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ORIGINAL ARTICLE

Effects of fluctuating thermal regimes on cold survival and life history traits of the spotted wing *Drosophila* (*Drosophila suzukii*)

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Abstract *Drosophila suzukii* is an invasive pest causing severe damages to a large panel of cultivated crops. To facilitate its biocontrol with strategies such as sterile or incompatible insect techniques, *D. suzukii* must be mass-produced and then stored and transported under low temperature. Prolonged cold exposure induces chill injuries that can be mitigated if the cold period is interrupted with short warming intervals, referred to as fluctuating thermal regimes (FTR). In this study, we tested how to optimally use FTR to extend the shelf life of *D. suzukii* under cold storage. Several FTR parameters were assessed: temperature (15, 20, 25 °C), duration (0.5, 1, 2, 3 h), and frequency (every 12, 24, 36, 48 h) of warming intervals, in two wild-type lines and in two developmental stages (pupae and adults). Generally, FTR improved cold storage tolerance with respect to constant low temperatures (CLT). Cold mortality was lower when recovery temperature was 20 °C or higher, when duration was 2 h per day or longer, and when warming interruptions occurred frequently (every 12 or 24 h). Applying an optimized FTR protocol to adults greatly reduced cold mortality over long-term storage (up to 130 d). Consequences of FTR on fitness-related traits were also investigated. For adults, poststorage survival was unaffected by FTR, as was the case for female fecundity and male mating capacity. On the other hand, when cold storage occurred at pupal stage, poststorage survival and male mating capacity were altered under CLT, but not under FTR. After storage of pupae, female fecundity was lower under FTR compared to CLT, suggesting an energy trade-off between repair of chill damages and egg production. This study provides detailed information on the application and optimization of an FTR-based protocol for cold storage of *D. suzukii* that could be useful for the biocontrol of this pest.

Key words cold storage; fluctuating thermal regimes; life-history traits; recovery; spotted wing drosophila

Introduction

Insects exposed to stressful low temperature often exhibit high mortality or sublethal effects affecting their life history traits (Denlinger & Lee, 2010). To tolerate and survive deleterious conditions, insects can use behavioral strategies, like avoidance (Hawes *et al.*, 2008), or acclimation-related physiological adjustments, such as heat shock proteins synthesis or cryoprotectant accumulation (Clark & Worland, 2008; Denlinger & Lee, 2010; Colinet *et al.*, 2013). A growing number of studies

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have shown that interrupting the exposure to constant low temperatures (CLT) with periodic warm phases (referred to as fluctuating thermal regimes, FTR) can mitigate, or even offset cold-induced mortality (see Colinet *et al.*, 2015b, 2018 for review). Although FTR treatments are not ecologically relevant, they may be used as an efficient protocol for prolonging insect survival during cold storage (Colinet & Hance, 2010; Rinehart *et al.*, 2011; Koštál *et al.*, 2016; Colinet *et al.*, 2018). Despite being artificial, FTR may trigger processes similar to naturally fluctuating temperatures; therefore, their study may also help understanding insect response to natural thermal variations. Insect's chill-injuries are linked to metal ions, water and metabolic homeostasis deregulations, as well as potential alterations of proteins and cell membranes (Koštál *et al.*, 2004; Denlinger & Lee, 2010; MacMillan & Sinclair, 2011). Many researchers have proposed that FTR benefits are due to physiological repair or recovery during recurrent warm pulses (Colinet *et al.*, 2018).

The present study focuses on the spotted wing drosophila, *Drosophila suzukii*, an alien species, originating from Southeast Asia, that has been introduced in both Europe and North America in 2008 (Hauser *et al.*, 2009; Raspi *et al.*, 2011; Calabria *et al.*, 2012) and that is now widely spread in these areas (Hauser, 2011; Cini *et al.*, 2012). This fly is a pest of soft fruits. Indeed, females oviposit in ripe fruits that larvae consume (Kanzawa, 1939; Mitsui *et al.*, 2006). Consequently, *D. suzukii* provokes important economic losses in a large range of cultivated crops (Goodhue *et al.*, 2011; Walsh *et al.*, 2011; Asplen *et al.*, 2015). To biologically control this pest, classical biocontrol, as well as sterile insect technique (SIT) and incompatible insect technique (IIT) are currently under deep investigation (Nikolouli *et al.*, 2018). These environment-friendly methods require recurrent inundative releases of massive numbers of sterile or incompatible insects. In this regard, a cost-effective mass-production needs to be developed. Low-temperature storage and cold treatment are integral part of mass-rearing and release protocols. Insects are frequently exposed to low temperature at several critical steps (e.g., egg collection, rearing, shipping, handling, and release), and these stressful treatments often cause loss of quality and/or mortality, which can seriously compromise the success of these programs (Mutika *et al.*, 2014). As *D. suzukii* is a chill-susceptible species (Kimura, 2004; Dalton *et al.*, 2011; Jakobs *et al.*, 2015; Plantamp *et al.*, 2016; Ryan *et al.*, 2016; Enriquez & Colinet, 2017), implementing cold storage protocols without loss of performance may be challenging. Consequently, applying FTR could be a suitable option to mitigate negative effects of cold storage. With SIT or IIT, released flies have to compete with wild flies, and therefore, preserving their performances after cold storage is essential.

The first aim of this study was to optimize FTR protocol for short-term cold storage of *D. suzukii* adults and pupae by sequentially changing different parameters, such as temperature, duration and frequency of warming interruptions. It has previously been showed in other species that an increased recovery duration associated with optimal recovery temperature can nearly counterbalance chilling damages (Colinet *et al.*, 2011; Yocum *et al.*, 2012; Rinehart *et al.*, 2016). Increasing the frequency of warming intervals has also been associated with reduced mortality in various species (Colinet *et al.*, 2006; Yocum *et al.*, 2012). Hanč and Nedvěd (1999) showed, however, that cold survival does not necessarily increase linearly with recovery temperature or duration. Thus, it is important to explore a range of conditions to optimally use FTR. In the present study, we tested whether (i) beneficial effect of FTR increases with recovery temperature and duration, (ii) there is an optimal combination of recovery temperature × duration above which no additional benefit is observed, and (iii) the more frequently insects have the opportunity to recover the better the cold survival.

Fluctuating thermal regime is thought to allow repair mechanisms, but these processes likely require energy, which would normally be allocated to other biological functions. Warming pulses under FTR are known to be associated with an overshoot in metabolic rate, which suggests a higher energy consumption under FTR than under CLT (Lalouette *et al.*, 2011; Yocum *et al.*, 2011; Boardman *et al.*, 2013). As a result, it can be assumed that physiological repair under FTR is related to fitness costs. There are only some few evidences for this in *Bactrocera latifrons* (Takano, 2014), where FTR treatment reduced the number of insects laying viable eggs. Fecundity was also reduced after fluctuating treatment in *Zeiraphera canadiensis* (Carroll & Quiring, 1993) and in *Ceratitis capitata* (Basson *et al.*, 2012), but these treatments were not proper FTR (we refer to "proper" FTR as prolonged cold periods interrupted with recurrent short warming intervals). Fitness costs of FTR treatments thus remain poorly investigated (Colinet *et al.*, 2018). The second aim of this work was precisely to test the impact of CLT and FTR on several life history traits of *D. suzukii*. We hypothesized that (i) prolonged cold stress may induce latent damage that could manifest several days after the end of cold storage and that may express as late mortality, especially under CLT and (ii) higher energetic costs of FTR may translate into reduced reproductive traits (i.e., fecundity, mating capacity).

Materials and methods

Flies origin and rearing

To ensure that the effect of FTR on *D. suzukii* cold tolerance was robust and consistent, all experimentations were performed twice with two different wild-type lines. The "line 1" was a population from infested fruits collected from different locations in Trentino (Italia), and brought to the Vigalzano station of the Edmund Mach foundation (46.042574 N, 11.135245 E) in 2011. This line was sent to our laboratory (Rennes, France) in early 2016. Flies from the "line 2" were collected from infested blueberries and raspberries in Thorigné Fouillard, France (48.0616 N, –1.2387 E) in September 2016. Flies were reared in glass bottles (100 mL) and supplied *ad libitum* with artificial diet (agar: 15 g, sucrose: 50 g, organic carrot powder: 50 g, brewer yeast: 30 g, cornmeal: 20 g, kalmus: 8 g [Kalmus, 1943], Nipagin: 8 mL, water: 1 L). At least 15 bottles of 100–300 flies per line were used for continuous maintenance and emerging flies from different bottles were crossed at each generation to limit inbreeding. Bottles were placed in incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan) under standard conditions: 25 °C, 65%–70% RH, and 12 L : 12 D. Pupae and adults randomly taken from the rearing stock were used in all experiments. Pupae were collected 48 h after pupation (i.e., corresponding to 8 d after egg laying at 25 °C). Adults used in all experiments were 4 or 5 d old when tested to limit age-related differences in stress tolerance (Colinet *et al.*, 2015a). Males were separated from females visually (with an aspirator) without CO_2 to avoid stress due to anesthesia (Colinet & Renault, 2012).

