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1 **Cold tolerance of third-instar *Drosophila suzukii* larvae**

2

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16

17

18 **Abstract**

19 *Drosophila suzukii* is an emerging global pest of soft fruit; although it likely overwinters as an
20 adult, larval cold tolerance is important both for determining performance during spring and
21 autumn, and for the development of temperature-based control methods aimed at larvae. We
22 examined the low temperature biology of third instar feeding and wandering larvae in and out of
23 food. We induced phenotypic plasticity of thermal biology by rearing under short days and
24 fluctuating temperatures (5.5-19 °C). Rearing under fluctuating temperatures led to much slower
25 development (42.1 days egg-adult) compared to control conditions (constant 21.5 °C; 15.7 d),
26 and yielded larger adults of both sexes. *D. suzukii* larvae were chill-susceptible, being killed by
27 low temperatures not associated with freezing, and freezing survival was not improved when ice
28 formation was inoculated externally via food or silver iodide. Feeding larvae were more cold
29 tolerant than wandering larvae, especially after rearing under fluctuating temperatures, and
30 rearing under fluctuating temperatures improved survival of prolonged cold (0 °C) to beyond 72
31 h in both larval stages. There was no evidence that acute cold tolerance could be improved by
32 rapid cold-hardening. We conclude that *D. suzukii* has the capacity to develop at low
33 temperatures under fluctuating temperatures, but that they have limited cold tolerance. However,
34 phenotypic plasticity of prolonged cold tolerance must be taken into account when developing
35 low temperature treatments for sanitation of this species.

36

37 **Keywords:** spotted wing drosophila; cold tolerance; chill susceptible; overwintering; phenotypic
38 plasticity; fluctuating thermal regimes

39

40

41 **Introduction**

42 Spotted wing drosophila, *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), is an
43 emerging global pest of soft fruit (Cini *et al.*, 2014; Lee *et al.*, 2011; Walsh *et al.*, 2010). *D.*
44 *suzukii* lays eggs in unripe fruit. The entry wound and larval development promote fruit
45 degradation, resulting in significant losses to blueberry, strawberry and cherry crops (Bolda *et*
46 *al.*, 2010). As with most *Drosophila* except *D. lutescens*, which may overwinter as a larva or
47 pupa in Japan (Kimura, 1988), *D. suzukii* appears to overwinter as an adult, and there is a well-
48 described 'winter morph' that is darker than the summer morph (Zerulla *et al.*, 2015). This
49 winter morph has some improved tolerances to environmental stress (Plantamp *et al.*, 2016;
50 Shearer *et al.*, 2016; Toxopeus *et al.*, 2016; Wallingford *et al.*, 2016). However, larvae appear to
51 be significantly less cold tolerant than adults, being killed by short exposures to sub-zero
52 temperatures (Dalton *et al.*, 2011) and longer exposures to temperatures near 0 °C (Kanzawa,
53 1939).

54

55 Insect cold tolerance strategies are usually divided into freeze tolerance (those that can withstand
56 internal ice formation) and freeze avoidance, wherein individuals can survive cold as long as
57 they do not freeze, but are killed when ice formation occurs (the supercooling point, SCP;
58 Sinclair *et al.*, 2015). The majority of insects, however, are chill-susceptible, killed by processes
59 unrelated to ice formation at temperatures above the SCP (Sinclair *et al.*, 2015). Strachan et al.
60 (2011) found that larvae of 18 of 27 *Drosophila* were chill-susceptible, with another eight freeze-
61 avoidant. Larvae of the closely-related *Chymomyza costata* and *C. amoena* are freeze tolerant
62 when sufficiently cold-acclimated and with external ice inoculation (Košťál *et al.*, 2011; Sinclair
63 *et al.*, 2009). However, no *Drosophila* larvae are currently thought to be freeze tolerant. Cold
64 tolerance can also be phenotypically plastic. *D. melanogaster* larvae exhibit a rapid cold-

65 hardening response (Czajka and Lee, 1990), as well as responding to longer-term acclimation
66 (Rajamohan and Sinclair, 2009).
67
68 We observed that some late-instar *D. suzukii* larvae in field cages survived a cold snap in
69 November 2014 that reached -6.9 °C and killed all the adult flies. This led us to hypothesise that
70 acclimation or hardening may make larvae more cold-tolerant than previously reported.
71 Moreover, because the host fruit are often exported, cold tolerance of the larvae is relevant for
72 determining the capacity of larvae to survive chilling during processing and transport. Thus, our
73 objective was to better characterise the cold tolerance of *D. suzukii* larvae. We measured growth
74 and development, SCP, cold tolerance strategy and acute and chronic lethal temperatures of
75 third-instar feeding and wandering larvae with and without an acclimation under fluctuating
76 temperatures. For feeding larvae, we conducted experiments both within food (replicating likely
77 field conditions) and without food (which allows us to better control the conditions and get a
78 more precise measure of lethal limits).
79

80 Methods

81 *Animal rearing and treatment groups*

82 We established a *Drosophila suzukii* population from approximately 200 individuals collected in
83 the Halton Hills region, Ontario, Canada (43°34'N 79°57'W). We reared flies on a banana-
84 cornmeal-agar medium (Markow and O'Grady, 2005), at 21.5 ± 1 °C and 60 ± 5 % relative
85 humidity under 13:11 L:D, as described elsewhere (Jakobs *et al.*, 2015; Nyamukondiwa *et al.*,
86 2011; Toxopeus *et al.*, 2016). We used 3.7 L population cages containing approximately 300
87 adult flies that were two to six days post-eclosion (to reduce any parental age effect). Flies laid
88 eggs on Petri dishes of banana food that had been dyed green with food colouring, which allowed

us to separate feeding and non-feeding larvae. We removed the plates from the population cages every 24 h, and reared larvae on the Petri dishes.

To induce phenotypic plasticity in *D. suzukii* larvae, we placed the food plates with the eggs into two different rearing conditions (treatment). Eggs were placed under either control conditions (21.5 °C, 13:11 L:D) or exposed to a fluctuating thermal regime (FTR; 5.5 °C/19 °C, 11.5:12.5 L:D), simulating the average photoperiod and daily minimum and maximum temperatures from late September in London, Ontario.

