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Jantina Toxopeus

Ruth Jakobs

Laura V Ferguson

Tara D Gariepy

Brent J Sinclair

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

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

6

7   Jantina Toxopeus[1]*, Ruth Jakobs[1,3]*, Laura V. Ferguson[1], Tara D. Gariepy[2] and Brent J.

8   Sinclair[1]†

9   [1]Department of Biology, University of Western Ontario, London, ON, Canada

10   [2]Agriculture and Agri-Food Canada, Southern Crop Protection and Food Research Centre,

11   London, ON, Canada

12   [3]Present Address: Department of Chemical Ecology, Bielefeld University, Germany

13

14   *Contributed equally

15   †Author for correspondence: Dept. Biology, University of Western Ontario, London, ON,

16   Canada N6A 5B7. Tel. +1-519-661-2111 ext. 83138; fax +1-519-661-3935; email

17   bsincla7@uwo.ca.

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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

2

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

67 cold tolerance (Teets and Denlinger, 2013). By contrast, longer term responses such as

68 acclimation may occur over days-to-weeks, or across development. One seasonally-plastic

69 response is diapause, a state of developmental arrest characterised by reduced metabolism and

70 enhanced stress tolerance that is initiated, maintained, and terminated by specific environmental

71 cues (Koštál, 2006). Many temperate and polar insects overwinter in diapause, a state that is

72 often induced by short photoperiods, and is promoted by low temperatures (e.g. Lankinen et al.,

73 2013; Spielman and Wong, 1973; Tatar and Yin, 2001). Diapause termination often requires

74 specific cues, which may be some combination of temperature and photoperiod, and diapause is

75 often only terminated after a required time period of dormancy (Tauber et al., 1986).

76

77   Overwintering adult insects may enter reproductive diapause, usually with high lipid

78 reserves to fuel overwinter metabolism (e.g. Ohtsu et al., 1992; Sim and Denlinger, 2013),

79 suppressed ovarian development (e.g. Sim and Denlinger, 2009), and enhanced tolerance of cold

80 (e.g. Vesala and Hoikkala, 2011) and desiccation (e.g. Benoit and Denlinger, 2007). The onset of

81 reproductive diapause is accompanied by suppressed insulin and juvenile hormone (JH)

82 signalling (Denlinger, 2002), causing an upregulation of *foxo* (*forkhead transcription factor*)

83 (Sim et al., 2015) and altered transcription of genes pertinent to vitellogenesis (e.g. Baker and

84 Russell, 2009; Sim and Denlinger, 2009), fat accumulation (e.g. Sim and Denlinger, 2013) and

85 stress tolerance (e.g. Baker and Russell, 2009). The latter includes genes that encode heat shock

86 proteins (e.g. King and MacRae, 2015; Rinehart et al., 2000) and antioxidant enzymes (e.g. Sim

87 and Denlinger, 2011). Diapause may also enhance immunity through upregulation of immune-

88 responsive genes, possibly protecting against infections over winter (e.g. Poelchau et al., 2013;

89 Ragland et al., 2010). In most species, patterns of gene expression in diapause are markedly

4

90 different to those associated with temperature-induced quiescence, for example, *Drosophila*

91 *montana* upregulate *actin*, *catalase*, *period*, and *Thor* when in diapause compared to quiescence

92 (Salminen et al., 2015).

93

94   Adult *Drosophila* are generally chill-susceptible (Nyamukondiwa et al., 2011), but often

95 have phenotypically-plastic stress tolerance. For example, combinations of hardening, rearing,

96 and acclimation conditions lead to significant within-population variation in CT_{min} and chill

97 coma recovery time in *Drosophila melanogaster* (Ransberry et al., 2011). Many *Drosophila* spp.

98 overwinter in apparent reproductive diapause with suppressed ovarian development (Carson and

99 Stalker, 1948; Kimura, 1988; Lumme et al., 1975; Muona and Lumme, 1981; Schmidt et al.,

100 2005; Tyukmaeva et al., 2011; Watabe, 1983), higher lipid reserves (Ohtsu et al., 1992; Schmidt

101 et al., 2005) and enhanced cold tolerance (Schmidt and Paaby, 2008). However, diapause

102 incidence can vary considerably within a species, with diapause-inducing conditions failing to

103 suppress ovarian development in Australian *D. melanogaster* populations (Lee et al., 2011).

104 Often, this variation occurs along a cline, as has been observed for several *Drosophila* spp.

105 (Lankinen et al., 2013; Schmidt and Paaby, 2008; Tyukmaeva et al., 2011). In *Drosophila* spp.,

106 the term "reproductive diapause" has been used to describe a general ovarian dormancy, without

107 necessarily requiring that the dormancy be maintained and terminated by specific cues. In this

108 paper we will adhere to the more stringent definition of diapause outlined by Koštál (2006),

109 which requires that the dormancy be maintained by environmental cues other than temperature.

110

111   Many overwintering *Drosophila* adults have enhanced melanisation (Gibert et al., 2007;

112 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

136 et al., 2015). However, it has not been established whether this is a true diapause, and tolerance

137 to other stresses pertinent to overwintering biology (e.g. desiccation) have not been investigated

138 in either morph of this species.

139

140 　　　　Here we show that pupal development under low temperature and short day conditions

141 ('winter-acclimation') induces a dark morph similar to the winter morph. Our goal was to

142 determine whether these winter-acclimated *D. suzukii* have the physiological and molecular

143 characteristics of a true diapause, and whether they have enhanced stress tolerance relevant to

144 overwintering. We examine ovarian development in response to different temperature and

145 photoperiod cues, as well as energy stores, longevity, tolerance of cold and desiccation stress,

146 immunity and expression of stress- and reproduction-related genes. We show that low

147 temperatures delay reproductive maturity, and that winter-acclimated flies are more stress-

148 tolerant than summer-acclimated *D. suzukii*. Because changes in winter-acclimated flies appear

149 to be regulated largely by temperature, we express doubt that this is a 'true' diapause.

150

151 **2. Materials and methods**

152 *2.1. Animal rearing and treatment groups*

153 We used the laboratory population of *D. suzukii* described by Jakobs et al. (2015) which was

154 established from approximately 200 individuals collected in the Halton Hills region, Ontario,

155 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%

156 relative humidity under 13:11 L:D, at a constant density (c. 70-100 individuals per 35 ml vial) on

157 a banana medium (Markow and O'Grady, 2005), with a large outbred population maintained

158 using population cages (Rajamohan and Sinclair, 2008). We also maintained a laboratory

159 population of *D. suzukii* collected in Agassiz, British Columbia, Canada (49°24'N 121°76'W, c.

