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Reproductive arrest and stress resistance in winter-acclimated *Drosophila suzukii*

Running title: **Winter-acclimated *D. suzukii***

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Abstract

Overwintering insects must survive the multiple-stress environment of winter, which includes low temperatures, reduced food and water availability, and cold-active pathogens. Many insects overwinter in diapause, a developmental arrest associated with high stress tolerance. *Drosophila suzukii* (Diptera: Drosophilidae), spotted wing drosophila, is an invasive agricultural pest worldwide. Its ability to overwinter and therefore establish in temperate regions could have severe implications for fruit crop industries. We demonstrate here that laboratory populations of Canadian *D. suzukii* larvae reared under short-day, low temperature, conditions develop into dark 'winter morph' adults similar to those reported globally from field captures, and observed by us in southern Ontario, Canada. These winter-acclimated adults have delayed reproductive maturity, enhanced cold tolerance, and can remain active at lower temperatures, although they do not have the increased desiccation tolerance or survival of fungal pathogen challenges that might be expected from a more heavily melanised cuticle. Winter–acclimated female *D. suzukii* have underdeveloped ovaries and altered transcript levels of several genes associated with reproduction and stress. While superficially indicative of reproductive diapause, the delayed reproductive maturity of winter-acclimated *D. suzukii* appears to be temperature-dependent, not regulated by photoperiod, and is thus unlikely to be 'true' diapause. The traits of this 'winter morph', however, likely facilitate overwintering in southern Canada, and have probably contributed to the global success of this fly as an invasive species.

Key words

Spotted wing drosophila, winter morph, reproductive diapause, stress tolerance, overwintering

1. Introduction

Winter imposes cold, desiccation, immune challenge and food limitation on ectotherms, and is a key determinant of the biology and life history of temperate, montane, and polar insects (Williams et al., 2015). As temperature decreases, insects enter a reversible state of neuromuscular paralysis, known as chill coma, at the critical thermal minimum (CT_{min}) (MacMillan and Sinclair, 2011), before freezing at the supercooling point (SCP) (Lee, 2010). Many insects are chill-susceptible, dying of cold injuries unrelated to freezing, with a lethal temperature (LT) above the SCP (Bale, 1996). More cold-tolerant insects are generally categorised as either freeze-tolerant, surviving internal ice formation, or freeze-avoidant, maintaining a low SCP, but being killed by internal ice (Lee, 2010). Overwintering insects usually need to survive long periods of cold exposure – often at mild temperatures if they overwinter under the snow (Petty et al., 2015; Sømme, 1996), and because they usually cannot feed, they must either depress metabolic rate or accumulate energy reserves pre-winter (Sinclair, 2015). Because they may be exposed to vapour-pressure deficits and cold-active pathogens, they are also likely to have enhanced desiccation tolerance and immune function (Sinclair et al., 2013). Thus, we expect a suite of enhanced stress tolerances to allow overwintering insects to survive the multiple-stress environment of winter (Williams et al., 2015).

Physiological responses by ectotherms to stress are often plastic, varying across developmental stages and seasons. Within a life stage, stress tolerance can change through hardening, acclimation, or acclimatization responses (e.g. Ransberry et al., 2011). Hardening may occur on short time scales, such as rapid cold hardening (RCH), where brief exposures to low temperatures (e.g. 1 h at 0°C) induce a suite of physiological changes that confer improved

cold tolerance (Teets and Denlinger, 2013). By contrast, longer term responses such as acclimation may occur over days-to-weeks, or across development. One seasonally-plastic response is diapause, a state of developmental arrest characterised by reduced metabolism and enhanced stress tolerance that is initiated, maintained, and terminated by specific environmental cues (Koštál, 2006). Many temperate and polar insects overwinter in diapause, a state that is often induced by short photoperiods, and is promoted by low temperatures (e.g. Lankinen et al., 2013; Spielman and Wong, 1973; Tatar and Yin, 2001). Diapause termination often requires specific cues, which may be some combination of temperature and photoperiod, and diapause is often only terminated after a required time period of dormancy (Tauber et al., 1986).

Overwintering adult insects may enter reproductive diapause, usually with high lipid reserves to fuel overwinter metabolism (e.g. Ohtsu et al., 1992; Sim and Denlinger, 2013), suppressed ovarian development (e.g. Sim and Denlinger, 2009), and enhanced tolerance of cold (e.g. Vesala and Hoikkala, 2011) and desiccation (e.g. Benoit and Denlinger, 2007). The onset of reproductive diapause is accompanied by suppressed insulin and juvenile hormone (JH) signalling (Denlinger, 2002), causing an upregulation of *foxo* (*forkhead transcription factor*) (Sim et al., 2015) and altered transcription of genes pertinent to vitellogenesis (e.g. Baker and Russell, 2009; Sim and Denlinger, 2009), fat accumulation (e.g. Sim and Denlinger, 2013) and stress tolerance (e.g. Baker and Russell, 2009). The latter includes genes that encode heat shock proteins (e.g. King and MacRae, 2015; Rinehart et al., 2000) and antioxidant enzymes (e.g. Sim and Denlinger, 2011). Diapause may also enhance immunity through upregulation of immune-responsive genes, possibly protecting against infections over winter (e.g. Poelchau et al., 2013; Ragland et al., 2010). In most species, patterns of gene expression in diapause are markedly

different to those associated with temperature-induced quiescence, for example, *Drosophila montana* upregulate *actin*, *catalase*, *period*, and *Thor* when in diapause compared to quiescence (Salminen et al., 2015).

Adult *Drosophila* are generally chill-susceptible (Nyamukondiwa et al., 2011), but often have phenotypically-plastic stress tolerance. For example, combinations of hardening, rearing, and acclimation conditions lead to significant within-population variation in CT_{min} and chill coma recovery time in *Drosophila melanogaster* (Ransberry et al., 2011). Many *Drosophila* spp. overwinter in apparent reproductive diapause with suppressed ovarian development (Carson and Stalker, 1948; Kimura, 1988; Lumme et al., 1975; Muona and Lumme, 1981; Schmidt et al., 2005; Tyukmaeva et al., 2011; Watabe, 1983), higher lipid reserves (Ohtsu et al., 1992; Schmidt et al., 2005) and enhanced cold tolerance (Schmidt and Paaby, 2008). However, diapause incidence can vary considerably within a species, with diapause-inducing conditions failing to suppress ovarian development in Australian *D. melanogaster* populations (Lee et al., 2011). Often, this variation occurs along a cline, as has been observed for several *Drosophila* spp. (Lankinen et al., 2013; Schmidt and Paaby, 2008; Tyukmaeva et al., 2011). In *Drosophila* spp., the term "reproductive diapause" has been used to describe a general ovarian dormancy, without necessarily requiring that the dormancy be maintained and terminated by specific cues. In this paper we will adhere to the more stringent definition of diapause outlined by Koštál (2006), which requires that the dormancy be maintained by environmental cues other than temperature.

Many overwintering *Drosophila* adults have enhanced melanisation (Gibert et al., 2007; Stephens et al., 2015). Darker cuticles in *Drosophila* are associated with reduced water loss rates

113 (Parkash et al., 2014; Rajpurohit et al., 2008; Ramniwas et al., 2013) and increased immune
114 function (Dubovskiy et al., 2013; Kutch et al., 2014). The upregulation of the immune-related
115 gene *Thor* (Bernal and Kimbrell, 2000) in *D. montana* diapause (Salminen et al., 2015) also
116 suggests enhanced immunity in association with reproductive diapause.
117
118 *Drosophila suzukii* Matsumara (Diptera: Drosophilidae), commonly known as spotted
119 wing drosophila, has recently emerged as a global pest of soft fruit (Walsh et al., 2011). *D.*
120 *suzukii* females have a serrated ovipositor, which allows them to lay eggs in unripe fruit, causing
121 major economic losses, particularly in stone fruit (cherry, peach, plum, nectarine), raspberry,
122 blackberry, blueberry, strawberry, and grape crops (Deprá et al., 2014; Hauser, 2011; Walsh et
123 al., 2011), causing severe economic damage (Bolda et al., 2010). *D. suzukii* originates from
124 Southeast Asia with first descriptions from Japan (Kanzawa, 1939), where it mainly inhabits
125 warm-temperate regions (Kondo and Kimura, 2008). Since first recorded in North America in
126 California in 2008, *D. suzukii* has spread to Oregon, Washington, Florida and British Columbia
127 (Canada) in 2009, and was recorded in Utah, Louisiana, North Carolina, South Carolina,
128 Wisconsin, Michigan, Alberta, Manitoba, Ontario and Quebec in 2010 (reviewed by Hauser,
129 2011). Thus, it has spread to temperate climates where it is exposed to more severe winter
130 conditions. While *D. suzukii* adults reared under summer-like conditions have limited cold
131 tolerance (Jakobs et al. 2015), 'winter morph' adults with darkened cuticles have been captured
132 in temperate regions in autumn (Stephens et al., 2015), and are probably the overwintering life
133 stage (Kanzawa, 1939). Winter morph females have reduced ovarian development (Mitsui et al.,
134 2010) and better tolerate brief exposure to low temperatures (Stephens et al., 2015) than their
135 summer morph counterparts, and have been described as being in reproductive diapause (Zerulla

et al., 2015). However, it has not been established whether this is a true diapause, and tolerance to other stresses pertinent to overwintering biology (e.g. desiccation) have not been investigated in either morph of this species.

Here we show that pupal development under low temperature and short day conditions (‘winter-acclimation’) induces a dark morph similar to the winter morph. Our goal was to determine whether these winter-acclimated *D. suzukii* have the physiological and molecular characteristics of a true diapause, and whether they have enhanced stress tolerance relevant to overwintering. We examine ovarian development in response to different temperature and photoperiod cues, as well as energy stores, longevity, tolerance of cold and desiccation stress, immunity and expression of stress- and reproduction-related genes. We show that low temperatures delay reproductive maturity, and that winter-acclimated flies are more stress-tolerant than summer-acclimated *D. suzukii*. Because changes in winter-acclimated flies appear to be regulated largely by temperature, we express doubt that this is a ‘true’ diapause.