We used third instar feeding and wandering larvae for experiments. We checked the food plates for larvae on a daily basis and removed larvae with a soft paintbrush. Banana food medium was carefully removed from larvae with tap water and larvae were blotted dry with a tissue. The life stage of a subset of larvae on each collection day was identified using the morphology of the mouth hooks (Figure 1A-C) and anterior spiracles (Figure 1D), based upon Demerec's (1965) descriptions for *D. melanogaster*. In addition, feeding third instar individuals appeared green as they still carried green food in their gut, while wandering-stage instars were transparent and lacked food in the gut (Figure 1E).

To determine the effect of the treatments on developmental time, eggs were reared into adults under control conditions, FTR or a constant low temperature (11 °C, 10:14 LD). We removed pieces of the banana medium carrying approximately ten eggs, and transferred them into 35 mL vials containing banana medium (n=6 vials/treatment). We collected the adults that developed from these eggs daily and stored them at -20 °C. When emergence had ended, we

112 dried the flies over silica gel for approximately 48 h. Flies were sexed and weighed (MX5
113 microbalance, Mettler Toledo, Columbus, OH, USA) as a measure of offspring dry mass.

114

115 *Cold tolerance*

116 We determined cold tolerance parameters using the approach described by Sinclair et al. (2015).
117 To determine the supercooling point (SCP), we placed larvae individually into 1.7 mL
118 microcentrifuge tubes in contact with a 36-AWG type-T copper-constantan thermocouple
119 (Omega, Laval, Quebec, Canada) connected to a computer via a TC-08 interface and Picolog
120 v5.20.1 software (Pico Technology, Cambridge, UK), which recorded the temperature at 0.5 s
121 intervals. The tubes were placed into holes in an aluminium block cooled by methanol (diluted c.
122 50 % in water) circulated from a refrigerated bath (Lauda Proline 3530, Würzburg, Germany).
123 Larvae were equilibrated at 0 °C and cooled to -30 °C at 0.1 °C/min. The SCP was defined as
124 the lowest temperature before the exotherm caused by the latent heat of crystallisation.

125

126 To determine the cold tolerance strategy, larvae were placed into microcentrifuge tubes and
127 cooled, as described for the SCPs. After half the larvae had frozen (indicated by the exotherm),
128 all individuals were removed quickly to room temperature and placed individually into the wells
129 of 6-well cell culture plates with a ca. 1 cm^3 piece of banana food. Survival was assessed as the
130 ability to develop into adults. Flies were considered chill susceptible if both unfrozen and frozen
131 flies died, freeze-avoidant if all unfrozen flies survived (but those that froze died), and freeze
132 tolerant if individuals that froze survived.

133

134 Because some insects are freeze tolerant only with external ice inoculation, (e.g. Shimada and
135 Riihimaa, 1988) we applied an external ice nucleator (silver iodide) to initiate freezing (Strachan

136 *et al.*, 2011). We dipped larvae into a silver iodide/water slurry and determined the SCP and cold
137 tolerance strategy as described above.

138

139 We estimated the acute lethal temperature (LT) of third feeding and wandering larvae of the
140 control and FTR group by exposing these larvae to a range of low temperatures for 1 h. Groups
141 of ten larvae were placed into a 0.65 mL microcentrifuge tube (n=3 groups/ temperature/ stage/
142 treatment combination). These tubes were placed into a pre-cooled aluminium block (described
143 above) and held for 1 h at temperatures ranging from -15 °C to 0 ° C (encompassing 0-100 %
144 mortality). Temperature during exposure was recorded in two blank tubes that were directly
145 placed next to the tubes with larvae in the cooling block using thermocouples as above.
146 Following the low temperature exposure we placed each opened tube into a 35 mL vial
147 containing banana medium and reared the larvae to eclosion under control conditions. Survival
148 was determined as the ability to eclose as adult.

149

150 Because larvae might be exposed to low temperatures inside their food, we also determined acute
151 low-temperature survival of larvae in 35 mL vials containing banana food. Groups of 20 larvae
152 were placed into each vial, which was exposed to low temperatures for 1 h as described above.
153 Temperature was determined by placing the thermocouples inside the food medium (2 cm below
154 the food surface). After cold exposure, food vials containing the larvae were placed under control
155 conditions and the number of adults that eclosed counted as a measure of survival. The
156 temperature measured inside and outside the food differs during a 1 h exposure. Thus,
157 temperature was recorded inside and outside the food during an exposure to -9 °C (n=10). SCP of
158 the banana food was determined during this exposure and used in analyses.

159

160 To determine survival of prolonged exposure to milder cold temperatures (see Sømme (1996) for
161 rationale), we placed groups of ten larvae into food vials (n=3 groups/ stage/ treatment/ time),
162 and assessed survival after exposure for 6, 12, 18, 24, 36, 48, 60, 72 and 120 h to 0 °C/ 60% RH
163 in a Tenney ETCU16 chamber (Thermal Product Solutions, White Deer, PA, USA). Survival
164 was assessed as successful eclosion after the vial was returned to 21.5 °C.

165

166 To test for a rapid cold-hardening response, larvae were pre-exposed to 0 °C or 4 °C for one hour
167 with one hour recovery at 21.5 °C (cf. Ransberry *et al.*, 2011) and survival was determined at
168 temperatures close to the previously estimated $LT_{80\text{-}1h}$ (temperature at which 80 % of the
169 individuals die after 1 h exposure; control feeding -4.6 °C; FTR feeding: -8.7 °C; control
170 wandering: -6.6 °C; FTR wandering -8.8 °C). Ten larvae were placed into 0.65 mL
171 microcentrifuge tubes, each tube was placed into a 50 mL vial, which was immersed in a cooling
172 bath set to the $LT_{80\text{-}1h}$ for 1 h (n=5 groups/ stage/ treatment combination). After cold exposure,
173 the tubes were placed into food vials kept under rearing conditions; survival was assessed as
174 successful eclosion.

175

176 *Data analysis*

177 All analyses were conducted in in R version 3.0.1 (R Core Team, 2012). SCPs and dry mass
178 were compared among treatments and stages or sex using a two-way ANOVA, for which model
179 assumptions were checked. Survival after exposure to the $LT_{80\text{-}1h}$ was compared among
180 treatments using Kruskal-Wallis test. We used accelerated failure time models (AFT) from the
181 survival package in R to determine time at which 80 % of the individuals developed from eggs
182 into adults (Dt_{80}). The best-fit models used a log-logistic error distribution and treatment and
183 stage as factors. Developmental time was compared among treatments using a Kuskal-Wallis test

184 and between sex using a Wilcoxon rank sum test. The effect of the interaction of the treatments
185 and sex was analysed with a Kruskal-Wallis test followed by a Wilcoxon pairwise comparison
186 with Bonferroni-Holm correction.