160 12 m a.s.l.). Unless otherwise specified, we used the Ontario population for the experiments

161 described below.

162

163   We also opportunistically collected winter morph flies from the field. At dusk on October

164 17, 2014, one of us (LVF) observed hundreds of wild *D. suzukii* on the windows of Nature

165 Conservancy of Canada's Ivey Research Station on Pelee Island, Ontario, Canada (41°48'N

166 82°37'W). We collected males and females and later confirmed the identification as *D. suzukii*

167 using wing and genital morphology (Fig. 1A). We returned the animals to the laboratory,

168 photographed whole flies and dissected ovaries from three females.

169

170   Summer-acclimated flies (Fig. 1B, D) were held under the rearing conditions (21.5±1°C,

171 60±5% relative humidity, 13:11 L:D). To induce a phenotype similar to the winter morph, vials

172 containing wandering third instar larvae were transferred into 11°C, 10:14 L:D (MIR153, Sanyo,

173 Bensenville, Illinois, USA). These winter-acclimated adults had a darkened cuticle (Fig. 1C), and

174 ovaries were absent or underdeveloped 10 d post-eclosion, while summer morph females had

175 fully-developed ovaries at this time (Fig. 2A). Flies were collected on the day of eclosion and

176 sorted by sex under brief CO_2 exposure either immediately or 3 d after eclosion; we allowed flies

177 at least three days under the rearing conditions to recover from CO_2 exposure prior to use in

178 experiments, so most experiments were begun on flies 5-6 d post-eclosion (Nilson et al., 2006).

179 All quantitative experimental data is available in a supplementary Excel spreadsheet

180 (metadata.xlsx).

181

182   *2.2. Ovarian development*

183   We examined reproductive development in Ontario laboratory winter-acclimated flies by

184   visually assessing ovarian maturity in females reared under 11ºC and 15ºC, at both short (10:14

185   L:D) and long (14:10 L:D) day lengths. We dissected ovaries from five females at 5, 10, 15, 20,

186   and 30 d post-eclosion. We classified ovarian development into three stages: no development, in

187   which the ovaries contained no visible yolk; partial development, in which the ovaries contained

188   eggs partially filled with yolk; and full development, in which all eggs contained complete yolk

189   (Fig. 2A-C). We also examined ovaries from winter-acclimated flies originating from British

190   Columbia at 5 and 30 d post-eclosion, and from the flies collected from Pelee Island upon return

191   to the laboratory.

192

193   *2.3. Energy stores*

194   We quantified neutral lipid and carbohydrate content of summer and winter-acclimated flies 8 h

195   post-eclosion, following Williams (2012). Briefly, we homogenised groups of three flies in 1 ml

196   chloroform: methanol (2:1 v/v; lipids) or ten flies in 200 µl 30% KOH (carbohydrates). We

197   extracted neutral lipids from the homogenate using a modification of the Folch method and

198   quantified them using TLC-FID (Iatroscan MK-6, Shell-USA, Fredericksburg, VA, USA), with

199   cholesterol (not detectable in these flies) as an internal standard to correct for loss during

200   extraction (Williams et al., 2011). We quantified total carbohydrates using the anthrone method

201   (Carroll, 1955); briefly, we extracted glycogen from homogenised tissue suspensions with heat

202   (100ºC, 20 min), added 300 µl ethanol to 150 µl of homogenate and precipitated the glycogen

203   from solution with 7.5 µl of saturated $NaSO_4$. We dried and resuspended the glycogen pellet in

204   water, added 750 µl of anthrone reagent (0.05% anthrone, 1% thiourea, 1 l 72% H_2SO_4), cooled

205 samples in an ice bath, heated samples at 100ºC for 15 min, and re-cooled samples in an ice bath.

206 Absorbance of samples and standards were measured at 620 nm.

207

208   We expressed lipid and carbohydrate content as µg/mg body tissue. We compared both

209 quantities between summer- and winter-acclimated flies in R version 3.0.1 (R Development Core

210 Team, 2013) using ANOVA with a square-root transformation of lipid content to meet

211 assumptions of normality. Interactions between sex and acclimation were not significant and

212 were removed to improve the fit of the model (Crawley, 2012).

213

214 *2.4. Longevity and offspring production*

215 To determine if winter-acclimated flies have a longer lifespan than summer-acclimated flies, we

216 placed groups of 8-10 flies (n= 3-10 groups/treatment) into vials containing banana medium at

217 21.5°C and 13:11 L:D, and counted the number of surviving flies for each vial every 3-4 d for

218 152 d. Because winter-acclimated females had undeveloped ovaries when isolated, and are thus

219 unlikely to reproduce, we accounted for the effect of a decreased longevity through reproduction

220 in case of the mated summer-acclimated females by also using virgin summer females. Thus the

221 six experimental groups included mated and virgin summer-acclimated males and females; and

222 winter-acclimated males and females. Summer-acclimated virgins were separated by sex within

223 8 h of eclosion, and all other flies were sorted into vials 3 d post-eclosion. Flies were transferred

224 to vials with fresh medium at each observation. Vials were retained for one month post-transfer

225 to count the number of offspring produced by each group of females. We arcsine-square-root-

226 transformed the proportion of surviving flies at each time point and used a non-linear mixed

10

227 effects models in R with "vial" as a random effect to compare survival curves between

228 experimental groups.

229

230 *2.5. Gene expression*

231 We used reverse transcriptase quantitative PCR (RT-qPCR) to quantify transcript abundance of

232 genes associated with reproductive development, diapause, stress tolerance, and melanisation

233 (Table 1) in summer- and winter-acclimated flies during development. We collected individuals

234 at the following developmental stages: summer- and winter-acclimated wandering larvae, early

235 pupae (within 24 h of pupation), female late pupae (24 h before eclosion), 1- and 5-d-old female

236 adults; and winter-acclimated female adults 30 and 60 d post-eclosion. For each life stage, we

237 extracted total RNA from three samples of 8-10 individuals using TRIzol (Invitrogen, Burlington

238 ON, Canada), according to the manufacturer's instructions. We synthesised cDNA (qScript

239 cDNA Supermix; Quanta Biosciences, Gaithersburg, MD, USA) from 500 ng of DNase-treated

240 RNA (PerfeCTa DNase I; Quanta Biosciences), according to the manufacturer's protocols.

241

242   We designed primers (Table S1) to amplify 150 bp products for qPCR based on

243 sequences from the *D. suzukii* genome (Chiu et al., 2013), with the exception of *cpo*. Because

244 this sequence was unavailable from the *D. suzukii* genome assembly, we aligned *cpo* sequences

245 available in NCBI from other *Drosophila* spp. using Clustal Omega (Sievers et al., 2011), and

246 restricted primer design to conserved regions of this gene (see Fig. S1, Table S1 for details). We

247 sequenced the amplified *cpo* product at the London Regional Genomics Centre (Robarts

248 Research Institute, London ON, Canada), confirming that it had 99% identity to *D. melanogaster*

249 *cpo* sequences.

