

Cold tolerance of *Drosophila suzukii* (Diptera: Drosophilidae)

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Abstract

Drosophila suzukii Matsumura, or spotted wing drosophila (Diptera: Drosophilidae), is a fly that is believed to be native to Japan. Spotted wing drosophila females lay eggs into ripe and ripening soft skinned fruits, where larvae develop. The species was first found in the continental United States in 2008, where it was reported to cause extensive economic damage in many fruit crops, including raspberry, blackberry, blueberry and cherry.

Spotted wing drosophila is believed to overwinter as a seasonally acclimated winter-morph adult. While spotted wing drosophila is found in northern states with colder climates, the ability of it to establish and overwinter in these regions is unknown. Likelihood of spotted wing drosophila overwintering survival was hypothesized by reviewing literature on other *Drosophila* species cold tolerance. Cold tolerance of spotted wing drosophila was then measured by comparing the supercooling points and lower lethal temperatures of seasonally acclimated (winter-morphs) and non-acclimated adults (summer-morphs). The ability of winter-morphs and summer-morphs to rapidly cold-harden was also studied to determine if the fly can quickly acclimate to new cold temperatures.

Table of Contents

Acknowledgements.....	i
Abstract.....	ii
List of Figures.....	v
Chapter 1: Overwintering ecology and cold tolerance in <i>Drosophila</i>	1
Introduction to <i>Drosophila suzukii</i>	2
Introduction to insect cold tolerance.....	3
Introduction to <i>Drosophila</i> cold tolerance research.....	6
Cold Acclimation.....	7
Geographic Origin and Cold Tolerance.....	10
Dark Morphs in <i>Drosophila</i>	12
Overwintering in <i>Drosophila</i>	13
Conclusions.....	14
Chapter 2: Cold hardiness of winter acclimated <i>Drosophila suzukii</i> (Diptera: Drosophilidae) adults.....	18
Summary.....	19
Introduction.....	20
Methods.....	23
Colony Source.....	23
Supercooling Point Determination.....	24
Lower Lethal Temperature Determination.....	26
Results.....	29

Supercooling Points.....	29
Lower Lethal Temperature.....	29
Discussion.....	30
Chapter 3: Rapid Cold-Hardening in winter-morph and summer-morph <i>Drosophila</i>	
<i>suzukii</i> (Diptera: Drosophilidae).....	38
Summary.....	39
Introduction.....	40
Methods.....	42
Colony Source.....	42
Assays for Rapid Cold-Hardening.....	44
Cold Exposure Assays.....	45
Two Hour Cold Exposure Assay.....	45
Lower Lethal Temperature Assay.....	49
Results.....	50
Two Hour Cold Exposure Assay.....	50
Lower Lethal Temperature Assay.....	51
Discussion.....	52
References.....	58

List of Figures

Figure 1.1: Predicted habitat suitability of North America to <i>D. suzukii</i>	16
Figure 1.2: Types of insect overwintering strategies	17
Figure 2.1: <i>Drosophila suzukii</i> in a microcentrifuge tube with thermocouple.....	35
Figure 2.2: Supercooling points of <i>Drosophila suzukii</i> winter-morphs, summer-morphs, pupae, and <i>D. melanogaster</i>	36
Figure 2.3: <i>Drosophila suzukii</i> winter and summer-morph lower lethal temperature and cumulative proportion frozen.....	37
Figure 3.1: Survival of <i>D. melanogaster</i> , <i>D. suzukii</i> winter and summer-morphs with short-term acclimation and without short-term acclimation.....	56
Figure 3.2: Effect of short-term acclimation of lower lethal temperature of summer and winter-morph <i>D. suzukii</i>	57

**Chapter 1: Overwintering Ecology and Cold Tolerance in
*Drosophila***

Introduction to *Drosophila suzukii*

Drosophila suzukii Matsumura (Diptera: Drosophilidae) from the *D. melanogaster* Meigen species group, was first described from specimens collected in Japan in the 1920s (Kanzawa 1939). *Drosophila suzukii*, or spotted wing drosophila, was first discovered in the United States in Hawaii in the 1980's, but was not reported to cause any damage. Extensive damage was noted when this insect was found in 2008 in California, the first continental U.S. state found to be infested (Walsh et al. 2011). *Drosophila suzukii* oviposition and larval feeding in soft-skinned fruits damage crops extensively, especially grapes and other berries, and renders fruit unmarketable (Walsh et al. 2011).

Drosophila suzukii is found on nearly every continent, with the notable exceptions being Oceania and Antarctica (Commonwealth of Australia 2013). This insect was discovered in Spain in 2008 and has spread rapidly across Europe (Cini et al. 2012). In 2012, *D. suzukii* was first discovered in South America in Brazil (Depra et al. 2014). In North America, *D. suzukii* now has been found in British Columbia and most US states (NAPIS, 2014).

Widespread occurrences of *D. suzukii* in North America, including areas with cold climates such as Minnesota, Washington State, British Columbia (NAPIS, 2014), suggest that *D. suzukii* may survive winter temperatures to persist in these areas. However, CLIMEX modeling done before the widespread invasion of *D. suzukii*, suggested that many areas of central and northern North America are unsuitable or only marginally suitable for the species (Fig. 1.1, Damaus 2009). Since 2008, *D. suzukii* has

been found in almost every U.S. state, including in areas which are were classified as unsuitable such as North Dakota, Wyoming, and Colorado (NAPIS 2015).

The ability of *D. suzukii* to survive cold is contested. Kimura (2004) reported that this insect could not survive 24 hours at any sub-zero temperature, and therefore suggested that overwintering in Japan may be limited to man-made structures. More recent research which allowed *D. suzukii* to briefly acclimate to cold before experiments showed similar results (Dalton et al. 2011). The majority of previous research, however, has focused on *D. suzukii* summer-morphs. Winter-morphs can be produced by introducing summer-morphs eggs to autumn and winter conditions such as low temperatures and low light photoperiods (see chapter 2). Winter-morphs are larger, darker, and have longer wings than summer-morph *D. suzukii* (P.W. Shearer, personal communication). *D. suzukii* is hypothesized to overwinter as an adult winter-morph in reproductive diapause (Kanzawa 1939). While summer-morphs cannot survive longer than a week at 1°C (Dalton et al. 2011), winter-morphs are able to survive several months at 1°C (P.W. Shearer, personal communication). *Drosophila suzukii* overwintering behavior is unknown, but they may be winter active (Uchino 2005). Additional research on the cold tolerance and winter behavior of *D. suzukii* winter-morphs is necessary.

Introduction to insect cold tolerance

Insects, as poikilothermic animals, cannot survive exposure to extreme temperatures without physiological adaptations. Therefore, low temperature is often an important indicator of potential geographic range and abundance patterns of insects (Denlinger and Lee 2010). Research has attempted to define insect cold limits, especially

in the field of cold tolerance, which focuses on an insect's ecophysiological responses to cold. An insect's response to cold is often indicative of their overwintering strategy and can allow for estimates of their thermal limits. The temperature at which the insect's body fluids begin to freeze (usually below 0°C) is called the supercooling point. Due to cryoprotectants (e.g., salts, sugar alcohols, amino acids, and proteins), insects are able to “supercool”, or cool past the melting point/freezing point of their bodily fluids (Denlinger and Lee 2010).

An insect's ability to withstand exposure to cold temperatures can be measured in several ways, including mortality, continued development, and sublethal effects. While mortality is a common indicator of cold tolerance, the time an insect requires to recover after cold exposure may also be ecologically important. David et al. (1998) developed a regime which tested for cold tolerance without mortality by exposing adult *D. melanogaster* to 0°C for varying times and, upon removal, measured the time until they could stand. This measure of cold tolerance, called the “chill-coma recovery time”, increases with longer cold-exposure periods and is greater in tropical than temperate species (David et al. 1998).

Lower lethal temperature can be measured by briefly introducing insects to a temperature around the supercooling point and measuring mortality (e.g., Morey et al. 2012). These results can be used to create a model of predicted mortality from exposure to a given temperature. A hypothesis of the insect population overwintering strategy (i.e., determining whether the insect population is chill intolerant, freeze intolerant, or freeze

tolerant) can be formed by comparing this model of cumulative mortality to the cumulative supercooling point.

Insect chill injuries and death can result from prolonged exposure to a temperature which is not immediately lethal (Sømme 1996). The lower lethal time, or the time at which an insect dies after constant exposure to a single temperature, can indicate the ability of an insect to overwinter. Although it does not incorporate natural fluctuations in temperature that insects would most likely be exposed to, the development of a lower lethal time for a few ecologically relevant temperatures creates a base knowledge of the effect of prolonged cold on insect survivorship.

An insect which dies from chill injury before it freezes is considered “chill intolerant” (Fig. 1.2). Most tropical and temperate insect species are chill intolerant, including *Drosophila melanogaster* and most other *Drosophila* spp. (Strachan et al 2011).

Insects that die when ice forms within their body tissues are considered “freezing intolerant” (Fig. 1.2, Denlinger and Lee 2010). Insects that are freeze intolerant keep their body fluids from freezing (Sinclair et al. 2003). The accumulation of cryoprotectants also lowers their supercooling point. A freezing intolerant strategy is common among insects from temperate environments (Sinclair et al. 2003). Few members of Diptera are freeze intolerant, but it has been reported that adults of *Diamesa mendotae* Muttkoski (Diptera: Chironomidae) may be freeze intolerant (Bouchard et al. 2006).

Insects that are able to survive freezing are considered “freeze tolerant” (Fig. 1.2, Denlinger and Lee 2010). Often, freeze tolerant insects have a much warmer

supercooling point than chill intolerant insects. The formation of ice in freeze tolerant insects is restricted to extracellular compartments (Sinclair et al. 2003). More insects from the Southern hemisphere are freeze tolerant (77% of those studied) than those from the Northern hemisphere (29% of those studied) (Chown et al. 2005). A few species of drosophilids possess larvae that have been shown to survive partial freezing, including *Chymomyza costata* Zetterstedt (Shimada and Riihimaa 1988, Stachan et al 2011, Košťál et al 2012).

Knowledge of an insect's cold tolerance strategy is particularly important to determine its overwintering abilities (Denlinger and Lee 2010). Freeze tolerant insects are more likely to be found in poleward regions, while chill intolerant insects may be limited to tropical or sub-tropical regions or human modified structures (Sinclair et al. 2003).

The purpose of this review is to summarize previous research on *Drosophila* cold tolerance that might be relevant to *D. suzukii*. Understanding the effects of cold on *Drosophila* spp. is crucial to interpret *D. suzukii* cold tolerance results and refine a future research agenda. Four main topics are covered: overwintering biology of *Drosophila*, and the effects of acclimation, pigmentation, and geographic origin on cold hardiness of *Drosophila*.

Introduction to *Drosophila* cold tolerance research

Drosophila melanogaster is often used as a model organism in ecology and genetics studies, and increasingly in insect cold tolerance research. Although little is known of how and where *D. melanogaster* overwinters (Izquierdo 1991), the study of its

ability to survive cold is well described. All life stages of *D. melanogaster* have a supercooling point between -17°C and -20° (Czajka and Lee 1990) and are naturally chill intolerant (Strachan et al. 2011). Studies have shown, however, that *D. melanogaster* larvae can be made to be freeze-tolerant by feeding them a proline supplemented diet; these larvae can survive even if their body reaches 50% crystallization (Košťál et al. 2011).