Survival to thermal treatments (experiments 1–3)

Figure 1A illustrates the experimental scheme for the optimization of FTR protocol for short-term cold storage of *D. suzukii*. Both thermal treatments (CLT and FTR) were applied to both lines and both stages (pupae and adults). Temperatures for CLT were 5 and 7.5 °C for pupae and 5 °C for adults. In all experiments (1–7), the thermal conditions used in the different treatments were controlled by programmed incubators (Model MIR-154-PE; PANASONIC, Healthcare Co., Ltd. Gunma, Japan). In FTR, incubators took approximately 30 min to increase temperature from 5 or 7.5 °C to 15, 20, or 25 °C. In pupae, a preliminary test at 10 °C yielded to approximately 100 % survival after 15 d; therefore, this temperature was not used for subsequent experiments because flies were unaffected by cold. For the same reason, 7.5 °C was not used in adults. Indeed, in a previous work we showed that exposure to 7.5 °C only weakly affected adult's survival (Enriquez & Colinet, 2017). For all assays, individuals were placed in vials and supplied with food. For pupae exposed to 5 °C, survival was monitored after 1, 2, 3, 4, 5, 6, or 7 d and for those exposed to 7.5 °C after 1, 2, 3, 4, 6, 8, or 10 d. For each time point, three tubes of 10 pupae were removed from cold condition and replaced at 25 °C. Survival was then scored as the number of emerged flies (partially emerged flies were considered as not emerged). For adults exposed to 5 °C, from both sexes separately, survival was monitored after 1, 2, 3, 5, 7, 9, or 12 d of cold exposure. For each time point, three tubes of 10 males and three tubes of 10 females were removed

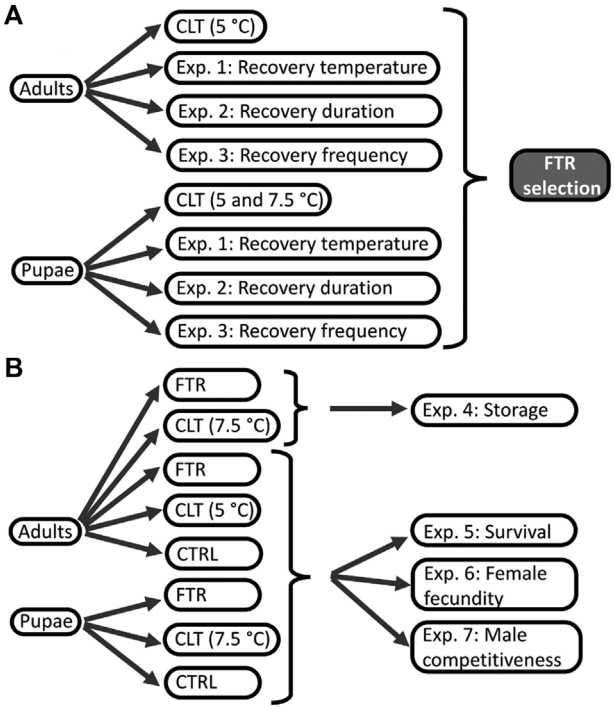

Fig. 1 Experimental plan. A: Survival assays. Pupae were exposed to constant low temperature (CLT) at 5 and 7.5 °C and adults were exposed to CLT at 5 °C. Cold exposures were also performed using fluctuating thermal regimes (FTR), where different recovery temperature (exp. 1), duration (exp. 2), and frequency (exp. 3) were tested. B: Impact of thermal treatments on cold storage tolerance and life history traits. Adults were exposed to either CLT (7.5 °C) or optimized FTR protocol, and long-term cold storage survival was followed for several weeks (exp. 4). Pupae and adults were subjected to optimized FTR or CLT or not exposed (control: CTRL). After treatments, post-storage survival (exp. 5), female fecundity (exp. 6), and male competitiveness (exp. 7) were assayed in both exposed adults and in adults emerging from exposed pupae.

from cold and replaced at 25 °C. Adult survival was assessed 24 h after flies were replaced to standard conditions.

For FTR, recovery temperature, duration and frequency were studied sequentially. For all FTR assays, the procedure was similar to that of CLT, except that the cold period was interrupted by recurrent recovery periods varying in (i) temperature: the cold period was interrupted daily by a 2 h break at 15, 20, or 25 °C (experiment 1); (ii) duration: the cold exposure was interrupted daily by a 20 °C break of 30 min, 1, 2, or 3 h (experiment 2); and (iii) frequency: the cold period was interrupted by 3 h breaks at 20 °C applied every 12, 24, 36, or 48 h (experiment 3). Each experiment was conducted on different cohorts from different generations (see Fig. 1A).

Long-term cold storage as adult (experiment 4)

After we selected the optimal parameters for short-term cold storage (experiments 1 to 3), an additional experiment was carried out to assess whether this optimized FTR protocol might be used to extend adult shelf life over much longer period (i.e., several weeks or months). For this purpose, adults (from the line 1 only) were exposed to either CLT at 7.5 °C or FTR where the cold period (7.5 °C) was interrupted twice a day by a break at 20 °C for 3 h (i.e., considered as optimal parameters). In this experiment, we choose 7.5 °C because this temperature is not too stressful for adults *D. suzukii*, and therefore more appropriate for longer storage (Enriquez & Colinet, 2017). Males and females were separately placed in tubes of 20 flies and supplied with food. The experiment lasted 129 d. Approximately every 7 d, three tubes of 20 males and 20 females were removed from cold incubators, placed at 25 °C and survival was assessed after 24 h.

Poststorage survival (experiment 5)

Poststorage survival under standard conditions was monitored for 20 consecutive days following a short-term cold storage, in order to assess fitness consequences and putative latent damage resulting from cold treatment. Individuals were subjected to cold treatment either as pupae or as adult. Both lines were tested. The short-term cold treatment consisted of five consecutive days either under CLT (5 °C for adults and 7.5 °C for pupae) or under FTR, where the cold exposure (the same as under CLT) was interrupted every 12 h for 3 h at 20 °C. A control group (CTRL) was maintained under standard rearing condition at 25 °C (see Fig. 1B). For pupae, after 5 d under CLT or FTR, cold-exposed individuals were replaced to standard conditions (25 °C) for emergence. Emerged males and females from CLT, FTR, and CTRL were then placed in food vials and their survival was monitored daily for 20 d. For adults, after 5 d under CLT or FTR, surviving flies were replaced to standard conditions in food vials and separated by sex. Survival was then monitored daily for 20 d. For each treatment, line and sex, five replicates of 10 flies were used. For both pupae and adult assays, food vials were changed every 2 d.

Female fecundity (experiment 6)

To assess the reproductive cost of a short-term cold treatment, fecundity was scored in cold-exposed females, as well as in females emerging from cold-exposed pupae. Both lines were tested. Pupae and females were exposed to CLT (7.5 °C), FTR (7.5 °C with 3 h breaks at 20 °C every 12 h), or unexposed to cold (CTRL) for five consecutive days (see Fig. 1B). Fecundity of 15 females from each treatment was then monitored daily for 15 consecutive days. Females were placed with two untreated males for the first 48 h of the experiment to allow mating. All females were kept individually into 50 mL falcon tubes under standard conditions. Tubes were placed vertically, plugs facing down. Plugs were filled with a medium composed of 3.5 g agar, 12.5 g sucrose, 20 g brewer yeast, 2 g Kalmus, and 2 mL Nipagin in 250 mL of water. Food plugs were changed daily, and eggs laid by females were counted under stereomicroscope.

Sexual competitiveness (experiment 7)

Male mating competitiveness was assessed following a short-term cold storage applied to both pupae and adults. All individuals used were isolated as pupae before emergence to avoid mating in order to get virgins. Both lines were tested. Males and pupae were exposed to CLT or FTR treatment or not cold exposed (CTRL) for five consecutive days, following the same method as described in "Female fecundity" section (Fig. 1B). Males were marked either on the left or the right wing to differentiate them during mating trials. To do so, males were briefly anesthetized under CO_2 (<1 min) and the last part of the marginal and submarginal wing cells was cut under stereomicroscope using forceps. A preliminary assay was performed to test a possible deleterious effect of the side of the cut wing on male competitiveness in CTRL flies, but no difference was observed between males marked on the right or the left wing (see results section). Within each treatment, half of the males were marked on the right wing, and the other half on the left wing. Mating competitiveness assays were

conducted 24 h after marking to avoid any deleterious effect due to anesthesia. All flies used in assays were marked (either on left or right wing) to avoid any confounding effect due to wing cut. In each trial, a piece of raspberry was placed at the bottom of a tube together with a virgin untreated female. Two marked virgin males from two different treatments were then simultaneously introduced in the vial for mating competition trials: (i) CTRL versus CLT (pupae: $n = 17$ replicates for the line 1 and 27 for the line 2; adults: $n = 30$ for the line 1, and 38 for the line 2), (ii) CTRL versus FTR (pupae: $n = 29$ replicates for the line 1 and 29 for the line 2; adults: $n = 23$ for the line 1, and 32 for the line 2). Tubes were then continuously inspected for 4 h. When a mating was observed, the treatment of the successful male was identified thanks to its wing cuts. Assays were conducted in the morning (from 8:00 to 12:00), corresponding to the period when *D. suzukii* is the most active (Hardeland, 1972; Evans *et al.*, 2017).

Statistical analyzes

All analyzes were performed with R (R Core Team, 2016). Data from each survival experiment (exp. 1 to 3) and from long-term cold storage experiment (exp. 4) were analyzed using generalized linear models (GLMs) with logit link function for proportions outcome (i.e., number of dead/alive individuals per vial). For long-term cold storage (exp. 4), the 50% median lethal time (Lt_{50}) for each treatment and sex was calculated as follows (Venables & Ripley, 2002):

$$Lt_{50} = \frac{\text{logit}\,(0.5) - a}{b},$$

where a and b, respectively, correspond to the intercept and the slope of GLM prediction for each condition. GLM parameters were then resampled (1000 iterations, thanks to "arm" package; Gelman & Sue, 2014). Thereafter, the Lt_{50} values obtained from each condition were compared using a two-way ANOVA followed by Tukey's *post hoc* tests (package "multcomp"; Hothorn *et al.*, 2008). For poststorage survival (exp. 5), mixed effects generalized models (GLMMs) with logit link function for proportions outcomes were applied. As the same flies within a vial were monitored every day, the vial identity was used as a random variable to account for repeated measures. Female fecundity (exp. 6) was analyzed using a GLMM following a Poisson error family, with a Log link function. As females were individually followed for 15 d, female identity was considered as a random variable. In all experiments, models were simplified by removing nonsignificant interactions (Crawley, 2007). The effects of each variable and their interactions were analyzed via the analysis of deviance ("ANOVA" function in "car" package; Fox & Weisberg, 2011). Then, differences among thermal treatments were analyzed by comparing least-squares means using the "lsmeans" package (Rusell, 2016). Finally, to help interpreting all the terms of models, effect plot function in the package "effects" (Fox, 2003) was used. The effect plots generated show the conditional coefficients ("marginal effects") for all variables and interaction terms. Finally, data from competitiveness assays (exp. 7) were analyzed using exact binomial tests based on the null hypothesis that both competitors had equal probability of success ($P = 0.5$).

Results

For all the experiments, data from adults and pupae were analyzed separately. In order to facilitate the reading, we only provide in the main document a simplified version of the statistical outputs in Table 1, in which only the effect of the main variables of interest are presented. Comprehensive statistical outputs that show the significance of all model's variables and all their interactions are available in Table S1, for all the experiments separately. Outcomes from effects plots illustrating the main effects and interactions are also available in supplementary figures for each experiment separately (Figs. S1–S11). Outcomes from multiple comparisons between thermal treatments are shown within these figures by different lettering (Figs. S1–S11). Furthermore, to simplify the main message, the graphical representations of models show results from both lines pooled, although both lines were always considered separately in all analyses. Graphical representations that differentiate both lines can still be found in supplementary figures (Fig. S12).