11

250

251   We conducted qPCR in 20 μl reactions with the PerfeCTa SYBR Green FastMix (Quanta

252   Biosciences) using 0.2 μl cDNA as template and primers at 0.4 mM in a Bio-Rad CFX96 Real-

253   Time System with a C1000 Thermal Cycler (Bio-Rad, Mississauga, ON, Canada). We conducted

254   three technical replicates of each of the three biological replicates. We used the following

255   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

256   melt-curve determination in increments of 0.5°C from 65°C to 95°C. For quality control, we only

257   used groups of technical replicates whose standard deviation in Ct was less than 0.2. We used the

258   Bio-Rad CFX Manager (version 3.1) to determine the Ct value for each biological replicate,

259   normalise expression against two housekeeping genes, *Ef1α48D* and *TBP* (Zhai et al., 2014), and

260   calculate mean relative normalised expression ($\Delta\Delta Ct$) of each gene for each developmental

261   stage. We compared relative transcript abundance for each gene among acclimation regime and

262   developmental stages using two-way ANOVAs in R.

263

264   *2.6. Cold tolerance*

265   We measured CT_{min} using a glass knock-down column as previously described (Ransberry et al.,

266   2011). We transferred 500-800 summer- or winter-acclimated flies into the column and after 15

267   min equilibration at 21°C the column was cooled to -15°C at 0.1°C min^{-1}. As each fly reached its

268   CT_{min} and lost coordination, it fell out of the column and into a 50 ml plastic vial containing

269   soapy water. This vial was changed every 1°C and flies were filtered out, sorted by sex, and

270   counted. The temperature at which 80% of the flies enter chill coma (CT_{min80}) was calculated and

271   compared among sexes and acclimation regimes using accelerated-failure-time (AFT) models in

272   R (Jakobs et al., 2015). Because the data were not normally-distributed, CT_{min} was compared

12

273 between sex and acclimation regime using a Wilcoxon rank sum test and the effect of the

274 interaction of the acclimation regime and sex was analysed with a Kruskal-Wallis test followed

275 by a Wilcoxon pairwise comparison with Bonferroni-Holm correction.

276

277   To determine the supercooling point (SCP), we placed adult flies individually into 1.7 ml

278 microcentrifuge tubes in contact with a 36-AWG type-T copper-constantan thermocouple

279 (Omega, Laval, Quebec, Canada). We placed the tubes an aluminium block cooled by 50%

280 methanol circulated from a programmable refrigerated bath (Lauda Proline 3530, Würzburg,

281 Germany). Flies were equilibrated at 0°C and cooled to -30°C at 0.1°C min^{-1}. Temperature was

282 recorded at 0.5 s intervals by Picolog v5.20.1 software (Pico Technology, Cambridge, UK) via a

283 Pico Technology TC-08 interface. The SCP was defined as the lowest temperature before the

284 exotherm caused by the latent heat of crystallisation (Lee, 2010). SCPs were compared among

285 the acclimation regimes and sex using a two-way ANOVA and Tukey's post-hoc test in R.

286

287   We determined the cold tolerance strategy according to Sinclair et al. (2015). Flies were

288 cooled as described for SCP determination (n = 10/morph/sex). After half the flies had frozen

289 (indicated by the exotherm), all individuals were transferred individually to wells of 6-well cell

290 culture plates at room temperature with a ca. 1 cm^3 piece of banana medium on three layers of

291 paper towel. Survival was assessed as the ability to stand and move in a coordinated fashion after

292 24 h. Flies were classified as chill-susceptible (died due to chilling injuries unrelated to freezing)

293 if both unfrozen and frozen flies died, freeze-avoidant (died upon freezing) if only unfrozen flies

294 survived, or freeze-tolerant if frozen flies survived.

295

296   The acute lethal temperature (LT) of *D. suzukii* was estimated using 1 h exposures to sub-

297   zero temperatures for each acclimation regime and sex. Groups of 7-10 flies were placed into a

298   1.7 ml microcentrifuge tube and placed directly into a pre-cooled block and held for 1 h at

299   temperatures ranging from -13°C to 0°C (encompassing 0-100% mortality). Temperature during

300   exposure was recorded in every tube using thermocouples as above. All flies from each tube

301   were placed into one well of a 6-well cell culture plate at room temperature containing ca. 1 cm^3

302   banana food and the proportion of surviving flies was assessed after 24 h.

303

304   The lethal time (Lt) of *D. suzukii* was determined under chronic cold exposure to 0°C by

305   submerging three groups of ten flies of each acclimation regime and sex in an ice slurry for 1-24

306   d (encompassing 0-100% mortality). Vials containing banana medium were enclosed in sealed

307   plastic bags to avoid flooding, and the ice slurry was kept in a 20 L styrofoam container in a 4°C

308   cold room, with ice added as needed to maintain the slurry temperature at 0°C. Three vials of

309   summer-acclimated flies were removed daily to assess survival, while vials of winter-acclimated

310   flies were removed twice per week. Survival was assessed after 24 h at the flies' rearing

311   temperature. In addition, we placed two vials each of summer- and winter-acclimated adults

312   under the snow for two weeks in London, Ontario (43°00'N 81°29'W, c. 250 m a.s.l.) from

313   January 26 to February 9, 2015 to assess survival in a potential overwintering habitat.

314

315   The $LT_{80\text{-}1h}$ (temperature at which 80% of flies will die after a 1 h exposure) and Lt_{80}

316   (lethal time at which 80% die) were calculated for winter- and summer-acclimated females and

317   males using a generalised linear model with a binary error distributions and logit link function

14

318 and the fit was tested with Wald's χ^2 using the Glht() function in the package MASS in R

319 (Venables and Ripley, 2002).

