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Invasion Potential and Overwintering Biology of the Redbay Ambrosia Beetle (Coleoptera: Curculionidae) in the United States

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By

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Several native species of Lauraceae (e.g. sassafras) in the southeastern United States are being eradicated by laurel wilt disease. Laurel wilt is caused by a highly invasive and cryptic ambrosia beetle, *Xyleborus glabratus*, and its fungal symbiont. The symbiont pathogen is spread during colonization of native Lauraceae. *Xyleborus glabratus* and the pathogen are remarkably effective at colonizing and killing healthy populations of Lauraceae in a brief time period. Control methods have been unable to slow the spread of laurel wilt disease and *X. glabratus* populations have been spreading into northern latitudes. Presently, cold temperatures may be the only factor limiting establishment of the beetle in interior populations of sassafras. Empirically derived physiological data from this study were combined with climatic, microhabitat, and host data to model the invasive potential/hazard rate of *X. glabratus* and laurel wilt in sassafras forests of the United States. Sharing this model data will help land managers, forest health specialists, urban foresters, and landowners make informed proactive management decisions regarding laurel wilt disease.

Keywords: *Xyleborus glabratus*, laurel wilt disease, cold hardening, invasion modeling.

DEDICATION

Dedicated to my mother, Lucy Formby, who said “I don’t care if you get a degree in basket weaving, just get a degree”. Well, how about a doctorate in insects? Thanks for your constant support and encouragement through many, many years of academia and my other pursuits.

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CHAPTER I

INTRODUCTION

1.1 Preface

The redbay ambrosia beetle, *Xyleborus glabratus* Eichhoff (Coleoptera: Curculionidae), is native to forests of southern Asia (Rabaglia 2006). In Asia, *X. glabratus* is essential to hardwood forest succession (Wood and Bright 1992;); however, in the southeastern United States, the beetle is the primary vector of laurel wilt disease (LWD). Laurel wilt is caused by a fungal pathogen (*Raffaelea lauricola* T. C. Harr., Fraedrich & Aghayeva) carried and cultivated by *X. glabratus*. Laurel wilt is lethal to 11 species of North American trees in the laurel family (Lauraceae; Kendra et al. 2013). The female *X. glabratus* introduces the pathogen during colonization (tunnel and gallery excavation) of mature, apparently healthy trees. Interestingly, colonization of healthy hosts may be a newly derived behavior since the beetle's North American establishment (Kendra et al. 2013). Following inoculation, the pathogen is capable of killing mature redbay trees in as little as 4 weeks (Mayfield et al. 2008).

Laurel wilt disease has spread extensively throughout the southeastern United States (confirmed in 9 states) and has reached epidemic levels in many areas (Kendra et al. 2013; USDA Forest Service Laurel Wilt Distribution Map 2016). Laurel wilt has more commonly killed redbay (*Persea borbonia* (L.) Spreng.) and swampbay (*Persea palustris* (Raf.) Sarg.); however, sassafras (*Sassafras albidum* (Nutt.) Nees) and avocado (*Persea*

americana Mill.) are increasingly being attacked and killed (Kendra et al. 2013). According to Frank Koch (2016), research ecologist with the U. S. Forest Service-Eastern Forest Environmental Threat Assessment Center, the insect-pathogen disease complex has led to the death of ~430 million redbay trees since its establishment and subsequent spread from Port Wentworth, Georgia in ~2002. In Miami-Dade County, Florida, where avocado revenue accounted for ~$23.5 million in the 2011-2012 crop year (National Agricultural Statistics Service 2013), entire avocado groves are being killed (Kendra et al. 2013). Moreover, the National Germplasm Repository for avocado, which is viewed as a source for genetic diversity to support crop improvement and botanical research, is threatened by LWD (Ayala-Silva et al. 2004; Kendra 2013).

Control options (e.g. insecticides, fungicides) have been unable to slow the spread of the insect or the pathogen (Kendra et al. 2013). However, cold is a crucial abiotic factor that determines survival and distribution of insect species (Alford et al. 2016). Therefore, low temperatures may be the only factor that limits the expansion of *X. glabratus* in North America. Many studies have examined the impacts of laurel wilt on tree health and forest structure (Goldberg and Heine 2009; Gramling 2010; Evans et al. 2014; Cameron et al. 2015; Nielson and Rieske 2015), and a few studies have examined the basic biology of *X. glabratus,* such as its life cycle (Brar et al. 2013). No studies, to date, have examined the overwintering physiology or the effect of low temperatures on the survival of *X. glabratus*. The following research was intended to examine the effects of low temperatures on *X. glabratus* to predict the beetle's range potential in the Unites States. Predicting range potential, not only helps determine the likely ecosystem impacts

of LWD, but could help land managers and owners formulate management strategies, determine resource logistics, or plan detection surveys.

1.2 Invasion Modeling

One of the primary objectives for researchers interested in understanding the potential impacts of a newly established pest is predicting its distribution potential using a geographic information systems (GIS; Andrewartha and Birch 1954; Kolar and Lodge 2001). Numerous models are available to researchers interested in modeling biological invasions and predicting range expansion, e.g. maximum entropy, genetic algorithm for rule-set prediction (GARP), and species distribution models (Peterson 2003; Phillips et al. 2006; Kearney and Porter 2009). Some models are complex, time consuming, and require specific software defined inputs (e.g. GARP), whereas others can be simplistic, quick, and require one to several user defined inputs (e.g. species distribution models). However, due to the complexity of biological systems and/or data limitations, each model has advantages and disadvantages over another (Kearney and Porter 2009). As a result, a researcher must determine the best model for the organism and associated data, but perhaps more importantly, a researcher must choose a model that will convey the results to both peers and the general public. For many reasons (e.g. funding/grant purposes, survey and trap deployments, and publication quotas), however, some researchers must produce quick models at the expense of accurate and meaningful results. Unfortunately, rapidly produced models are often built upon assumptions and can contain little empirical data (Guisan and Thuiller 2005), and as a result, they can become irrelevant in a short period of time (Koch and Smith 2008).

The use of species distribution models (SDMs) to predict range potential of ecological invaders has grown immensely in the last two decades (Guisan and Thuiller 2005). The primary goal of a researcher who use SDMs is to map an organisms' potential habitat in space and time (Guisan and Thuiller 2005). Most SDMs use geo-referenced presence-absence data to make inferences regarding environmental suitability. These are known as correlative SDMs (Guisan and Zimmermann 2000; Kearney and Porter 2009). In other words, data that are associated with favorable (presence) or unfavorable (absence) conditions are extracted from larger datasets and are used as predictor or constraint variables in geographic information systems (Guisan and Thuiller 2005; Bomford 2009; Kearney and Porter 2009). However, in correlative SDMs, it is hard to determine whether a predictor variable directly contributes to a constraint or is in response to another variable outside the model (MacNally 2000), but under the right circumstance(s) (i.e. if the organism is well-studied and the researcher is confident with the locality records or ecological validation can be tied to a predictor variable) correlative SDMs can lead to quick and relatively accurate results given that the predictor variable directly contributes to an species' limits (Guisan and Thuiller 2005).

Inherent problems in correlative SDMs still exist (e.g. historical climate data may not resemble current or future trends, temperature data may be a crude resolution, physiological tolerance of the organism in invaded range may have changed, and incorrect locality data) and within a few years models using correlative data can be outdated or incorrect (Kearney and Porter 2009). Researchers are more recently integrating physiological data of organisms and their environments to correct the data and increase the predictive power of SDMs (Ungerer et al. 1999; Yoshino and Ishii 2001;

Kearney and Porter 2009; Formby et al. 2013). This type of modeling is known as mechanistic SDMs (Kearney and Porter 2009).

Bebber et al. (2013) reports that many pest species are expanding into higher latitudes and global climate change may be responsible for this phenomenon; however, in some instances (i.e. newly established pest species), the mechanisms driving range expansion are largely unknown (Lehmann et al. 2015). The physiological adaptations organisms, especially insects, use to survive unfavorable conditions may have a greater impact on range expansion than a changing global climate alone. Understanding the mechanisms that allow insects to tolerate and adapt to unfavorable climatic conditions is a central tenet of invasion biology (Lehmann et al. 2015). In recent years several approaches for modeling physiological tolerance are on the rise, e.g. the ecophysiological comparative approach (Lehmann et al. 2015) and the artificial acclimation approach (Koštál et al. 2011; Bemani et al. 2012). The comparative approach examines the physiological tolerance of a closely related species that lives in an area that may be eventually invaded by a well-known pest species. The data from the native species then can be used with climate match or niche modeling to predict range expansion of the pest (Lehman et al 2015). The acclimation approach exposes a pest to artificial conditions to test for physiological limits associated with more extreme environmental conditions than a pest is normally exposed (Kostal et al. 2011; Formby et al. 2013). The experimental data (e.g. supercooling point, lower lethal temperature) then can be used in niche models or GIS models (Ungerer et al. 1999). The following research followed the acclimation approach and experimentally determined the low temperature limits of *X. glabratus*. Following data collection the data (i.e. lower lethal temperature) were combined with

host species and several climate datasets to generate several mechanistic SDMs to predict the range potential of *X. glabratus* in the United States.

1.3 Biology of Scolytinae

Bark and ambrosia beetles (Curculionidae: Scolytinae) are a large, cosmopolitan group of insects (~7500 described species world-wide) and can be found anywhere woody plants grow (Wood 1982; Bright and Skidmore 2002). Bark beetles live between the bark and woody tissues of trees and feed on the soft phloem tissue (phloeophagous). Ambrosia beetles, on the other hand, live within the wood and cultivated one or more species symbiotic fungi as their primary food source (xylomycetophagous; Wood 1982). Subsequently, since ambrosia beetles carry and cultivate their food source within each new host, they are mostly generalists and can utilize multiple families and/or many species of hosts (Wood 1982). However, most bark beetles are specialists and have specific nutritional larval requirements; therefore, they usually prefer only a select few species in one family of trees as hosts (Wood 1982). It is very common to find bark and ambrosia beetles living and reproducing only in recently felled, injured, stressed, or moribund woody plant tissues; however, some species attack apparently healthy trees and others only live and reproduce inside mature fruit or seed (Wood 1982; Wood 2007; Coulson and Klepzig 2011; Formby et al. 2013). Regardless of the preferred host material, the target tissue(s) must contain an adequate amount moisture and nutrients for larval development or fungal cultivation, however, host tissues can quickly dry or lose nutrients following colonization (Wood 2007). As a result, most bark and ambrosia beetles complete only one generation in host material before emerging to search for a fresh, more suitable host (Wood 1982).

Since the early Cretaceous (~145 mya), bark and ambrosia beetles have provided beneficial ecosystem services worldwide (Wood 1982). In fact, scolytines are so good at removing low vigor trees and thinning the forest that they have been described as nature's "principle foresters" (Wood 2007). Regardless of biology, survival and reproduction of ambrosia beetles are dependent upon host detection, sometimes over long distances. Ambrosia beetles, unlike bark beetles, do not use pheromones to locate mates, nor do they aggregate to overcome tree defenses. Instead, ambrosia beetles are attracted to the volatile chemicals (e.g. ethanol, terpenes) emanating from trees that have compromised defense systems (Wood 2007). Moreover, each ambrosia beetle species can recognize a host not only through olfaction, but through visual recognition and ultrasonic acoustical emissions (Wood 1982; Haack et al. 1988; Mayfield and Brownie 2013).

Ecologically, ambrosia beetles help precondition trees for decomposition through their tunneling and fungal inoculation activities (Wood 1982). This cryptic ecological service benefits forest ecosystems worldwide (Wood 1982). However, because of rapid, global movement of host material, monoculture agroforestry practices, and increasingly warmer global temperatures ambrosia beetles are spreading and establishing in novel habitats (Haack 2001, Haack 2006). As a result, they are increasingly responsible for threatening the health of naïve forest and urban trees worldwide (Wylie et al. 1999; Kubono and Ito 2002; Lieutier et al. 2004; Haack 2001; Haack 2006; Kendra et al. 2013).

1.4 Biology and spread of the redbay ambrosia beetle and laurel wilt

The first *Xyleborus glabratus* detected in the United States was trapped in Port Wentworth, Georgia in May 2002 (Rabaglia et al. 2006). In 2003, widespread mortality of redbay trees had been reported around several counties surrounding Savannah, Georgia

(Kendra et al. 2013). By 2004, the association between *X. glabratus* and the pathogen, *Raffaelea lauricola*, had been discovered (Kendra et al. 2013). However, since 2004, the geographic range of *X. glabratus* has expanded beyond those predicted from spatio-temporal estimates (Koch and Smith 2008). In March 2010, the first beetle was trapped in Miami-Dade County, Florida five years before the predicted date. Subsequently, the disease rapidly spread into nearby commercial avocado groves and stands of swampbay trees on Tree Islands in the Everglades National Park (Kendra et al. 2013).

The *Xyleborus glabratus* female is a small (~2 mm in length), cylindrical, dark brown wood-boring ambrosia beetle endemic to southern Asia (Taiwan, Japan, Myanmar, India, and Bangladesh; Rabaglia et al. 2006). In Asia, *X. glabratus* is unassociated with tree disease or mortality; however, it has been associated with stem dieback in Asia (Kendra et al. 2013). Female *X. glabratus* use both olfactory and visual cues to find hosts (Mayfield and Brownie 2013). The female also is capable of producing female offspring via sexual reproduction and male offspring via asexual reproduction (i.e. haplo-diploidy) (Kendra et al. 2013). *Xyleborus glabratus* males have fused elytra and are flightless, and rarely, if ever, leave their natal tree (Wood 1982; Kendra et al. 2013). The males do, however, tend to the fungal gardens within the host, mate with siblings, and even mate with their mother (Kendra et al. 2013).

The gallery system of *X. glabratus* is constructed perpendicular to the trunk and consists of one main entrance tunnel that eventually branches into 2-5 secondary tunnels, each with 0-3 tertiary tunnels (Brar et al. 2013). Eggs are laid continuously in groups of 1-8 at the distal ends of the secondary and tertiary tunnels. Pupation also occurs in the distal ends of these tunnels, indicating they function also as brood galleries (Brar et al.

2013). In redbay, eggs are first observed at 11 days, larvae at 20 days (with a total of 3 larval instars), pupae at 26 days, and teneral adults at 31 days after gallery initiation (Brar et al. 2013).

In the United States, Hanula et al. (2008) found no significant difference in the beetle's attraction to uninfected or *R. lauricola* infected bolts. This result indicates that there are no pheromones or fungal volatiles important to *X. glabratus* host finding. Mayfield and Brownie (2013) reported that stem silhouette diameter has a significant effect on host finding; moreover, an analysis of terpeniod emissions from host Lauraceae found several sesquiterpenes (kairomones) important to *X. glabratus* host finding. A synthetic blend of the fungal volatiles was not attractive to *X. glabratus* alone; however, it increased attraction when paired with synthetic host volatiles (Kuhns et al. 2014). The pathogenic symbiont of *X. glabratus*, *Raffaelea lauricola* T.C. Harrington, Aghayeva, & Fraedrich (Ophiostomatales: Ophiostomataceae), is a newly described fungal species and the only known ambrosia beetle symbiont to cause complete tree mortality (Hanula et al. 2008). Mortality has been confirmed in redbay (*Persea borbonia* (L.) Sprengel), swampbay (*Persea palustris* (Rafinesque) Sargent), sassafras (*Sassafras albidum* (Nuttall) Nees), northern spicebush (*Lindera benzoin* (L.) Blume), and avocado (*Persea americana* Miller) among other North American lauraceous species (Fraedrich et al. 2008; Mayfield et al. 2008).

Since initial detection, *X. glabratus* has spread inland and along the coast into the Carolinas and Florida (USDA Forest Service Laurel Wilt Distribution Map; Cameron et al. 2015). In Duval County, Florida, mortality of mature redbay increased 92% within two years of initial beetle infestations (Mayfield et al. 2008). As *X. glabratus* moves

outside the range of redbay and swampbay, laurel wilt has caused extensive sassafras mortality (Fraedrich et al. 2008; Smith et al. 2009; Riggins et al. 2011; Bates et al. 2013, Fraedrich et al. 2015). Disjunct populations are now established in Mississippi, Alabama, Texas, Arkansas, and Louisiana (Riggins et al. 2010; Formby et al. 2012; Bates et al. 2013; Fraedrich et al. 2015). Anthropogenic movement of infested wood is suspected in the establishment of these disjunct populations (Florida Department of Agriculture and Consumer Services 2012, Kendra et al. 2013, Fraedrich et al. 2015). The infection site in Marengo County, Alabama is ~200 km from the nearest infestation site in Mobile County, Alabama and exceeds a previous temporal estimation of invasion by 12 years (Koch and Smith 2008).

From 2011 to 2014, Marengo County was the only documented site where redbay is not found, and where sassafras was the only lauraceous species killed by laurel wilt (Bates et al. 2013). In 2012, more than 20 sassafras trees were reported in various stages of wilt in Marengo County, up from one tree the previous year (Bates et al. 2013). The susceptibility of sassafras to laurel wilt offers a potential avenue for the beetle and disease to spread throughout the central and inland northern forests of the eastern United States. Due to its widespread geographic range, mortality of sassafras could have myriad ecological consequences that negatively impacts taxa across several Kingdoms (Griggs 1990).

Control measures have been unable to slow the spread of the beetle and mortality from cold temperatures may be the only factor limiting of *X. glabratus* expansion within the United States. Determination of the supercooling point is an essential starting point for physiological investigations and limitations of cold tolerance in any insect (Salt 1961;

Bale and Walters 2001; Bale 2002; Renault et al. 2002). Supercooling is a form of protection against cold 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). Supercooling point (SCP) is the temperature at which spontaneous ice formation occurs in the insect body and represents the limit of supercooling (Lee 2010). An exothermic reaction (heat spike) occurs from the crystallization of body fluids when the SCP is reached (Lee 2010). The heat spike, or SCP, is identified through the use of thermocouples.

