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## Low temperature tolerance of adult *Drosophila suzukii* (Diptera: Drosophilidae)

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Biology

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LOW TEMPERATURE TOLERANCE OF ADULT *DROSOPHILA SUZUKII*  
(Diptera: Drosophilidae)

(Thesis format: Monograph)

by

Ruth Jakobs

Graduate Program in Biology

A thesis submitted in partial fulfillment  
of the requirements for the degree of  
Master of Science

The School of Graduate and Postdoctoral Studies  
The University of Western Ontario  
London, Ontario, Canada

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# Abstract

The crop pest *Drosophila suzukii*, native to Southeast Asia, has been found in Ontario since 2010. This raises concern with respect to it establishing permanent populations, however, little is known about its cold tolerance. I investigated the low-temperature tolerance, including phenotypic plasticity, of *D. suzukii*. While acclimation increased cold tolerance, there was no evidence of short-term cold-hardening. Chill coma occurs at -1.2 °C, which will limit winter activity. Cold shock decreased the reproductive output of females, but this negative effect may be mitigated by re-mating. *Drosophila suzukii* is chill-susceptible and 80 % of the flies die after exposure to -7.5 °C (females) and -7 °C (males). Even acclimated flies could not survive at 0 °C for more than seven days, or in overwintering field cages. These results suggest that *D. suzukii* might not be able to survive winter conditions in the field in Ontario.

## Keywords

*Drosophila suzukii*, spotted wing drosophila, overwintering, cold tolerance, phenotypic plasticity

## **Co-Authorship Statement**

This work was conducted under the supervision of Dr. Brent J. Sinclair and Dr. Tara D. Gariepy. All the aspects of sampling design and analysis were planned in cooperation with Drs. Sinclair and Gariepy, and any publications subsequent to the completion of this work will be co-authored with them.

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## List of Abbreviations

CA	Constant acclimation
FA	Fluctuating acclimation
AFT	Accelerated-failure-time model
CCR	Chill coma recovery time
CCR <sub>80</sub>	Time at which 80 % of the flies recovered from chill coma
CT <sub>min</sub>	Critical thermal minimum
CT <sub>min80</sub>	Temperature at which 80 % of the flies enter chill coma
Lt <sub>80</sub>	Lethal time at which 80 % die
LT <sub>80-1h</sub>	Lower temperature at which 80 % die after 1 h exposure
LT <sub>90-2h</sub>	Lower temperature at which 90 % die after 2 h exposure
RCH	Rapid cold-hardening
SCP	Supercooling point

# 1 Introduction

Low temperatures impair development and performance, and may cause mortality of insects and thus can be a major determinant of drive population dynamics and geographic ranges, particularly in temperate regions (Sinclair et al., 2003a). Invasive species have been introduced to a new habitat out of their normal geographic range and therefore may encounter new climates. Thus understanding overwintering biology is important to predict if an invasive species can establish in an area that experiences low temperatures (Bale and Hayward, 2010; Williams et al., in press a). *Drosophila suzukii* Matsumura (Diptera: Drosophilidae) is native to Southeast Asia and has been found in Ontario, Canada since 2010 (Fraser et al., 2011). This pest species has the potential to cause severe economic damage to fruit crops, because it has a wide host range and fast generation time (Kanzawa, 1939). However as its potential to overwinter in Ontario's cold winter climate remains unclear, I assessed the tolerance of *D. suzukii* to low temperature stress.

## 1.1 Low temperatures limit northern range distribution

Abiotic factors, such as temperature, are thought to limit the poleward distributions of species (Gaston, 2009). As insects are small ectotherms and their body temperature is mainly determined by the environmental temperature, low temperatures not only determine survival but also population growth (Boggs and Inouye, 2012). Thus, winter influences individual fitness and range distribution (Addo-Bediako et al., 2000), so species living at higher latitudes generally show increased physiological tolerance to low temperatures, than those closer to the equator. For example the swallowtails, *Papilio canadensis* (Rotschild & Jordan) and *P. glaucus* L. (Lepidoptera: Papilionidae), (Kukal et al., 1991; Williams et al., in press b) and southern pine beetle, *Dendroctonus frontalis* Zimmermann (Coleoptera: Scolytidae), are limited in their northern range distribution by poor survival at low temperature (Ungerer et al., 1999). Winter temperatures also determine the ability of invasive pest species to establish (Bale, 2002). The emerald ash borer, *Agrilus planipennis* Fairmaire (Coleoptera: Buprestidae), is invasive to Canada and was able to establish as a pest species due to its cold tolerance (Crosthwaite et al., 2011).

## 1.2 Insects at low temperatures

An insect's body temperature declines with the environmental temperature, and after passing a lower thermal threshold, insects might enter chill coma, a state of paralysis (Hazell and Bale, 2011; MacMillan and Sinclair, 2011). The critical thermal minimum ( $CT_{min}$ ) describes the chill coma onset temperature and depending on the duration of cold exposure, chill coma is a reversible state (MacMillan & Sinclair 2011). Chill coma recovery time (CCR) quantifies recovery as the time it takes for an insect to recover from standardized chill coma inducing conditions (David et al., 1998). Both  $CT_{min}$  and CCR are commonly used measures to determine low-temperature performance (Andersen et al., 2014; David et al. 1998; Gibert et al. 2001; Hoffmann et al., 2003 b). The  $CT_{min}$  and CRR are lower in temperate *Drosophila* species than in tropical ones (Gibert and Huey, 2001; Gibert et al., 2001).

### 1.2.1 Cold tolerance strategy

Insects show different abilities to survive sub-zero temperatures and their cold tolerance strategies are divided into freeze tolerance and freeze avoidance, depending on the ability to survive internal ice-formation. The temperature at which internal ice formation occurs is called the supercooling point (SCP) (Lee and Denlinger, 1991). However most insects are chill-susceptible and die of injuries unrelated to freezing (Lee and Denlinger, 1991)

Freeze-tolerant species, such as the woolly bear caterpillar, *Pyrrharctia isabella* (Smith) (Lepidoptera: Arctiidae), (Marshall and Sinclair, 2012a), survive freezing by inducing ice-nucleation in the extracellular spaces (Lee, 2010; Sinclair, 1999). Freeze-avoiding insects, such as the emerald ash borer, survive low temperatures by maintaining their body fluids in a liquid state, but die upon freezing (Lee and Denlinger, 1991). Most insect species are chill-susceptible and die of chilling injuries (Bale, 1996). For example, adults of *Drosophila melanogaster* Meigen (Diptera: Drosophilidae) are chill-susceptible, because only 50 % survive an exposure to -5 °C for two hours, even though they remain unfrozen until -20 °C (Czajka and Lee, 1990). The temperature at which *Drosophila* die varies between species depending on their geographical range. While 50 % of *D. montana* die after exposure to -13.2 °C for 2 h, 50 % of *D. birchii* die at -3.3 °C (Andersen et al. 2014).

The majority of insect species switch strategies between seasons and display the same strategy every winter (Zachariassen, 1985); however there are species that switch strategies between years like *Dendroides canadensis* Latreille (Coleoptera: Pyrochroidae) (Horwath and Duman, 1984).

It is likely that chill susceptibility is the ancestral state for insects, and that freeze avoidance and freeze tolerance are derived state, which have evolved within various insect taxa multiple times (Sinclair et al., 2003b; Strachan et al., 2011). Freeze-tolerant and freeze-avoiding species may overwinter in the same habitat and share biochemical components; thus it remains unclear why one strategy seems favourable over the other in temperate regions. There are several different hypotheses that address the question of why a particular species exhibits a certain cold tolerance strategy. According to an overwintering energetics model, freeze avoidance is advantageous when pre-winter energy stores are high and supercooling costs are low, whereas freeze tolerance is favourable when energy stores and the costs of freeze-thaw cycles are low (Voituron et al., 2002). The temperature in the Southern Hemisphere is less predictable than the Northern Hemisphere and shows a high variance in temperature around 0 °C, which results in repeated freeze-thaw cycles and could explain the higher abundance of freeze-tolerant species in the Southern Hemisphere (Sinclair and Chown, 2005). Freeze tolerance is also an advantage in microhabitats where external ice nucleation precludes freeze avoidance (Sinclair et al., 2003b).

### **1.2.2 Injuries related to low temperature exposure**

Chill-susceptible insects can accumulate chilling injuries, which can result in malfunction of development, reduced fitness and mortality (Shreve and Lee, 2004; Turnock, 1993; Turnock et al., 1983). Chilling injuries can be divided into acute chilling injuries, which occur on a short time scale (< 6 h, acute cold exposure) and chronic chilling injuries that accumulate over a longer period of time (> 6 h, chronic cold exposure) (Lee, 2010; Nedvěd et al., 1998; Rajamohan and Sinclair, 2008). The intensity of chilling injury increases with longer exposure time and/or lower temperatures (Nedvěd et al., 1998). Chilling injuries can result in mortality (Turnock et al., 1983), as well as reduce courtship and mating activity (Shreve and Lee, 2004) and fecundity (Marshall and Sinclair, 2010).

Heat shock proteins are upregulated after chilling in *Pyrrhocoris apterus* L. (Hemiptera: Pyrrhocoridae), suggesting that protein damage during or after chilling (Košťál and Tollarová-Borovanská, 2009). However studies with *D. melanogaster* only reveal small changes in heat shock protein expression (Burton et al., 1988; Sinclair et al., 2007; Zhang et al., 2011), indicating protein damage after low temperature exposure does not occur in all species. Direct chilling injuries are thought to be associated with apoptosis in the flight muscles of *D. melanogaster* (Yi et al., 2007) and apoptosis in the gut, Malpighian tubules and the fat body in the flesh fly *Sarcophaga crassipalpis* Macquart (Diptera: Sarcophagidae) (Yi and Lee, 2004). Membrane phase-transitions (Ramløv, 2000), oxidative stress (Rojas and Leopold, 1996), and loss of ion and water balance all cause chronic chilling injuries (Košťál et al., 2006). Chilling injuries can decrease neuromuscular function which negatively affects coordination in *S. crassipalpis*, causing a failure of eclosion (Yocum et al., 1994). Thus, chilling injuries are problematic at many different levels of the organism, which can affect the individual and may drive population dynamics.

The formation of ice crystals can mechanically damage membranes and proteins (Storey and Storey, 1988). In addition, cellular water leaves the cell due to osmotic dehydration and can join the growing ice lattice, causing a decrease of cell volume and an increase in the intra- and extracellular solute concentration (Lee, 2010). The loss of cell volume causes membrane damage (Lee and Denlinger, 1991), and the increase of solute concentration denatures proteins resulting in an impairment of the metabolism in both freeze-avoiding and freeze-tolerant insects (Lee, 2010). In addition, freezing can damage the brain, muscle and Malpighian tubules (Collins et al., 1997) as well as cause hypoxia (Joanisse and Storey, 1996). During thawing, reactive oxygen species can damage tissues (Zachariassen, 1985). Additionally, the lack of reestablishment of ion homeostasis can be lethal during thawing (Boardman et al. 2011).

### **1.2.3 Phenotypic plasticity of cold hardiness**

Insects have the ability to increase their cold hardiness to adjust to seasonal temperature changes. These plastic responses occur on different time scales: Rapid cold-hardening is

a response to short-term cold exposure, and acclimation is a response to longer-term low-temperature and short day length exposure (Lee and Denlinger, 1991).

In temperate regions, air temperature can fluctuate by more than 20 °C daily (Irwin and Lee, 2003). To contend with rapid and severe decreases in temperature, some insects are able to induce physiological changes on short time scales (30 min - 2 h) that increase cold-hardiness (Lee et al., 1987; Ransberry et al., 2011). This phenomenon, known as rapid cold-hardening (RCH), was first described in *S. crassipalpis* (Lee et al., 1987) and has since been described in a wide range of chill-susceptible, freeze-avoiding and freeze-tolerant species (Teets and Denlinger, 2013). Rapid cold-hardening can increase survival at low temperatures (Czajka and Lee, 1990; Lee et al., 1987) and decrease CT<sub>min</sub> and CCR (Ransberry et al. 2011). Rapid cold-hardening also improves courtship and mating behaviour at low temperatures (Shreve and Lee, 2004), and increases reproductive output after cold shock (Overgaard et al., 2007).

Seasonal changes in photoperiod and/or temperature can induce acclimatization (in the field), which is mimicked by acclimation in the lab (Teets and Denlinger, 2013). While many studies use constant conditions for acclimation, others use ecologically-based thermoperiods to simulate natural conditions (Colinet et al., in press; Kelty and Lee, 2001). Cold acclimation increases survival after cold exposure (Sinclair and Roberts, 2005) and decreases CT<sub>min</sub> and CCR (Ransberry et al., 2011) allowing insects to remain active at lower temperatures. Insects might also suppress their metabolic rate when overwintering to conserve energy reserves (Williams et al., 2012b). Changes in the lipid membrane composition increase fluidity of the membrane at low temperatures (Pruitt and Lu, 2014). Many insects accumulate biochemicals to increase cold tolerance; e.g. freeze-avoiding species accumulate anti-freeze proteins, polyols and sugars to lower the SCP (Han and Baue, 1995; Zachariassen, 1985). However the accumulation of these cryoprotectants can be energetically-costly (Storey, 1997).

Rapid cold-hardening and acclimation both increase cold tolerance; however these two phenomena appear to be driven by different mechanisms, as the combination of RCH and acclimation can further increase cold tolerance (Rajamohan and Sinclair, 2008; Shintani and Ishikawa, 2007). Furthermore, RCH enhanced cold tolerance in some



Drosophilidae, while acclimation did not (Strachan et al., 2011). RCH does not appear to carry ecological costs in terms of development, longevity or fecundity (Powell and Bale, 2004).

#### **1.2.4 Diapause**

Diapause and cold-hardening are both important to winter survival, but it is unclear whether they are independent mechanisms or whether acclimation is part of diapause (Denlinger, 1991). Diapause is a state of arrested development that is induced in a specific life stage by stimuli that signal the onset of unfavourable conditions (e.g. short day length and low temperatures prior to winter), and allows survival of stressful seasons (see Danks, 2006; Hahn and Denlinger, 2011 for reviews). After induction of diapause, insects may accumulate energy stores for overwintering and increase their stress tolerance (Hahn and Denlinger, 2011), and after initiation of diapause insects stop their development (Košťál, 2006).

Univoltine insect species have one generation per year, and therefore a particular developmental stage is always associated with overwintering. Some univoltine species overwinter in an obligatory diapause, like the spruce budworm, *Choristoneura fumiferana* Clemens (Lepidoptera: Tortricidae), which overwinters as a second-instar larva (Han and Bauce, 1998). Bivoltine and multivoltine species have more than one generation per year, and therefore environmental cues like photoperiod are important for inducing diapause only in overwintering generations (Bradshaw et al., 2004). The overwintering life stage is usually species-specific (Sinclair et al., 2003a) and thus post-winter life history may vary greatly among species. Insects that overwinter as eggs or larvae can replace consumed energy reserves in the spring, while species that overwinter as pupae or adults may depend on the energy reserves that they consume as larvae to overwinter and reproduce, if the adults do not feed (Sinclair, in press).

Some insect species do not display a full diapause with a decrease in metabolic rate, but only reproductive diapause as adults (Tatar and Yin, 2001). Females show ovarian arrest, which is advantageous for these species, because it increases survival under unfavourable conditions and females only oviposit eggs in periods of favourable conditions that allow survival and development of progeny (Pener, 1992). While males of

some species inseminate females prior to overwintering and die before the winter, like *Culex pipiens* L. (Diptera: Culicidae), other males do not show a reproductive diapause, but are able to overwinter like *Anacridium aegyptium* L. (Caelifera: Acrididae) (Denlinger and Armbruster, 2014; Norris and Richards, 1965; Pener, 1992). In the monarch butterfly, *Danaus plexippus* L. (Lepidoptera: Nymphalidae), both sexes migrate in reproductive diapause (Herman, 1981).

Some *Drosophila* species are thought to overwinter in reproductive diapause with an arrest of ovarian development triggered by photoperiod and/or temperature (Kimura, 1988a). In *D. melanogaster* reproductive diapause is indicated by an absence of yolk deposition in the ovarian follicles (Saunders et al., 1989). Warm-temperate *Drosophila* species (*D. lutescens* and *D. rufa*) and cool-temperate *Drosophila* species (*auraria* complex) show reproductive diapause while tropical and subtropical relatives (*D. melanogaster* and *D. takahashii*) do not. Cool-temperate species enter reproductive diapause earlier than warm-temperate species (Kimura, 1988b). The ability to enter reproductive diapause is not only dependent on the species, but also on latitude. With an increase in latitude and therefore a decrease in temperature and photoperiod, the proportion of diapausing *D. melanogaster* increased in North America (Schmidt et al., 2005; Williams and Sokolowski, 1993). *Drosophila melanogaster* in reproductive diapause have higher energy reserves than non-diapausing individuals (Ohtsu et al., 1992; Ohtsu et al., 1993) and cool-temperate species in reproductive diapause have improved low temperature tolerance compared to warm-temperate species (Kimura, 1988b). Thus, *Drosophila* in reproductive diapause might be better prepared to overwinter in colder regions than non-diapausing *Drosophila*.

