# Host influence on the cold hardiness of the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae)

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## Dedication

I'm dedicating this thesis to my big brother, John Christianson, because after he bought me a couple beers on his most recent trip to Minneapolis, I promised him I would.

But in all seriousness, John, you always set a high bar and made sure it wasn't easy to meet your standard.  You've had a much bigger influence on me than I'll ever admit to your face, but you've (generally) been a pretty good influence.  So thank you.

# Abstract

The emerald ash borer, *Agrilus planipennis* Fairmaire, is an invasive insect in North America that has caused extensive damage to ash trees throughout its invaded range. The range of *Fraxinus* spp., specifically black ash (*Fraxinus nigra*) extends farther north than the known *A. planipennis* distribution. The ability of an insect to withstand cold, or its cold hardiness, is important in limiting its northern distribution. The cold hardiness of *A. planipennis* from green ash and black ash was assessed using two laboratory measures, supercooling points and lower lethal temperature, in laboratory- and naturally-infested *A. planipennis* larvae.

Supercooling points of *A. planipennis* larvae from black and green ash, with medians ranging from -25.5 to -32.6°C and -24.0 to -34.2°C respectively, were not significantly different during the winters of 2012-13 and 2013-14, although the laboratory infested larvae tested in November 2011 had significantly warmer supercooling points. Kaplan-Meier estimates of the likelihood of freezing of larvae from black ash and green ash were not significantly different between tree species or between years.

A high proportion of larvae died after being frozen, but freezing did not kill all larvae. Lower lethal temperatures for 50% of the population ($LT_{50}$) were estimated after accounting for the proportion of larvae that would survive freezing. $LT_{50}$s of larvae from green ash were -35.2°C (95% CI -35.9°C, -34.7°C) in the winter of 2012-13 and -33.4°C (-34.1°C, -32.1°C) in the winter of 2013-14. Larvae from black ash had estimated lower lethal temperatures of -33.4°C (-34.4°C, -32.3°C) in 2012-13 and -33.1°C (≤-37.5°C, -35.5°C) in 2013-14.

*Agrilus planipennis* larvae overwinter under the bark of ash trees, so larvae may not be experiencing winter air temperatures. To determine temperatures under the bark of ash trees, temperature probes were placed under the bark of both green and black ash at 1.4m high and at the base of the tree on the north and south faces. Weather stations recorded air temperature. Daily minimum temperatures under the bark of green ash ranged from 0.5 to 4.7°C warmer than daily minimum air temperatures, and temperatures were 1.2 to 5 degrees warmer under the bark of black ash. Temperatures at the base of the trees provided the most insulation, possibly due to any snow cover adding to the insulative effect of the bark.

During the time in which we took measurements, the air temperature fell below -30°C for an average of 16 hours during the winter of 2012-13. Temperatures under the bark of green ash did not reach -30°C, and temperatures under the bark of black ash fell below -30°C, on average, for 2.6 hours. In 2013-14, air temperature was -30°C or colder for an average of 146.1 hours, 78.1 hours under the bark of green ash, and 118.7 hours under the bark of black ash.

Because of the differences in air temperatures and temperatures under the bark of trees, we cannot use air temperature to directly predict *A. planipennis* mortality. Because under-bark temperatures do not reach the temperature required to kill 50% of the population of *A. planipennis* every year, further research should consider how the larvae survive sublethal temperatures for longer periods of time.

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## Review of the Literature

In 2002, the Michigan Department of Natural Resources identified an exotic wood-boring beetle that is now considered one of the most destructive forest pests in North America: the emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae). *Agrilus planipennis* larvae chew through the bark to feed in a characteristic serpentine pattern in the cambium, phloem, and outer xylem layers of ash trees (*Fraxinus* spp.). Larval feeding can girdle a tree and kill it in 1-4 years (Yu 1992, Cappaert et al. 2005). *Agrilus planipennis* has killed tens of millions of ash trees in North American forests and urban areas (McCullough et al. 2009).

*Agrilus planipennis* has yet to achieve its ultimate distribution in North America. Cold often determines the northern limits of an invading insect's range. Although Venette and Abrahamson (2010), Crosthwaite et al. (2011), Sobek-Swant et al. (2012), and Vermunt et al. (2012) have studied the cold tolerance of *A. planipennis*, the influence of different host species on the overwintering ability of *A. planipennis* remains unknown. The potential geographic range extension by *A. planipennis* into northern Minnesota where black ash is abundant in lowland black ash-American elm-red maple forests is a concern (Erdmann et al. 1987). Black ash thrives on wet and poorly drained mineral soils and is often the only tree species that successfully establishes in these environments. If *A. planipennis* reaches northern Minnesota, the potential impact on black ash stands could be significant. *Agrilus planipennis* was confirmed in Superior, WI, in August 2013, just across the Saint Louis Bay of Lake Superior from Duluth, MN (Abrahamson 2014). *Agrilus planipennis* is a threat to ash trees and other arthropod species that rely on ash trees. Gandhi and Herms (2010) identified 44 arthropod species that are “highly associated” with ash trees in North America and are therefore are at risk of disappearing along with the ash trees.

This thesis describes my studies of the effects of green ash (*Fraxinus pennsylvanica* Marshall) and black ash (*F. nigra* Marshall) on the cold tolerance and microhabitat of overwintering larvae of *A. planipennis.* This chapter begins with a discussion of the status and distribution of *A. planipennis* in Asia, its native range, and North America, its invaded range. The discussion is followed by a short, general history of research on insect cold hardiness. I then describe the natural history and basic biology of *A. planipennis*, including taxonomy, identification, and life cycle with an emphasis on overwintering. Hosts, both native and novel, of *A. planipennis* are reviewed with respect to their evolutionary relatedness, geographic distribution, and attractiveness to *A. planipennis*. North American ash species currently affected by this insect are emphasized, particularly the two most common ash species in Minnesota, green ash and black ash. The chapter concludes with a review of previous research on *A. planipennis* cold hardiness and discusses the importance of further cold hardiness research for the management of *A. planipennis*.

## I. The Problem

*Agrilus planipennis* is a flat-headed borer that is native to northeastern China, far eastern Russia, Taiwan, Korea, and Japan (Liu et al. 2003, Cappaert et al. 2005, Poland and McCullough 2006). It was first identified in North America in 2002 near Detroit, Michigan and in Windsor, Ontario (Liu et al. 2003). As of September 2014, *A.*

*planipennis* has been confirmed in 24 states and two Canadian provinces. The current distribution of *A. planipennis* in North America overlaps with green ash, black ash, white ash (*F. americana* L.), blue ash (*F. quadrangulata* Michx.), and pumpkin ash (*F. profunda* (Bush) Bush), and all of these species have been confirmed as susceptible hosts (Lindell et al. 2008). Kovacs et al. (2010) estimated that cities and states affected by *A. planipennis* could spend almost $11 billion through 2019 in ash tree removal, replacement, and treatment. The economic impact increases to $25 billion if all ash trees, including uninfested trees, are removed and replaced at once. Early detection surveys for *A. planipennis* can identify new infestations before damage is extensive, providing more time for management. More time lowers costs because of an economic principle known as discounting (i.e., the value of dollar spent today is more than a dollar spent tomorrow). However, early detection is difficult because a delay exists between the initial infestation of a tree and the appearance of signs and symptoms (Cappaert et al. 2005).

*Agrilus planipennis* affects the appearance of a tree, and these signs can be used to identify trees that might be infested. Woodpeckers are natural predators of *A. planipennis* larvae, and woodpecked holes in a tree indicate the presence of insects under the bark (Lindell et al. 2008). Woodpeckers may also flake off the bark. Splits in the bark may expose the characteristic serpentine gallery, also characteristic of a beetle in the genus *Agrilus* Dahl. D-shaped exit holes are specific to members of *Agrilus* (Katovich et al. 2000, Haavik et al. 2012, Sever et al. 2012), and *A. planipennis* are the only known *Agrilus* spp. to attack ash in North America. Epicormic shoots and branch dieback are also general signs of underlying problems in the tree, and in conjunction with other symptoms, may point to an infestation of *A. planipennis* (Cappaert et al. 2005).

Ash trees are an important part of forests in North America, and all North American ash species are considered susceptible to *A. planipennis* (Cappaert et al. 2005, Poland and McCullough 2006, Lindell et al. 2008). Ash trees provide seeds and browse for game birds, songbirds, small mammals, deer, and moose (Erdmann et al. 1987, Wright et al. 1990). Ghandi and Herms (2010) found 43 native North American arthropod species that are monophagous on ash trees, and 238 native and exotic species that are associated with North American ash trees at some point during their life cycle. Species that rely on ash are at risk of endangerment because *A. planipennis* has the potential to extirpate the North American ash resource (Gandhi and Herms 2010). Besides being a resource for timber, pulpwood, and furniture-grade lumber, black ash in particular is an important cultural resource for Native American tribes that use the wood for traditional basket weaving (Benedict and Frelich 2008, Diamond and Emery 2011). Maas et al. (2006) found that in cities the vicinity of green space was positively correlated with perception of general health, especially in low income, urban neighborhoods. Donovan et al. (2013) found that as losses of ash trees due to *A. planipennis* infestation increased, so did mortality due to cardiovascular and lower-respiratory-tract illnesses.

*Agrilus planipennis* was found in Minnesota for the first time in Saint Paul in 2009, and is now known to be in Hennepin, Ramsey, Houston, Winona, and Olmsted counties (Minnesota Department of Agriculture et al. 2012, Abrahamson 2014, Sommerfeld 2014). In August 2013, *A. planipennis* was detected in Superior, WI, just across the Saint Louis Bay from Duluth, MN (Abrahamson 2014). If detected in Duluth, this infestation would be the northernmost in Minnesota.

Minnesota has more than 900 million ash trees (Miles et al. 2011), about 75% of which are black ash. Black ash and green ash make up almost half of Minnesota's timber resource by volume in lowland forests and about one quarter of Minnesota's total forest resource (Miles et al. 2011, Vanderschaaf 2012). Black ash is abundant in northern lowland hardwood forests, one of the only native tree species that grows in bogs and poorly drained soils (Telander 2013). Green ash is a popular boulevard tree in communities throughout the state and is a common native species to the central and southern part of Minnesota. *Agrilus planipennis* has infested green and black ash in the state. White ash and blue ash have been infested in the Minneapolis-Saint Paul Metro Area (M. Abrahamson and J. Osthus, personal communication), but these species represent less than 2% of the total number of all ash trees in Minnesota (Vanderschaaf 2012).

State and local governments in Minnesota have taken aggressive approaches to manage *A. planipennis*. Early detection, pre-emptive removal of ash trees in urban areas, prompt removals of infested trees, insecticide treatments, implementation of public outreach programs, and quarantines that restrict the movement of ash material from known infested counties collectively have helped minimize the spread of *A. planipennis* within Minnesota (Poland and McCullough 2006, McCullough and Mercador 2012, Minnesota Department of Agriculture et al. 2012). The Minnesota Department of

Agriculture has an active biocontrol program, which has included the introduction of three parasitic wasps: *Spathius agrili*, *Tetrastichus planipennisi*, and *Oobius agrili* (Duan et al. 2011). Insecticidal treatments, such as trunk injections of imidacloprid and soil drenches, are also recommended for healthy-looking urban ash trees as an alternative to tree removal and replacement with non-ash species (Mota-Sanchez et al. 2009; Herms et al. 2014). Unrelated to management, northern Minnesota's cold climate may also be a factor that is limiting the northward and westward spread of *A. planipennis*.

## II. Taxonomy and Morphology of *A. planipennis*

*Agrilus planipennis* is a flat-headed borer in the Buprestidae, Leach 1815, a family of insects commonly known as the jewel beetles (Grimaldi and Engel 2005). Buprestid larvae, which bore under the bark of trees, into stems, or leaf-mine, are soft-bodied with an expanded thorax. Adult jewel beetles range in size from 3-100mm long, have 11 antenomeres, are bullet-shaped, metallic, and brilliantly colored (Bellamy and Nelson 2002).

*Agrilus* Curtis 1825 has about 2,800 species, more than any other insect genus (Bellamy 2008). *Eupristocerus* and *Agrilus* are the only genera in Buprestidae in which larvae have urogomphi (Burke 1917).

*Agrilus planipennis* larvae are colored cream to light yellow can be distinguished from any other North American *Agrilus* spp. by the bell shape of the abdominal segments (Chamorro et al. 2012). Four instars can be distinguished by the length of the urogomphi (Wang et al. 2005) or the width of the peristoma (Lyons and Jones 2005). The fourth instar excavates a pupal cell. Once the pupal cell is complete, the larva folds itself into the cell in what is commonly referred to as the “j-stage”, which is a mostly inactive or quiescent stage. As a late fourth instar in the pupal cell and just prior to pupation, the larva shortens into a rounded prepupa, which is not another instar, but is occasionally termed a “fat head” (Duan et al. 2010). Adult beetle color can range from a vivid emerald green to almost black, while the ventral side of the abdomen is purple-red to copper.

Jendek (1994) synonymized *Agrilus feretrius* Obenberger, *A. marcopoli* Obenberger, and *A. marcopoli ulmi* Kurosawa under *Agrilus planipennis* Fairmaire. However, genetic analysis suggests that *A. marcopoli ulmi*, a population found in a Japan, is substantially different from *A. planipennis* and should remain a separate species (Bray et al. 2011). Bray et al. (2011) found little genetic diversity among North American populations, suggesting a single introduction from one Asian population, most likely from Tianjin City province or Hebei province. Keever et al. (2013) used microsatellite population genetics to determine that the population in Quebec was sufficiently genetically distinct from populations in Ontario and Michigan to suggest another introduction.

## III. Life cycle of *A. planipennis*

Adult *A. planipennis* begin to emerge at approximately 230-260 degree days above a base temperature of 10°C, which often occurs in Mid-May into early June (Poland and McCullough 2006). Emergence is most likely on warm sunny days (Wang et al. 2010). Adults live approximately 30 days, including time spent in the pupal cell (Wang et al. 2010). Adults move into the canopy to feed on foliage, but damage to the tree from foliage feeding is negligible. Adults mature sexually after feeding and will begin mating about 5 days after emergence (Wei et al. 2007). On average females will lay 30 eggs, singly or in small clusters (i.e., ≤ 7 eggs) under bark flaps or in bark crevices, typically from mid-May through early June (Wei et al. 2007). After approximately two weeks, neonates chew through the chorion into the bark. Larvae feed on the phloem, cambium, and outer xylem, creating serpentine galleries, typically meandering towards the base of the tree. However, factors such as high larval densities or low host quality can lead to irregularly shaped galleries (Wei et al. 2007). By late summer or early autumn, late instars begin excavation of a pupal cell, typically at or just below the surface of the sapwood. Once in a pupal cell, the late instar folds into a "j"-shape and overwinters. The entire larval stage requires at least 300 days to develop to pupae (Wei et al. 2007). Each larva pupate in a pupal cell. This stage lasts about 20 days. Teneral adults stay in the pupal cell. Fully sclerotized adults emerge from the tree after about 5 days (Wei et al. 2007).

*Agrilus planipennis* is univoltine in most of its native and invaded range (Xia et al. 2004, Wei et al. 2007). In a univoltine cycle, a larva will reach the "j-stage" and overwinter in a pupal cell. In a semivoltine life cycle, which has been observed in northern parts of its native range and in new and low-density infestations in North America (Wei et al. 2007, Wang et al. 2010), *A. planipennis* takes two years to fully develop. Eggs are typically laid later in the summer season by late-emerging adults, leading to earlier instars overwintering. The first winter, larvae typically overwinter as second instars, although first and third instars are occasionally found, and as late fourth instar or j-stage larvae the second winter. Overwintering larvae appear to be in a quiescent state, although larvae may be entering facultative diapause (Bauer et al. 2004, Wang et al. 2010).