Although the majority of cold tolerance research on *Drosophila* has focused on *Drosophila melanogaster*, many other *Drosophila* species have been studied in depth. Most species of *Drosophila* are temperate or tropical (Gibert et al. 2007). Strachan et al. (2011) measured mean supercooling points of first instar *Drosophila* from 27 different species, and most varied between -17 and -22°C , similar to *D. melanogaster*. The warmest mean supercooling point, however, was reported at -11.2°C for *D. virilis* and the coldest mean supercooling point was -24.9°C for *D. affinis* (Strachan et al. 2011). *Drosophila* cold tolerance research has been reviewed in detail by Hoffmann et al. (2003), and in general *D. melanogaster* adults survive colder temperatures when they are reared at cooler temperatures, heat hardened, cold-hardened, or crowded as larvae.

Cold Acclimation

While insects may appear to have an innate response to cold, many adapt to cold temperatures. Some insects can improve their cold hardiness in a matter of hours (a phenomenon known as rapid cold-hardening), or over longer times during development (i.e., long term acclimation) (e.g., Sinclair and Roberts 2005, Colinet and Hoffmann 2012). The ability of an insect to increase cold tolerance varies by species and by

population (Hoffmann et al 2002). In particular, several species of *Drosophila*, including *Drosophila melanogaster*, have greater survivorship after a cold acclimation regime (e.g., Hoffmann et al 2002, Czajka and Lee 1990). Although *D. melanogaster* has a supercooling point of -20°C , it does not survive exposure to temperatures below 0°C , even for short periods of time (Czajka and Lee 1990). In order for *D. melanogaster* to survive winters, it must experience some kind of acclimation (Czajka and Lee 1990).

Rapid cold-hardening allows diapausing and non-diapausing insects to survive non-freezing temperatures that would otherwise be lethal (Lee et al. 1987). This response is induced when the temperature of an insect is lowered to a non-lethal temperature, typically below the developmental threshold for the insect, for as little as 30 minutes. When the temperature of the insect subsequently is lowered to a level that is lethal to non-acclimated individuals, more individuals survive exposure. Lee et al. (1987) hypothesized that this response is caused by the ability of the insect to rapidly accumulate cryoprotectants, such as glycerol and phosphorylase, while at the non-lethal temperature. This result is consistent with results from Chen et al. (1987), who found that within two hours after transfer to 0°C , glycerol concentrations doubled or tripled in the flesh fly, *Sarcophaga crassipalpis* Macquart. The rapid cold-hardening response may also be related to the heat-shock response, which is characterized by an increase in heat-shock proteins in cells following an environmental stress (reviewed in Lee 1987). When *S. crassipalpis* is transferred from 25°C to 36°C and held there temporarily, survivorship at -10°C is greater than if it had just been exposed to -10°C directly (Chen et al. 1987).

The phenomenon of rapid cold-hardening has been studied extensively in *D. melanogaster*. *Drosophila melanogaster* cannot survive a 2 h exposure at -5°C , but 100% of individuals survive exposure to this temperature if they are acclimated beforehand for 2 h at 5°C (Czajka and Lee 1990). More ecologically relevant rapid cold-hardening has also been observed in *D. melanogaster* by cooling the insect at a slow rate, or creating a thermoperiodic cycle based on natural conditions (Kelty and Lee 1999, 2001). In addition, the ability of *D. melanogaster* to rapidly cold-harden in a field setting has been verified. When adults are cooled from 22°C to 10°C in a field overnight, 68% survive a one hour cold assay at -6°C ; *D. melanogaster* without an overnight cooling have a survival rate of 10% (Kelty 2007).

Developmental acclimation appears to have a stronger effect on cold tolerance than rapid cold-hardening. While rapid cold-hardening can lower the LT_{50} (i.e., the lethal temperature after brief exposure for 50% of the population) of *D. melanogaster* by 1 to 2°C (Sinclair and Roberts 2005), developmental acclimation may lower the LT_{50} by up to 6°C (Goto 2000). A rearing temperature of 5°C lower than room temperature increases survivorship of *D. melanogaster* after 2 h at -5°C by nearly 80%, while rapid cold-hardening alone causes a 20% increase in survivorship (Colinet and Hoffmann 2008). These results have been confirmed in studies with a longer stress. *Drosophila melanogaster* reared at 19°C , as opposed to room temperature (25°C), showed $>30\%$ increased survivorship after 4 h at -5°C (Rako and Hoffmann 2007).

Other *Drosophila* spp. also have a greater resistance to cold after individuals undergo developmental acclimation. When eight species of the *D. melanogaster* species

subgroup reared at 15°C and room temperature (23°C) were exposed to a cold shock, chill coma recovery was significantly shorter in flies reared in the developmental acclimation regime (Hori and Kimura 1998).

Induction of developmental acclimation in a laboratory is closely associated with mimicking natural fall and winter conditions, including temperature and photoperiod. Photoperiod alone can affect cold tolerance. Rearing *D. melanogaster* at a low light photoperiod (10:14 (L:D) h vs. 14:10 (L:D) h) shortened chill coma recovery time after exposure to near-freezing temperatures (Lanciani et al 1992). Similar results were reported in *D. montana*, where males and females reared in a short photoperiod were more cold tolerant than those reared in a long photoperiod (14:10h L:D vs. 16:8h L:D) (Vesala and Hoikkala 2011). This phenomenon has also been shown to occur in other members of Diptera, including the northern blowfly, *Protophormia terraenovae* Robineau-Desvoidy (Fischer et al. 2011).

Geographic Origin and Cold Tolerance

In temperate insect species, the lower developmental limits (or the lowest temperature where an insect can continue its growth) are much more variable than the higher temperature limits, and thus are a more accurate measure of the range of the insect (e.g., Addo-Bekiako et al. 2000). Insect response to environmental stresses, such as cold, varies both among species and among populations of the same species (e.g., Hoffmann and Watson 1993).

The relationship between cold tolerance and geographic origin of drosophilid species has been well-researched (e.g., Hoffmann et al. 2003, Hoffmann and Watson

1993, Gilbert and Huey 2001). *Drosophila* species from more poleward latitudes and higher elevations experience cold more often than their tropical counterparts. Insect species from more equatorial latitudes are less cold tolerant than insect species from higher latitudes, possibly as a consequence from selection by cold (Hoffmann et al. 2002). Tropical and subtropical populations of *D. subobscura* have been reported as significantly less cold tolerant than more northern populations of the same species (David et al. 2003). This pattern has been shown to exist in different species of *Drosophila*, including North American populations of *D. melanogaster* (Schmit and Paaby 2008). In addition, widely distributed, generalist species of *Drosophila* (such as *D. melanogaster*) appear to have a wider range of cold resistance than narrowly distributed, specialist species (Kellermann et al. 2009).

A substantial amount of research on *Drosophila* species has focused on chill coma recovery. After exposure to cold, *Drosophila* species of temperate origin have a much quicker recovery time (< 10 minutes) than *Drosophila* species of tropical origin (between 20 and 120 minutes) (Gibert et al. 2007). Interestingly, of ten cosmopolitan species studied, seven were of tropical origin and should be unable to survive cold temperatures despite being widespread (Gibert et al. 2007).

According to Hopkin's Law of Bioclimatics (1919), one degree of latitude has the same effect on insects as does 400 ft of altitude. It can be assumed then, that insects from higher altitudes would be more cold tolerant than insects from low altitude, similar to the effects of latitude. A study done on copper butterflies showed that high-altitude populations had greater cold stress resistance than low-altitude populations, although they

had decreased heat resistance (Fischer et al. 2010). In *Drosophila*, a comparison of cold hardiness of four different species in the *Drosophila takahashii* Sturtevant species subgroup showed that *Drosophila* found in higher altitudes were more cold tolerant than species in lower altitudes (Kimura 1994). This pattern also occurs in populations of the same species; *D. melanogaster* from higher altitudes appear to be more cold tolerant than those from low altitudes (Collinge et al. 2006, Parkash et al. 2010). In addition, altitude is correlated with pigmentation, with higher altitude flies having darker pigmentation (Parkash et al. 2010).

Dark Morphs in *Drosophila*

Insects reared in cold and arctic conditions are often darker than those that are reared at more temperate conditions (Leather et al. 1993). Previous research on cold tolerance of *D. melanogaster* has shown that adult females reared at a low temperature have a dark pigmentation and a large wing: thorax ratio (Ayrinhac et al. 2004). The reason for this darker morph is poorly understood but a few studies have explored potential advantages. One potential advantage of the darker pigmentation is found in adults of *D. melanogaster* and *D. jambulina* Parshad and Paika, where darker pigmentation is associated with a higher desiccation resistance (Parkash et al. 2008, 2009). Another potential explanation is that darker pigmentation allows insects to absorb radiation more efficiently to warm up more quickly (Leather et al. 1993). Darker pigmentation appears to be a side effect of low rearing temperature, and although the coloration does not cause *Drosophila* to be more cold tolerant, the two traits may

coincide. *Drosophila suzukii* occurs in larger, darker morphs with longer wings in the late fall and winter (Kanzawa 1939).

Overwintering in *Drosophila*

Most species of *Drosophila* overwinter as adults in diapause, although a few may overwinter as larvae (e.g., Strachan et al. 2011, Lumme et al. 1974, Kimura et al. 1988). Diapause is an endocrine-mediated dormancy that has long been associated with an increased cold tolerance in insects (Denlinger 1991, Bale and Hayward 2009). Whether the association between diapause and cold hardiness is causal or coincidental is unknown (Denlinger 1991). All species of the *D. melanogaster* species group where diapause has been studied overwinter as adults in diapause, while those in the *D. subobscura* species group have more variation and may overwinter as pupae or adults in diapause or not, depending on the species (Kimura 1987, Goto et al. 1999).

The ability of *D. melanogaster*, a tropical species, to enter diapause and overwinter in cold climates has been extensively studied (e.g., Izquierdo 1991, Hoffmann et al. 2003, Kimura 1988). Although *D. melanogaster* has been long established in temperate climates, overwintering *D. melanogaster* have not been found in the field (Izquierdo 1991). Diapause has been induced in *D. melanogaster* under laboratory conditions involving a short photoperiod while maintaining insects at room temperature (Lanciani et al. 1991, Lanciani et al. 1992). A few studies have confirmed the ability of adult *D. melanogaster* in diapause to overwinter in northern climates (Izquierdo 1991, Hoffmann et al. 2003).

Conclusions

Drosophila spp. vary in their levels of cold tolerance, but all species studied possess the ability to increase their cold hardiness in a variety of ways. *Drosophila melanogaster* is one of the most researched insects in cold hardiness studies, and the knowledge on its cold hardiness provides a basis for future *Drosophila* research. *Drosophila melanogaster* is chill intolerant and appears to have a low base level of cold tolerance, but is able to persist in poleward regions due to its ability to become cold acclimated.

Because *D. melanogaster* possesses the ability to rapidly and developmentally acclimate, enter diapause, have darker morphs, and have varied base cold tolerance dependent on geographic origin, it seems likely that *D. suzukii* could do the same. If *D. melanogaster* is able to overwinter as an adult in diapause, and other species of the *D. melanogaster* species group tend to overwinter as an adult in diapause, it seems reasonable to speculate that *D. suzukii* will also overwinter as an adult in diapause. We predict that *Drosophila suzukii*, which is a widely distributed and generalist fruit pest from high latitudes, will be more cold tolerant than other *Drosophila* species that are not as widely distributed. *D. suzukii* reared at cooler conditions will likely have darker pigmentation and be more cold tolerant than *D. suzukii* reared at warmer conditions, as has been shown by P.W. Shearer (personal communication). *Drosophila suzukii* is most likely chill intolerant, but should be able to survive in poleward regions, especially if it undergoes rapid cold hardening and developmental acclimation. Future studies should

focus on attempting to increase *D. sukii* cold tolerance in order to understand the coldest regions *D. sukii* should be able to survive.

