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Abstract

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

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

Introduction

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

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

hardening response (Czajka and Lee, 1990), as well as responding to longer-term acclimation (Rajamohan and Sinclair, 2009).

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

Methods

Animal rearing and treatment groups

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

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

91

92 To induce phenotypic plasticity in *D. suzukii* larvae, we placed the food plates with the eggs into
93 two different rearing conditions (treatment). Eggs were placed under either control conditions
94 (21.5 °C, 13:11 L:D) or exposed to a fluctuating thermal regime (FTR; 5.5 °C/19 °C, 11.5:12.5
95 L:D), simulating the average photoperiod and daily minimum and maximum temperatures from
96 late September in London, Ontario.

97

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

106

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

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

Cold tolerance

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

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

Because some insects are freeze tolerant only with external ice inoculation, (e.g. Shimada and 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

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

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

Data analysis

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

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

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

Results

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

Supercooling points ranged from -23.3 °C in a wandering larva reared under FTR to -7.3 °C in a feeding control larva (Table 1). Feeding third-instar larvae had higher SCPs than wandering third-instar larvae ($F_{1,176}=76.612$, $p<0.001$), and while FTR treatment led to a slight increase in 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

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

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

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

Discussion

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

Most insects follow a ‘temperature-size rule’ such that the rate of development increases, but 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

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

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

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

However, FTR acclimation had only a limited impact on acute cold tolerance, improving acute cold tolerance by less than 2 °C in feeding larvae when they were exposed to cold without food, but not modifying acute cold tolerance in other groups. Similarly, we did not detect a rapid cold-hardening response in acute cold tolerance; however, we did not try a range of induction conditions, and it is possible that the RCH response is only elicited at lower temperatures (Sinclair and Chown, 2006). By contrast, FTR acclimation more than doubled survival time at 0 °C in both wandering and feeding larvae. Thus, although *D. suzukii* larvae appear to have limited plasticity for tolerance of absolute temperature, the limits for survival of long exposures are very plastic and need to be considered carefully when developing temperature-based treatments using mildly cold temperatures.

In conclusion, we show that *D. suzukii* larvae are not substantially cold tolerant, and that although there is plasticity in their tolerance to prolonged low temperatures, they have only limited ability to modify their acute cold tolerance. Thus, it could be possible to develop low temperature treatments that could control late-instar *D. suzukii* larvae without damaging fruit.

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Figure Captions

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

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

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

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 ($LT_{80\text{-}1h}$). 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,

11.5:12.5 L:D; B: feeding, D: wandering) were pre-exposed to 0°C or 4 °C with one hour recovery at 21.5 °C and then exposed to temperatures close to the $LT_{80\text{-}1h}$ (control feeding to -4.6 °C, FTR feeding to -8.7 °C, control wandering to -6.6 °C and FTR wandering to -8.8 °C). There was no difference in survival among any of the treatment groups.

Tables

Table 1. Supercooling points and cold tolerance strategy of third instar larvae of *Drosophila suzukii*. Mean ± SEM (sample size in 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 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 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

Table 2. Mortality after acute and prolonged low-temperature exposure for third feeding and wandering larvae of *D. suzukii*. LT_{80} (° C, 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 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 the same letters are not significantly different from each other (see Table 4 for statistics, Tukey's HSD).

			Feeding larvae			Wandering larvae		
	Group	Treatment	LT_{80}/ Lt_{80}	curve fit		LT_{80}/ Lt_{80}	curve fit	
				Wald χ^2	P		Wald χ^2	p
$LT_{80\text{-}1h}$ (°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