320

321   To determine if winter-acclimated flies exhibit a rapid cold hardening (RCH) response,

322 survival following acute exposure (as described above) to the $LT_{80\text{-}1h}$ was determined in 3-6

323 replicates of ten winter-acclimated flies per sex exposed to a pre-treatment of 1 h at 0°C,

324 followed by 1 h recovery at the rearing temperature, as well as winter-acclimated flies that

325 experienced no pre-treatment (Jakobs et al., 2015). The arcsine square root transformation of the

326 proportion of surviving flies was compared among the two treatments and sexes using a two-way

327 ANOVA in R.

328

2.7. Desiccation tolerance

330 The time to death of flies exposed to desiccating conditions was determined by sealing groups of

331 flies in vials with silica gel (Gibbs and Matzkin, 2001). We placed ten replicates of 7-10 flies per

332 acclimation regime and sex in empty 35 mL vials (n=10), with a foam stopper overlaid with 3 g

333 of silica gel and sealed with Parafilm. We counted the number of surviving flies in each vial

334 every 3 h from 0 h to 24 h (encompassing 0-100% mortality) at room temperature (c. 23°C). We

335 compared survival among groups over time as for the longevity analysis. The Lt_{80} for desiccation

336 was determined via a generalised linear model as for chronic cold exposure.

337

2.8. Fungal infections

339 To determine if cold-acclimation increased the immunity of winter-acclimated flies, we gently

340 shook groups of ten 6-d-old virgin male and female flies of each morph for 30 s on an agar plate

15

341 containing sporulating *Metarhizium brunneum*, following Le Bourg et al. (2009). Non-infected

342 controls of each sex and acclimation regime were shaken on a sterile plate. We returned groups

343 of ten flies to food vials at either 12ºC or 21.5ºC, and recorded the proportion of surviving flies

344 every 24 h. We compared survival over time of summer- and winter -acclimated males and

345 females at each temperature using non-linear mixed effects models as described for the longevity

346 analysis.

347

348 **3. Results**

349 *3.1. Winter-acclimated adults delay ovarian development*

350 Adults from laboratory populations from Ontario and British Columbia developed a darkened

351 cuticle when reared at 11°C (Fig. 1), and ovarian development was delayed by low temperatures

352 (Fig. 2). Field-collected flies also displayed dark cuticles and reduced ovarian development,

353 similar to flies reared at 11°C in the laboratory (Fig. 1). At 21.5°C, summer-acclimated females

354 complete ovarian development by 10 d post-eclosion (representative ovaries in Fig. 2A). At

355 15ºC, ovarian development was complete by 20 d post-eclosion, and was complete in most flies

356 reared at 11ºC by 30 d post-eclosion. The ovarian development at 11°C and 15°C did not vary

357 with photoperiod (Fig. 2), nor did cuticular darkening (data not shown). Females transferred

358 from 11°C (10:14 L:D) to 21.5°C (13:11 L:D) at 10 d post-eclosion became reproductively

359 active within three days. Further, we observed eggs and larvae in food vials of flies at 11ºC

360 (10:14 L:D) 30 to 60 d post-eclosion.

361

16

362 *3.2. Winter- and summer-acclimated flies have similar lipid and carbohydrate contents*

363 The only neutral lipid class we detected in *D. suzukii* <8 h post-eclosion was triacylglycerols

364 (Fig. S2). Triacylglycerol content (summer acclimation: 82.2±14.4 µg·mg tissue^{-1}; winter

365 acclimation: 81.3±17.7 µg·mg tissue^{-1}) and carbohydrate content (summer acclimation: 4.3±0.9

366 µg·mg tissue^{-1}; winter acclimation: 4.8±0.9 µg·mg tissue^{-1}) did not differ between morphs or by

367 sex (TAG: sex: $F_{1,16}$=0.004, P=0.95; acclimation regime: $F_{1,16}$=0.02, P=0.88; Carbohydrate: sex:

368 $F_{1,22}$=0.05, P=0.81; acclimation regime: $F_{1,22}$=0.31, P=0.58).

369

370 *3.3. Acclimation regime and reproduction alter longevity*

371 Isolated winter-acclimated females and virgin summer-acclimated females produced no

372 offspring, while mated summer-acclimated females laid viable eggs for up to 85 d post-eclosion.

373 When maintained at 21.5 °C, winter-acclimated females had higher survival than summer-

374 acclimated females (acclimation regime: t_{702}=4.65, P<0.001; time: t_{702}=54.43, P<0.001;

375 acclimation regime × time: t_{702}=0.95, P=0.34), with mated summer-acclimated female mortality

376 occurring 24 d before summer-acclimated virgin and winter-acclimated females (reproductive

377 status: t_{702}=1.04, P=0.30; time: t_{702}=47.84, P<0.001; reproductive status × time: t_{702}=4.16,

378 P<0.001; Fig. 3A, Table 2). Summer-acclimated males survived for approximately 15 d longer

379 than winter-acclimated males (acclimation regime: t_{820}=2.05, P=0.04; time: t_{820}=65.51, P<0.001;

380 acclimation regime × time: t_{820}=1.03, P=0.30; Fig. 3B, Table 2).

381

382 *3.4. Winter-acclimated females differentially express stress- and reproduction-related genes*

383 Transcript levels of all genes varied during development (Table 3). Several stress-related gene

384 transcript levels increased in winter-acclimated, but not summer-acclimated, flies post-eclosion,

17

385 including *Cat* and *Sod,* which encode oxidative stress enzymes, and *smp-30* (Fig. 4A-C, Table

386 3). Winter-acclimated females upregulated two heat shock protein genes, *Hsp83* and *Hsc70-4*, at

387 all developmental stages, while *Hsp27* did not differ between winter- and summer-acclimated

388 flies, and *Hsp23* had lower transcript levels in winter-acclimated pupae (Fig. 4D-G, Table 3).

389 *PPO1* expression increased late in pupal development, but transcript levels did not differ

390 between winter- and summer-acclimated groups (Fig. 4H, Table 3).

391

392   Transcript abundance of the regulatory genes *Jheh1* and *Thor* increased in wandering

393 larvae after 24 h at 11°C, and in 6 d winter-acclimated females, whereas *foxo* was upregulated in

394 1 d winter-acclimated females (24 h post-eclosion) (Fig. 4I-K, Table 3). Downregulation of *cpo*

395 in summer-acclimated adults coincided with expression of *Yp1*, while winter-acclimated adults

396 maintained high *cpo* transcript levels and did not express *Yp1* (Fig. 4L,M, Table 3).