The reason for the variation in voltinism is not known. Some hypotheses include host quality (Tluczek et al. 2011), larval density, and shorter warm seasons for development in more northern regions (Wei et al. 2007).

## IV. Hosts of *A. planipennis*

In its native range, *A. planipennis* is known to feed on four tree genera. *Fraxinus* spp., a monophyletic group in the family Oleaceae (Wallander 2008), is the most common host genus, including Asian species such as *F. chinensis* Roxb., *F. mandshurica* Rupr., *F. rhynchophylla* Hance., as well as introduced North American species *F. pennsylvanica*, *F. americana*, and *F. velutina* (Anulewicz et al. 2008). It has also been found attacking *Ulmus* spp., *Juglans* spp., and *Pterocarya* spp., but these genera are believed to be hosts for *A. marcopoli ulmi* rather than *A. planipennis* (Anulewicz et al. 2008, Bray et al. 2011). In North America *A. planipennis* has only been found on *Fraxinus* species (Anulewicz et al. 2008). Oleaceae also includes the *Olea*, *Syringa*, *Osmanthus*, *Forsythia*, *Jasminum*, and *Ligustrum* genera. Mountain ash (*Sorbus* spp.) is in the Rosaceae family and is not affected by *A. planipennis* (Hardin et al. 2001).

Adult *A. planipennis* begin feeding on foliage after initial flight (Wei et al. 2007). Anulewicz et al. (2008) found in a field choice study that adult beetles will land on other non-ash species, such as American elm (*Ulmus americana* L.), black walnut (*Juglans nigra* L.), hickory (*Carya ovata* (Mill.) K. Koch), and hackberry (*Celtis occidentalis* L.). However, the greatest number of adults landed on ash species. Among ash species, adults spend more time on green, black, and white ash over blue, Manchurian, and

European ash (*F. excelsior* L.) (Pureswaran and Poland 2009). McCullough et al. (2009) showed that adults prefer stressed ash trees and ash that receive full sun exposure. Although adult *A. planipennis* prefer ash as a host, they will oviposit on non-ash hosts in field and laboratory no choice experiments. In no choice assays, *A. planipennis* oviposited on green, white, and black ash species, as well as swamp privet (*Forestiera acuminate* (Michx.)Poir.), Chinese privet (*Ligustrum sinense* Lour.), glossy privet (*L. lucidum* Ait.), black walnut, elm, hickory, and hackberry (Anulewicz et al. 2006). However, in field experiments they will oviposit significantly less frequently on non-ash tree species than on ash species (Anulewicz et al. 2008).

While eggs were laid on non-ash trees and a small number of larvae attempted to feed on these hosts, *A. planipennis* larvae were not able to develop on American elm, black walnut, hickory, or hackberry (Anulewicz et al. 2006, Anulewicz et al. 2008). Larvae successfully fed and formed galleries on privet in the no choice oviposition experiment (Anulewicz et al. 2006), however *A. planipennis* has not been found on privet species in North America. In North America *A. planipennis* has been found on green, black, white ash, blue ash, and pumpkin ash (Lindell et al. 2008). Although adult landing preferences and oviposition did not differ among ash species, larval density in green ash was significantly greater than in other host species (Anulewicz et al. 2008). Chen et al. (2012) found that larvae extract more essential and non-essential amino acids from green ash than from white and black ash, suggesting they are better able to use higher concentrations of nutrients when feeding on green ash.

Green ash and white ash are consistently found to be preferred hosts for adults (Anulewicz et al. 2006, Anulewicz et al. 2008, Pureswaran and Poland 2009). These two species are closely related in the *Fraxinus* clade Melioides (Wallander 2008). Black ash falls into the Fraxinus clade along with Manchurian ash, which has been argued to be a subspecies of black ash.  Chinese ash and *F. rhynchophylla*, both Asian species, are synonymized, and are within the Ornus clade. Blue ash, typically the least preferred novel ash host of *A. planipennis* (Anulewicz et al. 2008), is in the group Dipetalae, and is more closely related to non-ash species within Oleaceae, such as privet (Wallander 2008).

Sixteen ash species occur in North America; four of these species are currently affected by the invasion of *A. planipennis* – green ash, black ash, white ash, and blue ash – but all are considered susceptible to attack. *Agrilus planipennis* is considered a significant threat to other species such as pumpkin ash (*F. profunda*) and velvet ash (*F. velutina* Torr.). Pumpkin ash is concentrated in the mid-Atlantic states but is scattered through the Gulf states and southern Midwest states (Hardin et al. 2001). Because the relatively limited range of pumpkin ash matches with the distribution of *A. planipennis*, New Jersey (Snyder 2013) and Pennsylvania (PNHP 2014) have designated it an endangered species, and Michigan has listed it as "threatened" (Michigan Natural Features Inventory 1999). Iowa (Natural Resource Commission 2009) and Wisconsin (WI DNR 2014) have assigned blue ash "threatened" status and Pennsylvania (PNHP 2014) has designated it as endangered. Rhode Island (Enser 2007) listed black ash a species of special concern. Minnesota has not declared special status for any ash species in the state (Division of Fish and Wildlife 2013).

## V. Cold Hardiness

### *A history of studies of insect cold hardiness*

Payne (1928) described insect cold hardiness as a two-part problem: 1) an insect's ability to survive extreme cold as measured by its supercooling point ("intensity factor"), and 2) an insect's ability to survive moderately cold temperatures for extended periods of time ("quantity factor"). These concepts remain central to the study of insect cold hardiness today. However, she was not the first to study how insects survive exposure to extreme cold temperatures. Scientists have been studying how insects respond to cold for hundreds of years. In this section I focus on measures that have been used to determine insect responses to cold exposure and some of factors that influence how insects survive cold.

Réaumur's *Mémoires pour servir à l'histoire des insects* (vol. 2 of 6) (1740) detailed a study where he observed that insect larvae froze below the freezing temperature of water and noted that not all larvae freeze at the same temperature. Although the chemicals that led to this depression of the freezing points were still unknown, he hypothesized that "liquors" in the hemolymph were responsible (Egerton 2006). Beyond just the temperatures at which insects were freezing, Réaumur continued observations and noted that some larvae recovered after freezing, while others died.

Technology progressed in the 19th Century and scientists were able to measure insect body temperatures and at what temperatures insects freeze more precisely with the advent of the thermocouple (Seebeck 1826). Bachmetjew (1901, WMW 1902), a Russian physicist, found that when supercooling an insect, the body temperature reaches a “critical point”, the lowest temperature reached before ice nucleation of body fluids begins, indicated by an increase in heat, i.e., the latent heat of fusion. Critical or undercooling points (Payne 1926, Robinson 1926, Payne 1927a, 1929, Mail and Salt 1933) are now commonly referred to as “supercooling points”, the lowest temperature reached before the body fluids spontaneously freeze (Payne 1927a, Lee and Denlinger 1985, 1991).

Bachmetjew (1899) described the process of controlling the cooling rate on an insect’s vital minimum temperature (the point at which the insect dies), and was the first to publish a description of the exotherm in an insect, or the release of the latent heat of crystallization, when it freezes (Bachmetjew 1901). Bachmetjew also outlined conditions that can influence the supercooling point, such as the cooling rate, the insect’s developmental stage and sex, whether or not the insect was fed or starved, the exposure time, how many times the insect was cooled, and the amount of liquid in the insect’s body (i.e., the “sap coefficient”).

The study of seasonal changes in supercooling points became common in the early 1900s. Payne (1926) reviewed earlier works that found varying supercooling points for the same species throughout the year, with the lowest values occurring in the cold winter months, and insects losing some cold hardiness with rising spring temperatures. However, she also mentioned that some insects found in environments with relatively stable temperatures, specifically aquatic insects and stored-product pests, typically do not exhibit variability in their cold hardiness. Payne (1927a) found in her study of oak borers – *Synchroa punctata* Newman., *Dendroides canadensis* Latreille, *Elater* sp., *Chrysobothris femorata* (Olivier), *Romaleum rufulum* Haldeman (now *Enaphalodes rufulus* (Haldeman)), *Xylotrechus colonus* (Fabricius), *Elaphidion mucrunotatum* Fab. [sic], *Graphisurus fasciatus* (Degeer), and *Nycotobates pennsylvanica* Deg. [sic] – in Minnesota that cold hardiness is seasonal, with supercooling points decreasing to stay below air temperatures in the fall. Payne (1927a) noted that the seasonal trend of supercooling points was consistent across species, but mean supercooling points varied among species. She also reported two more important factors to consider in insect cold hardiness: 1) winter-acclimated insects, those that would exhibit the extreme minimum supercooling point, can lose their cold hardiness with prolonged exposure to warmer temperatures, and 2) insects collected in the summer that are not cold acclimated can be held at a moderately low temperatures, and increase cold hardiness (although not to the extent of a winter-acclimated insect).

Salt's work in the 1930s considered the importance of the microhabitat where insects overwinter and how that influences how cold hardiness studies are applied and how relevant laboratory techniques may be. Mail and Salt (1933) studied supercooling points, which were known to be the lower lethal temperature, of winter acclimated and artificially cold-hardened *Leptinotarsa decemlineata* Say, the Colorado potato beetle, in relation to soil temperatures they experience while overwintering in order to predict the pest's northern range. Salt (1936) discussed the difficulty of obtaining a consistent cooling rate in laboratory experiments, how different cooling rates led to different supercooling points in *Ephestia kühniella* Zeller, and how the different cooling rates resulted in different sizes of the "rebound" (now termed the "exotherm"). Robinson

(1926), Salt (1936), and Hodson (1937) all tried to explain how environmental water and internal water content was related to insects' ability to overwinter.

However, water is not the only important liquid to be considered for insect overwintering and cold hardiness. Wilbur and McMahan (1958) returned to Réaumur's idea of "liquors" in insect hemolymph as a physiological mechanism for cold hardening in tissues and reported that a 10-20% concentration of glycerol in the heart of *Popilis disjunctus* (Illiger), now *Odontotaenius disjunctus* (Illiger) protected the heart from freezing, increased recovery rates of the insect compared to insects with lower concentrations of glycerol, and protected the tissue from freezing for longer periods of time at low temperatures above the freezing point. Wyatt and Meyer (1959) identified increased concentrations of glycerol in diapausing pupae of *Hyalophora cecropia* L., with special note that the insect overwinters in this stage. Salt (1959) correlated lower supercooling points in *Bracon cephi* (Gahan) with higher concentrations of glycerol, but determined that the presence of glycerol did not prevent mortality from freezing. Sømme (1964) noted that concentrations of glycerol that lower supercooling points differ among species and are seasonal. While glycerol is relatively common in insects that experience cold temperatures, this compound is just one of several carbohydrate cryoprotectants, which are small, stable, water-soluble, non-toxic molecules that work to depress an insect's supercooling point to avoid freezing and, at higher concentrations, may reduce ice formation and resulting cell damage (Denlinger and Lee 2010). Ice-nucleator proteins and antifreeze proteins are also important insect cryoprotectants, but glycerol was found to be the major component in *A. planipennis* (Crosthwaite et al. 2011), so I will not be discussing the others in this section.

Cooling rate, developmental stage, sex, winter acclimation, and cryoprotectants are still considered factors in cold hardiness experiments, as well as seasonal variability of supercooling points, and the influence of different host species on herbivores (reviewed by Uvarov 1931, Sinclair et al. 2003, Denlinger and Lee 2010). Salt (1966) proposed using a standard cooling rate of 1°C/minute; this rate is considered the standard cooling rate for experiments (Lee and Denlinger 1985, Sinclair et al. 2003, Denlinger and Lee 2010).

### *Nutrition and host influence on insect cold hardiness*

Insect nutrition and feeding status were recognized as important factors to insect overwintering (Baumberger 1917, Leathers 1922). Robinson (1926) reported that differences in the water content of food given to the granary weevil *Sitophilus granarius* (L.) led to differences in its ability to withstand exposure to low temperatures. *Dendroctonus monticolae* Hopkins (now *Dendroctonus ponderosae* Hopkins) were less cold hardy when they came from sugar pine as opposed to lodgepole pine and ponderosa pine (Yuill 1941). *Megachile rotundata* (F.) froze at higher temperatures when they fed on honey “contaminated” with dust versus “clean” honey (Krunic 1971); the difference in supercooling points was attributed to possible ice nucleators in the contaminated honey. Liu (2007) observed different supercooling points from overwintering *Helicoverpa armigera* Hübner pupae when they had been reared on different host plants, and seasonal differences in supercooling points and glycogen content were reported among those different hosts (Liu et al. 2009). Hiessaar (2009) found that *Phyllotreta undulata* Kutschera fed oilseed rape and white cabbage had lower supercooling points than individuals fed white mustard and Indian mustard; this difference held for flea beetles that were tested shortly after feeding, and those that were starved for four days.

Host plant can play a role in survival of the individual that is feeding and its offspring. Trudeau (2010) reared *Malacosoma disstria* Hübner on trembling aspen or sugar maple for an entire generation, then compared the supercooling points and overwintering survival of unfed overwintering progeny. Supercooling points from the sugar maple group was significantly lower than the trembling aspen group in November, in general supercooling points did not significantly differ between groups. However, >80% of progeny of parents that were reared on sugar maple survived, which was significantly greater than the 30% survival of progeny of parents reared on trembling aspen.

### *Classifying insect cold hardiness*

Supercooling points became the basis for categorizing physiological strategies that insects use to survive cold. If an insect dies when it freezes, it is “freezing-susceptible” (Salt 1961) or “freeze intolerant” (Denlinger and Lee 2010). If an insect can recover after being frozen, it is “freezing-tolerant” (Salt 1961, Denlinger and Lee 2010).

However, insect cold hardiness is not as simple as whether or not an insect dies when it freezes. Along with supercooling points, “critical minimums” – or what we now would call lower lethal temperatures – were of interest to scientists studying economically important insects (Sanderson 1908, Payne 1927a, Salt 1936). Many insects overwinter in locations that never experience temperatures so extreme as to cause freezing (Payne 1927b, Mail 1932). However, insects may be exposed to more moderately cold temperatures for extended periods of time, such as those that overwinter under snowpack, in leaf litter, or in the soil where temperatures usually do not fall much below 0°C (Mail 1932).

Modern lower lethal temperature studies focus on instantaneous exposure to temperatures at and surrounding the supercooling point, the “intensity factor”; lower lethal time studies, which will not be discussed at length here, focus on the “quantity” factor by adding a time factor to low temperature exposure (Payne 1928, Denlinger and Lee 2010). Lower lethal temperature studies add one more commonly accepted category to insect cold hardiness: chill intolerant. In lower lethal temperature studies, insects are cooled to temperatures below and above the supercooling point and then warmed for survivorship to be determined (Carrillo et al. 2004, Morey et al. 2012, Hanson et al. 2013). Exposure temperatures above the supercooling point are important because some insects die from cold injury incurred before freezing. Lower lethal temperature observations may complicate categorizing insect cold hardiness strategies even further. By observing mortality at and below the supercooling points, a few individuals in a so-called “freeze-intolerant” species may exhibit freeze tolerance. These species that exhibit such a mixed strategy may be termed “partially freeze tolerant” (Bale 1993, Sinclair 1999, Morey et al. 2013).

