $\pm\text{SE}$ );  $n=48$ ) *Xyleborus glabratus* tested in Jackson County, Mississippi. Different letters denote significant differences ( $\alpha = 0.05$ ).

were correlated with a  $\sim 12^{\circ}\text{C}$  increase in the SCP (Strong-Gunderson et al. 1990). Ice-nucleating bacteria have been found in field-collected insects (Lee et al. 1987), but it is unknown what effects 31 d of artificially cold hardening may have had on gut bacteria abundance and diversity in *X. glabratus*. The depressed mean SCP of artificially cold hardened beetles may also be attributed to a decrease in lipid content, as seen in the related scolytine, *Ips pini* (Say) (Lombardero et al. 2000), and this should be substantiated in *X. glabratus*. However, by comparing the significant differences between the mean SCPs of beetles field-collected during the summer months to the mean SCPs of cold hardened beetles, it is apparent that *X. glabratus* is capable of a high degree of thermal plasticity.

Insect size is another factor that can influence SCP (Sømme 1982; Pugh 1994; David & Vannier 1994; Colinet et al. 2006; Hahn et al. 2008). Colinet et al. (2006) found a negative trend between SCP and size in the aphid parasitoid, *Aphidius colemani* Viereck (Hymenoptera: Braconidae). The smaller sized parasitoid supercooled to lower temperatures even though it was reared at a higher temperature ( $25^{\circ}\text{C}$ ) than the larger sized braconid reared at  $15^{\circ}\text{C}$ . Hahn et al. (2008) found that larger body size of red imported fire ant workers supercooled to temperatures  $\sim 3^{\circ}\text{C}$  higher than the smallest workers.

To determine if the body size of *X. glabratus* females were correlated with SCPs, the width of the pronotum was measured. The width of the pronotum is a good metric to overall beetle body size (Amman 1982; Simmons et al. 1999). Mean pronotal width of the female *X. glabratus*

sampled was  $0.701 \pm 0.003\text{mm}$  ( $\pm\text{SE}$ ). The linear regression between SCPs and pronotal width produced a weak model with very low correlation, indicating no significant relationship ( $P = 0.23$ ;  $R^2 = 0.03$ ). Mean dry biomass (another measure of relative individual body size) of all samples was  $0.283 \pm 0.006\text{ mg}$  ( $\pm\text{SE}$ ). The linear regression of SCP to dry biomass also produced a model indicating no significant relationship between SCP and dry biomass ( $P = 0.38$ ;  $R^2 = 0.02$ ). The multivariate linear regression analysis of the dependent variable (SCP) to the independent variables (pronotal width and dry biomass) suggests that there was no statistically significant multicollinearity between SCP, pronotal width, and dry biomass ( $P = 0.47$ ;  $F_{1,45} = 0.78$ ;  $R^2 = 0.03$ ). The low  $R^2$  and high  $P$  values of the biometric regressions indicate that body size has no effect on SCPs of *X. glabratus*.

It remains unknown if or when *X. glabratus* enters diapause. Many tropical scolytines ignore the seasonal changes observed by other insects and continue their normal physiological activity throughout the year (Wood 1982). Events leading to diapause (e.g., gut purging), or production of substances important to diapause (e.g., thermal hysteresis proteins) may influence SCP, chill injury, or other physiological systems of cold tolerance (Sømme 1982; Denlinger 1991; Pullin 1992, 1996). In the mountain pine beetle, *Dendroctonus ponderosae* Hopkins, a lack of diapause makes it less dependent on hormonal controls than in diapausing insects (Merivee 1978; Sømme 1982; Hodkova & Hodek 1994). As mentioned earlier, Maner et al. (2013) found *X. glabratus* females emerging in all months of the year; therefore, it is unlikely this species enters diapause. However, this requires further investigation to accurately model the invasion potential of *X. glabratus* in North America.