397

398 *3.5. Winter-acclimated flies are active at lower temperatures and more cold-tolerant than*

399 *summer-acclimated flies*

400 The temperature at which 80% of flies had entered chill coma (CT_{min80}) ranged from 0.5°C to -

401 5.5°C, and was approximately 5°C lower in winter- than summer-acclimated flies (acclimation

402 regime: $W = 163640$, $P < 0.001$; sex: $W = 78912$, $P < 0.001$, acclimation regime × sex: Kruskal-

403 Wallis $\chi^2 = 369.23$, df= 3, $P<0.001$; Table 2).

404

405   SCP ranged from -22.4°C in a summer-acclimated male to -14.3°C in a summer-

406 acclimated female. The mean SCP of summer-acclimated males was lower than the other groups

407 (sex: $F_{1,72}=27.367$, $P<0.001$; acclimation regime: $F_{1,72}=22.395$, $P<0.001$, sex × acclimation

18

408 regime: $F_{1,72}=9.81$, $P<0.001$; Table 2). However no fly from any group survived freezing or

409 temperatures close to the SCP, thus we consider all groups to be chill-susceptible (Table 2).

410

411　　　An acute (1 h) low-temperature exposure caused mortality below -5.2°C for summer-

412 acclimated flies and below -7.8 °C for winter-acclimated flies, with winter-acclimated flies

413 surviving temperatures approximately 3°C lower than summer-acclimated flies (Fig. 5A,B;

414 Table 2,4). When exposed to 0°C, all summer-acclimated *D. suzukii* were dead within 1 week, a

415 time period that almost all winter-acclimated adults survived (Fig. 5C,D). Based on the Lt_{80},

416 winter-acclimated adults survived for approximately 10 d longer at 0°C than summer-acclimated

417 adults (Table 2, Table S2). Summer-acclimated flies under the snow cover during the London,

418 ON, winter also had 100% mortality after one week, while winter-acclimated flies had 70%

419 mortality after one week under the snow, and 100% mortality after two weeks. During this

420 period, the minimum temperature below the snow during the first week was -1.8°C, and -1.1°C

421 during the second week. The RCH treatment increased the proportion of winter-acclimated flies

422 surviving a 1 h exposure at the LT_{80} by 35% (treatment: $F_{1,18}=13.626$, $P=0.002$; sex: $F_{1,18}=0.101$,

423 $P=0.78$, treatment $\times$ sex: $F_{1,18}=0.013$, $P=0.91$; Table 2).

424

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

426 All *D. suzukii* exposed to desiccating conditions at c. 23°C died within 24 h (Fig. 6). Females

427 survived for 3 to 4 h longer than males of the same acclimation regime (sex: $t_{320}=3.24$, $P=0.001$;

428 time: $t_{320}=32.86$, $P<0.001$; sex $\times$ time: $t_{320}=0.92$, $P=0.36$; Fig. 6; Table 2). Winter-acclimated

429 flies under desiccating conditions did not survive significantly longer than summer-acclimated

19

430   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;

431   Table 2).

432

433   *3.7. Winter-acclimated females are more resistant to fungal infections than summer-acclimated*

434   *females at warm temperatures*

435   Non-infected flies had 100% survival for the duration of fungal infection experiments. At

436   21.5°C, most fungal-infected flies died within two weeks, while survival at 12°C exceeded 30 d

437   (Fig. 7). Female winter-acclimated flies had better survival over time following fungal infection

438   than summer-acclimated flies at 21.5°C (acclimation regime: t_{172}=2.30, P=0.02; time: t_{172}=22.99,

439   P<0.001; acclimation regime × time: t_{172}=2.50, P=0.01; Fig. 7). However, we observed no

440   difference between infected winter- and summer-acclimated females at 12°C (acclimation

441   regime: t_{288}=1.89, P=0.06; time: t_{288} =16.58, P<0.001; acclimation regime × time: t_{288}=0.03,

442   P=0.97; Fig. 7). Male survival of fungal infection did not vary between acclimation regimes at

443   either 21.5°C (acclimation regime: t_{130}=1.48, P=0.14; time: t_{130}=21.37, P<0.001; Fig. 7) or 12°C

444   (acclimation regime: t_{289}=0.03, P=0.98; time: t_{289}=20.38, P<0.001; Fig. 7).

445

446   **4. Discussion**

447   When wandering larvae are reared under short days and low temperatures (i.e. winter

448   acclimation), laboratory populations of *D. suzukii* adults from both Ontario and British Columbia

449   exhibited a 'winter morph' – characterized by a darkened cuticle and underdeveloped or absent

450   ovaries – comparable to specimens observed by us in the field in Ontario (Pelee Island) and

451   reported elsewhere (e.g. Zerulla et al., 2015). We observed this cuticular darkening when larvae

452   were exposed to both short and long days at 11°C, but not at 15°C or 21.5°C (data not shown).

453 This suggests that low temperature, rather than photoperiod, is the cue for increased melanisation

454 in the winter-acclimated flies.

455

456   The delay in ovarian development we observed in winter-acclimated flies also appears to

457 be primarily regulated by temperature. Flies had fully-developed ovaries by 10 d post-eclosion at

458 21.5°C, 20 d post-eclosion at 15°C, and 30 d post-eclosion at 11°C, regardless of day length.

459 Temperature-dependence of ovarian development in our study is consistent with gene expression

460 data: the vitellogenic *Yp1* transcripts were present in summer-acclimated adults 1 and 6 d post-

461 eclosion, whereas winter-acclimated adults of the same age lacked the transcript, indicating that

462 winter-acclimated flies are not producing yolk in the period immediately after eclosion. *Yp1*

463 transcript levels remain low in 30 and 60 d-old winter-acclimated females, suggesting that egg

464 production is slow at 11°C. *Jheh1* expression increased when wandering larvae were transferred

465 to 11°C, which is consistent with a model of suppressed JH signalling causing a reproductive

466 delay (Sim et al., 2015). The suppression of ovarian development in winter-acclimated females

467 coincided with high levels of *foxo* and *cpo* transcripts, whose products are hypothesized to

468 contribute to suppression of vitellogenesis (Schmidt et al., 2008; Sim et al., 2015). *Hsp23*

469 expression is associated with vitellogenic *D. montana* females (Salminen et al., 2015), which

470 may explain its reduced expression in winter-acclimated *D. suzukii* females.