### **1.2.5 Overwintering microclimate and microhabitat**

Insects are not only able to undergo physiological changes in the season but also behavioural changes, as they migrate to warmer habitats or select an overwintering microhabitat. Insects overwintering in Ontario use a wide variety of different overwintering habitats: for example the prepupae of the emerald ash borer overwinters beneath tree bark (Wang et al., 2010), the acorn weevil, *Curculio glandium* Marsham (Coleoptera: Curculionidae), borrows 5 cm deep into the soil (Udaka and Sinclair, 2014)

and the woolly bear caterpillar, overwinters beneath the snow cover (Marshall and Sinclair, 2012a).

A good overwintering habitat must allow for easy access in the fall, suitable overwintering conditions and be easy to leave in the spring. While well-buffered microhabitats provide less fluctuation in temperature and warmer temperatures, they might be harder to find and access. In addition, insects overwintering in well-buffered environments (such as beneath snow cover) experience a difference in season length, because the snow has to melt before they can be active (reviewed in Danks, 1991). The well-buffered microhabitat prevents exposure to lethal air temperatures, but the exposure temperature in this overwintering microhabitat not only affects survival, but also fitness, because warm winter temperatures can increase metabolic rate. Increased metabolic rates may then lead to a more rapid decrease of energy stores during the winter and an reduced fecundity in the spring (Irwin and Lee, 2003; Marshall and Sinclair, 2012a). As well, microhabitats not only affect the temperature but also humidity. At sub-zero temperatures a moist habitat might induce freezing, but a protected microhabitat might also limit water loss (Danks, 2000).

Some insects lack the ability to survive outdoor conditions in winter and overwinter in human-made structures. Several invasive pest species in Ontario overwinter in association with houses, like the brown marmorated stink bug, *Halyomorpha halys* Stål (Hemiptera: Pentatomidae) (Lee et al., 2014), and the Eastern subterranean termite, *Reticulitermes flavipes* Kollar (Isoptera: Rhinotermitidae) (Clarke et al., 2013). Other insects overwinter in buildings associated with agriculture like the house fly, *Musca domestica* L. (Hanec, 1956). In these cases, reducing access to these refuges would allow for successful pest management.

### **1.3 *Drosophila suzukii***

*Drosophila suzukii* is commonly known as spotted wing drosophila (Fraser et al., 2011; Kanzawa, 1939). While most of the drosophilids are economically unimportant, *D. suzukii* is a pest, because the female has a serrated ovipositor allowing it to lay eggs in healthy thin-skinned fruits such as blueberries, raspberries, strawberries, peaches, grapes and tomatoes (Atallah et al., 2014; Kanzawa, 1939). The fruit is damaged as the three larval

instars develop inside the fruit (Kanzawa, 1939), and the oviposition scar allows entrance by secondary pests, fungal and bacterial pathogens leading to decay of unripe fruits (Hamby et al., 2012; Walsh et al., 2010).

*Drosophila suzukii* originates from Southeast Asia. It is unclear if *D. suzukii* is native to Japan; but populations have likely been established there since at least 1916, and are best-studied in Japan (Kanzawa, 1939; Mitsui et al., 2010). Therefore, Japan can be considered as the natural environment and observations can be used to predict behaviour in similar regions. *Drosophila suzukii* is mainly found in warm-temperate regions of Japan (Kondo & Kimura 2006). In the subtropical Yamanashi region (in Japan (35°35'N, 138°51'E), adults overwinter beneath leaf litter and leave the overwintering habitat in March (first findings during the year) (Kanzawa, 1939). *Drosophila suzukii* has not been found overwintering at high altitudes (Mitsui et al., 2010), which might indicate that this species is not very cold-hardy. In the Yamanashi region, the number of adults decreased with a decline in temperatures and food sources in October (Kanzawa, 1939), which suggests that adults seek shelter as winter approaches.

In the last few decades, *D. suzukii* has been recorded outside of Southeast Asia, including Hawaii since 1980 (Kaneshiro, 1983), Spain (Cinci et al., 2012) and California since 2008 (Walsh et al., 2010). *Drosophila suzukii* was reported in Oregon, Washington and British Columbia in 2009 (Hauser, 2011). In 2010 *D. suzukii* was first found in Ontario, in the Niagara region (Fraser et al., 2011), and in Alberta, Manitoba and Quebec (Hauser, 2011). While the total monetary loss of fruits caused by *D. suzukii* in Canada is unknown, this pest caused a loss of approximately 20-30 % of strawberries, blueberries, raspberries, blackberries and cherries in the United States resulting in USD \$511 million in damage in 2008 (Bolda et al., 2010). *Drosophila suzukii* is potentially a threat to crops in Canada, where blueberries, strawberries and cherries were worth CAD \$27.5 million in 2011 (Statistics Canada, 2012).

In Ontario, the phenology of *D. suzukii* differs from that in Japan. Since 2010 there has been regular summer trapping of *D. suzukii* in Ontario, where the first flies of the year were captured in June (2012, 2013, 2014) and August (2011) (OMAFRA, 2014). These captures are relatively late in comparison to captures in Japan, where the first flies

were captured in March (Kanzawa, 1939). The number of trapped *D. suzukii* decreases in Ontario in October (OMAFRA, 2014). Although *D. suzukii* has been found during the summer in multiple years, its ability to overwinter in Ontario has not been examined.

The potential of *D. suzukii* to establish permanent populations in Ontario depends on its ability to withstand low temperature exposure, because winters in Ontario are longer and colder than winters in Yamanashi, Japan (World Weather Online, 2014). However little is known about the overwintering biology of *D. suzukii* anywhere. Laboratory experiments suggest that that *D. suzukii* is relatively cold-intolerant, because 75 % of flies die after a 24h-exposure to -1.8 °C (females) or -0.7 °C (males) (Kimura, 2004). Adults survived exposure to temperatures ranging from 1 to 10 °C longer than pupae, but did not survive longer than 17 days at 1°C (Dalton et al., 2011). However these few studies did not determine increases in low-temperature tolerance due to phenotypic plasticity and did not cover all potential metrics of low-temperature tolerance. In addition the few existing studies on *D. suzukii* cold tolerance only used flies from Japan (Kimura, 2004) or Oregon (Dalton et al., 2011). Because cold tolerance can vary geographically, and newly-introduced populations can undergo severe genetic bottlenecks and selection events, it is necessary to explore the cold tolerance limits of *D. suzukii* caught in Ontario.

## 1.4 Objectives

The potential of *D. suzukii* to establish permanent populations in Ontario might be limited by low temperatures. However little is known about the ability of *D. suzukii* to withstand low temperature stress. In this thesis, I examine the low temperature tolerance of adult *D. suzukii*, because they are thought to be the overwintering life stage (Kanzawa, 1939). I assessed the phenotypic plasticity of low temperature tolerance of males and females by manipulating conditions in the laboratory to produce summer- and winter-like adult *D. suzukii*.

My main objective was to determine the low temperature tolerance of *D. suzukii*, including the phenotypic plasticity of its cold hardiness. I measured chill coma onset to learn about activity boundaries in fall and spring. I assessed cold tolerance strategy and survival after acute, chronic, and semi-field cold exposure to determine lethal

temperature limits. In addition, I explored the effect of cold exposure on reproduction to learn how cold can affect reproduction of overwintering flies.

## 2 Methods

### 2.1 Study animals

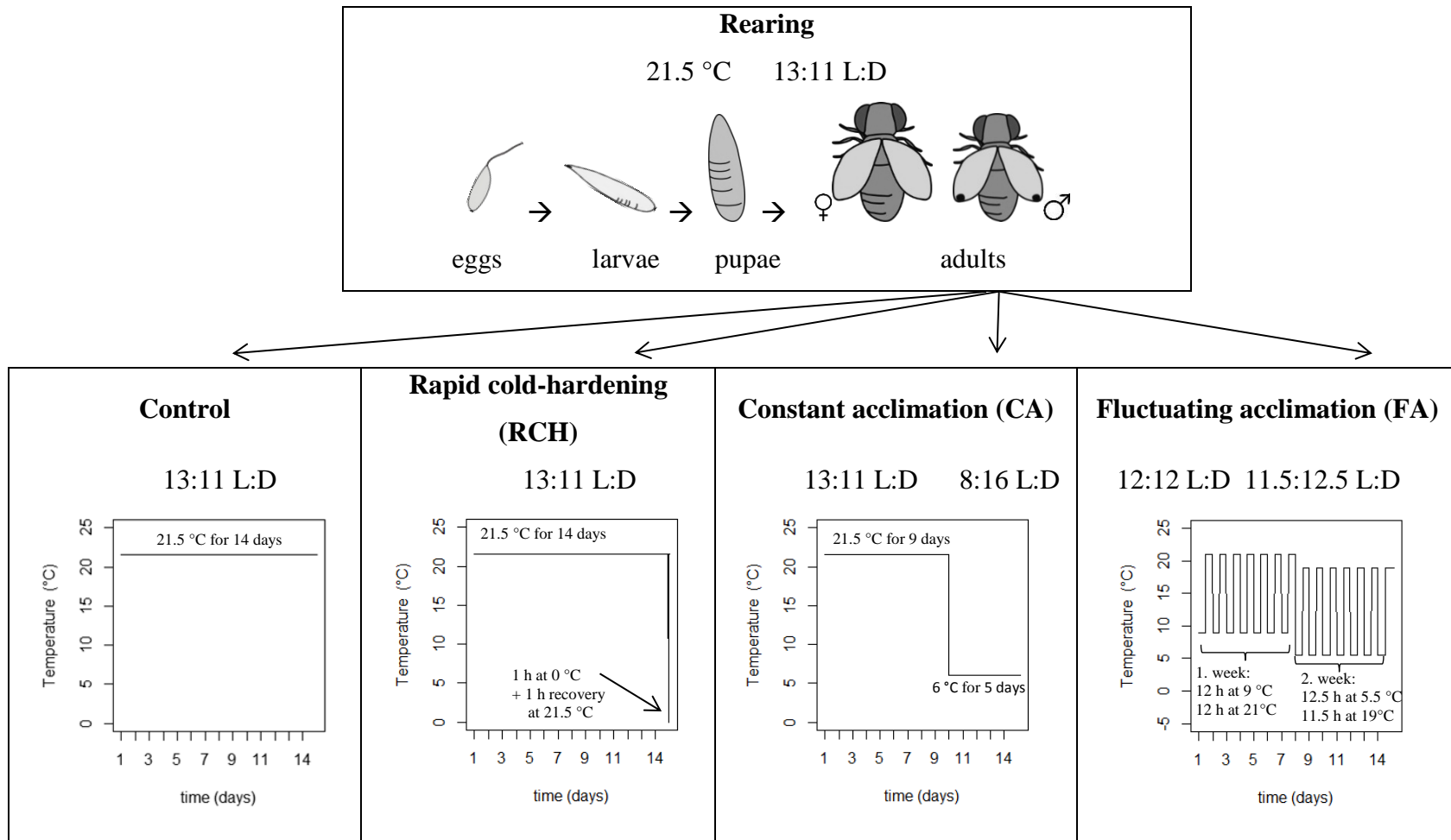
The *D. suzukii* population was established from approximately 200 individual flies originally collected in the Halton Hills region, Ontario, Canada (43°37 N 79°57 W). Flies were fed on a banana medium (containing 1 L water, 112.5 g banana, 47.5 g corn syrup, 30 g barley malt, 27.5 g active yeast, 6.5 g agar, 3 ml propionic acid, 2 g methylparaben) (Markow and O'Grady, 2006) and reared in a walk-in temperature-controlled growth chamber (M-25, Environmental Growth Chambers, Chagrin Falls, Ohio, USA), at  $21.5 \pm 1$  °C and  $60 \pm 5$  % relative humidity under 13:11 L:D. Population cages were built out of a 3.8 L PET plastic jar (23 cm × 15 cm × 13 cm) with a piece of medical stockinette closed with a clip to allow access to the cage. A Petri dish containing 35 mL of banana food, topped with active yeast paste (to stimulate oviposition) was placed into the population cage. After approximately 16 h, the food plate was removed and cut in pieces containing approximately 75 eggs, which were then transferred into 35 mL vials containing approximately 10 mL banana food. On the day of eclosion, adult flies were transferred into new food vials using a funnel. Flies were left to mate for approximately 24 h, then were anaesthetized using CO<sub>2</sub> for less than 10 min and sorted by sex. For experiments where virgin females were needed, flies were collected six hours after eclosion and separated by sex using CO<sub>2</sub> for less than 10 min.

### 2.2 Treatment groups

Seasonal changes and rapid responses to low temperature can induce phenotypic plasticity in insect cold tolerance (Lee and Denlinger, 1991). To test for phenotypic plasticity, *D. suzukii* adults were divided into four different treatment groups. The control group represents flies during the summer, while rapid cold-hardening (RCH), constant acclimation (CA) and fluctuating acclimation (FA) prepared the flies for winter (Figure 2.1). The control group contained adult flies held at the rearing conditions (21.5 °C, 13:11 L:D). For the RCH pre-treatment, adult flies were held at 21.5 °C for 14 days and were then transferred into empty 35 mL fly vials with a moist cotton stopper (to prevent desiccation) on the day of the experiment. These vials were placed in ice-water slurry (0 °C) for one hour and flies were subsequently returned to 21.5 °C for 1 h in new fly vials

containing approximately 10 mL fly food. Flies of the CA group were held at 21.5 °C for 9 d and then exposed to 6 °C and a light regime of 8:16 L:D for 5 d in an incubator (MIR153, Sanyo, Bensenville, Illinois, USA). Fluctuating fall conditions for the FA group were simulated by exposing the flies to two weeks with average weekly minimum and maximum temperatures of late September in London, Ontario (data from 2012), with the corresponding light conditions. In the first week, FA flies were exposed to 9 °C for 12 hours (darkness) and 21 °C for 12 hours (light). In the second week, 5.5 °C for 12.5 hours (darkness) and 19 °C for 11.5 hours (light) were used.





**Figure 2.1 Temperature and light conditions for fly rearing and treatments.**

## **2.3 Effect of low temperatures on survival**

### **2.3.1 Supercooling points (SCP)**

Individual adult flies from each treatment group were transferred into 1.7 mL microcentrifuge tubes. A 36-AWG type-T copper-constantan thermocouple (Omega, Laval, Quebec, Canada) was inserted through the lid and the tip of the thermocouple was held against the fly's body using cotton wool. The tubes were placed into holes in an aluminium block, which was connected to a methanol (50-100 %) circulator (Lauda Proline 3530, Würzburg, Germany) allowing the flies inside the tubes to be cooled from 0 °C to -30 °C at 0.1 °C/min. The thermocouples were connected to PicotechTC-08 interfaces with connection to the computer program Picolog v5.20.1 (Pico Technology, Cambridge, UK). The SCP was defined as the lowest temperature before exothermic reaction indicated by a sudden temperature increase. The SCPs of the different treatment groups were compared using a one-way ANOVA in R version 3.0.1 (R Development Core Team, 2013).

### **2.3.2 Cold tolerance strategy**

The cold tolerance strategy (freeze tolerance, freeze avoidance or chill susceptibility) of an insect can be determined by assessing survival after cold exposure and ice formation (Crosthwaite et al., 2011). To determine the cold tolerance strategy of female and male *D. suzukii* of treatment groups, ten individuals of each group and sex were placed separately into microcentrifuge tubes with a thermocouple attached to the body and cooled, as described for the SCPs. After half the flies had frozen (indicated by the exotherm), all individuals were removed quickly to room temperature and placed individually into 6-well plates with a ca. 1 cm<sup>3</sup> piece of banana food. Survival was assessed after 24 h. Insects that died due to chilling injuries unrelated to freezing (both unfrozen and frozen flies died) are considered chill-susceptible, those dying upon freezing (only unfrozen flies survived) are considered freeze-avoiding and those surviving freezing are freeze-tolerant (Table 2.1).

**Table 2.1 Determining cold tolerance pattern in insects.** Freeze-tolerant species survive freezing, while freeze-avoiding species die upon freezing and chill-susceptible species die at temperatures above the freezing point of their bodies.

<b>Frozen</b>	<b>Unfrozen</b>	<b>Cold tolerance pattern</b>
survival	survival	freeze tolerance
no survival	survival	freeze avoidance
no survival	no survival	chill susceptibility

### 2.3.3 Survival after acute low-temperature exposure

The lower temperature (LT) of control male and female *D. suzukii* was estimated using an acute exposure (1 h). To do so, groups of ten females or males were placed into a 1.7 mL microcentrifuge tube and were held at separate test temperatures ranging from -13 °C to 0 °C (resulting in mortality from 0 to 100 %) for 1 h using a methanol circulator (Lauda Proline 3530, Würzburg, Germany). Temperatures were recorded using thermocouples. Flies from each tube were placed into one well of a 6-well cell culture plate containing ca. 1 cm<sup>3</sup> banana food and survival was assessed after 24 h. A generalized linear model with a binary error distributions and logit link function was used to calculate the LT<sub>80-1h</sub> (lower temperature at which 80 % die after 1 h exposure) for both females and males and the fit was tested with Wald's statistics using the package *MASS* in R version 3.0.1 (R Development Core Team, 2013).