2. Materials and methods

2.1. Animal rearing and treatment groups

We used the laboratory population of *D. suzukii* described by Jakobs et al. (2015) which was established from approximately 200 individuals collected in the Halton Hills region, Ontario, Canada (43°34’N 79°57’W, c. 200 m a.s.l.). The population was reared at 21.5±1°C and 60±5% relative humidity under 13:11 L:D, at a constant density (c. 70-100 individuals per 35 ml vial) on a banana medium (Markow and O'Grady, 2005), with a large outbred population maintained using population cages (Rajamohan and Sinclair, 2008). We also maintained a laboratory

population of *D. suzukii* collected in Agassiz, British Columbia, Canada (49°24'N 121°76'W, c. 12 m a.s.l.). Unless otherwise specified, we used the Ontario population for the experiments described below.

We also opportunistically collected winter morph flies from the field. At dusk on October 17, 2014, one of us (LVF) observed hundreds of wild *D. suzukii* on the windows of Nature Conservancy of Canada's Ivey Research Station on Pelee Island, Ontario, Canada (41°48'N 82°37'W). We collected males and females and later confirmed the identification as *D. suzukii* using wing and genital morphology (Fig. 1A). We returned the animals to the laboratory, photographed whole flies and dissected ovaries from three females.

Summer-acclimated flies (Fig. 1B, D) were held under the rearing conditions (21.5±1°C, 60±5% relative humidity, 13:11 L:D). To induce a phenotype similar to the winter morph, vials containing wandering third instar larvae were transferred into 11°C, 10:14 L:D (MIR153, Sanyo, Bensenville, Illinois, USA). These winter-acclimated adults had a darkened cuticle (Fig. 1C), and ovaries were absent or underdeveloped 10 d post-eclosion, while summer morph females had fully-developed ovaries at this time (Fig. 2A). Flies were collected on the day of eclosion and sorted by sex under brief CO_2 exposure either immediately or 3 d after eclosion; we allowed flies at least three days under the rearing conditions to recover from CO_2 exposure prior to use in experiments, so most experiments were begun on flies 5-6 d post-eclosion (Nilson et al., 2006). All quantitative experimental data is available in a supplementary Excel spreadsheet (metadata.xlsx).

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4 182 *2.2. Ovarian development*
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7 183 We examined reproductive development in Ontario laboratory winter-acclimated flies by
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9 184 visually assessing ovarian maturity in females reared under 11ºC and 15ºC, at both short (10:14
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12 185 L:D) and long (14:10 L:D) day lengths. We dissected ovaries from five females at 5, 10, 15, 20,
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14 186 and 30 d post-eclosion. We classified ovarian development into three stages: no development, in
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17 187 which the ovaries contained no visible yolk; partial development, in which the ovaries contained
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19 188 eggs partially filled with yolk; and full development, in which all eggs contained complete yolk
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22 189 (Fig. 2A-C). We also examined ovaries from winter-acclimated flies originating from British
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24 190 Columbia at 5 and 30 d post-eclosion, and from the flies collected from Pelee Island upon return
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27 191 to the laboratory.
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31 193 *2.3. Energy stores*
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34 194 We quantified neutral lipid and carbohydrate content of summer and winter-acclimated flies 8 h
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36 195 post-eclosion, following Williams (2012). Briefly, we homogenised groups of three flies in 1 ml
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39 196 chloroform: methanol (2:1 v/v; lipids) or ten flies in 200 µl 30% KOH (carbohydrates). We
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41 197 extracted neutral lipids from the homogenate using a modification of the Folch method and
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44 198 quantified them using TLC-FID (Iatroscan MK-6, Shell-USA, Fredericksburg, VA, USA), with
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46 199 cholesterol (not detectable in these flies) as an internal standard to correct for loss during
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49 200 extraction (Williams et al., 2011). We quantified total carbohydrates using the anthrone method
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51 201 (Carroll, 1955); briefly, we extracted glycogen from homogenised tissue suspensions with heat
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53 202 (100ºC, 20 min), added 300 µl ethanol to 150 µl of homogenate and precipitated the glycogen
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56 203 from solution with 7.5 µl of saturated $NaSO_4$. We dried and resuspended the glycogen pellet in
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58 204 water, added 750 µl of anthrone reagent (0.05% anthrone, 1% thiourea, 1 l 72% H_2SO_4), cooled
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4 205 samples in an ice bath, heated samples at 100ºC for 15 min, and re-cooled samples in an ice bath.
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12 208 We expressed lipid and carbohydrate content as μg/mg body tissue. We compared both
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14 209 quantities between summer- and winter-acclimated flies in R version 3.0.1 (R Development Core
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17 210 Team, 2013) using ANOVA with a square-root transformation of lipid content to meet
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19 211 assumptions of normality. Interactions between sex and acclimation were not significant and
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22 212 were removed to improve the fit of the model (Crawley, 2012).
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27 214 *2.4. Longevity and offspring production*
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29 215 To determine if winter-acclimated flies have a longer lifespan than summer-acclimated flies, we
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31 216 placed groups of 8-10 flies (n= 3-10 groups/treatment) into vials containing banana medium at
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34 217 21.5°C and 13:11 L:D, and counted the number of surviving flies for each vial every 3-4 d for
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36 218 152 d. Because winter-acclimated females had undeveloped ovaries when isolated, and are thus
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39 219 unlikely to reproduce, we accounted for the effect of a decreased longevity through reproduction
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41 220 in case of the mated summer-acclimated females by also using virgin summer females. Thus the
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44 221 six experimental groups included mated and virgin summer-acclimated males and females; and
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46 222 winter-acclimated males and females. Summer-acclimated virgins were separated by sex within
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49 223 8 h of eclosion, and all other flies were sorted into vials 3 d post-eclosion. Flies were transferred
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51 224 to vials with fresh medium at each observation. Vials were retained for one month post-transfer
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53 225 to count the number of offspring produced by each group of females. We arcsine-square-root-
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56 226 transformed the proportion of surviving flies at each time point and used a non-linear mixed
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effects models in R with "vial" as a random effect to compare survival curves between experimental groups.

2.5. Gene expression

We used reverse transcriptase quantitative PCR (RT-qPCR) to quantify transcript abundance of genes associated with reproductive development, diapause, stress tolerance, and melanisation (Table 1) in summer- and winter-acclimated flies during development. We collected individuals at the following developmental stages: summer- and winter-acclimated wandering larvae, early pupae (within 24 h of pupation), female late pupae (24 h before eclosion), 1- and 5-d-old female adults; and winter-acclimated female adults 30 and 60 d post-eclosion. For each life stage, we extracted total RNA from three samples of 8-10 individuals using TRIzol (Invitrogen, Burlington ON, Canada), according to the manufacturer's instructions. We synthesised cDNA (qScript cDNA Supermix; Quanta Biosciences, Gaithersburg, MD, USA) from 500 ng of DNase-treated RNA (PerfeCTa DNase I; Quanta Biosciences), according to the manufacturer's protocols.

We designed primers (Table S1) to amplify 150 bp products for qPCR based on sequences from the *D. suzukii* genome (Chiu et al., 2013), with the exception of *cpo*. Because this sequence was unavailable from the *D. suzukii* genome assembly, we aligned *cpo* sequences available in NCBI from other *Drosophila* spp. using Clustal Omega (Sievers et al., 2011), and restricted primer design to conserved regions of this gene (see Fig. S1, Table S1 for details). We sequenced the amplified *cpo* product at the London Regional Genomics Centre (Robarts Research Institute, London ON, Canada), confirming that it had 99% identity to *D. melanogaster* *cpo* sequences.

We conducted qPCR in 20 µl reactions with the PerfeCTa SYBR Green FastMix (Quanta Biosciences) using 0.2 µl cDNA as template and primers at 0.4 mM in a Bio-Rad CFX96 Real-Time System with a C1000 Thermal Cycler (Bio-Rad, Mississauga, ON, Canada). We conducted three technical replicates of each of the three biological replicates. We used the following reaction conditions: 2 min at 95°C, 40 cycles of 15 s at 95°C, 15 s at 60°C, and 30 at 72°C, with melt-curve determination in increments of 0.5°C from 65°C to 95°C. For quality control, we only used groups of technical replicates whose standard deviation in Ct was less than 0.2. We used the Bio-Rad CFX Manager (version 3.1) to determine the Ct value for each biological replicate, normalise expression against two housekeeping genes, *Ef1α48D* and *TBP* (Zhai et al., 2014), and calculate mean relative normalised expression (ΔΔCt) of each gene for each developmental stage. We compared relative transcript abundance for each gene among acclimation regime and developmental stages using two-way ANOVAs in R.

2.6. Cold tolerance

We measured CT_{min} using a glass knock-down column as previously described (Ransberry et al., 2011). We transferred 500-800 summer- or winter-acclimated flies into the column and after 15 min equilibration at 21°C the column was cooled to -15°C at 0.1°C min^{-1}. As each fly reached its CT_{min} and lost coordination, it fell out of the column and into a 50 ml plastic vial containing soapy water. This vial was changed every 1°C and flies were filtered out, sorted by sex, and counted. The temperature at which 80% of the flies enter chill coma (CT_{min80}) was calculated and compared among sexes and acclimation regimes using accelerated-failure-time (AFT) models in R (Jakobs et al., 2015). Because the data were not normally-distributed, CT_{min} was compared

between sex and acclimation regime using a Wilcoxon rank sum test and the effect of the interaction of the acclimation regime and sex was analysed with a Kruskal-Wallis test followed by a Wilcoxon pairwise comparison with Bonferroni-Holm correction.

To determine the supercooling point (SCP), we placed adult flies individually into 1.7 ml microcentrifuge tubes in contact with a 36-AWG type-T copper-constantan thermocouple (Omega, Laval, Quebec, Canada). We placed the tubes an aluminium block cooled by 50% methanol circulated from a programmable refrigerated bath (Lauda Proline 3530, Würzburg, Germany). Flies were equilibrated at 0°C and cooled to -30°C at 0.1°C min^{-1}. Temperature was recorded at 0.5 s intervals by Picolog v5.20.1 software (Pico Technology, Cambridge, UK) via a Pico Technology TC-08 interface. The SCP was defined as the lowest temperature before the exotherm caused by the latent heat of crystallisation (Lee, 2010). SCPs were compared among the acclimation regimes and sex using a two-way ANOVA and Tukey's post-hoc test in R.