471

472   While many insects accumulate considerable energy stores (e.g. lipids or carbohydrates)

473 prior to overwintering (Sim and Denlinger, 2013), newly-eclosed winter-acclimated *D. suzukii*

474 had similar triacylglycerol and carbohydrate content to summer-acclimated flies. We note that

475 these energy stores could be adequate to fuel overwintering if metabolic rate is decreased in

21

476 winter-acclimated flies (Hahn and Denlinger, 2007). However, we shifted only wandering larvae

477 to the winter-acclimation regime, which meant that there was no additional opportunity to

478 accumulate lipid reserves during larval development, which may explain why our flies did not

479 have increased body size described elsewhere for 'winter morphs' (Zerulla et al. 2015). In

480 addition, we did not determine whether adult winter-acclimated flies accumulate additional

481 energy stores when feeding. Adults from the *D. auraria* complex reared under low temperatures

482 and short photoperiods accumulate triacylglycerol contents two-fold higher than summer-

483 acclimated flies during a period 8 to 32 d post-eclosion (Ohtsu et al., 1992). Thus, measuring

484 energy accumulation and use both during larval development and in adult flies post-eclosion will

485 be necessary to fully determine whether *D. suzukii* employs an energy conservation or

486 accumulation strategy to fuel overwinter energetic demands.

487

488 In addition to delayed reproductive maturity, winter-acclimated *D. suzukii* had enhanced

489 stress tolerance and altered gene expression suggestive of increased stress tolerance. Winter-

490 acclimated flies were more cold-tolerant than summer-acclimated flies, with a lower chill coma

491 onset temperature (CT_{min80}), and lethal temperature (LT_{80}), consistent with Stephens et al.

492 (2015). *D. suzukii* SCPs did not differ with acclimation regime, were comparable to SCPs of

493 other *Drosophila* spp. (Andersen et al., 2015), and likely do not reflect cold tolerance in this

494 chill-susceptible species. Winter-acclimated adults also displayed a robust rapid cold-hardening

495 response compared to summer-acclimated adults as examined by Jakobs et al. (2015).

496

497 The CT_{min} of winter- and summer-acclimated *D. suzukii* are within the range of CT_{min}

498 observed for other mid-latitude (38-50°) *Drosophila* spp. (Andersen et al., 2015). However,

22

499 although winter-acclimated adults had a lower CT_{min} and survived for longer at 0°C than

500 summer-acclimated flies, they were killed after three weeks at 0°C. This is consistent with

501 mortality we observed in field-deployed winter-acclimated flies, and suggests that the winter-

502 acclimated flies reared in this study would be unlikely to survive for long enough at 0°C to

503 successfully overwinter in Ontario. We expected winter-acclimated flies to be more desiccation-

504 tolerant than summer-acclimated flies, because darker cuticles are associated with reduced water

505 loss rates in other species of *Drosophila* (Parkash et al., 2014; Rajpurohit et al., 2008; Ramniwas

506 et al., 2013). However, flies from both acclimation regimes were similarly desiccation-sensitive

507 at room temperature, although we did not measure water loss rates or cuticular water loss, which

508 means we cannot entirely rule out melanisation-related reduced cuticular permeability in winter-

509 acclimated *D. suzukii*. In addition, are expected to be lower at lower temperatures, so water

510 balance under overwintering conditions is an avenue for further study.

511

512   Some of the enhanced cold tolerance of the winter-acclimated could be attributed to

513 increased expression of oxidative stress enzymes (*Sod*, *Cat*), which are upregulated in several

514 diapausing insects (Salminen et al., 2015; Sim and Denlinger, 2011). Sim and Denlinger (2011)

515 demonstrated that *Cat* expression is required for longevity and prevention of ovarian follicle

516 degeneration in diapausing mosquitoes, and it is therefore possible that antioxidant enzyme

517 expression in winter-acclimated *D. suzukii* contributes to enhanced survival at 0°C. At higher

518 temperatures (21.5°C), however, longevity was primarily determined by reproductive activity in

519 female *D. suzukii*, with similar survival in virgin summer-acclimated flies and non-reproductive

520 winter-acclimated flies. Mated summer-acclimated females had lower longevity, likely due to

521 energy expended in producing offspring (Flatt and Kawecki, 2007). However, male winter-

522 acclimated flies had shorter lifespans at 21.5°C than summer-acclimated flies, in contrast to *D.*

523 *melanogaster* that survive similarly at 25°C whether acclimated at 11°C or 25°C (Tatar et al.,

524 2001). We did not measure longevity at moderately low temperatures (e.g. 4 °C, as is commonly

525 found beneath snow), and speculate that longevity may be adequate to survive the winter if

526 measured at low temperatures where flies may not be accumulating chilling injuries, as has been

527 reported for *D. melanogaster* by Tatar et al. (2001).

528

529 Heat shock proteins can considerably enhance stress tolerance (King and MacRae, 2015).

530 Indeed, *Hsp83* was upregulated throughout development of the winter-acclimated flies. By

531 contrast, *Hsp27* transcript levels did not differ between summer and winter-acclimated flies,

532 similar to several heat shock proteins during *D. triauraria* reproductive diapause (Goto and

533 Kimura, 2004). The gene *smp-30* is induced by cold acclimation in *D. melanogaster* (Goto,

534 2000), expressed in diapausing *D. montana* females (Kankare et al., 2010), and is also

535 upregulated in winter-acclimated *D. suzukii*. *smp-30* has similar functional motifs to *regucalcin*,

536 which is upregulated during initiation of *D. montana* reproductive diapause (Salminen et al.,

537 2015), but is not induced following chill coma in *D. melanogaster* (Reis et al., 2011). The

538 upregulation of *smp-30* in winter-acclimated *D. suzukii* appears to be a response to cold

539 acclimation, however the role of this gene in conferring cold tolerance remains unclear in

540 *Drosophila*.

541

542 Fungal or bacterial pathogens can be a key source of overwinter mortality (Colinet et al.,

543 2015; Hokkanen, 1993; Sinclair et al., 2013; Williams et al., 2015). Because the melanisation

544 cascade is associated with both cuticle darkening and encapsulation responses (Dubovskiy et al.,

545 2013; Kutch et al., 2014), we expected winter-acclimated flies to have improved

546 immunocompetence in association with the darkened cuticle. However, male winter-acclimated

547 flies had no survival advantage over their summer-acclimated counterparts, and female winter-

548 acclimated flies only had improved survival to fungal infection in warm (21.5°C) but not cool

549 (12°C) conditions. Increased melanisation alone was therefore insufficient to increase

550 immunocompetence. The greater fungal resistance of winter-acclimated compared to summer-

551 acclimated females at 21.5°C suggests that winter-acclimated females can take advantage of

552 warm spells to fight infection (Colinet et al., 2015). These results are consistent with *PPO1*

553 expression patterns, which did not differ between summer- and winter-acclimated *D. suzukii;*

554 *PPO1* is important for melanin-based immune function in *D. melanogaster* (Binggeli et al.,

555 2014). The elevated *Thor* expression in 6-d-old winter-acclimated females may contribute to the

556 enhanced immunocompetence exhibited by these females under warm conditions (Bernal and

557 Kimbrell, 2000).

558

559 *4.1. Are winter-acclimated* D. suzukii *in diapause?*

560   The winter-acclimated *D. suzukii* in our study had enhanced stress tolerance and apparent

561 suppression of ovarian development; however, we do not believe this represents diapause *sensu*

562 *stricto*. Photoperiod had no effect on either reproductive development or cuticular darkening

563 (which appeared to be entirely temperature-dependent), and the ovarian developmental delay we

564 observed appeared to simply be due to slower growth at low temperatures rather than induced by

565 a particular environmental cue (Koštál, 2006). This differs from early work on *D. melanogaster*,

566 which found that low temperatures and short days are both required to induce suppression of

567 ovarian development (Saunders et al. 1989), but is consistent with later *D. melanogaster* work, in

25

568   which low temperatures caused ovarian suppression under both long and short days (Tatar et al.