187 The $LT_{80\text{-}1h}$ (temperature at which 80 % of flies will die after a 1 h exposure) and Lt_{80} (lethal
188 time at which 80 % of the individuals die during chronic low-temperature exposure) were
189 calculated for both third instar feeding and wandering larvae from the control and FTR groups
190 via a generalized linear model (Venables and Ripley, 2002) with a binomial error distribution
191 and logit link function (fit was tested with Wald's χ^2) using the package MASS in R . Differences
192 between groups were compared using a generalized linear model. We used the ghlt() function of
193 the package multcomp in R (Bretz *et al.*, 2011) to run a Tukey's post-hoc comparison using the
194 treatment × stage interaction.

195

196 **Results**

197 The rearing conditions altered the developmental time from egg to adult (Wald χ^2 = 1246.41, df=
198 2, p < 0.001). The DT_{80} (time taken for 80 % of the individuals to eclose as adults) was shortest
199 in control flies (15.7 ± 0.1 days), followed by FTR flies (42.1 ± 0.5 days). Flies reared under
200 constant low temperatures had the longest development time (62.4 ± 0.6 days, Figure 2). Females
201 were consistently heavier than males, and flies reared under FTR and constant low temperatures
202 were larger than controls (Figure 3).

203

204 Supercooling points ranged from -23.3 °C in a wandering larva reared under FTR to -7.3 °C in a
205 feeding control larva (Table 1). Feeding third-instar larvae had higher SCPs than wandering
206 third-instar larvae ($F_{1,176}$=76.612, p<0.001), and while FTR treatment led to a slight increase in
207 SCP of feeding larvae, it did not change the SCP of the wandering stage (treatment × stage:

208 $F_{1,176}$= 2.968, p=0.087; Table 1). No larvae survived internal ice formation, indicating that they
209 are not freeze-tolerant. Further, larvae did not survive temperatures slightly above the SCP,
210 indicating that the flies are chill-susceptible (Table 1). Application of an external ice nucleator
211 (AgI) significantly increased the SCP ($F_{1,176}$= 127.098, p<0.001), but did not lead to freeze
212 tolerance (Table 1). There was no significant interaction between external ice nucleation and
213 rearing conditions on SCP (treatment × AgI: $F_{1,176}$= 0.114, p=0.736).

214

215 We determined acute low temperature tolerance of control and FTR feeding and wandering
216 larvae by exposing them to a range of temperatures between -15 and 0 °C with food (in food
217 vials) and without food (in tubes). The temperature inside the food decreased more slowly than
218 outside the food, and the food froze at -8.2 ± 0.4 °C (n=10, example shown in Figure 3 froze at at
219 -8 °C after 32 min). Overall survival decreased with the temperature. Acute low-temperature
220 survival of larvae without food was affected by the treatment (Table 2). Feeding larvae of the
221 FTR group survived lower temperatures than feeding larvae of the control group with no overlap
222 of the survival curves (Figure 5A); whereas the survival curves of all the groups overlapped in
223 wandering larvae (Figure 5B). In addition, survival was affected by the life stage (Table 2). The
224 $LT_{80\text{-}1h}$ (temperature at which 80 % of the individuals die after 1 h exposure) was the lowest in
225 FTR feeding larvae (-8.9 ± 0.3 °C) and FTR wandering larvae (-8.4 ± 0.4 °C), followed by the
226 control wandering larvae (-6.6 ± 0.1 °C, Table 3). Control feeding larvae had the highest $LT_{80\text{-}1h}$
227 (-4.8 ± 0.3 °C). Acute low-temperature survival determined with food was affected by the life
228 stage (Table 2). FTR feeding larvae and FTR wandering larvae had a lower $LT_{80\text{-}1h}$ (feeding: -9.6
229 ± 0.3 °C, wandering: -8.7 ± 0.3 °C) than control feeding and wandering larvae (feeding -8.0 ± 0.3
230 °C, wandering: -7.2 ± 0.2 °C) (Table 3). Survival curves of feeding larvae from different
231 treatments did not overlap (Figure 5C), whereas they did among groups of wandering larvae

232 overlapped (Figure 5D). Feeding larvae show a lower LT_{80} when exposed to low temperatures
233 with food than without food, whereas there is no difference for wandering larvae (Figure 5).

234

235 We checked larval survival after exposing them to 0 ºC for up to 120 h. Mortality began after
236 6 h at 0 °C in both control and FTR wandering larvae and FTR feeding larvae, but after 12 h at 0
237 °C for control feeding larvae (Figure 6). However, mortality accumulated more slowly in FTR
238 larvae: all the control wandering larvae died after 72 h, whereas there was still some survival of
239 FTR larvae at the 72 h timepoint (Figure 6; Table 2). Survival was affected by the interactions of
240 time, treatment and life stage (except treatment × stage, Table 3).

241

242 To test for a rapid cold-hardening response, we exposed both FTR and control larvae to
243 different pre-treatments followed by a 1 h exposure to a discriminating temperature. We did not
244 observe any increase in acute cold tolerance by either larval stage under any rearing or pre-
245 treatment condition (Figure 7).

246

247 **Discussion**

248 Understanding low temperature survival by *D. suzukii* larvae could facilitate the development of
249 temperature-based treatment of fruit or packaging for export, and reveals the potential for *D.*
250 *suzukii* to overwinter in the larval stage, perhaps in waste fruit in orchards and vineyards. Here
251 we show that third instar *D. suzukii* larvae are chill-susceptible, have limited plasticity of cold
252 tolerance, and develop more slowly, but into larger adults, if reared under cool conditions.