Figures:

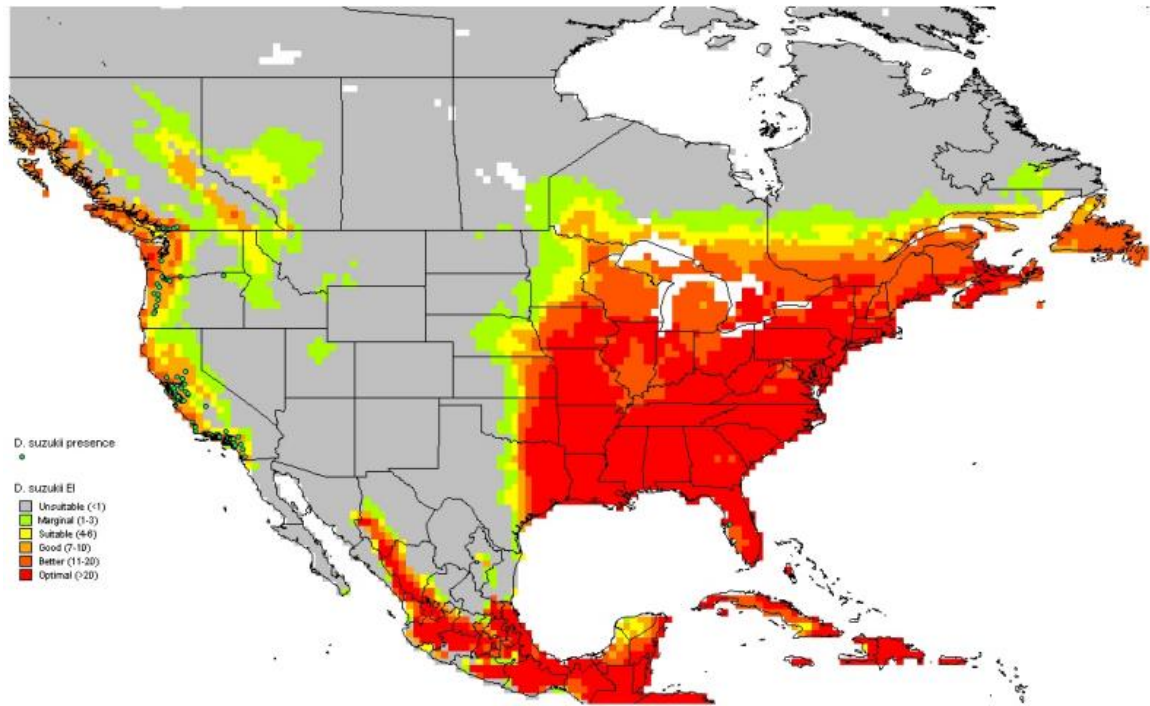


Figure 1.1: CLIMEX model of predicted habitat suitability of North America to *D. suzukii* (reproduced from Damaus 2009)

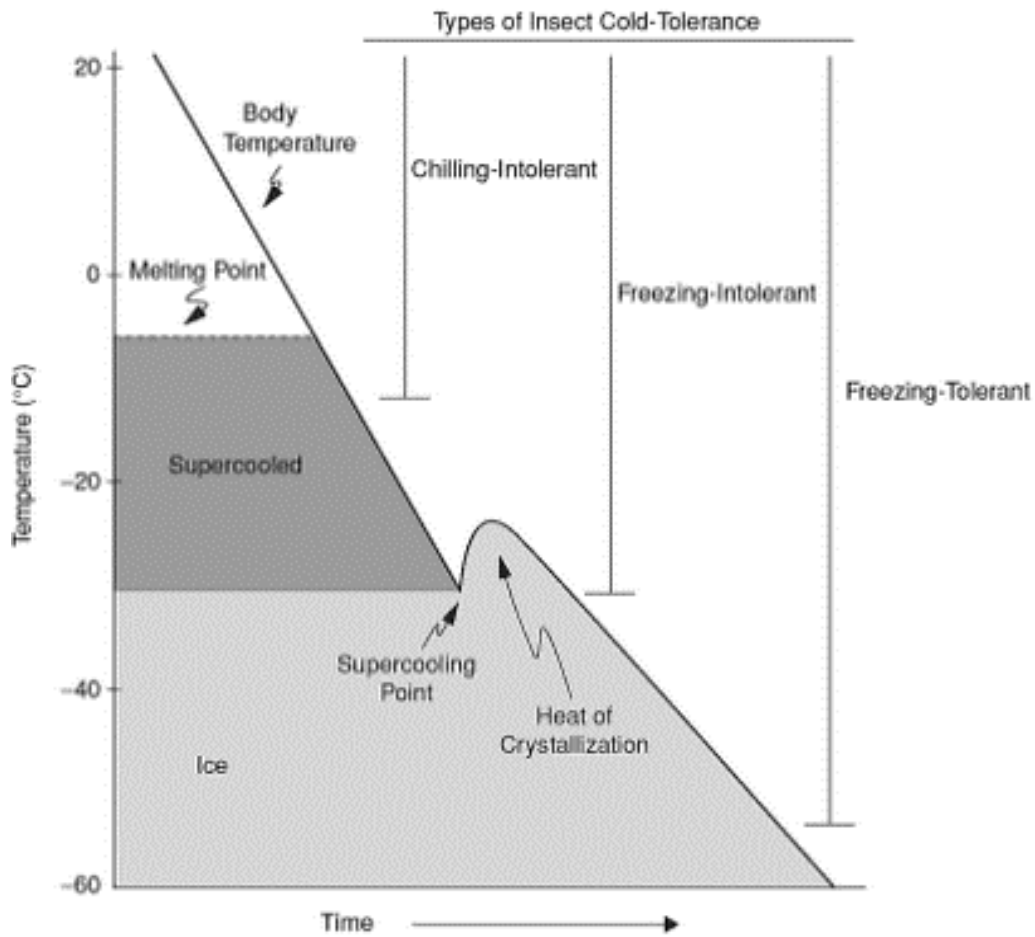


Figure 1.2: Types of insect overwintering strategies, shown as an insect cools at a constant rate. (Reproduced from Denlinger and Lee 2010)

**Chapter 2: Cold hardiness of winter acclimated *Drosophila*
suzukii (Diptera: Drosophilidae) adults**

Summary

Drosophila suzukii Matsumura, spotted wing drosophila, is an exotic fruit fly that is native to Southeast Asia and was first detected in the continental United States in 2008. Previous modeling studies have suggested that *D. suzukii* might not survive in portions of the northern United States or southern Canada due to the effects of cold. As a result, we measured two aspects of insect cold tolerance, the supercooling point and lower lethal temperature, for summer-morph *D. suzukii* adults and winter-morph adults. Supercooling points were compared to adults of *D. melanogaster* Meigen and summer-morph *D. suzukii* pupae. The lower lethal temperature of *D. suzukii* winter-morph adults was significantly colder than for *D. suzukii* summer-morph adults, while supercooling points of *D. suzukii* winter-morph adults were actually warmer than for *D. suzukii* summer-morph adults and pupae. *D. suzukii* summer-morph adult supercooling points were not significantly different than those for *D. melanogaster* adults. These measures indicate that *D. suzukii* is a chill intolerant insect, and winter-morph adults are the most cold tolerant life stage. These results can be used to improve predictions of where *D. suzukii* might be able to establish overwintering populations and cause extensive damage to spring fruit crops.

Introduction

Non-native insects have the potential to cause widespread damage to both natural and cultivated systems (e.g., Manchester and Bullock 2000, Pimentel et al. 2000). Spotted wing drosophila, *Drosophila suzukii* Matsumura, is native to Southeast Asia and was first discovered in the continental United States in 2008 (Hauser 2011). Female *D. suzukii* can oviposit into intact soft-skinned fruits including *Rubus* spp., *Prunus* spp., and *Vitis* spp. (Walsh et al. 2011). Larval feeding further damages the fruit and renders it unmarketable. The combined effects of lower crop yield and increased costs to control *D. suzukii* have led to substantial local reductions in revenue from berry crops (Goodhue et al. 2011).

Drosophila suzukii is found on nearly every continent, with the notable exceptions being Oceania and Antarctica (Commonwealth of Australia 2013). This insect was discovered in Spain in 2008 and has spread rapidly across Europe (Cini et al. 2012). In 2012, *D. suzukii* was first discovered in South America in Brazil (Depra et al. 2014). In North America, *D. suzukii* has been found in British Columbia and most US states (NAPIS, 2014).

Most species of *Drosophila* overwinter as adults in diapause, although some may overwinter as pupae or larvae (Hoffmann et al. 2003, Strachan et al. 2011). *Drosophila suzukii* is believed to overwinter as adult winter-morphs underneath leaf litter in forested areas (Kanzawa 1939). Winter-morphs are larger and darker than summer-morphs and have longer wings (P.W. Shearer, personal communication). They have been found in the fall in northern climes (Kanzawa 1939) and have been successfully reared under

laboratory conditions (P.W. Shearer, personal communication). Winter-morphs of *D. suzukii* are triggered by introducing eggs and larvae to autumn/winter conditions such as low temperatures and short days, so these morphs are thought to be cold acclimated. Large, dark morphs associated with overwintering conditions have been described in other *Drosophila* spp., including *D. melanogaster* Meigen (e.g., Aryinhac et al. 2004, Gibert et al. 2007). Overwintering females of *D. suzukii* may be in diapause (Zerulla et al. 2015, Mitsui et al. 2010) and more cold tolerant than males (Zerulla et al. 2015, Kanzawa 1939).

Cold temperature is a limiting factor in the geographic range of several *Drosophila* spp. (Kimura 1988). If pupae or adults of *D. suzukii* overwinter beneath leaf litter and snow in northern climes of North America, individuals must withstand extended exposure to low temperatures (i.e., months at 0°C), with brief exposures to harsh cold shocks. Previous studies have suggested that summer-morph *D. suzukii* must have temperatures above 7.2°C for development to occur (Tochen et al. 2014) and are unable to survive for three months at 10°C (Dalton et al. 2011) while winter-morphs are able to withstand several months at 1°C (P.W. Shearer, personal communication). This result suggests that *D. suzukii* is as cold tolerant or more cold tolerant than non-acclimated *D. melanogaster*, which is able to survive >45 days at 4°C (Izquierdo et al. 1991). In addition, a CLIMEX model created for *D. suzukii* suggests that much of the northern United States and southern Canada provides a suboptimal climate (Damus 2009). There is a lack of knowledge, however, about the ability of *D. suzukii* to survive brief exposures to harsh cold temperatures.

Insect cold tolerance frequently is quantified by using the supercooling point and the lower lethal temperature. The supercooling point is the temperature at which the insect's body fluids begin to freeze, typically below 0°C due to the colligative properties of salts, amino acids, proteins and sugar-alcohols in the hemolymph (Denlinger and Lee 2010). Insects that succumb to chill injury before their body fluids freeze are considered 'chill intolerant' (Denlinger and Lee 2010), a cold response common to many tropical species, including most species of *Drosophila* (Strachan et al. 2011). Insects that survive chilling but die due to the formation of ice within their body tissues are considered 'freeze intolerant', while those that are able to survive freezing are considered 'freeze tolerant' (Denlinger and Lee 2010). Among temperate insects, freeze intolerance is more common than freeze tolerance (Sømme 1996). A few species of *Drosophila* larvae have been shown to survive partial freezing (Shimada and Riihimaa 1988, Strachan et al. 2011, Košťál et al. 2012).