569   2001), with lower temperatures resulting in more severe ovarian suppression (Lee et al., 2011).

570   We did not, however, examine an extensive range of photoperiods (cf. Lankinen et al., 2013), so

571   it is possible that reproductive diapause could be induced under a different combination of day

572   lengths and temperatures, or with exposure to short days earlier in development (Saunders,

573   2014). Furthermore, we found no evidence that ovarian development was suppressed by cues

574   other than temperature. Winter-acclimated flies transferred from 11°C to 21.5°C resumed

575   ovarian development almost immediately, similar to *D. melanogaster* transferred from 11°C to

576   25°C (Tatar et al., 2001), suggesting that reproduction is not actively suppressed. Flies resumed

577   reproduction once ovarian development was complete at 11°C even under short days, similar to

578   spontaneous 'diapause' termination observed in *D. melanogaster* within 6-7 weeks post-eclosion

579   (Lee et al., 2011), implying that no particular environmental cue was required to terminate the

580   reproductive developmental delay (Koštál, 2006). Thus, the lack of ovarian development

581   associated with winter-acclimated *D. suzukii* is not consistent with a true diapause, but likely

582   reflects slow development at low temperatures.

583

584   The expression levels of candidate diapause-related genes are also not strongly

585   supportive of a true diapause in winter-acclimated *D. suzukii*. Based on the current model that JH

586   and insulin suppression leads to expression of the transcription factor *foxo* and its targets (Sim et

587   al., 2015), we expect JH esterases (e.g. Jheh1) to initiate JH suppression and subsequently

588   increase *foxo* expression (Poelchau et al., 2013). However, *Jheh1* is only substantially

589   upregulated after *foxo* transcript abundance peaks in *D. suzukii* winter-acclimated flies. The

590   putative targets of FOXO, *Thor* and *cpo*, which are upregulated in *D. montana* diapause

26

591 (Kankare et al., 2010; Salminen et al., 2015), are also upregulated in *D. suzukii* winter-

592 acclimated adults. However, although *foxo* upregulation coincides with the increase in *cpo*

593 transcript abundance, further supporting that FOXO induces *cpo* expression in *D. suzukii*,

594 increased *Thor* expression does not coincide with *foxo* transcript abundance. Thus, it appears that

595 transcription factors other than *foxo* could regulate *Thor* in *D. suzukii*. *Hsc70-4*, which is

596 upregulated during *D. montana* diapause termination (Salminen et al., 2015), is also expressed in

597 *D. suzukii* winter-acclimated females, but its expression is not restricted to the time at which

598 ovarian development resumes. Thus, although we observed some upregulation of diapause-

599 related genes in winter-acclimated *D. suzukii*, the timing of this upregulation is inconsistent with

600 diapause as characterised in other *Drosophila* species.

601

602   Nevertheless, the enhanced stress tolerance of winter-acclimated *D. suzukii* means they

603 are more likely to successfully overwinter than summer-acclimated flies. The low CT_{min} of

604 winter-acclimated flies suggests they could remain active under snow cover during winter, when

605 80% of summer-acclimated flies would be in chill coma and unable to feed or select beneficial

606 microhabitats. Although winter-acclimated flies from the laboratory did not survive under the

607 snow for more than two weeks, they exhibited a RCH response that increased survival at the

608 LT_{80-1h}. Therefore, cold tolerance of winter-acclimated adults is plastic during and after exposure

609 to low environmental temperatures, improving the probability of successful overwintering. The

610 acclimation conditions we used to induce the 'winter morph' (11°C, 10:14 L:D) were

611 representative of typical autumn conditions in southern Ontario, but do not capture the thermal

612 variability that *D. suzukii* would experience in nature. While Stephens et al. (2015) suggest

613 neither summer nor winter morph *D. suzukii* are likely to survive winter conditions, it is possible

27

614 that developmental or adult acclimation (or repair of accumulated damage) under a fluctuating

615 thermal regime would promote increased stress tolerance (Colinet et al., 2015), extending

616 survival (and possibly longevity) of *D. suzukii* during overwintering conditions.

617

5. Conclusions

619 While not an example of true diapause, *D. suzukii* stress tolerance increases due to both

620 developmental and adult acclimation at low temperatures, improving the cold tolerance and low

621 temperature activity of this chill-susceptible species. This stress tolerance in winter-acclimated

622 flies coincides with increased cuticle melanisation and female reproductive delay. Higher

623 melanisation does not appear to improve desiccation tolerance or immunocompetence. However,

624 stress-related genes upregulated in winter-acclimated flies, such as those encoding antioxidants,

625 heat shock proteins, and smp-30, may underlie the increase cold tolerance we observed. The

626 temperature-induced delay in reproductive development is likely mediated through suppression

627 of *Yp1* expression by high levels of foxo and cpo. Future studies should examine the extent to

628 which these genes affect the stress tolerance and reproductive physiology of *D. suzukii*, and the

629 impact of other acclimation treatments (e.g. fluctuating thermal regimes) on the ability of this

630 pest to withstand overwintering stresses.

631

28

641

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Figure legends

892

893 **Fig. 1. Summer and winter morphs of *Drosophila suzukii*.** Dorsal view of (A) field-collected

894 winter morph *D. suzukii* female from Pelee Island, Ontario, Canada and (B) lab-reared, summer-

895 acclimated *D. suzukii*. Lateral view of lab-reared (C) winter-acclimated and (D) summer-

896 acclimated *D. suzukii* females.

897 **Fig. 2. The effect temperature and photoperiod on ovarian development in *D. suzukii*.**

898 Representative ovaries dissected from *D. suzukii* females with (A) zero, (B) partial, or (C) full

899 ovarian development. The proportion of each class of ovaries in groups of five females reared

900 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

901 long days, 14:10 L:D, and (G) 15°C and long days, 14:10 L:D.