In a subsequent experiment, survival among the different treatment groups was compared after a 1 h exposure to the LT<sub>80-1h</sub>. Female and male flies of the different treatment groups were exposed to -7.2 °C (females) and -7 °C (males) for 1 h and then placed in 6-well plates as described above. Survival was assessed after 24 h and compared among the treatment groups using a one-way ANOVA in R.

### 2.3.4 Survival after chronic low temperature exposure

To determine the effect of chronic cold exposure on *D. suzukii*, female or male flies of the control and FA group were exposed to 0 °C for 10 days, during which survival was assessed every 12 h.

Ten female or male flies were placed into 1.7 mL microcentrifuge tubes. Three tubes of each sex and treatment were placed into a 16.5 × 14.9 cm sealed plastic bag. Four of these small bags were placed into a 26.8 × 27.3 cm sealed plastic bag with an iButton (Model 1920 L, Maxim Integrated, San Jose, CA, USA). These bags were then placed into a 30 × 40 × 30 cm insulated container filled with ice water slurry (0 °C). One 16.5 × 14.9 cm sealed plastic bag containing three tubes of each sex and treatment was sampled every 12 h. Flies from each tube were placed into one well from a 6-well plate, which contained about 1 cm<sup>3</sup> banana food containing. Survival was assessed after 24 h. The Lt<sub>80</sub>

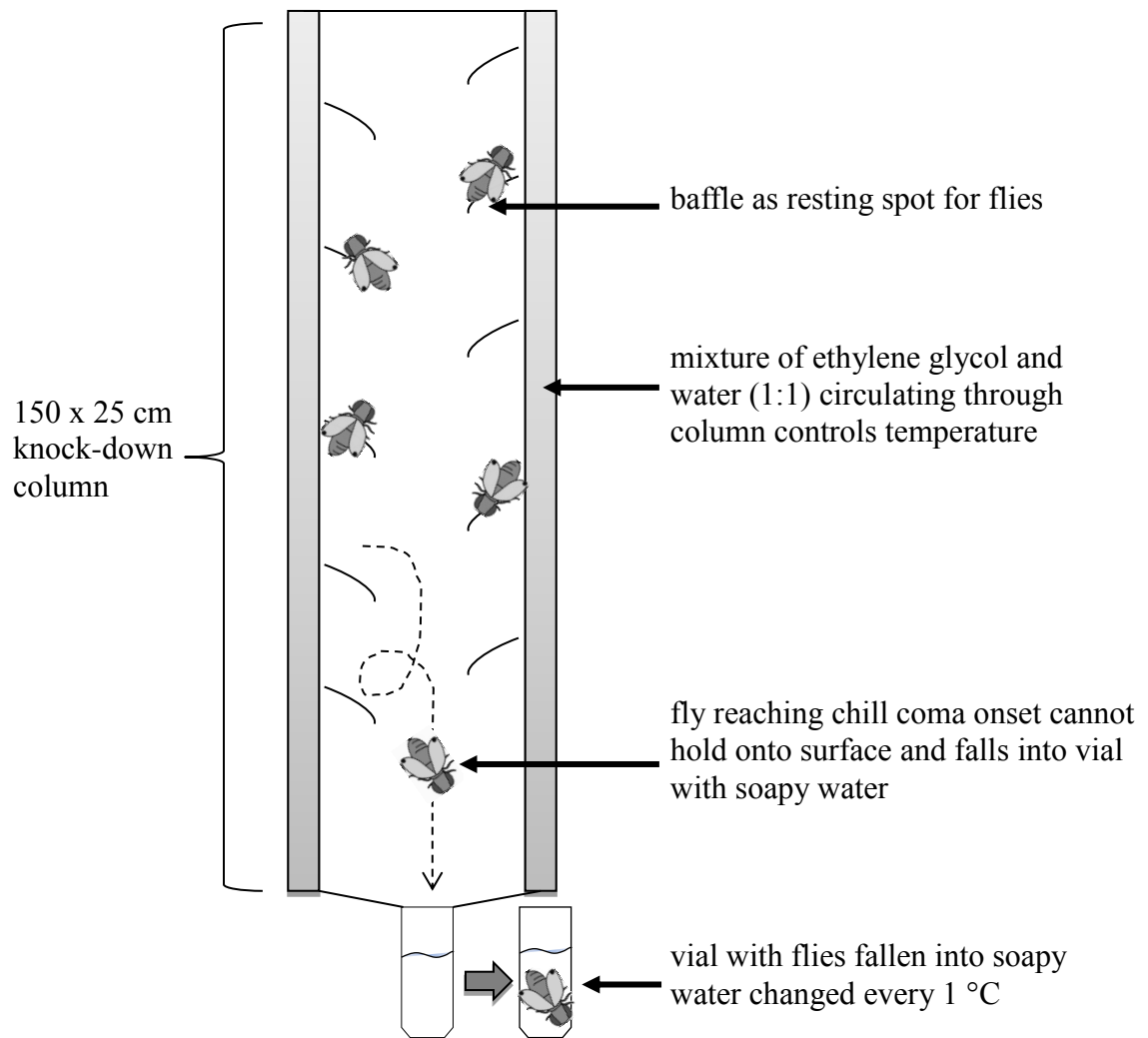
(lethal time at which 80 % die) was calculated for both females and males using a generalized linear model with a binary error distributions and logit link function and the fit was tested with Wald's statistics using the package *MASS* in R (Venables and Ripley, 2002).

## **2.4 Effect of low temperatures on activity**

### **2.4.1 Critical thermal minimum ( $CT_{min}$ )**

The critical thermal minimum ( $CT_{min}$ ) is the measure of chill coma onset and was determined for all treatment groups using a  $150 \times 25$  cm glass knock-down column (Figure 2.2). A refrigerated circulator (model 1157P, VWR International, Radnor, PA, USA) circulated a mixture of ethylene glycol and water (1:1) through the outer layer of the column, allowing for temperature control (Ransberry et al., 2011). Aluminium baffles in the column provided surfaces on which flies could rest. Temperature was monitored by three thermocouples at the top of the column and three at the bottom of the column. Approximately 500-800 flies were transferred into the column and after 15 min at 21 °C the temperature was decreased to -15 °C at 0.1 °C/min. As each fly reached its  $CT_{min}$ , it could not cling onto the surface and fell into a 50 mL plastic vial containing soapy water. This vial was changed every 1 °C and flies were filtered out of the water and stored frozen until sex of the collected flies was determined.

The accelerated-failure-time (AFT) model included in the *survival* package in R (Therneau and Grambsch, 2000) was used to compare the effects of sex and treatment on the  $CT_{min}$ . The temperature at which 80% of the flies enter chill coma ( $CT_{min80}$ ) was calculated using the AFT models in R (Ransberry et al., 2011).  $CT_{min}$  of the different treatment groups was compared using a one-way ANOVA in R.



**Figure 2.2 Knock-down column used to measure the critical thermal minimum.**

### **2.4.2 Chill-coma recovery time (CCR)**

Chill coma recovery time is the time it takes an insect to recover movement after a standardized cold exposure causing chill coma (Ransberry et al., 2011). With an increase in exposure time to the chill-coma-inducing temperature, CCR increases and reaches a plateau phase, after which insects may accumulate chilling injuries (MacMillan and Sinclair, 2011). To minimize variation in CCR an exposure time within the plateau phase has to be determined. To do so groups of 10 female or male adult flies of the control group were transferred into 1.7 ml microcentrifuge tubes. Six tubes containing either females or males were placed into sealed plastic bags, which were immersed into ice-water slurry (0 °C). After 1, 2, 3, 4, 5, 6, 7, 8 and 10 h, flies were transferred into empty 6-well plates. All flies were in chill coma and the time until flies were able to stand on their legs was measured as chill coma recovery time.

For subsequent experiments, an exposure time of 8 h was chosen, because it lies at a point where the recovery time and exposure time relationship reached a plateau (see section 3.2). To compare CCR among the different treatment groups, six microcentrifuge tubes containing either ten males or females of each treatment group were placed in a sealed plastic bag, which was immersed in ice-water slurry for 8 h. Recovery time for each fly was assessed as described above.

The effect of sex and treatment on the CCR was compared using an accelerated-failure-time (AFT) model. The time at which 80 % of the flies recovered from chill coma was calculated using the AFT models ( $CCR_{80}$ ) and CCR was compared among treatments using a one-way ANOVA in R.

## **2.5 The effect of low temperature exposure on reproduction**

Long-term low temperature exposure can induce reproductive diapause through acclimation (Saunders et al., 1990), whereas acute low temperature exposure can decrease reproductive output due to cold shock (Marshall and Sinclair, 2010; Shreve and Lee, 2004). To test whether adults showed an adaptive response regarding low temperature exposure, ovaries of females were dissected to assess the potential of ovarian diapause. To examine the passive response to low temperatures regarding reproduction,

female and male flies were exposed to a non-lethal temperature and reproductive output was assessed.

Ten females of each treatment group (all at least 15 days old) were dissected to assess ovarian development and therefore the incidence of ovarian diapause. Females of the different treatment groups were anaesthetized using brief exposure to CO<sub>2</sub>. Flies were transferred into a 1:1 mixture of distilled water and PBS (phosphate buffered saline). Under the microscope the head was removed using dissecting pins. The abdomen was held with one dissection pin while the other pin was used to remove the last two abdominal segments. While pulling away the last two abdominal segments the gut and the ovaries were removed from the body. Photographs of the ovaries were taken using a camera (Nikon digital sight DS-Fi1, Tokyo, Japan) installed on a stereomicroscope (Nikon SMZ 1500, Tokyo, Japan) and edited in NIS-Elements (D3.22.14, Nikon, Tokyo, Japan). To count the number of chorionated eggs per pair of ovaries, the ovaries were placed in 5% Tween20 and the eggs teased out using dissecting pins. The number of eggs per pair of ovaries was compared among the treatment groups using a one-way ANOVA in R.

To determine the effect of low temperature on female reproduction, females from each treatment were placed individually into 1.7 mL microcentrifuge tubes. These flies were exposed to -3.5°C for 1 h (showed no lethal injuries after acute cold exposure) using a Lauda refrigerated circulator or held at 21.5 °C. After exposure, female flies were placed separately into food vials with one male (remated) or without a male (not remated), that was held at control conditions. Flies were transferred to new food vials every two days for 18 days in total. Eggs were reared to adults and sex and dry mass was determined to assess offspring quality.

The sum of all offspring produced within 20 days was compared among treatments, exposure types (cold-exposed vs. not cold-exposed), mating types (remated vs. not remated) and their interaction using a three-way ANOVA for the different treatment groups separately. To determine if there is any trade-off between reproductive output and investment in the offspring the dry mass of the total number of offspring was compared among the treatment groups using a three-way ANOVA in R.



To determine the effect of low temperatures on male reproductive output, males of the different treatment groups were exposed to -3.5°C for 1 h or held at 21.5 °C as described above. Males were placed individually into food vials with a single virgin female. Food vials were changed every second day for 18 days in total. The total number of female and male offspring per vial was determined after eclosion of all offspring.

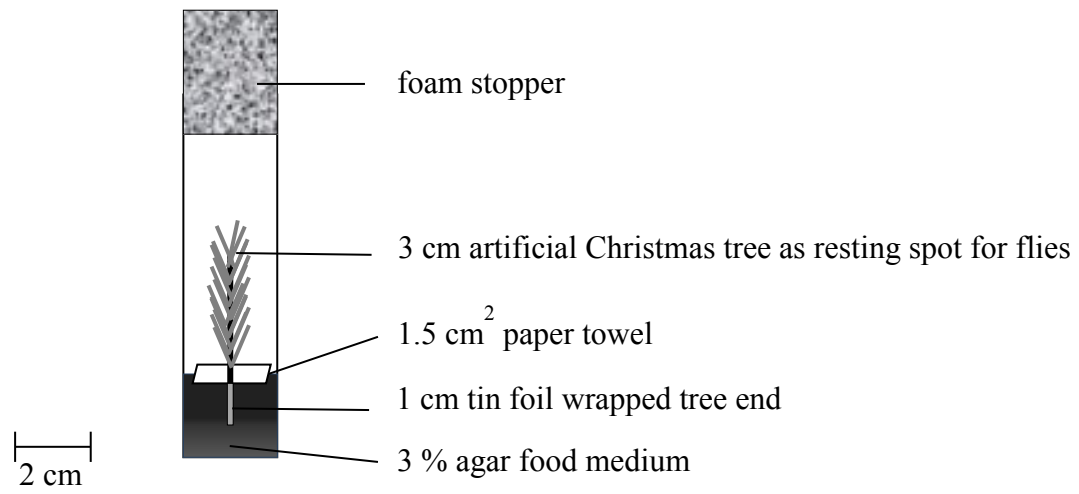
The cumulative number of offspring per male (exposed or unexposed) was plotted over time with a loess smoothing curve using the R packages *ggplot2* (Wickham, 2009) and *MASS*. An overlap of the loess smoothing curve indicated no significant difference between the groups. The sex ratio of the total offspring was compared among the different treatment groups and exposure types using a two-way ANOVA in R.

## **2.6 Reproductive output in a semi-field environment in late fall**

The effect of natural fall conditions (decreased temperature and photoperiod) on reproduction of *D. sukii* was determined by comparing flies that were held in field cages to flies that were held under control conditions in the lab.

Five female and five male flies per vial were acclimated on banana food to October conditions (10 h at 10.5 °C and light, 14 h at 6.5 °C and darkness) in an incubator (MIR153, Sanyo, Bensenville, Illinois, USA). Flies were then transferred into field cage vials (Figure 2.3), which contained banana food medium (described above) that was modified using 36 g agar (per 1 L of food) to create a more solid food medium. Each 35 mL vial contained approximately 10 mL of the modified banana food. A piece of artificial vegetation and a piece of paper towel was used to prevent flies from sticking in the food. Ten vials were placed inside a walk-in growth chamber at 21.5 °C (laboratory control), while ten vials were placed into wire fly racks in a 47.5 × 38.9 × 27.7 cm plastic container (field cage). An iButton data logger (Model 1920 L, Maxim Integrated, San Jose, California, USA) was placed into an empty vial with a foam stopper to monitor temperature in a vial and placed into the field cage. The cage was placed in a garden in London, Ontario (43°00'N 81°15'W) on 1 October, 2013. Additional sets of ten vials with flies were placed outdoors in the same location on 15 October and 29 November to account for any effect of senescence on the reproductive output. Flies from vials from the

field cages and in the incubator were transferred to new vials on a weekly basis. Old fly vials were placed in an incubator at 21.5 °C and 13:11 L:D to rear eggs into adults. The number of offspring per fly was compared between laboratory and field cage with a one-way ANOVA in R.



**Figure 2.3 Design of a field cage fly vial.**

## **2.7 Survival at different overwintering microhabitats in a semi-field environment**

To test whether *Drosophila suzukii* can overwinter outside, field cages were buried beneath leaf litter, placed in a garden shed, or directly exposed to outdoor conditions starting on 26 November 2013 in the same garden as the reproduction field cage (above). The overwintering habitat buried beneath leaf litter was chosen as it is the overwintering site of *D. suzukii* in Japan (Kanzawa, 1939). Overwintering in a shed is representative for overwintering in an unheated structure associated with an orchard. Flies directly exposed to air temperature experience the most severe temperature. Two hundred field cage vials each with ten October-acclimated females or males were placed in each condition with 100 vials in each plastic container, resulting in two containers with 100 vials for each sex in every condition (total of 600 vials and 6000 flies). Temperature was recorded in each field cage every 30 min using Hobo data loggers (Onset, Bourne, Massachusetts, USA). On 18 December 2013 20 vials of each location and sex were placed into an incubator at 21.5 °C and 13:11 L:D and survival was assessed after 24 h.

### 3 Results

#### 3.1 Effect of low temperatures on survival

In adults of *D. sukuzii*, the SCPs ranged from -16.1 °C in FA females to -23.3 °C in FA males (Table 3.1), and had a unimodal distribution. Neither treatment nor sex had an effect on the supercooling point ( $F_{1,112} = 2.823$ ,  $p = 0.096$ ). Flies of all treatment groups and sexes were unable to survive internal ice formation, which indicates that they are not freeze-tolerant. Individuals that were exposed to temperatures slightly above the SCP did not survive either, which shows that flies were not freeze-avoiding, but chill-susceptible (Table 3.2).