Lower lethal temperature can be measured by briefly introducing insects to a temperature around the supercooling point and determining mortality (e.g., Morey et al. 2012). This parameter can be used to understand instant mortality from cold shocks, although prolonged exposure to a temperature which is not immediately lethal can also result in chill injury and death (Sømme 1996).

This study quantifies cold tolerance of adult female and male summer-morph and winter-morph *D. sukii* by measuring the supercooling point and lower lethal temperature. The effects of morph and sex on supercooling points were compared. Supercooling points for adult *D. sukii* were further compared to adult *D. melanogaster*

(a species known to persist in northern climates) and pupae of *D. suzukii*. We hypothesized that the adult female winter-morph of *D. suzukii* would be more cold tolerant than male winter-morph *D. suzukii*, adult summer-morphs and non-acclimated pupae of *D. suzukii* of both sexes, and that the cold hardiness of summer-morph *D. suzukii* would be similar to *D. melanogaster*.

Materials and Methods

Colony Source.

Drosophila suzukii were obtained from a colony at Michigan State University in East Lansing, MI in December, 2013. This colony was established from adult flies found in a blueberry field in Allegan County, Michigan in August 2013 (Steve Van Timmeren, personal communication). Adults were kept in St. Paul, MN, in narrow fly vials stoppered with foam plugs (Genesee Scientific, San Diego, CA) and where larvae were fed on a traditional agar-cornmeal-yeast diet as described in Dalton et al. (2011).

Summer-morph *D. suzukii* were reared in programmable growth chambers (Percival Scientific Inc., Perry, IA), typically at $24 \pm 2^\circ\text{C}$ with a photoperiod of 14:10 (L:D) h. Under these conditions, summer-morph adults lived for approximately 2-3 weeks after eclosion. Winter-morph adult *D. suzukii* were produced by placing vials with 1-3-d-old eggs laid by summer-morph *D. suzukii* into a Percival programmable growth chamber at $10 \pm 1^\circ\text{C}$ with a photoperiod of 12:12 (L:D) h (protocol modified from P.W. Shearer, personal communication). The colder temperatures slowed growth, and adults emerged from pupae 1 to 2 months after being placed in the growth chamber. Recently emerged adult flies (<48 h old or between 17 and 33 degree days (base temp = 7.2°C) for summer-

morph flies, and <120 h old or under 14 degree days for winter-morphs flies) were used for all experiments.

Oregon-R strain of *Drosophila melanogaster* was obtained from the University of Minnesota in June, 2014. *Drosophila melanogaster* were kept in narrow fly vials stoppered with a foam plug (Genesee Scientific, San Diego, CA) and fed the same artificial agar-cornmeal-yeast diet as *D. suzukii* (Dalton et al. 2011). *Drosophila melanogaster* were reared at room temperature ($24 \pm 2^\circ\text{C}$) on a benchtop in St. Paul, MN, in a separate lab from *D. suzukii* to prevent cross-contamination, because *D. melanogaster* has previously outcompeted *D. suzukii* in mixed cultures (MKA, personal observation). *Drosophila melanogaster* adults <48 h-old were used for all experiments.

Supercooling Point Determination.

Supercooling points were measured with custom-built thermocouples. These were created by threading 0.127 mm diameter copper and constantan wires through a milled plastic dowel fitted with an O-ring, and then inserting the dowel into a microcentrifuge tube. The O-ring sealed the tube and prevented the insect from escaping (Fig. 2.1). The entire apparatus was placed in the center of a 20 cm polystyrene cube plugged with a rubber stopper, and the polystyrene cube was placed in a -80°C freezer, which cooled the insect at approximately $1^\circ\text{C}/\text{minute}$ (Carrillo et al. 2004). Thermocouples were connected to a computer via an analog data acquisition unit (USB-TC, Measurement Computing, Norton, MA), and temperature was recorded once per second by using TracerDAQ Pro (Measurement Computing Corporation, Norton, MA).

The supercooling point was measured as the lowest temperature before the release of the latent heat of freezing, indicated by a spike in temperature (Denlinger and Lee, 2010).

Supercooling point experiments followed a completely randomized design, with 12 – 16 individuals per batch, and at least two batches per treatment. Treatments consisted of species, stage or morph, and were typically run in separate batches due to availability. When sex was of interest, equal numbers of males and females were tested in the same batch. Because treatment and batch were confounded factors, potential treatment differences must be interpreted with caution. All analyses were conducted in R 3.0 (R Core Team, Vienna, Austria). Details of each analysis are provided below.

Adult Drosophila suzukii.

A total of 30 winter-morph and 60 summer-morph adult *D. suzukii*, half of which were females, were tested. To separate *D. suzukii* from the colony, adults were anesthetized with CO₂ using a FlyPad (Genesee Scientific, San Diego, CA) and one fly was placed into each 1.5-ml microcentrifuge tube. Summer-morph adults were measured in six batches, while winter-morph adults were measured in two batches.

Summer-morph supercooling points were not normally distributed, so all data compared to summer-morphs were transformed by using an x^2 transformation as recommended by a Box-Cox analysis (Box and Cox 1964). Data was back-transformed to present the mean and 95% CI of the supercooling points. A single ANOVA was used to test the effects of sex, morph, and their potential interaction on supercooling points of winter-morph and summer-morph *D. suzukii*.

Adult *Drosophila melanogaster*.

The supercooling points of 26 adult *D. melanogaster* were measured to compare with summer-morph *D. suzukii*. The supercooling points were measured in two batches, each containing 13 adults. To separate *D. melanogaster* from the colony, adults were confined on a tray in an ice bath for < 5 min and transferred into individual 1.5 ml microcentrifuge tubes. *Drosophila melanogaster* were statistically compared to *D. suzukii* adult summer-morphs by using a Welch two sample t-test (R command: t.test).

Drosophila suzukii Pupae.

Some species of *Drosophila* overwinter as pupae, so supercooling points of pupae were tested and compared to supercooling points of adults. *Drosophila suzukii* pupae were reared at $24 \pm 1^\circ\text{C}$ with a photoperiod of 16:8 (L:D) h, as for summer-morphs. A total of 58 pupae, < 24 h old, were transferred from the colony by using a camelhair brush and transferred to individual 1.5mL microcentrifuge tubes. Pupae were tested in 3 batches. Supercooling points were determined as for adults and compared to adult *D. suzukii* summer-morphs by using a Welch two sample t-test.

Lower Lethal Temperature Determination.

These experiments followed a randomized complete block design. *Drosophila suzukii* were cooled in a Thermo Scientific refrigerated bath circulator (A40, Thermo Fisher Scientific, Newington, NH) with SIL-180 silicon oil as a coolant. Temperature of the adults was monitored using thermocouples as described for supercooling point determination. Summer-morph and winter-morph adult *D. suzukii* were cooled in separate batches (at a rate of $1^\circ\text{C}/\text{minute}$) to -10, -15, -20, and $-25^\circ\text{C} \pm 1^\circ\text{C}$ and held at the

temperature for approximately 1 sec. Adults that supercooled were not treated differently, and cooled to the target temperature. Control flies were held at room temperature (approximately $24 \pm 2^\circ\text{C}$) in individual microcentrifuge tubes until experimental flies were finished cooling. Experimental adults were returned immediately to room temperature and all adults were transferred to individual microcentrifuge 1.5mL tubes that contained approximately 0.5mL artificial diet and a ventilation hole. Four adults of each cooling treatment were run per each block, half female, for a total of 20 flies per block (including controls), with five blocks for winter-morphs and 11 blocks for summer-morphs. Mortality was measured after 24 h at room temperature to allow adults to recover, as has been used in previous studies of *Drosophila* (Czajka and Lee 1990). Survival was determined by movement and the ability of the adult to upright itself (e.g., Czajka and Lee 1990, Dalton et al., 2011). Sex was recorded at the same time as mortality by looking for spots on wings or a pointed abdomen indicating an ovipositor (Hauser 2011).

All analyses were conducted in R (R Core Team, Vienna, Austria). Abbot's correction ($P' = P - P_0 \frac{100-P}{100-P_0}$; Healy 1952), was applied to remove mortality that could not be attributed to cold exposure, where P' is the corrected percentage of adults that died because of the effects of the cold treatment, P is the percentage of adults that died in the treatment, regardless of the cause, and P_0 is the percentage of control adults that died without experiencing cold. The percentages were then multiplied by the number of flies tested for a specific treatment prior to analysis.

The relationships between mortality and temperature for winter and summer-morphs were analyzed by using general linearized models with a logit-link in R. Survival of each individual (coded as 0 = death, 1 = survival) was the response variable and block, treatment temperature, sex, and interaction effects were explanatory variables. The LT_{50} and LT_{90} (the temperature at which 50% or 90% of the population dies after exposure for one second) were determined with R- function “dose.p(glm, p=0.5 or p=0.1)” in the MASS package. The predicted mortality curves for summer- and winter-morph *D. sukikii* were compared using a third generalized linear model with survival of each individual (coded as 0 = death, 1 = survival) as the response variable and treatment temperature as the explanatory variable. Winter-morphs and summer-morphs were analyzed separately.

In a final set of analyses, the supercooling point was compared to the lower lethal temperature data to determine the cold tolerance strategy. The supercooling point data and lower lethal temperature data were combined into one dataset. The elements of the dataset were experiment (coded as SCP = supercooling point, LLtemp = lower lethal temperature), treatment temperature (i.e., exposure temperature for lower lethal temperature experiments or temperature at which the exotherm was detected for supercooling point experiments), positive outcomes (i.e., number of individuals that survived a given temperature in lower lethal temperature experiments or cumulative number of individuals that did not freeze by the given temperature in supercooling point experiments) and negative outcomes (i.e., number of individuals that did not survive a given temperature in lower lethal temperature experiments or cumulative number of

individuals that froze by the given temperature in supercooling point experiments). A generalized linear model was then created with probability of observing a positive outcome as the response variable and treatment temperature, experiment and the interaction of treatment temperature and experiment as the explanatory variables. Winter-morphs and summer-morphs were analyzed separately.

Results

Supercooling Points.

Sex did not significantly affect the supercooling point of adult winter-morph ($F = 1.8$, $df = 1$, 25 , $P = 0.19$) or summer-morph *D. sukuzii* ($F = 2.9$, $df = 1$, 41 , $P = 0.10$; results were pooled for analysis). Winter-morph adults had a significantly warmer supercooling point ($F = 33.9$, $df = 1$, 77 , $P < 0.0001$) than summer-morph adults. Summer-morph adult *D. sukuzii* had a mean supercooling point of -20.2°C (95% CI: -20.9 , -19.6 ; Fig. 2.2), while winter-morph *D. sukuzii* had a mean supercooling point of -17.5°C (95% CI: -17.7 , -17.2 ; Fig. 2.2). Pupae had a mean supercooling point of -21.9°C (95% CI: -22.6 , -21.1 ; Fig. 2.2). Supercooling points for pupae were significantly cooler than summer-morphs ($t = -4.1$, $df = 107$, $P < 0.0001$). The mean supercooling point of *D. melanogaster* was -19.6°C (95% CI: -20.1 , -19.0 ; Fig. 2.2). Summer-morph *D. sukuzii* and *D. melanogaster* supercooling points were not significantly different ($t = 1.4$, $df = 78.1$, $P = 0.2$)

Lower Lethal Temperature.