253

254 Most insects follow a 'temperature-size rule' such that the rate of development increases, but
255 body size decreases, with increasing temperature (Kingsolver and Huey, 2008). This appears to

256 be true for *D. melanogaster* (Partridge *et al.*, 1994), and our data show it is also the case for *D.*
257 *suzukii.* Fluctuating temperatures are most consistent with the conditions experienced in nature,
258 and development rate increases under FTR conditions, likely because of the effects of Jensen's
259 inequality on development (Colinet *et al.*, 2015). The outcomes of larval growth of *Manduca*
260 *sexta* depend on both mean and fluctuations of temperature (Kingsolver *et al.*, 2015), but our
261 single fluctuating regime does not allow us to dissect these more subtle effects for *D. suzukii.*
262 We did not determine whether this increased adult mass is due to increased energy reserves, as
263 observed in adults from the *D. auraria* complex reared under fall conditions accumulated more
264 triacylglycerol than summer morph flies (Ohtsu *et al.*, 1993). If they do have increased energy
265 stores, then this is likely due to acquisition during the larval period, since *D. suzukii* adults from
266 this population that were transferred to fall-like conditions as wandering larvae did not have
267 increased body size or triacylglycerol and carbohydrate content compared to those that developed
268 under summer conditions (Toxopeus *et al.*, 2016). Thus, the thermal sensitivity of larvae
269 determines not only their cold tolerance, but also their potential performance as adults, and we
270 speculate that in nature, the body size differences of the winter morphs likely results from larval
271 responses, not the temperature/photoperiod effect.
272
273 Similar to adults of this species (Jakobs *et al.*, 2015), both feeding and wandering *D. suzukii*
274 larvae were chill susceptible, regardless of acclimation treatment or ice nucleation environment.
275 Chill-susceptibility appears to be the ancestral state of cold tolerance for *Drosophila*, and is the
276 only strategy reported in the melanogaster subgroup, to which *D. suzukii* belongs (Kimura, 2004;
277 Strachan *et al.*, 2011). Chill susceptible insects are killed by both cold and freezing, so deliberate
278 inoculation of ice formation is one possible way to enhance low temperature control of insects
279 using this strategy (Strong-Gunderson *et al.*, 1992). Because they are chill-susceptible, the SCP
280 has limited ecological relevance (Sinclair *et al.*, 2015), although changes in SCP can indicate
281 modifications to gut contents (in this case perhaps explaining the shift in SCP with acclimation in

282 feeding, but not wandering larvae), or to other physiological parameters (Coleman *et al.*, 2014).
283 Larvae of some drosophilids survive internal ice formation only when it is inoculated externally
284 (e.g. by ice in the food; Shimada and Riihimaa, 1988), however we show that externally
285 inoculated freezing is lethal in *D. suzukii*, and freeze tolerance is therefore unlikely under natural
286 conditions, as well as in the lab.

287

288 Wandering larvae were more tolerant of acute cold exposure than feeding larvae, whereas the
289 opposite was true during long-term exposure. Acute cold exposure likely causes direct injury to
290 cells, while chronic cold exposure appears to be more related to long-term loss of homeostasis
291 (MacMillan and Sinclair, 2011; Rajamohan and Sinclair, 2008; Sinclair and Roberts, 2005; Teets
292 and Denlinger, 2013). The presence of food substantially increased acute low temperature
293 survival in feeding larvae, possibly because the food may have substantially buffered the
294 temperature exposure (Figure 4), effectively reducing the time for which feeding larvae were
295 exposed to each temperature (Nedvěd *et al.*, 1998). Wandering larvae have left the food, so even
296 when food is present, they likely do not benefit from this buffering, which means that the
297 presence of food cannot modify their tolerance. Feeding larvae tolerated 0 °C for approximately
298 40 % longer than wandering larvae, which is surprising, since we would expect wandering larvae
299 to be more resistant to environmental conditions – including temperature – since they have left
300 the buffered environment of the food. Nevertheless, our results suggest that wandering larvae
301 could be particularly susceptible to prolonged cold exposure, perhaps in the context of cold-
302 storage of fruit.

303

304 Insects can increase their tolerance of low temperatures through plasticity via acclimation over
305 long periods (including during development), or rapidly through hardening responses (Teets and
306 Denlinger, 2013). Acclimation responses are usually especially robust under fluctuating
307 temperature conditions (Colinet *et al.*, 2015), including in adult *D. suzukii* (Jakobs *et al.*, 2015).

308 However, FTR acclimation had only a limited impact on acute cold tolerance, improving acute
309 cold tolerance by less than 2 °C in feeding larvae when they were exposed to cold without food,
310 but not modifying acute cold tolerance in other groups. Similarly, we did not detect a rapid cold-
311 hardening response in acute cold tolerance; however, we did not try a range of induction
312 conditions, and it is possible that the RCH response is only elicited at lower temperatures
313 (Sinclair and Chown, 2006). By contrast, FTR acclimation more than doubled survival time at 0
314 °C in both wandering and feeding larvae. Thus, although *D. suzukii* larvae appear to have
315 limited plasticity for tolerance of absolute temperature, the limits for survival of long exposures
316 are very plastic and need to be considered carefully when developing temperature-based
317 treatments using mildly cold temperatures.
318
319 In conclusion, we show that *D. suzukii* larvae are not substantially cold tolerant, and that
320 although there is plasticity in their tolerance to prolonged low temperatures, they have only
321 limited ability to modify their acute cold tolerance. Thus, it could be possible to develop low
322 temperature treatments that could control late-instar *D. suzukii* larvae without damaging fruit.
323
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331
332

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434

435 **Figure Captions**

436 **Figure 1. Identification of larval stages of *Drosophila suzukii*.** Mouthparts of first (A), second
437 (B), and third (C) larval instars vary in size and shape (scale bar: 50 µm). Third-instar wandering
438 larvae (D) have well-developed anterior spiracles (scale bar 500 µm), while third-instar feeding
439 larvae do not (not shown). Dyed food (green in colour, appears dark in figure) is apparent in the
440 gut of larvae that are feeding, whereas third-instar wandering larvae have cleared their gut and
441 are transluscent (E).

442

443 **Figure 2. Distribution of developmental time of *Drosophila suzukii* during different**
444 **treatments.** Histograms of developmental time of females (A) and males (B) reared under
445 control conditions (white; 21.5 °C, 13:11 L:D), fluctuating thermal regime (= FTR; light grey;
446 5.5 °C/19 °C, 11.5:12.5 L:D) and constant low temperatures (dark grey; 11 °C, 10:14 L:D). The
447 DT_{80} (time at which 80% of the flies eclosed, see text for details) for the control group is
448 represented by the dotted line, for the FTR group by the dashed line and for the constant low
449 temperatures by the solid line. Lines with different letters denote significantly different
450 developmental times across both A and B (treatment: $\chi^2 = 265.48$, $p < 0.001$, sex: $W = 14510$, $p =$
451 0.07, treatment × sex: $\chi^2 = 268.37$, $p < 0.001$).

452

453 **Figure 3. Weight of adult *Drosophila suzukii* reared under different treatments.** Dry mass ±
454 SE (mg) was affected by sex and treatment of the flies (Treatment $F_{2,314} = 4.437$, $p < 0.05$, Sex:
455 $F_{1,314} = 119.551$, $p < 0.001$, Treatment × Sex: $F_{1,314} = 0.65$, $p = 0.523$). Tukey's HSD was run
456 without the interaction, because it was non-significant.