To assess lower lethal temperature limits of adults of *D. sukuzii*, flies of the control group were exposed to a range of temperatures (-13 °C to 0°C) for 1 h (acute low temperature exposure). An exposure between -4 °C and 0 °C did not kill control flies; however there was a sharp decline in survival between -8 and -5 °C. All flies died when exposed to temperatures below -10 °C (Figure 3.1). The  $LT_{80-1h}$  (temperature at which 80 % die after 1 h exposure) was  $-7.5 \pm 0.1$  °C for control females (Wald's statistic = 14.51,  $p < 0.001$ ) and  $-7.2 \pm 0.1$  °C for control males (Wald's statistic = 15.16,  $p < 0.001$ , Figure 3.1).

Survival was assessed after an acute exposure to temperatures close to the  $LT_{80-1h}$  for females (-7.2 °C) and males (-7 °C), in each of the treatment groups, to test for phenotypic plasticity. The CA and FA treatments increased survival by around 80 % after a one hour exposure to -7.2 °C in female ( $F_{3,20} = 215.7$ ,  $p < 0.001$ , Figure 3.2 A) and to -7 °C in male adult *D. sukuzii* ( $F_{3,20} = 138.5$ ,  $p < 0.001$ , Figure 3.2 B); however, the RCH treatment did not improve survival (females:  $F_{3,20} = 215.7$ ,  $p = 0.2$ , Figure 3.2 A; males:  $F_{3,20} = 138.5$ ,  $p = 0.99$ , Figure 3.2 B).

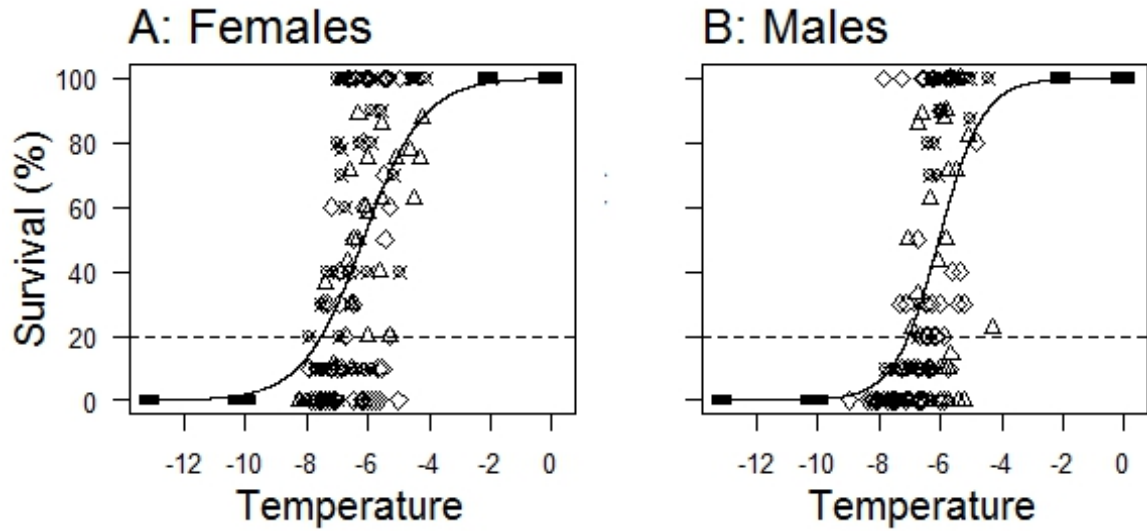
Flies of the control and FA group were exposed to 0 °C for several days (chronic cold exposure), survival of subsamples of the treatment groups was assessed every 12 hours (Figure 3.3.). The  $Lt_{80}$  (lethal time at which 80 % die) ranged from 70 h in control males to 165 h in FA females (Table 3.3) and increased by around three days in FA females ( $\chi^2 = 11.54$ ,  $p < 0.001$ ) and two days in FA males in comparison to the control group ( $\chi^2 = 8.9$ ,  $p < 0.001$ ).

**Table 3.1. Supercooling points of male and female adult *Drosophila suzukii*.** Mean ( $\pm$  SEM) supercooling point ( $^{\circ}\text{C}$ ) of the different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA and fluctuating acclimation = FA).

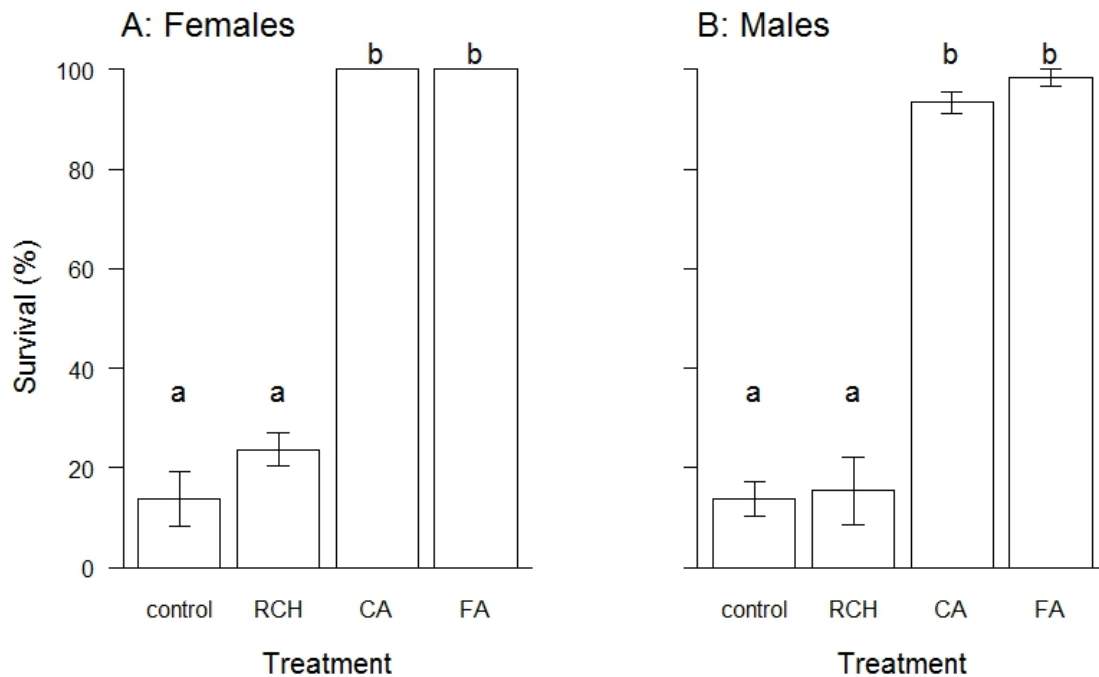
Treatment	Supercooling point ( $^{\circ}\text{C}$ )	
	Females	Males
Control	$-20.4 \pm 0.6$	$-20.0 \pm 0.4$
RCH	$-21.3 \pm 0.3$	$-20.8 \pm 0.4$
CA	$-19.7 \pm 0.3$	$-21.0 \pm 0.3$
FA	$-18.8 \pm 2.1$	$-22.1 \pm 0.2$

**Table 3.2 Cold tolerance pattern of female and male adults of *Drosophila suzukii* in different treatment groups.** Adult flies of four different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA and fluctuating acclimation = FA) were cooled at  $0.1^{\circ}\text{C}/\text{min}$  to  $-19.5^{\circ}\text{C}$ , the temperature when half of the flies were frozen (indicated by SCP) and the other half remained unfrozen.

Treatment group	Females			Males		
	Number of flies dead		Cold tolerance pattern	Number of flies dead		Cold tolerance pattern
	Unfrozen	Frozen		Unfrozen	Frozen	
Control	5/5	5/5	chill-susceptible	5/5	5/5	chill-susceptible
RCH	5/5	5/5	chill-susceptible	5/5	5/5	chill-susceptible
CA	5/5	5/5	chill-susceptible	5/5	5/5	chill-susceptible
FA	5/5	5/5	chill-susceptible	5/5	5/5	chill-susceptible

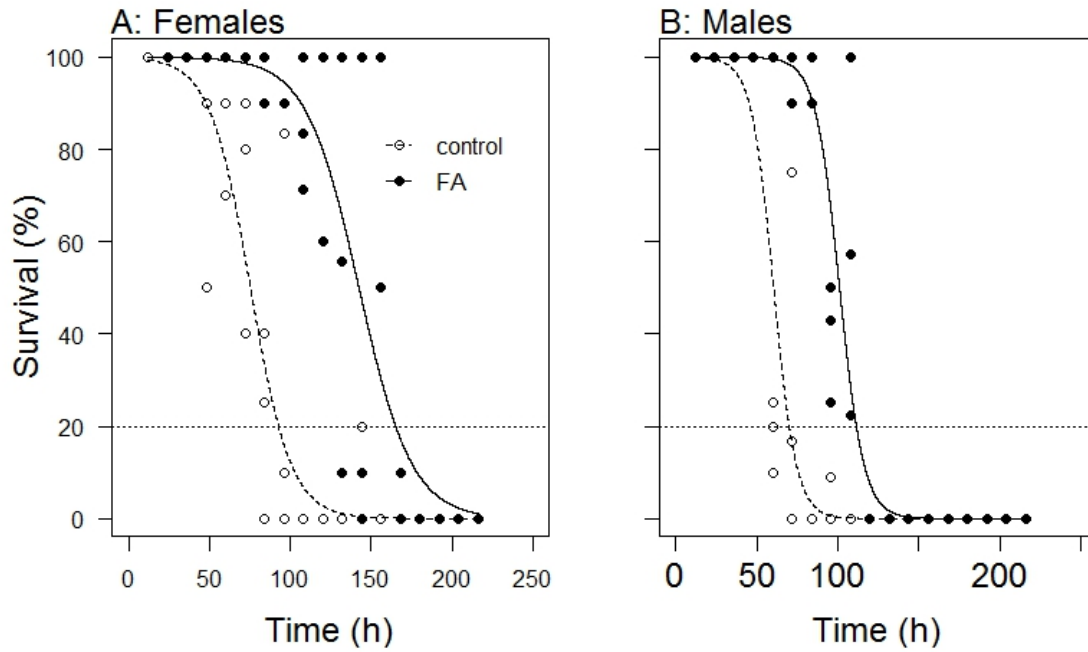


**Figure 3.1 Survival of *Drosophila suzukii* after acute low temperature exposure.** Female (A) and male (B) adult *Drosophila suzukii* of the control group were exposed to a single temperature ranging from -13 °C to 0 °C for a one hour. The different symbols indicate different experimental dates. Each point represents the proportion of flies that survived in a group of seven to ten flies. The solid line represents the survival curve calculated with a generalized linear model (binary error distributions, logit link function) (Wald's statistic: female = 14.51,  $p < 0.001$ ; male = 15.16,  $p < 0.001$ ), the dotted line shows 80 % mortality (LT<sub>80-1h</sub>).



**Figure 3.2 Survival of *Drosophila suzukii* after acute low-temperature exposure following different pre-treatments.** Mean survival  $\pm$  SEM ( %) after one hour exposure to  $-7.2^{\circ}\text{C}$  in female (A) and to  $-7^{\circ}\text{C}$  in male (B) adult *D. suzukii* of the treatment groups control, rapid cold-hardening (RCH), constant acclimation (CA) and fluctating acclimation (FA). Treatments with the same letter are not significantly different ( $p > 0.05$ ) according to Tukey's test (one-way ANOVA).





**Figure 3.3 Survival of *Drosophila suzukii* during chronic cold exposure.** Female (A) and male (B) adult *D. suzukii* of the control (open symbols, dashed line) and fluctuating acclimation (FA; closed symbols, solid line) group were exposed to 0 °C, sampled every 12 hours and survival was assessed. Each point represents the proportion of flies that survived in a group of ten flies. Survival curves were calculated by a generalized linear model (binary error distribution and logit link function); statistics shown in Table 3.3. The dotted line shows 80 % mortality ( $Lt_{80}$ ).

**Table 3.3 Lethal time after chronic cold exposure to 0 °C of *Drosophila suzukii*.** Female and male adult *Drosophila suzukii* of the control and fluctuating acclimation (FA) group were exposed to 0 °C, sampled every 12 hours and survival was assessed. Time at which 80% were dead ( $Lt_{80} \pm SE$ ) was calculated with a generalized linear model (binary error distribution and logit link function) and tested with Wald's statistics.

Treatment	Females		Males	
	$Lt_{80}$ (h)	Wald's statistics	$Lt_{80}$ (h)	Wald's statistics
control	$92.7 \pm 2.9$	df=43, $\chi^2=-9.75$ , p<0.001	$70 \pm 2.2$	df=68, $\chi^2=-7.37$ , p<0.001
FA	$165 \pm 3.3$	df=49, $\chi^2=-10.8$ , p<0.001	$111.5 \pm 2.3$	df=56, $\chi^2=-7.77$ , p<0.001

### 3.2 Effect of low temperatures on activity

The temperature at which 80% of the flies enter chill coma is the  $CT_{min80}$ . FA flies had the lowest  $CT_{min80}$  of  $-1.7 \pm 0.1$  °C, followed by control ( $-1.2 \pm 0.1$  °C) and RCH flies ( $0.1 \pm 0.1$  °C;  $\chi^2 = 849.47$ ,  $df = 3$ ,  $p < 0.001$ ;

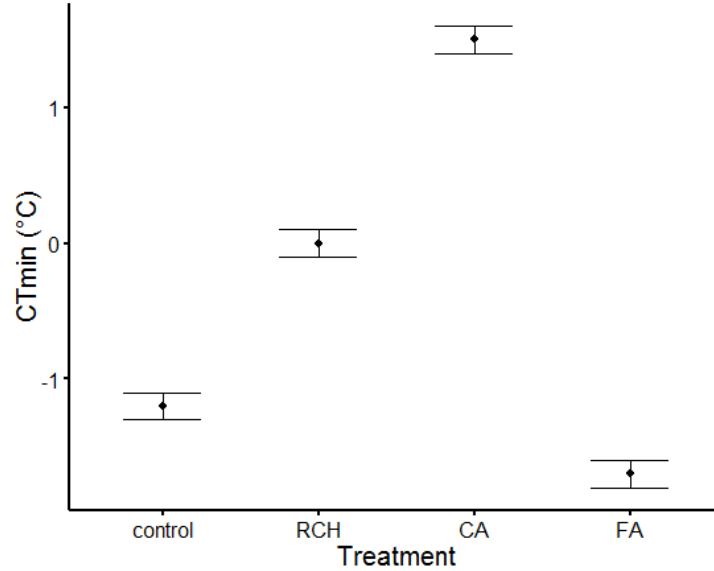
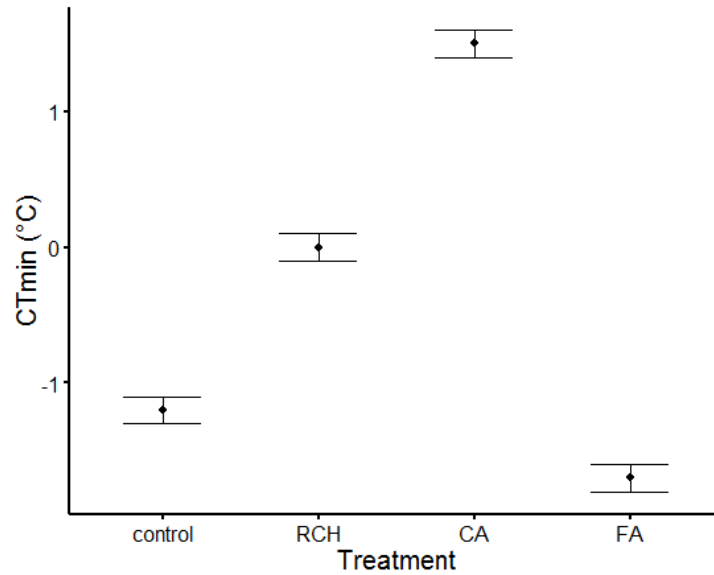
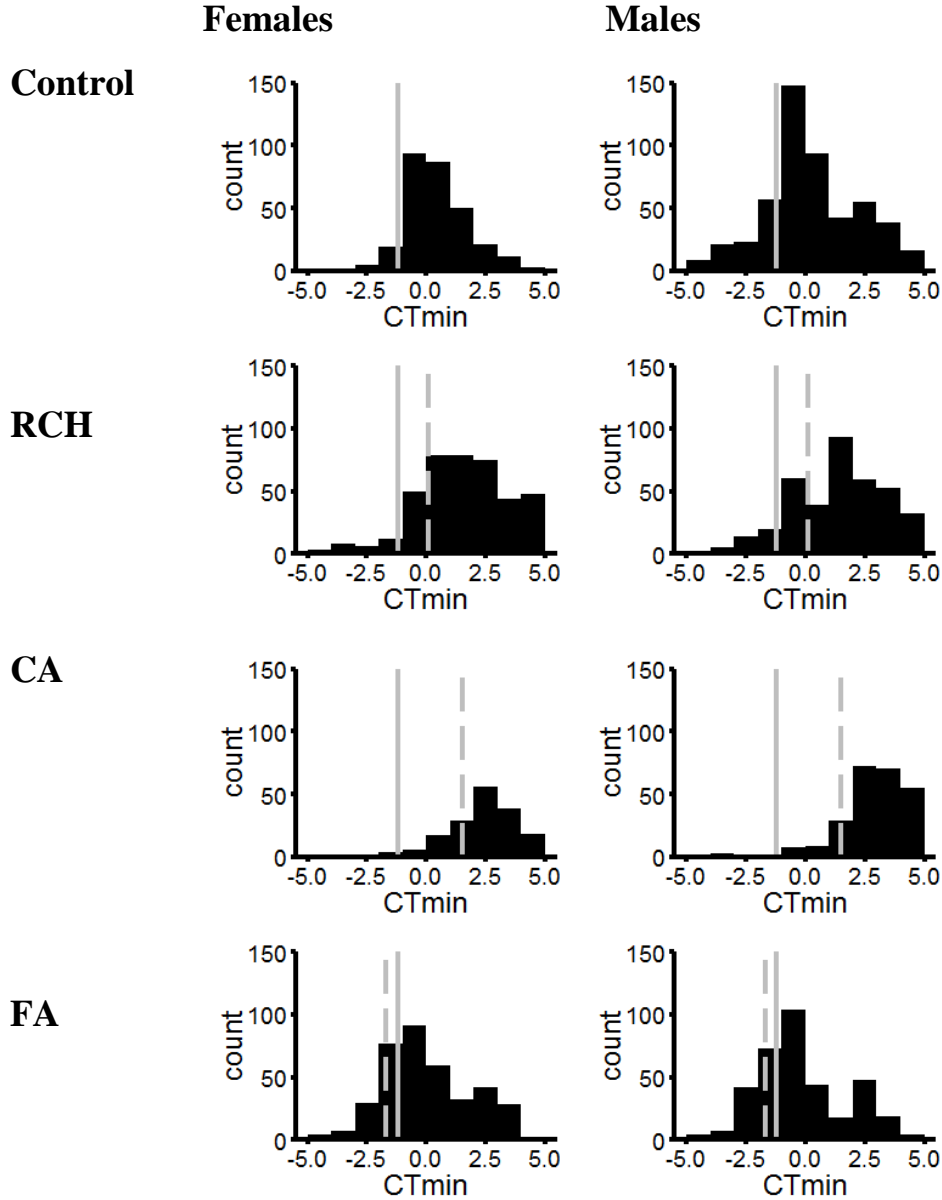