Supercooling point has been investigated in other scolytine beetles to help determine the geographic limits of pest species. For example, Ungerer et al. (1999) utilized SCP to formulate the models used for the northern distribution limits of *Dendroctonus frontalis* Zimmerman and Régnière and Bentz (2007) applied the SCP of *D. ponderosae* Hopkins to model the spatial distribution of their population as a function of daily changes in the temperature. However, apart from general life cycle and behavioral information (Ngoan et al. 1976; Weber and McPherson 1983; Hanula et al. 2008; Brar et al. 2013), little to nothing is known regarding ambrosia beetle physiology (Chapman 1958), especially the mechanisms behind cold hardening (Formby et al. 2013).

1.5 Overwintering biology of insects

1.5.1 Cold hardening

Cold hardening is just one of the strategies insects use to mitigate the lethal and sub-lethal effects of low temperatures (Danks 2005; Denlinger and Lee 2010). Some insects survive low temperatures by migrating long-distances to areas of warmer temperatures (e.g. monarch, *Danaus plexippus* L.) or short distances to protected

hibernacula (e.g. lady bird beetles, *Coccinella* spp.). Insects unable to move to more favorable conditions prepare for the onset of cold temperatures by cold hardening (i.e. modify physiological systems; Danks 2005). Cold hardening modifications can include, but are not limited to; synthesis of cryoprotectants (e.g. glycerol; Storey and Storey 1991; Denlinger and Lee 2010), synthesis of antifreeze proteins (e.g. glycoproteins; Duman 2001; Zachariassen and Kristiansen 2000), and removal or translocation of ice nucleating proteins and substances to extracellular spaces (Salt 1953; Salt 1961; Ramlov 2000). More recently, heat shock proteins have been proposed as an additional mechanism contributing to cold hardiness in insects (Rinehart et al. 2007). The physiological modification(s) and concentration(s) used by an insect depends on its cold hardiness strategy (Chown and Sinclair 2010).

Insects initiate cold hardening processes prior to the onset of cold weather through the recognition of environmental cues. These include; changes in thermoperiod, photoperiod, and/or water availability (Baust 1985; Allmen and Zettel 1984; Leather et al. 1993; Meier and Zettel 1997; Layne and Kuharsky 2000). Lee (1989) reported the process of seasonal cold hardening in insects requires at least several weeks to complete; however, a variety of insect species can cold harden in a matter of minutes to hours, i.e. rapid cold hardening (Lee et al. 1987; Sinclair et al. 2003; Danks 2005). Several other forms of cold hardiness have been described recently (Bale 1996; Sinclair 1999; Denlinger and Lee 2010) proving the process is dynamic, complex, and still little understood. Understanding the physiological mechanisms insects use to tolerate cold is of interest to laboratory researchers and field scientists, alike. For example, the discovery of a novel ice-nucleating microorganism or protein may be of interest to researchers in

insect biological control systems (Fields 1993; Lee et al. 1993; Lee at al. 1996); and in invasion ecology, the discovery of specific insect limitations, mechanisms, or responses to low temperatures can be used as an aid to predict invasion potential (Lehmann et al. 2015) or determine any number of ontogenetic processes (Convey 1997; Hicke et al. 2006; Jamieson et al. 2012).

Every insect species requires a specific range of temperatures to regulate biological and physiological processes (Vannier 1987; Brar et al. 2013) and any fluctuations outside the optimal range can damage physiological systems, alter development, and/or reduce survival (Lee 1989; Lee 1991; McMillan et al. 2005; Jaramillio et al. 2009; Brar 2012; Papanikolaou et al. 2013). It can therefore be assumed that ambrosia beetles survive low temperatures by initiating behavioral and physiological adjustments prior to winter. The limited data suggest that most ambrosia beetles forgo diapause (Wood 1982; Wood 2007; Hanula et al. 2008; Maner et al. 2013) and cold hardening may be their primary survival mechanism against low temperatures. However, ambrosia beetles spend their entire life cycle, except for host seeking flights in optimal conditions protected within host refugia (Wood 1982). Living within a host may provide ambrosia beetles with enough protection from cold temperatures that they do not need to cold harden. For example, Hoshikawa et al., (1988) estimated that the migration of beetle larvae into 10 cm soil provides thermal buffering equivalent to a southerly migration of 100 km. Alternately, a study of ash trees in Canada found bark provided very little buffering (1.0°C buffering; Vermunt et al. 2012). However, a study conducted during the winter in the western Unites States found daily minimum temperatures under the bark on the south side of pine trees were on average 2.1°C warmer than ambient winter air

temperatures (Bolstad et al. 1997). This suggests that ambrosia beetles maybe slightly more protected than bark beetles due to the increased amount of woody material insulating them from ambient air temperatures.

Low temperatures, together with the characteristics of a new habitat (e.g. environmental cues), often limit the establishment of insects outside their native range, even in insects that cold harden (Dank 2005). However, as the global climate changes, native and non-native insects are readily establishing in new habitats and expanding into higher latitudes (Logan et al. 2003, Powell and Bentz 2009, Bebber et al. 2013), and to make matters worse, non-native species are more likely to be pests than native species due to the lack of predators and other natural biocontrols in the invaded habitats (Simberloff 1986; di Castri 1989; Stauss et al. 2006). In North America, the number of successfully introduced non-native forest insects, such as wood-boring beetles, has risen significantly in the last few decades and this trend is predicted to continue (Leibhold et al. 1995; Haack 2001; McCullough et al. 2005; Haack 2006; Langor et al. 2009; Bertheau at al. 2010).

1.5.2 Lower lethal temperature and cold tolerance strategies

The lower lethal temperature (LLT) of an insect is the temperature that can kill the entire population of that species (Sinclair et al. 2015). The LLT should be measured only after other studies of cold tolerance have been conducted, e.g. supercooling point (Sinclair et al. 2015). Lower lethal temperature provides information regarding the survival of a population or species under certain environmental circumstances by empirically testing the insect's thermal limitations (Hatherly et al. 2005). The LLT is a factor that helps describe an insects species' cold tolerance strategy when its supercooling

point is unrelated to the insect's survival, e.g. in chill-susceptible or freeze tolerant species (Baust and Rojas 1985; Sinclair et al. 2015). Furthermore, when used in combination with supercooling point, lower lethal temperature can be used to indirectly determine an insects' cold tolerance strategy (Denlinger and Lee 2010).

There are numerous cold tolerance strategies in insects and each strategy describes a particular physiological response to cold and internal ice formation (Sinclair et al. 2015). Chill susceptible species succumb to the effects of cold temperatures even before ice is formed within the insect body (Bale 1993). This is sometimes called chill- or cold-intolerance (Lee 2010; Sinclair et al. 2015). An example of a chill-susceptible insect is the false codling moth, *Thaumatotibia leucotreta* (Meyrick). Larvae of the false codling moth freeze at -13°C to -22°C, but are killed by short-term exposure to -8°C to -12°C (Boardman at al. 2012). These insects are killed as a result of chill-injury (i.e. cold shock), which causes irrevocable damage to cell membranes (Lee and Denlinger 2010). Chill susceptibility characterizes the majority of insects, most notably those insects that are from the tropics (Sinclair et al. 2015). Because many non-native pests originate from tropical regions, studying the long-term effects of low temperatures (chill injury) on chill-susceptible insects is an important factor in predicting their establishment and spread (Lee 2010).

Freeze intolerant species, unlike chill-susceptible insects, can survive exposure to cold temperatures as long as body tissues do not freeze (Sinclair et al. 2015). However, freeze tolerant species can tolerate sub-zero temperatures and cannot be chill injured (Lee 2010). Most insects exposed to sub-zero temperatures during the winter are freeze intolerant and, as a result, they prepare for cold temperatures by increasing cold tolerance

and/or remain unfrozen by supercooling, sometimes as low as -40°C to -60°C (Ring and Tesar 1981). Finally, freeze tolerant species can survive the formation of ice within the insect body (Lee 2010). For example, prepupae of goldenrod gall fly *Eurosta solidaginis* (Fitch) have an average supercooling point (SCP) of -8°C and can survive with 60% of its body frozen; however, mortality does not begin until -25°C is reached (Morrissey and Baust 1976). Defining the cold tolerance strategy of an insect helps; 1) interpret the meaning SCP (e.g. does the SCP equal the LLT) and, 2) determine the correct method for measuring lethal temperature; however, it does not help predict mortality during winter, nor does it directly define the level of cold tolerance in an insect (Bale 1993; Sinclair 1999; Sinclair et al. 2015).

Table 1.1 Cold tolerance strategies of insects as determined by survival of internal ice formation.

	Internal Ice Formed	**No internal Ice Formed**
Alive	Freeze-tolerant	Chill-susceptible or freeze-avoidant
Dead	Chill-susceptible or freeze-avoidant	Chill-susceptible

Insects that are chill-susceptible die of injuries unrelated to freezing, freeze-avoidant insects die upon internal ice formation, and freeze-tolerant species are able to survive internal ice formation (modified from Sinclair et al. 2015).

1.5.3 Scolytinae

There are 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, it was discovered that the ambrosia beetle *Xylosandrus germanus* (Blandford) overwinters in the adult stage (Weber and McPherson 1983), and the bark beetles *Ips grandicollis* Eichhoff and *Dendroctonus ponderosea* Hopkins overwinter in all stages (Yousef et al. 2014) and the larval stage (Régnière and Bentz 2007), respectively. 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. Most scolytines overwinter deep within host material, however, a few overwinter outside of host material. For example, Kinghorn and Chapman (1959) found that the striped ambrosia beetle *Trypodendron lineatum* (Olivier) leaves host logs and overwinters no more than ~1.3 cm deep in leaf litter up to 304 meters from its host. The bark beetles *I. grandicollis* and *D. frontalis*, on the other hand, remain just under the bark (Wood 1982; Bentz and Mullins 1999; Lombadero et al. 2000). Morgan (1967) reported that scolytine adults surviving winter can produce spring brood and many ambrosia beetles, such as *X. glabratus* are parthenogenetic, thus if one female survives winter in a new area it can be readily colonized in the spring (Wood 1982).

1.5.4 Overwintering biology in the redbay ambrosia beetle

This study is the first to investigate supercooling point or any aspect of overwintering physiology in an ambrosia beetle, probably because of their cryptic behavior. *Xyleborus glabratus* may have a high degree of thermal plasticity because it is native to the tropics, yet has no problem colonizing hosts in the subtropical climate of the southeastern U.S. This may mean that *X. glabratus* could be a model organism with

which to begin ambrosia beetle cold tolerance studies, especially from the invasion ecology perspective. Examining the overwintering biology of *X. glabratus* will help to describe its cold tolerance strategy, increase the understanding of cold tolerance in ambrosia beetles in general, and form the basis of building models to predict the invasion potential and ecological impacts of laurel wilt in the United States.

Thus, to better understand *X. glabratus* cold tolerance and invasion potential, this study; 1) measured a particular set of biomolecules (sugars, lipids, and glycogen) related to insect cold hardening, 2) measured supercooling points and the lower lethal temperature of artificially acclimated and naturally overwintering *X. glabratus*, 3) determined the cold hardiness strategy of *X. glabratus*, and 4) created species distribution models that represent the invasion potential of *X. glabratus* in the United States under different climate scenarios.

CHAPTER II

METHODS FOR DETERMINING COLD TOLERANCE AND INVASION POTENTIAL OF THE REDBAY AMBROSIA BEETLE

2.1 Methods

2.1.1 Summer collected *Xyleborus glabratus*

Female *Xyleborus 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 were placed in slash pine dominant stands with a laurel wilt symptomatic redbay understory (Hanula & Sullivan 2008). 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. 2.1). Monthly (Jun, Jul, and Aug 2011) maximum and minimum mean temperatures (Fig. 2.2) during the trapping season in Jackson County, Mississippi were approximately 32.5 and 22.5°C, respectively. The weather station, which recorded the temperature data, was located in the center of Jackson County, MS and was approximately 10km from all collection sites (NOAA-NERRS 2012). No males were captured or tested because they rarely, if ever, leave their natal tree (Wood 1982). All living and apparently healthy female *X. glabratus* were transported to the laboratory immediately following daily trap checks. Supercooling point tests were conducted on

captured beetles within 2 h of trap collection. In total, 55 live *X. glabratus* female beetles were captured and transported to the laboratory for testing.

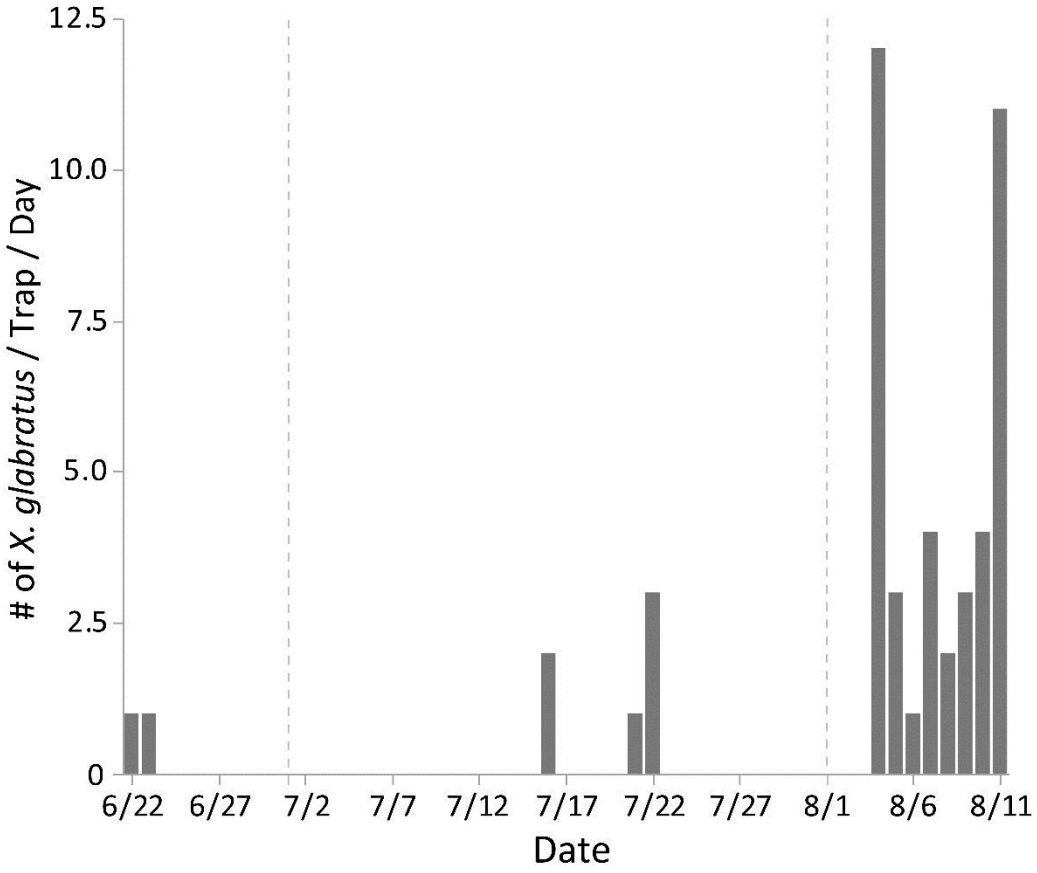

Figure 2.1 Frequency of summer-collected *Xyleborus glabratus* females collected during 2011 in Jackson County, Mississippi

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 baited with manuka oil and placed in symptomatic redbay stands. Beetles were transported to the lab and tested within 2 hours of capture.

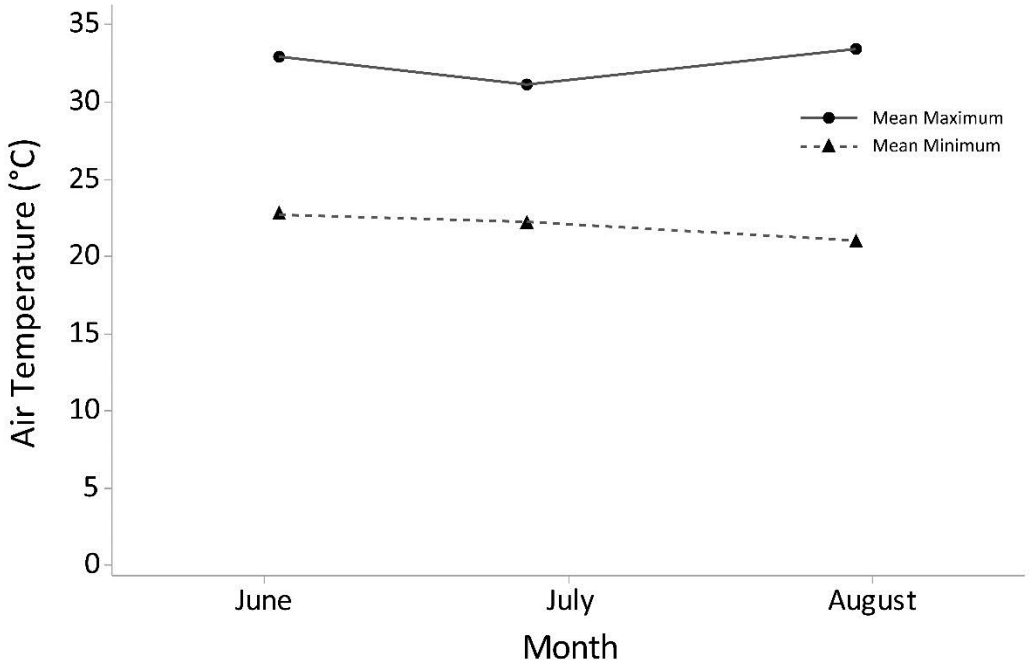

Figure 2.2 Maximum and minimum average air temperatures encountered by host seeking *Xyleborus glabratus* females during the summer of 2011 (June-August) in Jackson County, MS

The weather station, which recorded the data, was located in the center of Jackson County, MS.