Recovery temperature (exp. 1)

Adult's cold survival was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR at 15, 20, or 25 °C) (Table 1; Fig. 2A, B). Males had higher survival than females (Table 1; Figs. 2A, B, S1B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 2A, B, S1C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 2A, B), and *post hoc* comparisons showed that recovery temperature of 20 and 25 °C provided the highest survival (Fig. S1D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 2A, B). In females, survival gradually increased

Table 1 Statistical outputs from GLMs and GLMMs. GLMs were used to analyze data from experiments 1, 2, 3, and 4: variation of recovery temperature, duration, frequency, and long-term storage. Data from experiment 5 and 6 (poststorage survival and female fecundity) were analyzed with GLMMs. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; NS: not significant. TRT stands for thermal treatment (i.e., CLT, FTR, and CTRL). The parameter "temp" refers to temperature of cold period (i.e., 5 or 7.5 °C for pupae). TRT: treatment (CLT: constant low temperature; FTR: fluctuating thermal regime; CTRL: control).

Experiment:	Exp. 1: Recovery temperature						Exp. 2: Recovery duration						Exp. 3: Recovery frequency						Exp. 4: Long-term cold storage			Exp. 5: Poststorage survival						Exp. 6: Females fecundity					
	Adults			Pupae			Adults			Pupae			Adults			Pupae			Adults			Adults			Pupae			Adults			Pupae		
Parameter:	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value	χ^2	df	*P* value
Line	153.93	1	***	0.13	1	NS	21.73	1	***	131.3	1	***	134.52	1	***	40.53	1	***		NA		516.14	1	***	0.06	1	NS	334.54	1	***	2432.04	1	***
Temp		NA		190.38	1	***		NA		460.13	1	***		NA		249.14	1	***		NA			NA			NA			NA			NA	
Sex	33.42	1	***		NA		32.1	1	***		NA		95.76	1	***		NA		66.47	1	***	1.52	1	NS	1.82	1	NS		NA			NA	
Time	1077.69	1	***	581.84	1	***	1102.96	1	***	1167.05	1	***	947.14	1	***	713.34	1	***	519.95	1	***	293.47	1	***	145.33	1	***	286.88	1	***	663.67	1	***
TRT	350.52	3	***	166.45	3	***	1486.87	4	***	221.24	4	***	647.22	4	***	254.67	4	***	303.67	1	***	32.6	2	***	962.22	2	***	2072.35	2	***	988.22	2	***
TRT:line	44.13	3	***	49.17	3	***	43.38	4	***	2.17	4	NS	12.39	4	*	25.74	4	***		NA		1.65	2	NS	33.38	2	***	666.81	2	***	255.86	2	***
TRT:temp		NA		19.41	3	***		NA		27.99	4	***		NA		22.31	4	***		NA			NA			NA			NA			NA	
TRT:sex	40.98	3	***	NA			23.9	4	***	NA			87.98	4	***	NA			8.38	1	**	6.39	2	*	25.88	2	***		NA			NA	
Time:TRT	408.1	3	***	221.14	3	***	291.13	4	***	135.6	4	***	505.21	4	***	237.39	4	***	0.41	1	NS	9.06	2	*	19.09	2	***	652.6	2	***	119.88	2	***

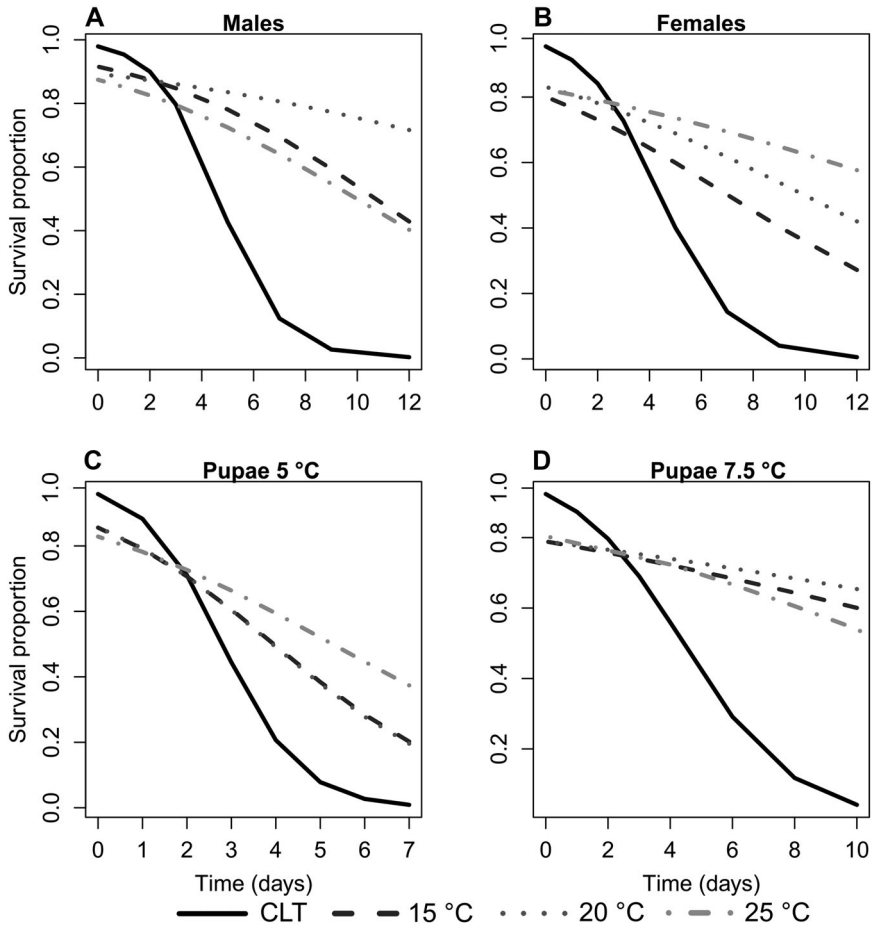

Fig. 2 Survival responses to changes in recovery temperature. Solid black curve: insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a daily recovery period of 2 h at 15, 20 or 25 °C. The curves correspond to model predictions (Binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

with recovery temperature, while in males, survival was the highest with a recovery temperature of 20 °C (Table 1; Figs. 2A, B, S1I). Finally, temporal decrease in survival was much faster under CLT than under FTR treatments (Table 1; Figs. 2A, B, S1J).

Cold survival of pupae was significantly affected by temperature, time, and thermal treatment (Table 1; Fig. 2C, D). Survival was higher when the cold period was 7.5 compared to 5 °C (Table 1; Figs. 2C, D, S2B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 2C, D, S2C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 2C, D), and *post hoc* comparisons showed that all recovery temperatures were equivalent (Fig. S2D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 2C, D). When the cold period was 5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 2C, D, S2I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 2C, D, S2J).

Recovery duration (exp. 2)

Cold survival of adults was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR for 30 min, 1, 2 or 3 h) (Table 1; Fig. 3A, B). Males had higher survival than females (Table 1; Figs. 3A, B, S3B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 3A, B, S3C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 3A, B), and *post*

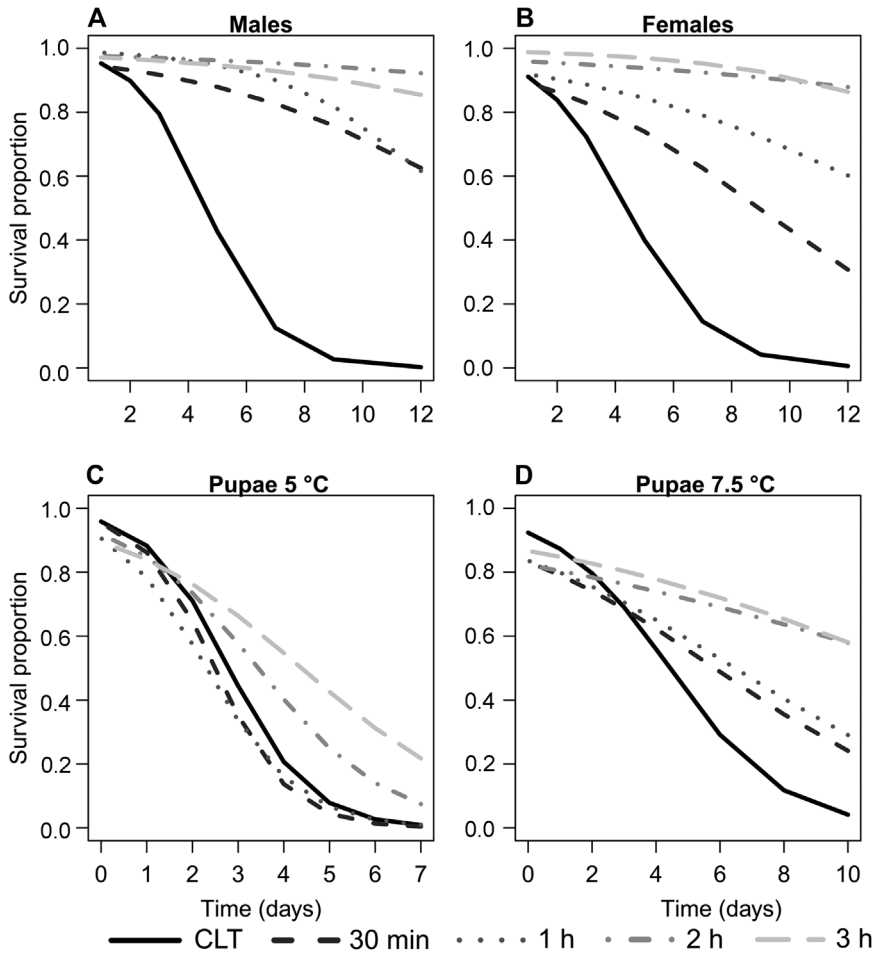

Fig. 3 Survival responses to changes in recovery duration. Solid black curve: Insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a daily recovery period at 20 °C for 30 min, 1, 2, or 3 h. The curves correspond to model predictions (Binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

hoc comparisons showed that recovery duration of 2 and 3 h provided higher survival than duration of 0.5 and 1 h (Fig. S3D). It should be noted that with FTR treatments allowing 2 and 3 h recovery, adult cold mortality was nearly fully compensated (Figs. 3A, B). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 3A, B). In females, survival gradually increased with recovery duration, while in males, survival was highest with 2 h of recovery (Table 1; Figs. 3A, B, S3I). Finally, temporal decrease in survival was much faster under CLT than under FTR treatments. Among FTR conditions, survival decreased with time more slowly when recovery duration was 2 and 3 h (Table 1; Figs. 3A, B, S3J).