We determined the cold tolerance strategy according to Sinclair et al. (2015). Flies were cooled as described for SCP determination (n = 10/morph/sex). After half the flies had frozen (indicated by the exotherm), all individuals were transferred individually to wells of 6-well cell culture plates at room temperature with a ca. 1 cm^3 piece of banana medium on three layers of paper towel. Survival was assessed as the ability to stand and move in a coordinated fashion after 24 h. Flies were classified as chill-susceptible (died due to chilling injuries unrelated to freezing) if both unfrozen and frozen flies died, freeze-avoidant (died upon freezing) if only unfrozen flies survived, or freeze-tolerant if frozen flies survived.

296 The acute lethal temperature (LT) of *D. suzukii* was estimated using 1 h exposures to sub-zero temperatures for each acclimation regime and sex. Groups of 7-10 flies were placed into a 1.7 ml microcentrifuge tube and placed directly into a pre-cooled block and held for 1 h at temperatures ranging from -13°C to 0°C (encompassing 0-100% mortality). Temperature during exposure was recorded in every tube using thermocouples as above. All flies from each tube were placed into one well of a 6-well cell culture plate at room temperature containing ca. 1 cm^3 banana food and the proportion of surviving flies was assessed after 24 h.

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304 The lethal time (Lt) of *D. suzukii* was determined under chronic cold exposure to 0°C by submerging three groups of ten flies of each acclimation regime and sex in an ice slurry for 1-24 d (encompassing 0-100% mortality). Vials containing banana medium were enclosed in sealed plastic bags to avoid flooding, and the ice slurry was kept in a 20 L styrofoam container in a 4°C cold room, with ice added as needed to maintain the slurry temperature at 0°C. Three vials of summer-acclimated flies were removed daily to assess survival, while vials of winter-acclimated flies were removed twice per week. Survival was assessed after 24 h at the flies' rearing temperature. In addition, we placed two vials each of summer- and winter-acclimated adults under the snow for two weeks in London, Ontario (43°00'N 81°29'W, c. 250 m a.s.l.) from January 26 to February 9, 2015 to assess survival in a potential overwintering habitat.

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315 The $LT_{80\text{-}1h}$ (temperature at which 80% of flies will die after a 1 h exposure) and Lt_{80} (lethal time at which 80% die) were calculated for winter- and summer-acclimated females and males using a generalised linear model with a binary error distributions and logit link function

and the fit was tested with Wald's χ^2 using the Glht() function in the package MASS in R (Venables and Ripley, 2002).

To determine if winter-acclimated flies exhibit a rapid cold hardening (RCH) response, survival following acute exposure (as described above) to the $LT_{80\text{-}1h}$ was determined in 3-6 replicates of ten winter-acclimated flies per sex exposed to a pre-treatment of 1 h at 0°C, followed by 1 h recovery at the rearing temperature, as well as winter-acclimated flies that experienced no pre-treatment (Jakobs et al., 2015). The arcsine square root transformation of the proportion of surviving flies was compared among the two treatments and sexes using a two-way ANOVA in R.

2.7. Desiccation tolerance

The time to death of flies exposed to desiccating conditions was determined by sealing groups of flies in vials with silica gel (Gibbs and Matzkin, 2001). We placed ten replicates of 7-10 flies per acclimation regime and sex in empty 35 mL vials (n=10), with a foam stopper overlaid with 3 g of silica gel and sealed with Parafilm. We counted the number of surviving flies in each vial every 3 h from 0 h to 24 h (encompassing 0-100% mortality) at room temperature (c. 23°C). We compared survival among groups over time as for the longevity analysis. The Lt_{80} for desiccation was determined via a generalised linear model as for chronic cold exposure.

2.8. Fungal infections

To determine if cold-acclimation increased the immunity of winter-acclimated flies, we gently shook groups of ten 6-d-old virgin male and female flies of each morph for 30 s on an agar plate

containing sporulating *Metarhizium brunneum*, following Le Bourg et al. (2009). Non-infected controls of each sex and acclimation regime were shaken on a sterile plate. We returned groups of ten flies to food vials at either 12ºC or 21.5ºC, and recorded the proportion of surviving flies every 24 h. We compared survival over time of summer- and winter -acclimated males and females at each temperature using non-linear mixed effects models as described for the longevity analysis.

3. Results

3.1. Winter-acclimated adults delay ovarian development

Adults from laboratory populations from Ontario and British Columbia developed a darkened cuticle when reared at 11°C (Fig. 1), and ovarian development was delayed by low temperatures (Fig. 2). Field-collected flies also displayed dark cuticles and reduced ovarian development, similar to flies reared at 11°C in the laboratory (Fig. 1). At 21.5°C, summer-acclimated females complete ovarian development by 10 d post-eclosion (representative ovaries in Fig. 2A). At 15ºC, ovarian development was complete by 20 d post-eclosion, and was complete in most flies reared at 11ºC by 30 d post-eclosion. The ovarian development at 11°C and 15°C did not vary with photoperiod (Fig. 2), nor did cuticular darkening (data not shown). Females transferred from 11°C (10:14 L:D) to 21.5°C (13:11 L:D) at 10 d post-eclosion became reproductively active within three days. Further, we observed eggs and larvae in food vials of flies at 11ºC (10:14 L:D) 30 to 60 d post-eclosion.

3.2. Winter- and summer-acclimated flies have similar lipid and carbohydrate contents

The only neutral lipid class we detected in *D. suzukii* <8 h post-eclosion was triacylglycerols (Fig. S2). Triacylglycerol content (summer acclimation: 82.2±14.4 μg·mg tissue^{-1}; winter acclimation: 81.3±17.7 μg·mg tissue^{-1}) and carbohydrate content (summer acclimation: 4.3±0.9 μg·mg tissue^{-1}; winter acclimation: 4.8±0.9 μg·mg tissue^{-1}) did not differ between morphs or by sex (TAG: sex: $F_{1,16}$=0.004, P=0.95; acclimation regime: $F_{1,16}$=0.02, P=0.88; Carbohydrate: sex: $F_{1,22}$=0.05, P=0.81; acclimation regime: $F_{1, 22}$=0.31, P=0.58).

3.3. Acclimation regime and reproduction alter longevity

Isolated winter-acclimated females and virgin summer-acclimated females produced no offspring, while mated summer-acclimated females laid viable eggs for up to 85 d post-eclosion. When maintained at 21.5 °C, winter-acclimated females had higher survival than summer-acclimated females (acclimation regime: t_{702}=4.65, P<0.001; time: t_{702}=54.43, P<0.001; acclimation regime × time: t_{702}=0.95, P=0.34), with mated summer-acclimated female mortality occurring 24 d before summer-acclimated virgin and winter-acclimated females (reproductive status: t_{702}=1.04, P=0.30; time: t_{702}=47.84, P<0.001; reproductive status × time: t_{702}=4.16, P<0.001; Fig. 3A, Table 2). Summer-acclimated males survived for approximately 15 d longer than winter-acclimated males (acclimation regime: t_{820}=2.05, P=0.04; time: t_{820}=65.51, P<0.001; acclimation regime × time: t_{820}=1.03, P=0.30; Fig. 3B, Table 2).

3.4. Winter-acclimated females differentially express stress- and reproduction-related genes

Transcript levels of all genes varied during development (Table 3). Several stress-related gene transcript levels increased in winter-acclimated, but not summer-acclimated, flies post-eclosion,

including *Cat* and *Sod,* which encode oxidative stress enzymes, and *smp-30* (Fig. 4A-C, Table 3). Winter-acclimated females upregulated two heat shock protein genes, *Hsp83* and *Hsc70-4*, at all developmental stages, while *Hsp27* did not differ between winter- and summer-acclimated flies, and *Hsp23* had lower transcript levels in winter-acclimated pupae (Fig. 4D-G, Table 3). *PPO1* expression increased late in pupal development, but transcript levels did not differ between winter- and summer-acclimated groups (Fig. 4H, Table 3).

Transcript abundance of the regulatory genes *Jheh1* and *Thor* increased in wandering larvae after 24 h at 11°C, and in 6 d winter-acclimated females, whereas *foxo* was upregulated in 1 d winter-acclimated females (24 h post-eclosion) (Fig. 4I-K, Table 3). Downregulation of *cpo* in summer-acclimated adults coincided with expression of *Yp1*, while winter-acclimated adults maintained high *cpo* transcript levels and did not express *Yp1* (Fig. 4L,M, Table 3).

3.5. Winter-acclimated flies are active at lower temperatures and more cold-tolerant than summer-acclimated flies

The temperature at which 80% of flies had entered chill coma (CT_{min80}) ranged from 0.5°C to -5.5°C, and was approximately 5°C lower in winter- than summer-acclimated flies (acclimation regime: W = 163640, P < 0.001; sex: W = 78912, P < 0.001, acclimation regime × sex: Kruskal-Wallis χ^2=369.23, df= 3, P<0.001; Table 2).

SCP ranged from -22.4°C in a summer-acclimated male to -14.3°C in a summer-acclimated female. The mean SCP of summer-acclimated males was lower than the other groups (sex: $F_{1,72}$=27.367, P<0.001; acclimation regime: $F_{1,72}$=22.395, P<0.001, sex × acclimation

regime: $F_{1,72}$=9.81, P<0.001; Table 2). However no fly from any group survived freezing or temperatures close to the SCP, thus we consider all groups to be chill-susceptible (Table 2).

An acute (1 h) low-temperature exposure caused mortality below -5.2°C for summer-acclimated flies and below -7.8 °C for winter-acclimated flies, with winter-acclimated flies surviving temperatures approximately 3°C lower than summer-acclimated flies (Fig. 5A,B; Table 2,4). When exposed to 0°C, all summer-acclimated *D. suzukii* were dead within 1 week, a time period that almost all winter-acclimated adults survived (Fig. 5C,D). Based on the Lt_{80}, winter-acclimated adults survived for approximately 10 d longer at 0°C than summer-acclimated adults (Table 2, Table S2). Summer-acclimated flies under the snow cover during the London, ON, winter also had 100% mortality after one week, while winter-acclimated flies had 70% mortality after one week under the snow, and 100% mortality after two weeks. During this period, the minimum temperature below the snow during the first week was -1.8°C, and -1.1°C during the second week. The RCH treatment increased the proportion of winter-acclimated flies surviving a 1 h exposure at the LT_{80} by 35% (treatment: $F_{1,18}$=13.626, P=0.002; sex: $F_{1,18}$=0.101, P=0.78, treatment × sex: $F_{1,18}$=0.013, P=0.91; Table 2).