2.1.2 Naturally acclimatized *Xyleborus glabratus*

Naturally acclimatized adult, female *X. glabratus* were collected monthly (September 2013 to May 2014) from freshly felled laurel wilt symptomatic redbay. The symptomatic redbay used in this study were felled in the Grand Bay National Estuarine Research Reserve (Grand Bay NERR) in Jackson County, Mississippi (N 30.4297° W -88.4279°; 2 m a.s.l.). Once felled, symptomatic redbay were cut into 33.0 cm bolts and immediately transported ≤ 3.0 km to the field station at Grand Bay NERR. To expedite the extraction process at the field station, the bolts were offloaded in a central work area with a concrete floor. During beetle extraction, a piece of white cardboard (~1.5 cm thick) was placed under each bolt. Each bolt was individually set up on the cardboard and

split (~halved) with a hatchet along its length repeatedly until it was reduced to small kindling. After each swing of the hatchet both the bolt and cardboard were examined for female *X. glabratus*. Beetles within galleries near the surface could be removed without damage, usually after a few hard taps on the spilt bolt with a hammer. Beetles found during extraction were carefully examined under a 10x hand lens (Baush and Lomb Model 81-61-71) for damage caused by the extraction process. Only beetles that appeared undamaged were collected for laboratory experimentations. The extraction process was repeated until all the bolts from a single tree were reduced to kindling and examined for the presence of *X. glabratus*.

Once the extraction process was complete, the beetles were taken immediately from the work area into the laboratory and allowed to warm to room temperature (~21 °C) for 10 minutes. The beetles were then examined under stereomicroscope (Leica, brand, etc.) to check for physical condition and mobility. Only beetles that appeared healthy and were able to crawl were used for experimentation (this was to avoid analyzing dead or dying specimens). Beetles used in the supercooling point study were immediately tested. Beetles intended for biochemical analysis were immediately placed in a -15 °C freezer. If a single redbay produced only a few adult female beetles, then they were frozen for biochemical analysis and another tree was felled and the process was repeated until enough specimens were collected for supercooling point determination.

2.1.3 Artificial acclimation of *Xyleborus glabratus*

Female *X. glabratus* were reared from symptomatic redbay bolts in Jun 2012. All symptomatic redbay bolts were obtained in the same forest locations as the 2011 study. The beetles were reared from 33.0 cm bolts of redbay cut from infested trees. Trees were

felled during the spring of 2012 and were promptly placed in 50 gallon rearing containers after being cut to proper bolt length. During the rearing process, the rearing containers were kept outside in a shaded area and checked daily. On 14 Jul 2012, approximately 300 apparently healthy, mature female *X. glabratus* were collected from the rearing containers. Beetles were placed in a low temperature incubator (VWR International, Model 2015, Radnor, Pennsylvania) for 31 days and acclimated with a thermo-photoperiod of 7 ºC:2 ºC (10:14 h L:D). This thermo-photoperiod was meant to simulate a natural cycle during the late autumn months near the northerly, inland limits of sassafras (e.g. North-Central Pennsylvania).

Initially, female *X. glabratus* were introduced into a 20 ºC incubator. Immediately following introduction, the temperature was lowered to 7 ºC at a rate of 1.0 ºC/day and when 7 ºC was reached (day 14) the thermoperiod was initiated (Fig. 2.3). At 31 days, 200 beetles were removed from the incubator and warmed to ambient temperatures (~21 ºC) for 2 hours. At the end of the 2 hours each beetle was given a survival rating (dead, limited response, or highly responsive) based on a response to stimulus (i.e. a small, fine-tipped paintbrush). Supercooling was performed only on highly responsive beetles, in total, 111 highly responsive and apparently healthy *X. glabratus* were successfully acclimatized and used for SCP determination.

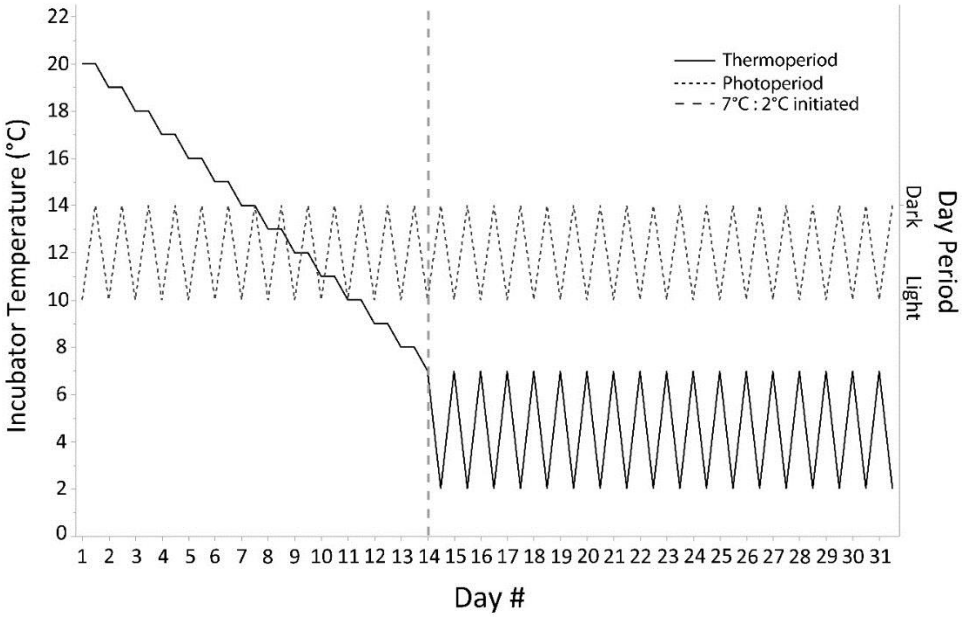

Figure 2.3 Artificial acclimatization cycle of *Xyleborus glabratus*.

Beetles initially were introduced into a 20 ºC incubator. Following introduction, the temperature was lowered to 7 ºC at a rate of 1.0 ºC/day and when 7 ºC was reached (day 14) the thermoperiod (7 ºC:2 ºC) was initiated.

2.1.4 Supercooling point determination

The supercooling points of individual *X. glabratus* were measured in real-time using a type-T (copper constantan; ±1.0°C standard error) thermocouple connected to a Picotech TC-08 data logger (Pico Technology®, Cambridge, UK). Temperatures from the data logger were recorded and graphed using PicoLog software for Windows (v. 5.2; Pico Technology®, Cambridge, UK) at 1 second intervals. Each specimen was placed in a 1.5 ml microcentrifuge tube and a type-T thermocouple was positioned such that the exposed wire was in direct contact with the insect cuticle. The beetle and thermocouple were held securely in place at the bottom of the microcentrifuge tube by a foam stopper. Each microcentrifuge tube was inserted into a flat foam float (6 mm thick) until the bottom of the tube protruded 2.5 cm beyond the float (to ensure the beetles were fully

submerged during testing). The flotation apparatus was then placed in a refrigerated liquid bath (PolyScience® Model 8006 6L) containing potassium formate (Dynalene® HC 50) (Andreadis et al. 2005; Ansart et al. 2007). The initial temperature of the bath was based on the ambient temperature at time of collection (to minimize stress to the insects). The potassium formate was cooled from the initial temperature to -30 °C at a rate of -1.0 °C /min. This cooling rate was used for several reasons; 1) it is the most common cooling rate in the literature, and 2) cooling rate has little effect on supercooling point (Salt 1966; Hahn et al. 2008; Crosthwaite et al. 2011). The supercooling point of each beetle was the lowest temperature recorded before the release of exotherm (Lee 1989; Koštal and Šimek 1996). After supercooling points were reached, the beetles were removed from the liquid bath, allowed to warm to room temperature (~21 °C) for 10 minutes and checked for survival. Each beetle was then placed into a new 1.5 ml microcentrifuge tube containing damp filter paper (to minimize desiccation) and held at room temperature for 24 hrs. The final survival rating of each beetle was confirmed and recorded 24 hours after testing.

Supercooling points were tested on naturally acclimatized beetles that had been exposed to natural climatic variations and artificially acclimatized beetles that had been subjected to a colder temperature cycle. The artificially acclimatized beetles were tested over the course of a few days; however, every month eight naturally acclimatized beetles were collected from symptomatic redbay trees and tested. The seasonal data in the results section were pooled and average every 3 months from monthly data.

2.1.5 Determination of lower lethal temperature

The lower lethal temperature of *Xyleborus glabratus* was determined by exposing female beetles to a variety of temperatures (5, 0, -5, and -10°C) and a variety of exposure times (6, 8, 10, and 24 hours). Tests were conducted on naturally acclimatized (section 2.1.2) reared (section 2.1.3), and artificially acclimatized female *Xyleborus glabratus*. However, due to time and travel constraints, not all physiology types were exposed to every time and temperature. Temperatures on the day of collection (12 December 2012) averaged 7.8°C with a maximum and minimum of 15.5°C and 0.6°C, respectively. In all, 34 beetles were naturally acclimatized and collected on 12 December and exposed to 24 hours of 0.0, -5.0, and -10.0°C. Artificially acclimated beetles, on the other hand, were subjected to 6 hours of -10.0°C only because of time and location complexities. In all, twenty beetles were artificially acclimated and tested for lower lethal temperature tolerance. A total of 350 were reared from containers and tested for low temperature mortality.

Lower lethal temperatures of *X. glabratus* were tested in a low temperature incubator (VWR International, Model 2015, Radnor, Pennsylvania) programmed to lower the temperature at a rate of -1.0°C/minute. Beetles were introduced to the incubator set at an initial temperature matching the conditions of the cohort prior to testing. For example, naturally acclimatized beetles collected on 12 December 2012 were introduced to an incubator set at 8.0°C; whereas, artificially acclimated beetles were placed into a 7.0°C incubator. This was meant to limit the amount of stress placed on the beetles immediately before testing. The exposure time started when the experimental temperature for that cohort was reached. After the exposure time was reached, the beetles were removed from

the incubator, warmed to room temperature (~21 °C) for 10 minutes and checked for survival. Each beetle was then placed into a 1.5 ml microcentrifuge tube containing damp filter paper (to minimize desiccation) and held at room temperature for 24 hrs. The survival rating recorded for each beetle was reconfirmed 24 hrs after removal from the incubator.

2.2 Biochemical analyses

2.2.1 Preparation of *Xyleborus glabratus* whole body homogenates for analyses

Homogenate preparation and biochemical assays were derived from methods described by Bemani et al. (2012). Female X. glabratus (n = 8 from each month) were individually macerated in 150 µl of 2% Na_2SO_4 using a motor driven pestle homogenizer in 1.5 ml microcentrifuge tubes for 1 minute. 500 µl of chloroform: methanol (1:2) was added to each homogenate and vortexed rapidly for 1 minute. The samples were then centrifuged at 8000 x g for 10 minutes at 4°C in an angle-head rotor of an Eppendorf 5430 R table top centrifuge. The resulting supernatant and pellet were analyzed to determine the amount of total body sugars, glycogen, and lipids in each insect. All results are reported as µg of biomolecular content/insect.

2.2.2 Total body sugars (mono and disaccharides)

150µl of the supernatant from the initial procedure (section 2.2.1) was extracted from each sample and placed into fresh 1.5 ml microcentrifuge tubes. 100µl of deionized H_20 was then added to the supernatant and vortexed for 1 min. 500µl of anthrone reagent (500mg anthrone dissolved in 500ml of concentrated H_2SO_4) was added to the mixture. Samples were reacted in a dry bath for 10 min at 90 °C. 200µl of each sample was

pipetted in triplicate (600µl total from each sample) into a 96-well microtitre plate. The samples were read at 630 nm on a microplate spectrophotometer (BioTek Synergy H1M). The amount of sugars in the sample were calculated from raw values using a D-glucose standard curve (Benmani et al. 2012).

2.2.3 Glycogen

The pellet resulting from the initial homogenate procedure (section 2.2.1) was washed with 400µl of 80% methanol in a microcentrifuge to remove any remnants of sugars. The sample was then vortexed for 1 minute. The resulting mixture was centrifuged at 6000 x gravity for 5 minutes at 4°C. Following the centrifugation process the supernatant was discarded and 250µl of deionized H_20 was added to the pellet. The pellet and water was vortexed for 1 minute and heated for 5 minutes at 70°C. Following heating, 200µl of the solution was removed and put into fresh microcentrifuge tubes. The sample was reacted for 10 minutes at 90°C with 500µl of anthrone reagent (500mg anthrone dissolved in 500ml of concentrated H_2SO_4). 200µl of each sample was pipetted in triplicate (600µl total from each sample) on a 96-well microtitre plate. The samples were read at 630 nm on a microplate spectrophotometer (BioTek Synergy H1M). The amount of glycogen in each sample was calculated from raw values using a glycogen standard curve (Benmani et al. 2012).

2.2.4 Lipids

5µl of the supernatant from the original procedure (section 2.2.1) was extracted and placed into fresh Eppendorf tubes. 100µl of H_2SO_4 was added to the 5µl of supernatant and vortexed for 1 minute. The resulting mixture was heated in a dry bath for

10 minutes at 100°C. The solution was then cooled for 10 minutes after which 500µl of vanillin solution was added. The mixture was vortexed for 1 min and allowed to react for 30 min. 200µl of each sample was pipetted in triplicate (600µl total from each sample) on a 96-well microtitre plate. The samples were read at 540 nm on a microplate spectrophotometer (BioTek Synergy H1M). The amount of lipids in the sample were calculated from raw values using an Oleic acid standard curve (Benmani et al. 2012).

2.3 Statistical analyses

Monthly, seasonal, and artificially acclimated beetle supercooling points and biochemical data were tested for normality using a Shapiro-Wilk test. Data from normal distributions were analyzed across all months using one-way ANOVA and means were compared using a Tukey-Kramer HSD *post hoc* test. Nonparametric data were analyzed using a Kruskall-Wallis test and means were compared using the Steel-Dwass all pairs method.

Linear regressions were performed to determine the relationship, if any, between temperatures, biochemicals, and supercooling points. The temperature data were downloaded through the NOAA NERRS Centralized Data Management Office website (Fig. 2.4; accuracy: ± 0.2 °C at 20°C). The meteorological station that recorded the temperature data was located ~3 km from the symptomatic redbay used for beetle collection. The data were also fit to non-linear and generalized linear regressions, however, linear regressions produced better statistical estimates.

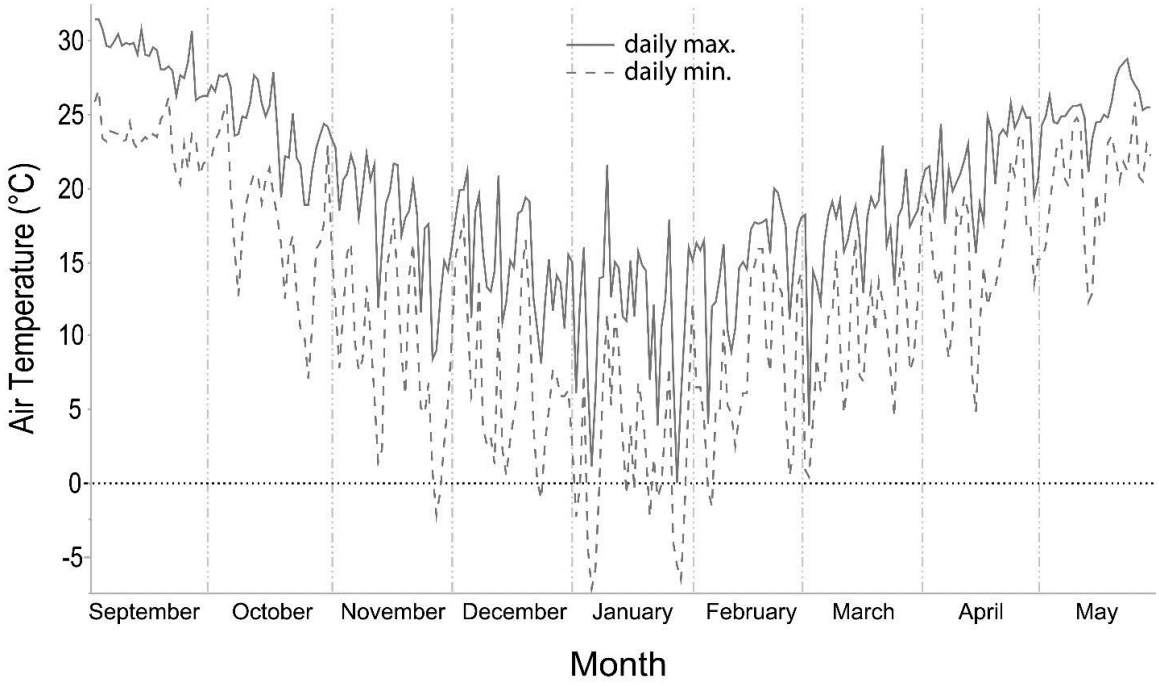

Figure 2.4 Temperature data (accuracy: ± 0.2 °C at 20°C) from the Grandbay National Estuarine Research Reserve redbay/*Xyleborus glabratus* collection site.

The meteorological station that recorded the temperature data was located ~3 km from the symptomatic redbay used for beetle collection. Monthly average temperatures were regressed against supercooling points and biochemicals to determine if any relationships between variables existed.

Biochemical concentrations (X) were regressed against SCPs (Y) to determine if X were correlated to Y. Air temperatures (X) were independently regressed against supercooling points (Y) and biochemical concentrations (Y) to determine if X were correlated to Y. Linear regression were used to determine if body size and weight had an effect on supercooling points. Furthermore, multivariate regressions (i.e. standard least squares) were used to determine if there was a combined influence of the predictor variables on the response. When two predictor variables were used, a full factorial design was incorporated (to create an interaction variable) into the analysis. Three multivariate regressions independently measured the influence of mean monthly air temperatures (X_1),

a mean monthly biochemical concentration (X_2), and the interaction between X_1 and X_2 on supercooling points (Y). Another multivariate regression measured the influence of mean monthly biochemical concentrations ($X_1, X_2, X_3,$) on SCPs (Y), while the final multivariate regression measured the influence of mean monthly air temperature (X_1) and mean monthly biochemical concentrations (X_2, X_3, X_4) on supercooling points (Y). All statistical tests and analyses were performed in JMP Pro 11.0 (SAS Institute Inc., Cary, NC, USA). Mean ± standard error is reported throughout.

2.4 Determination of Body Size and Mass

Following the determination of supercooling points, the pronotal width of naturally acclimatized *Xyleborus glabratus* collected in June, July, and August were measured to the nearest 0.001 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 70ºC for 24 h (Riggins et al. 2009). The beetles were then weighed to the nearest 0.001mg in a covered Mettler UMT2 Micro-Balance® analytical scale (Mettler-Toledo International, Inc.).