Figure 3.4). Acclimated flies had the highest  $CT_{min80}$  of  $1.5 \pm 0.1$  °C, which was driven by the positively skewed distribution, found only in the CA group (Figure 3.5). There was no effect of sex on  $CT_{min80}$  of *D. sukuzii*.

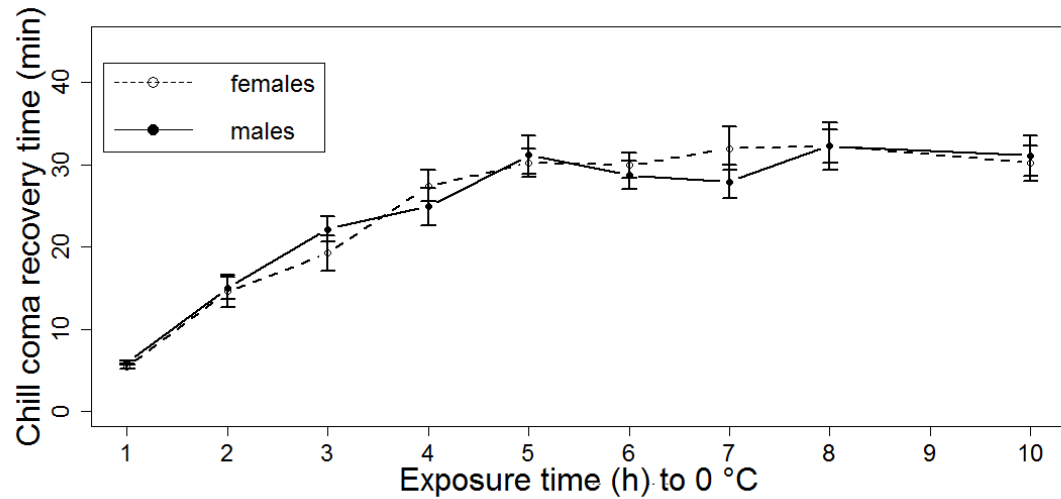
To test for phenotypic plasticity in chill coma recovery time, flies were exposed to 0 °C for eight hours, because this exposure time lies within the plateau phase of the recovery time-exposure time relationship (Figure 3.6).  $CCR_{80}$  ranged from 8.5 minutes in CA flies to 40.5 min in control flies. Males took as long to recover from chill coma as females of the same treatment group; however treatment had a significant effect on CCR ( $\chi^2 = 1.8 \times 10^{-59}$ ,  $df = 3$ ,  $p < 0.001$ , logistic error distribution). FA and CA flies had a  $CCR_{80}$  approximately 30 minutes shorter than control and RCH flies ( $F_{3,139} = 216.83$ ,  $p < 0.001$ ) (Figure 3.7).



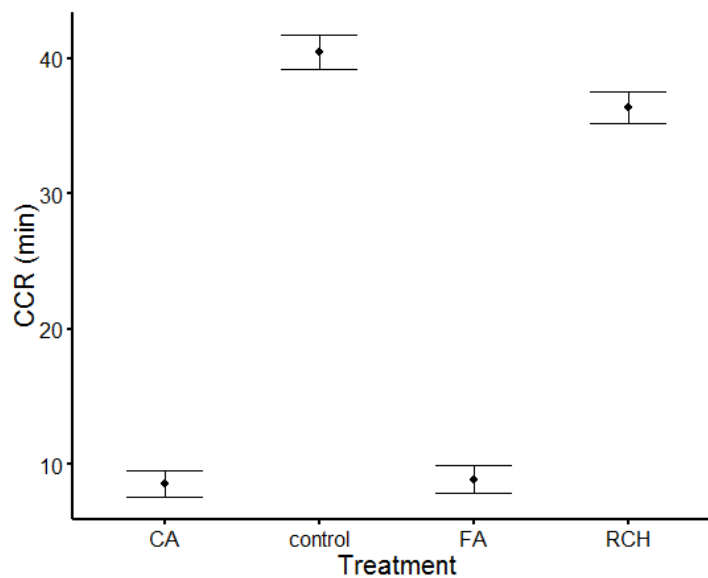
**Figure 3.4 Critical thermal minimum of *Drosophila suzukii* following different pre-treatments.** CT<sub>min80</sub> (temperature at which 80% of the flies entered chill coma)  $\pm$  SEM (°C) of flies of different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA and fluctuating acclimation = FA) was calculated using an accelerated failure time model (logistic error distribution)); the calculated CT<sub>min80</sub> was the same for females and males. Treatment had a significant effect on CT<sub>min80</sub> ( $\chi^2 = 1.8 \times 10^{-59}$ , df= 3,  $p < 0.001$ , logistic error distribution). Treatments with the same letter are not significantly different (one-way ANOVA  $F_{3,2793}=267.1$ ,  $p < 0.001$ ).



**Figure 3.5. Distribution of the critical thermal minimum of *Drosophila suzukii* following different pre-treatments.** Critical thermal minimum ( $CT_{min}$ ) for control, rapid cold-hardened (RCH), constant acclimated (CA) and fluctuation acclimated (FA) female (left) and male (right) adults in *Drosophila suzukii*. The solid grey line represents the  $CT_{min80}$  (temperature at which 80% of the flies entered chill coma) of control flies (shown on every panel) and the dashed line the  $CT_{min80}$  of the treatment group in the panel.  $CT_{min80}$  was calculated using the AFT model, which showed no significant effect of sex and a significant effect of Treatment on  $CT_{min80}$  ( $\chi^2 = 849.47$ ,  $df = 3$ ,  $p < 0.001$ , logistic error distribution).



**Figure 3.6 Chill coma recovery time of control *Drosophila suzukii*.** Chill coma recovery time (CCR) was determined for control females and males after an exposure to 0 °C for 1 to 10 h with ten flies at each time point.



**Figure 3.7 Plasticity of chill coma recovery time in *Drosophila suzukii*.** CCR values  $\pm$  SEM for control, rapid cold-hardened (RCH), constant acclimated (CA) and fluctuating acclimated (FA) flies display the chill coma recovery time at which 80 % of the flies recovered from an 8 h exposure to 0 °C, according to an AFT model (logistic error distribution). There was no significant difference between female and male flies according to the AFT. Treatments with the same letter are not significantly different (one-way ANOVA  $F_{3,143}=207.1$ ,  $p < 0.001$ ).

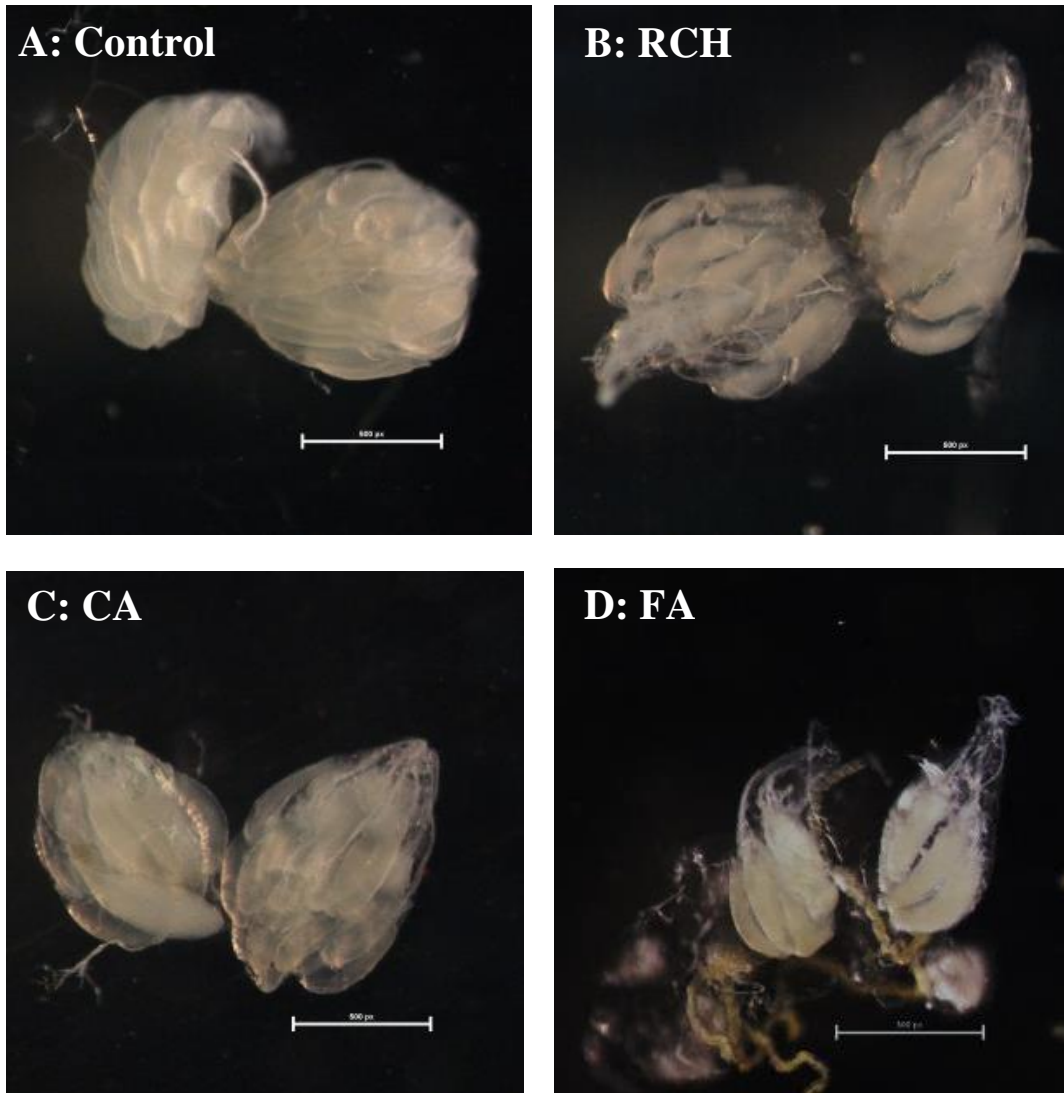
### 3.3 Effect of low temperature exposure on reproduction

The ovaries of *D. sukii* females of all treatment groups were dissected to see whether any of the treatments induced reproductive diapause. Females of the control, RCH and CA group had fully developed ovaries of the same size, while FA females had smaller ovaries (Figure 3.8). The ovaries of FA females contained significantly fewer chorionated eggs ( $14.9 \pm 2.8$ ) than ovaries of control ( $28.8 \pm 2.2$ ), RCH ( $28.3 \pm 1.2$ ) and CA ( $29.1 \pm 1.5$ ) females ( $F_{3,36} = 11.86$   $p < 0.001$ ).

Acute low temperature exposure can affect the number of offspring of mated females by cold shock. Low temperatures ( $-3.5$  °C) impaired the reproductive output (measured as total number of female offspring) in *D. sukii* females of the control, CA and FA, but not the RCH flies. Remating significantly increased the reproductive output by approximately 70% in control and FA, but not in RCH and CA flies (Figure 3.9 & Table 3.4). Dry mass of the female offspring was used as a measure of investment of each female in her offspring, but was not affected by cold exposure; however treatment and remating had an effect (Figure 3.10. & Table 3.5). Offspring from control flies weighed less than offspring from CA and FA flies and remating increased weight by 3-11 %.

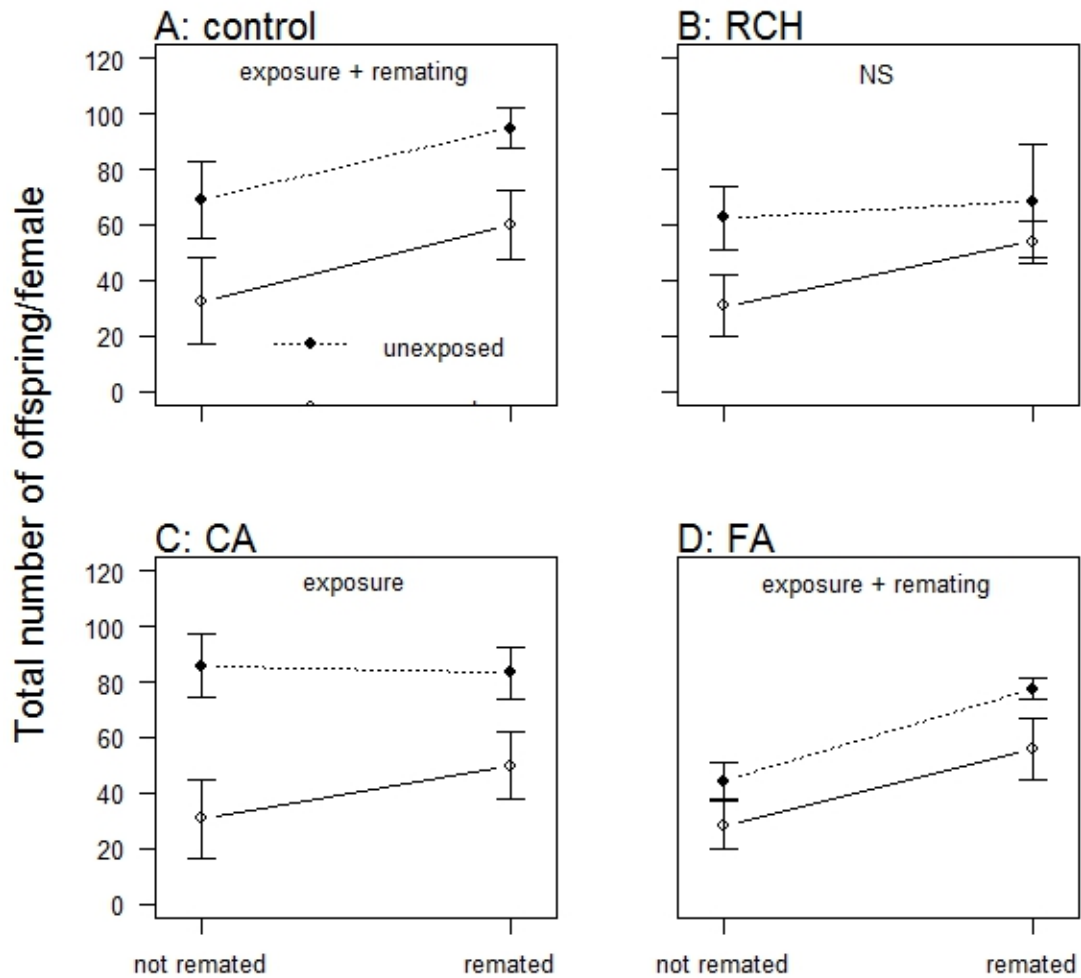
Cold-exposed males of the control and CA group had the same number of offspring as non-exposed males; however RCH and FA males that were cold exposed had 25 - 50 % fewer offspring than males that were not cold exposed (Figure 3.11.).