Cold survival of pupae was significantly affected by temperature, time and thermal treatment (Table 1; Fig. 3C, D). Survival was higher when the cold period was 7.5 compared to 5 °C (Table 1; Figs. 3C, D, S4B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 3C, D, S4C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 3C, D), and *post hoc* comparisons showed that recovery duration had marked effect on pupal survival with 3 h being the best, followed by 2 h, and durations of 1 or 0.5 h were not different from CLT (Fig. S4D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 3C, D). When the cold period was 5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 3C, D, S4I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 3C, D, S4J).

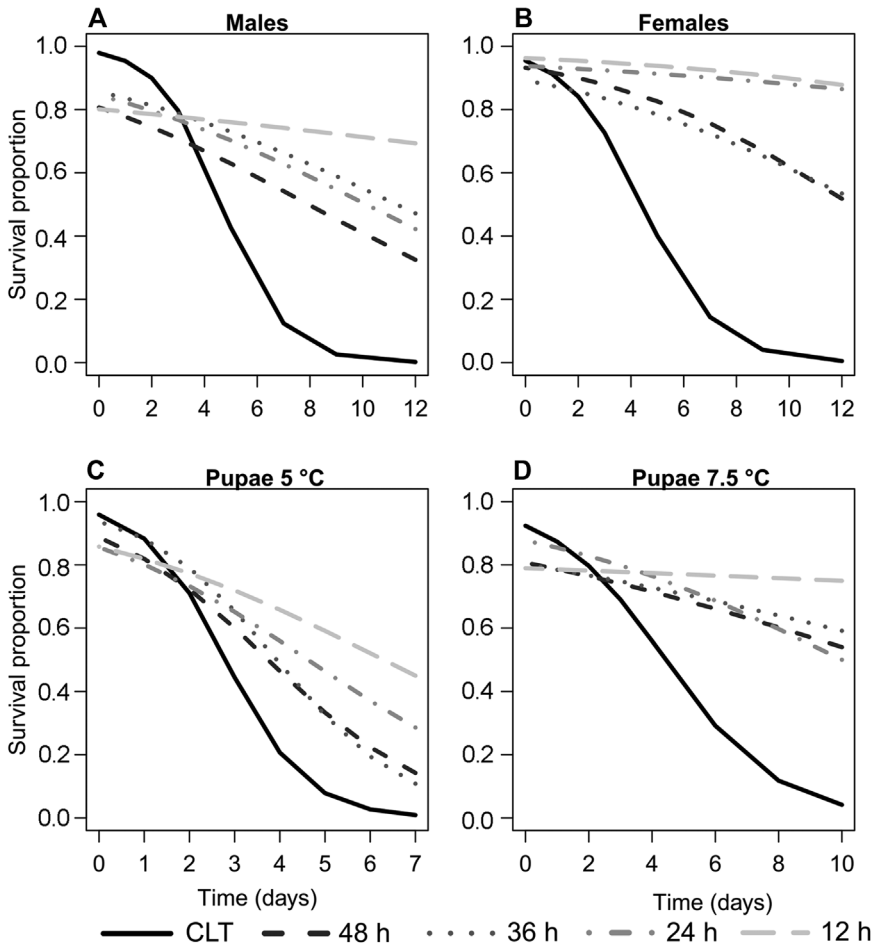

Fig. 4 Survival responses to changes in recovery frequency. Solid black curve: insects subjected to constant low temperature (CLT). Dashed gray curves: FTR where the cold exposure was interrupted by a 3 h recovery period at 20 °C, applied every 48, 36, 24, or 12 h. The curves correspond to model predictions (binomial GLM, logit link function). A: males; B: females; C: pupae exposed to 5 °C; D: pupae exposed to 7.5 °C.

Recovery frequency (exp. 3)

Cold survival of adults was significantly affected by all the main effects: sex, time, and thermal treatment (i.e., CLT or FTR every 12, 24, 36, or 48 h) (Table 1; Fig. 4A, B). Males had lower survival than females (Table 1; Fig. 4A, B, S5B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 4A, B, S5C). Adults survival was globally much higher under FTR than under CLT (Table 1; Fig. 4A, B), and *post hoc* comparisons showed that recovery frequency of 12 and 24 h provided highest survival, followed by 36 and 48 h (Fig. S5D). With FTR treatments cycling every 12 h, cold mortality was nearly fully compensated, especially in females (Fig. 4A, B). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 4A, B). In females, survival was clearly higher with recovery frequencies of 12 and 24 h, while in males, effects were less contrasted (Table 1; Figs. 4A, B, S5I). Finally, temporal decrease in survival was much more rapid under CLT than under FTR treatments, and among FTR conditions, survival decreased with time more slowly when recovery periods were frequent (Table 1; Figs. 4A, B, S5J).

Cold survival of pupae was significantly affected by temperature, time and thermal treatment (Table 1; Fig. 4C, D). Survival was higher when the cold period was 7.5 compared to 5 °C (Table 1; Figs. 4C, D, S6B). Overall, cold survival decreased with increasing time of exposure (Table 1; Figs. 4C, D, S6C). Pupal survival was globally much higher under FTR than under CLT (Table 1; Fig. 4C, D), and *post hoc* comparisons showed that recovery frequency of 12 h provided highest survival, followed

by 24, 36 and then 48 h (Fig. S6D). The effect of thermal treatment also interacted with temperature and time (Table 1; Fig. 4C, D). When the cold period was 5 °C, pupal survival under both FTR and CLT was much lower than when it was 7.5 °C (Table 1; Figs. 4C, D, S6I). Finally, temporal decrease in pupal survival was much faster under CLT than under FTR treatments (Table 1; Figs. 4C, D, S6J).

Long-term cold storage of adults (exp. 4)

Fig. 5 shows the results from long-term cold storage of adults exposed to two thermal treatments: CLT (7.5 °C) or FTR (7.5 °C alternating with breaks of 20 °C for 3 h occurring twice a day). Cold survival was significantly affected by sex, time, and thermal treatment (Table 1; Fig. 5A, B). Females were globally more cold tolerant than males (Table 1; Figs. 5A, B, S7A). Cold survival decreased with increasing time of exposure (Table 1; Figs. 5A, B, S7B). Survival was higher under FTR than under CLT (Table 1; Figs. 5A, B, S7C). The effect of thermal treatment also interacted with sex but not with time (Table 1; Fig. 5A, B). Males benefited more from FTR than females (Table 1; Figs. 5A, B, S7E). Temporal decrease in survival did not differ among treatments (Table 1; Figs. 5A, B, S7F). Under CLT, males and females Lt_{50} values were, respectively, 26.14 and 54.36 d, while under FTR, they were, respectively, 45.61 and 118.32 d (Fig. 5C). Lt_{50} values were all significantly different ($F = 50\,828$, df = 3, $P < 0.001$; all Tukey's P values < 0.001).

Poststorage survival (exp. 5)

Survival was recorded for 20 d (at 25 °C) following three treatments: 5-d cold exposure (CLT or FTR), or no cold exposure (CTRL). For adults, survival was significantly affected by time and thermal treatment, but not by sex (Table 1; Fig. 6A, B). Survival decreased with time (Table 1; Figs. 6A, B, S8C). Overall, CLT showed the lowest survival rate, followed by CTRL and then FTR (Table 1; Figs. 6A, B, S8D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 6A, B). The effect of thermal treatment was not pronounced in females (because survival was globally high), while in males, positive effect of FTR was more evident (Table 1; Figs. 6A, B, S8I). Temporal decrease in survival varied according to treatments and was faster under CLT than under FTR or CLTR (Table 1; Figs. 6A, B, S8J).

When exposure occurred at pupal stage, subsequent 20-d adult survival was affected by time and thermal treatment but not by sex (Table 1; Fig. 6E–H). Survival decreased with time (Table 1; Figs. 6C, D, S9C). CLT showed the lowest survival, followed by FTR, and then CTRL (Table 1; Figs. 6C, D, S9D). The effect of thermal treatment also interacted with sex and time (Table 1; Fig. 6C, D). Survival under FTR did not differ from CTRL in females but was lower than CTRL in males (Table 1; Figs. 6C, D, S9I). Temporal decrease in survival differed among treatments, with CLT showing the fastest decrease (Table 1; Figs. 6C, D, S9J).

Female fecundity (exp.6)

Cumulated fecundity (over 15 d) of females that were cold exposed as adults varied with time and thermal treatment (Table 1; Fig. 7A). Cumulated fecundity increased with time (Table 1; Figs. 7A, S10B). Females exposed to CLT produced the lowest number of eggs, followed by females exposed to FTR, then, unexposed CTRL females were the most productive (Table 1; Figs. 7A, S10C). The effect of thermal treatment on fecundity also interacted with time (Table 1; Fig. 7A). Temporal egg-laying patterns differed among treatments: after FTR or CLT treatment, the beginning of the egg-laying phase was delayed in comparison with the CTRL group (Table 1; Figs. 7A, S10F).

Cumulated fecundity of females emerging from cold-exposed pupae was affected by time and thermal treatment (Table 1; Fig. 7B). Cumulated fecundity increased with time (Table 1; Figs. 7B, S11B). Pupae exposed to FTR resulted in the lowest fecundity, followed by CLT then CTRL (Table 1; Figs. 7B, S11C). The effect of thermal treatment on fecundity also interacted with time (Table 1; 7B). Finally, temporal egg-laying patterns differed among treatments: start of the egg-laying phase was slightly delayed under FTR in comparison with CTRL (Table 1; Figs. 7B, S11F).