2.5 Sassafras Buffering Study

This experiment was conducted in northwest Mississippi because sassafras is virtually non-existent along the Gulf Coast (USDA Forest Service, Forest Inventory and Analysis). Additionally, even after intensive field surveys, no specimens could be located at the Grand Bay National Estuarine Research Reserve (Grand Bay NERR) site. Permission was obtained in late July 2013 to use a large, mature sassafras on private

property in northwest Mississippi (Desoto County, Mississippi). This specimen likely is a better representation of sassafras occurring along higher latitudes of the United States than a sassafras located along the Gulf Coast of Mississippi. For example, the research site in northwest Mississippi averages extreme minimum temperatures between -15.0 to -12.2ºC (USDA Zone 7a) annually, compared to the coastal research site at Grand Bay NERR which averages extreme minimum temperatures between -6.7 to –3.9ºC (USDA Zone 9a) annually. The temperatures at the northwest Mississippi site not only are lower during the winter, but are lower for longer duration; therefore, the tree in northwest Mississippi should provide temperature data more representative of higher latitudes of the United States.

This specific tree was chosen because it grows entirely within a forest stand, like many of the other naturally regenerated sassafras trees spread across the eastern United States. There were other mature sassafras trees in the area; however, they were growing along fence lines or in open fields. The topography of the site was mostly flat and tree was sheltered on all sides from direct wind by codominant, intermediate, and overtopped/understory trees. The tree was surrounded by mature oaks, other mature broadleaf trees, and some mature and juvenile eastern red cedars (*Juniperus virginiana* L.); thus, the canopy around the tree was closed to direct sunlight, even during the winter months.

Internal tree temperatures were recorded at two different depths, 5.1 cm and 12.7 cm, within a live, ~25.0 cm DBH sassafras (*Sassafras albidum* (Nutt.) Nees) tree (Fig. 2.5). Ambient data were recorded 920 cm away, and not on the surface of the tree, to minimize any radiant heat effect from the bark. Each data logger was connected to a

temperature sensor via a 1.8 meter data cable. All three data loggers were attached to a mounting board (5.1 cm x 10.2 cm x 1.4 m) that was driven into the ground ~1.0 meter away from the tree. The holes created (13 mm drill bit) for the thermocouples entered on the south side of the tree and silicon was placed around the data cable at the entrance of the hole to minimize temperature loss/gain and protect the thermocouples.

Data collection began August 2013 and continued for one year; however, only winter (December – February) data were used for analyses. Temperatures were recorded at 1 hour intervals for the entire sampling period. At the end of the sampling period, the data on each logger were transferred to a computer via a HOBO® Waterproof Shuttle (Onset Corp., U-DTW-1). The shuttle has the capacity to read and store all logger information (serial number, deployment number, data, and battery status) in the field until it is transferred to a computer program and analyzed (HOBOware® Pro, Onset Corp., Version 3.0).

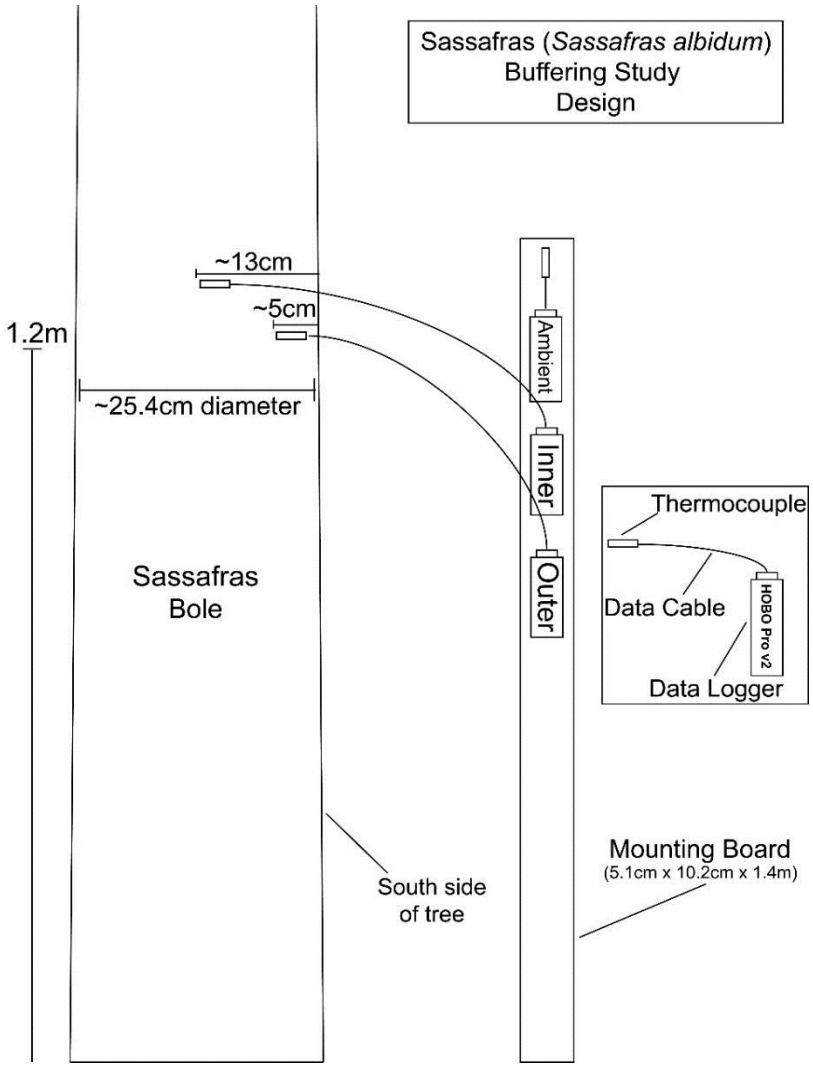

Figure 2.5 Design of the sassafras buffering study

Internal tree temperatures were recorded at two different depths (5.1 cm and 12.7 cm) within a live, ~25.0 cm diameter sassafras (*Sassafras albidum* (Nutt.) Nees) tree. Ambient data were recorded 920 cm away from the sassafras, and not on the surface of the tree, to minimize any radiant heat effect from the bark. All three data loggers were attached to a mounting board which was driven into the ground ~1.0 meter away from the tree. The thermocouples were placed on the south side of the tree and silicon sealant was placed around the data cable.

Quantifying the differences between ambient air temperatures and temperatures within a living sassafras helped define the amount of thermal protection sassafras offers *X. glabratus* during the winter. Additionally, these buffering data (section 3.1.7) were

used to correct the ambient air temperature data used in the invasion potential models (i.e. species distribution models). Sassafras was chosen for this study because it is more widely distributed and ranges into higher latitudes than any other species susceptible to laurel wilt, perhaps with the exception of northern spicebush. Northern spicebush has a distribution comparable to sassafras, but the majority of stem diameters on northern spicebush are small. Small stems are not as attractive to *X. glabratus* as large stems (Mayfield and Brownie 2013), nor do small stems support brood development as well as large stems (Brar et al. 2013; Maner et al. 2013). Therefore, northern spicebush may not be biologically relevant to *X. glabratus* spread.

2.6 Modeling *Xyleborus glabratus* invasion potential

All invasion potential modeling was completed using a geographic information system (ESRI®). One temperature dataset was used to model *Xyleborus glabratus* invasion potential in the United States. The dataset covered the conterminous United States and excluded Alaska and Hawaii (projection: Mercator Auxiliary Sphere). The dataset was a 30-year (1976-2005) mean annual "extreme" minimum temperature raster data file (signed integer; Copyright© PRISM Climate Group, July 2006, Oregon State University). This dataset had a standard deviation of ±6.56°C. The map datum and spatial resolution of both datasets were NAD83 and 800 meters (30 arc-seconds), respectively.

Temperature data originated from a collection of approximately 8,000 major climate network stations across the United States, Canada, and Mexico. Monthly averages were calculated from daily data. The PRISM (Parameter-elevation Relationships on Independent Slopes Model) interpolation method (nearest neighbor) for both datasets used a 1 degree (3 arc-second) digital elevation model (DEM) as a predictor grid. The

accuracy of the DEMs was 130 meter circular error with 90% probability. This PRISM interpolation method calculated a climate–elevation regression for each DEM cell, which is well suited to regions with mountainous terrain because it incorporates a framework that addresses the spatial scale and pattern of orographic processes.

The station data were assigned weights based on the geographical and physical similarity of the station to the grid cell. The geographical and physical attributes considered were location, elevation, coastal proximity, slope, and aspect. Aspect (the azimuth of a grid cell in degrees), in particular, is one of the attributes that increases the interpolation power of PRISM in mountainous terrain because it is directly related to temperature and moisture levels, especially when slope is greater than zero, e.g. northern aspect in the Northern Hemisphere (0 or 360°) is cooler and more moist than a southern aspect (180°), which is warmer and drier. The goal of the PRISM interpolation method was to generate repeatable climate estimates that closely simulate the current state of climate patterns in the United States (Daly et al. 2008). As a result, the datasets have been heavily peer reviewed, and in fact, the PRISM dataset is used in the USDA Plant Hardiness Zone Map to determine the distribution of plant species (Bishop et al. 1998; USDA-NRCS 1998; Daly and Johnson 1999; Vogel et al. 1999; Daly et al. 2001).

The PRISM temperature data (-39.0°C to 20.0°C) were reclassified (Spatial Analyst, Reclassify Tool) into three classes to create each hazard map. Reclassification was based on the *X. glabratus* lower lethal temperature and artificially acclimatized cold hardened data from this study. The three temperature classes for the current hazard rating were; 4 (temperatures > -10.0°C, no survival limitations); 2 (temperatures -13°C to -10°C, limited survival); 0 (temperatures < -13°C, no survival), and the 3 temperature

classes for the future, climate change (+1.5°C based on current IPCC estimates) hazard rating model were; 4 (temperatures > -11.5°C, no survival limitations); 2 (temperatures -14.5°C to -11.5°C, limited survival); 0 (temperatures < -14.5°C, no survival).

Sassafras distribution data (trees/hectare) were downloaded from the USDA Forest Service Forest Inventory and Analysis database. Redbay, California laurel, pondspice (*Litsea aestivalis*), silkbay (*Persea humulis* Nash), and avocado (*P. americana* Mill.)) were excluded from the analyses because they grow in regions where minimum winter temperatures are too warm to limit establishment. Other species, such as northern spicebush (*Lindera benzoin* (L.) Blume), were excluded because there were no reliable raster datasets of their distribution. The sassafras dataset was reclassified and individually multiplied (Raster Calculator) by each temperature dataset. The sassafras data (1-100 trees/hectare) were reclassified (Spatial Analyst, Reclassify Tool) into three classes based on studies examining *X. glabratus* attractiveness to sassafras stands of differing densities (e.g. Cameron et al. 2015; Fraedrich et al. 2015). The three sassafras density values used for determining hazard rating were; 3 (>60 trees/hectare); 2 (30 to 60 trees/hectare); 1 (< 30). Final hazard rating models were created by multiplying (Spatial Analyst, Raster Calculator) the reclassified temperature data by the reclassified sassafras data (Fig. 2.6).

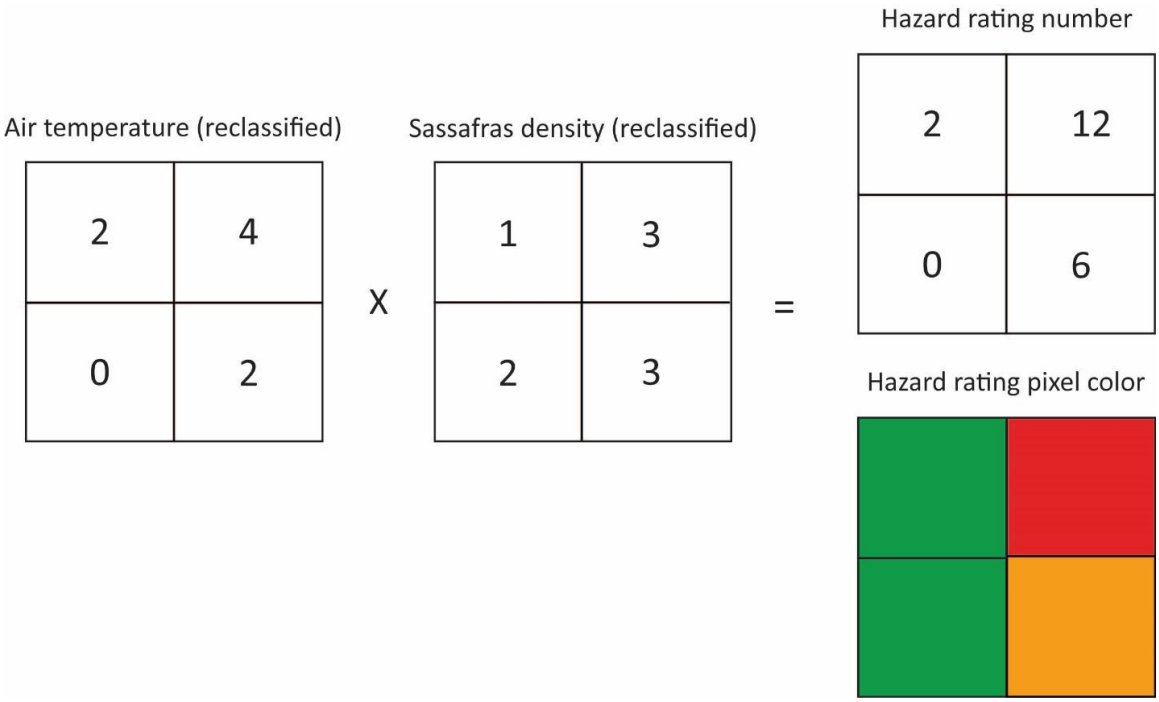

Figure 2.6 Example raster calculator of air temperatures (°C; reclassified values) multiplied by sassafras density (trees/hectare; reclassified values).

The original air temperature data was reclassified based on the *X. glabratus* lower lethal temperature, sassafras buffering, and artificially acclimatized cold hardened data from this study. The original sassafras data were reclassified based on studies examining *X. glabratus* attractiveness to sassafras stands of differing densities (Cameron et al. 2015; Fraedrich et al. 2015).

CHAPTER III

RESULTS OF COLD TOLERANCE STUDIES AND REDBAY AMBROSIA BEETLE INVASION MODELING

3.1 Results

3.1.1 Supercooling points

Supercooling points (SCPs) of naturally acclimatized beetles were significantly warmer than artificially acclimatized beetles, i.e. beetles artificially acclimatized had significantly colder mean supercooling point than naturally acclimatized beetles (X^2 = 46.0, $P < 0.0001$; Fig. 3.1). However, there was no significant difference in naturally acclimatized SCPs across the seasons ($X^2 = 0.97$, $P = 0.91$). There was a significant difference between naturally acclimatized (monthly tested) and artificially acclimated in SCPs ($X^2 = 74.4$, $P < 0.0001$; Fig. 3.2); however, there was no significant monthly change in SCPs among naturally acclimatized *X. glabratus* ($X^2 = 12.0$, $P = 0.15$). The warmest SCP (-7.82°C) was recorded from an artificially acclimatized beetle and the coldest SCP (-26.98°C) was recorded from a naturally acclimatized beetle. Any number of physiological or behavioral differences could be driving this phenomenon.

Mean pronotal width of the female *X. glabratus* collected from June, July, and August of 2011 was 0.701 ± 0.003mm (± SE). The results from a generalized linear model regression indicated no significant correlation between SCPs and pronotal width

(P = 0.23; R^2 = 0.03). Mean dry biomass of all samples from June, July, and August 2011 was 0.283 ± 0.006 mg (± SE). A linear regression of SCP to biomass also indicated no significant relationship between SCP and dry biomass (P = 0.38; R^2 = 0.02). A multivariate linear regression of the dependent variable (SCP) to the independent variables (pronotal width and biomass) suggested that there was no statistically significant pronotal width x biomass interaction on SCPs (P = 0.47; F_{145} = 0.78; R^2 = 0.03).

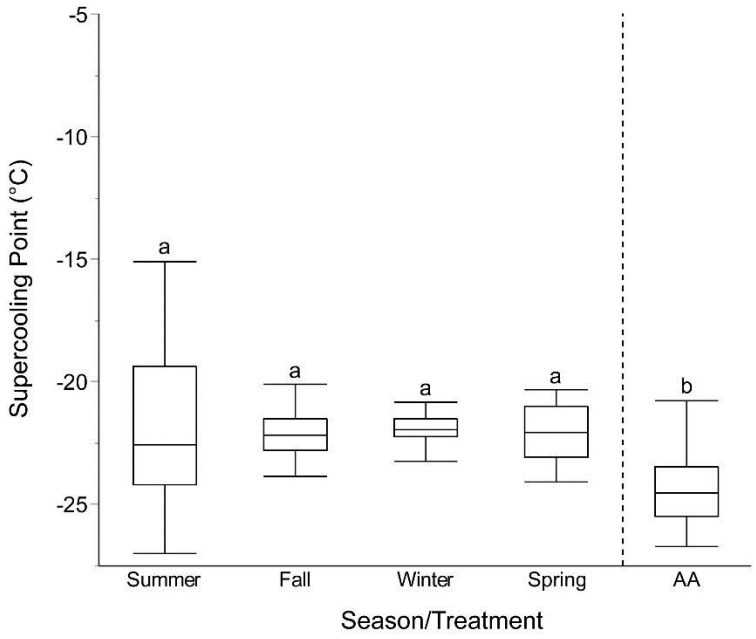

Figure 3.1 Mean supercooling point temperature (°C) of naturally acclimatized (seasonally tested) and artificially acclimatized (AA) specimens

Data were collected from 2011 to 2014 in Jackson County, MS. Mean data with the same letters are not significantly different (α = 0.05; Wilcoxon Test).