Although chill intolerant, freeze intolerant, and freeze tolerance are widely used to categorize degrees of insect cold hardiness, other categories have been suggested. Bale

(1993) suggested six categories: 1) freeze tolerant, high supercooling points and low lower lethal temperatures, 2) freeze avoiding, low supercooling points and little to no mortality for long periods of time at low temperatures as long as the insect does not freeze, 3) highly chill tolerant, find some level of environmental protection from extreme temperatures (e.g., snow pack) and are able to survive for long periods of time at moderately low temperatures, 4) moderately cold tolerant, similar to highly chill tolerant species except these insects die after shorter durations at moderately low temperatures, 5) chill susceptible, potentially low supercooling points, but mortality occurs after short exposure periods to temperatures above the supercooling point, and 6) opportunistic survival, which, like chill susceptible species, die at temperatures above the supercooling point, but these insects use warm overwintering sites. Sinclair (1999) proposed dividing Bale's freeze tolerant category into partial freeze tolerance (surviving some ice formation in the body), moderate freeze tolerance (surviving freezing to about 10°C below the supercooling point), strong freeze tolerance (surviving freezing to 30°C or more below the supercooling point), and freeze tolerance with low supercooling point (surviving freezing and having a low supercooling point). Other categories based on causes of cold injury (i.e., freezing, cold shock, and chilling) have also been suggested (Nedved 2000), but chill intolerant, freeze intolerant, and freeze tolerant are most commonly used.

## VI. Conclusion

Crosthwaite et al. (2011) studied cold hardiness of *A. planipennis* from green ash in southern Ontario; they measured seasonal supercooling points and lower lethal temperatures. Larval supercooling points were tested at 2-4 week intervals between

September 2008 and mid-April 2009, and September 2009 and mid-April 2010 using 5 to 12 larvae at each interval. In both study periods, supercooling points significantly declined from September through December, with the lowest supercooling point of -35.3°C recorded in November 2009. The concentration of glycerol, the major cryoprotectant in *A. planipennis*, increases at the same time as supercooling points decrease (Crosthwaite et al. 2011). This study determined that *A. planipennis* was freeze intolerant and that mortality above the supercooling point was not due to cold exposure. Supercooling points increase with early spring temperatures in March, but *A. planipennis* is not quick to lose cold acclimation. Sobek-Swant et al. (2012) found that once cold acclimated, *A. planipennis* supercooling points increased only marginally after one week at 10°C and only after two weeks at 0-4°C. Vermunt et al. (2012) measured under-bark temperatures on north and south sides of ash trees in Canada and found that wood-borers would experience about a 2°C- and 4°C-buffer, respectively, from the minimum air temperature.

While these studies address important parts of *A. planipennis* overwintering biology, many questions remain to be answered. The second chapter of this thesis will focus on the cold hardiness of field collected *A. planipennis* from Minnesota, which tend to experience colder winter temperatures than *A. planipennis* in other currently infested regions in North America. *Agrilus planipennis* larvae from green and black ash, the two most common ash trees in Minnesota, will be tested for cold hardiness and overwintering mortality, since influence of host species on cold hardiness has not yet been shown. Chapter three will focus on temperatures taken underneath the bark of both green ash and black ash to determine if larvae experience different temperatures in different hosts, and how different host ranges may determine where *A. planipennis* can survive winter, establish, and appear as an important pest.

# Chapter 2

# Host Influence on the Cold Hardiness of *Agrilus planipennis* Fairmaire

## I. Introduction

The emerald ash borer, *Agrilus planipennis* Fairmaire, is a flat-headed borer that is native to east Asia (Liu et al. 2004; Cappaert et al. 2005; Poland and McCullough 2006; Duan et al. 2012) and first identified in North America in 2002 (i.e., in Michigan, USA, and Ontario, Canada; Liu et al. 2004). As of September 2014, *A. planipennis* has been confirmed in 24 states and two Canadian provinces (USDA APHIS PPQ and CFIA 2014). *Agrilus planipennis* is highly specific to true ash trees (*Fraxinus* spp.) in North America (Anulewicz et al. 2006, 2008). All 20 species of ash in North America are considered susceptible to *A. planipennis* infestation (Wallander 2008), although blue ash is less preferred than other North American ash species (Pureswaran and Poland 2009; Tanis and McCullough 2012). Larval feeding in the cambium, phloem, and outer xylem (Bauer et al. 2004; Cappaert et al. 2005) can girdle a tree and kill it in 1-4 years (Yu 1992; Cappaert et al. 2005). *Agrilus planipennis* has killed tens of millions of ash trees in North American forests and urban areas (McCullough et al. 2009; Smith et al. 2009), is endangering native arthropods (Gandhi and Herms 2010), and is costing cities and states billions of dollars to manage (Kovacs et al. 2010). Due to the delays between an initial infestation, symptom development, and tree death, *A. planipennis* can be established in an area for years before it is detected (Siegert et al. 2007).

*Agrilus planipennis* was found in Minnesota for the first time in Saint Paul in 2009, and as of September 2014 is known to be in Hennepin, Ramsey, Houston, Winona, and Olmsted counties (Minnesota Department of Agriculture et al. 2012; Abrahamson

2014; Sommerfeld 2014). In August 2013, *A. planipennis* was detected in Superior, WI, just across the Saint Louis Bay from Duluth, MN (Gilson and Dick 2013; Abrahamson 2014). If detected in Duluth, this infestation would be the northernmost in Minnesota.

Minnesota has more than 900 million ash trees, about 75% of which are black ash (*F. nigra* Marshall; Miles et al. 2011). Black ash and green ash (*F. pennsylvanica* Marshall) comprise almost half of Minnesota's timber resource by volume in lowland forests and about one quarter of Minnesota's total forest resource (Miles et al. 2011; Minnesota Department of Natural Resources 2011). Black ash is abundant in northern lowland hardwood forests, one of the few native tree species that grows in bogs and poorly drained soils (Palik et al. 2012; Telander 2013). Green ash is a popular boulevard tree in communities throughout the state and is a common native species to the central and southern part of Minnesota (VanderSchaaf 2012). *Agrilus planipennis* has infested green and black ash in the state. White ash (*F. americana* L.) and blue ash (*F. quadrangulata* Michx.) have been infested in the Minneapolis-Saint Paul Metro Area (M. Abrahamson and J. Osthus, personal communication), but these species represent less than 2% of the total number of all ash trees in Minnesota (VanderSchaaf 2012).

Cold often determines the northern limits of an invading insect's range (e.g., Ungerer et al. 1999, Crozier 2003). *Agrilus planipennis* has yet to achieve its ultimate distribution in North America. The potential geographic range expansion by *A. planipennis* into northern Minnesota where black ash is abundant in lowland black ash-American elm-red maple forests (Erdmann et al. 1987) is a concern. Supercooling points, the lowest temperature an individual reaches before the release of the latent heat of fusion when body fluids begin to crystallize, and lower lethal temperature, the measure of mortality in a population after a brief exposure to a low temperature, are commonly used to assess the ability of an insect to withstand cold temperatures, i.e., its cold hardiness (Sømme 1982, reviewed in Denlinger and Lee 2010). If the lower lethal temperature is above the supercooling point, the insect is classified as chill-intolerant. If the insect dies when it freezes (i.e., the lower lethal temperature equals the supercooling point), the insect is chill tolerant/freeze intolerant. Freeze tolerance occurs when an insect can survive the freezing of its body fluids.

Crosthwaite et al. (2011) studied some aspects of cold hardiness in *A. planipennis* from green ash in southern Ontario. A seasonal supercooling point trend was found, with the lowest mean supercooling points occurring from November to February, at approximately -30°C. A lower lethal temperature study found that *Agrilus planipennis* is freeze intolerant (Crosthwaite et al. 2011). The concentration of glycerol, the major cryoprotectant in *A. planipennis*, increases in concurrence with decreasing supercoiling points (Crosthwaite et al. 2011). *Agrilus planipennis* is also not quick to lose cold acclimation. Sobek-Swant et al. (2012) found that once cold acclimated, *A. planipennis* supercooling points only marginally increased after one week when exposed to 10°C or warmer, and only after two weeks at 0-4°C. Vermunt et al. (2012) found that overwintering *A. planipennis* larvae under the bark do not experience the minimum air temperature, but 4°C warmer on average.

Previous work on cold tolerance and overwintering ability of *A. planipennis* (Venette and Abrahamson 2010, Crosthwaite et al. 2011, Sobek-Swant et al. 2012, Vermunt et al. 2012) did not consider the influence of different host species on overwintering ability, which could alter the predicted geographic distribution of this insect. Different host plants are associated with different supercooling points and overwintering ability in some insects (Yuill 1941, Liu et al. 2007, 2009; Hiiesaar et al. 2009, Trudeau et al. 2010).

*Agrilus planipennis* adults consistently prefer green ash over black ash (Anulewicz et al. 2006, 2008; Pureswaran and Poland 2009), and Chen et al. (2012) found that larvae extract more essential and non-essential amino acids from green ash than from black ash, suggesting they are better able to digest nutrients from green ash. Because of the differences in host preference and usage, we expect to see a difference in cold hardiness between *A. planipennis* larvae from green ash and black ash. In this study, we use artificially-infested and field-collected larvae to measure supercooling points, and field-collected larvae to measure lower lethal temperature to determine if host plant affects the cold hardiness of *A. planipennis* larvae.

## II. Materials and Methods

### *Laboratory rearing of A. planipennis.*

Because of the low numbers of infested black ash within the current distribution of *A. planipennis* in Minnesota, we artificially infested cut logs with laboratory reared larvae. Rearing of *A. planipennis* was modified from the methods outlined in Keena et al. (2010). Green ash, naturally-infested with *A. planipennis* from Minneapolis and Saint Paul, MN, were felled in late May and early June 2011, cut into 38-56 cm lengths, and placed in 66.8 (length) by 31.75 cm (diameter) cardboard rearing tubes with vented plastic lids in a controlled temperature greenhouse. Rearing tubes were checked daily for newly emerged adults. Adults were transferred to 1.9L plastic jars (United States Plastic Corporation, Lima, OH) with a mesh lid (aluminum window screen with an opening size of 1.41 x 1.59 mm or stainless steel wire mesh with an opening size of 6.4 x 6.4 mm overlapped for openings approximately one quarter the size). The bottom of the jar and the lid were lined with basic grocery store bleached coffee filter paper. Adults were also given a water pick and fresh green ash foliage from a mature tree. Jars were cleaned every other day at which time filter paper, water, and foliage were replaced. Eggs that were laid on the filter paper were counted and transferred to 37 mL sealed plastic cups (Solo Cup Company, Highland Park, IL). Rearing jars and egg cups were stored in an illuminated growth chamber at 26°C with a photoperiod of 16:8 (L:D) h. Cups were checked daily for newly emerged neonates, which were infested into cut logs on the same day.

Green and black ash logs from Grand Rapids, MN, far from any known infestations of *A. planipennis*, were cut in July 2011. Two green ash and two black ash logs were used in 2011. Logs ranged in size from 12-16 cm in diameter and 90-94 cm in length. A total of 26 neonates, all that were available, were infested from 11 July – 18 July 2011, 13 neonates in each species of ash (no more than 1 larva per 205 $cm^2$ of log surface area). Neonates were only placed in the top 60 cm of the log because each log was set in a plastic potting dish with 2.5-5 cm of water. Notches 2-3 cm wide and down to the phloem were made in the bark with a 1.25 cm chisel, and a neonate was placed at the bottom of each notch. Laboratory infested logs were kept in a greenhouse through

August (mean temperature June - August 2011 = 27.6 ± 2.6°C SD, mean temperature June - August 2012 = 28.4 ± 3.3°C) before they were moved into an unheated garage in preparation for winter temperature acclimation. The bark was peeled from the logs, and larvae were extracted in November 2011. The rearing process was repeated in 2012. Uninfested logs were cut in July 2012 from Grand Rapids, MN, and ranged in size from 25.5-33 cm in diameter and 118-125 cm in length. Logs were artificially infested from 16 July – 19 July 2012, with 28 neonates infested into green ash and 29 infested into black ash. Artificially infested logs were peeled in January 2013.

### *Cold exposure.*

*Agrilus planipennis* larvae were cooled following protocols modified from Carrillo et al. (2004). Each larva was weighed to the nearest tenth of a milligram before being placed in a trimmed 9 mm (diameter) x 27 mm (length) gelatin capsule (size 000) (Capsuline, Pompano Beach, FL). Larvae were placed in capsules to prevent contact with the high vacuum grease (Dow Corning, Midland, MI) that was used to attach the capsule to the thermocouple. Copper-constantan thermocouples were attached to multi-channel data loggers (USB-TC, Measurement Computing, Norton, MA) so that up to 16 insects could be cooled individually at a time. Temperatures were recorded at the first point where the two metals met (Carrillo et al. 2004) once per second by using TracerDAQ software (Measurement Computing, Norton, MA). Coiled thermocouples, as described in Hanson et al. (2013), were insulated with a polystyrene cube designed to keep the cooling rate of the insect at a constant 1°C min$^{-1}$ in a -80°C freezer (Carrillo et al. 2004).

### *Supercooling points of laboratory-infested larvae.*

Supercooling points of laboratory-infested *A. planipennis* larvae were measured in November 2011 and January 2013. Bark was peeled from study logs. All recovered larvae had reached the late fourth instar, sometimes called the "j"-stage (Chamorro et al. 2012). In 2011 six of thirteen infested *A. planipennis* larvae had successfully developed in black ash, and five were extracted without injury. Seven of thirteen larvae developed in green ash, and four were successfully recovered. In 2013 seven of 29 larvae developed in black ash, and four were extracted. Eight of 28 larvae developed in green ash, and four were successfully recovered. Larvae were cooled until an exotherm was detected.

All data were analyzed in R version 3.1.1 (R Development Core Team 2008). Linear regression was used to assess the relationship between supercooling point and larval mass. Larval supercooling points were compared between hosts in each year and between years for each host by using multiple Welch's t-test with a Bonferroni corrected α of 0.0125, to maintain an overall α of 0.05, after we confirmed equal variances with an F-test.

### *Field-collected A. planipennis larvae.*

*Agrilus planipennis* larvae from naturally-infested trees were used for cold hardiness assessments in the winters of 2012-13 and 2013-14. In January 2013, black and green ash trees were removed from the Fort Snelling Golf Course, in Minneapolis, MN (44.886286° latitude, -93.195189° longitude). These trees had been planted and maintained by the City of Minneapolis and by Fort Snelling Golf Course personnel. Logs ranging in size from 5-20 cm in diameter and approximately 90 cm in length were stored outside in Saint Paul on the north side of a building and hidden from view of regular traffic. Logs were peeled and larvae extracted between 10 January and 28 February, 2013. In the winter of 2013-14, green and black ash logs were removed from Great River Bluffs State Park (approximately 43.944479° latitude, -91.38496° longitude) in Winona County, MN, in November and December 2013 and January and early February 2014 (Permit: Minnesota Department of Agriculture, State Formal Quarantine No. RF-1 036, RF-1076, RF-2074 Emerald Ash Borer, Section VI: Special Exemptions). Logs 5-15 cm in diameter and approximately 120 cm in length were peeled between 30 December, 2013, and 27 March, 2014. The stages of all larvae that were found were noted as either fourth instar (including the "j-stage") or early (first - third) instar, whether or not the larva was recovered without injury. The proportions of individuals that were fourth instars were compared between hosts in the same year and within the same host between years using a z-test of proportions.

Extracted larvae from both seasons were stored at 0°C for 24-96 h before measuring larval mass and exposing larvae to cold. This time allowed minor injuries from extraction to become visible, but not long enough for the overwintering larvae to lose cold acclimation (Sobek-Swant et al. 2012).