The sex ratio of the offspring of cold-exposed vs non-cold-exposed and remated vs not-remated females and males from the different treatment groups was compared to see if cold exposure or treatment had an effect on the sex of their offspring. Neither treatment nor cold-exposure of males had an effect on the sex ratio of the offspring (Table 3.6).



**Figure 3.8 Ovaries of female *Drosophila suzukii* following different pre-treatments.** Ovaries of females of the treatment groups control (A), rapid cold-hardening (B), constant acclimation (C) and fluctuating acclimation (D) were dissected at the age of 15 days. The scale bar indicates 500  $\mu\text{m}$ .

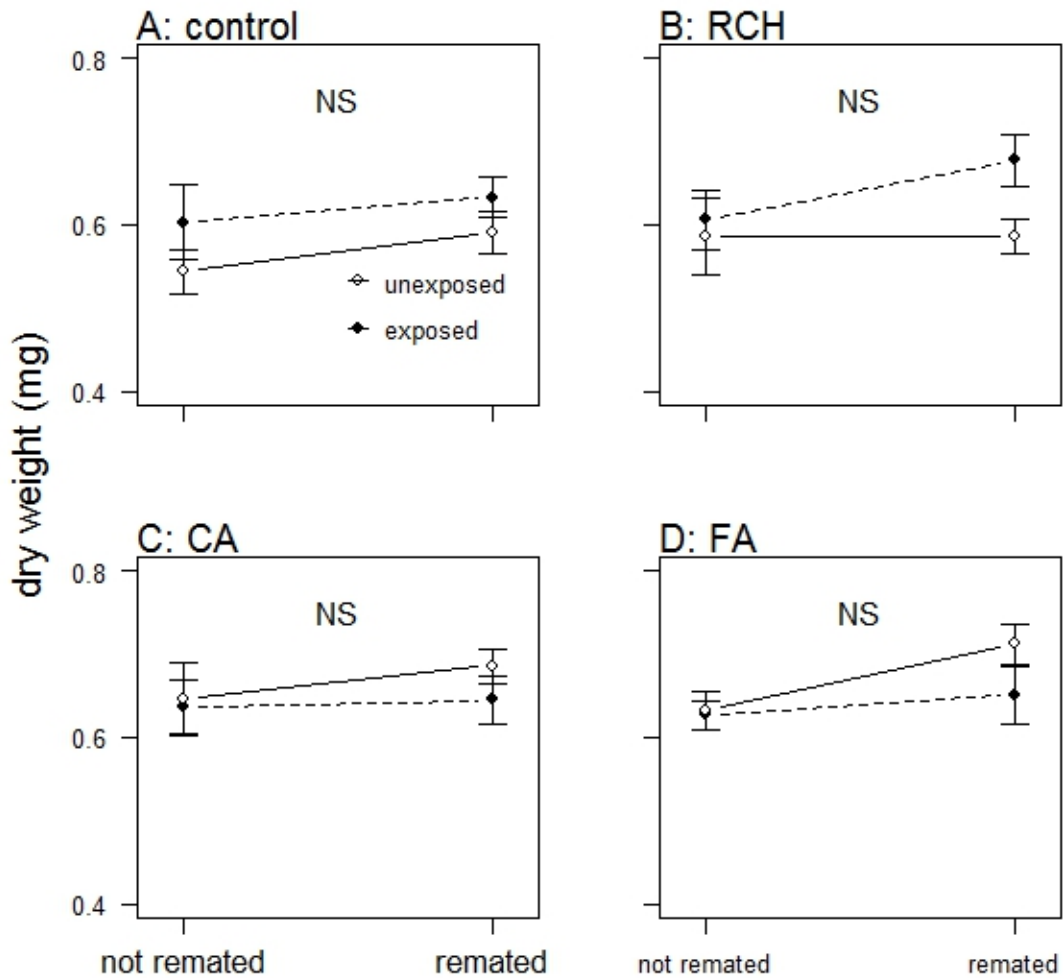




**Figure 3.9 Effect of low temperature exposure and remating on the reproductive output of female *Drosophila suzukii*.** The total number of offspring per female is the sum of all offspring after 18 days for females of the control (A), rapid cold-hardening = RCH (B), constant acclimation = CA (C) and fluctuating acclimation = FA (D) group. Mated female flies were unexposed or exposed to  $-3.5^{\circ}\text{C}$  for an hour and then either not remated or remated with a single male at  $21.5^{\circ}\text{C}$  and 13:11 L:D. Error bars represent the SEM. Significant effects of exposure type, remating, or their interaction are indicated at the top of the figure; NS = no significant effect (see Table 3.4 for statistics).

**Table 3.4. ANOVA statistics describing the effect of low temperature exposure on the female reproductive output of *Drosophila suzukii*.** Mated females of the different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA, fluctuating acclimation = FA) were unexposed or exposed to -3.5 °C for an hour (exposure) and then not remated or remated (remating) with a single male. Retained terms with significant p-values ( $p < 0.05$ ) are bolded.

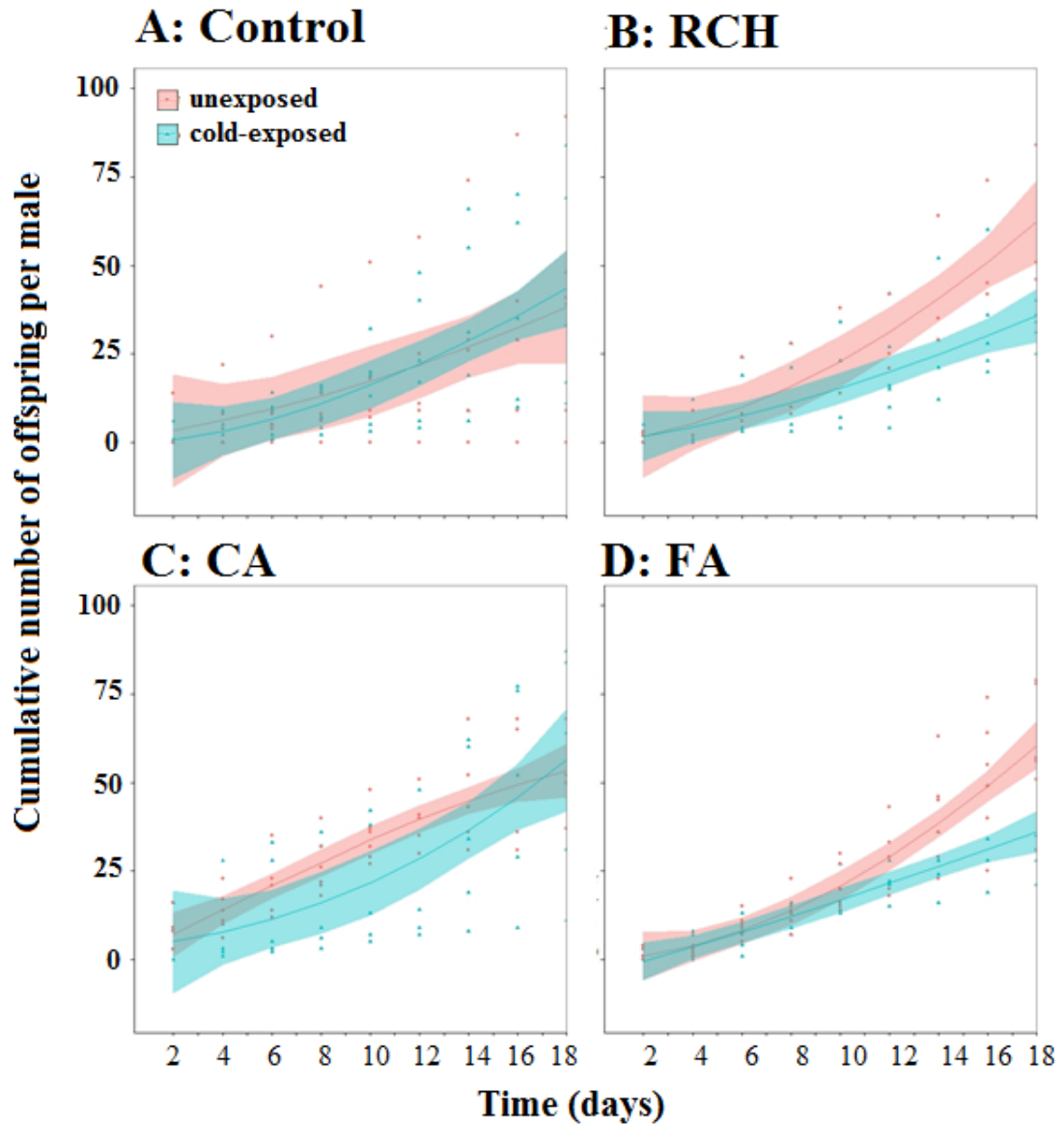
<b>Treatment</b>	<b>Term</b>	<b>df</b>	<b>F</b>	<b>P</b>
Control	<b>Exposure</b>	1	7.542	<b>0.015</b>
	<b>Remating</b>	1	4.7	<b>0.0494</b>
	Exposure $\times$ remating	1	0.004	0.95
	Error	15		
RCH	Exposure	1	3.37	0.083
	Remating	1	1.95	0.18
	Exposure $\times$ remating	1	0.54	0.48
	Error	15		
CA	<b>Exposure</b>	1	13.766	<b>0.0014</b>
	<b>Remating</b>	1	0.49	0.5
	<b>Exposure <math>\times</math> remating</b>	1	0.828	0.37
	Error	20		
FA	<b>Exposure</b>	1	4.68	<b>0.0471</b>
	<b>Remating</b>	1	14.649	<b>0.0017</b>
	<b>Exposure <math>\times</math> remating</b>	1	0.127	0.726
	Error	15		



**Figure 3.10 Effect of low temperature exposure of *Drosophila suzukii* females on mass of female offspring.** Dry mass ( $\pm$  SEM) of female offspring of *D. suzukii* females of the control (A) rapid cold-hardening = RCH (B), constant acclimation = CA (C) and fluctuating acclimation = FA group was determined after rearing eggs that were laid within the first two days into adults at 21.5 °C. Mated females (mothers) of the different treatment groups were unexposed or exposed to -3.5 °C for an hour (exposure) and then not remated or remated (remating) with a single male.

**Table 3.5 ANOVA statistics describing the effect of low-temperature exposure of *Drosophila suzukii* females on the mass of female offspring.** Mated females of the different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA, fluctuating acclimation = FA) were unexposed or exposed to -3.5 °C for an hour (exposure) and then not remated or remated (remating) with a single male. Retained terms with significant p-values ( $p < 0.05$ ) are bolded.

Term	Df	F	P
<b>Treatment</b>	<b>3</b>	<b>3.977</b>	<b>0.009</b>
Exposure	1	0.644	0.424
<b>Remating</b>	<b>1</b>	<b>5.745</b>	<b>0.018</b>
Treatment × exposure	3	2.362	0.074
Treatment × remating	3	0.131	0.942
Exposure × remating	1	0.064	0.801
Treatment × exposure × remating	3	0.775	0.510
Error	141		



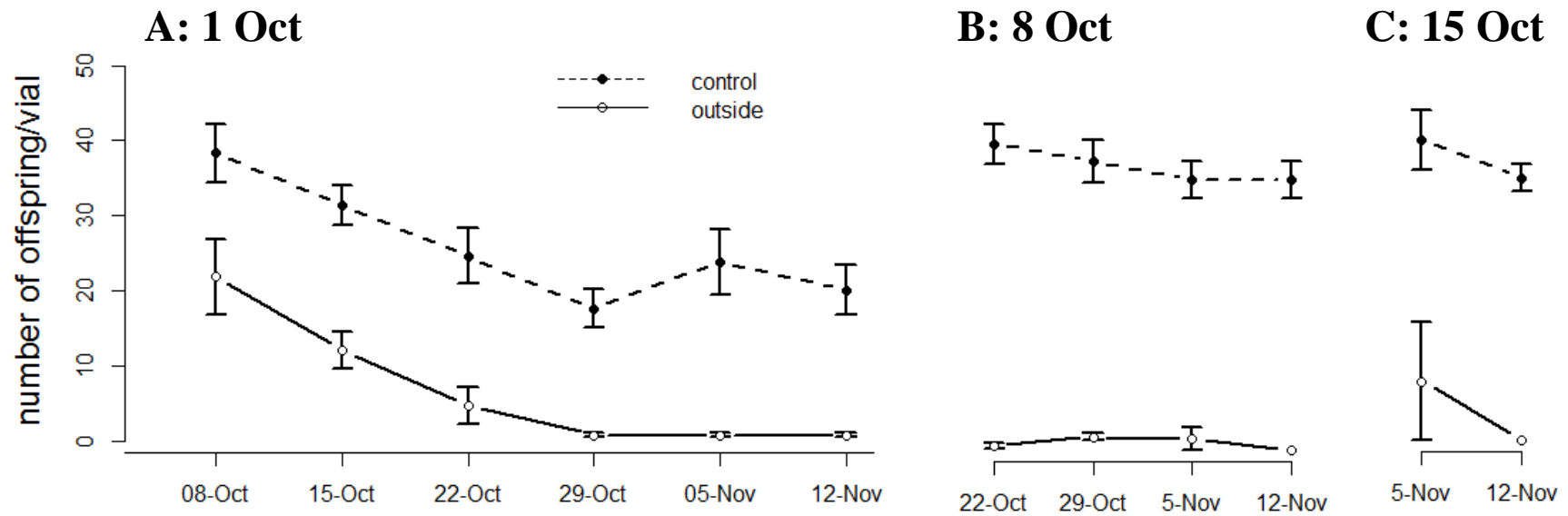
**Figure 3.11 Effect of low temperature exposure on reproductive output of *Drosophila suzukii* males.** Males of the treatment groups control (A), rapid cold-hardening = RCH (B), constant acclimation = CA (C) and fluctuating acclimation = FA (D) were unexposed or exposed to  $-3.5^{\circ}\text{C}$  for an hour and mated with one virgin female afterwards. The reproductive output as cumulative number of offspring was monitored over 20 days after the exposure. The dots indicate the cumulative number of offspring/male at each time point. The shaded area shows the 95 % confidence interval of the loess smoothed fit curve.

**Table 3.6 ANOVA statistics describing the effect of low-temperature exposure of *Drosophila suzukii* on the sex ratio of offspring.** Males or mated females of the different treatment groups (control, rapid cold-hardening = RCH, constant acclimation = CA, fluctuating acclimation = FA) were unexposed or exposed to -3.5 °C for an hour (exposure). Females were not remated or remated (remating) with a single male. Retained terms with significant p-values ( $p < 0.05$ ) are bolded.

Sex	Term	df	F	P
Female	Treatment	3	2.569	0.061
	Exposure	1	0.051	0.821
	Remating	1	1.187	0.279
	Treatment $\times$ exposure	3	1.418	0.244
	Treatment $\times$ remating	3	1.383	0.254
	Exposure $\times$ remating	1	0.037	0.847
	Treatment $\times$ exposure $\times$ remating	3	0.877	0.457
	Error	76		
Male	Treatment	3	2.244	0.0832
	Exposure	1	0.467	0.4947
	Treatment $\times$ exposure	3	1.35	0.2583
	Error	299		

### 3.4 Reproductive output in a semi-field environment in late fall

The effect of the semi-field environment in the fall (temperature and photoperiod) on the number of offspring produced by five females and males during one week was determined and compared to the reproductive output of flies held in an incubator at 21.5 °C. In the semi-field environment in fall, *D. suzukii* produced fewer offspring than under controlled, indoor conditions (21.5 °C and 13 L: 11 D) (Figure 3.12). In the semi-field environment *D. suzukii* produced very few offspring after 22 October and completely ceased egg laying after 12 November (date  $F_{5,242} = 7.025$ ,  $p < 0.001$ ; treatment  $F_{1,242} = 453.687$ ,  $p < 0.001$ ; date×treatment  $F_{5,242} = 2.555$ ,  $p < 0.05$ ). All flies were dead on 19 November. Temperature and day length decreased over time (Table 3.7). After 15 October, the temperature dropped below 0 °C. In the weeks before 29 October and 5 November the minimum temperature was -4.4 °C and -3.4 °C, respectively.



**Figure 3.12 Effect of semi-field cage conditions on the reproductive output of *Drosophila suzukii*.** Ten vials of five females and males were acclimated to October conditions and either placed outside in London, Ontario (43°00'N 81°15'W) or into an incubator at 21.5 °C and 13:11 L:D (control) on 1 October (A), 15 October (B) and 29 October (C). Flies were able to lay eggs for one week until vials were replaced (dates on x-axis). Eggs were reared into adults 21.5 °C and 13:11 L:D.