Mating competitiveness (exp. 7)

Outcomes of male mating competitiveness are shown in Fig. 8. First, effect of the side of the wing cut on mating success in CTRL flies was tested (using exact binomial tests) and no effect was found (left vs. right, $P = 0.56$). Next, we tested effect of cold treatments on male mating success. Males cold-treated as adult, whether under CLT or FTR, showed similar mating success as CTRL males (CTRL vs. FTR, $P = 1$; CTRL vs. CLT, $P = 0.90$). The only significant effect detected was in males subjected to CLT as pupae that had lower mating success than CTRL males (CTRL vs. CLT, $P < 0.05$). Males exposed to FTR

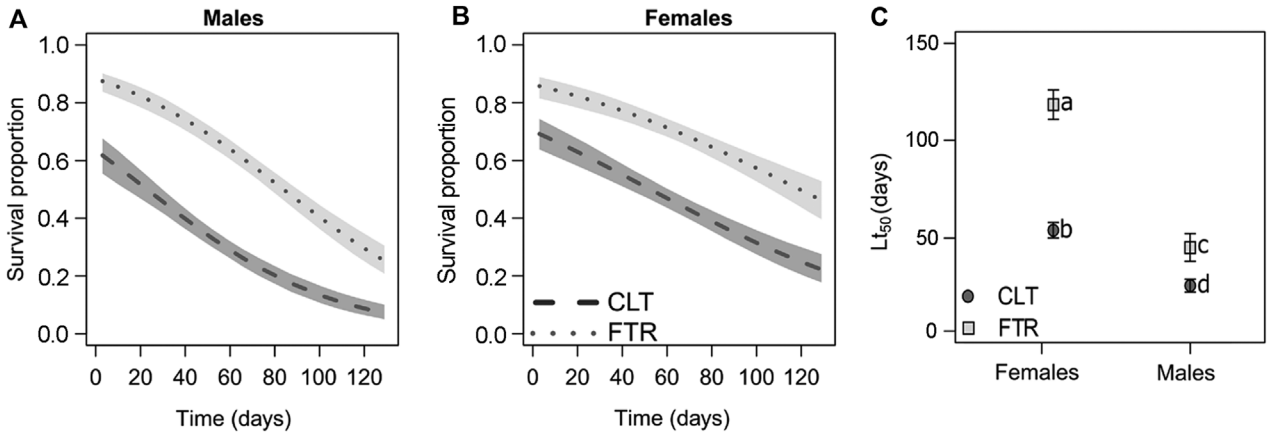

Fig. 5 Survival responses to long-term cold storage. Dashed gray curve: CLT (7.5 °C); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. The curves correspond to model predictions (binomial GLM, logit link function), and shaded areas to 95% interval confidence. A: males; B: females. C: estimated Lt_{50} values from GLMs. Symbols with different letters are significantly different ($P < 0.001$).

as pupae had similar mating success as CTRL males (CTRL vs. FTR, $P = 0.35$).

Discussion

FTR effect on cold survival

In this study, we analyzed cold storage of *D. suzukii* under CLT and FTR, and explored consequences on survival and fitness-related traits. Concerning effects of FTR on survival, we hypothesized that beneficial effect of FTR should increase with recovery temperature, duration, and frequency, and that there should be an optimal combination of recovery temperature × duration above which no additional benefit is observed. Most of the tested FTR treatments led to significant reduction in cold-induced mortality compared to CLT, corroborating earlier observations about the positive effect of FTR (e.g., Renault *et al.*, 2004; Colinet *et al.*, 2006; Koštál *et al.*, 2007; Javal *et al.*, 2016; Torson *et al.*, 2017). Although, the benefits of FTR were of different magnitude depending on the various experimental conditions. Beneficial effects of FTR were robust, as it was consistently observed in both lines, sexes and life stages. Other studies showed that FTR has positive effects on pupal survival (Colinet *et al.*, 2006; Dollo *et al.*, 2010; Rinehart *et al.*, 2011; Yocum *et al.*, 2012, 2016), but in *D. melanogaster* a positive response of FTR at pupal stage was not clearly observed (Javal *et al.*, 2016). The temperatures tested by Javal *et al.* (2016) were lower than 5 °C, and at these temperatures, chilling damage might be harder to recover.

We aimed to identify an optimal FTR protocol for short- or long-term cold storage from the best combination of recovery temperature, duration and frequency in both pupae and adults. Among the three recovery temperatures tested, 20 and 25 °C provided roughly similar benefits. Using 15 °C as a recovery temperature also allowed insects to survive cold storage longer than their counterparts exposed to CLT, but benefits were slightly less evident than at 20 and 25 °C. Previous studies have found that increasing FTR recovery temperature results in better survival (Nedvěd *et al.*, 1998; Renault *et al.*, 2004; Colinet *et al.*, 2011; Yocum *et al.*, 2012), but we found that an increase of 5 °C (from 20 to 25 °C) did not significantly promoted survival. Furthermore, a too high recovery temperature may eventually become deleterious (Hanč & Nedvéd, 1999). Moreover, when the recovery temperature was set at 25 °C, during decreasing temperature phases, relative humidity could reach 100%, resulting in condensation of water inside incubator and within vials. Condensation can be deleterious for flies, as they may easily get stuck on droplets and died. Moreover, fungal development is favored by high relative humidity, which may compromise insect storage (Colinet *et al.*, 2018). Consequently, only 20 °C was conserved for the follow-up experiments. Increasing the recovery duration and frequency resulted in a gradual decrease in mortality. In most cases, the longer and the most frequent the recovery duration, the lower the mortality, in both pupae and adults. This is consistent with earlier data on other insects (Hanč & Nedvéd, 1999; Colinet *et al.*, 2006; Yocum *et al.*, 2012). Interestingly, even a very short recovery duration (30 min) decreased cold-induced mortality, suggesting

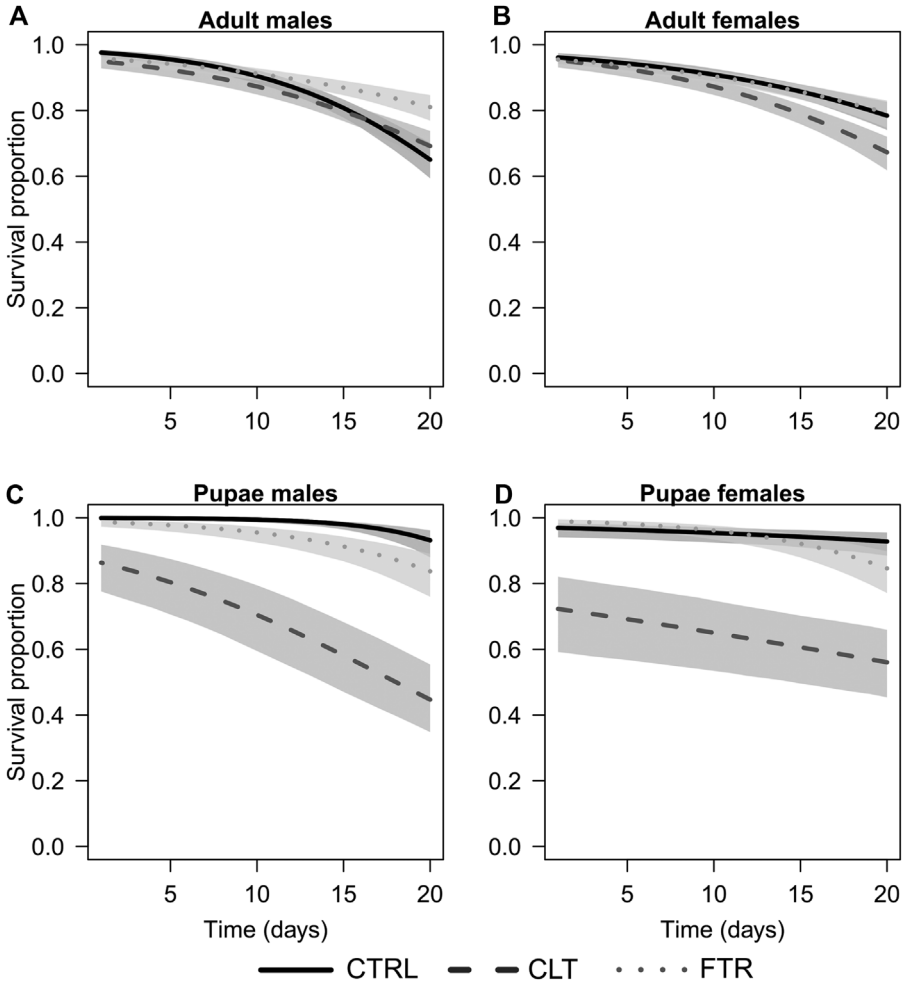

Fig. 6 Survival at 25 °C subsequent to a short cold-exposure under different regimes. Solid black curve: control (CTRL: 25 °C for 5 d); dashed gray curve: CLT applied to adults (5 °C for 5 d) and to pupae (7.5 °C for 5 d); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 d. After exposure to CTRL, CLT, and FTR for 5 d, survival was monitored for 20 d under standard conditions. The curves correspond to model predictions (binomial GLMM, logit link function), and shaded areas to 95% interval confidence. A: adult males; B: adult females; C: males from cold-exposed pupae; D: females from cold-exposed pupae.

that, as many other species, *D. suzukii* has the capacity to quickly recover from cold stress when temperature turns favorable.

By systematically changing FTR parameters, we could define what we consider to be the optimal settings for *D. suzukii*: interruption of the cold period every 12 h by warming intervals at 20 °C for 3 h. This optimized treatment clearly allowed adults and pupae to remain alive at cold during a much longer period than insects exposed to CLT. When applied to adults, these optimal FTR settings almost doubled Lt_{50} values reaching 54 d in males and 118 d in females. Maximum longevity of *D. suzukii* under laboratory standard conditions does not exceed 35 d at 25 °C (Tochen *et al.*, 2014; Kim *et al.*, 2015). In this regard, the application of FTR could also be used as life-span extension protocol to maintain lines of interest, such as those used in SIT and IIT programs, with a low impact on fly's survival. In addition, this FTR protocol offers real opportunity for short-term cold storage of pupae that can be safely conserved for at least 10 d.

Impact of FTR on life history traits

The generally accepted explanation for the promoting effects of FTR is that warm interruptions allow repair

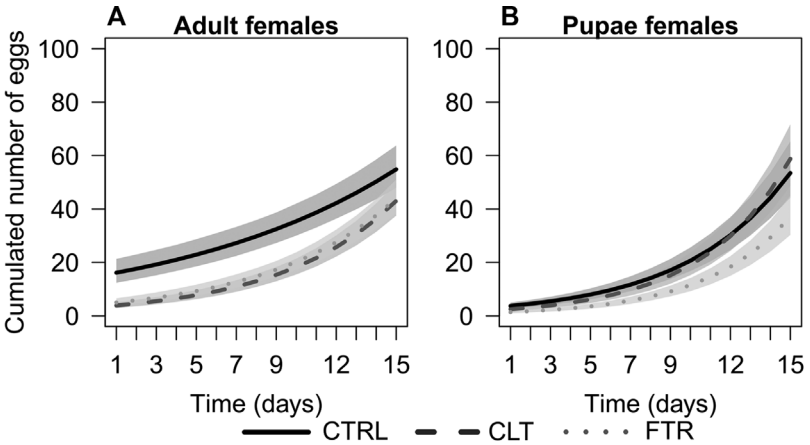

Fig. 7 Fifteen-day cumulated fecundity subsequent to a short cold-exposure under different regimes. Solid black curve: control (CTRL: 25 °C for 5 d); dashed gray curve: CLT applied to adults (5 °C for 5 d) and to pupae (7.5 °C for 5 d); dotted gray curve: FTR where the cold exposure was interrupted by breaks of 3 h at 20 °C every 12 h. FTR was applied to adults and pupae for 5 d. After exposure to CTRL, CLT, and FTR for 5 d, the fecundity was monitored for 15 d under standard conditions. The curves correspond to model predictions (Poisson GLMM, log link function), and shaded areas to 95% interval confidence. A: adult females; B: females from cold-exposed pupae.