Laboratory cooling experiments of field-collected larvae were performed from 11 January - 1 March 2013, and 3 January- 20 March 2014. In January 2013, 13 larvae from green ash and 15 larvae from black ash were cooled until all gave supercooling points. Results from these supercooling point blocks were used to determine cold exposure treatments for lower lethal temperature studies. These cold exposure studies were conducted following a randomized complete block experimental design. Up to ten larvae from black and green ash (i.e., up to 20 larvae total per block) were randomly assigned to one of five temperature treatments (yielding two larvae from each host per temperature). A total of 111 larvae from green ash and 100 larvae from black ash were used in the winter of 2012-13; 47 larvae from both green and black ash were used in the winter of 2013-14. Treatment temperatures in the winter of 2012-13 were -20°C, -25°C, -30°C, -35°C, and a room temperature control (~25°C). Treatment temperatures in the winter of 2013-14 were -25°C, -30°C, -35°C, -40°C, and a room temperature control. In both years, larvae selected for controls were processed in exactly the same way, including being placed in individual capsules and attached to copper-constantan thermocouples with high vacuum grease. Larvae with a mass less than 1 mg were tested for supercooling points but were not observed for survival because of apparent sensitivity to handling. For all other larvae, if an exotherm was observed as the individual cooled to its target temperature, the supercooling point was noted, but the individual was not removed from the freezer until the target temperature was reached. At that time, the larva was promptly removed from the freezer and held at room temperature.

To determine if larvae had survived, cold-exposed and control larvae were removed from capsules and held in covered 24-well cell culture plates (Corning, Tewksbury, MA) at room temperature in the dark. Culture plates were held in a plastic storage container with a loose fitting lid for gas exchange and wet paper towels to keep humidity high. First observations were taken 72 h after cold exposure, and then every other day for 10 days. Color and movement were noted. Only fourth instars could develop without feeding, so signs of development (e.g., molting) were noted for this stage. Larvae that were active during the observation period or had pupated were classified as having survived. Larvae that had not molted or pupated, did not move, and were discolored were categorized as dead.

*Analysis of the likelihood of freezing.*

The relationship between supercooling point and mass, a proxy for development, was analyzed by linear regression in R. Because no relationship between these two measures was found ($F_{1,\,129} = 0.654$, $P = 0.420$), supercooling points were not analyzed by instar. Supercooling point frequency distributions by host were tested for normality with the Shapiro-Wilk test of normality in R (function *shapiro.test*). Because larval supercooling point distributions from both green and black ash in the winter of 2012-13 were not normally distributed (Table 1), supercooling point data were analyzed by using non-parametric survival analysis in the survival package in R (Therneau 2014). This package also allowed us to account for censoring in the data. In our case, temperature replaced time in the survival analysis. A *Surv* object was created, which categorized each larva as having an exotherm or not. When exotherms were observed, the supercooling points provided the exact temperatures at which freezing began, and these data are considered non-censored. A larva that did not give an exotherm before reaching the target temperature was considered a right-censored observation (i.e., we presumed that the supercooling point occurred at an unspecified temperature colder than the removal temperature). To estimate the probability that at individual would begin to freeze, given that it had been cooled to a particular temperature, non-parametric Kaplan-Meier curves were estimated from non-censored and right-censored observations by using the *survfit* function in the R survival package (Therneau 2014). We used the *survdiff* function to test for differences (α = 0.05) in Kaplan-Meier curves between each host in the same year or between years for the same host.

### *Analysis of mortality*.

Host influence on larval mortality was compared by a Z-test of proportions. Next, the total proportion mortality of frozen individuals from each host was compared using the Z-test. The proportions of mortality from individuals that did not freeze from both hosts were compared in the same way. Z-scores were calculated in Microsoft Excel (version 14.0.7128.5000, Microsoft Corporation 2010) and associated P-values were determined using a standard normal probabilities Z-score table (α = 0.05).

### *Analysis of cold-tolerance strategy.*

The determination of a species' cold-tolerance strategy (i.e., chill intolerant, chill tolerant/freeze-intolerant, or freeze tolerant) depends on a comparison between the likelihood of freezing and the likelihood of dying at a particular temperature (Sinclair 1999). In our experiments, relatively high mortality (20.5% in 2012-13, and 34.9% in 2013-14) among larvae that had not been exposed to cold (i.e., room-temperature controls) complicated the interpretation of results from parametric or non-parametric survival analysis. As a result, we performed an analysis to determine whether observed patterns of mortality were consistent with the classification of overwintering *A. planipennis* larvae as chill-tolerant/freeze-intolerant (Crosthwaite et al. 2011).

Chill-tolerant/freeze-intolerant insects are able to survive exposure to low temperatures but die once freezing begins. We began by estimating the probability that a larva would be dead, $D_t(+)$, or alive, $D_t(-)$, after exposure to a particular temperature, $t$. For a chill-tolerant/freeze-intolerant species, an individual would be dead either because it froze or because it was killed by handling or some other agent. If the likelihood that an individual larva would freeze after exposure to a particular temperature is expressed as $p_t$, the sensitivity of the insect to freezing is $Se$, and the likelihood that an unfrozen larva survives processing is $Sp$, the probability that an individual would die at a particular temperature is given by:

$$D_t(+) = p_t Se + (1 - p_t)(1 - Sp),$$

where $Se$ and $Sp$ assume values from 0 – 1. For a perfectly freeze intolerant species, $Se = 1$ and for a partially freeze-tolerant species, $0 < Se < 1$. Conversely,

$$D_t(-) = p_t(1 - Se) + (1 - p_t)(Sp).$$

Values for $p_t$, $Se$, and $Sp$ are estimated empirically: $p_t$ comes from a Kaplan-Meier curve, $Se$ is the observed fraction of individuals with an exotherm that died, and $Sp$ is the observed survivorship from the room temperature controls.

When the number of individuals ($n$) exposed to a particular temperature is finite, the probability of observing exactly $x$ deaths can be determined from the binomial distribution, which, generically written, is $\binom{n}{x} p^x (1 - p)^{n-x}$. Substituting $D_t(+)$ for $p$ and $D_t(-)$ for (1-$p$), we obtain:

$$P(D^+ = x) = \binom{n}{x} (p_t Se + (1 - p_t)(1 - Sp))^x (p_t(1 - Se) + (1 - p_t)Sp)^{n-x}$$

We utilized this equation in a Microsoft Excel worksheet to calculate the expected probability of observing exactly *x* deaths (allowed to vary from 0 to *n*) during our cold exposure experiments. In these calculations, *n* was the number of insects from black or green ash that were cooled to a target temperature. Host- and year-specific estimates of $p_t$, *Se*, and *Sp* were used in the calculations. The expected number of deaths at a given temperature was $\sum_{x=0}^{n} xP(D^+ = x)$. Approximate 95% confidence intervals were identified as the values of *x* that corresponded to the 2.5% and 97.5% quantiles of the cumulative distribution function. Expected (± 95% CI) and observed values were expressed as a proportion of *n* and compared graphically.

To estimate temperatures that would kill 50% or 90% of *A. planipennis* larvae (i.e., the $LT_{50}$ and $LT_{90}$, respectively), we needed to account for the relationship between freezing and death. The proportion of interest (i.e., 0.5 or 0.9) was divided by the appropriate *Se* to get the adjusted proportion for each year and host. The temperatures (within 95% confidence intervals) required to cause that adjusted proportion of mortality were calculated from the Kaplan-Meier supercooling point curves using the *quantile* function in R. When the corrected proportion was greater than 0.95, 95% confidence intervals for the $LT_{90}$ could not be estimated. When the corrected proportion was greater than 1.0, an $LT_{90}$ could not be estimated.

## III. Results

### *Supercooling points of laboratory reared A. planipennis larvae.*

The supercooling points of larvae from artificially infested green and black ash did not have equal variances in 2011 ($F_{4,3} = 0.085$, P = 0.038) but were equal in 2013

($F_{3,3} = 0.81$, $P = 0.86$). Mean supercooling points were not different between hosts in 2011 ($t = -0.8$, $df = 3.4$, $P = 0.49$; Table 1) or 2013 ($t = 1.6$, $df = 6$, $P = 0.16$; Table 1). The mean supercooling point of larvae from black ash was significantly lower in January 2013 than in November 2011 ($t = 3.5$, $df = 7$, $P = 0.001$). The mean supercooling points of larvae from green ash were not significantly different between years with the Bonferroni corrected $\alpha = 0.0125$ ($t = 3.3$, $df = 6$, $P = 0.016$).

*Field-collected A. planipennis larvae.*

*Stage distributions of larvae*. In the winter of 2012-13, 99.4% of *A. planipennis* larvae from naturally infested green ash were fourth instars, which was significantly more ($Z = 3.64$, $P < 0.001$) than the 92.3% of larvae from naturally infested black ash that were fourth instars (Figure 1). In the winter of 2013-14, 46.8% of larvae found in green ash were fourth instars, which was not statistically different ($Z = 1.30$, $P = 0.099$) from the 53.5% of larvae from black ash that were fourth instars. In the winter of 2012-13, significantly more fourth instars were found in green ash ($Z = 14.80$, $P < 0.001$) and black ash ($Z = 9.14$, $P < 0.001$) than in the following winter.

*Supercooling points of field-collected A. planipennis larvae*. Supercooling points were not significantly different between hosts in the winter of 2012-13 ($\chi^2 = 1.9$, $df = 1$, $P = 0.17$; Figure 2) or in 2013-14 ($\chi^2 = 0$, $df = 1$, $P = 0.90$; Figure 2). Supercooling points of larvae from black ash were not significantly different between years ($\chi^2 = 1.2$, $df = 1$, $P = 0.28$; Figure 2), but were significantly different between years for larvae from green ash ($\chi^2 = 6.4$, $df = 1$, $P = 0.01$; Figure 2). In the winter of 2012-13, the coldest supercooling points recorded from black ash and green ash were -37.5°C and -36.2°C, respectively, and in the winter of 2013-14, -37.3°C and -38.0°C (Figure 2).

Supercooling point distributions between hosts were not significantly different from the winter of 2012-13 ($\chi^2$ = 1.9, df = 1, P = 0.17; Figure 2) or 2013-14 ($\chi^2$ = 0, df = 1, P = 0.90; Figure 2). Supercooling points of larvae in black ash between years were not significantly different ($\chi^2$ = 1.2, df = 1, P = 0.28; Figure 2), but were significantly different for larvae from green ash ($\chi^2$ = 6.4, df = 1, P 0.0113; Figure 2).

*Analysis of mortality.* Significantly more mortality occurred for larvae from black ash than green ash in two temperature treatments from the winter of 2012-13: -35°C (Z = 2.13, P = 0.017), and -25°C (Z = 2.50, P = 0.0062). There were no significant statistical differences in proportion of mortality between hosts at any other temperature treatments or during the winter of 2013-14 (Table 2).

*Analysis of cold tolerance strategy*. The proportion of frozen larvae that died (*Se*) was not different between green and black ash in either winter or between the winters of 2012-13 and 2013-14 (Table 3). Proportions of larvae that did not freeze but died (*Sp*) were not significantly different between green and black ash for either year or between years for either host (Table 3). Observed proportion of larval mortality for each treatment temperature fell within the expected 95% confidence intervals (Figure 3). In general, mortality increased non-linearly as temperatures declined.

To determine the lower lethal temperature for 50% and 90% of larvae from black ash in the winter of 2012-13, the proportions of larvae that needed to freeze to result in 50% or 90% mortality were 0.519 and 0.934, respectively, which gave an $LT_{50}$ of

-33.4°C (95% CI: -34.4°C, -32.3°C) and an $LT_{90}$ of -36.2°C (≤-37.5°C, -35.5°C). For the winter of 2013-14, the required proportions of frozen larvae from black ash were 0.562 and 1.01 to give an $LT_{50}$ of -33.1°C (-34.5°C, -31.5°C). Because the proportion of frozen larvae required for 90% mortality was greater than 1.0, the $LT_{90}$ could not be estimated.

To determine the lower lethal temperature for 50% and 90% of larvae from green ash in the winter of 2012-13, the proportions of larvae that needed to freeze to result in 50% or 90% mortality were 0.552 and 0.994, respectively, which gave an $LT_{50}$ of -35.2°C (95% CI: -35.9°C, -34.7°C) and an $LT_{90}$ of -36.8°C (95% CI could not be estimated). For the winter of 2013-14, the required proportion of frozen larvae from green ash for 50% mortality of the population was 0.593 for an $LT_{50}$ of -33.4°C (-34.1°C, -32.1°C). The adjusted proportion for 90% mortality of larvae from green ash was 1.05, so an $LT_{90}$ could not be estimated.

## IV. Discussion

Understanding the cold tolerance of insects is often more complicated than just measuring supercooling points (Salt 1964, Bale 1996, Denlinger and Lee 2010); comparisons of the extent of mortality at a given temperature to the proportion of insects that may have frozen at that temperature provide a more robust characterization of insect cold tolerance (Sinclair 1999). Host can occasionally affect the lower lethal temperature of overwintering *A. planipennis* as shown by the differences in $LT_{50}$ between larvae from green ash and black ash in the winter of 2012-13. While host did not consistently affect mortality, where there were differences, larvae from black ash were less cold hardy than larvae from green ash. Host did not affect supercooling points, but the supercooling points of *A. planipennis* larvae that we recorded generally were lower than previously reported. Our comparison of lower lethal temperatures and supercooling point distributions suggests *A. planipennis* may be partially freeze tolerant, where a proportion of individuals can freeze and survive (Wharton and Hawes 2010), even though the species is otherwise classified as freeze intolerant.

For overwintering *A. planipennis* larvae, Wu et al. (2007) reported supercooling points in China between -23.0 and -26.4°C. Venette and Abrahamson (2010) reported a mean supercooling point of -25.0°C for winter acclimated larvae. Crosthwaite et al. (2011) reported that larvae of *A. planipennis* change supercooling points seasonally and are at their maximum supercooling capacity from November through February, when the mean supercooling points were reported to be -27 to -28°C. Our artificially infested larvae had supercooling points in November 2011 that were similar to these previously reported values. However, the mean supercooling points of artificially-infested larvae in January 2013 and field-collected larvae in January-March 2013 and January-March 2014 were 1.8 to 5.8°C lower than previously reported mean supercooling points and were significantly colder than those from November 2011. Because Minnesota experiences colder winters than many other North American regions affected by *A. planipennis*, it is possible that geography plays a role in the variation of reported cold hardiness in *A. planipennis*. For example, in China, cold hardiness of the migratory locust *Locusta migratoria* L. varied between northern and southern regions, where overwintering eggs in northern regions could survive exposure to colder winter temperatures than overwintering eggs from warmer southern regions (Jing and Kang 2003). In North America the hemlock woolly adelgid, *Adelges tsugae* Annand, maintains its overwintering cold tolerance longer in colder regions than in southern regions where air temperatures increase earlier in the year (Skinner et al. 2003).

The differences in supercooling points from previous studies and ours could also be related to the extent of cold acclimation that occurs during the fall and early winter. The warmer supercooling points we recorded in November 2011 could have been because testing was performed too early in the winter season before the insects had reached their maximum cold hardiness or the insects were responding to a warmer than average fall, which preceded a warmer than average winter (Ault et al. 2013). This possibility suggests that, at least to a certain extent, *A. planipennis* is able to adjust its response to cold temperatures depending on the temperatures it experiences during the fall acclimation period. If *A. planipennis* larvae are able to respond to varying fall and early winter temperatures for cold temperature acclimation, our data seem to show the lower limit of *A. planipennis*' capacity to cold harden. The fall and winter of 2013-14 was colder than average, but supercooling points and the mortality from freezing were not statistically different from the results collected during the more typical Minnesota winter of 2012-13. A possibility also exists that there were multiple North American introductions, and a cold hardier population persisted in colder regions, but current genetic testing suggests that the U.S. populations most likely stem from a single introduction (Bray et al. 2011).