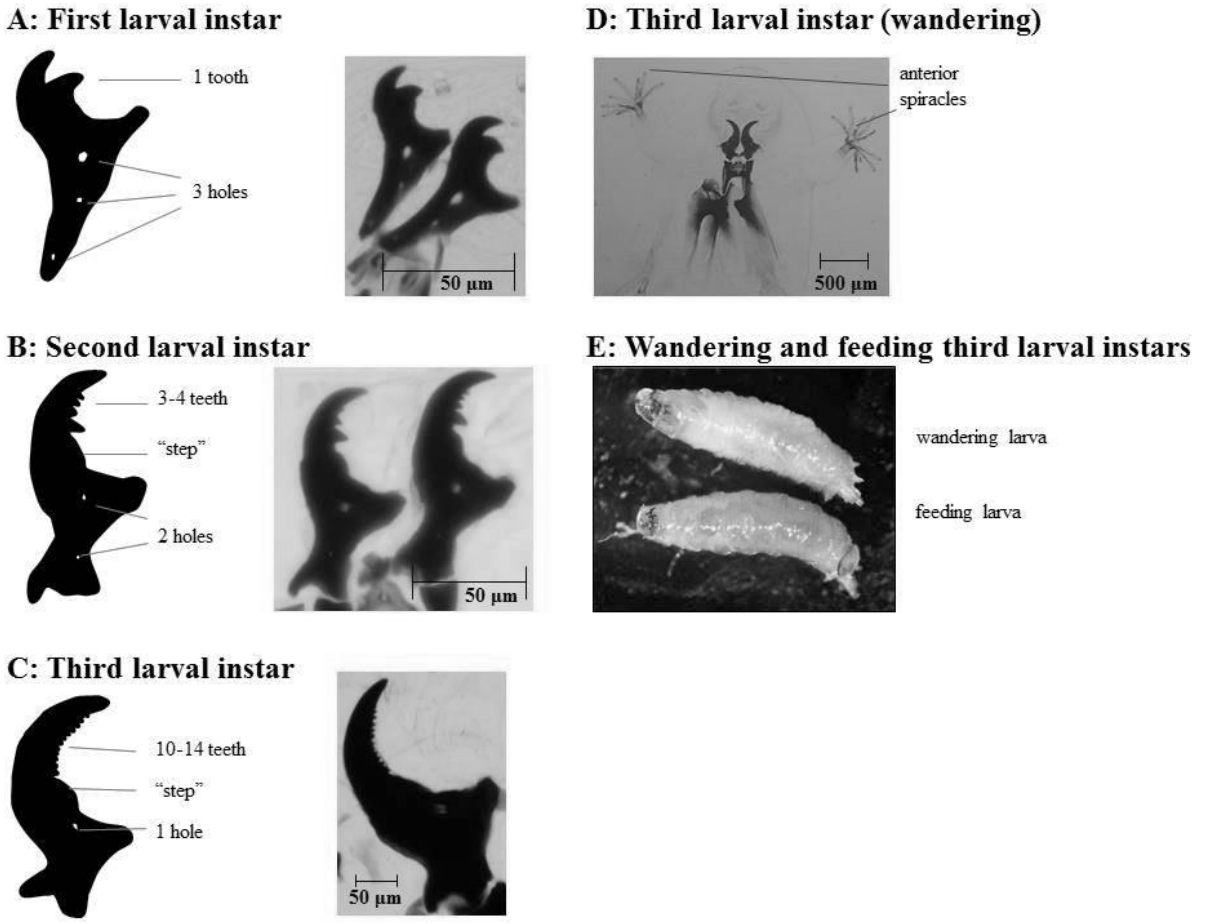
Table 3. Statistics for the generalized linear model for chronic low temperature survival of third feeding and wandering larvae of *D. suzukii* reared under different conditions.

The generalized linear model was calculated with a binomial error distribution and logit link function (fit was tested with Wald's χ^2). Bold *P*-values indicate a significant effect of the model term on survival. Treatments are rearing under control conditions (21.5 °C, 13:11 L:D) or a fluctuating thermal regime (FTR; 5.5 °C/19 °C, 11.5:12.5 L:D), and we used two life 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**