3.6. Winter- and summer-acclimated flies are desiccation-sensitive

All *D. suzukii* exposed to desiccating conditions at c. 23°C died within 24 h (Fig. 6). Females survived for 3 to 4 h longer than males of the same acclimation regime (sex: t_{320}=3.24, P=0.001; time: t_{320}=32.86, P<0.001; sex × time: t_{320}=0.92, P=0.36; Fig. 6; Table 2). Winter-acclimated flies under desiccating conditions did not survive significantly longer than summer-acclimated

flies (morph: t_{320}=0.29, P=0.77; time: t_{320}=32.23, P<0.001, sex × time: t_{320}=1.32, P=0.19; Fig. 6; Table 2).

3.7. Winter-acclimated females are more resistant to fungal infections than summer-acclimated females at warm temperatures

Non-infected flies had 100% survival for the duration of fungal infection experiments. At 21.5°C, most fungal-infected flies died within two weeks, while survival at 12°C exceeded 30 d (Fig. 7). Female winter-acclimated flies had better survival over time following fungal infection than summer-acclimated flies at 21.5°C (acclimation regime: t_{172}=2.30, P=0.02; time: t_{172}=22.99, P<0.001; acclimation regime × time: t_{172}=2.50, P=0.01; Fig. 7). However, we observed no difference between infected winter- and summer-acclimated females at 12°C (acclimation regime: t_{288}=1.89, P=0.06; time: t_{288} =16.58, P<0.001; acclimation regime × time: t_{288}=0.03, P=0.97; Fig. 7). Male survival of fungal infection did not vary between acclimation regimes at either 21.5ºC (acclimation regime: t_{130}=1.48, P=0.14; time: t_{130}=21.37, P<0.001; Fig. 7) or 12ºC (acclimation regime: t_{289}=0.03, P=0.98; time: t_{289}=20.38, P<0.001; Fig. 7).

4. Discussion

When wandering larvae are reared under short days and low temperatures (i.e. winter acclimation), laboratory populations of *D. suzukii* adults from both Ontario and British Columbia exhibited a 'winter morph' – characterized by a darkened cuticle and underdeveloped or absent ovaries – comparable to specimens observed by us in the field in Ontario (Pelee Island) and reported elsewhere (e.g. Zerulla et al., 2015). We observed this cuticular darkening when larvae were exposed to both short and long days at 11°C, but not at 15°C or 21.5°C (data not shown).

This suggests that low temperature, rather than photoperiod, is the cue for increased melanisation in the winter-acclimated flies.

The delay in ovarian development we observed in winter-acclimated flies also appears to be primarily regulated by temperature. Flies had fully-developed ovaries by 10 d post-eclosion at 21.5°C, 20 d post-eclosion at 15°C, and 30 d post-eclosion at 11°C, regardless of day length. Temperature-dependence of ovarian development in our study is consistent with gene expression data: the vitellogenic *Yp1* transcripts were present in summer-acclimated adults 1 and 6 d post-eclosion, whereas winter-acclimated adults of the same age lacked the transcript, indicating that winter-acclimated flies are not producing yolk in the period immediately after eclosion. *Yp1* transcript levels remain low in 30 and 60 d-old winter-acclimated females, suggesting that egg production is slow at 11°C. *Jheh1* expression increased when wandering larvae were transferred to 11°C, which is consistent with a model of suppressed JH signalling causing a reproductive delay (Sim et al., 2015). The suppression of ovarian development in winter-acclimated females coincided with high levels of *foxo* and *cpo* transcripts, whose products are hypothesized to contribute to suppression of vitellogenesis (Schmidt et al., 2008; Sim et al., 2015). *Hsp23* expression is associated with vitellogenic *D. montana* females (Salminen et al., 2015), which may explain its reduced expression in winter-acclimated *D. suzukii* females.

While many insects accumulate considerable energy stores (e.g. lipids or carbohydrates) prior to overwintering (Sim and Denlinger, 2013), newly-eclosed winter-acclimated *D. suzukii* had similar triacylglycerol and carbohydrate content to summer-acclimated flies. We note that these energy stores could be adequate to fuel overwintering if metabolic rate is decreased in

winter-acclimated flies (Hahn and Denlinger, 2007). However, we shifted only wandering larvae to the winter-acclimation regime, which meant that there was no additional opportunity to accumulate lipid reserves during larval development, which may explain why our flies did not have increased body size described elsewhere for ‘winter morphs’ (Zerulla et al. 2015). In addition, we did not determine whether adult winter-acclimated flies accumulate additional energy stores when feeding. Adults from the *D. auraria* complex reared under low temperatures and short photoperiods accumulate triacylglycerol contents two-fold higher than summer-acclimated flies during a period 8 to 32 d post-eclosion (Ohtsu et al., 1992). Thus, measuring energy accumulation and use both during larval development and in adult flies post-eclosion will be necessary to fully determine whether *D. suzukii* employs an energy conservation or accumulation strategy to fuel overwinter energetic demands.

In addition to delayed reproductive maturity, winter-acclimated *D. suzukii* had enhanced stress tolerance and altered gene expression suggestive of increased stress tolerance. Winter-acclimated flies were more cold-tolerant than summer-acclimated flies, with a lower chill coma onset temperature (CT_{min80}), and lethal temperature (LT_{80}), consistent with Stephens et al. (2015). *D. suzukii* SCPs did not differ with acclimation regime, were comparable to SCPs of other *Drosophila* spp. (Andersen et al., 2015), and likely do not reflect cold tolerance in this chill-susceptible species. Winter-acclimated adults also displayed a robust rapid cold-hardening response compared to summer-acclimated adults as examined by Jakobs et al. (2015).

The CT_{min} of winter- and summer-acclimated *D. suzukii* are within the range of CT_{min} observed for other mid-latitude (38-50°) *Drosophila* spp. (Andersen et al., 2015). However,

although winter-acclimated adults had a lower CT_{min} and survived for longer at 0°C than summer-acclimated flies, they were killed after three weeks at 0°C. This is consistent with mortality we observed in field-deployed winter-acclimated flies, and suggests that the winter-acclimated flies reared in this study would be unlikely to survive for long enough at 0°C to successfully overwinter in Ontario. We expected winter-acclimated flies to be more desiccation-tolerant than summer-acclimated flies, because darker cuticles are associated with reduced water loss rates in other species of *Drosophila* (Parkash et al., 2014; Rajpurohit et al., 2008; Ramniwas et al., 2013). However, flies from both acclimation regimes were similarly desiccation-sensitive at room temperature, although we did not measure water loss rates or cuticular water loss, which means we cannot entirely rule out melanisation-related reduced cuticular permeability in winter-acclimated *D. suzukii*. In addition, are expected to be lower at lower temperatures, so water balance under overwintering conditions is an avenue for further study.

Some of the enhanced cold tolerance of the winter-acclimated could be attributed to increased expression of oxidative stress enzymes (*Sod*, *Cat*), which are upregulated in several diapausing insects (Salminen et al., 2015; Sim and Denlinger, 2011). Sim and Denlinger (2011) demonstrated that *Cat* expression is required for longevity and prevention of ovarian follicle degeneration in diapausing mosquitoes, and it is therefore possible that antioxidant enzyme expression in winter-acclimated *D. suzukii* contributes to enhanced survival at 0°C. At higher temperatures (21.5°C), however, longevity was primarily determined by reproductive activity in female *D. suzukii*, with similar survival in virgin summer-acclimated flies and non-reproductive winter-acclimated flies. Mated summer-acclimated females had lower longevity, likely due to energy expended in producing offspring (Flatt and Kawecki, 2007). However, male winter-

acclimated flies had shorter lifespans at 21.5°C than summer-acclimated flies, in contrast to *D. melanogaster* that survive similarly at 25°C whether acclimated at 11°C or 25°C (Tatar et al., 2001). We did not measure longevity at moderately low temperatures (e.g. 4 °C, as is commonly found beneath snow), and speculate that longevity may be adequate to survive the winter if measured at low temperatures where flies may not be accumulating chilling injuries, as has been reported for *D. melanogaster* by Tatar et al. (2001).

Heat shock proteins can considerably enhance stress tolerance (King and MacRae, 2015). Indeed, *Hsp83* was upregulated throughout development of the winter-acclimated flies. By contrast, *Hsp27* transcript levels did not differ between summer and winter-acclimated flies, similar to several heat shock proteins during *D. triauraria* reproductive diapause (Goto and Kimura, 2004). The gene *smp-30* is induced by cold acclimation in *D. melanogaster* (Goto, 2000), expressed in diapausing *D. montana* females (Kankare et al., 2010), and is also upregulated in winter-acclimated *D. suzukii*. *smp-30* has similar functional motifs to *regucalcin*, which is upregulated during initiation of *D. montana* reproductive diapause (Salminen et al., 2015), but is not induced following chill coma in *D. melanogaster* (Reis et al., 2011). The upregulation of *smp-30* in winter-acclimated *D. suzukii* appears to be a response to cold acclimation, however the role of this gene in conferring cold tolerance remains unclear in *Drosophila*.

Fungal or bacterial pathogens can be a key source of overwinter mortality (Colinet et al., 2015; Hokkanen, 1993; Sinclair et al., 2013; Williams et al., 2015). Because the melanisation cascade is associated with both cuticle darkening and encapsulation responses (Dubovskiy et al.,

2013; Kutch et al., 2014), we expected winter-acclimated flies to have improved immunocompetence in association with the darkened cuticle. However, male winter-acclimated flies had no survival advantage over their summer-acclimated counterparts, and female winter-acclimated flies only had improved survival to fungal infection in warm (21.5°C) but not cool (12°C) conditions. Increased melanisation alone was therefore insufficient to increase immunocompetence. The greater fungal resistance of winter-acclimated compared to summer-acclimated females at 21.5°C suggests that winter-acclimated females can take advantage of warm spells to fight infection (Colinet et al., 2015). These results are consistent with *PPO1* expression patterns, which did not differ between summer- and winter-acclimated *D. suzukii; PPO1* is important for melanin-based immune function in *D. melanogaster* (Binggeli et al., 2014). The elevated *Thor* expression in 6-d-old winter-acclimated females may contribute to the enhanced immunocompetence exhibited by these females under warm conditions (Bernal and Kimbrell, 2000).