Due to a lack of ambrosia beetle cold tolerance literature, the closest studies we can compare our results to are those of bark beetles (Gehrken 1984; Bentz & Mullins 1999; Lombardero et al. 2000). Lombardero et al. (2000) found no significant differences between SCPs of bark beetles (*D. frontalis*, *I. pini*, *I. grandicollis* (Eichoff), *I. perroti* Swaine) taken from winter locations or those cold hardened in an incubator at  $0^{\circ}\text{C}$  for 4 mo. Unlike Lombardero et al. (2000), our study saw significant differences in SCPs between field-collected and artificially cold hardened specimens. Our findings do follow the results of *D. ponderosae* cold acclimatization studies. These studies found that adequate acclimation to low temperatures significantly increased *D. ponderosae* cold hardiness and survival (Wygant 1940; Yuill 1941; Sømme 1964). The use of artificially cold hardened *X. glabratus* as an analog for naturally winter hardened specimens from the upper latitudes of the eastern U.S. could be more realistic



when modeling for distribution potential. Due to the mild winter temperatures encountered by *X. glabratus* within its current range, using winter hardened specimens from these areas may greatly underestimate the cold hardening ability and therefore underestimate the invasion potential. Nevertheless, no other supercooling studies of *X. glabratus* or any other ambrosia beetle could be located, so differences in SCPs between artificially cold hardened and naturally cold hardened ambrosia beetles are unknown, demanding intensive future research.

Fig. 4 is a preliminary map offering a graphic representation of where the observed *X. glabratus* mean SCPs occur in North America. The map depicts mean minimum winter air temperature isotherms, sassafras distribution, and the zone of temperatures where both experimentally determined mean SCPs occur. Due to a lack of inventory data, this map omits northern spicebush,

but sassafras (shown on map) shares a similar northern distribution with northern spicebush. Based on the cold hardened SCP data, *X. glabratus* could spread into southern Ontario, Canada; however, SCP is not the sole determinant of cold tolerance or absolute mortality (Salt 1961; Baust & Rojas 1985; Knight et al. 1986; Lee & Denlinger 1985; Lee 1991; Bale 1996; Sinclair 1999; Bale 2002; Renault et al. 2002).

Furthermore, it is important to consider the refugia of wood-boring insect microhabitat, which provides protection from ambient air temperatures and extreme weather events (Wood 1982; Bolstad et al. 1997; Poland & McCullough 2006; Tran et al. 2007). The bark and wood act as a buffer which aids in regulating the internal thermoclimate relative to air temperatures. Temperatures within the heartwood can take several hours to days to respond to changing environmental conditions (Derby & Gates 1966), and in many