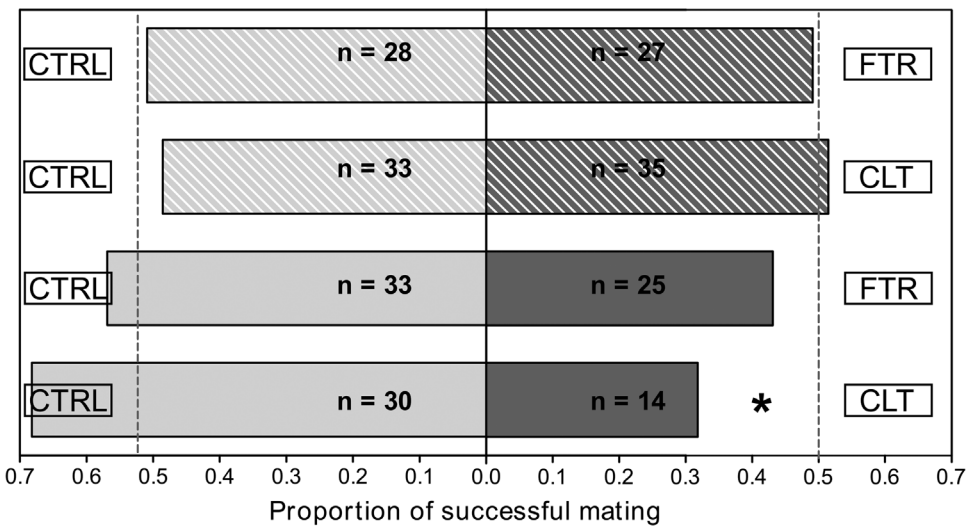

Fig. 8 Male competitiveness subsequent to a short cold-exposure under different regimes. Light-gray bars: control males (CTRL: 25 °C for 5 d); dark-gray bars: males exposed to CLT (5 or 7.5 °C for 5 d in adults and pupae, respectively) and males exposed to FTR for 5 d (cold interrupted by breaks of 3 h at 20 °C every 12 h). Full bars correspond to individuals exposed to thermal treatments at pupal stage, and dashed bars correspond to individuals exposed as adults. After exposure to CTRL, CLT, and FTR for 5 d, male competitiveness was monitored in mating assays. Numbers of successful males are indicated inside their respective bars. The sign (*) corresponds to $P < 0.05$.

of chilling-injuries that otherwise accumulate under CLT (Renault *et al.*, 2004; Koštál *et al.*, 2007; Colinet *et al.*, 2018). Several repair mechanisms have been supposed to be involved, such as reestablishment of ion homeostasis (Koštál *et al.*, 2007), restoration of membrane lipids composition (Colinet *et al.*, 2016), activation of antioxidant system (Lalouette *et al.*, 2011; Torson *et al.*, 2017), accumulation of cryoprotectants, and production of heat shock proteins (Pio & Baust, 1988; Colinet *et al.*, 2007; Lalouette *et al.*, 2007; Boardman *et al.*, 2013). These adjustments are probably associated with energy cost for the organisms (Colinet *et al.*, 2018), consequently, the second aim of this work was to explore fitness consequences of cold storage under CLT versus FTR, assuming that

CLT would induce deleterious effects that would carry over into adult life, and that FTR would generate cost that could alter life history traits.

Survival after cold treatment Survival of adults was followed for 20 consecutive days after a cold exposure of 5 d (CLT vs. FTR) applied either at the adult or pupal stage, and this was compared to patterns of flies kept at 25 °C (CTRL). Data revealed that CLT markedly decreased subsequent adult survival compared to unexposed CTRL or FTR-exposed flies, especially when individuals were treated at the pupal stage. By contrast, flies' survival was largely superior under FTR than under CLT, especially when FTR was applied to adults. When applied to pupae, benefits of FTR were slightly less evident. It is possible that FTR applied to adults allows efficient repair of chilling injuries, but when applied to pupae, cold damage may not be fully recovered and carry-over in the next stage. Our results show that pupae are globally more cold susceptible than adults. Previous works on *D. suzukii* haves highlighted these differences (Dalton *et al.*, 2011; Ryan *et al.*, 2016; Enriquez & Colinet, 2017). We found that exposure to stressing CLT in pupae had lasting effects on the adults emerging from these pupae. Metamorphosis is ongoing during pupal stage and a prolonged cold stress during this critical phase might induce latent damages with a knock-on effect in adults. In the butterfly *Bicyclus anynana*, heat stress experienced early in life carried over to later stages, reducing subsequent fitness (Klockmann *et al.*, 2017). In *Plutella xylostella*, heat injury in eggs and 3rd-instar larvae also carried over to the adult stage and led to a more rapid rate of egg laying (Zhang *et al.*, 2015). The impact of cold stress on developing stages and the resulting consequences on adult survival and fitness remain poorly documented. Here, we provide evidence of a carry-over effect on adult's survival, resulting from cold stress at pupal stage, particularly when pupae were exposed to prolonged cold stress without opportunity to recover.

FTR impact on female fecundity Effects of FTR on fecundity have been poorly documented. In the present study, cold-exposed females, under both FTR and CLT, showed a delayed and reduced cumulated egg production compared to unexposed CTRL flies. This result suggests a negative impact of low temperature on subsequent reproductive potential. Stressful temperature, like heat stress, can alter egg development and can induce oviposition delays due to hormonal imbalance (Gruntenko *et al.*, 2003). Cold stress may also cause damages to diverse tissues in insects, leading to impairment of reproductive abilities (Rinehart *et al*., 2000) or delayed ovarian development (Jones & Kunz, 1998). These phenomena may have led to the observed decrease in fecundity. We also observed that *D. suzukii* flies cold-treated as pupae showed lower fecundity than controls, particularly when exposed to FTR. Ismail *et al.* (2010) did not observe any fecundity decrease after FTR exposition in *Aphidius ervi* females. Likewise, Murdoch *et al.* (2013) tested the effect of warming interruptions applied once or thrice during a cold storage period of 8-week and found no differences between constant or fluctuating storage on fecundity of the leek moth *Diadromus pulchellus*. Allocation of limited energy often results in life-history trade-offs, for instance between reproduction and longevity (Zera & Harshman, 2001; Attisano *et al.*, 2012). Contrary to CLT-exposed pupae, we found no sign for decreased longevity (i.e., 20 d survival poststress) of FTR-exposed pupae in comparison with CTRL pupae. On the other hand, we found evidence for reduced egg production in females exposed at pupal stage to FTR compared to CTRL, but not in pupae exposed to CLT. Energy used for repair mechanisms under FTR might have led to this reduced egg production.

Mating competitiveness Concerning sexual competitiveness, when males were subjected to CLT or FTR as adults, no difference was observed with control. However, when cold treatment was applied to pupae, FTR males showed similar mating performance as control males, but CLT males showed a decreased mating success. Sub- and supra-optimal temperatures decrease sperm production and viability, and can cause sterility (Rinehart *et al.*, 2000; Araripe *et al.*, 2004; David *et al.*, 2005; Porcelli *et al.*, 2017). In the parasitoid wasp *Dinarmus basalis*, a cold stress during pupal development had detrimental effects on eclosion of pupae and males showed a reduced sperm stock at emergence. These cold-stressed males were at a disadvantage in accessing females and inseminated fewer females than control wasps (Lacoume *et al.*, 2007). Although we did not check the sperm production neither the sperm viability of cold-exposed *D. suzukii* males, we may hypothesize that CLT (i.e., the most stressful treatment according to our data) may have induced an alteration of spermatogenesis, which may have influenced mating behavior and male's success. Colinet and Hance (2009) also showed that exposing parasitoids pupae to cold storage reduced male's mating success when exposed to CLT, but not to FTR. CLT-exposed wasps had altered mobility and velocity, probably due to muscles impairments. In *Drosophila* flies, male courtship success is correlated with running speed and success in aggressive interactions with competitive males (Partridge & Farquhar, 1983). Therefore, we can also assume that a reduction in mating performances of *D. suzukii* males exposed to CLT may be, at least in part, a consequence of a reduced mobility.

Several studies that looked at the cold stress effect on pupae observed pharate adults unable to fully emerge from puparium (Lacoume *et al.*, 2007). This phenomenon has been attributed to muscle contraction deficiency (Yocum *et al.*, 1994; Kelty *et al.*, 1996), resulting from neuromuscular dysfunctions due to low temperature (Koštál *et al.*, 2004; 2007). Since FTR allows the restoration and maintenance of ion homeostasis (Koštál *et al.*, 2007, 2016), FTR-exposed flies were possibly less subjected to neuromuscular and mobility disfunctions.

Concluding remarks

The present work conclusively shows that storage at 5 or 7.5 °C interrupted by recovery periods at 20 °C for 3 h every 12 h is particularly appropriate for storage of adults. Pupae were globally more sensitive to cold than adults, but FTR allowed this stage to be stored with minimum impact on survival for approximately 10 d. Even if 7.5 °C under FTR seems promising for relatively short storage durations of pupae, caution needs to be taken with longer periods, as development can still proceed under these conditions and may lead to undesirable emergence during cold storage. In the present study, we focused on thermal-related parameters of FTR, but other parameters could be important for cold storage, such as relative humidity, which can affect thermal tolerance of insects, including *D. suzukii* (Boardman *et al.*, 2013; Enriquez & Colinet, 2017; Eben *et al.*, 2018). Even if FTR offer promising results in laboratory scaled experiments, implementation of such technique in industrial scale may be costly and challenging (Colinet *et al.*, 2018). Works are still required to study and to adapt FTR to face these constraints. Finally, from a fundamental aspect, the bioenergetics of FTR remain poorly explored, and future studies should aim to determine whether or not the energy depleted during cold storage drives life-history trade-offs.

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Disclosure

The authors declare there are no competing interests.

References

Araripe, L.O., Klaczko, L.B., Moreteau, B. and David, J.R. (2004) Male sterility thresholds in a tropical cosmopolitan drosophilid. *Zaprionus indianus*. *Journal of Thermal Biology*, 29, 73–80.