Sex did not significantly affect the lower lethal temperature of winter-morph ($F = 0.4$, $df = 1$, 85 , $p = 0.5$) or summer-morph *D. sukuzii* ($F = 0.8$, $df = 1$, 87 , $p = 0.4$; results

were pooled for analysis). In general, for the same temperature, mortality of winter-morphs was less than summer-morphs (Fig. 2.3). Logistic regression models describing mortality as a function of temperature for winter-morph and summer-morph adults had significantly different intercepts ($z = 3.4$, $p = 0.02$), though the slopes were not significantly different ($z = 1.1$, $p = 0.280$). The intercept of 7.9 ± 1.4 (\pm SE) for winter-morphs and 3.8 ± 1.13 for summer-morphs suggests that summer-morphs begin dying at a warmer temperature than winter-morphs. The predicted LT_{50} for summer-morph adults was $-10.01^{\circ}\text{C} \pm 0.8$ (\pm SE), and for winter-morph adults was $-15.3^{\circ}\text{C} \pm 0.6$ (Fig. 2.3). The predicted LT_{90} for summer-morph adults was -15.6 ± 1.1 , and for winter-morph adults was $-19.55^{\circ}\text{C} \pm 0.9$.

Logistic regression models to determine the overwintering strategy of winter-morphs had significantly different slopes ($z = 11.1$, $P < 0.001$) and intercepts ($z = 10.5$, $P < 0.001$). Winter-morphs began dying before they supercooled, and supercooled at a much faster rate than they died (Fig. 2.3). Logistic regression models to determine the overwintering strategy of summer-morphs had significantly different slopes ($z = -2.6$, $P = 0.007$) and intercepts ($z = -3.7$, $P < 0.001$). Summer-morphs began dying before they supercooled, and supercooled at a faster rate than they died (Fig. 2.3).

Discussion

Previous research on *D. sukikii* cold tolerance has suggested that summer-morph adults should not be able to overwinter in cold climates (Kimura et al. 2004, Dalton et al. 2011), but winter-morph adults might (P.W. Shearer, personal communication). However, while winter-morphs have been reported to survive long periods at mild

temperatures (P.W. Shearer, personal communication), it is likely that they lack the ability to survive brief exposure to extreme temperatures. This study provides a basis for which temperatures can be considered extreme for *D. suzukii* winter-morphs and summer-morphs.

Most mortality occurs before the supercooling point in both summer-morphs and winter-morphs of *D. suzukii*, although mortality in the summer-morph begins several degrees warmer than for winter-morphs. The difference between the cumulative supercooling point curves and mortality curves of *D. suzukii* suggest adults are chill intolerant, or that they die before freezing. Chill intolerance is common in temperate insects, and suggests that *D. suzukii* must avoid extreme cold temperatures to overwinter. Winter-morph *D. suzukii* may have a mixture of both a chill intolerant strategy (i.e., death occurs before an individual freezes) and a freeze-intolerant strategy (i.e., death occurs once an individual freezes). The predicted mortality curve begins at temperatures before supercooling, but it follows the supercooling point curve very closely in cooler temperatures (Fig. 2.3). This suggests that some winter-morph *D. suzukii* may be freeze intolerant, and some may be chill intolerant. In addition, it appears that winter-morphs may not invest in types of cryoprotectants that prevent freezing, but rather invest in resources which allow them to survive chill injury.

A possible explanation for the warmer supercooling points of winter-morph adult *D. suzukii* than summer-morph adult *D. suzukii* could be an effect of suboptimal rearing conditions. O'Doherty and Bale (1985) described a warmer supercooling point of the peach-potato aphid *Myzus persicae* Sulzer that were reared at 5°C for 7 d than insects

reared at 20°C. The authors hypothesized that this decrease in supercooling ability was due to suboptimal conditions. *Drosophila suzukii* reared at 10°C may have an increased ability to survive over time at milder temperatures at the cost of a warmer supercooling point. Future studies are needed to investigate why cold-acclimated winter-morph *D. suzukii* adults have a warmer supercooling point despite their higher survival at cooler temperatures.

Our results suggest that the supercooling point of *D. suzukii* pupae is cooler than summer-morph *D. suzukii* adults. This result could have important ecological implications. *Drosophila suzukii* winter-morphs may not emerge until late in the fall, and as a result, pupae must be able to survive cold shocks before adults overwinter. In addition, it has been suggested that immobile stages of *Drosophila* may be more cold tolerant because they are unable to behaviorally avoid damaging temperatures (e.g., Hoffman et al. 2003). These results suggest pupae may be able to overwinter, but previous research has suggested that although pupae are able to withstand winter temperatures for a short period of time, they will emerge as adults even at low temperatures (i.e. 1°C or 3°C) and subsequently die (Dalton et al. 2011). In addition, pupae which overwinter in soil or rotting berries may be susceptible to inoculative freezing, and pupae are freeze intolerant (A.R. Stephens, unpublished data).

Previous studies have indicated that the supercooling point of *D. melanogaster* may vary with age, but 2-d-old *D. melanogaster* should be expected to have a supercooling point between -19°C and -21°C, which is consistent with our results (Czajka and Lee, 1990). In addition, *D. melanogaster* is a chill intolerant insect. The lack of a

significant difference between the supercooling point of *D. melanogaster* and *D. sukii* summer-morphs suggests that other measures of cold tolerance, such as the lower lethal temperature of *D. sukii* summer-morphs may be similar to *D. melanogaster*. If chill intolerant *D. melanogaster* can overwinter in northern, temperate climates as has been predicted in previous studies (e.g., Izquierdo 1991), *D. sukii* might be able to overwinter in these climates as well. However, the ability of *D. melanogaster* to overwinter in cold climates is debated by some authors (e.g., Izquierdo 1991).

Overwintering *D. sukii* will only survive cold shocks in temperate climates of the northern U.S. and Canada if it has a way to escape the cold, such as under snow or indoors. However, *D. sukii* may not spend winter beneath snow, as previous studies have shown that *D. sukii* is a winter active species (Uchino 2005). The ability of winter-morphs of *D. sukii* to survive an extended period at low temperatures has yet to be measured. Although *D. sukii* may not be able to survive extreme cold shocks, insect survival of cold also varies based on the length of time an insect is exposed to a cold temperature (Sømme 1996). Future research on the lower lethal time, or the time it takes *D. sukii* to die while constantly exposed to a cold stress is needed.

The lack of effect of sex on supercooling point and lower lethal temperature of *D. sukii* does not confirm previous results. Previous studies have indicated that females enter a reproductive diapause and are more likely to be found in the spring than males, suggesting that females are more cold hardy than males (Zerulla et al. 2015). While male and female *D. sukii* can survive cold shocks at a similar rate, however, it may be that females have the ability to withstand a longer cold exposure.

Understanding the supercooling point and lower lethal temperatures of invasive pests can help refine predicted risk and control recommendations and may even lead to new management approaches, such as temperature-based treatment of fruit. Ultimately, these results indicate that both summer and winter-morph adult *D. suzukii* should not be able to successfully overwinter in cold climates without a way to buffer the cold. Future research should examine winter indoor activity of *D. suzukii*. Our results confirm a significant difference in cold tolerance of summer and winter-morphs of *D. suzukii*, suggesting that future ecologically relevant research on *D. suzukii* cold tolerance should focus on winter-morphs. The arrival of *D. suzukii* in poleward regions may be facilitated by other means and our results highlight the need to improve our understanding of the movement patterns and mechanisms of dispersal of *D. suzukii*. If the main form of dispersal is through fruit imports, it may be prudent to limit or monitor fruit imports from infested regions to areas where *D. suzukii* is not able to establish. In addition, future research should focus on the ability of *D. suzukii* to acclimate to colder temperatures. If *D. suzukii* is able to rapidly acclimate to cold temperatures, it is possible that it may be able to survive winter cold shocks.

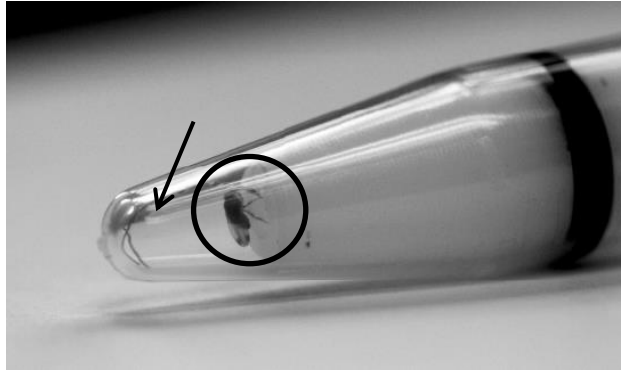


Figure 2.1: *Drosophila suzukii* male (circled) and thermocouple (arrow) in microcentrifuge tube.

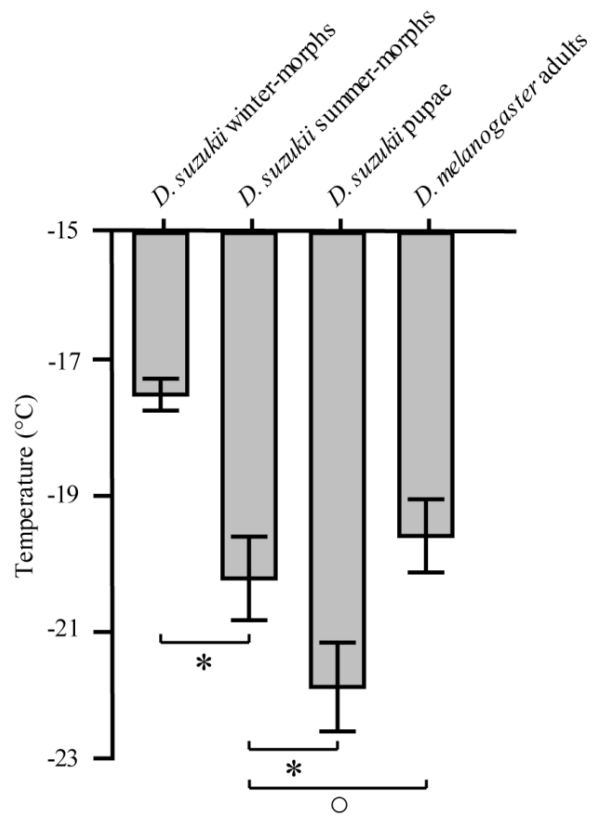


Figure 2.2: Supercooling points (mean \pm 95% confidence interval) of *Drosophila suzukii* and *D. melanogaster*. Brackets indicate statistical comparisons between two species or stages. Circles indicate no statistical difference ($P>0.05$); asterisks indicate a difference ($P<0.05$).