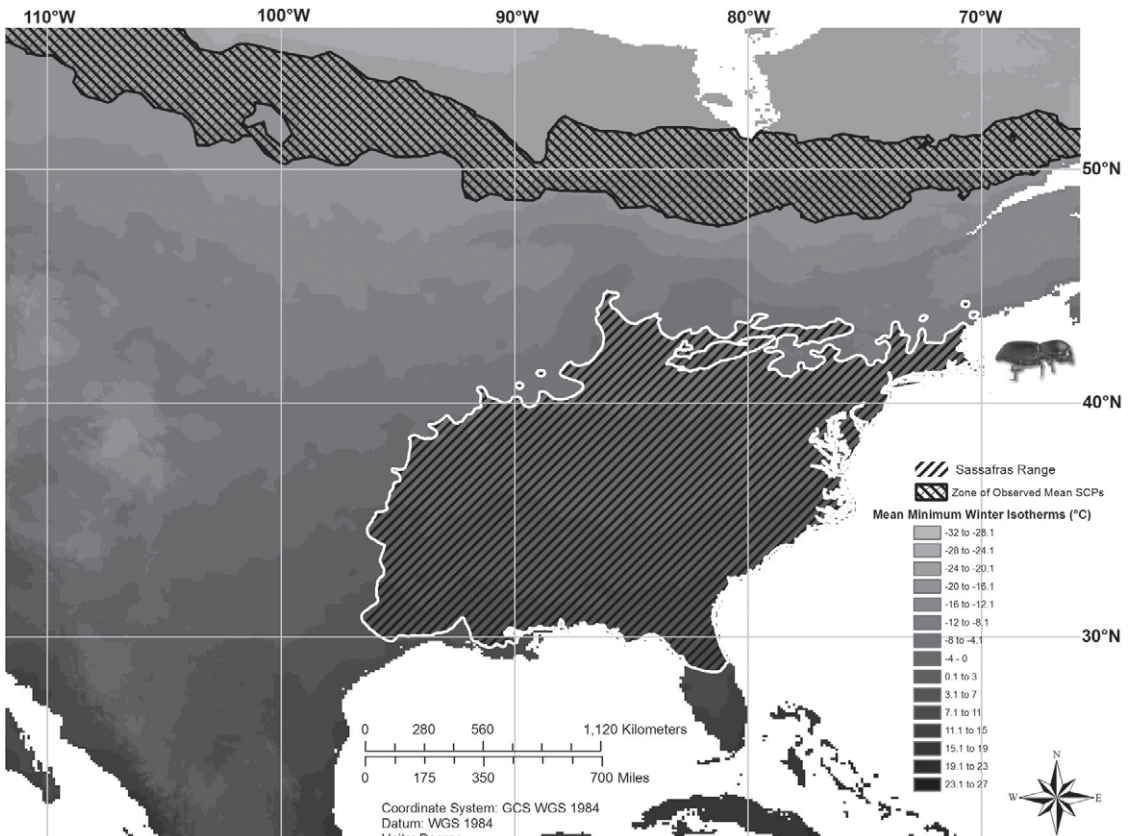


Fig. 4. Depiction of mean minimum winter temperatures (50 yr dataset) and sassafras distribution in the United States. The descending color intervals (light to dark) are increasing temperatures in degrees Celsius. The hatched area, outlined in white, signifies sassafras distribution in the U.S., but continues into southern Ontario, Canada (distribution not shown). The crosshatched region indicates where mean minimum winter air temperatures are cold enough to reach the SCP of both field-collected and artificially cold hardened *Xyleborus glabratus*. Solely based on SCP data, it is unlikely that temperature will limit *X. glabratus* distribution in any part of the native range of sassafras. However, this is preliminary data and mortality is likely at much warmer temperatures or with extreme winter temperature events.



instances, offer protection from short, intense temperature fluctuations. A 2 yr study found under-bark temperatures in ash (*Fraxinus* spp.) averaged ~2 °C warmer than winter air temperatures; however, buffering capacity is also dependent on several other factors (e.g. orientation of bole exposure, time of day, tree species and diam, wood and tissue moisture, depth of overwintering site) (Derby & Gates 1966; Bolstad et al. 1997; Vermunt et al. 2012). Additionally, during winter months, *X. glabratus* may be in direct contact with ice crystals from moisture stored within the wood or from that of *R. lauricola*. This proximity to external ice may have a direct effect on internal ice formation, but both Olsen et al. (1998) and Crosthwaite et al. (2011) show cuticular waxes contribute to a resistance against ice inoculation.

Even when factoring in the thermal buffering capacity of bark and wood, these SCP results may suggest a previous spatio-temporal model (Koch & Smith 2008) that used climate match data could have underestimated the potential distribution of *X. glabratus*. At the time of the Koch & Smith (2008) model, there was little evidence that *X. glabratus* would infest sassafras in the absence of redbay; since 2008 there has been mounting evidence to the contrary (Fraedrich et al. 2008; Smith et al. 2009; Riggins et al. 2011; Bates et al. 2013). Consequently, the model only included areas where redbay and sassafras occur together. Further studies examining extended and cyclical periods of low temperatures (i.e. repeated cold exposure (RCE), see; Marshall & Sinclair 2012) and the thermal buffering capacity of redbay and sassafras will also be needed to determine the lethal and sub-lethal effects (Bale 1987; Bale 2002; Carillo et al. 2005) of winter temperatures on *X. glabratus*. Future work will focus on collecting necessary *X. glabratus* cold tolerance data (i.e. sub-lethal effects, thermal buffering, RCE, LT<sub>50</sub>) and incorporating them with SCP and climate change variables to model the invasion potential of this significant tree killing insect-pathogen complex.

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