Figures

Figure 1



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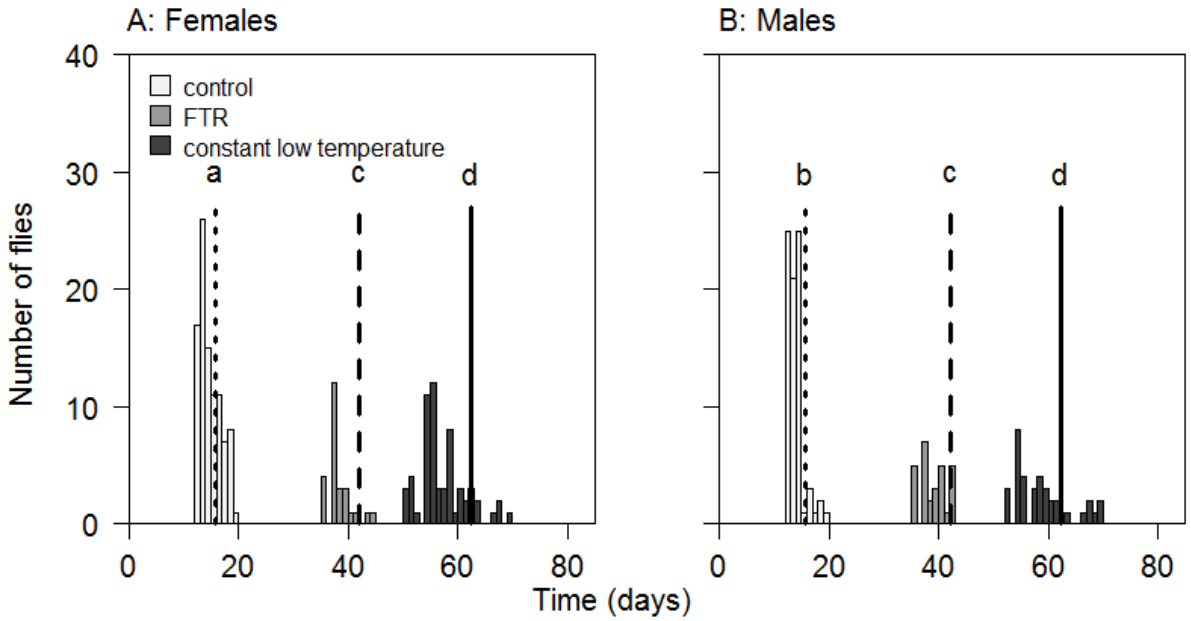