4.1. Are winter-acclimated D. suzukii *in diapause?*

The winter-acclimated *D. suzukii* in our study had enhanced stress tolerance and apparent suppression of ovarian development; however, we do not believe this represents diapause *sensu stricto*. Photoperiod had no effect on either reproductive development or cuticular darkening (which appeared to be entirely temperature-dependent), and the ovarian developmental delay we observed appeared to simply be due to slower growth at low temperatures rather than induced by a particular environmental cue (Koštál, 2006). This differs from early work on *D. melanogaster*, which found that low temperatures and short days are both required to induce suppression of ovarian development (Saunders et al. 1989), but is consistent with later *D. melanogaster* work, in

which low temperatures caused ovarian suppression under both long and short days (Tatar et al. 2001), with lower temperatures resulting in more severe ovarian suppression (Lee et al., 2011). We did not, however, examine an extensive range of photoperiods (cf. Lankinen et al., 2013), so it is possible that reproductive diapause could be induced under a different combination of day lengths and temperatures, or with exposure to short days earlier in development (Saunders, 2014). Furthermore, we found no evidence that ovarian development was suppressed by cues other than temperature. Winter-acclimated flies transferred from 11°C to 21.5°C resumed ovarian development almost immediately, similar to *D. melanogaster* transferred from 11°C to 25°C (Tatar et al., 2001), suggesting that reproduction is not actively suppressed. Flies resumed reproduction once ovarian development was complete at 11°C even under short days, similar to spontaneous 'diapause' termination observed in *D. melanogaster* within 6-7 weeks post-eclosion (Lee et al., 2011), implying that no particular environmental cue was required to terminate the reproductive developmental delay (Košťál, 2006). Thus, the lack of ovarian development associated with winter-acclimated *D. suzukii* is not consistent with a true diapause, but likely reflects slow development at low temperatures.

The expression levels of candidate diapause-related genes are also not strongly supportive of a true diapause in winter-acclimated *D. suzukii*. Based on the current model that JH and insulin suppression leads to expression of the transcription factor *foxo* and its targets (Sim et al., 2015), we expect JH esterases (e.g. Jheh1) to initiate JH suppression and subsequently increase *foxo* expression (Poelchau et al., 2013). However, *Jheh1* is only substantially upregulated after *foxo* transcript abundance peaks in *D. suzukii* winter-acclimated flies. The putative targets of FOXO, *Thor* and *cpo*, which are upregulated in *D. montana* diapause

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4 591 (Kankare et al., 2010; Salminen et al., 2015), are also upregulated in *D. suzukii* winter-
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7 592 acclimated adults. However, although *foxo* upregulation coincides with the increase in *cpo*
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9 593 transcript abundance, further supporting that FOXO induces *cpo* expression in *D. suzukii*,
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12 594 increased *Thor* expression does not coincide with *foxo* transcript abundance. Thus, it appears that
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14 595 transcription factors other than *foxo* could regulate *Thor* in *D. suzukii*. *Hsc70-4*, which is
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17 596 upregulated during *D. montana* diapause termination (Salminen et al., 2015), is also expressed in
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19 597 *D. suzukii* winter-acclimated females, but its expression is not restricted to the time at which
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24 599 related genes in winter-acclimated *D. suzukii*, the timing of this upregulation is inconsistent with
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31 602 Nevertheless, the enhanced stress tolerance of winter-acclimated *D. suzukii* means they
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34 603 are more likely to successfully overwinter than summer-acclimated flies. The low CT_{min} of
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36 604 winter-acclimated flies suggests they could remain active under snow cover during winter, when
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39 605 80% of summer-acclimated flies would be in chill coma and unable to feed or select beneficial
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41 606 microhabitats. Although winter-acclimated flies from the laboratory did not survive under the
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46 608 $LT_{80\text{-}1h}$. Therefore, cold tolerance of winter-acclimated adults is plastic during and after exposure
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49 609 to low environmental temperatures, improving the probability of successful overwintering. The
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51 610 acclimation conditions we used to induce the ‘winter morph’ (11°C, 10:14 L:D) were
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54 611 representative of typical autumn conditions in southern Ontario, but do not capture the thermal
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56 612 variability that *D. suzukii* would experience in nature. While Stephens et al. (2015) suggest
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58 613 neither summer nor winter morph *D. suzukii* are likely to survive winter conditions, it is possible
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that developmental or adult acclimation (or repair of accumulated damage) under a fluctuating thermal regime would promote increased stress tolerance (Colinet et al., 2015), extending survival (and possibly longevity) of *D. suzukii* during overwintering conditions.

5. Conclusions

While not an example of true diapause, *D. suzukii* stress tolerance increases due to both developmental and adult acclimation at low temperatures, improving the cold tolerance and low temperature activity of this chill-susceptible species. This stress tolerance in winter-acclimated flies coincides with increased cuticle melanisation and female reproductive delay. Higher melanisation does not appear to improve desiccation tolerance or immunocompetence. However, stress-related genes upregulated in winter-acclimated flies, such as those encoding antioxidants, heat shock proteins, and smp-30, may underlie the increase cold tolerance we observed. The temperature-induced delay in reproductive development is likely mediated through suppression of *Yp1* expression by high levels of foxo and cpo. Future studies should examine the extent to which these genes affect the stress tolerance and reproductive physiology of *D. suzukii*, and the impact of other acclimation treatments (e.g. fluctuating thermal regimes) on the ability of this pest to withstand overwintering stresses.

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Figure legends

Fig. 1. Summer and winter morphs of *Drosophila suzukii*. Dorsal view of (A) field-collected winter morph *D. suzukii* female from Pelee Island, Ontario, Canada and (B) lab-reared, summer-acclimated *D. suzukii*. Lateral view of lab-reared (C) winter-acclimated and (D) summer-acclimated *D. suzukii* females.

Fig. 2. The effect temperature and photoperiod on ovarian development in *D. suzukii*. Representative ovaries dissected from *D. suzukii* females with (A) zero, (B) partial, or (C) full ovarian development. The proportion of each class of ovaries in groups of five females reared under (D) 11°C and short days, 10:14 L:D, (E) 15°C and short days, 10:14 L:D, (F) 11°C and long days, 14:10 L:D, and (G) 15°C and long days, 14:10 L:D.

Fig. 3. The effect of acclimation regime and reproductive activity on longevity of *D. suzukii*. The proportion of surviving (A) females and (B) males over time at 21.5°C, 13:11 L:D. Each point represents the mean±s.e.m proportion of surviving flies from 3-9 vials (7-10 flies/vial). * indicates a significant effect of morph, † indicates a significant reproductive status × time interaction (non-linear mixed effects model details in text).

Fig. 4. Gene expression of summer- and winter-acclimated *D. suzukii* during development. Transcript abundance of target genes were normalised to the housekeeping genes *Ef1α48D* and *TBP*. (A) *smp-30*, (B) *Cat*, (C) *Sod*, (D) *Hsp83*, (E) *Hsc70-4*, (F) *Hsp27*, (G) *Hsp23*, (H) *PPO1*, (I) *Jheh1*, (J) *foxo*, (K) *Thor*, (L) *cpo*, and (M) *Yp1*. Each point represents the mean±s.e.m of three biological replicates of 8-10 individuals at a particular life stage: wandering larvae (Larva), pupae within 24 h of pupation (Early Pupa) or 24 h pre-eclosion (Late Pupa), and adults 1, 6, 30 and 60 d post-eclosion (1 d, 6 d, 30 d, 60 d, respectively). Open circles, dashed line: summer-

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4 914 acclimated flies; closed circles, solid line: winter-acclimated flies. * indicates a significant effect
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7 915 of acclimation regime, † indicates a significant acclimation regime × developmental stage
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9 916 interaction (two-way ANOVA, details in Table 3).
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13 917 **Fig. 5. Acute and chronic cold tolerance of summer- and winter-acclimated *D. suzukii*.**
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15 918 Survival of groups of 7-10 (A) female and (B) male flies 24 h after 1 h (acute) exposures to
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18 919 temperatures between 0°C and -13°C, and (C) female and (D) male flies following 1 to 24 d
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20 920 (chronic) exposures to 0°C. Each point represents the proportion of surviving flies in a single
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23 921 vial (7-10 flies/vial). The intersection between the dotted line and each curve indicates the
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25 922 temperature ($LT_{80\text{-}1h}$, A & B) or time (Lt_{80}, C & D) at which 80% mortality occurred. There was
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28 923 no effect of sex, so males and females were pooled for analysis. * indicates a significant effect of
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30 924 acclimation regime, † indicates a significant acclimation regime × temperature or time
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33 925 interaction, # indicates a significant acclimation regime × sex interaction (generalised linear
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35 926 model, details in Table S2).
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38 927 **Fig. 6. Desiccation tolerance of summer- and winter-acclimated flies.** The proportion of
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41 928 surviving (A) females and (B) males desiccated in vials containing Silica gel at room
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43 929 temperature. Each point represents the mean±s.e.m proportion of surviving flies from ten vials
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46 930 (8-10 flies/vial). There was a significant effect of sex, but no effect of acclimation regime (non-
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48 931 linear mixed effects model details in text).
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52 932 **Fig. 7. Survival following fungal infection of summer- and winter-acclimated flies.** The
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54 933 proportion of surviving (A) females and (B) males at 12°C and (C) females and (D) males at
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57 934 25°C following fungal infection with *Metarhizium brunneum*. Each point represents the
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935 mean±s.e.m proportion of surviving flies from ten vials (8-10 flies/vial). * indicates a significant

936 effect of acclimation regime (non-linear mixed effects model details in text).