Asplen, M.K., Anfora, G., Biondi, A., Choi, D.S., Chu, D., Daane, K.M. *et al*. (2015) Invasion biology of spotted wing drosophila (*Drosophila suzukii*): a global perspective and future priorities. *Journal of Pest Science*, 88, 469–494.

Attisano, A., Moore, A.J. and Moore, P.J. (2012) Reproduction-longevity trade-offs reflect diet, not adaptation. *Journal of Evolutionary Biology*, 25, 873–880.

Basson, C.H., Nyamukondiwa C. and Terblanche, J.S. (2012) Fitness costs of rapid cold-hardening in *Ceratitis capitata*. *Evolution*, 66, 296–304.

Boardman, L., Sørensen, J.G. and Terblanche, J.S. (2013) Physiological responses to fluctuating thermal and hydration regimes in the chill susceptible insect, *Thaumatotibia leucotreta*. *Journal of Insect Physioliogy*, 59, 781–794.

Calabria, G., Máca, J., Bächli, G., Serra, L. and Pascual, M. (2012) First records of the potential pest species *Drosophila suzukii* in Europe. *Journal of Applied Entomology*, 136, 139–147.

Carroll, A.L. and Quiring, D.T. (1993) Interactions between size and temperature influence fecundity and longevity of a tortricid moth, *Zeiraphera canadensis*. *Oecologia*, 93, 233–241.

Cini, A., Ioratti, C. and Anforta, G. (2012) A review of the invasion of *Drosophila suzukii* in Europe and a draft research agenda for integrated pest management. *Bulletin of Insectology*, 65, 149–160.

Clark, M.S. and Worland, M.R. (2008) How insects survive the cold: molecular mechanisms—a review. *Journal of Comparative Physiology B*, 178, 917–933.

Colinet, H. and Hance, T. (2009) Male reproductive potential of *Aphidius colemani* (Hymenoptera: Aphidiinae) exposed to constant or fluctuating thermal regimes. *Environmental Entomolology*, 38, 242–249.

Colinet, H. and Hance, T. (2010) Interspecific variation in the response to low temperature storage in different aphid parasitoids. *Annals of Applied Biology*, 156, 147–156.

Colinet, H. and Renault, D. (2012) Metabolic effects of CO_2 anesthesia in *Drosophila melanogaster*. *Biology Letters*, 8, 1050–1054.

Colinet, H., Chertemps, T., Boulogne, I. and Siaussat, D. (2015a) Age-related decline of abiotic stress tolerance in young *Drosophila melanogaster* adults. *Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 71, 1574–1580.

Colinet, H., Lalouette, L. and Renault, D. (2011) A model for the time–temperature–mortality relationship in the chill-susceptible beetle, *Alphitobius diaperinus*, exposed to

fluctuating thermal regimes. *Journal of Thermal Biology*, 36, 403–408.

Colinet, H., Nguyen, T.T.A., Cloutier, C., Michaud, D. and Hance, T. (2007) Proteomic profiling of a parasitic wasp exposed to constant and fluctuating cold exposure. *Insect Biochemistry and Molecular Biology*, 37, 1177–1188.

Colinet, H., Overgaard, J., Com, E. and Sørensen, J.G. (2013) Proteomic profiling of thermal acclimation in *Drosophila melanogaster*. *Insect Biochemistry and Molecular Biology*, 43, 352–365.

Colinet, H., Renault, D., Hance, T. and Vernon, P. (2006) The impact of fluctuating thermal regimes on the survival of a cold-exposed parasitic wasp, *Aphidius colemani*. *Physioligical Entomology*, 31, 234–240.

Colinet, H., Renault, D., Javal, M., Berková, P., Šimek, P. and Koštál, V. (2016) Uncovering the benefits of fluctuating thermal regimes on cold tolerance of *Drosophila* flies by combined metabolomic and lipidomic approach. *Biochimica et Biophysica Acta Molecular and Cell Biology of Lipids*, 1861, 1736–1745.

Colinet, H., Rinehart, J.P., Yocum, G.D. and Greenlee, K.J. (2018) Mechanisms underpinning the beneficial effects of fluctuating thermal regimes in insect cold tolerance. *Journal of Experimental Biology*, 221, jeb164806. https://doi.org/10.1242/jeb.164806.

Colinet, H., Sinclair, B.J., Vernon, P. and Renault, D. (2015b) Insects in fluctuating thermal environments. *Annual Review of Entomology*, 60, 123–140.

Crawley, M.J. (2007) *The R Book*. Wiley, Chichester, UK/ Hoboken, NJ.

Dalton, D.T., Walton, V.M., Shearer, P.W., Walsh, D.B., Caprile, J. and Isaacs, R. (2011) Laboratory survival of *Drosophila suzukii* under simulated winter conditions of the Pacific Northwest and seasonal field trapping in five primary regions of small and stone fruit production in the United States. *Pest Management Science*, 67, 1368–1374.

David, J.R., Araripe, L.O., Chakir, M., Legout, H., Lemos, B., Petavy, G. *et al*. (2005) Male sterility at extreme temperatures: a significant but neglected phenomenon for understanding *Drosophila* climatic adaptations. *Journal of Evolutionary Biology*, 18, 838–846.

Denlinger, D.L. and Lee, R.E. Jr. (2010) *Low Temperature Biology of Insects*. Cambridge University Press, Cambridge, UK.

Dollo, V.H., Yi, S.X. and Lee, R.E. Jr. (2010) High temperature pulses decrease indirect chilling injury and elevate ATP levels in the flesh fly, *Sarcophaga crassipalpis*. *Cryobiology*, 60, 351–353.

Eben, A., Reifenrath, M., Briem, F., Pink, S., and Vogt, H. (2018) Response of *Drosophila suzukii* (Diptera: Drosophilidae) to extreme heat and dryness. *Agricultural and Forest Entomology*, 20, 113–121.

Enriquez, T. and Colinet, H. (2017) Basal tolerance to heat and cold exposure of the spotted wing drosophila, *Drosophila suzukii. PeerJ*, 5, e3112, https://doi.org/10.7717/peerj.3112.

Evans, R.K., Toews, M.D. and Sial, A.A. (2017) Diel periodicity of *Drosophila suzukii* (Diptera: Drosophilidae) under field conditions. *PLoS ONE*, 12, e0171718. https://doi.org/10.1371/journal.pone.0171718.

Fox, J. (2003) Effect displays in R for generalized linear models. *Journal of Statistical Software*, 8, 1–27.

Fox, J. and Weisberg, S. (2011) *An {R} Companion to Applied Regression*. 2nd edn. Sage, Thousand Oaks.

Gelman, A. and Su, Y.S. (2014) Arm: data analysis using regression and multilevel/hierarchical models. *R package version* 1.7-07. Available at http://CRAN.R-project.org/package=arm, Access date: May-2018.

Goodhue, E.G., Bolda, M., Farnsworth, D., Williams, J.C. and Zalom, F.G. (2011) Spotted wing drosophila infestation of California strawberries and raspberries: economic analysis of potential revenue losses and control costs. *Pest Management Science*, 67, 1396–1402.

Gruntenko, N.E., Bownes, M., Terashima, J., Sukhanova, M.Z. and Raushenbach, I.Y. (2003) Heat stress affects oogenesis differently in wild-type *Drosophila virilis* and a mutant with altered juvenile hormone and 20-hydroxyecdysone levels. *Insect Molecular Biology*, 12, 393–404.

Hanč, Z. and Nedvéd, O. (1999) Chill injury at alternating temperatures in *Orchesella cincta* (Collembola: Entomobryidae) and *Pyrrhocoris apterus* (Heteroptera: Pyrrhocoridae). *European Journal of Entomology*, 96, 165–168.

Hardeland, R. (1972) Species differences in the diurnal rhythmicity of courtship behavior within the *melanogaster* group of the genus *Drosophila*. *Animal Behaviour*, 20, 170–174.

Hauser, M. (2011) A historic account of the invasion of *Drosophila suzukii* in the continental United States, with remarks on their identification. *Pest Management Science*, 67, 1352–1357.

Hauser, M., Gaimari, S. and Damus, M. (2009) *Drosophila suzukii* new to North America. *Fly*, 43, 12–15.

Hawes, T.C., Bale, J.S., Worland, M.R. and Convey, P. (2008) Trade-offs between microhabitat selection and physiological plasticity in the Antarctic springtail, *Cryptopygus antarcticus* (Willem). *Polar Biology*, 31, 681–689.

Hothorn, T., Bretz, F. and Westfall, P. (2008) Simultaneous inference in general parametric models. *Biometrical Journal*, 50, 346–363.

Ismail, M., Vernon, P., Hance, T. and van Baaren, J. (2010) Physiological costs of cold exposure on the parasitoid *Aphidius ervi*, without selection pressure and under constant or fluctuating temperatures. *BioControl*, 55, 729–740.

Jakobs, R., Gariepy, T.D. and Sinclair, B.J. (2015) Adult plasticity of cold tolerance in a continental-temperate population of *Drosophila suzukii*. *Journal of Insect Physiology*, 79, 1–9.

Javal, M., Renault, D. and Colinet, H. (2016) Impact of fluctuating thermal regimes on *Drosophila melanogaster* survival to cold stress. *Animal Biology*, 66, 427–444.

Jones, S.R. and Kunz, S.E. (1998) Effects of cold stress on survival and reproduction of *Haematobia irritans* (Diptera: Muscidae). *Journal of Medical Entomology*, 35, 725–731.

Kalmus, H. (1943) A factorial experiment on the mineral requirements of a *Drosophila* culture. *The American Naturalist*, 77, 376–380.

Kanzawa, T. (1939) Studies on *Drosophila suzukii* Mats. *Yamanashi: Kofu, Yamanashi Applied Experimental Station*, 1–49. Abstract in *The Review of Applied Entomology*, 29:622.

Kelty, J.D., Killian, K.A. and Lee, R.E. (1996) Cold shock and rapid cold-hardening of pharate adult flesh flies (*Sarcophaga crassipalpis*): effects on behaviour and neuromuscular function following eclosion. *Physiological Entomology*, 21, 283–288.

Kim, M.J., Kim, J.S., Park, J.S., Choi, D.S., Park, J. and Kim, I. (2015) Oviposition and development potential of the spotted-wing drosophila, *Drosophila suzukii* (Diptera: Drosophilidae), on uninjured Campbell Early grape. *Entomological Research*, 45, 354–359.

Kimura, M.T. (2004) Cold and heat tolerance of Drosophilid flies with reference to their latidudinal distributions. *Oecologia*, 140, 442–449.