**Table 3.7 Temperature and hours of light *Drosophila suzukii* was exposed to in the reproduction field cage.** Weekly average temperatures  $\pm$  (SE), weekly minimum temperature (both measured in cages) and median day length (Lammi, 2008) flies experienced in a cages placed outside in London, Ontario (43°00'N 81°15'W).

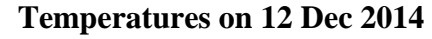
<b>Week</b>	<b>1 to 08 Oct</b>	<b>9 to 15-Oct</b>	<b>16 to 22 Oct</b>	<b>23 to 29 Oct</b>	<b>30 Oct to 05 Nov</b>	<b>06 to 12 Nov</b>
<b>Average temperature (°C)</b>	13.0 $\pm$ 0.3	11.3 $\pm$ 0.2	5.1 $\pm$ 0.1	6.6 $\pm$ 0.2	5.7 $\pm$ 0.2	5.3 $\pm$ 0.3
<b>Minimum temperature (°C)</b>	7.2	3.1	-0.9	-0.9	-4.4	-3.4
<b>Median day length</b>	11 h 36 min	11 h 15 min	10 h 55 min	10 h 36 min	10 h 17 min	9 h 58 min

### **3.5 Survival at different overwintering microhabitats in a semi-field environment**

The overwintering survival of female and male adult *D. suzukii* was determined in field cages at overwintering sites buried beneath leaf litter, in a shed, and directly exposed outside, starting on 26 November 2013. All female and male flies of all locations sampled on 18 December 2013 were dead after a cold snap on 12 December. The flies that were directly exposed outside experienced the lowest temperatures ( $T_{\min} = -14\text{ }^{\circ}\text{C}$ ), followed by the ones in the shed ( $T_{\min} = -7.4\text{ }^{\circ}\text{C}$ ) and the ones that were buried beneath leaf litter ( $T_{\min} = -5.6\text{ }^{\circ}\text{C}$ ) (Figure 3.13). On 12 December the flies directly exposed outside experienced more than 22 h below  $-8\text{ }^{\circ}\text{C}$  and the flies in the shed experienced  $-6\text{ }^{\circ}\text{C}$  for more than four hours.

## Temperatures on 12 Dec 2014

## Temperatures on 12 Dec 2014



## Temperatures on 12 Dec 2014

## 4 Discussion

The overall goal of my project was to delimit the tolerance of adult *D. suzukii* to the overwintering stress of low temperatures in Ontario, Canada. Evaluating whether *D. suzukii* can or cannot survive winter conditions in Ontario and establish permanently is important for planning successful pest management.

### 4.1 Acute effects of low temperatures on *D. suzukii* activity and survival

Low temperatures set a threshold for activity, including mating and feeding, in fall and spring, due to the onset of chill coma at the critical thermal minimum. The  $CT_{min80}$  ranged between +1.5 and -1.7 °C indicating that *D. suzukii* will not be active in winter (World Weather Online, 2014). Flies should be active and therefore found in traps at temperatures above the  $CT_{min}$ . However *D. suzukii* is only recorded in traps in Ontario between June and November (OMAFRA, 2014). The lack of *D. suzukii* in traps from March until June might be caused by mortality in the field and absence of repopulation from new sources.

$CT_{min}$  is used as a predictor for cold tolerance (Andersen et al., 2014) and is usually negatively-correlated with latitude (Gibert and Huey, 2001). The  $CT_{min80}$  of *D. suzukii* is in the range of known chill coma onset temperatures of *Drosophila*, which can range between -2.9 °C in *D. borealis*, a species found in cold regions (MacMillan, 2013; Markow and O'Grady, 2006), and 8.2 °C in *D. immigrans*, which are able to overwinter in cold-temperate regions (Kimura and Beppu, 1993; MacMillan, 2013). *Drosophila suzukii* has a similar  $CT_{min80}$  to *D. kanekoi* (-1.4 °C), which was originally collected in Hokkaido (Japan) (MacMillan, 2013), where the winter temperatures drop below -12 °C (World Weather Online, 2014); this could lead to the conclusion that the population of *D. suzukii* is from a cold-temperate region. However, *D. auraria* was also collected in same region as *D. kanekoi* and had a  $CT_{min80}$  of +2.5 °C (MacMillan, 2013) and thus  $CT_{min}$  might not be a good predictor for cold tolerance, because it is an estimate of activity and not survival.

Low-temperature survival depends on the ability to withstand freezing and chilling injury. Male and female adults of *D. suzukii* could not survive internal ice

formation or temperatures slightly above the SCP, which ranged between -17 °C and -23 °C. Thus, they are considered chill-susceptible (die of chilling injuries unrelated to freezing). Chill susceptibility and SCPs around -20 °C are common in *Drosophila* (Andersen et al., 2014; Czajka and Lee, 1990; Kimura, 1988b). The majority of insects are chill-susceptible and might therefore not be very cold hardy (Bale, 1996). However chill-susceptible species can survive winter conditions by overwintering in well buffered microhabitats, therefore minimizing low-temperature exposure (Danks, 1991). In the Yamanashi region in Japan *D. suzukii* overwinters beneath leaf litter (Kanzawa, 1939), yet winters in the Yamanashi region are warmer than winters in Ontario (World Weather Online, 2014). *Drosophila suzukii* might experience lower temperatures overwintering beneath leaf litter in Ontario, which causes mortality like that observed in the field cages.

Because *Drosophila suzukii* is chill-susceptible, low-temperature exposure can induce lethal chilling injuries. In control flies a one-hour exposure to around -5 °C led to lethal chilling injuries. Females and males had a LT<sub>80-1h</sub> of -7.5 °C and -7.2 °C respectively. The LT<sub>90-2h</sub> (lower temperature at which 90 % die after 2 h exposure) for various *Drosophila* species was between -3 °C in *D. ananassae* and -13 °C in *D. borealis* (Nyamukondiwa et al., 2011). Because *D. suzukii* would have to overwinter in habitats that are comparable to the native range of *D. borealis*, it would be expected that *D. suzukii* would need to have similar LT<sub>90-2h</sub> to *D. borealis*. It is difficult to compare the lower lethal temperatures among studies due to differences in rearing conditions and exposure time. However, extending the acute low-temperature exposure to two hours would be expected to increase the lethal temperature. This suggests that *D. suzukii* it is not as cold-hardy as *D. borealis* and not particularly cold-hardy relative to other *Drosophila* species.

Survival after acute cold exposure of control flies showed a sudden decline between -5 and -8 °C, which indicates that the mortality occurs over a relatively broad range. The high variation around the LT<sub>80</sub> in *D. suzukii* could have been caused by subtle differences in exposure or rearing conditions like differences in food quality (Colinet et al., 2012). However, I assessed survival after acute low-temperature exposure on several dates and there was no relation in survival to differences in rearing apparent in any trial,

indicating that subtle differences in exposure or rearing conditions did not affect  $LT_{80-1h}$ . Therefore, rearing or exposure conditions might not cause the variation I observed. Further variation in lethal temperature could be caused by genetic differences in the tested flies (Ayrinhac et al., 2004; Bubliy et al., 2002). A high genetic variation can be expected in this experiment, because I used an outbred population with (presumably) high genetic variation.

Neither females nor males of *D. suzukii* survived winter conditions in field cages which were directly exposed outside, stored in a shed or buried beneath leaf litter. Flies in the field cages in all locations experienced temperatures below -5 °C. The mortality under these conditions is not surprising, because mortality begins after 1 h exposure to -5 °C. Chilling injuries not only depend on the severity of low-temperature exposure, but also time period of exposure. While a one-hour exposure to 0 °C is non-lethal, a chronic cold exposure to 0 °C led to an  $Lt_{80}$  of 93 h (control) and 165 h (FA) in females and 70 h (control) and 112 h (FA) in males. As 0 °C is the temperature in a well-buffered overwintering habitat in the cold temperate regions, it indicates that *D. suzukii* is highly susceptible to accumulating lethal chilling injuries within a week of being outside, which explains the mortality of *D. suzukii* in the field cages along with the extreme cold snap.

#### **4.1.1 Plasticity of low-temperature tolerance in *D. suzukii***

Insects can change their cold hardiness in response to seasonal changes in fall. Rapid cold-hardening (RCH) increases cold tolerance after short term cold exposure and acclimation/acclimatization enhances cold hardiness on a longer term scale related to seasonal changes in temperature and photoperiod (Lee and Denlinger, 1991). Adult *D. suzukii* experienced a RCH treatment, constant acclimation (CA) and fluctuating acclimation (FA) to test for phenotypic plasticity of this species. Depending on the pre-treatment, *D. suzukii* adults displayed differences in the modification of low-temperature tolerance.

The RCH treatment did not change the cold tolerance strategy (chill-susceptible) or affect the SCP of *D. suzukii* adults. RCH increased  $CT_{min}$  in *D. suzukii*, although RCH decreased  $CT_{min}$  in *D. melanogaster* (Ransberry et al., 2011). The RCH group did not display an increase in acute low-temperature survival compared to the control group. This

lack in RCH response has been reported in a few drosophilids, that are not closely related to *D. suzukii*; however RCH increased survival by 14 to 90 % in a variety of other *Drosophila* species that are closely related to *D. suzukii* (Mitchell et al., 2013; Nyamukondiwa et al., 2011). *Drosophila suzukii* might not display an RCH response, because the right triggers for a RCH response were not provided in the experiment or *D. suzukii* does not have a RCH response. Rapid cold-hardening might only be induced in younger flies (Czajka and Lee, 1990), after pre-exposure to a lower temperature (Sinclair and Chown, 2006) or through ramping during the pre-treatment (Kelty and Lee, 1999). In a microclimate where the temperature drops below 0 °C often, an induction of RCH at 0 °C could be costly due to upregulation of heat shock proteins and selection may have acted against an RCH response at 0 °C (Sinclair and Chown, 2006). While *D. suzukii* did not exhibit an RCH response, acclimation did modify their cold tolerance, which shows that plasticity is not impossible.

Acclimation modified low-temperature performance in *D. suzukii*. Adults of the CA and FA groups were chill-susceptible and their SCP did not differ from the other treatment groups, but both CA and FA decreased chill coma recovery and mortality after acute cold exposure. This increase in low-temperature performance is common in *Drosophila* species (Rako and Hoffmann, 2006; Ransberry et al., 2011). Flies of the FA group survived an exposure to 0 °C three days longer than control flies. However, flies only survived for approximately a week at 0 °C. Surprisingly, flies of the CA group showed an increase in CT<sub>min</sub>, whereas flies of the FA group showed a decrease in CT<sub>min</sub> (as is expected for cold acclimation in *Drosophila*; Ransberry et al., 2011). While CA is beneficial for the flies, because it increases survival after acute cold exposure and decreases CCR, it could still be costly due to the physiological changes that increase cold tolerance (MacMillan and Sinclair, 2011; Storey, 1997). FA increases low-temperature performance in comparison to CA, which is not surprising, because ecologically-based thermoperiods are known to decrease mortality after acute cold exposure and CT<sub>min</sub> in *D. melanogaster* (Kelty and Lee, 2001). Fluctuating conditions simulate natural variation in photoperiod and temperature, which give cues, that are more likely to induce phenotypic plasticity (Colinet et al., 2014). In addition, the warm periods allow physiological

changes that insects cannot undergo during low-temperature periods (Colinet et al., 2014).

The lack of variation in SCP among treatment groups is not surprising, because a decrease in SCP would only be advantageous for freeze-avoiding insects that suppress the SCP to survive at lower temperatures (Lee and Denlinger, 1991). However some chill-susceptible insects depress the SCP due to physiological changes, like the increased glucose content in the chill-susceptible blow fly larvae *Calliphora vicina* Robineau-Desvoidy (Diptera: Calliphoridae) (Coleman et al., 2014). In the case of *D. suzukii*, because chilling injuries induce mortality at temperatures much higher than the SCP, modifying the SCP would be unlikely to alter cold tolerance.

The increased low-temperature performance observed in acclimated *D. suzukii* adults but not RCH adults likely occurred because long-term acclimation is based on different mechanisms than RCH (Teets and Denlinger, 2013). Acclimation induces accumulation of cryoprotectants (Lee and Denlinger, 1991), such as low molecular weight sugar alcohols like glycerol and sorbitol in *E. solidaginis* (Storey and Storey, 1983) and myo-inositol in *D. montana* (Vesala et al., 2012b). In addition, amino acids like proline increased cold tolerance in *D. melanogaster* larvae (Košťál et al., 2012), and several *Drosophila* species showed an increase in trehalose late fall (Kimura et al., 1992). RCH and acclimation both led to modifications of lipid membranes to increase membrane fluidity at low temperatures (Michaud and Denlinger, 2006; Overgaard et al., 2006; Pruitt and Lu, 2014). However modifications in the lipid membrane of *D. melanogaster* after RCH was not confirmed in all studies (MacMillan et al., 2009). Heat shock genes are only upregulated in acclimated, but not RCH-treated *Drosophila* (Vesala et al., 2012a). Cryoprotectants should be measured to investigate the mechanisms related to RCH and acclimation response in *D. suzukii*. If *D. suzukii* is able to accumulate cryoprotectants, it would suggest a mechanism by which low temperature tolerance is modified in these animals.

Cold-acclimated individuals not only perform better at low temperatures in the laboratory than non-acclimated individual, but also in the field at low temperatures (Kristensen et al., 2008; Terblanche, 2014). It was therefore expected that low



temperature acclimated *D. suzukii* would perform better under low-temperature conditions in the field. Although FA flies showed the highest cold tolerance of the tested flies, they could not survive a chronic cold exposure to 0 °C for more than a week. This indicated that acclimated adult *D. suzukii* have little potential to overwinter beneath leaf litter in Ontario, because the temperature beneath leaf litter is around 0 °C. The lack of overwintering potential in acclimated adult *D. suzukii* is supported by the mortality of flies that were held overwintering field cages beneath leaf litter.

While laboratory and semi-field experiments suggest *D. suzukii* is not sufficiently cold-hardy to overwinter in Ontario, a number of other factors may alter low-temperature performance. *Drosophila suzukii* is a multivoltine species, and therefore seasonal changes would not only affect adults, but also pre-adult stages. For example, cross-generation and developmental acclimation increased low-temperature performance in *D. melanogaster* (Bubliy et al., 2002; Ransberry et al., 2011; Watson and Hoffmann, 1995). To test the effect of cross-generation and developmental acclimation on low-temperature tolerance of *D. suzukii*, adults should be allowed lay eggs that are reared into adults (cross-generation and developmental acclimation; Watson and Hoffmann, 1995) or pre-adult stages should be reared into adults (developmental acclimation) under short day length and low temperatures (Ransberry et al., 2011). If cross-generation and developmental acclimation exists in *D. suzukii*, this should increase low-temperature performance of the offspring.

Food sources change with the season which could lead to changes in nutrient availability. Because food ingredients like yeast and sugars can affect cold tolerance (Colinet and Renault, 2014; Colinet et al., 2012), food ingredients could influence the thermal performance of *D. suzukii*. Also, *D. suzukii* is reared on artificial banana-based food, while *D. suzukii* is feeding on thin-skinned fruits such as blueberries in the field (Kanzawa, 1939), which could affect the low-temperature performance. Therefore the plasticity of low-temperature performance due to different food sources should be tested.

## **4.2 Effect of low-temperature exposure on reproduction**

Low temperatures not only affect survival and activity boundaries, but also reproduction (Hoffmann, 2010). Low-temperature exposure can decrease reproductive output as an

adaptive response through reproductive diapause (Kimura, 1988a) or a passive response due to low-temperature exposure, perhaps mediated by the direct effects of cold exposure on the reproductive system (Marshall and Sinclair, 2010). The effect of seasonal changes on ovarian development was determined by dissecting the ovaries of flies from the control, RCH, CA and FA group. To test the effect of low temperatures on reproduction of *D. sukukii*, flies were exposed to a non-lethal cold shock (-3.5 °C) and the reproductive output was determined.

Brief low-temperature exposure (2 h) can reduce the number of offspring in *D. melanogaster* (Marshall and Sinclair, 2010). In females of all treatment groups of *D. sukukii*, an exposure to -3.5 °C for 1 h decreased the number of offspring in comparison to non-cold exposed flies. Even though phenotypic plasticity increased low-temperature performance regarding survival and chill coma of *D. sukukii* from the FA and CA groups, none of the females of the different treatment groups showed an increase in low-temperature performance regarding reproductive output after cold exposure. The RCH treatment compounded the negative effect of low temperature on reproduction. The decrease in reproductive output due to acute low-temperature exposure could be caused by direct damage and effects on behaviour due to chilling injuries. Cold-exposed flies might allocate their resources to repairing the cold damage instead of reproduction (Marshall and Sinclair, 2010), and could display differences in courtship and mating behaviour (Shreve and Lee, 2004). Genes that control egg production are upregulated after cold exposure (Zhang et al., 2011) which could indicate that immature eggs are damaged during the cold exposure. In addition, low-temperature exposure led to damage or disposal of sperm stores of females in *D. melanogaster* (Ashburner et al. 2005). After cold exposure, remating increased the number of offspring, which might suggest a negative effect of cold on sperm stores. Thus, it appears that males would have to survive winter conditions to increase reproductive output and for *D. sukukii* to successfully establish in Ontario. However an increase in offspring can also be caused by the transfer of sex peptides in the seminal fluid, which increase the egg laying rate (reviewed by Kubli 2003).