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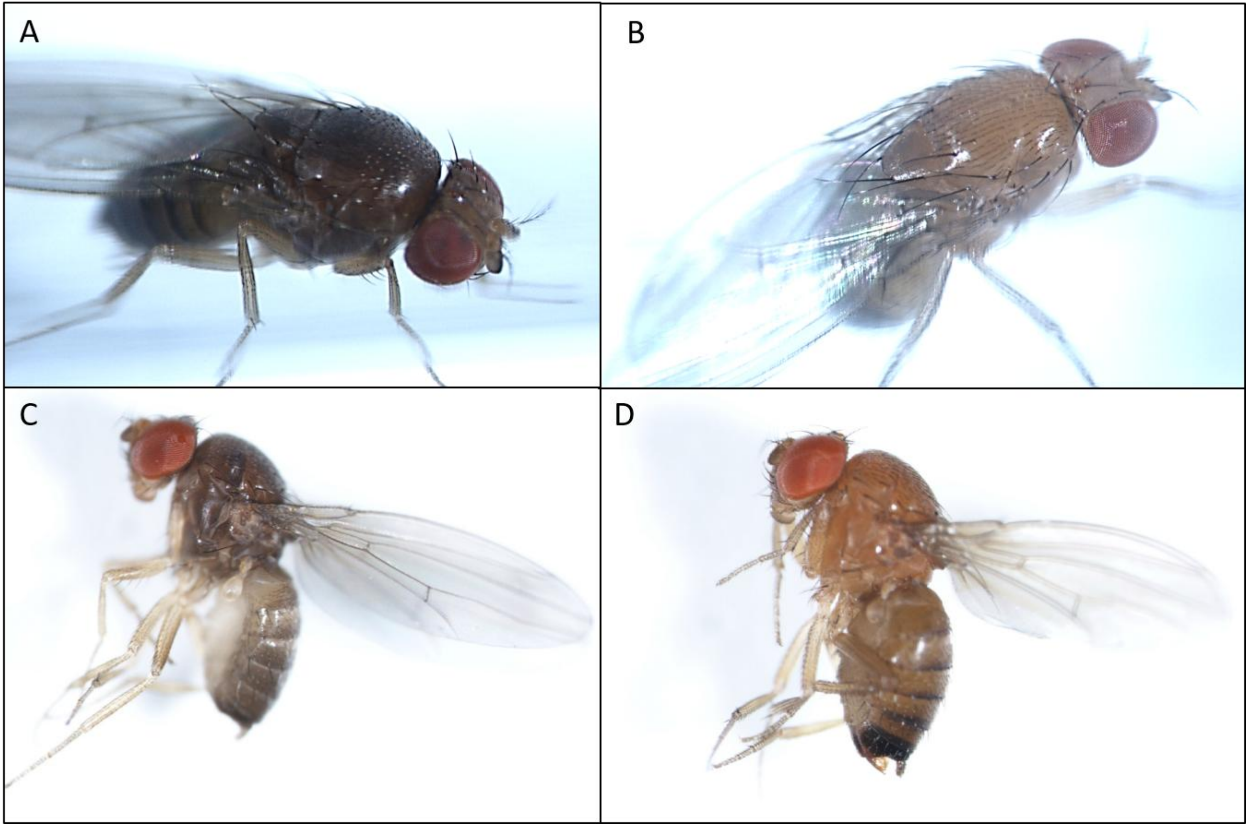

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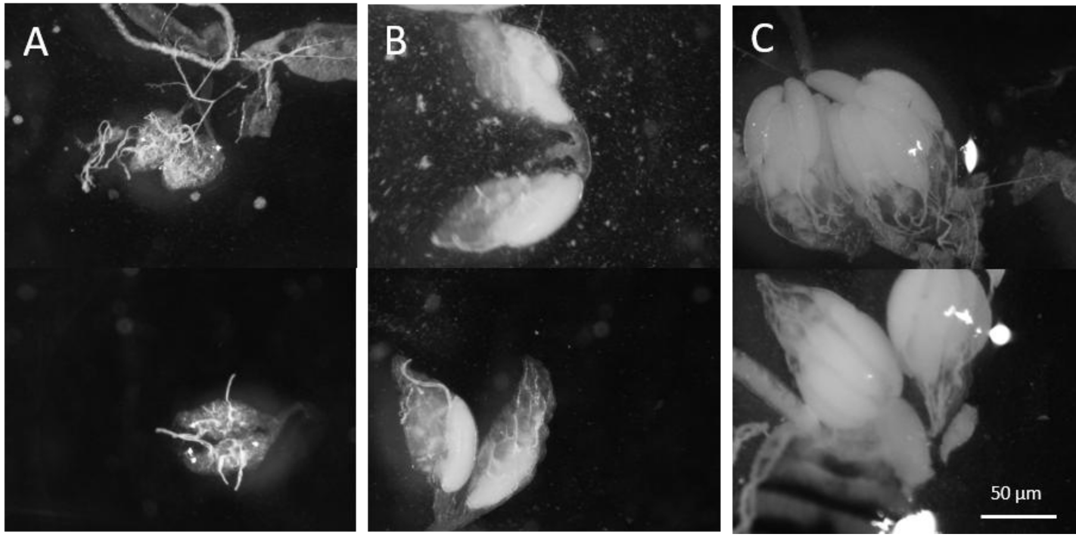

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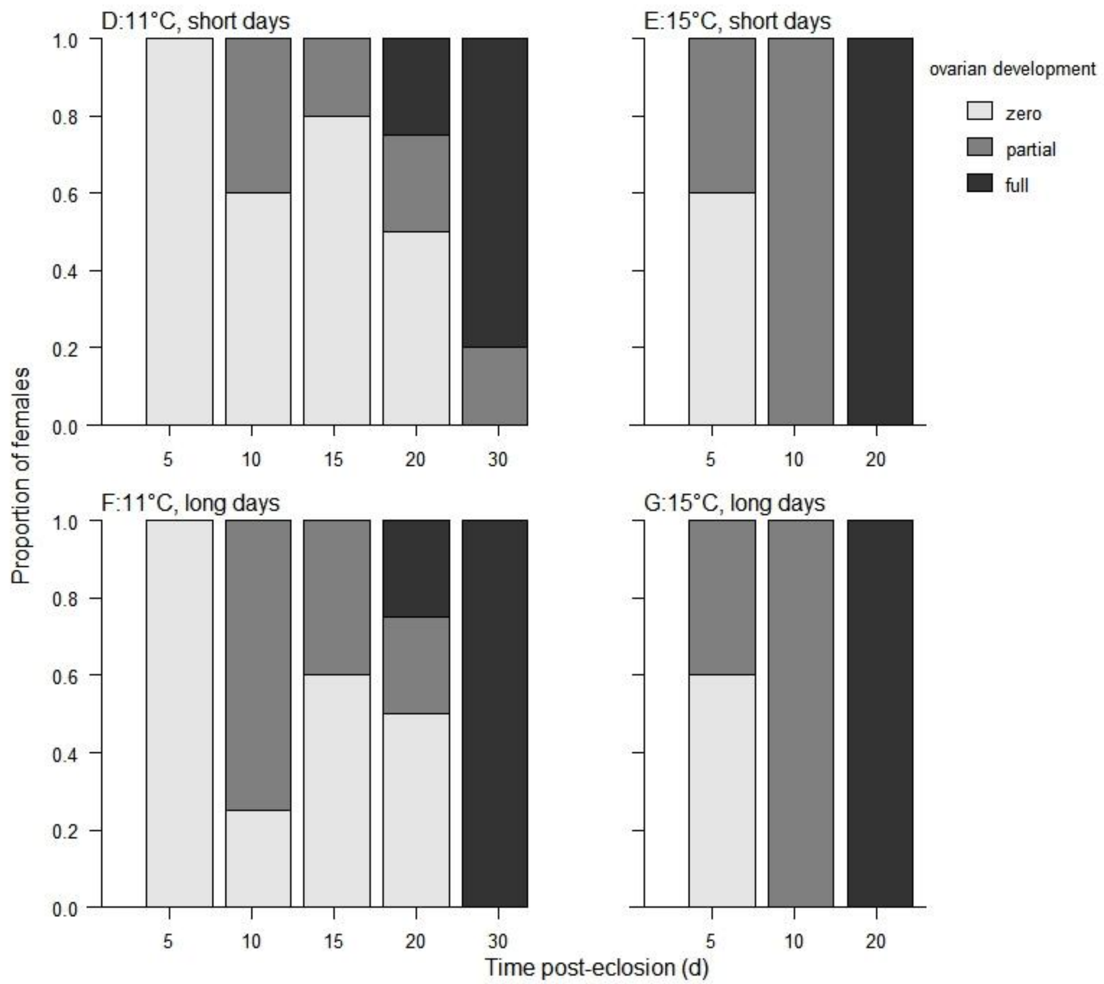

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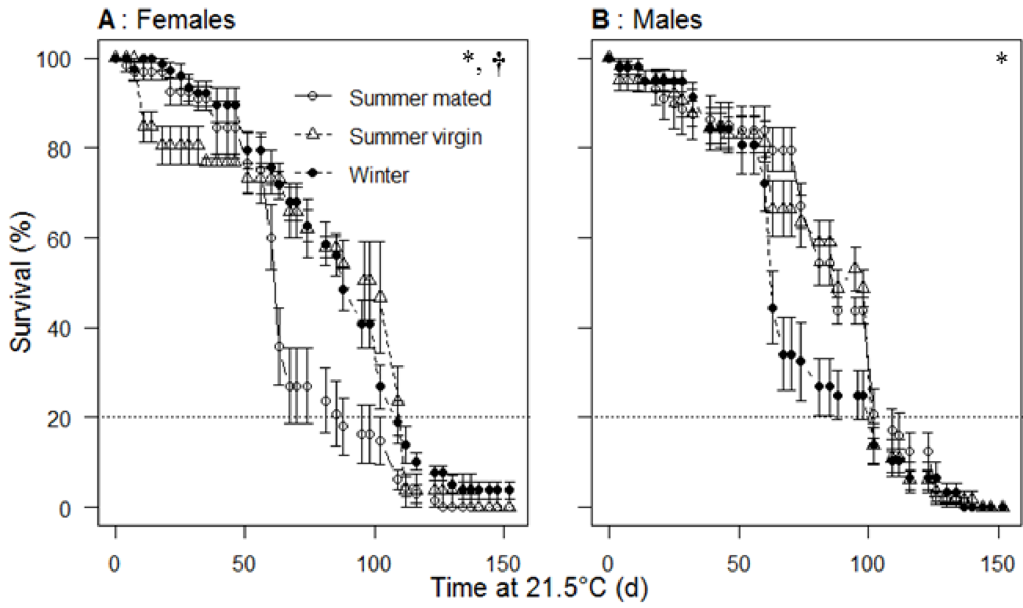