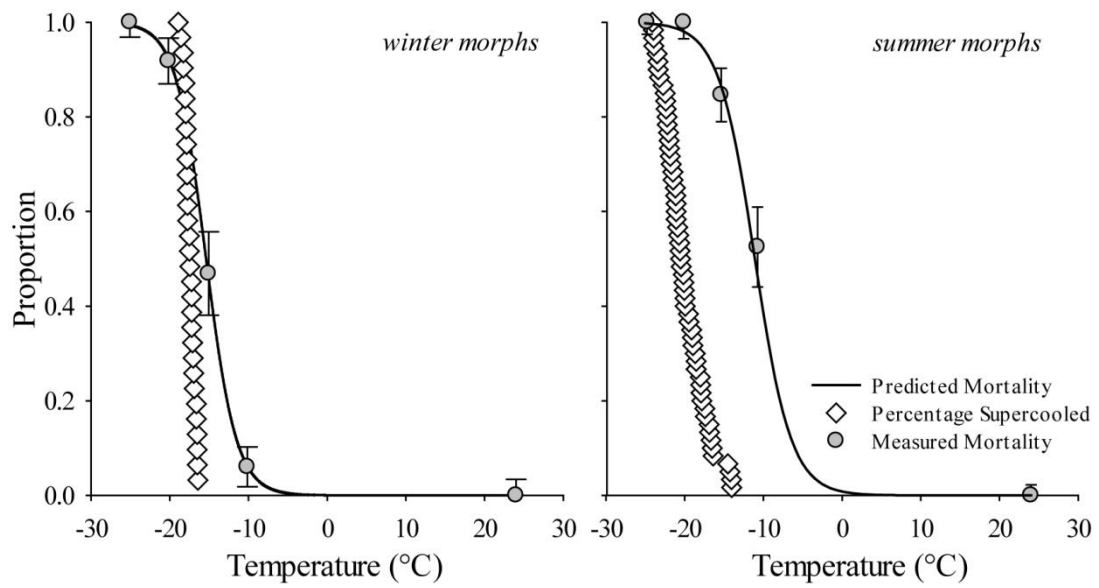


Figure 2.3: Measured and predicted cumulative proportion of winter- and summer-morph adults of *Drosophila sukii* that had supercooled (i.e., given an exotherm), summer-morphs died in response to acute exposure to cold temperatures. Mortality is mean \pm SE.

**Chapter 3: Rapid cold-hardening in winter-morph and
summer-morph *Drosophila suzukii* (Diptera: Drosophilidae)**

Summary

Rapid cold-hardening, where insects quickly accumulate cryoprotectants to survive cold exposure, occurs in several species, including the dipteran *Drosophila melanogaster* Meigen. In this study, we show that winter and summer-morphs of the invasive fruit fly *Drosophila suzukii* Matsumura (commonly known as spotted wing drosophila) are able to rapidly cold-harden when they are exposed to a short-term acclimation regime. Adult winter- and summer-morphs received a short-term acclimation by cooling individuals at 1°C/minute to 5°C or 0°C and holding them for two hours to induce rapid cold-hardening. Afterwards, adult cold tolerance was measured by determining survival of adults exposed to (i) -5°C or -10°C for 2 h, and (ii) -10°C, -15°C, -20°C, or -25°C for one second. Non-acclimated adult winter and summer-morphs also underwent cold exposure assays. Survival after cold exposure for *D. suzukii* was compared with *D. melanogaster*. We determined that *D. suzukii* is able to rapidly cold-harden, although there was an original lack of response when winter-morphs were introduced to the same temperature as summer-morphs. Lowering the acclimation and assay exposure temperatures winter-morphs experienced induced rapid cold-hardening, and winter-morph females were the most cold tolerant life stage. These results emphasize the importance of including the potential to acclimate when assessing the effects of cold on this invasive species.

Introduction

The ability of poikilothermic animals such as insects to survive cold temperatures has been studied with increasing interest to better characterize the effects of warming temperatures due to global climate change (e.g., Bale et al. 2010, Danks et al. 1996, Sinclair et al. 2003). Warming temperatures may allow for poleward range expansions of insects that would otherwise be unable to survive in cold regions. The cold limits that an insect can survive are dictated, in part, by the extent to which individuals may acclimate to extreme temperatures.

Research on the ability of insects to acclimate to cold, or cold-harden, focuses primarily on seasonal cold-hardening and rapid cold-hardening (reviewed in Teets and Denlinger 2013). Teets and Denlinger (2013) define seasonal cold-hardening as acclimation to cold that occurs over days or weeks in response to seasonal shifts in temperature and photoperiod. In contrast, rapid cold-hardening (i.e., the ability of an insect to quickly accumulate cryoprotectants to increase cold tolerance) can occur in as little as an hour (Teets and Denlinger 2013). Rapid cold-hardening has been studied in several insect species, including *Drosophila melanogaster* Meigen and *Sarcophaga crassipalpis* Macquart (e.g., Colinet et al. 2012, Czajka and Lee 1990, Chen et al. 1987, Lee et al. 1987). Adult *D. melanogaster* do not normally survive exposure to -5°C for 2 h, but if adults are exposed first to 5°C for 2 h, survival at -5°C approaches 100% (Czajka and Lee 1990).

Drosophila suzukii Matsumura, spotted wing drosophila, is an invasive fruit fly believed to be native to Southeast Asia. This species was first discovered in the United

States in 2008, and has since been found in most U.S. states and British Columbia (NAPIS 2014). *Drosophila suzukii* is of particular concern to small fruit and berry producers because the adult female oviposits into ripening fruit, where larvae develop and cause extensive damage (e.g., Kanzawa 1939, Walsh et al. 2011).

Drosophila suzukii is believed to overwinter as a seasonally cold-hardened adult winter-morph (Kanzawa 1939). Winter-morphs are larger, darker, and have longer wings than *D. suzukii* summer-morphs (Peter Shearer, personal communication; Kanzawa 1939), and most likely overwinter underneath leaf litter or bark (Kanzawa 1939). Field studies have demonstrated that winter-morph females are the first to emerge in the spring, suggesting that females are more cold tolerant than males (Kaur et al. 2013). Previous cold tolerance research on *D. suzukii* has shown that the summer-morph is unable to survive brief exposure to -15°C , but 50% of winter-morphs survive the same exposure (see chapter 2). Winter-morphs have been discovered in the field in late autumn and early winter and can be reared in a laboratory by mimicking these temperature and photoperiod conditions (P.W. Shearer, personal communication). Similar larger and darker morphs have been recorded in other species of *Drosophila*, including the closely related *D. melanogaster*, although it is unknown whether these morphs are the overwintering life stage (e.g. Ayrinhac et al. 2004, Gibert et al. 2007, Rajpurohit et al. 2008).

The objective of this study was to determine if summer-morph and/or winter-morph *D. suzukii* are able to rapidly cold-harden. Adult winter and summer-morphs underwent a short-term acclimation to induce rapid cold-hardening and were used in two cold tolerance assays. To determine if the short-term acclimation regime induced rapid

cold-hardening, the regime was first tested on a closely related species known to rapidly cold-harden, *D. melanogaster*. The ability of *D. melanogaster* and *D. suzukii* to rapidly cold-harden was tested by comparing the survivorship of adults which underwent a 2 h acclimation prior to a cold tolerance assay to adults which did not. The first cold tolerance assay compared acclimated and non-acclimated adult survivorship after a two hour sub-zero treatment, while the second assay compared acclimated and non-acclimated adult survivorship after one second of exposure to one of four sub-zero temperatures. The two separate assays were performed to compare the effects of rapid cold-hardening on both a short-term cold exposure and a longer cold exposure. We hypothesized that *D. suzukii* winter-morph females that had undergone the short-term acclimation regime would be more cold tolerant than *D. suzukii* winter-morph males that had undergone the same treatment, and both males and females would be more cold tolerant than those individuals that had not undergone short-term acclimation. In addition, we hypothesized that both summer-morph *D. suzukii* and *D. melanogaster* which had undergone the short-term acclimation would be more cold tolerant than adults that had not undergone the treatment.

Materials and Methods

Colony Sources.

A colony of *Drosophila suzukii* was established in St. Paul, MN in the fall of 2013 with 250 – 500 adults from a colony at Michigan State University. The colony at Michigan State University had been established from adult *D. suzukii* found in blueberry (*Vaccinium* spp.) fields in Allegan County, Michigan in the summer of 2013 (Steve

VanTimmeren, personal communication). Adults were kept in small vials stoppered with a foam cap (Genesee Scientific, San Diego, CA). The colony in St. Paul, MN, was maintained in vials containing a sugar-agar-cornmeal-yeast diet and transferred every two weeks, as described in protocols from Dalton et al. (2011). *Drosophila suzukii* were reared at $24 \pm 2^\circ\text{C}$ with a photoperiod of 14:10 (L:D) h. These rearing conditions produced summer-morph adults. Generation time at this temperature was approximately 2 weeks, and all summer-morph adults for experiments were used within 48 h of eclosion, or between 17 and 33 degree days old, where 7.2°C was considered the developmental base temperature (Tochen et al. 2014).

Oregon-R strain of *D. melanogaster* was obtained from the University of Minnesota in Minneapolis, MN in June, 2014. *Drosophila melanogaster* were kept in vials stoppered with a foam plug and reared on the same artificial sugar-agar-cornmeal-yeast diet as was used for *D. suzukii* (Dalton et al. 2011). *Drosophila melanogaster* were reared at room temperature ($24 \pm 2^\circ\text{C}$) on a benchtop in St. Paul, MN, in a separate building from *D. suzukii*. Great care was taken to prevent cross contamination with *D. suzukii* because *D. melanogaster* has contaminated and outcompeted cultures of *D. suzukii* (M.K. Asplen, personal communication). Adults ≤ 48 hours old were used for all experiments or under 24 degree days old, where 12°C was considered the developmental base temperature (Ashburner and Thompson 1978).

Adult winter-morph *D. suzukii* were produced by placing mated summer-morph females into diet vials and allowing them to oviposit for 2 days at $24^\circ\text{C} \pm 2^\circ\text{C}$. The diet with eggs was placed into a programmable growth chamber (Percival Scientific Inc.,

Perry, IA) maintained at $10^{\circ}\text{C} \pm 2^{\circ}\text{C}$ with a photoperiod of 12:12 (L:D) h, based on a protocol modified from P.W. Shearer (personal communication). The low temperature slowed development, and winter-morphs did not eclose until 1 - 2 months after eggs were laid. All winter-morph adults for experiments were used within 120 h, or under 15 degree days after eclosion.

Individual *D. suzukii* adults were anesthetized with CO_2 using a FlyPad (Genesee Scientific, San Diego, CA), while *D. melanogaster* were anesthetized by chilling adults for < 5 min on a tray in an ice bath. Time on ice was minimized to prevent the induction of a rapid cold-hardening response. *Drosophila suzukii* and *D. melanogaster* were maintained in separate buildings with different equipment to prevent cross-contamination of colonies. Individual flies of both species were placed into separate 1.5 ml microcentrifuge tubes.

Short-term Acclimation Conditions.

In all experiments, adults were held in individual microcentrifuge tubes with a custom built thermocouple made of 0.127 mm diameter copper and constantan wires. The wires were threaded inside of a milled plastic dowel fitted with a rubber O-ring which sealed the microcentrifuge tube and prevented the fly from escaping (Fig. 2.1). Temperature was measured where the wires crossed, near the tip of the microcentrifuge tube, and near or touching the adult. All adults were cooled in a ThermoScientific A40 Arctic Series Refrigerated/Heated Bath Circulator containing SIL-180 silicon oil (Thermo Fischer Scientific, Newington, NH). Thermocouples were connected to a computer using a data acquisition unit (USB-TC, Measurement Computing Corporation,

Norton, MA), and temperature was recorded once per second using TracerDAQ Pro (Measurement Computing Corporation, Norton, MA).