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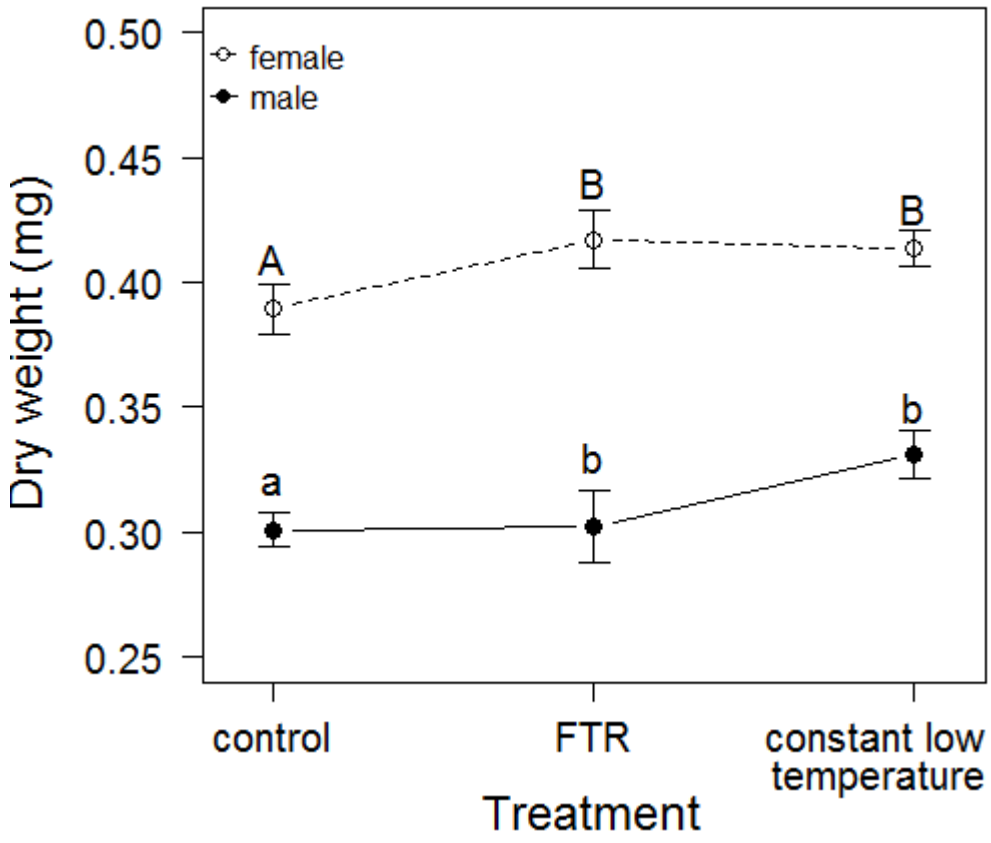
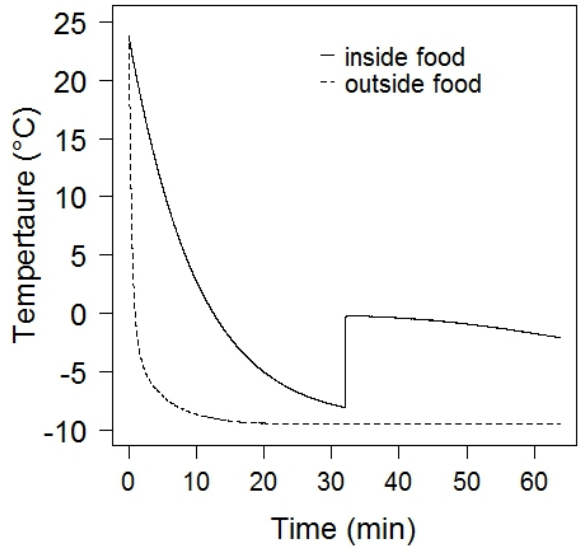
Figure 3