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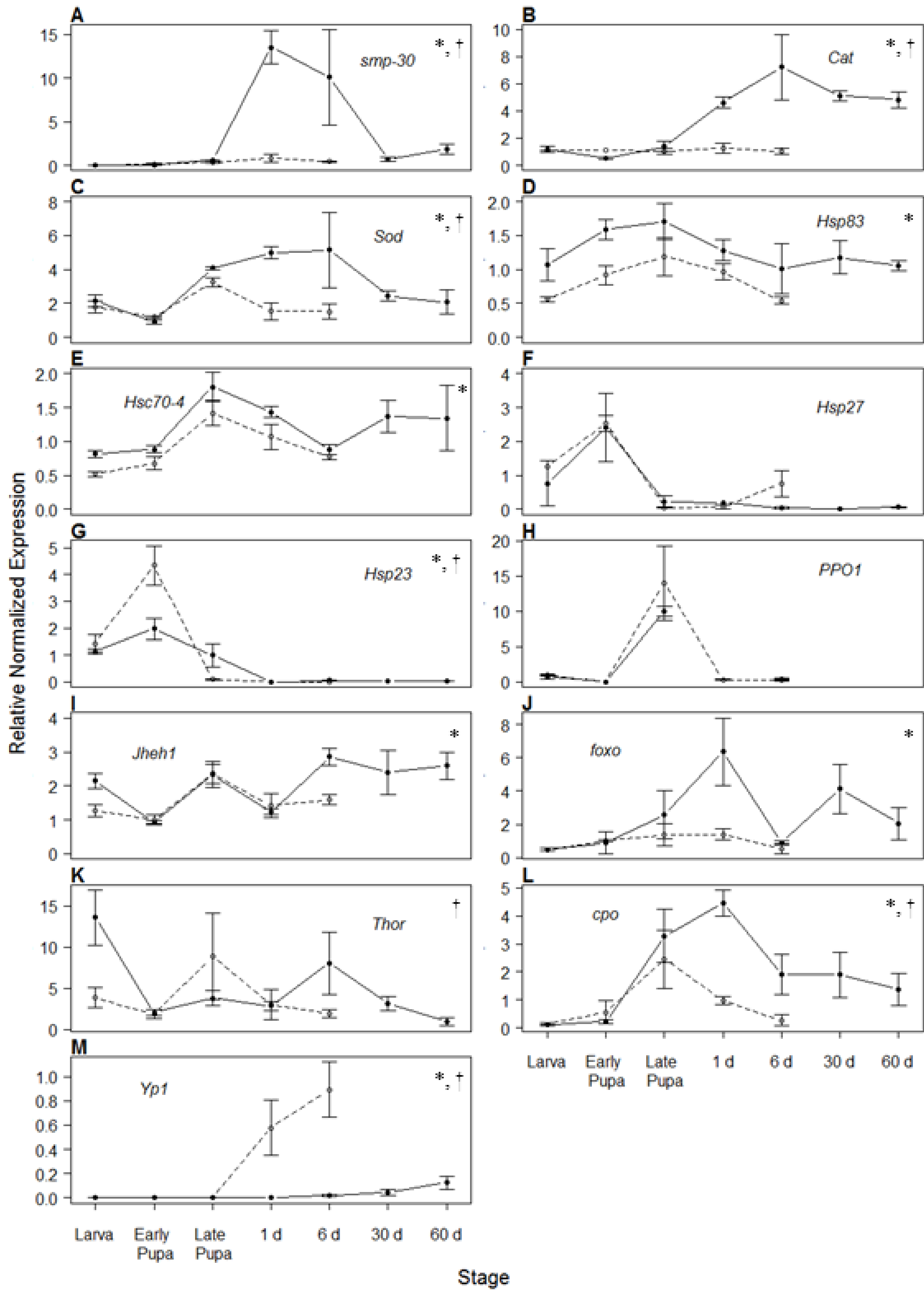

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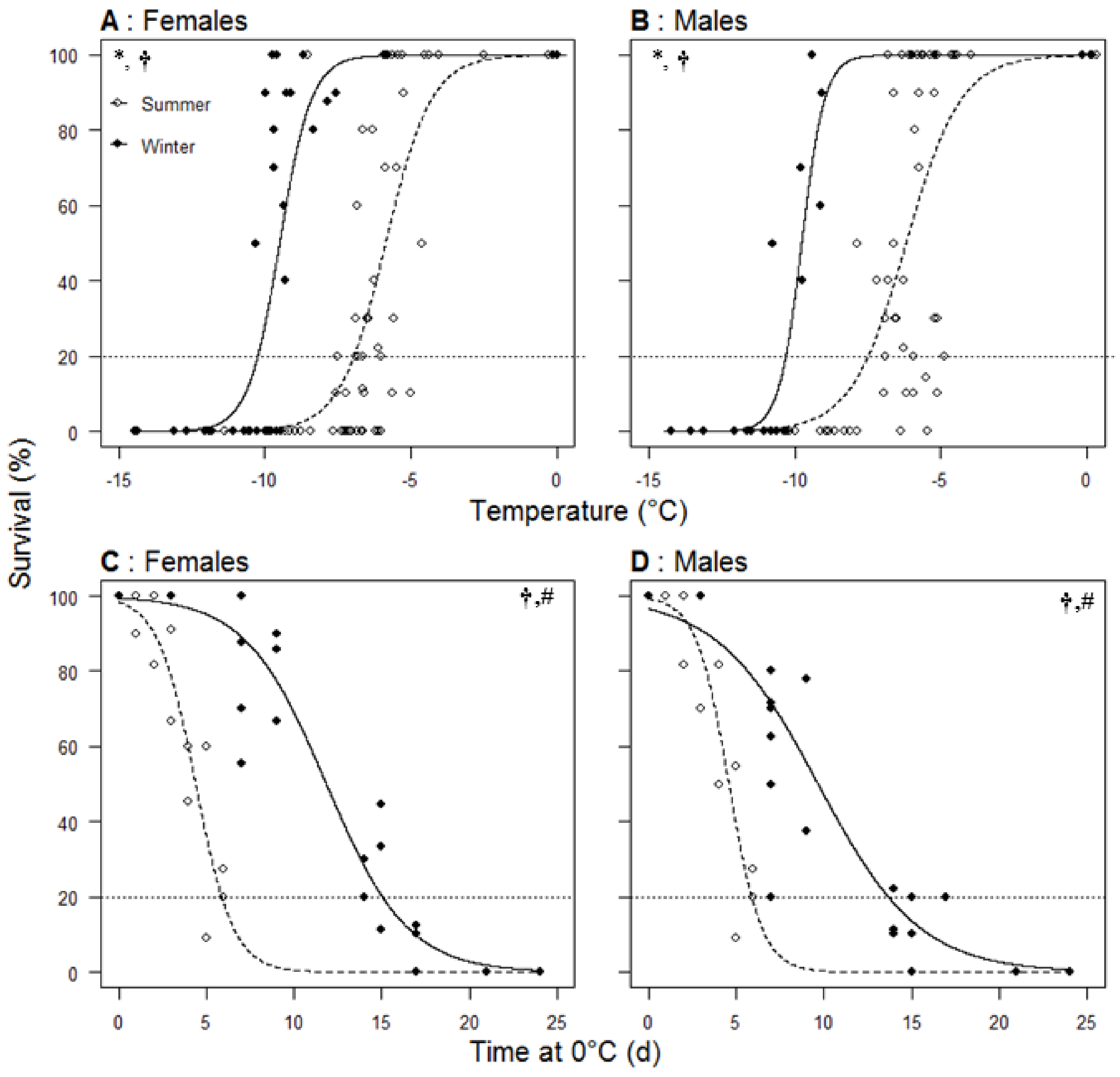

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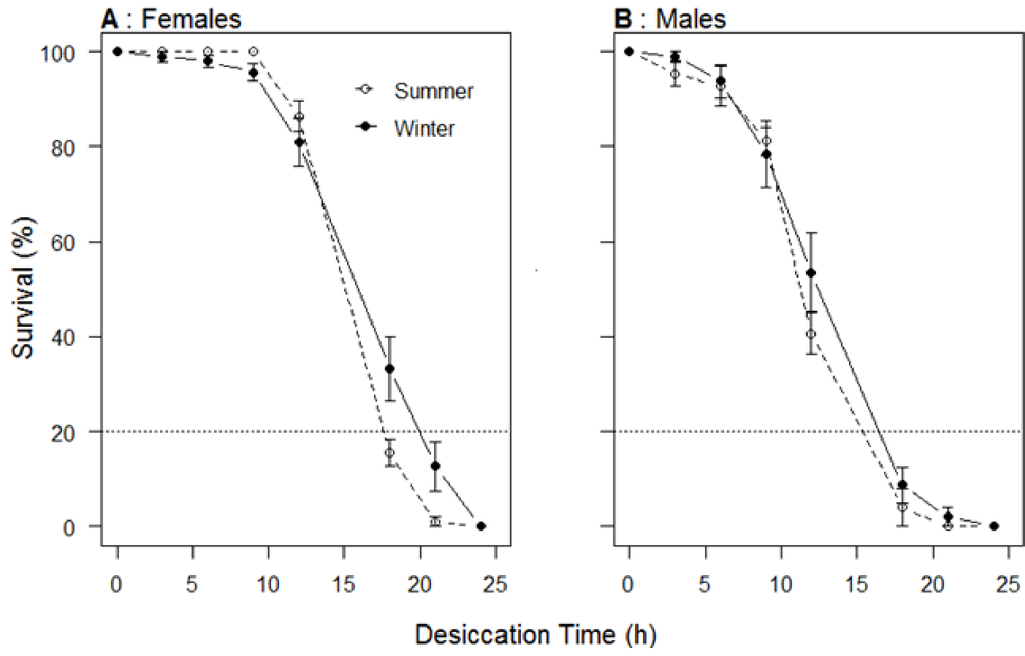