We attempted to induce a rapid cold-hardening response by exposing adults to a short-term cold acclimation regime. We began with *D. melanogaster*, a species previously proven to rapidly cold-harden (e.g., Czajka and Lee 1990, Kelty and Lee 1998, Kelty and Lee 2001). *Drosophila melanogaster* adults were cooled from 24°C to 5°C at 1°C/minute and held at 5°C for 2 h before undergoing cold tolerance assays. This method follows previous studies that induced rapid cold-hardening in *D. melanogaster* (Czajka and Lee 1990); however, it includes a cooling rate of 1°C/minute to the target temperature instead of instantaneous exposure. Summer-morph and winter-morph *D. suzukii* underwent the same regime as *D. melanogaster*. When all winter-morph *D. suzukii* survived initial cold tolerance assays, whether they experienced the short-term acclimation regime or not, we adjusted the short-term acclimation regime by cooling winter-morphs at a rate of 1°C/minute to 0°C and holding them at 0°C for 2 h before beginning cold-tolerance assays.

Cold Exposure Assays.

Two-hour, -5°C exposure assay.

To determine if each species and morph tested (*D. melanogaster*, *D. suzukii* winter-morphs and *D. suzukii* summer-morphs) could rapidly cold-harden, three separate experiments were conducted. Each experiment compared survivorship of individuals (of a single species or morph) that underwent a 2-h acclimation and those that did not. Within each experiment, adults that underwent short-term acclimation were tested in a

separate run from those that did not. A run contained 15-25 adults of one species or morph which were cooled at the same time. In all experiments, two runs, one with short-term acclimation and one without short-term acclimation, were conducted each day and comprised a block. The order of the acclimation treatments within a block was randomized. The group that was being tested (i.e., *D. melanogaster*, *D. suzukii* winter-morphs or *D. suzukii* summer-morphs) was not randomized. After we demonstrated that the modified approach to short-term acclimation induced rapid cold-hardening, the acclimation procedure was tested in summer-morph *D. suzukii*, followed by winter-morph *D. suzukii*. Morph and species were not compared.

Drosophila melanogaster.

This experiment involved three blocks. Three runs, each with 16-17 adult *D. melanogaster*, underwent a short-term acclimation regime for a total of 49 flies. After short-term acclimation, *D. melanogaster* were further cooled at a rate of 1°C/minute to -5°C and held for 2 h. Adults were removed and placed in individual 1.5mL microcentrifuge tubes with 0.5mL diet and a ventilation hole (as per chapter 2). To compare survivorship of flies which had undergone acclimation to those which had not, another three runs of 16-17 adult *D. melanogaster* (for a total of 51 flies) were cooled directly from room temperature (24°C ± 2°C) to -5°C and held for 2 h. Adult *D. melanogaster* were returned to room temperature and treated the same as those that had undergone short-term acclimation. After 24 h, survival was determined as the ability of the adult to upright itself, a measure used in previous studies of *Drosophila* (e.g., Kelty

and Lee 1999, Dalton et al 2011). Sex was not determined, as Czajka and Lee (1990) did not see a significant effect.

Drosophila suzukii summer-morphs.

This experiment involved three blocks. Three runs consisting of 16-17 adult summer-morph *D. suzukii* underwent a short-term acclimation regime, for a total of 52 flies. After short-term acclimation, summer-morphs were further cooled from 5°C to -5°C at 1°C/minute and held for 2 h. Summer-morphs were removed and treated as *D. melanogaster* had been. Another three runs of 16-17 adult summer-morph *D. suzukii* (for a total of 48 flies) were cooled directly from room temperature (~24°C ± 2°C) to -5°C and held for 2 h. These adults were returned to room temperature and treated the same as those that had undergone short-term acclimation. After 24 h, mortality and sex were determined. Mortality was determined as in *D. melanogaster*. Sex was determined at the same time by the presence of a spot on the wings (males) or a large ovipositor (Hauser 2011).

Drosophila suzukii winter-morphs.

The first experiment involved two blocks. Two runs of 18-20 adult winter-morph *D. suzukii* underwent a short-term acclimation regime, for a total of 38 flies. After short-term acclimation, winter-morphs were further cooled at 1°C/minute to -5°C and held there for 2 h. Winter-morphs were removed and treated the same as *D. melanogaster* and *D. suzukii* summer-morphs. Survivorship of winter-morphs which had undergone a short-term acclimation was compared to another two runs of 18-20 adult winter-morph *D. suzukii* (for a total of 40 flies) which were cooled directly from room temperature (24°C

$\pm 2^{\circ}\text{C}$) to -5°C and held there for 2 h. Winter-morph *D. suzukii* were returned to room temperature and treated the same as those who underwent short-term acclimation. After 24 h, mortality and sex were determined as previously described. Sex characteristics were no different for winter-morphs. All winter-morphs survived the assay temperature of -5°C regardless of treatment, so a second assay temperature was introduced.

The second experiment involved two blocks. Two runs consisting of 24-25 winter-morph *D. suzukii* underwent a short-term acclimation regime at 0°C , for a total of 49 flies. After short-term acclimation, winter-morphs were further cooled at a rate of $1^{\circ}\text{C}/\text{minute}$ to -10°C and held there for 2 h. Winter-morphs were removed and treated the same as *D. melanogaster* and *D. suzukii* summer-morphs. Survivorship of winter-morphs acclimated to 0°C were compared to winter-morphs cooled directly to -10°C . Another two runs of 24-25 adult winter-morph *D. suzukii* (for a total of 49 flies) were cooled directly from room temperature ($24^{\circ}\text{C} \pm 2^{\circ}\text{C}$) to -10°C and held there for 2 h. Winter-morph *D. suzukii* were returned to room temperature and treated the same as those who underwent short-term acclimation. After 24 h, sex and mortality were determined as previously described.

All statistical analyses were conducted in R 3.0 (R Core Team, Vienna, Austria) and Excel (Microsoft, Redmond, WA). In each of the four experiments (*D. melanogaster*, *D. suzukii* summer-morphs, and *D. suzukii* winter-morphs acclimated at 0°C and 5°C), individual ANOVAs were conducted to compare the effects of short-term acclimation and sex on survival. Summer-morphs, winter-morphs and *D. melanogaster* were not statistically compared to each other.

Lower Lethal Temperature Assay.

The purpose of these studies was to measure the effects of a short-term acclimation at -5°C or 0°C on survivorship of summer and winter-morphs of *D. sukikii* after a brief exposure to one of several sub-zero temperatures. Limitations on the timing and availability of adults and equipment required that each combination of acclimation and morph be tested at a different time. Methods and results for non-acclimated summer and winter-morphs were previously reported (see chapter 2). We follow those procedures with modifications noted below.

For summer-morphs, short-term acclimation was achieved by chilling individuals from $24 \pm 2^{\circ}\text{C}$ to 5°C at $1^{\circ}\text{C}/\text{minute}$ and holding them at 5°C for 2 h. Individuals were cooled further at $1^{\circ}\text{C}/\text{minute}$ to one of four randomly-assigned assay temperatures: -10, -15, -20, or -25°C . Exposure to the target temperature lasted approximately one second, after which the individual was immediately returned to room temperature, placed in a ventilated micro-centrifuge tube with diet, and allowed to recover for 24 h. Four to eight adults were tested per assay temperature per run. There were 7 runs of this test, which involved a total of 20 to 30 summer-morphs per each temperature.

For winter-morphs, short-term acclimation was achieved by chilling individuals from $24 \pm 2^{\circ}\text{C}$ to 0°C at a rate of $1^{\circ}\text{C}/\text{minute}$ and holding them at 0°C for 2 h. Individuals were cooled further at a rate of $1^{\circ}\text{C}/\text{minute}$ to one of four randomly assigned assay temperatures: -10, -15, -20, or -25°C . Exposure to the target temperature lasted for approximately one second before individuals were returned to room temperature and

placed in a ventilated microcentrifuge tube with diet and allowed to recover. Four to eight adults were tested per assay temperature per run.

Abbot's correction ($P' = P - P_0 \frac{100-P}{100-P_0}$; Healy 1952), was applied to remove mortality that could not be attributed to cold exposure, where P' is the percentage of the population that died due to treatment, P is the percentage of the treatment group that died, and P_0 is the percentage of adults held at room temperature that died for each morph.

Because sex did not have an effect on survival to lower lethal temperature exposures ($F = 0.40$, $df = 1$, 88 , $P = 0.53$), results for both sexes were pooled for analysis. Data were analyzed by using a binomial generalized lineal model (R command: `glm(Response Variable ~ Explanatory Variables, family=binomial)`), where survival was the response variable, and treatment temperature (-10, -15, -20, or -25°C) and the presence/absence of the short-term acclimation regime were the explanatory variables. Summer and winter-morphs were analyzed separately.

Results

Two Hour Cold Exposure Assay.

The short-term acclimation had a significant effect on survival after the 2 h assay for *D. melanogaster* and *D. suzukii* (Fig. 2). Survival of *D. melanogaster* without short-term acclimation was $14.3 \pm 4.9\%$ (\pm SE), but was significantly greater ($78.4 \pm 5.8\%$) for *D. melanogaster* that underwent the short-term acclimation ($F = 34.70$, $df = 1$, 93 , $P < 0.00001$; Fig. 3.1).

A significantly greater proportion of summer-morph *D. suzukii* survived a 2-h exposure to -5°C if they had received a short-term acclimation ($F = 49.86$, $df = 1$, 88 , $P <$

0.00001; Fig. 3.1); only $30.6 \pm 6.7\%$ of non-acclimated summer-morphs survived while $84.3 \pm 5.1\%$ of acclimated summer-morphs survived (Fig. 3.1). The effect of acclimation was not consistent among blocks (i.e., the interaction between block and short-term acclimation was significant; $F = 7.08$, $df = 2$, 88 , $P = 0.001$) because the non-acclimated summer-morphs in one block had a substantially higher survival after the 2 h cold shock.

Short-term acclimation at 5°C did not significantly increase survival of winter-morph *D. sukikii*, and adults survived exposure to -5°C ($F = 0.1617$, $df = 1$, 72 , $P = 0.68$; Fig. 3.1). However, after short-term acclimation at 0°C , winter-morph survival to 2 h of exposure to -10°C was affected by sex ($F = 13.06$, $df = 1$, 71 , $P < 0.001$; Fig. 3.1) and acclimation treatment ($F = 16.53$, $df = 1$, 71 , $P < 0.001$; Fig. 3.1). Females had greater survivorship than males (Fig. 3.1). A greater percentage of winter-morphs survived exposure to -10°C if individuals had a short-term acclimation at 0°C than if they did not (Fig. 3.1). Winter-morph males cooled from room temperature to -10°C had a survival rate of $20.0 \pm 0.8\%$, but when pre-treated with a short-term acclimation, $66.7 \pm 1.1\%$ survived (Fig. 3.1). Only $63.2 \pm 1.2\%$ of winter-morph females that had not been acclimated survived, but when treated with a short-term acclimation at 0°C , $89.5 \pm 0.5\%$ survived (Fig. 3.1). There was a significant interaction effect of the short-term acclimation and block ($F = 11.62$, $df = 1$, 71 , $P = 0.001$), demonstrating natural variation in the population's ability to acclimate.

Lower Lethal Temperature Assay.