Our observations suggest a portion (<13%) of the *A. planipennis* population might be partially freeze tolerant (Hawes and Wharton 2010). We observed some instances in which a clear exotherm was detected as a larva was cooled, evidence of the onset of freezing, but the larva survived when it was returned to room temperature. Survival was evidenced by development to a pupa (applicable only to fourth instars) or movement at the end of the observation period. Because each larva was not removed from the cold until it reached its target treatment temperature, the possibility exists that the insect was completely frozen (Sinclair 1999). Crosthwaite et al. (2011) reported that when *A. planipennis* larvae freeze, they die, a pattern consistent with other freeze intolerant insects.

Determining death of *A. planipennis* larvae from cold exposure can be difficult. Pupation provides clear evidence of survival but is only applicable to late fourth instars which do not need to feed to develop. Earlier instars need to feed to continue development, but larval survival on artificial diet is low (Wang et al. 2010). Movement is another indicator of survival. We frequently observed that within 72 h of cold exposure, larvae would not move or respond to touch. Thereafter, some larvae would "recover." Whether these moving larvae were moribund or healthy could not be determined. Discoloration is an indicator of death, but it may take several days at room temperature for discoloration to appear. To a point, the longer larvae were held at room temperature, the more likely clear evidence of mortality or survivorship would appear. However, it also became increasingly likely that mortality from a factor other than cold exposure (e.g., age, desiccation, or fungal infection) might occur. As a result, we focused on the color and activity of larvae at 13 d after cold exposure. In the winter of 2012-13, this observational period was long enough to determine survival to pupation in fourth instars or mortality with little uncertainty. Because earlier instars could not continue to develop without feeding, we had to rely more heavily on color and movement to determine if an individual larva survived or died due to freezing in the winter of 2013-14. In both winters, some larvae that froze were first observed as healthy (i.e., cream-to-yellow in color, moving), but freeze damage and death appeared later in the observations. For earlier instars, the observation period may not have been long enough to capture all freeze mortality. By potentially underestimating mortality due to freezing, we are providing a conservative estimate of mortality.

Finally, lower lethal temperatures should be considered in the context of temperatures *A. planipennis* would experience (Christianson 2014, Vermunt et al. 2012). Minimum temperatures under the bark of the tree are warmer than the minimum air temperature, ranging from approximately 1°C to >10°C, depending on the face of the tree (e.g., the south face tends to receive more warming from greater sun exposure) and height on the tree (e.g., the base of the tree is more likely to have additional insulation from snow cover) (Christianson 2014). Consequently, while winter air temperatures in northern Minnesota reach lethal temperatures more often than in areas where *A. planipennis* currently occurs, predictions based on air temperature alone will have some error. If *A. planipennis* spreads into northern Minnesota, larvae will likely experience greater mortality than in southern Minnesota; however, winter temperatures may not be enough of a barrier to prevent establishment.

**Table 1.** Median and mean (±SEM) supercooling points for larvae from green and black ash that were artificially infested (Lab) or naturally infested (Field) during the winters of 2011-12, 2012-13, or 2013-14.

| | n | Median (°C) | Mean ± SE (°C) | Shapiro-Wilk (W, *P*) |
|---|---|---|---|---|
| Lab 2011-12 | | | | |
| Black ash | 5 | -25.5 | -24.8 ± 0.97 | NA |
| Green ash | 4 | -24.0 | -22.1 ± 3.32 | NA |
| Lab 2012-13 | | | | |
| Black ash | 4 | -29.2 | -30.4 ± 1.42 | NA |
| Green ash | 4 | -34.2 | -33.8 ± 1.59 | NA |
| Field 2012-13 | | | | |
| Black ash | 41 | -32.6 | -31.2 ± 0.71 | 0.87, < 0.001 |
| Green ash | 30 | -32.9 | -29.8 ± 5.43 | 0.84, < 0.001 |
| Field 2013-14 | | | | |
| Black ash | 25 | -30.6 | -31.3 ± 0.63 | 0.95, 0.3 |
| Green ash | 36 | -32.1 | -31.4 ± 0.61 | 0.95, 0.1 |

Shapiro-Wilk tests for normality in the distribution of supercooling points.

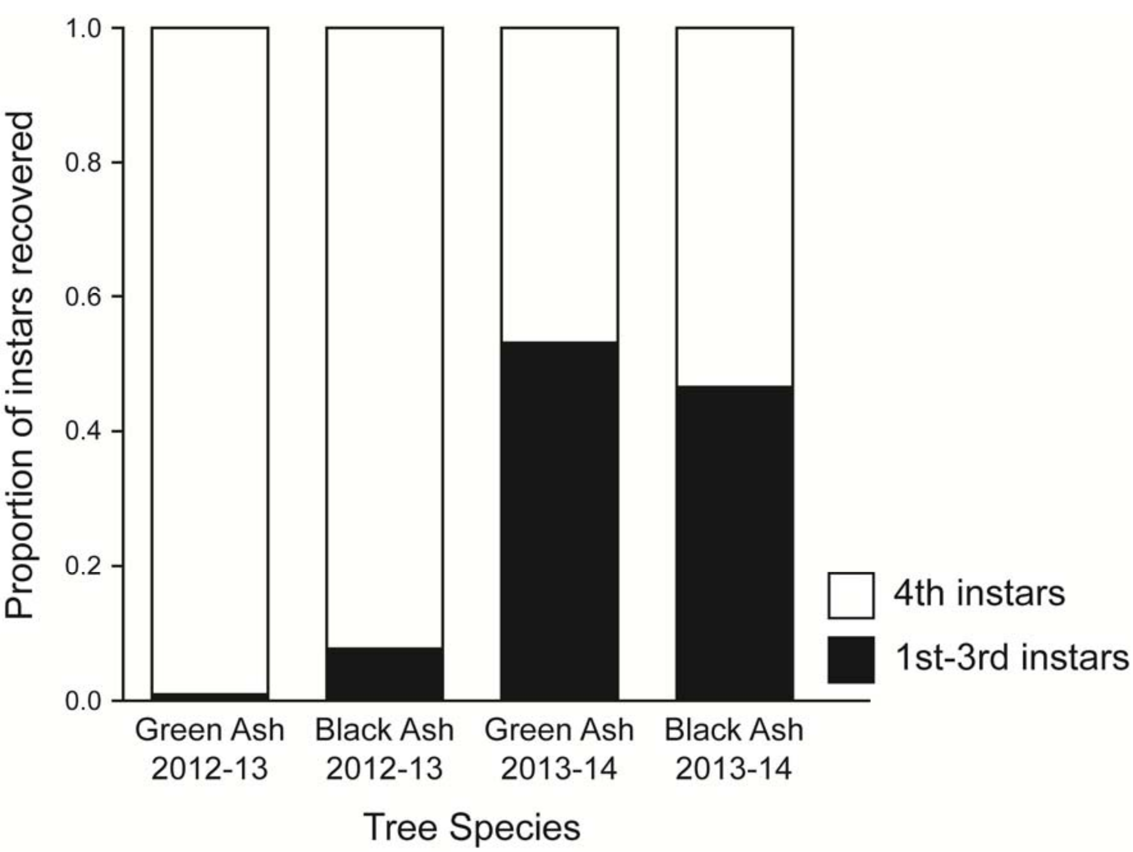

**Figure 1.** Stage distribution of *Agrilus planipennis* larvae recovered from green ash and black ash during the winters of 2012-13 and 2013-14.

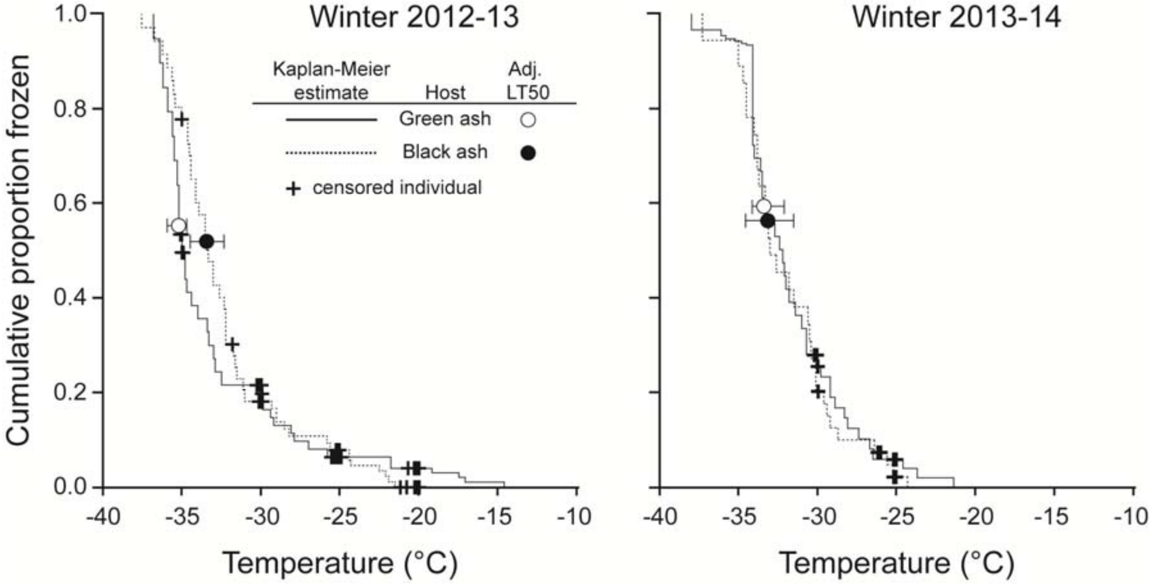

**Figure 2.** Kaplan-Meier estimates of the likelihood that an *A. planipennis* larva will begin to freeze (i.e., give a supercooling point) after exposure to a particular temperature, by tree species and year. The adjusted $LT_{50}$ (±95% confidence intervals) is 0.5 divided by the degree of mortality among frozen larvae. Censored individuals did not freeze by their lowest exposure temperature.

**Table 2.** Percent mortality of chilled larvae, compared between hosts at each treatment temperature.

| | | Black ash | | Green ash | | |
|---|---|---|---|---|---|---|
| Winter | Exposure Temperature (°C) | n | Mortality (%) | n | Mortality (%) | Z, P |
| 2012-13 | -20 | 14 | 28.6 | 18 | 27.8 | 0.049, 0.48 |
| | -25 | 22 | 54.6 | 24 | 20.8 | 2.50, 0.0062 |
| | -30 | 25 | 28.0 | 24 | 29.2 | 0.091, 0.18 |
| | -35 | 22 | 86.4 | 22 | 59.1 | 2.13, 0.017 |
| 2013-14 | -25 | 8 | 37.5 | 6 | 33.3 | 0.16, 0.44 |
| | -30 | 9 | 55.6 | 12 | 66. 7 | 0.52, 0.31 |
| | -35 | 13 | 84.6 | 10 | 80.0 | 0.21, 0.42 |
| | -40 | 6 | 83.3 | 7 | 85.7 | 0.12, 0.45 |

**Table 3.** Percent mortality of frozen larvae and control larvae (i.e., held at room temperature) from green and black ash in the winters of 2012-13 and 2013-14.

| | | Frozen larvae | | Control larvae | |
|---|---|---|---|---|---|
| Winter | Host | n | Mortality (%) | n | Mortality (%) |
| 2012-13 | Green ash | 21 | 90.5 | 24 | 29.2 |
| | Black ash | 28 | 96.4 | 20 | 40.0 |
| 2013-14 | Green Ash | 21 | 85.7 | 12 | 33.3 |
| | Black Ash | 18 | 88.9 | 11 | 36.4 |

No statistical differences were measured between hosts for a given year or between years for a given host in mortality of frozen larvae ($Z<0.89$, $P>0.21$) or control larvae ($Z<0.75$, $P>0.23$).

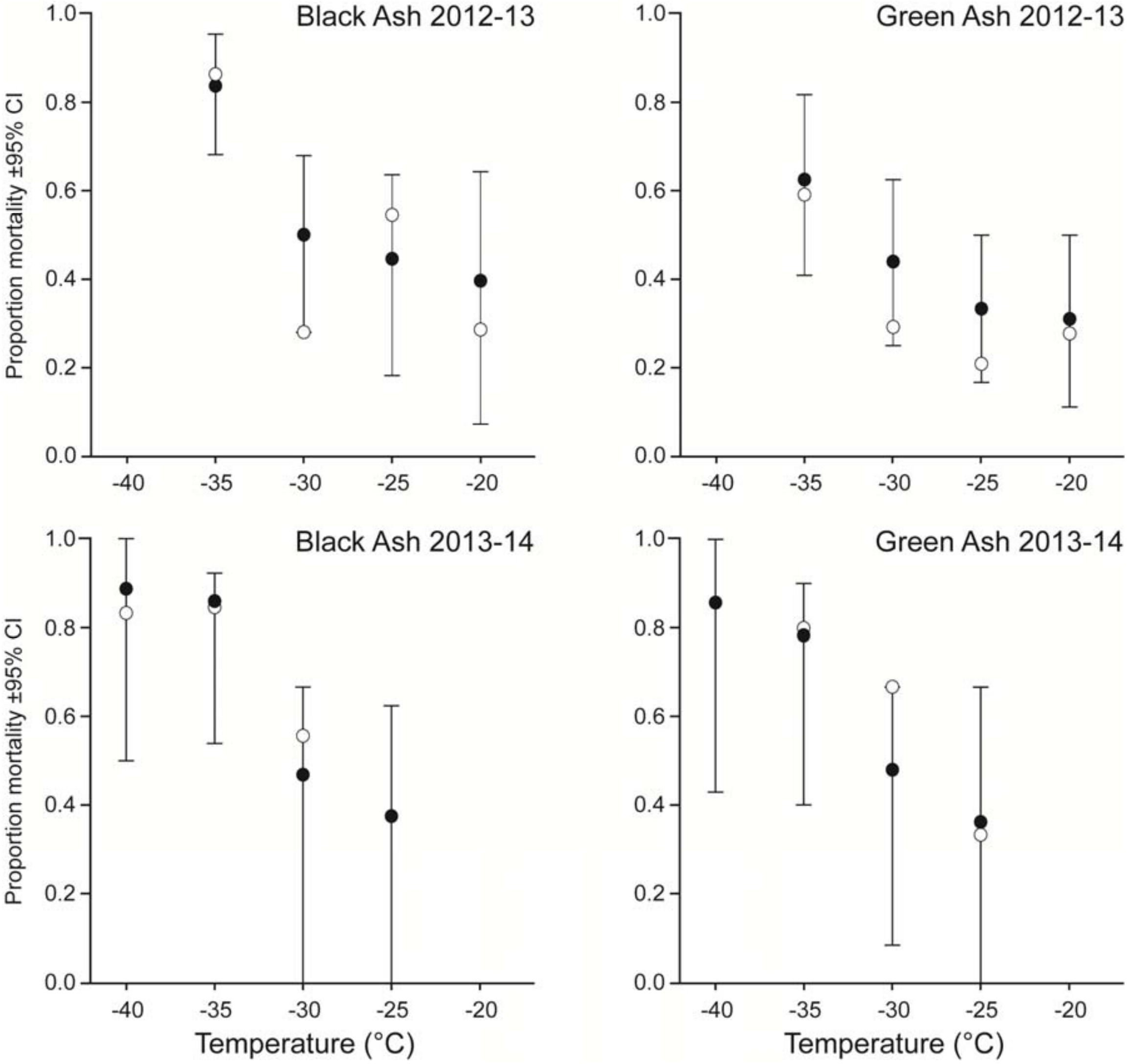

**Figure 3.** Observed proportion larval mortality (open circle) compared to expected proportion larval mortality (closed circle) with confidence intervals for each cold exposure treatment temperature, by host species and winter. When only one circle is seen, observed equals expected mortality.