902 **Fig. 3. The effect of acclimation regime and reproductive activity on longevity of *D. suzukii*.**

903 The proportion of surviving (A) females and (B) males over time at 21.5°C, 13:11 L:D. Each

904 point represents the mean±s.e.m proportion of surviving flies from 3-9 vials (7-10 flies/vial). *

905 indicates a significant effect of morph, † indicates a significant reproductive status × time

906 interaction (non-linear mixed effects model details in text).

907 **Fig. 4. Gene expression of summer- and winter-acclimated *D. suzukii* during development.**

908 Transcript abundance of target genes were normalised to the housekeeping genes *Ef1α48D* and

909 *TBP*. (A) *smp-30*, (B) *Cat*, (C) *Sod*, (D) *Hsp83*, (E) *Hsc70-4*, (F) *Hsp27*, (G) *Hsp23*, (H) *PPO1*,

910 (I) *Jheh1*, (J) *foxo*, (K) *Thor*, (L) *cpo*, and (M) *Yp1*. Each point represents the mean±s.e.m of

911 three biological replicates of 8-10 individuals at a particular life stage: wandering larvae (Larva),

912 pupae within 24 h of pupation (Early Pupa) or 24 h pre-eclosion (Late Pupa), and adults 1, 6, 30

913 and 60 d post-eclosion (1 d, 6 d, 30 d, 60 d, respectively). Open circles, dashed line: summer-

38

914 acclimated flies; closed circles, solid line: winter-acclimated flies. * indicates a significant effect

915 of acclimation regime, † indicates a significant acclimation regime × developmental stage

916 interaction (two-way ANOVA, details in Table 3).

917 **Fig. 5. Acute and chronic cold tolerance of summer- and winter-acclimated *D. suzukii*.**

918 Survival of groups of 7-10 (A) female and (B) male flies 24 h after 1 h (acute) exposures to

919 temperatures between 0°C and -13°C, and (C) female and (D) male flies following 1 to 24 d

920 (chronic) exposures to 0°C. Each point represents the proportion of surviving flies in a single

921 vial (7-10 flies/vial). The intersection between the dotted line and each curve indicates the

922 temperature (LT_{80-1h}, A & B) or time (Lt_{80}, C & D) at which 80% mortality occurred. There was

923 no effect of sex, so males and females were pooled for analysis. * indicates a significant effect of

924 acclimation regime, † indicates a significant acclimation regime × temperature or time

925 interaction, # indicates a significant acclimation regime × sex interaction (generalised linear

926 model, details in Table S2).

927 **Fig. 6. Desiccation tolerance of summer- and winter-acclimated flies.** The proportion of

928 surviving (A) females and (B) males desiccated in vials containing Silica gel at room

929 temperature. Each point represents the mean±s.e.m proportion of surviving flies from ten vials

930 (8-10 flies/vial). There was a significant effect of sex, but no effect of acclimation regime (non-

931 linear mixed effects model details in text).

932 **Fig. 7. Survival following fungal infection of summer- and winter-acclimated flies.** The

933 proportion of surviving (A) females and (B) males at 12°C and (C) females and (D) males at

934 25°C following fungal infection with *Metarhizium brunneum*. Each point represents the

39

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).

Figure 1

Figure 2

Figure 3

Figure 4

948   **Figure 5**

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953   **Figure 6**

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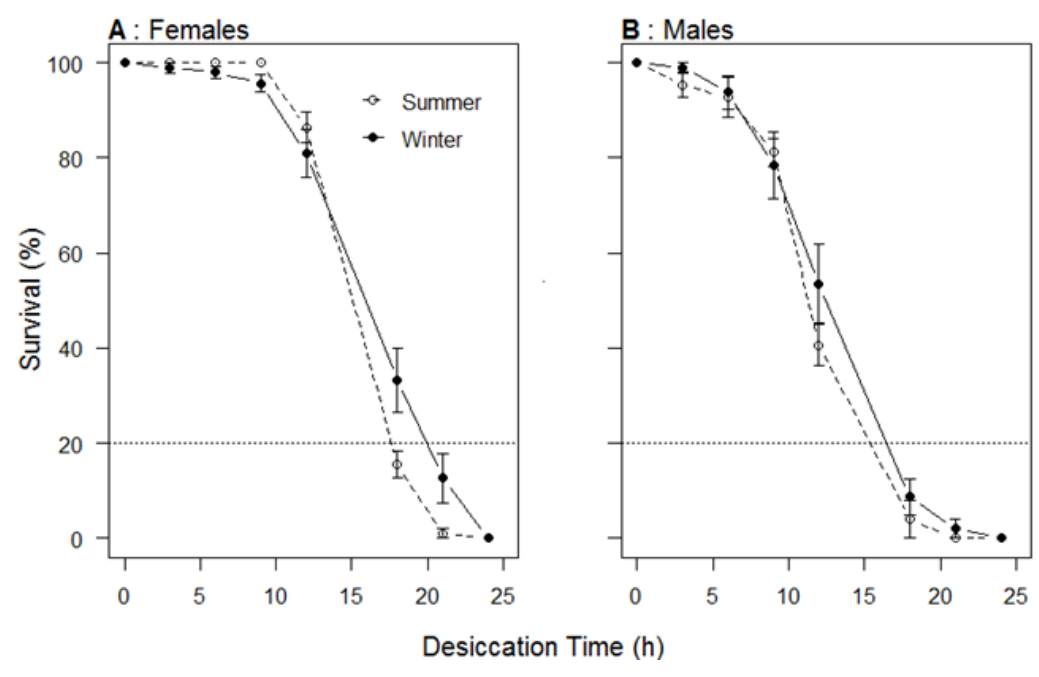
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956 **Figure 7**

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958

959 **Tables**

960 **Table 1. Genes examined via RT-qPCR.** Gene names and functions based on *D. melanogaster*

961 (flybase.org). Primer sequences available in supplementary material (Table S1).

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

962 [1] Housekeeping genes for RT-qPCR (Zhai et al., 2014)

48

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)

49

968 **Table 3. Two-way ANOVAs of relative transcript abundance for each target gene.**

969 Transcript abundance of each target gene was normalised against housekeeping genes *Ef1α48D*

970 and *TBP* in summer- and winter-acclimated flies. Bold *P*-values indicate a significant effect of

971 acclimation regime, stage, or the acclimation regime × stage interaction on transcript abundance.

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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