While cold-exposed control and CA males had the same number of offspring as non-cold-exposed males after 18 days, RCH and FA males did not. Low temperatures could have a negative effect on sperm quality as shown in *D. melanogaster* (Watson and Hoffmann, 1995), as well as on courtship and mating activity of male *D. suzukii*. During courtship, *Drosophila* males flick, vibrate, and wave their wings (Spieth, 1974). Because cold exposure can damage the flight muscles (Yi et al., 2007) it might decrease courtship performance, although RCH decreased damage to flight muscles in *D. melanogaster* (Yi et al., 2007) and therefore might improve courtship and mating behaviour (Shreve and Lee, 2004). Because sperm production is not very energetically-costly compared to egg production, the effect of low-temperature exposure on the reproductive output of males is not as severe as on female reproductive output. All males were able to reproduce after low-temperature exposure, which shows protection or recovery of the gonads.

Cold-exposed females had fewer offspring than non-cold exposed females, but there were no differences in dry mass of the offspring from cold exposed and non-cold exposed females, suggesting that there is no trade-off between quantity and quality of offspring. Measuring the dry weight of offspring of adults might not be a sufficient measure to indicate a trade-off as eggs were laid and reared into adults at 21.5 °C. These rearing conditions might not lead to a difference between the offspring from cold exposed and non-cold exposed females regarding weight. In *D. melanogaster* rearing flies at lower temperatures increased body size and wing area (Frazier et al., 2008; Partridge et al., 1994). Additional measures of performance of these offspring could include measuring effect of parental acclimation on cold performance of the offspring, because in *D. simulans* the exposure of acclimated females to non-lethal cold shock leads to an increase in low-temperature performance of their offspring (Watson and Hoffmann, 1995).

Low-temperature exposure of females or males did not affect the sex ratio of the offspring, which indicated that there is no effect on sperm regarding sex determination. Long and Pischedda (2005) hypothesize that females can bias sex ratio, because females of *D. melanogaster* seem to be able to bias sex ratio towards females, when they are mated with old males, because sons of older males performed worse in mating assays than sons of younger males. This could indicate that cold-exposed males are not less

attractive than non-cold-exposed males. However, a mating choice experiment needs to be performed to verify this. In *D. melanogaster* repeated cold exposure leads to a male-biased sex ratio, which could be adaptive, or could be because females are more costly to produce than males (Marshall and Sinclair, 2010). A single cold exposure, however, did not affect the sex ratio in *D. melanogaster* (Marshall and Sinclair, 2010). Thus, a single cold exposure might not affect sex ratio in *D. suzukii*, and the effect of repeated cold exposures on the sex ratio should be tested.

Long-term low-temperature exposure may induce diapause (state of arrested development) and thus energy might be allocated to storage and not reproduction (Hahn and Denlinger, 2007). In some *Drosophila* species, females can enter an ovarian or reproductive diapause that is induced by low temperatures and short photoperiod (Saunders et al., 1989; Williams and Sokolowski, 1993). In *D. suzukii*, none of the treatments led to reproductive diapause, but FA females had significantly smaller ovaries with fewer eggs than the other treatment groups. All flies still produced viable offspring. Flies of the FA treatment might undergo a trade-off between reproduction and accumulation of energy reserves to prepare for winter (Hahn and Denlinger, 2011). An increase in energy stores might help *D. suzukii* to cope with the winters in Ontario - particularly in mild microhabitats such as beneath snow or in buildings, and make them more likely to establish, which could be tested in future.

Reproductive diapause might not have been induced in my experiments due to a lack of capacity for diapause, or because the environmental cues inducing reproductive diapause were absent. Populations of *Drosophila* species and populations from higher latitudes entered reproductive diapause at longer days (earlier in the year) than populations from lower latitudes (Ichijo, 1986; Kimura et al., 1993). Thus, the cues to induce diapause are highly variable among species and populations, and the RCH, FA and CA treatments might not be sufficient to induce reproductive diapause in *D. suzukii*. In addition, reproductive diapause in *D. melanogaster* was induced during pre-adult stages (Williams and Sokolowski, 1993) or in newly-eclosed (< 8h) adults (Saunders et al., 1989). Because I only used females that were at least one day old, it is most likely that the flies had developed past the critical period for diapause determination.

Determining if developmental acclimation or acclimation of virgin females might trigger reproductive diapause would be necessary to determine overwintering survival because low-temperature tolerance was affected by ovarian diapause in cold-temperate species but not in warm-temperate species (Kimura, 1988b). In *D. melanogaster* the proportion of diapausing flies increased with latitude, which might indicate selection pressure under low-temperature stress towards diapause (Williams and Sokolowski, 1993). Drosophilids that are not able to enter reproductive diapause might have low overwintering potential under natural conditions and might only be able to overwinter in human-made structures (Kimura, 2004). If *D. suzukii* has no reproductive diapause, it could indicate that *D. suzukii* has low overwintering potential in cold regions.

There were no offspring from adults in field cages in late fall, which is most likely a passive cold effect rather than reproductive diapause, since the temperatures in the field cage dropped below -3.5 °C. It seems unlikely that the cessation of reproduction is caused by *D. suzukii* in the field entering reproductive diapause, because I only put adults into the field cages and adults in the laboratory did not enter reproductive diapause; however, ovaries of the flies in the semi-field cages were not dissected.

### **4.3 Ecological relevance**

Laboratory studies reveal limits for stress resistance, and the results can be extrapolated to field conditions (Terblanche, 2014). The critical thermal minimum may be a good predictor for activity in early fall and spring, and determining the cold tolerance strategy allows making predictions about the overwintering microhabitat. In addition, determining the effect of acute low-temperature exposure on reproduction and survival allows us to delimit the effects of low-temperature exposure on fitness. In the lab, chronic exposure to 0 °C led to an  $Lt_{80}$  of 165 h and 112 h in females and males of the FA group respectively. Chronic exposure to 0 °C simulates low-temperature exposure in a well-buffered overwintering habitat like beneath leaf litter and snow cover (Danks, 1991). Because *D. suzukii* died after eight days in the chronic cold experiment, it is not surprising *D. suzukii* beneath leaf litter in the semi-field cage died.

However, laboratory experiments cannot fully replicate natural conditions. In my experiment I used a single one-hour cold exposure to test the effect of low temperature on

survival. Under natural conditions flies would experience multiple cold exposures, which has resulted in less mortality in *D. melanogaster* than flies exposed to low temperatures for the same time period (Marshall & Sinclair, 2010). Low-temperature tolerance of *D. suzukii* might also increase under multiple cold exposures, therefore simulating this would allow for better prediction of survival of *D. suzukii* under natural conditions. Fluctuating temperatures that result in multiple cold exposures can have both negative and positive effects on low-temperature performance (Colinet et al. in press). Warm periods can be especially energetically costly, because they lead to an increase in metabolic rate (Williams et al., 2012a), and consumption of ATP to re-establish ion homeostasis to recovery from chill coma (MacMillan et al., 2012). Nonetheless, temperature fluctuations that result in low enough temperatures can induce RCH and increase survival at low temperatures (Colinet et al., in press). However *D. suzukii* might lack an RCH response, and therefore might not show an increase in low-temperature tolerance after repeated cold exposure. Because the responses to fluctuating temperatures and repeated cold can be idiosyncratic (Colinet et al., 2014; Marshall and Sinclair, 2012b), the effects of repeated cold on *D. suzukii* need to be explicitly explored in the laboratory.

In the field, insects experience multiple abiotic and biotic stressors that interact and vary on spatial and temporal scales, which can result in lower survival in the field than in the lab (Košťál et al., 2014). Overwintering insects experience desiccation stress, because they cannot acquire water when much of the environmental water is frozen, and dormant insects may lack the ability to adjust water balance (Danks, 2000). In addition, sub-zero temperatures create a desiccating environment, because the vapor pressure of the frozen environment is lower than the one in the insect's body (Danks, 2000). Desiccation stress not only affects survival but also reproduction, as shown in *C. pipiens*, where desiccation stress decreased energy stores and egg-laying capacity (Benoit et al., 2010). Overwintering insects also risk starvation as food resources are unavailable and insects might not feed during winter. The temperature in the overwintering habitat directly influences energy consumption, and therefore not only affects survival but also reproduction (Irwin and Lee, 2003; Williams et al., 2012a). Because overwintering insects experience these stressors simultaneously, it is not surprising that the protective

mechanisms (“cross-tolerance”) and signaling pathways (“cross-talk”) may overlap (Sinclair et al., 2013). However, there are few studies on the interaction of multiple stressors in *Drosophila*. To fully understand the effect of the interaction of multiple stressors during winter on survival of *D. suzukii*, flies should be exposed to related stressors such as desiccation or starvation at various temperatures and humidities.

Low-temperature performance can vary among populations (Sinclair et al., 2012). Depending on the origin, flies could display differences in cold tolerance as there is a latitudinal cline in cold tolerance for a wide variety of drosophilids (Hoffmann et al., 2001). For example, the cosmopolitan *D. melanogaster* recovers slightly faster from chill coma if the individual originated from a population in temperate, compared to tropical regions (Gibert et al., 2001). In *D. suzukii*, low-temperature tolerance measurements were only performed with adult flies originating from a population created by flies caught in the Halton Hills region. In other regions, populations of *D. suzukii* could originate from warmer or colder regions, and might therefore display differences in thermal performance. While it is unclear if the populations in Ontario differ in their origin, genetic differences between populations in the United States and Europe indicate different introduction events (Adrion et al., 2014). Populations in Ontario will likely undergo high selective pressure for low-temperature tolerance and might evolve higher tolerance over time if any survive and reproduce (Gibert et al., 2001; Hoffmann et al., 2001). Because *D. suzukii* shows genetic variation and phenotypic plasticity in low temperature performance, there could be selection towards an increased low-temperature tolerance leading to local adaptation in Ontario (Gibert and Huey, 2001; Hoffmann et al., 2003). Lab adaptations (Gilchrist et al., 2014) and inbreeding (Bechsgaard et al., 2013) could have changed the cold tolerance in the tested flies; however multiple studies indicate that among-species variation remains under laboratory conditions (Ayrinhac et al., 2004; Kellermann et al., 2009), and that cold tolerance is not affected (Strachan et al., 2011).

In this project only adults were used for experiments, as adults were the overwintering life stage in Japan (Kanzawa, 1939) and many drosophilids overwinter as adults (Kimura, 1988b). Low-temperature performance might be different for eggs,

larvae or pupae. Most of the *Drosophila* larvae are chill-susceptible; however larvae of some species are freeze-avoiding (Strachan et al., 2011), which might indicate that larvae of some species can survive low-temperature exposure and overwinter. Only five out of the 22 tested larvae species showed an increase in survival after RCH (Strachan et al., 2011), while the majority of adults showed a RCH response (Nyamukondiwa et al., 2011), which implies that adults are more likely to show a RCH response (Mitchell et al., 2013). Although it seems most likely that adults are the overwintering life stage, the low-temperature performance of pre-adult stages should be assessed to further delimit overwintering potential.

#### **4.4 Does *D. suzukii* have the potential to successfully overwinter in Ontario?**

Chill coma, and therefore activity boundaries, depend on the intensity and time period of low-temperature exposure (MacMillan and Sinclair, 2011). *Drosophila suzukii* entered chill coma after an exposure to 0 °C for approximately one hour, and thus it is expected to be in chill coma when temperatures drop below 0 °C for a longer period. In Ontario, the average air temperature can drop below 0 °C from November until March (World Weather Online, 2014), which limits *D. suzukii*'s activity during these months if the temperatures or time periods of low-temperature exposure sufficient. However the Ontario Ministry of Agriculture, Food and Rural Affairs has reported *D. suzukii* in traps from June until late November (OMAFRA, 2014), suggesting that the absence of *D. suzukii* is not only limited by activity.

*Drosophila suzukii* is chill-susceptible, not very cold tolerant, and cannot survive direct exposure to low air temperatures in winters regularly recorded in Ontario. As a consequence, they likely utilize microhabitats that do not expose them to extremely low temperatures. Such microhabitats could be beneath leaf litter as found in Japan (Kanzawa, 1939), or in human-made structures. Nonetheless, neither females nor males of *D. suzukii* survive winter conditions in field cages which were stored in a shed or buried beneath leaf litter.

In the Yamanashi region in Japan adults were found to be overwintering beneath leaf litter (Kanzawa, 1939), but the temperatures that *D. suzukii* might be experiencing in



that microhabitat might be higher as the average low temperature in the Yamanashi region is higher than in Ontario. In Ontario the temperatures in winter 2013/2014 were lower on average with more extreme minima, and temperatures remained lower for a longer period than in the years 2011/2012 and 2012/2013 in Ontario (Climate Canada, 2014), which probably led to mortality of *D. suzukii* in the field. In warmer years the survival might be higher.

In Ontario, the first captures of *D. suzukii* in the year are usually in June or July (OMAFRA, 2014). This relatively late capture in the season supports the claim that *D. suzukii* cannot survive in the winter conditions in Ontario, and suggests that *D. suzukii* re-invades Ontario from warmer regions, where they can overwinter. *Drosophila suzukii* could re-invade Ontario through passive migration in imported infested fruits. Therefore, limiting fruit transport from regions where *D. suzukii* might be overwintering could be important in limiting the economic impact of this pest in Ontario.

Low-temperature exposure has a negative impact on the reproductive output of females, which impedes their ability to establish a population in cold temperate climates. However remating increased the number of offspring after cold exposure, which indicates that if males survive winter and then reproduce, the population could recover following cold conditions. Males were able to reproduce after cold exposure, which might be caused by low costs of sperm production. While there was no evidence for reproductive diapause after acclimation of adults, it might be necessary to acclimate pre-adult stages to cease ovarian development (Saunders et al., 1989).

In Ontario, overwintering in human-made structures has been reported for various invasive pest species like the western conifer seed bug, *Leptoglossus occidentalis* Heidemann (Hemiptera: Coreidae), (Blatt, 1991), the Eastern subterranean termite (Clarke et al., 2013) and the brown marmorated stink bug (Lee et al., 2014). If *D. suzukii* overwinters in heated man-made structures, food and water sources might be limited, which thus may cause starvation and desiccation stress. Insects may tolerate desiccation through a higher initial water content, lower water loss rate and/or a lower lethal water limit (Danks, 2000). Therefore, determining survival, water content, lower water loss rate, and lower lethal water limit under desiccating conditions would be necessary to

delimit desiccation tolerance of *D. suzukii* and test its likelihood to survive in human-made structures. In order to survive starvation stress, insects accumulate energy store prior to overwintering. This energy supply must be sufficient to last through winter, as well as for metamorphosis and reproduction after winter (Hahn & Denlinger, 2011). As flies are ectotherms, their body temperature depends on the environmental temperature, which directly influences the metabolism and therefore energy utilization. Insects decrease their metabolic rates through ecological and physiological changes in order to conserve their energy stores. To delimit starvation resistance it is necessary to measure initial energy stores, the use of energy under starvation conditions, and lower lethal energy stores.

## 4.5 Conclusions and recommendations

Low winter temperatures in Ontario set boundaries for reproduction, activity and survival in *D. suzukii*. Cold shock reduces reproductive output of females; however, remating after cold shock increased the number of offspring, which indicates that both females and males have to survive winter conditions to successfully establish. The critical thermal minimum indicates that *D. suzukii* will not be active below 0 °C, which will restrict its activity from November until April in Ontario. This chill-susceptible species did not show a RCH response, and although acclimation of adults increased low-temperature performance, flies could not survive chronic cold exposure to 0 °C for more than eight days. Thus, it is not surprising that flies placed outside in field cages beneath leaf litter during winter 2013/2014 did not survive. Although developmental acclimation might induce reproductive diapause and could increase cold tolerance, we know that acclimated adult flies are unable to survive winter field conditions in Ontario. One consideration is that the winter of 2013/2014 was an extremely cold winter, and warmer winters might not kill *D. suzukii*. This could lead to a stochastic invasion of *D. suzukii*, due to successful overwintering in warm winters and mortality in cold winters.

*Drosophila suzukii* seems to be unable to survive winter conditions in Ontario. However these flies might be able to overwinter in heated, human-made structures. Additionally, or alternatively, they may be imported with infested fruits each spring. While future research investigating desiccation and starvation stress of *D. suzukii* is

needed to delimit their overwintering potential in human-made structures, it may be proactive to limit potential overwintering habitats. In addition, measures need to be adopted to reduce or manage the rate of importation of potentially infested fruits.

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# Curriculum Vitae

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