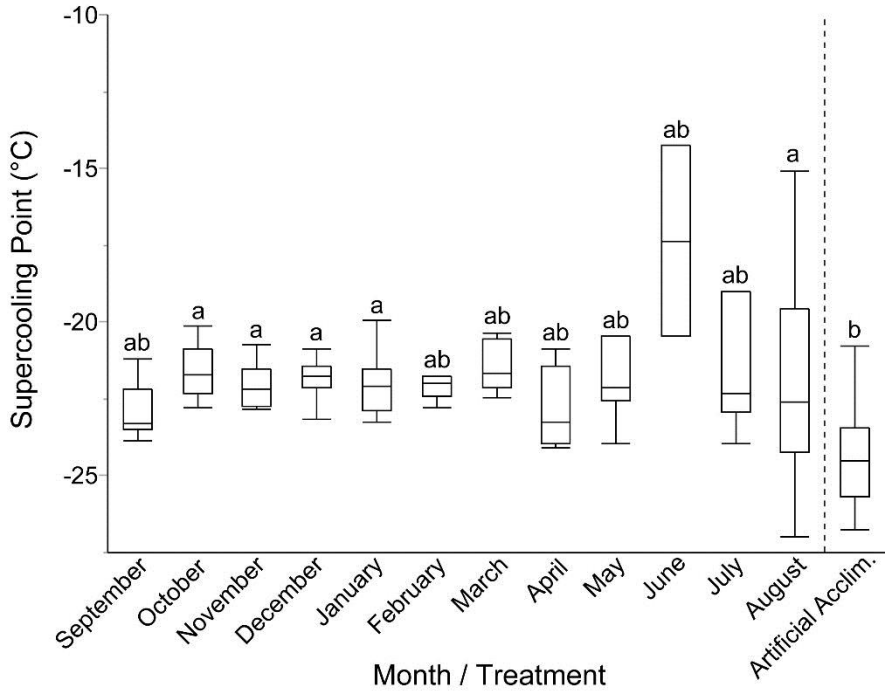

Figure 3.2 Mean supercooling point temperature (°C) of naturally acclimatized (monthly tested) and artificially acclimatized *Xyleborus glabratus*.

Data were collected from 2012 to 2014 in Jackson County, MS. Mean data with the same letters are not significantly different ($\alpha = 0.05$; Wilcoxon Test).

3.1.2 Total body sugars and glycogen

Seasonal total body sugar concentrations were significantly higher in the winter than in the fall or spring ($X^2 = 40.3$, $P < 0.0001$; Fig. 3.3); thus, seasonality had a significant effect on total body sugars. This seasonal trend was inversely related to the seasonal trend in glycogen concentrations, which were significantly lower in winter than in the fall or the spring ($X^2 = 23.5$, $P < 0.0001$; Fig. 3.3). Monthly total body sugars gradually increased September through December and peaked in January (143.56 ± 1.45 µg/insect; Fig. 3.4). However, after March, total body sugars declined (e.g. 51.21 µg/insect between March and April measurements) and returned to September levels by May (Fig. 3.4). On the other hand, monthly glycogen concentrations generally decreased

after September, but increased after February (i.e. 32.03 μg/insect increase between March and May measurements) and reached the highest levels in May (72.04 ± 1.27 μg/insect) and the lowest level in February (33.96 ± 4.10 μg/insect). The trend in monthly glycogen concentrations was also inversely related to the monthly trend in total body sugars from September to May, which was similar to the inverse relationship between seasonal trends of glycogen and total body sugars.

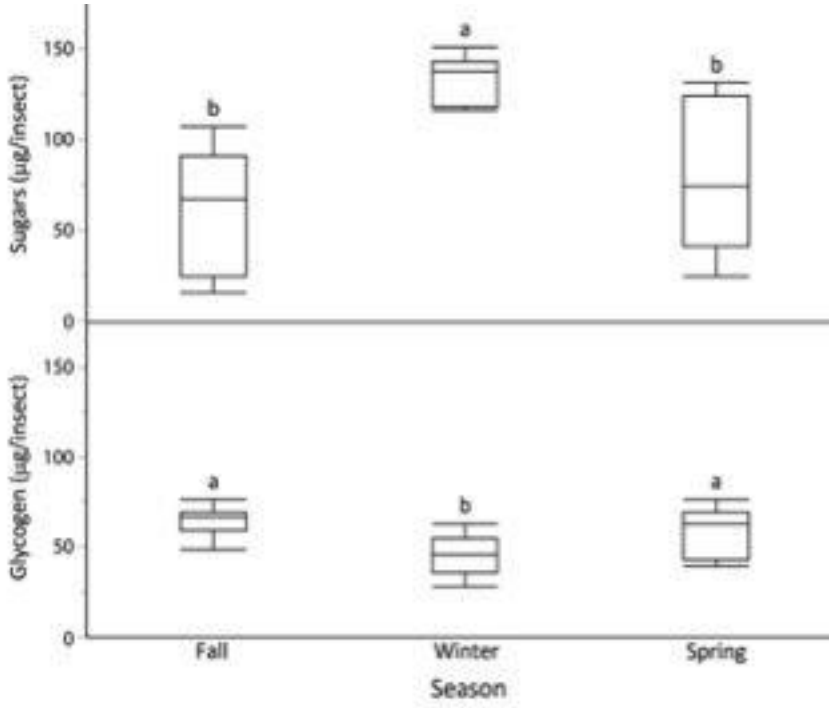

Figure 3.3 Mean total body sugar and glycogen concentrations (μg/insect) of naturally acclimatized (seasonally tested) *Xyleborus glabratus*

Data were collected from 2013 to 2014 in Jackson County, MS. Mean data with the same letters are not significantly different ($\alpha = 0.05$; Wilcoxon Test).

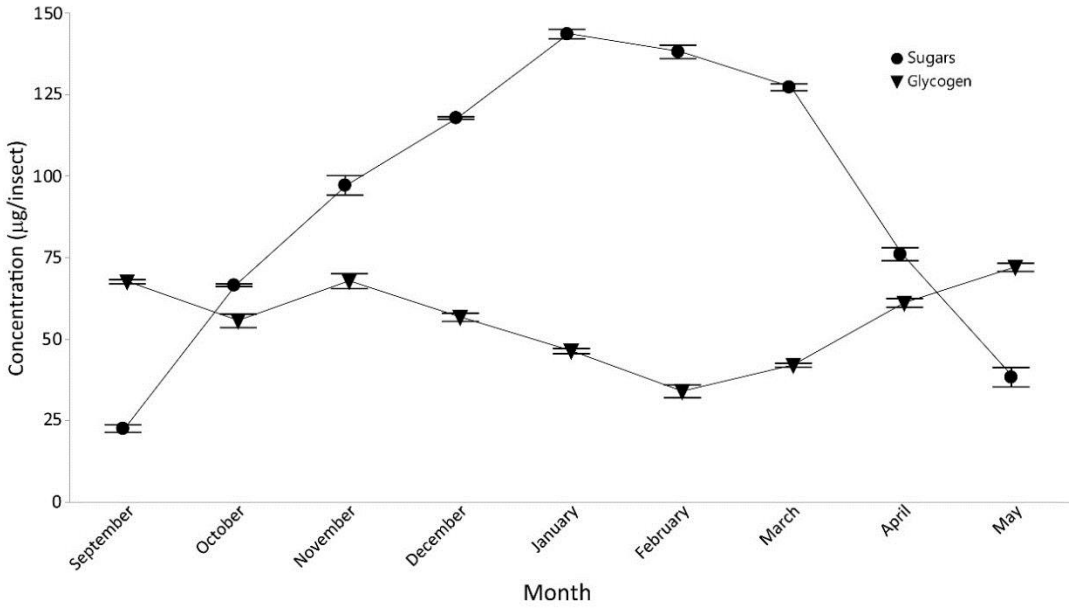

Figure 3.4 Mean total body sugar and glycogen concentrations (µg/insect) of naturally acclimatized (monthly tested) *Xyleborus glabratus*.

Data were collected from 2013 to 2014 in Jackson County, MS. Glycogen concentrations from September to May were inversely related to the monthly trend in total body sugar concentrations.

3.1.3 Lipid contents

Seasonality had a significant effect on lipids in *X. glabratus* ($R^2 = 0.33$, $P <$ 0.0001). The highest lipid concentrations (21.21 ± 0.50 µg/insect) occurred in winter, and the lowest levels occurred in the spring (Fig. 3.5). Monthly lipid levels were also significantly affected by temperatures ($F_{8, 63} = 11.74$, $P < 0.0001$), but with the exception of November, the levels generally remained stable from September-March (Fig. 3.6). After March, however, lipid levels declined (e.g. 5.17 µg/insect difference between March and April measurements) and reached the lowest levels (15.33 ± 0.30 µg/insect) in April. Lipids levels then increased slightly (1.38 µg/insect) from April to May.

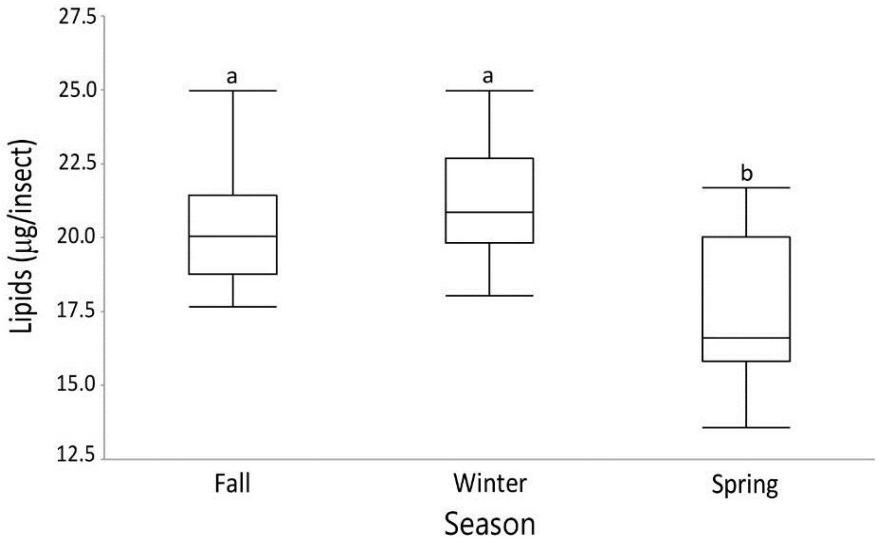

Figure 3.5 Mean lipid concentrations (µg/insect) of naturally acclimatized (seasonally tested) *Xyleborus glabratus*

Data were collected from 2013 to 2014 in Jackson County, MS. Mean data with the same letters are not significantly different ($\alpha = 0.05$; Tukey HSD).

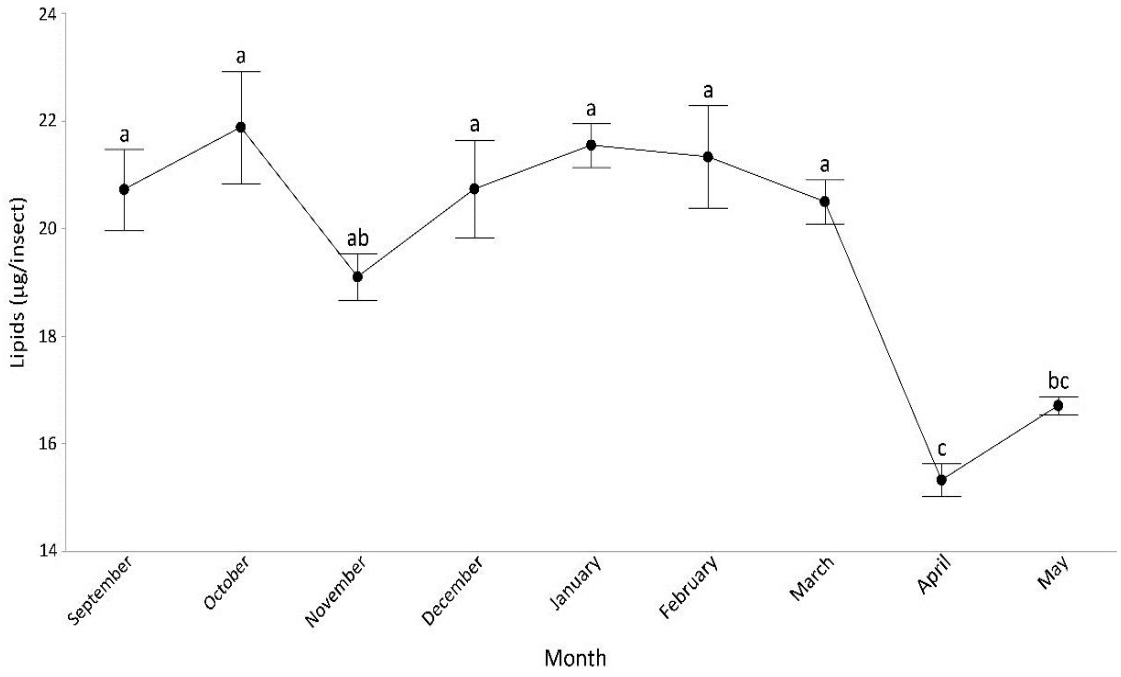

Figure 3.6 Mean lipid concentrations (µg/insect) of naturally acclimatized (monthly tested) *Xyleborus glabratus*.

Data were collected from 2013 to 2014 in Jackson County, MS. Mean data with the same letters are not significantly different ($\alpha = 0.05$; Tukey HSD).

3.1.4 Influence of temperature on supercooling points and biochemical concentrations

Air temperatures had no significant correlation to supercooling points (R^2 = 0.011, P = 0.79; Fig. 3.7) or lipids over the course of the study. However, air temperatures were negatively correlated to total body sugar (R^2 = 0.94, P < 0.0001) and positively correlated to glycogen (R^2 = 0.44, P = 0.05) concentrations in the insect body (Fig. 3.8).

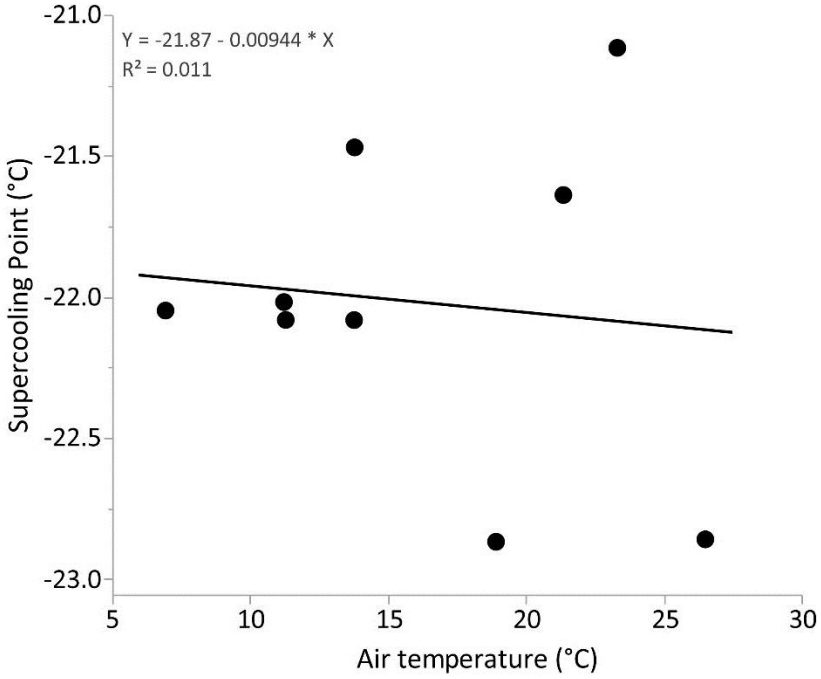

Figure 3.7 Relationship between mean monthly supercooling point (°C) and ambient air temperatures (°C

Data collected from 2013 and 2014 in Jackson County, MS. Air temperatures had no significant correlation to supercooling points (P = 0.79).

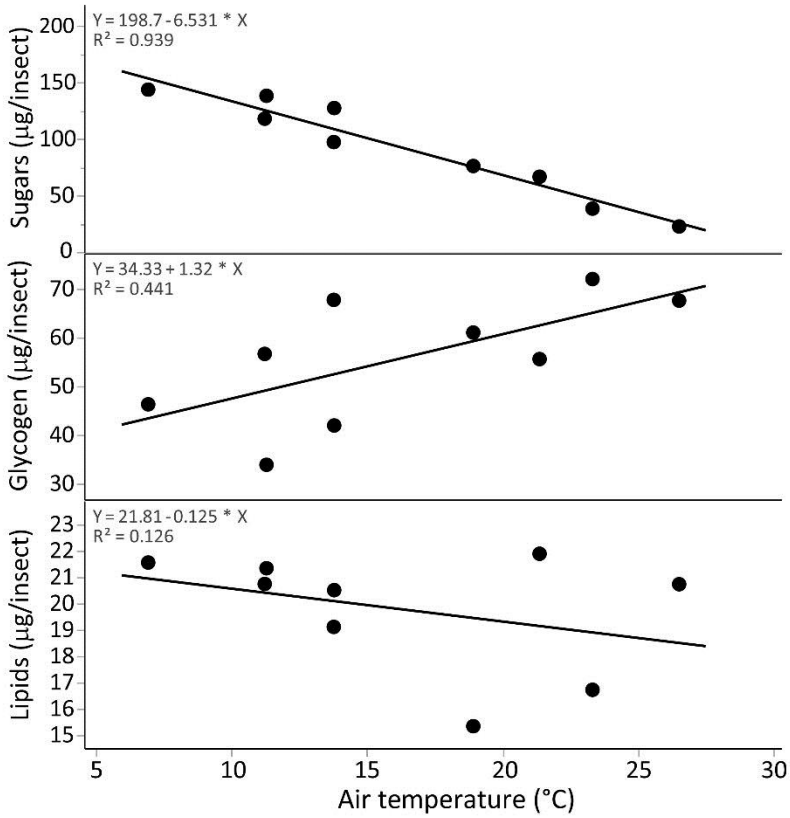

Figure 3.8 Relationships between mean monthly biochemical concentrations (µg/insect) and ambient air temperatures (°C)

Data collected from 2013 and 2014 in Jackson County, MS. Air temperatures had no effect on lipid concentrations ($P = 0.35$), but were found to have a significant effect on total body sugars ($P < 0.0001$) and glycogen ($P = 0.05$) concentrations.

3.1.5 Influence of biochemical concentrations on supercooling points

The amount of sugars in the insect body had no significance effect on supercooling points ($R^2 = 0.02$, $P = 0.72$; Fig. 3.9). Likewise, the amount of glycogen and lipids had little effect on supercooling points ($R^2 = 0.01$, $P = 0.78$ and $R^2 = 0.02$, $P = 0.75$, respectively; Fig. 3.9). The interaction among the lipid, sugar, and glycogen concentrations had a greater influence on the supercooling points than did any biochemical alone ($R^2 = 0.23$, $P = 0.18$).