Lower lethal temperature survival was significantly greater among summer-morphs of *D. sukikii* that received a short-term acclimation to 5°C than summer-morphs

that did not (Fig. 3.2). The slopes of rapidly cold-hardened summer-morphs and control summer-morphs were not significantly different ($z = -0.81$, $P = 0.42$, Fig. 3.2), but the intercepts were significantly different ($z = -1.96$, $P < 0.05$; Fig. 3.2). Rapid cold-hardening appears to affect the temperature summer-morphs began to succumb to chill injury, but not the rate at which they succumb to cold. Short-term acclimation did not significantly affect winter-morph survival after one second cold exposures (Fig. 3.2). Neither the slope nor the intercept were significantly different ($z = -0.51$, $P = 0.60$, $z = -0.81$, $P = 0.41$).

Discussion

The short-term acclimation assay successfully induced rapid cold-hardening in *D. melanogaster*, and our results confirm those in Czajka and Lee (1990). However, while Czajka and Lee reported that all non-acclimated *D. melanogaster* died after 2 h at -5°C , we found that approximately 14% of non-acclimated adults survived. A slower cooling rate of $1^{\circ}\text{C}/\text{minute}$ may have increased survival by allowing *D. melanogaster* to accumulate a small amount of cryoprotectants while cooling. Adults that underwent a short-term acclimation regime in Czajka and Lee (1990) had between 71 and 100% survival, and in our study, survival averaged 78%. This result suggests that short-term acclimation and a slower cooling rate do not have a cumulative effect on survival. The significant increase in survivorship when adults undergo a short-term acclimation confirms the rapid cold-hardening phenomenon occurs with a more gradual cooling rate (Fig. 3.1).

Drosophila suzukii summer-morph survival increased from 31% to 84% after 2 h at -5°C if they were first exposed to a short-term acclimation treatment of 2 h at 5°C (Fig. 3.1). This result suggests that summer-morphs have the ability to rapidly cold-harden. Both male and female summer-morphs have the ability to acclimate to cold temperatures, although they are not as cold tolerant as winter-morphs. The interaction effect between block and the effect of the short-term acclimation on survival implies that some natural variability exists in the ability of the *D. suzukii* summer-morph population to rapidly cold-harden and survive cold exposure.

The increased cold tolerance of summer-morph *D. suzukii* exposed to a short-term acclimation before the lower lethal temperature assay also demonstrates that the non-overwintering stage is still able to adjust physiologically to cooling conditions. This ability in summer-morphs could be important in seasons/climates which have abrupt shifts in weather patterns. Summer-morphs could be exposed to cold temperatures late in autumn, for example. In areas where it does not get cold enough for winter-morphs to develop naturally, summer-morphs may be able to survive some cold shocks in a mild winter.

The lack of a significant effect of a short-term acclimation in winter-morph *D. suzukii* that underwent short-term acclimation at 5°C prior to the 2 h assay at -5°C can be attributed to the increased cold hardiness of winter-morphs. Winter-morphs can survive a 2 h assay at 5°C without short-term acclimation because they are seasonally acclimated. Winter-morph *D. suzukii* survival significantly increased when introduced to a 2 h short-term acclimation at 0°C, showing that winter-morphs have the ability to rapidly cold-

harden. Female winter-morphs that underwent the short-term acclimation regime had the highest rate of survival to the two hour cold assay, and males that did not receive the short-term acclimation had the lowest rate of survival, as expected. There may be cumulative rapid cold-hardening effects of seasonal acclimation and short-term acclimation in *D. sukii*. The interaction effect between the short-term acclimation regime and block suggests the ability of winter-morphs to rapid cold-harden may be variable among the winter-morph population.

In the lower lethal temperature assay, there was no significant difference between acclimated and non-acclimated winter-morph *D. sukii*. Rapid cold-hardening increases pre-freeze survival, and winter-morphs may have already reached the limit of their ability to increase pre-freeze survival through seasonal acclimation. While survival does increase in a longer assay (2 h vs one-second in lower lethal temperature), the temperatures experienced in the lower lethal temperature assay are much closer to their supercooling point of -17.5°C (see chapter 2) and a possible limit for survival. Rapid cold-hardening may increase winter-morph survival to a longer term exposure to mild temperatures, but may not increase survival to a brief exposure to more extreme temperatures.

Our results establish that *D. sukii* has a plastic response to cold, similar to other *Drosophila* species. This is one of the first papers to use a rapid cold-hardening response to describe cold tolerance of an invasive species, which can be important for use in climate modeling and range map creation. Including this ecologically relevant and plastic

response to cold in future cold tolerance research is vital to understanding the true cold hardiness of an insect.

While previous research has shown that winter-morph *D. sukii* may not be able to survive winter temperatures in poleward regions (see chapter 2), this chapter demonstrates that winter-morph *D. sukii* could be able to use rapid cold-hardening to increase its ability to survive longer periods of cold temperatures, but not brief cold snaps. The rapid cold-hardening phenomenon may be even more relevant as it relates to *D. sukii* summer-morphs, because rapid cold-hardening increases their survival to both long term and short-term shocks. Summer-morphs must be able to survive some level of cold temperatures in the spring and fall, and if rapid cold-hardening is able to increase that ability, *D. sukii* populations may reach infestation levels earlier than hypothesized.

Figures:

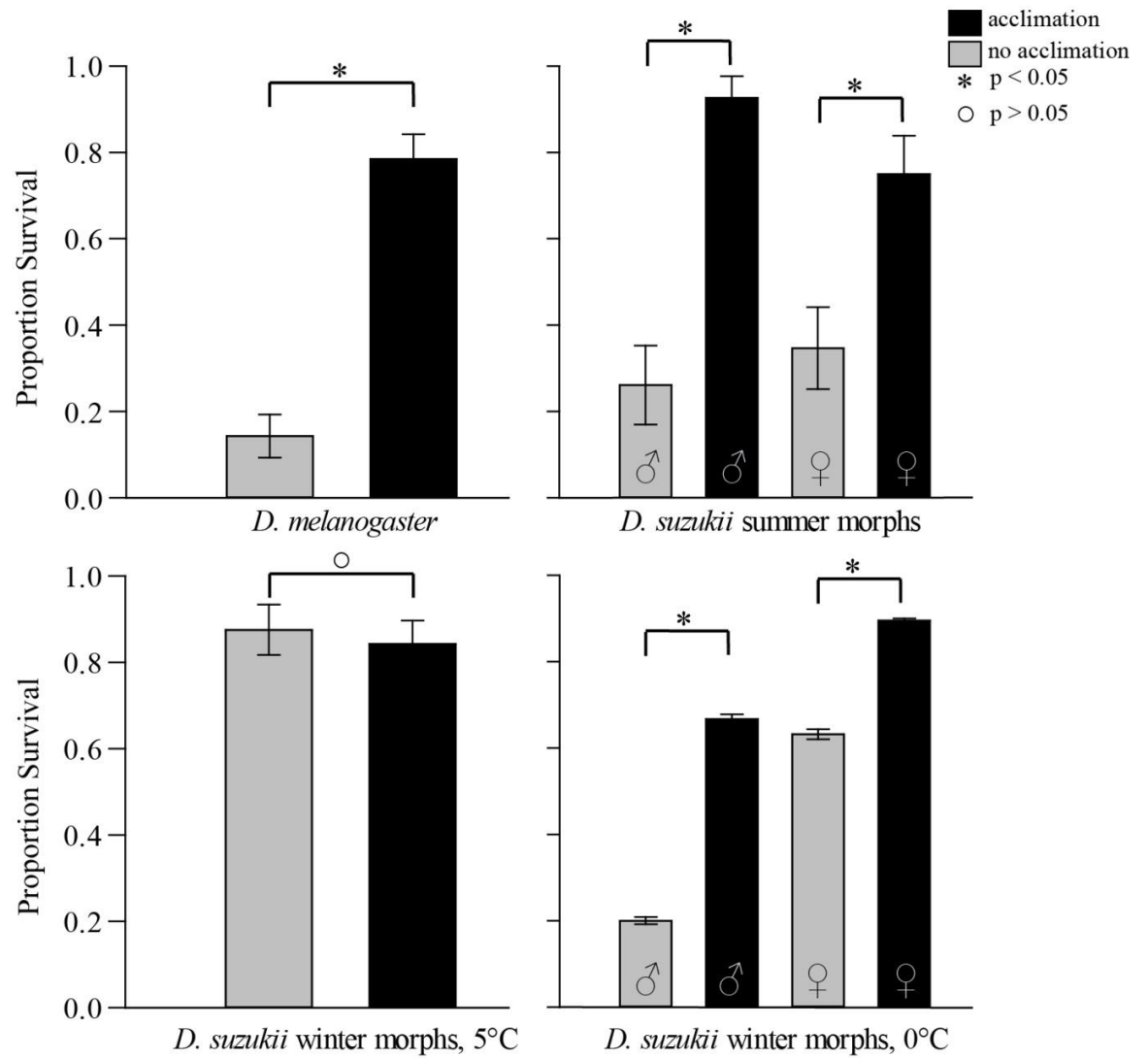


Figure 3.1: Survival of *D. melanogaster*, *D. sukikii* winter and summer-morphs with short-term acclimation (grey), and without short-term acclimation (black). Asterisk indicates significance ($p < 0.05$); open circle indicates no difference ($p > 0.05$).

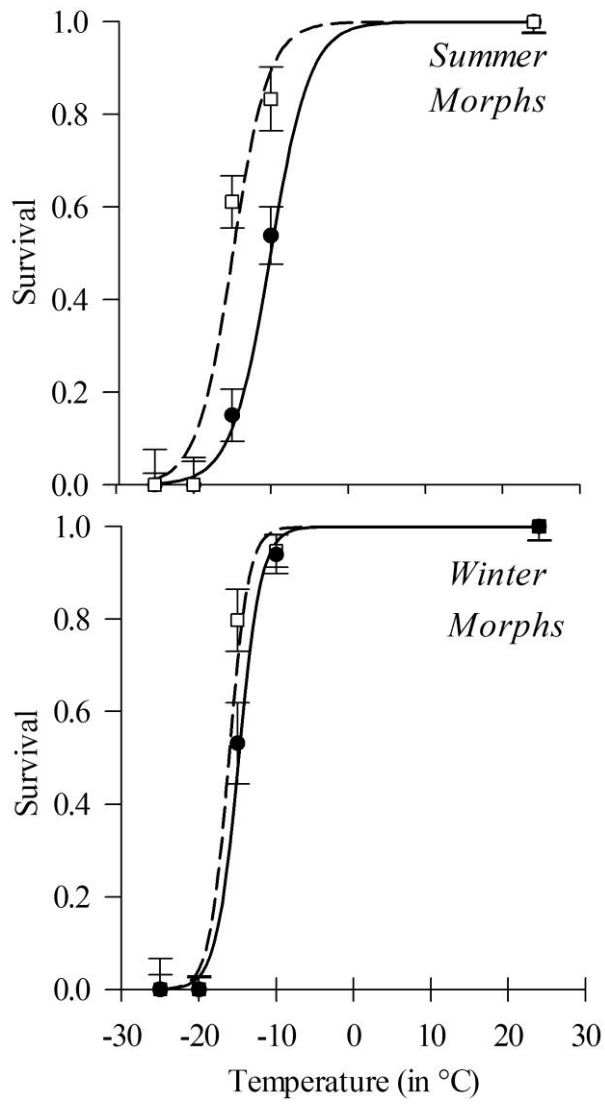


Figure 3.2: Effect of short-term acclimation of lower lethal temperature of summer and winter-morph *D. sukikii*. Dotted lines indicate predicted acclimated curves, straight lines are predicted curves cooled directly to each temperature. White squares are measured survival of unacclimated insects; black circles are measured acclimated survival.

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