# CHAPTER 3
# Estimated Temperatures Beneath Bark of Green and Black Ash in Minnesota

## I. Introduction

The emerald ash borer, *Agrilus planipennis* Fairmaire, is an invasive insect native to northeastern Asia that was first identified in North America in 2002 (Bauer et al. 2003). In North America, *A. planipennis* is specific to ash trees in the genus *Fraxinus* (Anulewicz et al. 2008, Pureswaran and Poland 2009) and has killed millions of ash trees (McCullough et al. 2009) in 24 states and two Canadian provinces.

*Agrilus planipennis* was found in Minnesota in 2009 in Saint Paul (Ramsey County) and Minneapolis (Hennepin County) and has since been confirmed in Houston, Winona, and Olmsted counties (Abrahamson 2014). In Minnesota, green ash, *Fraxinus pennsylvanica* Marshall, is the most abundant ash species where emerald ash borer currently occurs. Black ash, *F. nigra* Marshall, makes up approximately 80% of Minnesota's total ash resource (Miles et al. 2011), but this species occurs mostly in Minnesota's northern forests where *A. planipennis* has not yet been found. Black ash, often the only tree species that can grow in peat bogs or poorly drained soils (Erdmann et al. 1987, Telander 2013), is an important part of Minnesota's forests.

Cold temperatures can restrict a species' northern distribution (e.g., Crozier 2003, Hanson et al. 2013). Many insect species overwinter in locations that offer some protection from extreme low air temperatures, such as under soil, leaf litter, snow cover, or tree bark (Danks 2006, Morey et al. 2012, Vermunt et al. 2012). *Agrilus planipennis* overwinters approximately 1.5cm below the surface of the bark of ash trees (DeSantis et al. 2013).

In much of its native and invaded range, *A. planipennis* is univoltine (Xia et al. 2004, Wei et al. 2007). Fourth instar larvae excavate a pupal cell, typically in the outer sapwood. Once the cell is complete, the larva folds itself in the cell. This late fourth instar is commonly called the "j-stage" because of the folded shape (Wei et al. 2007, Chamorro et al. 2012). In a univoltine cycle, a larva reaches the j-stage and overwinters alone in a pupal cell. However in northern parts of its native range and in new and low-density infestations in North America (Wei et al. 2007, Wang et al. 2010), *A. planipennis* takes two years to fully develop (i.e., is semivoltine). In semivoltine life cycles, adults emerge and mate later in the summer, leading to earlier instars overwintering in galleries. Larvae typically spend the first winter as second instars, although first and third instars are occasionally found, and the second winter as late fourth instar or j-stage larvae (Bauer et al. 2003, Xia et al. 2004, Wang et al. 2010).

Vermunt et al. (2012) studied under-bark temperatures of green and white ash in urban and woodlot settings from six sites in Ontario, Canada. Under-bark temperatures were recorded from the north and south sides of the trees, and the daily minimum under-bark temperatures from the north and south sides of the trees were compared to daily minimum air temperatures at each site. They found very few trees even at their most northerly sites that reached under-bark temperatures of -25°C or colder (their threshold for killing approximately 10% of the *A. planipennis* population), and none that reached -30°C (approximately the supercooling point for *A. planipennis*). They also noted that there was approximately a 2°C buffering effect, but this was not consistent. However, they did not include an analysis of how microclimate temperatures may vary depending on the species of ash trees and only measured under bark temperatures at breast height, about 1.4m. In new infestations, larvae are more likely to be found higher in the tree where branches have small diameters and thinner bark, while in older infestations larvae are more densely distributed near the base of the tree (Timms et al. 2006). Green ash tends to have a thick, furrowed bark, and black ash bark tends to be smoother, with mature trees having shallow furrows (Hardin et al. 2001). Green ash bark is also heavier with a lower moisture content than black ash bark (Miles and Smith 2009). Because *A. planipennis* overwinters just under the bark of ash trees (Wei et al. 2007, Wang et al. 2010), these bark differences could create different microhabitats.

In this study we measure under bark temperatures of black and green ash and adjacent air temperatures at two sites near the Minneapolis-Saint Paul metro area in order to determine the winter temperatures *A. planipennis* might be experiencing in portions of Minnesota, and if winter temperatures, specifically extreme low temperatures, differ between ash species.

## II. Methods

### *Tree locations and probe placement*

Under-bark temperatures were monitored from 22 October 2012 to 27 February 2013 (from here on, referred to as Year 1) at Macalester College Katharine Ordway Natural History Study Area (approximately 44°48’41.5”N, 93°01’46.1”W) in two black ash and one green ash. All three trees were at the edge of the stand, though both black ash occurred closer to a lake shore. Under-bark temperatures were monitored from 13 November 2013 to 13 January 2014 and 1 March to 10 April 2014 (from here on, referred to as Year 2) at the Carlos Avery State Wildlife Management Area (approximately 45°21’09.0”N 93°02’42.9”W). Temperatures were not recorded between 14 January and 1 March 2014 due to equipment failure. A replication of green ash under-bark temperatures was lost in Year 2 due to interference with equipment (i.e., that tree’s data logger was vandalized with a shotgun). Monitored trees were located within the stand, but the stand was not densely populated. Both locations were chosen because green and black ash occur in close proximity at these sites; the monitored trees at each site were 10.5 m to 30.5 m apart from each other. Four 2-mm-diameter by 4-cm-long thermocouple probes model HYP-3 (Omega Engineering, INC., Stamford, CT) were installed in each tree. Holes 2.5 mm in diameter and approximately 4.5-5 cm deep were predrilled at an oblique angle so probes would record temperatures as close to the cambium as possible. Temperature probes were installed on the north and south face of the main stem of each tree at 1.4 m and no more than 15 cm above ground level. Air temperature was monitored next to one black ash and one green ash at each site using shielded 107B probes (Campbell Scientific, Logan, UT). Under-bark probes and air probes were connected to a CR10X data logger (Campbell Scientific, Logan, UT) that recorded temperatures every 15 minutes.

### *Statistical analysis*

Daily minimum and average temperatures were calculated for air and each probe location on each tree. We counted the number of days in which the air temperature or the under-bark temperature was ≤ -30°C and calculated the total approximate time (to the nearest 15 minutes) spent at temperatures below -30°C at each probe location for each tree over the study period. We chose -30°C because previous experiments suggest little mortality of *A. planipennis* occurs due to cold at temperatures warmer than -30°C (Crosthwaite et al. 2011, Christianson 2014).

We used multiple linear regression (*lm* function in R 3.1.1; R Development Core Team 2008) to determine the effect of tree species (green or black ash), tree face (north or south), and the interaction of these two variables on relationships between corresponding measures of air temperature and under-bark temperature (i.e., minimum or average temperatures). Backward elimination (*step* function in R 3.1.1) was used to remove non-significant terms from the model (P > 0.05) until only significant terms remained. Separate models were fit for temperatures under-bark temperatures recorded at 1.4 m and at the base of the tree to implicitly account for the effects of snow. Years 1 and 2 were analyzed separately.

## III. Results

The microclimate beneath the bark of an ash tree, where *A. planipennis* larvae overwinter, differs from air conditions. Conditions beneath the bark during the winter are generally warmer than air temperatures. Expected minimum under-bark temperatures in Year 1 at 1.4 m were 1.8 – 3.7°C higher than -33.5°C, the minimum observed air temperature. The minimum air temperature we recorded in Year 2 was -35.5°C, and expected under-bark temperatures at 1.4 m ranged from 3.1 – 4.1°C warmer.

### *Description of winter temperatures in 2012-2013 and 2013-2014*

The winter of 2013-2014 (Year 2) was on average colder than the winter of 2012-2013 (Year 1). According to the National Weather Service (2014), there were no days that reached -20°C or colder in December 2012 or March 2013, and only 1 day in February 2013 was colder than -20°C. The coldest minimum temperature recorded was on 1 February 2013 at -25°C. During Year 2, the months of December 2013, January, February, and March 2014 all had days recorded at or below -20°C. The minimum temperature on 6 January 2014 was the only recorded temperature below -30°C, at -30.56°C. These temperatures were measured at the National Weather Service station in Chanhassen, MN.

*Time spent under -30°C*

In the winter of Year 1, air temperatures at our study site were ≤-30°C for 13.0 hours over 3 days near black ash and 19.25 hours over 4 days near green ash (Table 4). Temperatures beneath the bark of black ash trees at 1.4 m reached -30°C on the north and south sides for an average time of 4.6 (± 3.0 SD) hours and 0.6 (± 0.5 SD) hours respectively, but never reached -30°C at the base of the black ash trees or beneath the bark of green ash (Table 4). In Year 2, air temperature during the study period was ≤-30°C for 142.0 hours over 17 days near black ash and 150.25 hours over 13 days near green ash. Due to equipment failure from 15 January 2014 to 28 February 2014, our recordings missed at least two more days that could have reached -30°C or colder according to the National Weather Service Forecast Office (2014). The north base of a black ash tree ("Black Ash #4" from Year 2 in Table 4) reached -30°C for a total of 40.5 hours over 6 days. The same black ash tree recorded 152.75 hours at or below -30°C over 17 days on the north face of the tree at 1.4 m, and 140.75 hours over 17 days on the south face at 1.4m. The temperature under bark measured on the north face of green ash at 1.4m spent 90.25 hours over 11 days at -30°C or lower, and 66 hours over 9 days on the south face. Black Ash #3 from Year 2 at 1.4 m spent 138.75 hours and 120.75 hours on the north and south faces respectively over 16 days.

*Relationship between air temperature and under-bark temperature*

*Winter 2012-2013 (Year 1).* Daily minimum temperatures beneath the bark of ash trees measured at 1.4 m were explained best by the tree species, the interaction of tree species and the face of the tree, and air temperature ($R^2 = 0.907$, $F_{4, 625} = 1529$, $P < 0.0001$, Table 5). When the daily minimum air temperature was 0°C, the daily minimum temperature beneath the bark of green ash was, on average, 1.2°C (± 0.16 SE) colder than black ash ($t = -7.31$, $df = 625$, $P < 0.0001$). On the south face of black ash, the under-bark temperature was 0.45°C (± 0.14 SE) warmer than on the north face ($t = 3.18$, $df = 625$, $P = 0.0015$). In green ash, the south face was 1.9°C (± 0.17) warmer than the north face under the bark ($t = 11.37$, $df = 625$, $P < 0.0001$). When the minimum air temperature was -30°C, green ash under-bark temperatures at 1.4 m were estimated to be 1.3 and 3.2°C warmer on the north and south faces, respectively, and 2.5 and 3°C warmer underneath the bark of black ash (Figure 4). For every 1°C that the daily minimum air temperature declined, temperature under the bark decreased by 0.9°C (± 0.01 SE, $t = 70.59$, $df = 625$, $P < 0.0001$).

At the base of the tree, tree species, face, the interaction of tree species and face, air temperature, and the interaction of tree species and air temperature were significant factors for describing daily minimum temperature under the bark ($R^2 = 0.824$, $F_{5, 624} = 584.5$, $P < 0.0001$) (Table 5). When daily minimum air temperature was 0°C, the temperature underneath the bark of green ash was, on average, at least 2.9°C (± 0.55 SE) warmer than black ash (t = 5.24, df = 624, P < 0.0001), and the south face of either tree species was at least 0.4°C (± 0.15 SE) warmer on average than the north face (t = 2.89, df = 624, P = 0.0040). With the interaction of tree species and face, the south face of green ash was, on average, at least 0.9°C (± 0.24 SE) warmer than any other face of either species (t = 3.56, df = 624, P = 0.00041). When the daily minimum air temperature reached -30°C, the north faces of green and black ash were estimated to be 2.8 and 4.0°C warmer than the south faces, respectively. The minimum under-bark temperatures on the south faces of green ash black ash are estimated to be 3.0 and 6.2°C warmer respectively than air temperature at -30°C (Figure 5). For a 1°C decrease in daily minimum air temperature, the under-bark temperature lowered, on average, by 0.6°C (± 0.02 SE, t = 33.53, df = 624, P < 0.0001). Because of the tree species and air temperature interaction, under-bark temperature of green ash decreased 0.2°C (± 0.03 SE) more slowly than black ash, i.e., at a rate of 0.8°C for every 1°C decline in air temperature (t = 8.17, df = 624, P < 0.0001).

Daily average temperatures under the bark measured at 1.4 m on the tree were best described by tree species, air temperature, the interaction of tree species and air temperature, and the interaction of air temperature and the face of the tree ($R^2 = 0.894$, $F_{4, 625} = 1313$, P < 0.0001, Table 5). Underneath the bark of green ash, the average temperature was 2.1°C (± 0.48 SE) warmer than black ash when air temperature was 0°C (t = 4.35, df = 625, P < 0.0001) (Figure 6). When daily average air temperature declined by 1°C, under-bark temperature declined, on average, by at least 0.84°C (± 0.02 SE) on the north faces of both species; the temperature under the bark on the south faces of the trees declined, on average, 0.03°C (± 0.02 SE) slower than the north face ($t = -5.22$, $df = 625$, $P < 0.0001$), i.e., by at least 0.81°C for each degree C decline in average air temperature.

The daily average under-bark temperatures at the base of the tree were best described by tree species, face, air temperature, the interaction of tree species and air temperature, the interaction of face and air temperature, and the three-way interaction of tree species, face, and air temperature ($R^2 = 0.851$, $F_{6, 623} = 594.3$, $P < 0.0001$, Table 5). At 0°C air temperature, temperatures under the bark of green ash were estimated to be 1.7°C (± 0.48 SE) warmer than temperatures under the bark of black ash ($t = 3.6$, $df = 623$, $P < 0.0001$, Figure 7). The south face on either tree was, on average, 2.3°C (± 0.46 SE) colder than north face of the tree ($t = -4.93$, $df = 623$, $P < 0.0001$) when air temperature was 0°C. When air temperature lowered by 1°C, temperature underneath the bark of black ash on the north face and green ash on the south face declined, on average, by 0.7°C (± 0.02 SE, $t = 36.73$, $df = 623$, $P < 0.0001$). The temperature on the south face of black ash declined 0.16°C (± 0.024 SE) slower than the north face for an adjusted rate of 0.54°C on average for every 1°C decrease in air temperature ($t = -6.58$, $df = 623$, $P < 0.0001$).

*Winter 2013-2014 (Year 2).* Daily minimum temperatures measured underneath the bark at 1.4 m were best described by tree species and air temperature ($R^2 = 0.83$, $F_{2, 487} = 1198$, $P < 0.0001$, Table 5). Under-bark temperatures in green ash during Year 2 were, on average, 0.97°C (± 0.21 SE) warmer than under-bark temperatures in black ash when air temperature was 0°C ($t = 4.65$, $df = 487$, $P < 0.0001$). When air temperature was -30°C, under-bark temperatures were estimated to be 4.4°C warmer in green ash and 2.4°C warmer in black ash (Figure 4). For every 1°C decline in air temperature, temperatures under the bark decreased by approximately 0.87°C (± 0.018 SE, $t = 48.25$, $df = 487$, $P < 0.0001$).