457

458 **Figure 3. Differences in temperatures exposure inside and outside the food.** The temperature
459 exposure during 1 hour at -9 °C in a food vial inside the food (dashed line) and outside the food
460 (solid line).
461
462 **Figure 5. Survival during acute low-temperature exposure of *D. suzukii* larvae.** Larvae
463 reared under control (21.5 °C, 13:11 L:D) conditions or a fluctuating thermal regime (=FTR; 5.5
464 °C/19 °C, 11.5:12.5 L:D) were exposed to a range of temperatures without food (A: feeding; B:
465 wandering) or with food (C: feeding; D: wandering). The size of the symbols reflects the number
466 of measurements of each group at this temperature (tubes: group of 10 larvae, food vial: group of
467 20 larvae, control = open symbols, FTR = crossed symbols). The dashed (control) and the solid
468 (FTR) lines are the survival curve calculated with a generalized linear model (see Table 3 for
469 statistics). The dotted line shows 80 % mortality (LT_{80-1h}). The grey box in C and D represent the
470 mean SCP of the food ± SE.
471
472 **Figure 6. Survival during chronic cold exposure of *D. suzukii* third instar larvae.** Larvae
473 reared under control conditions (21.5 °C, 13:11 L:D) or a fluctuating thermal regime (FTR; 5.5
474 °C/19 °C, 11.5:12.5 L:D) were exposed to 0 °C for up to 120 h (A: feeding; B: wandering). The
475 size of the symbols reflects the number of measurements of each group at this time point (n=3
476 groups of 10, control = open symbols, FTR = crossed symbols). The dashed (control) and the
477 solid (FTR) lines are the survival curve calculated with a generalized linear model (see Table 4
478 for statistics). The dotted line shows 80 % mortality (Lt_{80}).
479
480
481 **Figure 7. Survival following different short-term-hardening pre-treatments of *D. suzukii***
482 **larvae.** Third feeding and wandering larvae that were reared under control conditions (21.5 °C,
483 13:11 L:D; A: feeding, C: wandering) or a fluctuating thermal regime (= FTR; 5.5 °C/19 °C,

484 11.5:12.5 L:D; B: feeding, D: wandering) were pre-exposed to 0°C or 4 °C with one hour

485 recovery at 21.5 °C and then exposed to temperatures close to the $LT_{80\text{-}1h}$ (control feeding to -4.6

486 °C, FTR feeding to -8.7 °C, control wandering to -6.6 °C and FTR wandering to -8.8 °C). There

487 was no difference in survival among any of the treatment groups.

488 **Tables**

489 **Table 1. Supercooling points and cold tolerance strategy of third instar larvae of *Drosophila suzukii*.** Mean ± SEM (sample size in
490 parentheses). Control larvae were reared under 21.5 °C, 13:11 L:D, FTR (fluctuating thermal regime) under 5.5 °C/19 °C, 11.5:12.5 L:D. Silver
491 iodide (AgI) was used to externally inoculate ice formation. Groups with the same letter are not significantly different (p>0.05; Tukey's post-hoc
492 test); see text for statistics. See text for rationale for determining cold tolerance strategies.

Group	**Feeding**				**Wandering**			
	SCP (°C)	**Number of flies dead**		**Cold tolerance strategy**	**SCP (°C)**	**Number of flies dead**		**Cold tolerance strategy**
		unfrozen	**frozen**			**unfrozen**	**frozen**	
Control	-17.6 ± 0.6[b,c] (n=35)	5/5	5/5	chill-susceptible	-19.6 ± 0.4[c] (n=27)	5/5	5/5	chill-susceptible
FTR	-15.1 ± 0.7[b] (n=23)	5/5	5/5	chill-susceptible	-20.6 ± 0.5[c] (n=22)	5/5	5/5	chill-susceptible
Control + AgI	-9.4 ± 0.9[a] (n=21)	5/5	5/5	chill-susceptible	-16.4 ± 0.9[b] (n=21)	5/5	5/5	chill-susceptible
FTR + AgI	-8.5 ± 0.7[a] (n=18)	5/5	5/5	chill-susceptible	-14.8 ± 1.4[b] (n=17)	5/5	5/5	chill-susceptible

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495

496 **Table 2. Mortality after acute and prolonged low-temperature exposure for third feeding and wandering larvae of *D. suzukii*.** LT_{80} (° C,
497 temperature at which 80 % of the individuals die) was determined for larvae reared under control conditions (21.5 °C, 13:11 L:D) and under
498 fluctuating thermal regime (FTR; 5.5 °C/19 °C, 11.5:12.5 L:D) that were exposed to a range of temperatures with and without food. Groups with
499 the same letters are not significantly different from each other (see Table 4 for statistics, Tukey's HSD).

			Feeding larvae			**Wandering larvae**		
				curve fit			**curve fit**	
	Group	**Treatment**	**LT_{80}/ Lt_{80}**	**Wald χ^2**	**P**	**LT_{80}/ Lt_{80}**	**Wald χ^2**	**p**
LT_{80-1h} (°C)	without food	Control	-4.8 ± 0.3^{a}	6.63	<0.001	-6.6 ± 0.1^{b}	5.52	<0.001
		FTR	-8.9 ± 0.3^{c}	6.65	<0.001	-8.4 ± 0.4^{c}	5.75	<0.001
	with food	Control	-8.0 ± 0.3^{A}	10.29	<0.001	-7.2 ± 0.2^{B}	8.67	<0.001
		FTR	-9.6 ± 0.3^{C}	9.86	<0.001	-8.7 ± 0.3^{C}	11.73	<0.001
Lt_{80} (h) at 0°C	with food	Control	43.4 ± 2.9^{a}	8.62	<0.001	30.7 ± 1.94^{a}	-7.72	<0.001
		FTR	92.2 ± 7.2^{b}	7.83	<0.001	73 ± 5.2^{b}	-8.28	<0.001

500 **Table 3. Statistics for the generalized linear model for chronic low temperature survival**
501 **of third feeding and wandering larvae of *D. suzukii* reared under different conditions**.
502 The generalized linear model was calculated with a binomial error distribution and logit link
503 function (fit was tested with Wald's χ^2). Bold *P*-values indicate a significant effect of the
504 model term on survival. Treatments are rearing under control conditions (21.5 °C, 13:11 L:D)
505 or a fluctuating thermal regime (FTR; 5.5 °C/19 °C, 11.5:12.5 L:D), and we used two life
506 stages, feeding and wandering 3rd instar larvae.