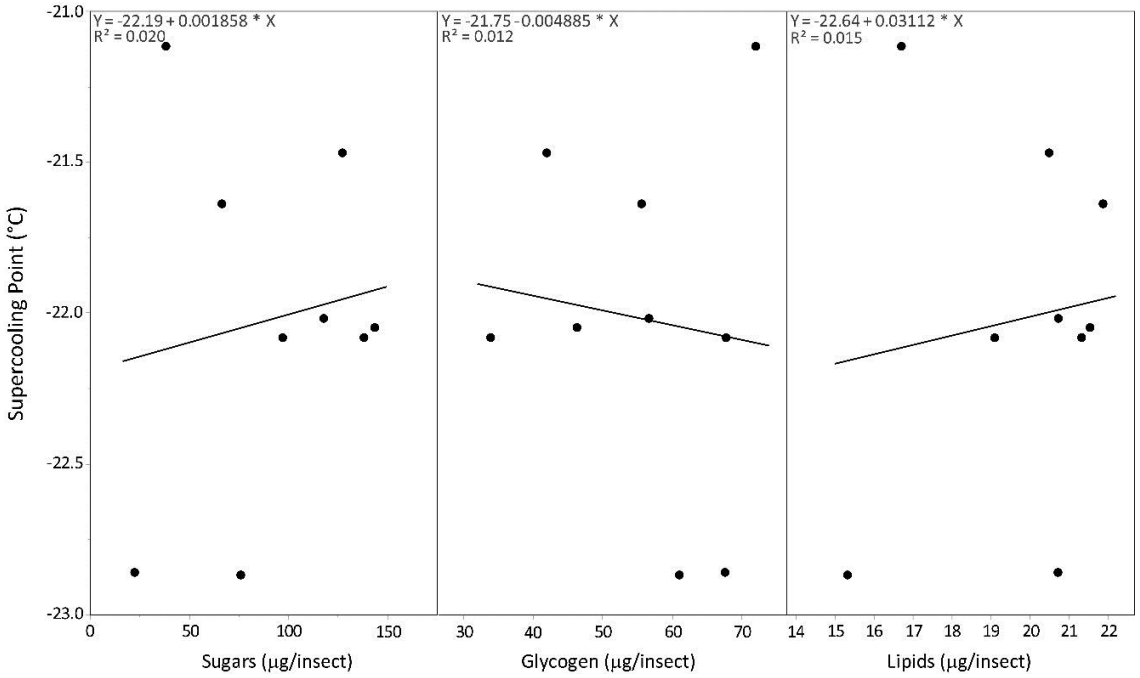

Figure 3.9 Relationships between mean monthly biochemical concentrations (µg/insect) and supercooling points (°C)

Data collected from 2013 and 2014 in Jackson County, MS. The amount of total body sugar, glycogen, and lipid concentrations had no influence ($P = 0.72$; $P = 0.78$; $P = 0.75$, respectively) on supercooling points.

3.1.6 Lower lethal temperatures

All beetles from each treatment were checked at 10 minutes and again at 24 hours after testing. All beetles, regardless of the time/temperature regime, which were categorized as "injured" at 10 minutes were dead 24 hours after the experiment. The results are summarized in Figure 3.10. *Xyleborus glabratus* mortality significantly decreased as temperatures increased ($R^2 = 0.57$, $df = 45$, $P < 0.0001$). Control treatments had significantly lower mortality than the other treatments ($F_{8, 63} = 2.83$, $R^2 = 0.17$, $P = 0.05$), but length of exposure time had no significant effect on mortality ($R^2 = 0.01$, $P = 0.66$; Fig. 3.11). Furthermore, there was no significant effect from an exposure time * temperature interaction on mortality ($R^2 = 0.58$, $df = 42$, $P = 0.36$) and -10.0°C was the

only temperature that had significantly higher mortality across all exposure times compared to the controls ($X^2 = 12.0$, $df = 3$, $P = 0.007$). However, naturally acclimatized beetles had significantly higher mortality than the controls when exposed to -5.0°C for 24 hours ($R^2 = 0.93$, $df = 30$, $P < 0.0001$).

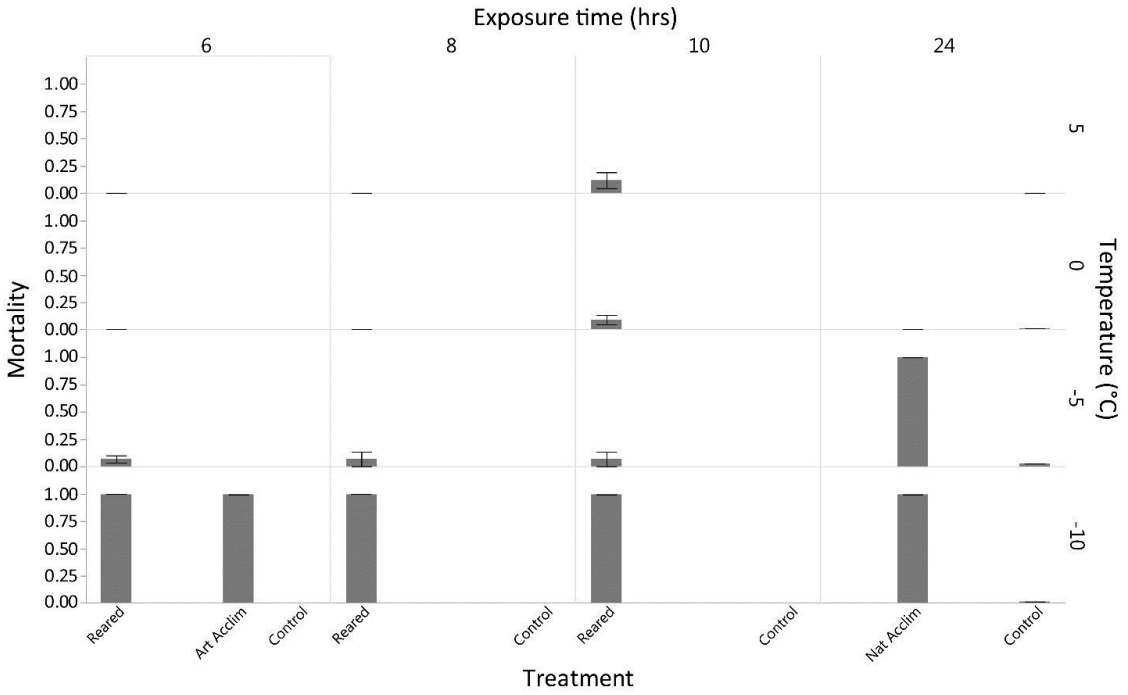

Figure 3.10 Mortality of reared, naturally and artificially acclimatized, and control groups of *Xyleborus glabratus* for each exposure time (hours) and temperature (°C)

Data were collected from 2012 to 2014 in Jackson County, MS. Due to logistics, not all treatments were tested at every exposure time and temperature combination. The only temperature that had significantly higher mortality across all exposure times and treatments compared to the controls was -10.0°C ($P = 0.007$). Naturally acclimatized beetles had significantly higher mortality than the controls when exposed to -5.0°C for 24 hours ($P < 0.0001$).

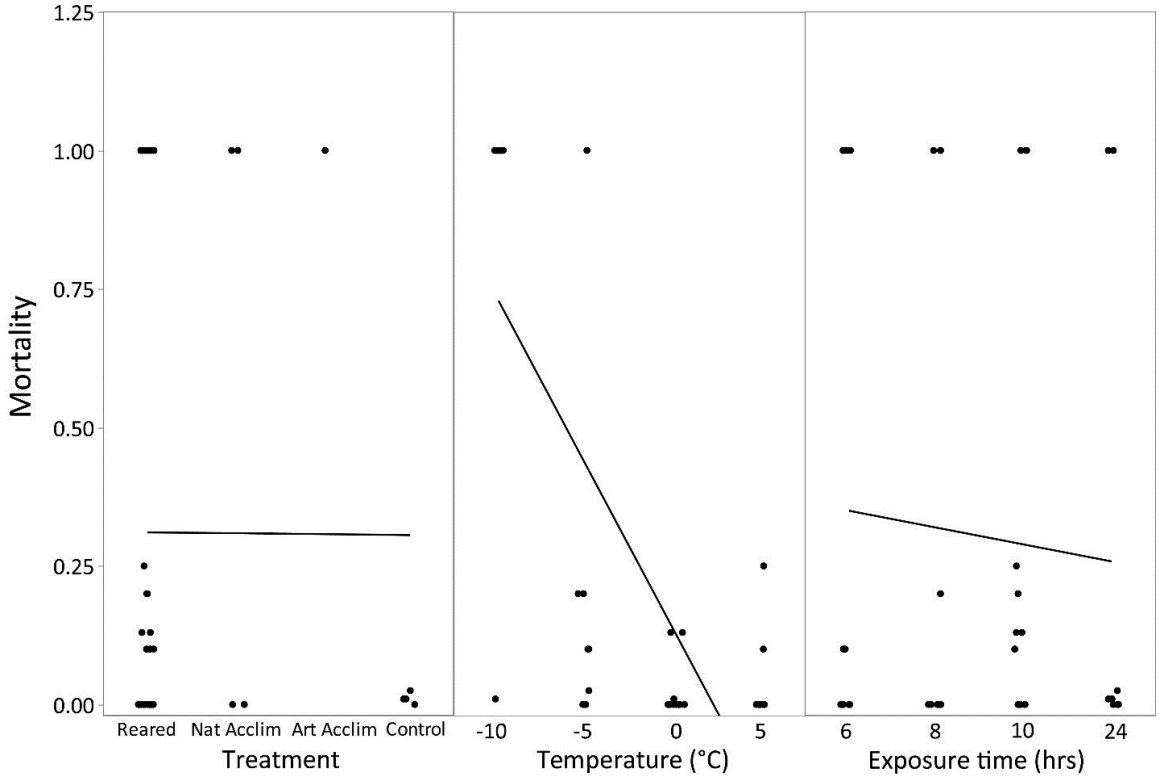

Figure 3.11 Relationships between treatment type, exposure time (hours), and temperature (°C) and mortality

Data collected from 2012 and 2014 in Jackson County, MS. *Xyleborus glabratus* mortality significantly decreased as temperatures increased ($R^2 = 0.57$, $P < 0.0001$). Control treatments had significantly lower mortality than the other treatments ($R^2 = 0.17$, $P = 0.05$); however, length of exposure time had no significant effect on mortality ($R^2 = 0.01$, $P = 0.66$).

3.1.7 Sassafras buffering

Mean ambient air, inner (depth of 13 cm), and outer wood (depth of 5 cm) temperatures are shown in Figure 3.12. The average difference (i.e. average buffering capacity) between ambient air temperatures and temperatures in the outer and inner wood during the winter was 0.3°C ±0.12 and 1.2°C ±0.13 (±SEM), respectively (Fig. 3.12). The buffering effect of the inner wood was used for correcting ambient temperature data because it offers more protection than the outer wood. Temperatures in both the outer (R^2

= 0.80, *df* = 1775, *P* = 0.0003) and inner tree (R^2 = 0.89, *df* = 1775, *P* < 0.0001) were significantly different than ambient air temperatures. The largest differences between ambient air and inner tree temperatures occurred on 8 January (+8.37°C) and 16 January 2015 (-9.86°C). Likewise, the largest differences between outer wood and ambient temperatures occurred on 8 January (+9.84°C) and 16 January 2015 (-13.34°C). Additionally, the temperatures in the inner wood were significantly buffered from air temperatures compared to temperatures in the outer wood (R^2 = 0.94, *df* = 1775, *P* < 0.0001). The relationship between the temperatures in the inner and outer wood and ambient air temperatures were significant and positively correlated (R^2 = 0.80, *df* = 1775, *P* < 0.0001 and R^2 = 0.64, *df* = 1775, *P* < 0.0001, respectively; Figs. 3.13, 3.15). It may be possible to predict inner and outer wood temperatures in sassafras from ambient temperatures (Figs. 3.14, 3.16); however, more sassafras should be measured to improve prediction estimates.

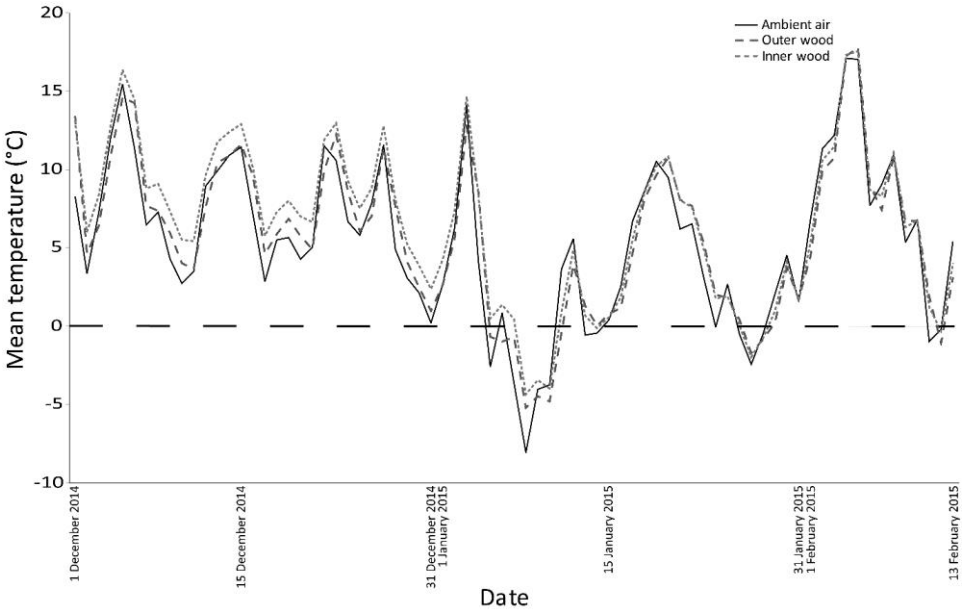

Figure 3.12 Mean ambient air, and inner (depth of 13 cm) and outer wood (depth of 5 cm) temperatures (°C) of a sassafras located in Desoto County, MS from 1 December 2014 to 13 February 2015.

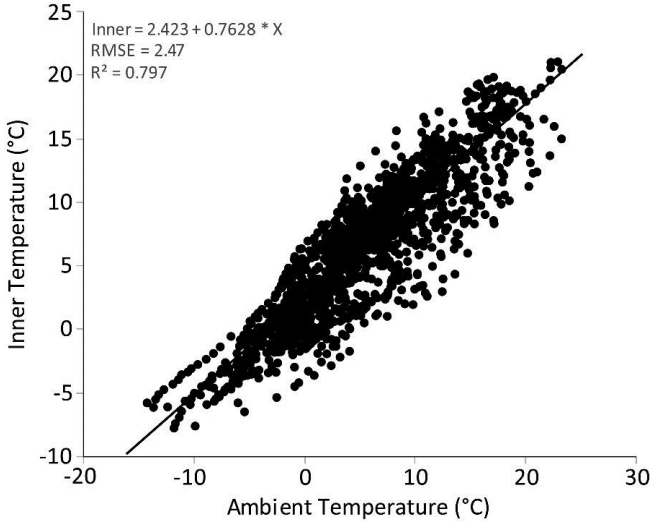

Figure 3.13 Relationship between the temperatures in the inner wood of sassafras (depth of 13 cm) and ambient air temperatures

Data collected from December 2014 to February 2015 in Desoto County, MS. The relationship between the data were significant and positively correlated ($R^2 = 0.80$, $P < 0.0001$). Ambient air temperatures were measured ~1 meter from the test tree.

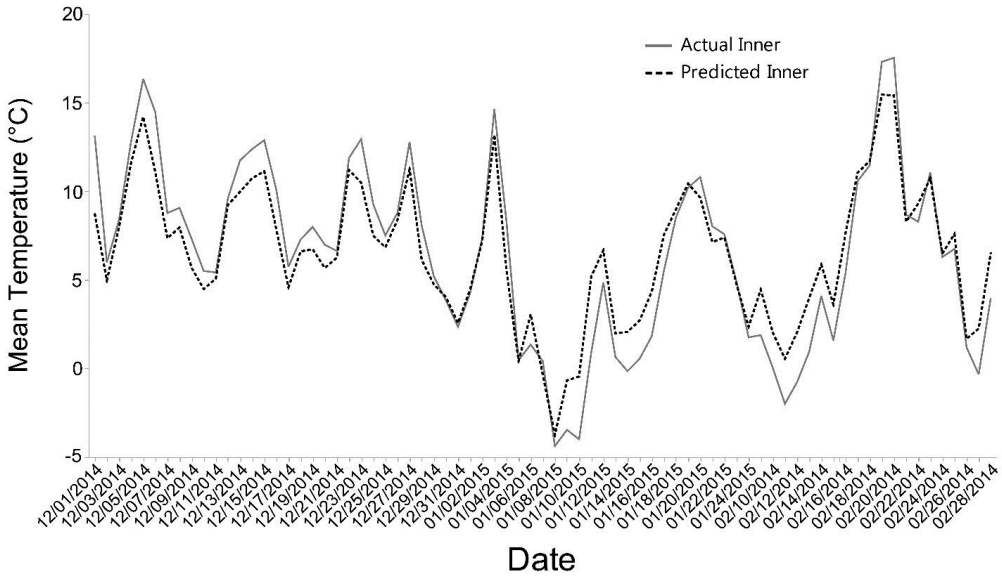

Figure 3.14 Predicted temperatures in inner sassafras wood (depth of 13 cm) in Desoto County, MS

Predicated data were calculated from regressions between actual inner wood and ambient air temperature data from December 2014 to February 2015. Ambient air temperatures were measured ~1 meter from the test tree.