Daily minimum temperatures underneath the bark at the base of the tree were best explained by face, the interaction of face and tree species, air temperature, the interaction of face and air temperature, and the three-way interaction of face, tree species and air temperature ($R^2 = 0.70$, $F_{7, 482} = 161.9$, $P < 0.0001$, Table 5). When air temperature was 0°C, the south face of black ash at the base of the tree was, on average, 3.9°C (± 0.86 SE) colder than the north face of the tree ($t = 21.48$, $df = 482$, $P < 0.0001$). On average, the south face of green ash was 4.9°C (± 1.1 SE) warmer than the south face of black ash when air temperature was 0°C ($t = 4.43$, $df = 482$, $P < 0.0001$). There was no significant difference between the north faces of black and green ash ($t = 1.04$, $df = 482$, $P = 0.301$). When the daily minimum air temperature was -30°C, the minimum under-bark temperatures on the north and south face of green ash were typically 5.5 and 5.9°C warmer than air temperature (Figure 5). The north and south face of black ash are estimated to be 5.8 and 8.2°C warmer than the -30°C air temperature. For every 1°C decline in air temperature, under-bark temperature declined, on average, by 0.5°C (± 0.02 SE, $t = 21.48$, $df = 482$, $P < 0.0001$). Under-bark temperature on the south face of the tree declined by an average rate of 0.3°C (-0.2°C ± 0.03 SE) for every decrease of 1°C air temperature ($t = -6.22$, $df = 482$, $P < 0.0001$). The south face of green ash declined by an additional 0.2°C (± 0.04 SE, t = 5.46, df = 482, P < 0.0001), i.e., at a rate of 0.5°C (Figure 5).

Daily average temperatures underneath the bark at 1.4 m were best described by tree species, face, and air temperature ($R^2 = 0.851$, $F_{3, 486} = 927.5$, $P < 0.0001$, Table 2). At 0°C air temperature, under-bark temperature of green ash was, on average, 0.7°C (± 0.18 SE) warmer than under-bark temperatures of black ash (t = 3.62, df = 486, P = 0.00033, Figure 5). The south faces of both tree species were approximately 0.5°C (± 0.16 SE) warmer than the north faces (t = 3.06, df = 486, P = 0.0024). For every 1°C decline in air temperature, temperatures under the bark lowered, on average, by 0.8°C (± 0.02 SE, t = 51.93, df = 486, P < 0.0001).

Daily average temperature under the bark at the base of the tree was best explained by tree face, the interaction of tree face and species, air temperature, the interaction of air temperature and tree species, the interaction of tree face and air temperature, and the three-way interaction of air temperature, tree face and species ($R^2 = 0.751$, $F_{7, 482} = 207.6$, $P < 0.0001$, Table 5). On average, the south face of black ash was 2.8°C colder than the north face of the tree when the average air temperature was 0°C (t = -3.99, df = 482, P < 0.0001, Figure 7). The south face of green ash was, on average, 1.3°C warmer than the north face of green ash, effectively 3.0°C (5.8°C ± 0.89 SE, t = 6.56, df = 482, P < 0.0001) warmer than average air temperature at 0°C. For every 1°C lowering in average air temperature, under-bark temperature on the north face at the base of black ash declined, on average, by 0.5°C (± 0.02 SE, t = 22.97, df = 482, P < 0.0001). The south face of black ash cooled at 0.2°C (± 0.03 SE) more slowly than the north face for every 1°C decline in air temperature (t = -5.57, df = 482, P < 0.0001). At the base of the tree, the north face of green ash cooled 0.1°C (± 0.04 SE) faster than the cooling rate on the comparable north face of black ash (t = 2.05, df = 482, P = 0.0407), i.e., at a rate of 0.6°Cfor each 1°C drop in daily average air temperature. The south face of green ash at the base of the tree cooled at a rate of 0.6°C for every 1°C decline in air temperature. In this case, the slower cooling rate for being on the south side of the tree (-0.17°C ± 0.03 SE, t = -5.57, df = 482, P < 0.0001) is offset by the three-way interaction between air temperature, tree species, and face (0.19°C ± 0.06, t = 3.35, df = 482, P = 0.000877).

## IV. Discussion

The temperatures recorded at our weather stations differed from the National Weather Service temperatures, likely due to effect of location. The Minneapolis-Saint Paul metro area exhibits a “heat island” effect (Malevich and Klink 2011), so the minimum temperatures recorded by the National Weather Service would be expected to be warmer than nearby, less-urbanized locations where our study sites were located.

Insects under the bark of ash trees will experience the greatest buffer from extreme low temperatures at the base of the tree, most likely due to snow cover that provides additional insulation. During Year 1, the Minnesota Department of Natural Resources (2014) reported an average snow depth of 11 cm south of Saint Paul, MN. The differences in under-bark temperatures at the base of the trees could be due to varying snow depth around the individual trees.

For under-bark temperatures measured at 1.4 m, minimum temperatures measured on the south side of the tree were typically 1-2°C warmer than temperatures on the north face of the tree. This result could be due to solar radiation having a greater warming effect on the south side of the tree than on the north side.

In Year 2, under-bark temperatures measured at 1.4 m showed similar buffer effects as in Year 1, but with less of a difference between the north and south faces of the trees. This could be due to a combination of the extreme low air temperatures and solar radiation having less of an effect on trees that were located within a stand, even though it was an open stand. The buffering effect at the base of the tree was also more consistent between the north and south sides of the tree, possibly due to heavy snow cover. For both years, the difference between under-bark temperatures and the air temperature was most apparent at extreme minimum air temperatures (i.e., the under-bark temperatures cooled more slowly and to a lesser extent than the air temperature).

Vermunt et al. (2012) show that temperatures in the air and beneath the bark of ash trees can differ, so modelling potential species distribution of *A. planipennis* based solely on air temperatures would overestimate winter mortality of *A. planipennis*. Our study suggests that tree species and location on the tree (i.e., height and face) should also be considered when determining the temperatures experienced by overwintering insects.

**Table 4.** Summaries of air temperatures and under-bark temperatures at two heights on the north (N) and south (S) faces of green ash (*F. pennsylvanica*) and black ash (*F. nigra*) measured in the winter of 2012-2013 (Year 1) and 2013-2014 (Year 2).

| Tree | Probe Location | Minimum °C (±SD) | Maximum °C (±SD) | Average °C (±SD) | Hours ≤-30°C | Days ≤-30°C |
|---|---|---|---|---|---|---|
| *Year 1* | | | | | | |
| Air | near Black Ash | -21.8 ±4.4 | -16.0 ±4.5 | -18.5 ±3.4 | 13.00 | 3 |
| | near Green Ash | -22.7 ±4.3 | -17.2 ±4.3 | -19.9± 4.1 | 19.25 | 4 |
| Green Ash #1 | N 1.4 m | -21.3 ±4.1 | -17.9 ±4.2 | -19.6 ±4.0 | - | - |
| | S 1.4 m | -21.1 ±4.0 | -15.9 ±4.0 | -18.9 ±4.0 | - | - |
| | N base | -20.3 ±3.2 | -18.0 ±3.8 | -19.1 ±3.3 | - | - |
| | S base | -20.0 ±3.4 | -16.7 ±4.0 | -18.5 ±3.4 | - | - |
| Black Ash #1 | N 1.4 m | -20.9 ±4.1 | -17.3 ±4.2 | -19.1 ±4.0 | 6.75 | 1 |
| | S 1.4 m | -20.2 ±4.0 | -17.0 ±4.0 | -18.6 ±3.8 | 0.25 | 1 |
| | N base | -19.9 ±3.2 | -17.5 ±3.8 | -18.7 ±3.3 | - | - |
| | S base | -19.9 ±3.4 | -16.3 ±4.0 | -18.3 ±3.4 | - | - |
| Black Ash #2 | N 1.4 m | -21.0 ±4.0 | -18.3 ±3.7 | -19.6 ±3.8 | 2.50 | 1 |
| | S 1.4 m | -20.8 ±4.0 | -17.8 ±3.7 | -19.3 ±3.7 | 1.00 | 1 |
| | N base | -19.7 ±2.7 | -17.7 ±3.2 | -18.7 ±2.8 | - | - |
| | S base | -18.8 ±2.1 | -17.1 ±3.2 | -18.0 ±2.4 | - | - |
| *Year 2* | | | | | | |
| Air | near Black Ash | -24.8 ±5.2 | -18.6 ±5.3 | -21.5 ±5.0 | 142.00 | 17 |
| | near Green Ash | -24.6 ±5.4 | -17.7 ±5.6 | -21.0± 5.2 | 150.25 | 13 |
| Green Ash #2 | N 1.4 m | -22.1 ±4.9 | -18.6 ±5.0 | -20.5 ±4.6 | 90.25 | 11 |
| | S 1.4 m | -21.8 ±4.9 | -16.8 ±5.5 | -19.7 ±4.6 | 66.00 | 9 |
| | N base | -21.4 ±3.1 | -18.7 ±4.1 | -20.2 ±3.2 | - | - |
| | S base | -19.9 ±3.4 | -16.3 ±4.0 | -18.3 ±3.4 | - | - |
| Black Ash #3 | N 1.4 m | -23.0 ±5.1 | -18.9 ±5.0 | -20.9 ±4.9 | 138.75 | 16 |
| | S 1.4 m | -22.9 ±5.1 | -17.7 ±5.2 | -20.4 ±4.9 | 120.75 | 16 |
| | N base | -20.1 ±2.0 | -18.6 ±2.7 | -19.4 ±2.1 | - | - |
| | S base | -19.8 ±1.8 | -18.1 ±3.0 | -19.0 ±2.1 | - | - |
| Black Ash #4 | N 1.4 m | -23.0 ±5.3 | -19.5 ±4.7 | -21.2 ±4.8 | 152.75 | 17 |
| | S 1.4 m | -22.9 ±5.3 | -19.0 ±4.6 | -20.9 ±4.8 | 140.75 | 17 |
| | N base | -22.3 ±4.1 | -19.5 ±4.1 | -20.9 ±4.1 | 40.50 | 6 |
| | S base | -20.3 ±2.4 | -18.3 ±3.6 | -19.4 ±2.7 | - | - |

Means are based on daily summaries and are given for the north and south faces measured at 1.4m and the base of each tree. The daily average temperature was calculated as the average of all temperatures recorded over a 24 hour period. Due to data logger failure, temperatures from 14 January – 28 February 2014 were not included.

**Table 5.** Multiple linear regression estimated coefficient values for air temperature versus under-bark temperatures measured from Year 1 and Year 2 at 1.4 m and at the base of the tree. Best fit regression models were determined by backward stepwise regression until remaining predictor variables were significant at α = 0.05, starting with a linear model that included all predictor variables and interactions.

| Predictor Variable | Estimated Coefficient | Standard Error | t | P |
|---|---|---|---|---|
| *Year 1* | | | | |
| Minimum Temperature at 1.4 m (R-squared = 0.904, F-statistic = 1529 on 4 and 625 DF) | | | | |
| Intercept | -1.67 | 0.29 | -5.87 | < 0.0001 |
| Tree sp. | -1.2 | 0.16 | -7.31 | < 0.0001 |
| Tree sp.(B) : Face | 0.45 | 0.14 | 70.59 | 0.00153 |
| Tree sp.(G) : Face | 1.91 | 0.17 | 3.18 | < 0.0001 |
| Air | 0.87 | 0.012 | 11.37 | < 0.0001 |
| Minimum Temperature at base of tree (R-squared = 0.824, F-statistic = 584.5 on 5 and 624 DF) | | | | |
| Intercept | -6.96 | 0.39 | -17.71 | < 0.0001 |
| Tree sp. | 2.9 | 0.55 | 5.24 | < 0.0001 |
| Face | 0.44 | 0.15 | 2.89 | 0.00398 |
| Tree sp. : Face | 0.85 | 0.24 | 33.53 | 0.00041 |
| Air | 0.58 | 0.02 | 3.56 | < 0.0001 |
| Air : Tree sp. | 0.22 | 0.027 | 8.17 | < 0.0001 |
| Average Temperature at 1.4 m (R-squared = 0.894, F-statistic = 1313 on 4 and 625 DF) | | | | |
| Intercept | -3.47 | 0.29 | -12.11 | < 0.0001 |
| Tree sp. | 2.1 | 0.48 | -4.35 | < 0.0001 |
| Air | 0.84 | 0.015 | 54.94 | < 0.0001 |
| Air : Tree sp. | 0.071 | 0.024 | 2.92 | 0.00361 |
| Air : Face | -0.027 | 0.0051 | -5.22 | < 0.0001 |
| Average Temperature at base of tree (R-squared = 0.851, F-statistic = 594.3 on 6 and 623 DF) | | | | |
| Intercept | -5.39 | 0.36 | -14.79 | < 0.0001 |
| Tree sp. | 1.73 | 0.48 | 3.61 | < 0.0001 |
| Face | -2.26 | 0.4 | -4.93 | < 0.0001 |
| Air | 0.71 | 0.19 | 36.73 | < 0.0001 |
| Air : Tree sp. | 0.07 | 0.25 | 2.69 | 0.00726 |
| Air : Face | -0.16 | 0.24 | -6.58 | < 0.0001 |
| Air : Tree sp. : Face | 0.091 | 0.1 | 8.81 | < 0.0001 |

(Table 5 cont.)

| | | | | |
|---|---|---|---|---|
| *Year 2* | | | | |
| Minimum Temperature at 1.4 m (R-squared = 0.831, F-statistic = 1198 on 2 and 487 DF) | | | | |
| Intercept | -1.48 | 0.47 | -3.14 | 0.00181 |
| Tree sp. | 0.97 | 0.21 | 4.65 | < 0.0001 |
| Air | 0.87 | 0.018 | 48.25 | < 0.0001 |
| Minimum Temperature at base of tree (R-squared = 0.702, F-statistic = 161.9 on 7 and 482 DF) | | | | |
| Intercept | -8.87 | 0.61 | -14.54 | < 0.0001 |
| Face | -3.92 | 0.86 | -4.55 | < 0.0001 |
| Face (N) : Tree sp. | 1.14 | 1.1 | 21.48 | 0.301 |
| Face (S) : Tree sp. | 4.89 | 1.1 | 1.04 | < 0.0001 |
| Air | 0.51 | 0.024 | 4.43 | < 0.0001 |
| Air : Face | -0.21 | 0.033 | -6.22 | < 0.0001 |
| Air : Face (N) : Tree sp. | 0.05 | 0.044 | 1.15 | 0.25 |
| Air : Face (S) : Tree sp. | 0.24 | 0.044 | 5.46 | < 0.0001 |
| Average Temperature at 1.4 m (R-squared = 0.851, F-statistic = 927.4 on 3 and 486 DF) | | | | |
| Intercept | -3.18 | 0.38 | -8.34 | < 0.0001 |
| Tree sp. | 0.66 | 0.18 | 3.62 | < 0.0001 |
| Face | 0.49 | 0.16 | 3.06 | 0.00237 |
| Air | 0.85 | 0.016 | 51.93 | < 0.0001 |
| Average Temperature at base of tree (R-squared = 0.751, F-statistic = 207 .6 on 7 and 482 DF) | | | | |
| Intercept | -9.52 | 0.5 | -19.06 | < 0.0001 |
| Face | -2.82 | 0.71 | -3.99 | < 0.0001 |
| Face (N) : Tree sp. | 1.68 | 0.89 | 22.97 | 0.0578 |
| Face (S) : Tree sp. | 5.81 | 0.89 | 1.9 | < 0.0001 |
| Air | 0.51 | 0.022 | 6.56 | < 0.0001 |
| Air : Tree sp. | 0.083 | 0.4 | 2.05 | 0.0407 |
| Air : Face | -0.17 | 0.31 | -5.57 | < 0.0001 |
| Air : Tree sp. : Face | 0.19 | 0.57 | 3.45 | 0.00088 |

Tree species were coded as green ash (G) = 1, black ash (B) = 0. Tree face was coded as north face (N) = 0, and south face (S) = 1. Significance of each model parameter was tested with a t-statistic (i.e., parameter estimate/standard error).