0.50
0.45
0.40
0.35
0.30
0.25
Dry weight (mg)
female
male
A
B
B
a
b
b
control
FTR
constant low temperature
Treatment

518 **Figure 4**



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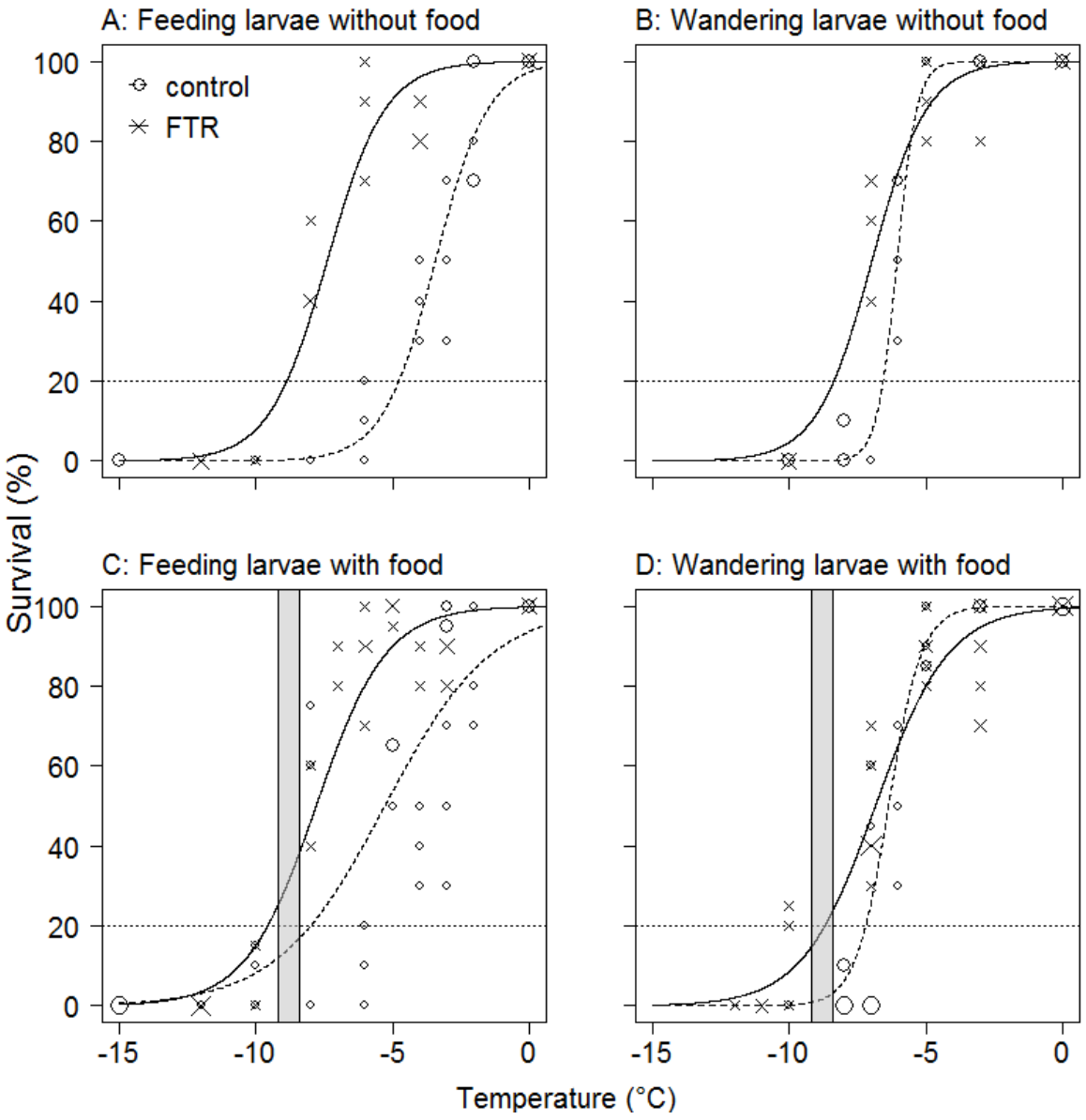
522 **Figure 5**



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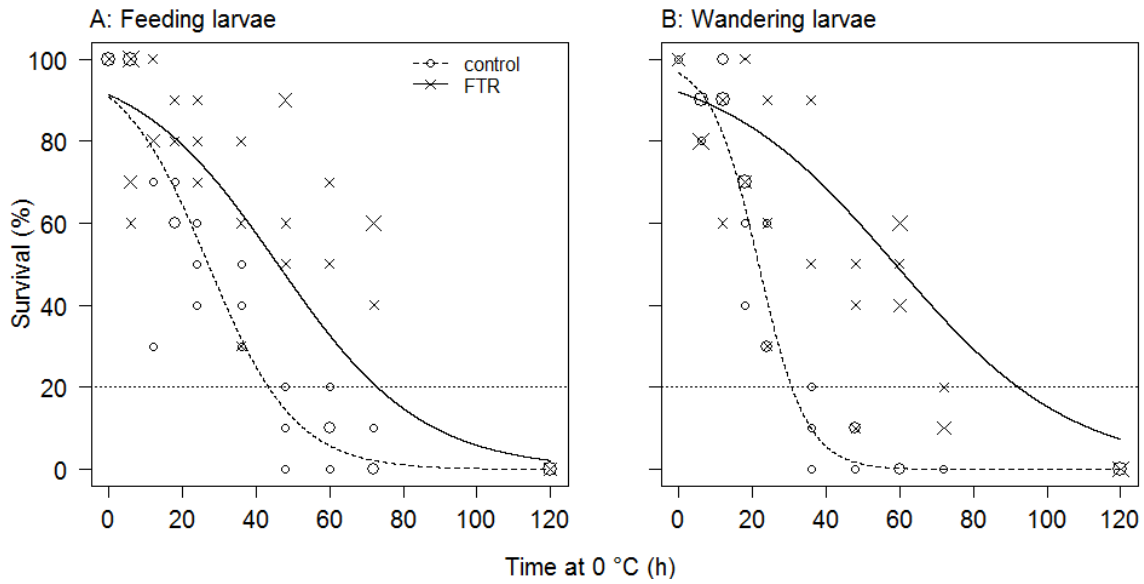
525 **Figure 6**