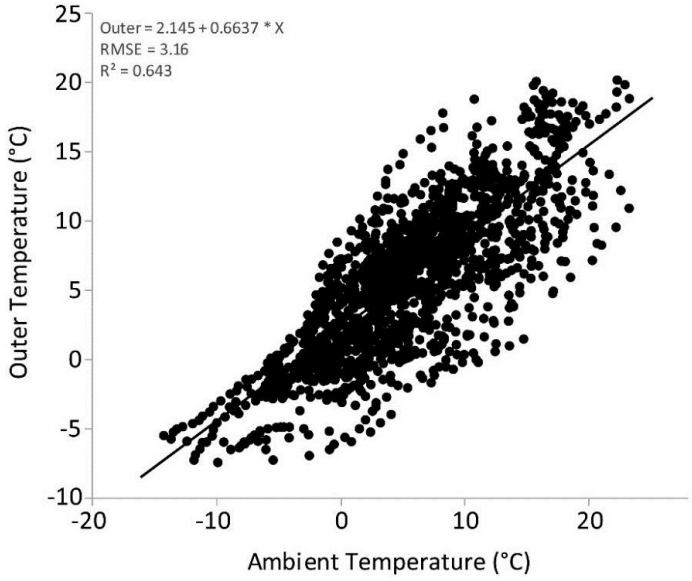

Figure 3.15 Relationship between the temperatures in the outer wood of sassafras (depth of 5 cm) and ambient air temperatures

Data collected from December 2014 to February 2015 in Desoto County, MS. The relationship between the data were significant and positively correlated ($R^2 = 0.64$, $P < 0.0001$). Ambient air temperatures were measured ~1 meter from the test tree.

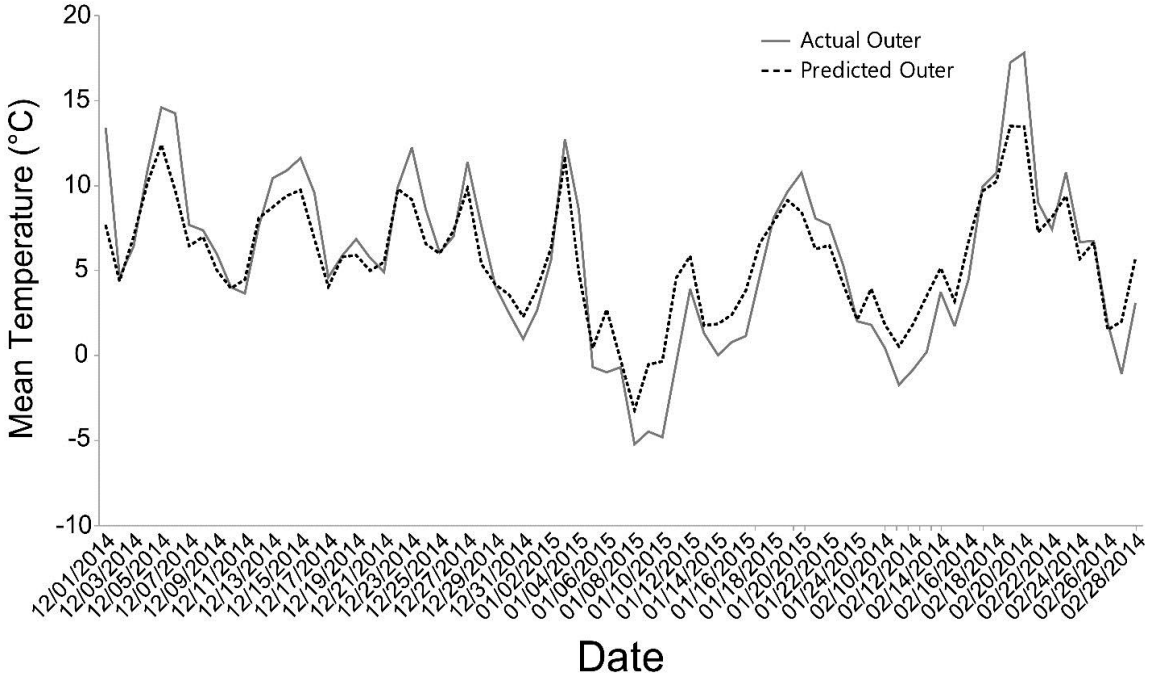

Figure 3.16 Predicted temperatures in outer sassafras wood (depth of 5 cm) in Desoto County, MS.

Predicated data were calculated from regressions between actual outer wood and ambient air temperature data from December 2014 to February 2015. Ambient air temperatures were measured ~1 meter from the test tree.

3.1.8 *Xyleborus glabratus* invasion potential

Figure 3.17 shows the current distribution of sassafras in North America. Two maps were created to illustrate the invasion potential/hazard rating of *Xyleborus glabratus* in the United States under different climate scenarios (Figs. 3.18, 3.19). All ambient air temperature data in these models were corrected with the buffering capacity of the inner sassafras wood (1.0°C). The first map depicts the current *X. glabratus*/laurel wilt hazard in the United States (Fig. 3.17). In this model, the northern spread of *X. glabratus*/laurel wilt is limited to approximately the 35°N latitude. The second map represents the *X. glabratus*/laurel wilt hazard in the United States after a 1.5°C increase in temperatures (Fig. 3.18). In this scenario, the beetle's spread potential increases from

approximately 35°N to 40°N. Under this model most sassafras populations below the 37.5°N latitude should be impacted to some degree, except local populations in the higher elevations of the southern Appalachians (e.g. Great Smokey Mountains, Blue Ridge Mountains). Redbay and California laurel will not be protected by low temperatures and, as a result, all mature populations of redbay and California laurel are at risk of being killed by laurel wilt disease.

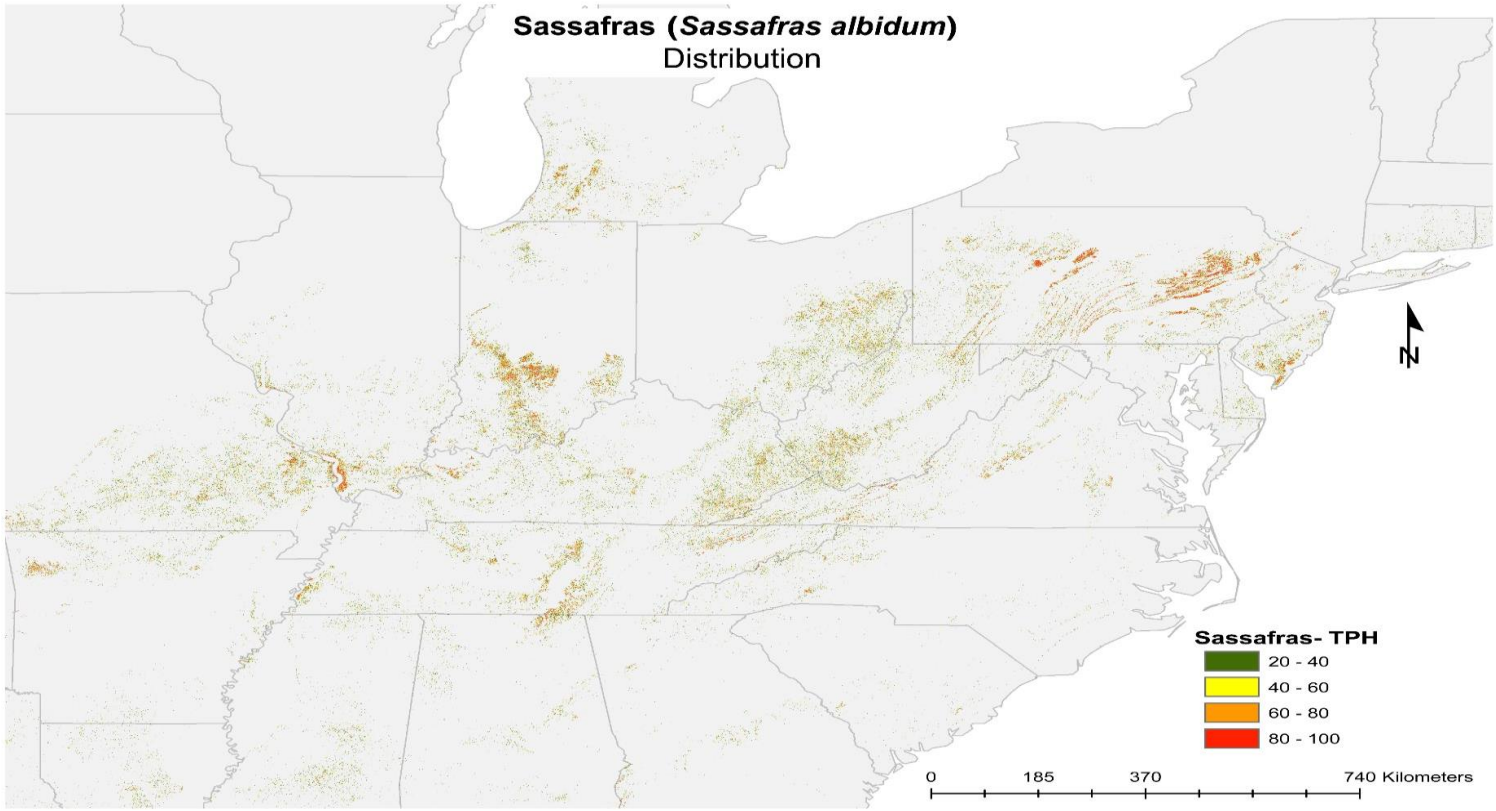

Figure 3.17 Current (2016) distribution of sassafras in the Unites States. Sassafras (trees/hectare) distribution from USDA Forest Inventory and Analysis survey data

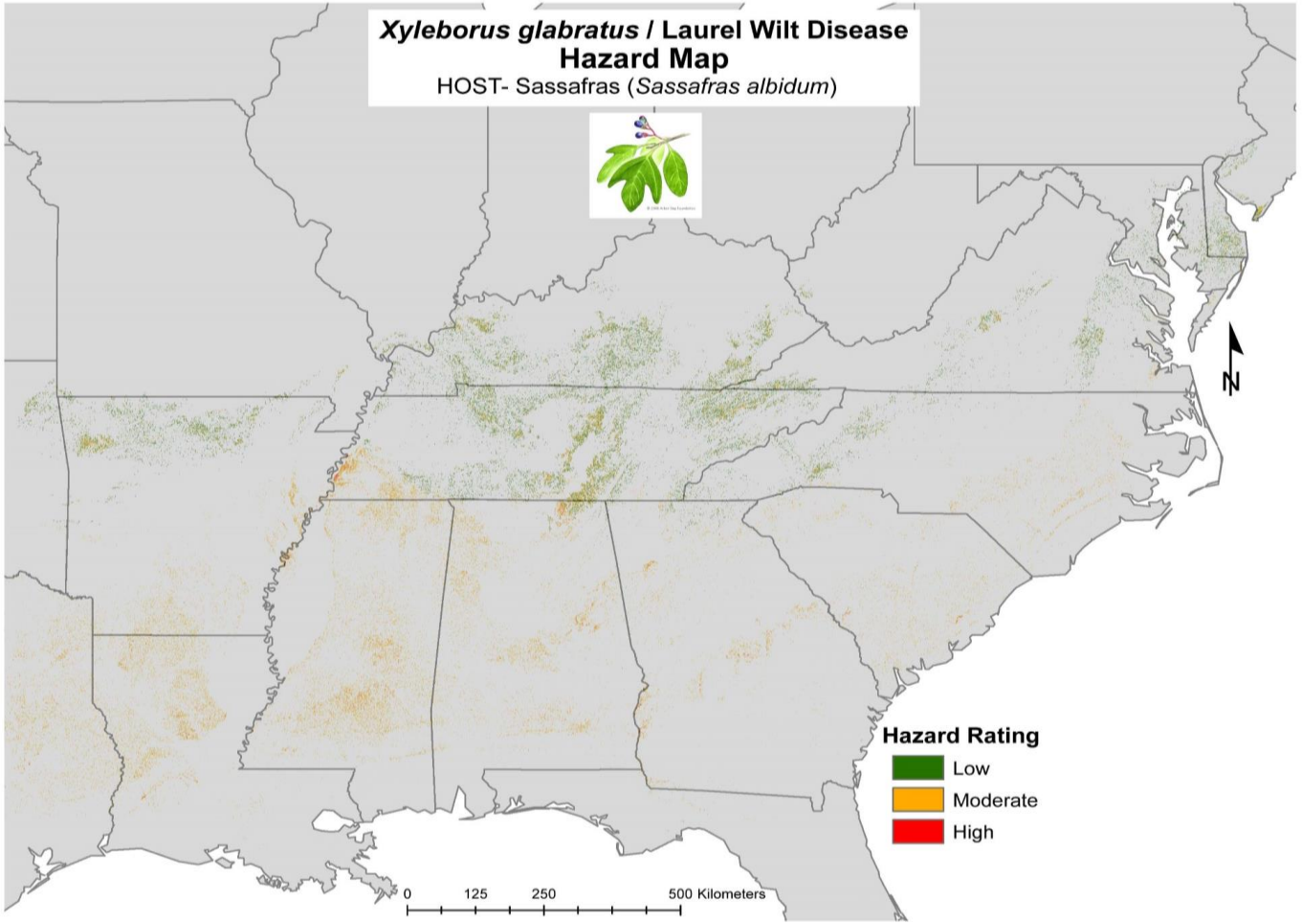

Figure 3.18 Current *Xyleborus glabratus*/laurel wilt hazard in the United States.

The northern spread of *X. glabratus* is limited to 35°N ±1° (±SEM) latitude. Hazard rating developed from host density (trees/hectare) and temperature (°C; 30 year extreme minimum mean temperature) data.

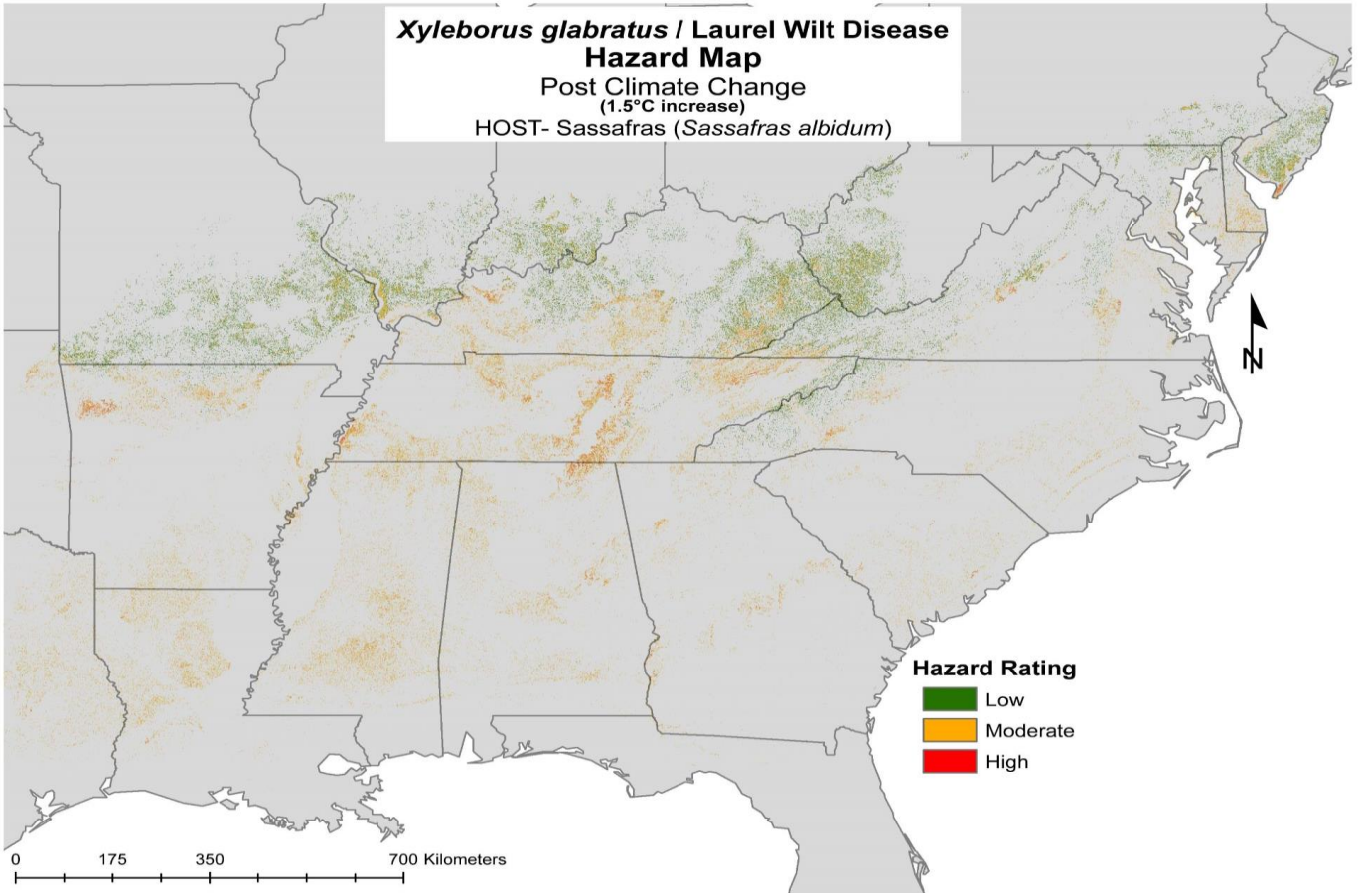

Figure 3.19 *Xyleborus glabratus*/laurel wilt hazard in the United States after a 1.5°C (IPCC estimate for the eastern US) increase in temperatures

Under this model, most sassafras populations south of the 37.5°N latitude should be impacted to some degree. Hazard rating developed from host density (trees/hectare) and temperature (°C; 30 year mean extreme minimum temperature) data

CHAPTER IV

DISCUSSION ON THE OVERWINTERING PHYSIOLOGY AND INVASION POTENTIAL OF THE REDBAY AMBROSIA BEETLE IN THE UNITED STATES

4.1 Discussion

4.1.1 Supercooling points and cold tolerance strategy

Cold tolerance strategy can be determined two ways; directly and indirectly. Direct measurement of a cold tolerance strategy involves exposing a set of individuals to cold temperatures until half of them have reached their supercooling points (Sinclair et al 2015). The cold tolerance strategy is determined by examining both the frozen and unfrozen individuals for survival, after all specimens have been warmed to room temperature (Sinclair et al 2015). The survival ratio of the frozen to unfrozen individuals determines the strategy (Table 4.1). Indirect measurement of cold tolerance strategy is measured by comparing the supercooling point (SCP) to the lower lethal temperature (LLT).