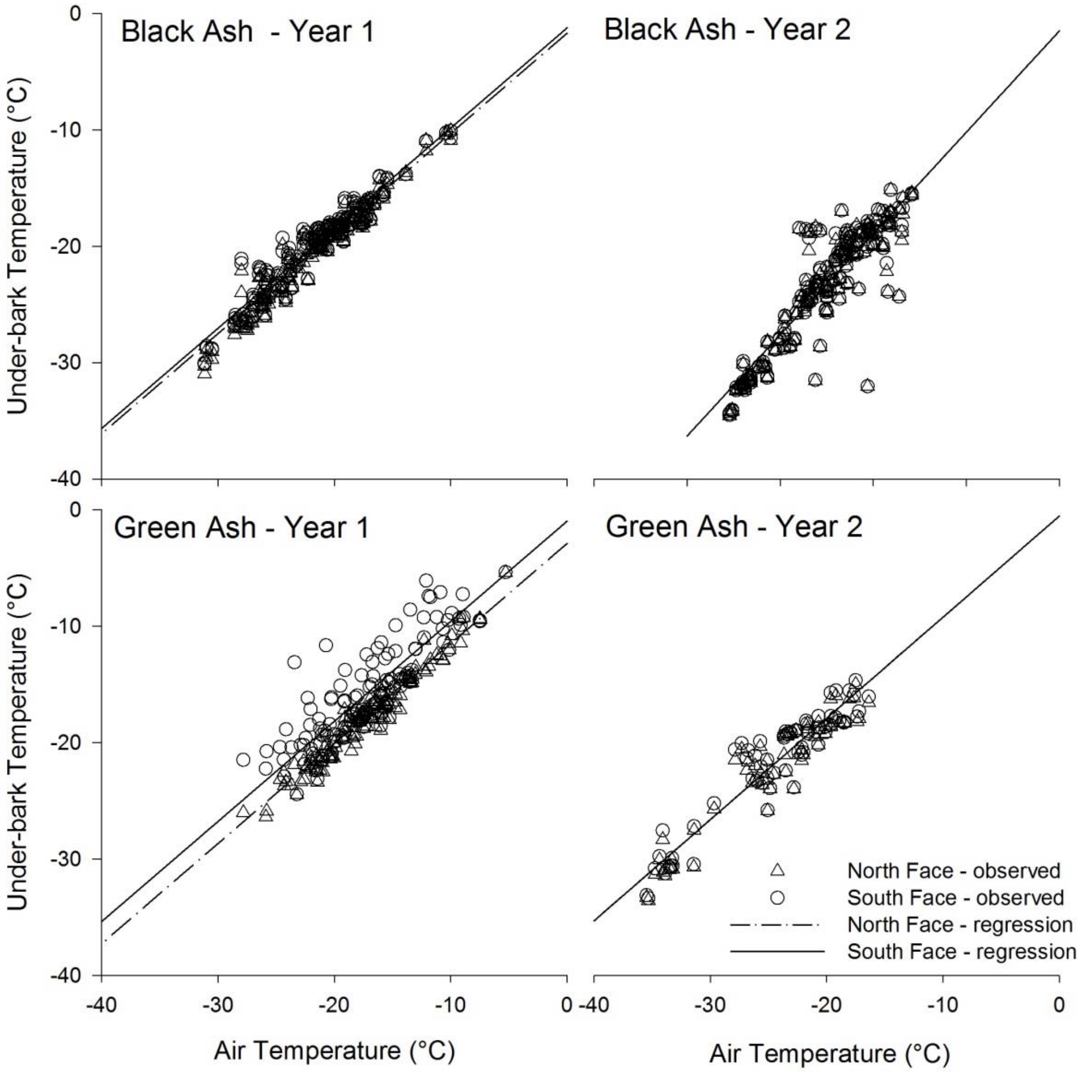

**Figure 4.** Daily minimum temperatures underneath the bark measured at 1.4 m vs. daily minimum air temperature. Observed temperatures for the north and south faces of each tree species are plotted with regression lines from each year. Regression lines were plotted using parameter estimates from multiple linear regression results.

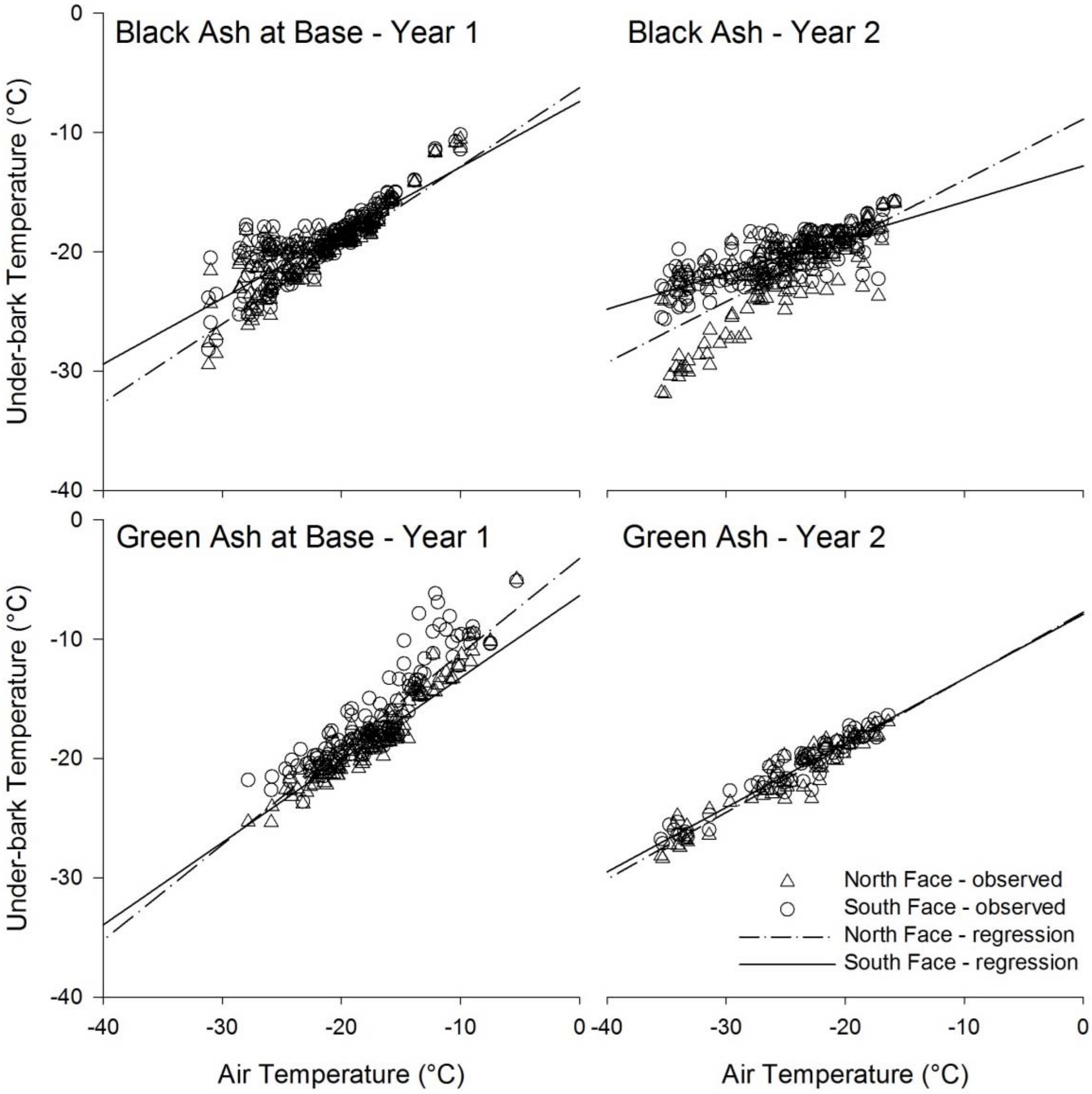

**Figure 5.** Daily minimum under-bark temperatures measured at the base of the tree vs. daily minimum air temperature. Observed temperatures for the north and south faces of each tree species are plotted with regression lines from each year. Regression lines were plotted using parameter estimates from multiple linear regression results.

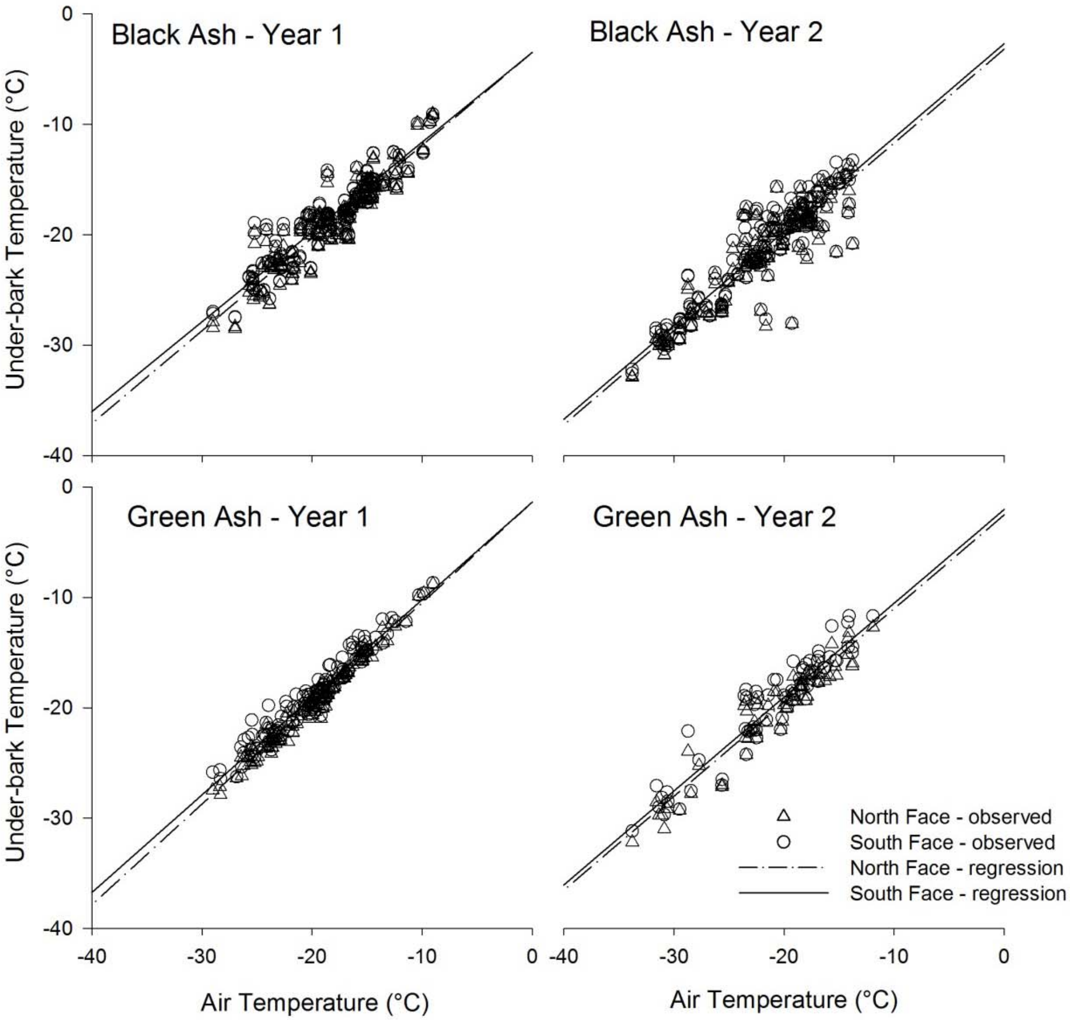

**Figure 6.** Daily average under-bark temperatures recorded at 1.4 m on the tree vs. daily average air temperatures. Observed temperatures for the north and south faces of each tree species are plotted with regression lines from each year. Regression lines were plotted using parameter estimates from multiple linear regression results.

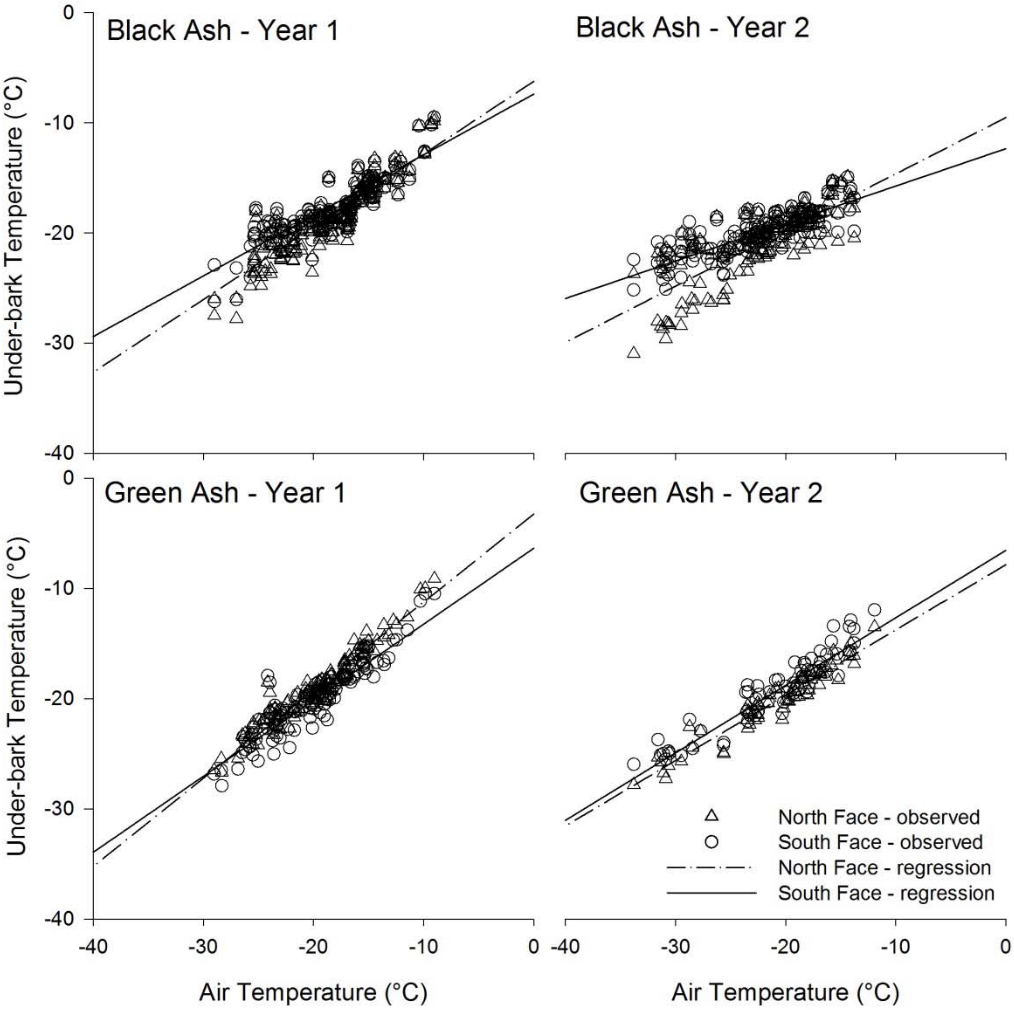

**Figure 7.** Daily average under-bark temperatures measured at the base of the tree vs. daily average air temperatures. Observed temperatures for the north and south faces of each tree species are plotted with regression lines from each year. Regression lines were plotted using parameter estimates from multiple linear regression results.

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