Term	**Group**			
	without food		**with food**	
Acute cold model	**Wald χ^2**	***P***	**Wald χ^2**	***P***
Temperature	6.63	**< 0.001**	11.586	**< 0.001**
Treatment	2.77	**< 0.01**	5.075	0.222
Life stage	4.23	**< 0.001**	6.231	**< 0.01**
Temperature × Treatment	0.47	0.636	3.003	0.578
Temperature × Life stage	3.26	**< 0.01**	5.852	**< 0.01**
Treatment × Life stage	3.75	**< 0.001**	5.962	**< 0.01**
Temperature × Treatment × Life stage	2.9	**< 0.01**	5.344	**< 0.01**
Chronic cold model				
Time			8.62	**<0.001**
Treatment			0.37	0.713
Life stage			1.99	**<0.05**
Time × Treatment			3.88	**<0.001**
Time × Life stage			3.12	**<0.01**
Treatment × Life stage			1.73	0.084
Time × Treatment × Life stage			2.52	**<0.05**

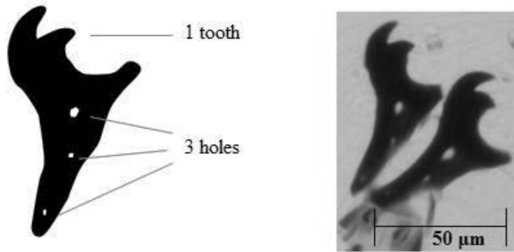
507

508

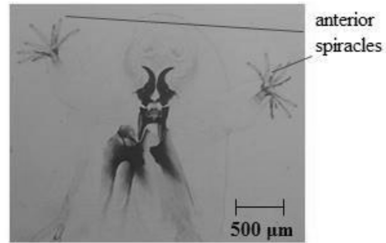
509 **Figures**

510 **Figure 1**

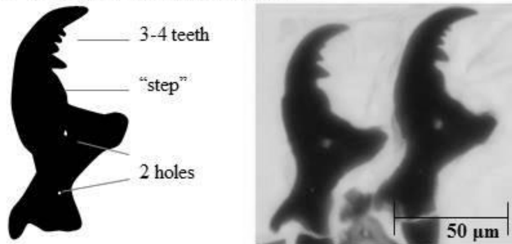
A: First larval instar



D: Third larval instar (wandering)

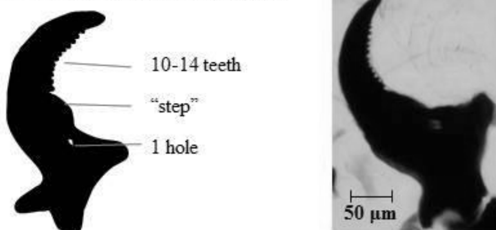


B: Second larval instar



E: Wandering and feeding third larval instars

C: Third larval instar



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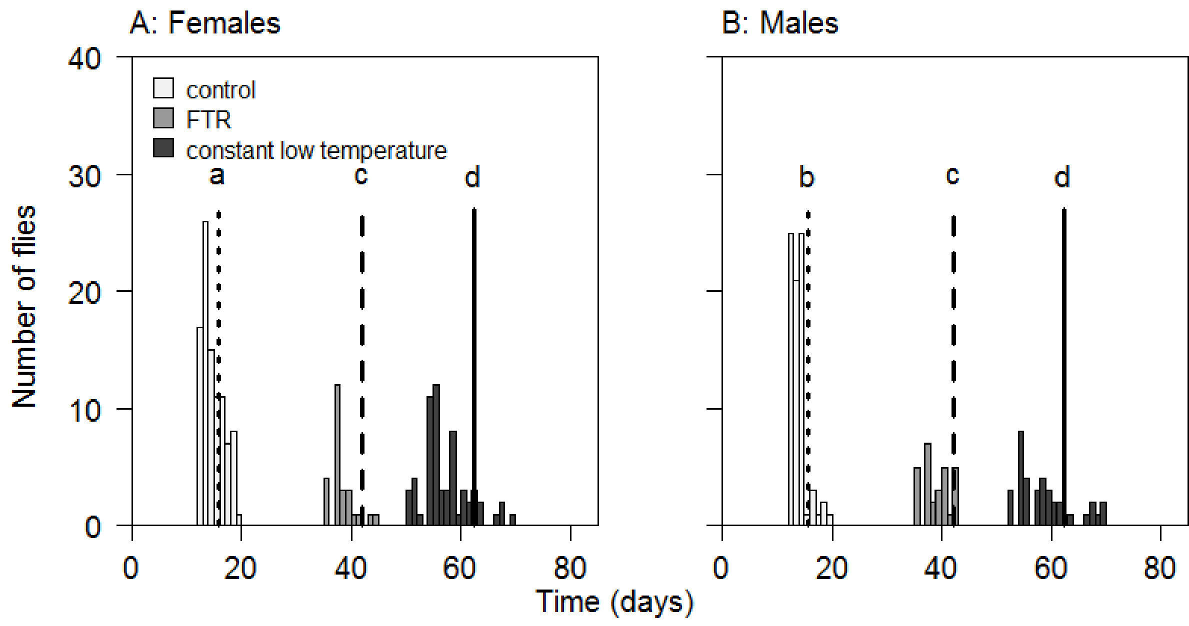
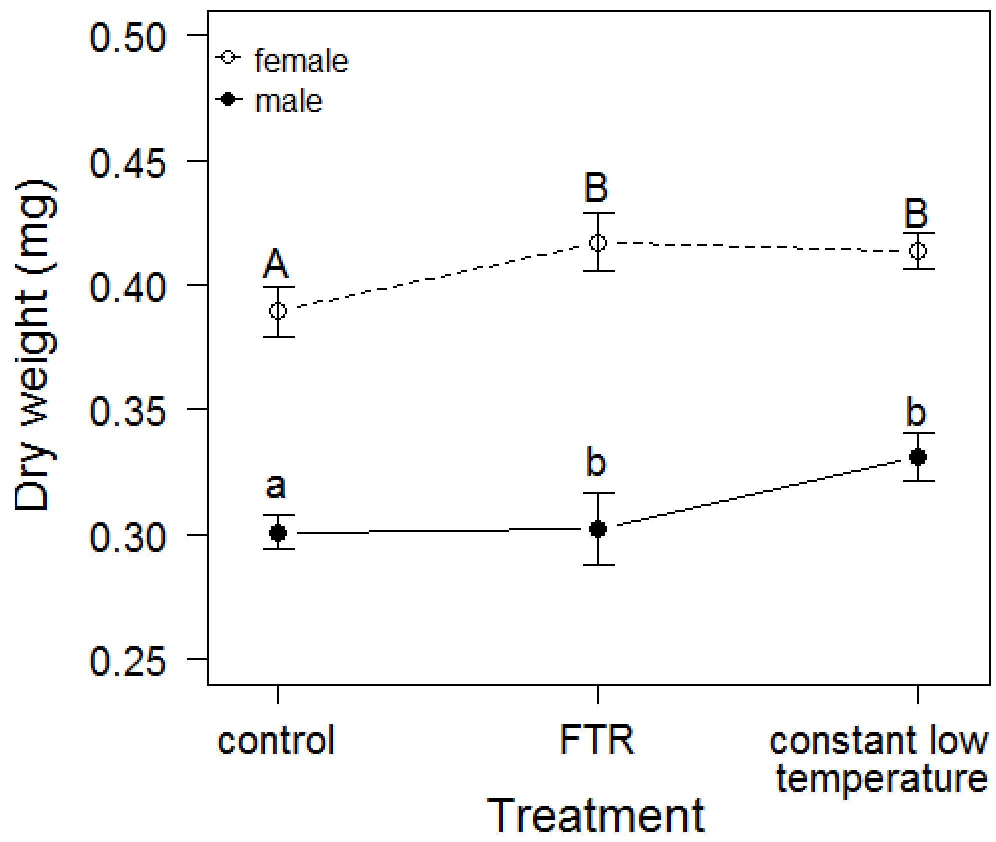
513 **Figure 2**