Table 4.1 Cold tolerance strategies in insects determined by survival post supercooling point studies

Species	*Drosophila suzukii*		*Reticulitermes flavipes*		*Perisphaeria sp.*	
Physiological State	Unfrozen	Frozen	Unfrozen	Frozen	Unfrozen	Frozen
Survival	0 %	0 %	100 %	0 %	100 %	100 %
Strategy	Chill-susceptible		Freeze-avoidant		Freeze-tolerant	

(modified from Sinclair et al. 2015).

In the case of *X. glabratus,* the SCP was lower than the LLT (SCP ≤ LLT), meaning the beetle is chill susceptible (Bale 1996; Fig. 4.1). Chill-susceptibility is just one of the subclasses within the freeze intolerant strategy. For example, the chill susceptible *Myzus persiae* (Hemiptera: Aphididae) has a SCP of -25.0°C, but the LLT occurs after exposure to several minutes below -5°C (Bale 1991). Even though supercooling points are biologically irrelevant to survival, the SCPs of freeze-intolerant insects generally decrease over the period of several weeks before the onset of winter (Rickards et al. 1987). However, it is hard to discern any trend in the SCPs of *X. glabratus* before winter. Moreover, the supercooling capacity of insects typically vary from one season to the next (Lee et al. 1996; Chown and Terblanche 2006), but in naturally collected *X. glabratus*, no significant variation was found among the seasons. The lack of variation among monthly and seasonal SCPs could be attributed to a few factors, discussed below.

4.1.2 Physiological effects on supercooling point capacity

Arguably one of the most important factors linked to insect supercooling capacity is body water content (Lee et al. 1996; Zachariassen et al. 2004). For example, insects with decreased body water content exhibit increased supercooling capacity (Gehrken and Somme 1987; Lee et al. 1996). In the case of overwintering *X. glabratus*, the protective microhabitat of host tissues and the availability of a cultivated food source may mitigate most effects from water loss. Therefore, it could be expected that the water content of naturally acclimatized *X. glabratus* remained steady over the course of the study; however, this would need to be verified.

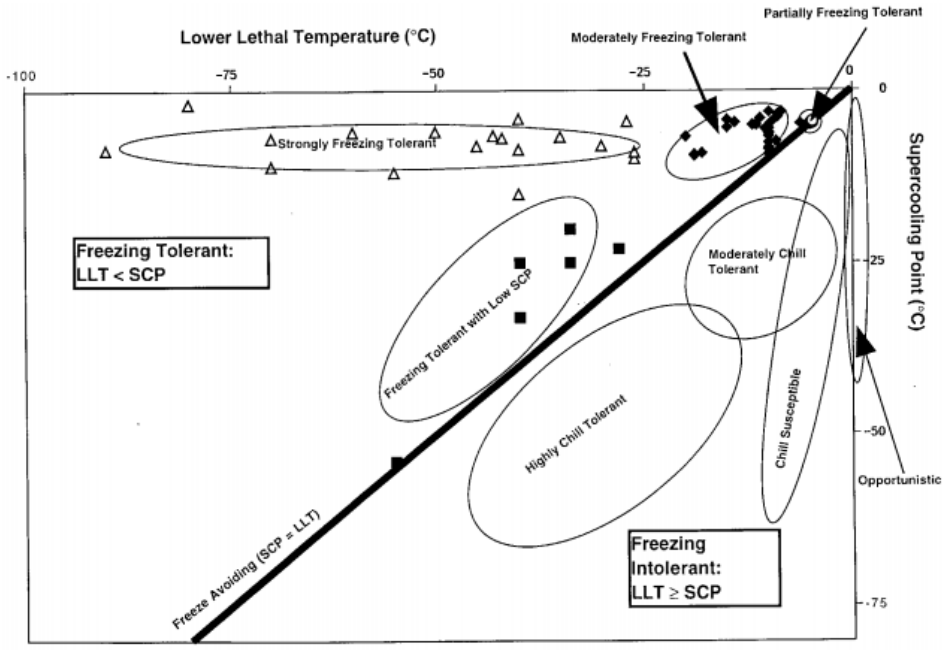

Figure 4.1 Relationship between supercooling point, lower lethal temperature, and cold tolerance strategies.

(modified from Sinclair 1999).

The ability of *X. glabratus* to consistently supercool to such low temperatures throughout the study is interesting given that insect is native to the tropics. Tropical insects rarely, if ever, experience cold temperatures in their native habitat (Wood 2007); however, given that *X. glabratus* is tiny and uniform in size (Formby et al. 2013), its ability to supercool may be solely due to small body size. For example, Angell (1982) discovered that tiny volumes of water remain unfrozen at -40°C below their equilibrium freezing point. This is supported by numerous studies that have found small insects, even smaller bodied insects of the same species, have lower supercooling points than their larger bodied counterparts (Block and Young 1979; Colinet et al. 2006; Hahn et al. 2008).

One of the most influential factors in initiating insect cold hardening is exposure to appropriate seasonal clues (e.g. decreasing temperatures); however, fall and winter temperatures along the Gulf Coast of Mississippi are mild (i.e. rarely fall below 0.0°C). For example, during the fall months (period of insect cold hardening) of this study, minimum temperatures averaged 16.7°C and maximum temperatures averaged 23.9°C, and in November, the coldest month of fall, minimum temperatures averaged 9.1°C and maximum temperatures averaged 18.2°C. Due to the lack of low temperatures, especially during the fall months, *X. glabratus* may have not cold hardened to maximum potential, and he lack of a seasonal trends in supercooling points may have reflected this phenomenon. The results from the artificially acclimatized study may support this conclusion further, i.e. artificial acclimatization significantly lowered the SCPs with respect to the beetles caught in southeastern Mississippi (~2.0 decrease in supercooling capacity).

4.1.3 Biochemicals

Temperature may have not influenced supercooling points in naturally acclimatized *X. glabratus*, but it did have an influence on two of the three biochemicals tested, i.e. an increase in total body sugar and decrease in glycogen concentrations were correlated to changes in temperature. Accumulation of cryoprotectants, such as sugars (trehalose and glucose), are known to enhance cold tolerance and supercooling capacity in many insects (Zachariassen et al. 2004; Lee 2010). On the other hand, Kostal et al. (2001) found accumulation of cryoprotectants in the hemipteran *Pyrrhocoris apterus* increased cold tolerance, but had no effect on supercooling capacity. Total body sugar concentrations increased from fall to winter in this study; therefore, it may be possible the phenomenon reported in *P. apterus* by Kostal et al. (2001) is true in *X. glabratus*. Renault et al. (2002) hypothesized that supercooling capacities found in tropical species are unrelated to cold hardiness, but are rather linked to ancestral mechanisms used for survival in tropical habitats (e.g. desiccation resistance). Pullin (1996) found mechanisms (e.g. carbohydrate accumulation) originally used by insects to combat desiccation and/or dehydration in tropical regions may have been selected later for their cryoprotective function. This theme is supported further by other studies that have examined the desiccation resistance-cold tolerance link (e.g. Ring and Danks 1994, 1998; Block 1996, 2002; Danks 2000; Bayley et al. 2001; Block and Zettel 2003). In fact, the desiccation resistance mechanisms in tropical species may help explain the influence of temperatures on biochemical changes in *X. glabratus*, but further analysis is needed to clarify this phenomenon.

There are other questions raised by the results of the biochemical analysis in *X. glabratus* that need clarification. For example, of the three compounds tested, lipids were not significantly influenced by temperatures and stayed relatively constant throughout the study; however, lipid concentrations were significantly lower in April than any other month but May. This raises the question: What mechanism(s) led to a significant decrease in lipid concentrations in April? It is well known that lipid reserves are used to meet energy needs after glycogen is depleted, but never were glycogen levels completely exhausted during the study. The sharpest decline in glycogen levels occurred in winter which coincided with an increase in sugars. Glycogen stores are often accumulated during late summer and early fall and used to synthesize carbohydrate cryoprotectants during low temperatures (Storey and Storey 1991; Thompson 2003). Lipid stores, however, are extremely critical to embryogenesis (Ziegler and Van Antwerpen 2006) and for maintaining prolonged periods of flight (Beenakkers et al. 1984). Spring (March-May) is a crucial time for *X. glabratus* host seeking and egg production (Brar et al. 2013; Maner et al. 2013). This could explain why naturally acclimatized beetles lipid concentrations were low in April and May. Interestingly, Maner et al. (2013) reported April to have the highest flight activity in 2012.

It is apparent that overwintering *X. glabratus* are not dependent on biochemicals to regulate supercooling, at least not the beetles collected in southeastern Mississippi where the winters are mild. To better understand *X. glabratus* overwintering biology more artificial acclimatization events, or natural acclimatization events at higher latitudes, will need to be conducted, along with additional analyses of specific sugars and polyhydric alcohols important in insect cold hardening. Nevertheless, as exemplified by

the lower supercooling points in the artificial acclimatization study, *X. glabratus* may become more cold tolerant as it continues to move north at an estimated rate of 54.8 km/year (Koch and Smith 2008). Additionally, the overwintering data provided in this study will help to enhance the understanding of tropical ambrosia beetle colonization in novel climates.

4.1.4 Lower lethal temperature

The proportion of reared *Xyleborus glabratus* that were chill injured (i.e. uncoordinated movement, unable to upright themselves, etc.) remained relatively constant across all exposure times and temperatures. The highest numbers of chill injured *X. glabratus* occurred in the naturally acclimatized and artificially acclimated treatments, and were especially high in artificially acclimated beetles. This type of injury, which occurs in the absence of extracellular ice formation, is known as cold shock, or direct chilling injury and results from brief (on the order of minutes to hours) exposures to cold (Chen et al. 1987; Chown and Nicolson 2004). Direct chilling injury can induce fluid-to-gel phase transitions in cell membranes which can lead to separation of protein and lipid membranes, changes in membrane permeability, and deactivation of membrane bound enzymes (Chown and Nicolson 2004). According to Hosler et al. (2000), the damage to plasma membranes from direct chilling injury can effect neurons and negatively impact neurological transmissions throughout the muscular system. Furthermore, direct chilling injury can inhibit enzyme activity, change protein structure and denaturation, and cause a loss of electrochemical potentials across cell membranes (Kostal et al. 2004, 2006 2007; Ramlov 2000), and there is a possibility that direct chilling injury can increase oxidative stress (Chown and Nicolson 2004).

Survival from direct chilling injury has never been reported to be improved by decreasing rearing temperatures (McDonald et al. 1997; Chown and Nicolson 2004). This finding is further supported by the lower lethal temperature results from this study, in which all chill injured beetles were dead 24 hours after testing. Survival against direct chill injury can be improved, however, by rapid cold hardening (Lee and Denlinger 2010). Rapid cold hardening (RCH) is a highly conserved trait which allows insects to swiftly adjust their physiological state in response to slight changes in temperature (Lee and Denlinger 2010). Rapid cold hardening is triggered by short term exposure (usually one to two hours) to sub-zero temperatures, and in some instances, even modest changes in temperatures (e.g. from 21 to 16°C; Shreve et al. 2004). Ecologically it is unlikely that the treatments used to induce RCH in the laboratory will occur in nature, especially in insects that are buffered from rapid temperatures changes, such a *X. glabratus*.

4.1.5 Sassafras buffering

It is important to consider the refugia of wood, which provides protection from ambient air temperatures and extreme weather events (Wood 1982; Bolstad et al. 1997; Poland and McCullough 2006; Tran et al. 2007). Temperatures within the heartwood of large trees can take several hours to days to respond to changing environmental conditions (Derby and Gates 1966). However, this study discovered that the buffering effect of sassafras heartwood (small tree; depth of 13cm) closely follows ambient air temperatures, and temperatures in the heartwood take only a few hours to respond to ambient temperatures (Fig. 3.12; section 3.1.7). As a result, the average buffering capacity of sassafras was used to correct air temperature data from weather station, such that any area with minimum air temperatures of -11.0°C represented the lower lethal

temperature of *X. glabratus* inside sassafras (-10.0°C). The results should not be considered absolute, as 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 and 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. However, this study was a biologically relevant starting point for the impacts of tree buffering on survival of *X. glabratus*.

Another issue to consider is the additional thermal buffering added by snowpack, especially in higher latitudes or elevations. This may not be an issue in species which occupy higher portions of a tree. However, *X. glabratus* occupies most large stems of a tree from the root collar to certain limbs in the crown. Pruitt (1957) recorded a temperature of 0°C under 40 cm of snow when the air temperature was -33.0°C; however, the buffering capacity of the snow is dependent upon its age and/or structure, i.e. fresh snow offers greater insulation than older, hard packed snow (Coulson et al. 1995). As a result, *X. glabratus* could potentially establish a few disjunct populations in higher latitudes or elevations considering the additional thermal buffering of winter snowpack.

With the lack of ambrosia beetle cold tolerance literature the most similar taxa to compare our results to are bark beetles (Gehrken 1984; Bentz and Mullins 1999;

Lombardero et al. 2000). Lombardero et al. (2000) found no significant differences between supercooling points of bark beetles (*Dendroctonus frontalis, Ips pini, I. grandicollis* (Eichoff)*,* and *I. perroti* Swaine) measured during the winter or of those cold hardened in an incubator at 0 °C for 4 months. However, unlike Lombardero et al. (2000), our study saw significant differences in supercooling points between naturally acclimatized, winter collected beetles and artificially acclimatized specimens. Our findings do follow the results of *D. ponderosae* cold acclimatization studies, which reported that adequate acclimation to low temperatures significantly increased *D. ponderosae* supercooling point (Wygant 1940; Yuill 1941; Sømme 1964).

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, *D. ponderosae* Hopkins, a lack of diapause makes it less dependent on hormonal controls than in diapausing insects (Merivee 1978; Sømme 1982; Hodkova and 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.

Several physiological factors may help explain the increased supercooling capacity of artificially acclimatized beetles. First, artificially acclimatized beetles may have experienced chill coma (i.e. the loss of locomotory capacity). Insects enter chill coma at a species-specific temperature (Mellanby 1939; Kerkut and Taylor 1959; Kostal

et al. 2006; Lee 2010), and in tropical insects, this temperature could be as high as 10 °C to 12 °C (Sehnal et al. 2003; Lee 2010). The artificially acclimatized beetles may have been unable to eat in a chill coma state, whereas naturally acclimatized beetles may have continued feeding throughout the study period. Termination of feeding and/or emptying of the gut decreases ice-nucleators and are often associated with cold hardening preparations in insects (Lee et al. 1996). These two mechanisms have been found to increase supercooling capacity (Block et al. 1990). Second, artificially acclimatized beetles may have entered chill coma, stopped feeding on their cultivated fungal food source, and became dehydrated. Dehydration can increase supercooling capacity without the need to accumulate cryoprotectants (Gerhken and Somme 1987). Lastly, due to the more extreme combination of time and temperature, the artificially acclimatized beetles may have been, in fact, more cold hardened through accumulations of other biochemicals (i.e. increased anti-freeze or heat shock proteins, cryoprotectants, and/or unsaturated fatty acids) than naturally acclimatized beetles.

The results from the biochemical analysis of naturally acclimatized *X. glabratus* may support this final hypothesis (i.e. SCPs were significantly influenced when all three biochemicals plus their interaction terms were included in regression analyses); however, other cold hardening preparations and/or mechanisms not analyzed in this study could be the primary driver. Several studies have reported active (i.e. emerging and flying) *X. glabratus* in every month of the year (Hanula et al. 2008; Maner et al. 2013); as a result, it is unlikely the beetle enters diapause and cessation of feeding or dehydration, or the combination of both, may have been the primary mechanism(s) behind the increase in the supercooling capacity of artificially acclimatized beetles. However, these two

mechanisms may not reflect the actual cold hardened processes in naturally acclimatized beetles.

4.2 Conclusion

The invasion potential results suggest that a previous spatio-temporal prediction model of *X. glabratus* spread that used climate match data could have underestimated the potential distribution of in the United States (Koch and Smith 2008). There was little evidence that *X. glabratus* would infest sassafras in the absence of redbay at the time Koch and Smith (2008) were creating their model. As a consequence, the model only included areas where redbay and sassafras occur together; but since 2008, enough evidence has been to conclude that *X. glabratus* can readily infest sassafras in the absence of redbay (Fraedrich et al. 2008; Smith et al. 2009; Riggins et al. 2011; Bates et al. 2013).

This study unfortunately reinforces the notion that laurel wilt has the potential to cause widespread mortality and may even cause the ecological extinction of several laurel species (Evan et al. 2010), including sassafras in the southern portion of its range. The cultural, economic, and ecological contribution of each laurel species may not be dependent upon the geographical extent of the species. Redbay, compared to sassafras and others, requires specialized habitat and occupies a small geographic extent. It occasionally grows inland, but never outside of the Atlantic and Gulf Coastal Plains. Although redbay has a small geographic extent it is tied to many species in the ecosystem, i.e. redbay is the primary food source for the larvae of the palamedes swallowtail (*Papilio palamedes* Drury; Lederhouse et al. 1992) and several newly described species of leaf-miners (*Phyllocnistis* spp.; Davis and Wagner 2011). Furthermore, the palamedes swallowtail is the primary pollinator of the endemic yellow-

fringed orchid (*Platanthera ciliaris* (L.) Lindl.). The yellow-fringed orchid is endemic to North America and prior to laurel wilt disease was already threatened with extinction due to habitat loss in several southeastern states (USDA PLANTS database). In other words, the decline of redbay could lead to a cascade of negative ecological effects that start with the decline of the palamedes swallowtail and yellow-fringed orchid.

This study has determined that temperatures above the beetle's supercooling point will be cold enough to limit its expansion throughout the entire range of sassafras, even though *X. glabratus* has spread unabated throughout much of the southeastern U.S., where it has caused widespread redbay mortality. This is good news for landowners living above the 35°N latitude (under current temperatures) and 40°N latitude (under a 1.5 °C warming) concerned with sassafras mortality and the negative effects on sassafras-associated wildlife. Furthermore, this study will help land managers and owners below the 35°N and 40°N latitude formulate preventative management strategies, determine if resources and logistics will be needed, and/or plan detection surveys.

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