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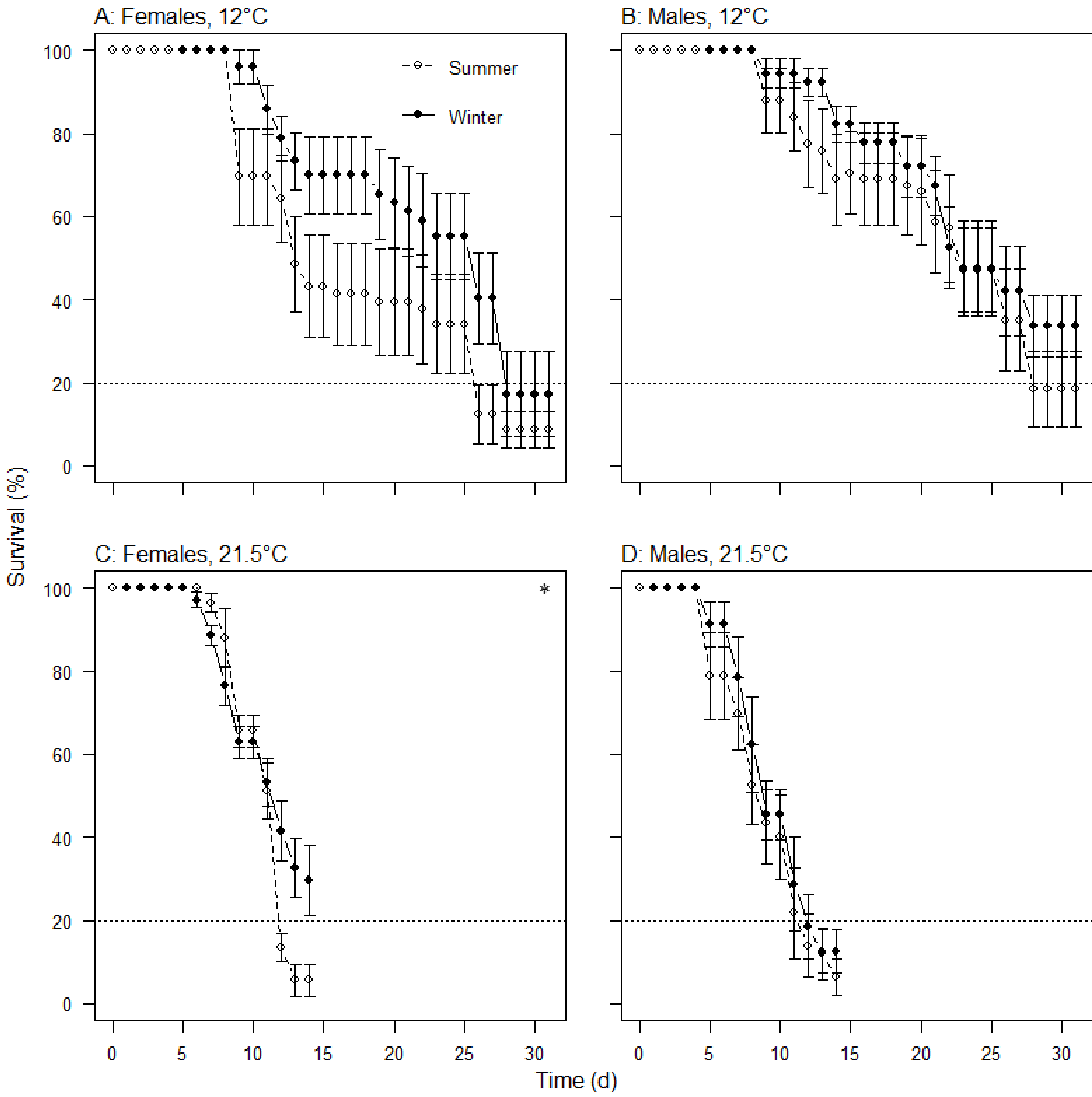

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4 959 **Tables**
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8 960 **Table 1. Genes examined via RT-qPCR.** Gene names and functions based on *D. melanogaster*
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10 961 (flybase.org). Primer sequences available in supplementary material (Table S1).
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Gene	Gene symbol	Function	Reference
Catalase	*Cat*	response to stress (oxidative)	Faust et al., 2012
couch potato	*cpo*	diapause regulation	Schmidt et al., 2008
Elongation factor 1α48D	*Ef1α48D*[1]	translation elongation factor	Lasko, 2000
forkhead box, sub-group O	*foxo*	transcription factor, diapause regulation	Sim et al., 2015
Heat shock protein cognate 70	*Hsc70-4*	chaperone binding, diapause termination	Wang and Brock, 2003, Shim and Lee, 2015
Heat shock protein 23	*Hsp23*	response to stress (hypoxia), cold acclimation	Azad et al., 2009, Qin et al., 2005
Heat shock protein 27	*Hsp27*	defense response to pathogens	Chen et al., 2010
Heat shock protein 83	*Hsp83*	response to stress (heat), cold acclimation	Neal et al., 2006, Qin et al., 2005
Juvenile hormone epoxide hydrolase 1	*Jheh1*	juvenile hormone catabolism	Campbell et al., 1992
Prophenoloxidase 1	*PPO1*	dopamine metabolism, melanisation	Li et al., 2012, Rämet et al., 2002
Senescence marker protein-30	*smp-30*	calcium ion binding, cold acclimation	Goto, 2000
Superoxide dismutase	*Sod*	response to stress (oxidative)	Ruan and Wu, 2008
TATA binding protein	*Tbp*[1]	transcription initiation	Davidson, 2003
Thor	*Thor*	immune response, response to stress (oxidative), diapause maintenance, lifespan determination	Bernal and Kimbrell, 2000, Tettweiler et al., 2005, Salminen et al., 2015
Yolk protein 1	*Yp1*	vitellogenesis	FlyBase

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50 962 [1] Housekeeping genes for RT-qPCR (Zhai et al., 2014)
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963 **Table 2. Longevity, low temperature performance and stress tolerance of adult *D. suzukii*.** All values are ± s.e.m., with *N* in
964 parentheses, indicating the number of flies (CT_{min80}, SCP, number of dead flies) or the number of groups of 7-10 flies ($LT_{80\text{-}1h}$, Lt_{80},
965 proportion of flies surviving $LT_{80\text{-}1h}$). We combined female and male data for survival at the $LT_{80\text{-}1h}$ because sex had no effect on the
966 RCH response. Groups with the same letter within an experiment are not different from each other (statistical details in text).

	Summer-acclimated		**Winter-acclimated**	
	Females	Males	Females	Males
Lt_{80} (d) at 21.5°C	mated: 84±4^{a} (7) virgin: 111±9^{c} (3)	mated: 108±4^{c} (9) virgin: 106±5^{c} (7)	109±4^{c} (8)	92±5^{b} (6)
CT_{min80} (°C)	-0.5±0.2^{a} (252)	0.4±0.2^{b} (276)	-5.5±0.2^{c} (213)	-5.1±0.3^{c} (141)
SCP (°C)	-17.9±0.5^{a} (18)	-20.3±0.4^{b} (20)	-17.3±0.1^{a} (24)	-17.7±0.1^{a} (14)
Number of flies dead	unfrozen: 5/5 frozen: 5/5	unfrozen: 5/5 frozen: 5/5	unfrozen: 5/5 frozen: 5/5	unfrozen: 5/5 frozen: 5/5
Cold tolerance strategy	chill-susceptible	chill-susceptible	chill-susceptible	chill-susceptible
$LT_{80\text{-}1h}$ (°C)	-7.0±0.1^{a} (60)	-7.5±0.2^{a} (54)	-10.2±0.1^{b} (36)	-10.3±0.1^{b} (21)
Lt_{80} (d) at 0°C	5.9±0.4^{a} (15)	5.9±0.4^{a} (15)	15.1±0.6^{b} (30)	13.7±0.7^{b} (30)
Proportion of flies surviving the $LT_{80\text{-}1h}$	no pre-treatment: 0.14±0.03* (12) RCH treatment: 0.20±0.04* (12)		no pre-treatment: 0.23±0.04 (12) RCH treatment: 0.58±0.06 (6)	
Lt_{80} (h) of desiccation	17.2±0.4^{a} (10)	15.3±0.4^{b} (10)	19.2±0.4^{a} (10)	14.3±0.4^{b} (10)
Lt_{80} (d) following fungal infection	21.5°C: 12.0±0.6 12°C: 24.7±1.6	21.5°C: 11.2±0.8 12°C: 30.2±2.0	21.5°C: 14.1±1.0 12°C: 30.5±2.2	21.5°C: 11.8±1.0 12°C: 32.0±2.3

967 *Values from Jakobs et al. (2015)

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5 968 **Table 3. Two-way ANOVAs of relative transcript abundance for each target gene.**
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7 969 Transcript abundance of each target gene was normalised against housekeeping genes *Ef1α48D*
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10 970 and *TBP* in summer- and winter-acclimated flies. Bold *P*-values indicate a significant effect of
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12 971 acclimation regime, stage, or the acclimation regime × stage interaction on transcript abundance.
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Target	acclimation		stage		acclimation × stage	
	F	*P*	*F*	*P*	*F*	*P*
Cat	23.861	**<0.001**	3.840	**0.013**	4.076	**0.017**
cpo	9.011	**0.007**	6.163	**<0.01**	3.037	**0.039**
foxo	9.649	**0.006**	2.837	**0.038**	2.218	0.106
Hsc70-4	8.636	**0.007**	5.988	**<0.001**	0.190	0.941
Hsp23	19.960	**<0.001**	23.414	**<0.001**	7.214	**<0.001**
Hsp27	1.654	0.219	6.434	**0.002**	0.289	0.880
Hsp83	13.487	**0.001**	2.984	**0.027**	0.198	0.937
Jheh1	9.321	**0.005**	5.850	**<0.001**	2.330	0.085
PPO1	0.053	0.823	7.166	**0.007**	0.259	0.853
smp-30	10.678	**0.004**	5.668	**0.001**	5.5416	**0.003**
Sod	8.612	**0.007**	3.720	**0.009**	3.112	**0.034**
Thor	0.584	0.452	2.745	**0.035**	3.299	**0.027**
Yp1	21.806	**<0.001**	6.808	**<0.001**	8.847	**<0.001**

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