514

515 **Figure 3**



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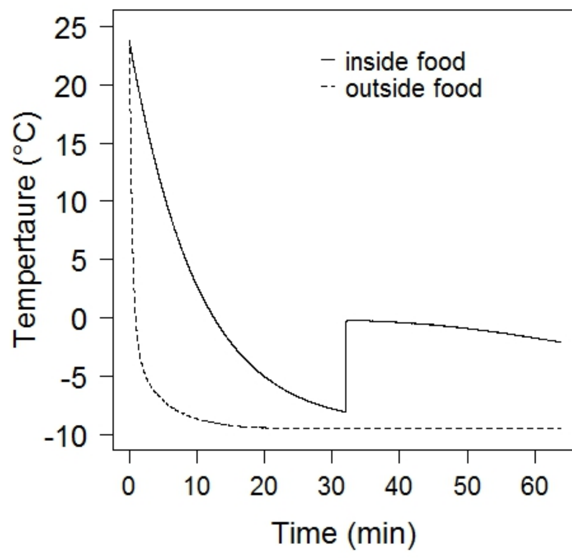
518 **Figure 4**



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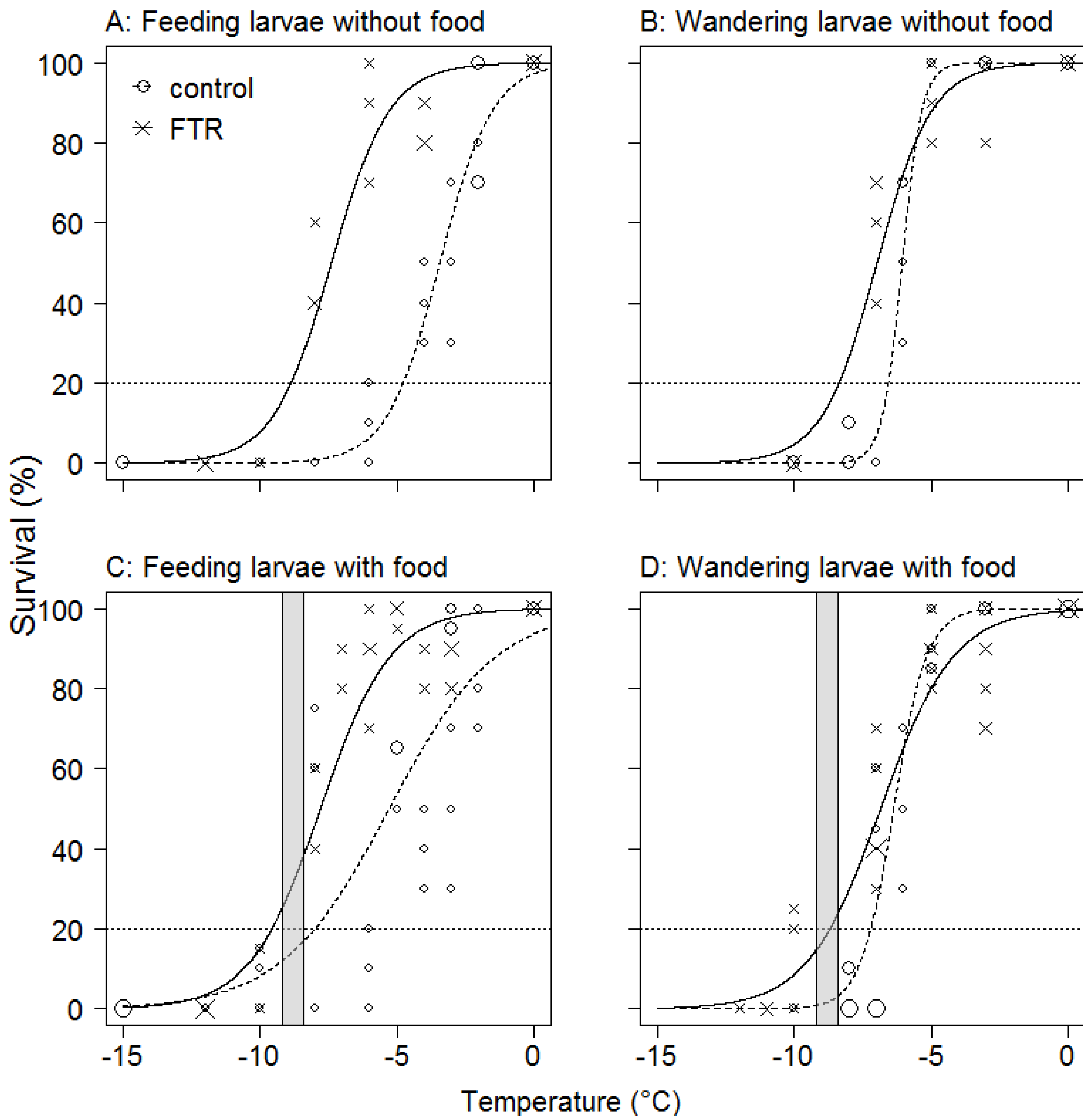
522 **Figure 5**