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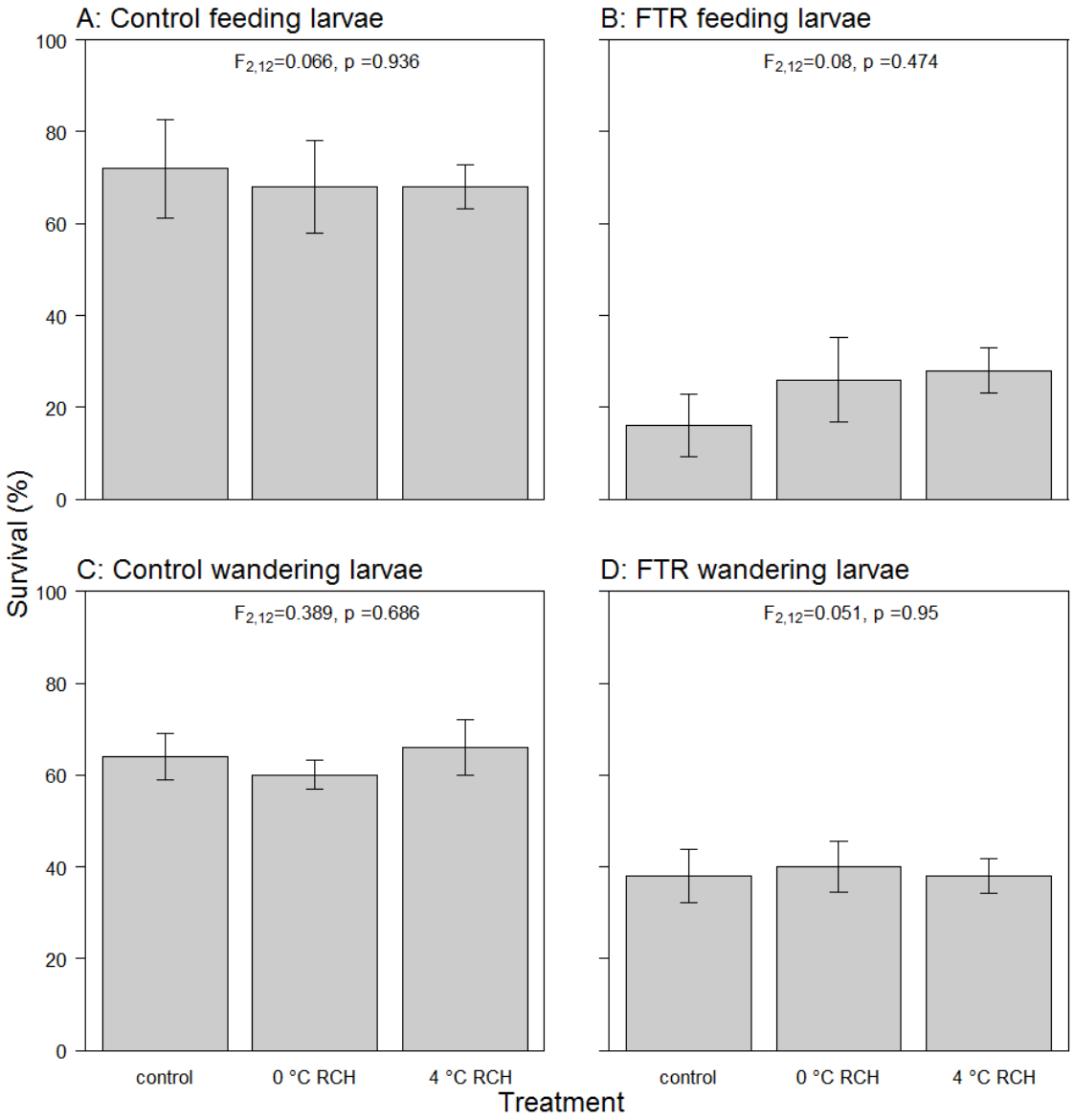
528 **Figure 7**



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