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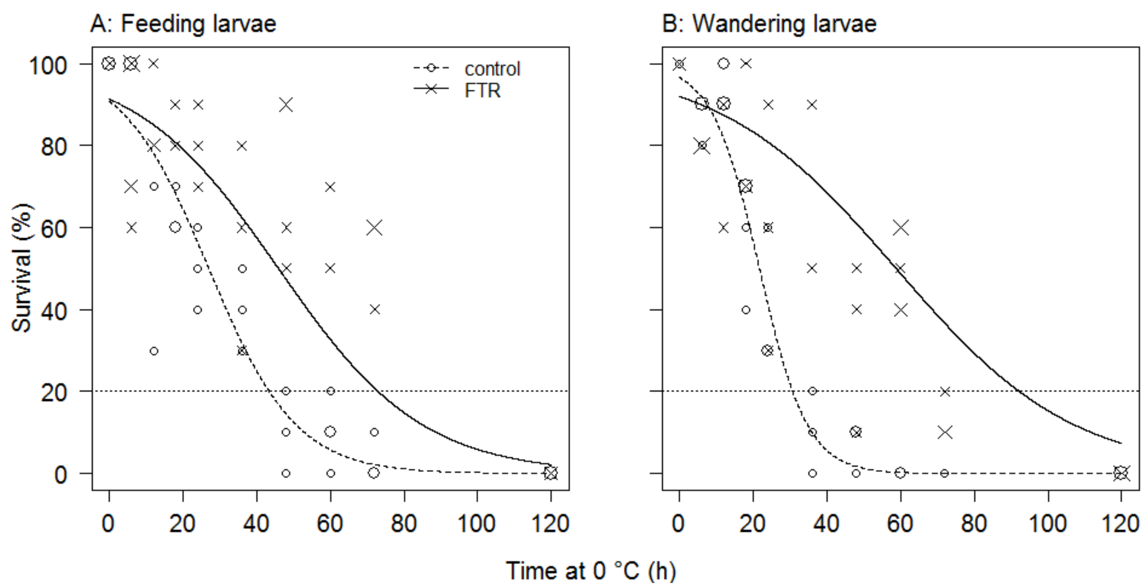
525 **Figure 6**



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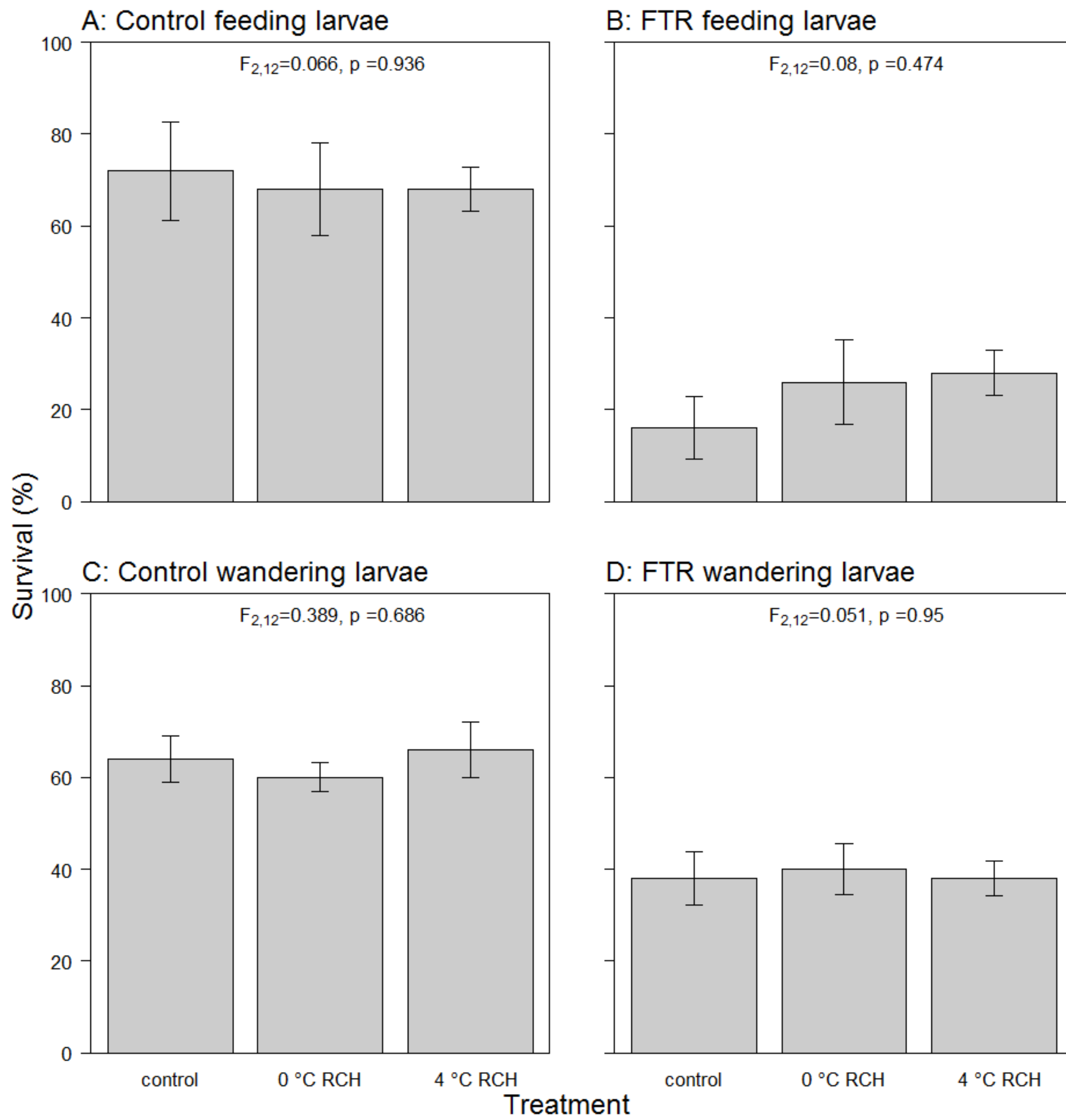
528 **Figure 7**



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