Klockmann, M., Kleinschmidt, F. and Fischer, K. (2017) Carried over: heat stress in the egg stage reduces subsequent performance in a butterfly. *PLoS ONE*, 12, e0180968, https://doi.org/10.1371/journal.pone.0180968.

Koštál, V., Korbelová, J., Štětina, T., Poupardin, R., Colinet, H., Zahradníčková, H. *et al*. (2016) Physiological basis for low-temperature survival and storage of quiescent larvae of the fruit fly *Drosophila melanogaster*. *Scientific Reports*, 6, 32346.

Koštál, V., Renault, D., Mehrabianova, A. and Bastl, J. (2007) Insect cold tolerance and repair of chill-injury at fluctuating thermal regimes: role of ion homeostasis. *Comparative Biochemistry and Physiology A*, 147, 231–238.

Koštál, V., Vambera, J. and Bastl, J. (2004) On the nature of pre-freeze mortality in insects: water balance, ion homeostasis and energy charge in adults of *Pyrrhocoris apterus*. *Journal of Experimental Biology*, 207, 1509–1521.

Lacoume, S., Bressac, C. and Chevrier, C. (2007) Sperm production and mating potential of males after a cold shock on pupae of the parasitoid wasp *Dinarmus basalis* (Hymenoptera: Pteromalidae). *Journal of Insect Physiology*, 53, 1008–1015.

Lalouette, L., Koštál, V., Colinet, H., Gagneul, D. and Renault, D. (2007) Cold exposure and associated metabolic changes in adult tropical beetles exposed to fluctuating thermal regimes. *FEBS Journal*, 274, 1759–1767.

Lalouette, L., Williams, C.M., Hervant, F., Sinclair, B.J. and Renault, D. (2011) Metabolic rate and oxidative stress in insects exposed to low temperature thermal fluctuations. *Comparative Biochemistry and Physiology A*, 158, 229–234.

MacMillan, H.A. and Sinclair, B.J. (2011) Mechanisms underlying insect chill-coma. *Journal of Insect Physiology*, 57, 12–20.

Mitsui, H., Takahashi, K.H. and Kimura, M.T. (2006) Spatial distributions and clutch sizes of *Drosophila* species ovipositing on cherry fruits of different stages. *Population Ecology*, 48, 233–237.

Murdoch, V.J., Cappuccino, N. and Mason, P.G. (2013) The effects of periodic warming on the survival and fecundity of *Diadromus pulchellus* during long-term storage. *Biocontrol Science and Technology*, 23, 211–219.

Mutika, G.N., Kabore, I., Parker, A.G. and Vreysen, M.J. (2014) Storage of male *Glossina palpalis gambiensis* pupae at low temperature: effect on emergence, mating and survival. *Parasites & Vectors*, 7, 465.

Nedvěd, O., Lavy, D. and Verhoef, H.A. (1998) Modelling the time–temperature relationship in cold injury and effect of high-temperature interruptions on survival in a chill-sensitive collembolan. *Functional Ecology*, 12, 816–824.

Nikolouli, K., Colinet, H., Renault, D., Enriquez, T., Mouton, L., Gibert, P. *et al*. (2018) Sterile insect technique and *Wolbachia* symbiosis as potential tools for the control of the invasive species *Drosophila suzukii*. *Journal of Pest Science*, 91, 489–503.

Partridge, L. and Farquhar, M. (1983) Lifetime mating success of male fruit flies (*Drosophila melanogaster*) is related to their size. *Animal Behaviour*, 31, 871–877.

Pio, C.J. and Baust, J.G. (1988) Effects of temperature cycling on cryoprotectant profiles in the goldenrod gall fly, *Eurosta solidaginis* (Fitch). *Journal of Insect Physiology*, 34, 767–771.

Plantamp, C., Salort, K., Gibert, P., Dumet, A., Mialdea, G., Mondy, N. and Voituron, Y. (2016) All or nothing: survival, reproduction and oxidative balance in spotted wing drosophila (*Drosophila suzukii*) in response to cold. *Journal of Insect Physiology*, 89, 28–36.

Porcelli, D., Gaston, K.J., Butlin, R.K. and Snook, R.R. (2017) Local adaptation of reproductive performance during thermal stress. *Journal of Evolutionary Biology*, 30, 422–429.

R Core Team (2016) *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical Computing, Vienna.

Raspi, A., Canale, A., Canovai, R., Conti, B., Loni, A. and Strumia, F. (2011) *Insetti delle aree protette del comune di San Giuliano Terme*. Felici editore. Pisa: San Giuliano Terme, Italy.

Renault, D., Nedvéd, O., Hervant, F. and Vernon, P. (2004) The importance of fluctuating thermal regimes for repairing chill injuries in the tropical beetle *Alphitobius diaperinus* (Coleoptera: Tenebrionidae) during exposure to low temperature. *Physiological Entomology*, 29, 139–145.

Rinehart, J.P., Yocum, G.D. and Denlinger, D.L. (2000) Thermotolerance and rapid cold hardening ameliorate the negative effects of brief exposures to high or low temperatures on fecundity in the flesh fly, *Sarcophaga crassipalpis. Physiological Entomology*, 25, 330–336.

Rinehart, J.P., Yocum, G.D., Kemp, W.P. and Bowsher, J.H. (2016) Optimizing fluctuating thermal regime storage of developing *Megachile rotundata* (Hymenoptera: Megachilidae). *Journal of Economic Entomology*, 109, 993–1000.

Rinehart, J.P., Yocum, G.D., West, M. and Kemp, W.P. (2011) A fluctuating thermal regime improves survival of cold-mediated delayed emergence in developing *Megachile rotundata* (Hymenoptera: Megachilidae). *Journal of Economic Entomology*, 104, 1162–1166.

Russell, V.L. (2016) Least-squares means: the R Package lsmeans. *Journal of Statistical Software*, 69, 1–33.

Ryan, G.D., Emiljanowicz, L., Wilkinson, F., Kornya, M. and Newman, J.A. (2016) Thermal tolerances of the spotted-wing drosophila *Drosophila suzukii*. *Journal of Economic Entomology*, 109, 746–752.

Takano, S.I. (2014) Survival of *Bactrocera latifrons* (Diptera: Tephritidae) adults under constant and fluctuating low temperatures. *Applied Entomology and Zoology*, 49, 411–419.

Tochen, S., Dalton, D.T., Wiman, N., Hamm, C., Shearer, P.W. and Walton, V.M. (2014) Temperature-related development and population parameters for *Drosophila suzukii* (Diptera: Drosophilidae) on cherry and blueberry. *Environmental Entomology*, 43, 501–510.

Torson, A.S., Yocum, G.D., Rinehart, J.P., Nash, S.A., Kvidera, K.M. and Bowsher, J.H. (2017) Physiological responses to fluctuating temperatures are characterized by distinct transcriptional profiles in a solitary bee. *Journal of Experimental Biology*, 220, 3372–3380.

Venables, W.N. and Ripley, B.D. (2002) *Modern Applied Statistics with S*. 4th edn. Springer, New York.

Walsh, D.B., Bolda, M.P., Goodhue, R.E., Dreves, A.J. and Lee, J.C. (2011). *Drosophila suzukii* (Diptera: Drosophilidae): invasive pest of ripening soft fruit expanding its geographic range and damage potential. *Journal of Integrated Pest Management*, 2, G1–G7.

Yocum, G.D., Greenlee, K.J., Rinehart, J.P., Bennett, M.M. and Kemp, W.P. (2011) Cyclic CO_2 emissions during the high temperature pulse of fluctuating thermal regime in eye-pigmented pupae of *Megachile rotundata*. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, 160, 480–485.

Yocum, G.D., Rinehart, J.P. and Kemp, W.P. (2012) Duration and frequency of a high temperature pulse affect survival of emergence-ready *Megachile rotundata* (Hymenoptera: Megachilidae) during low-temperature incubation. *Journal of Economical Entomology*, 105, 14–19.

Yocum, G.D., Žďárek, J., Joplin, K.H., Lee, R.E. Jr, Smith, D.C., Manter, K.D. *et al*. (1994) Alteration of the eclosion rhythm and eclosion behavior in the flesh fly, *Sarcophaga crassipalpis*, by low and high temperature stress. *Journal of Insect Physiology*, 40, 13–21.

Zera, A.J. and Harshman, L.G. (2001) The physiology of life history trade-offs in animals. *Annual Review of Ecology and Systematics*, 32, 95–126.

Zhang, W., Chang, X.Q., Hoffmann, A., Zhang, S. and Ma, C.S. (2015) Impact of hot events at different developmental stages of a moth: the closer to adult stage, the less reproductive output. *Scientific Reports*, 5, 10436.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Effect plots from GLM: temperature variation of FTR on adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S1D correspond to statistical differences between treatments (least-square means, $P < 0.05$)

Fig. S2 Effect plots from GLM: temperature variation of FTR on pupae. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S2D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S3 Effect plots from GLM: duration variation of FTR on adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S3D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S4 Effect plots from GLM: duration variation of FTR on pupae. The plots show the conditional coefficients ("marginal effects") of all variables included in the model

as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S4D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S5 Effect plots from GLM: frequency variation of FTR on adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S5D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S6. Effect plots from GLM: frequency variation of FTR on pupae. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), temperature, line, and their interactions. Different letters on Fig. S6D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S7 Effect plots from GLM: medium-term cold storage as adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, and their interactions.

Fig. S8 Effect plots from GLMM: poststorage survival of adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S8D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S9 Effect plots from GLMM: poststorage survival of pupae. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), sex, line, and their interactions. Different letters on Fig. S9D correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S10 Effect plots from GLMM: Female fecundity of adults. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line, and their interactions. Different letters on Fig. S10C correspond to statistical differences between treatments (least-square means, $P < 0.05$).

Fig. S11 Effect plots from GLMM: Female fecundity of pupae. The plots show the conditional coefficients ("marginal effects") of all variables included in the model as well as effects resulting from the interaction terms. The variables are time, treatment (TRT), line and their interactions. Different letters on Fig. S11C correspond to statistical differences between treatments (least-squares means, $P < 0.05$).

Fig. S12 Results from experiments 1 to 7 differentiate between lines 1 and 2. Each figure is associated with its caption.

Table S1: Detailed statistics from GLMs and GLMMs. Datasets from variation of temperature, duration, and frequency of the recovery period and long-term storage GLMs were used. Poststorage survival and female fecundity datasets were analyzed with GLMMs. $^{***}P < 0.001$; $^{**}P < 0.01$; $^{*}P < 0.05$; NS: not significant. TRT stands for thermal treatment (i.e., for experiments 1 to 4: CLT vs. FTR, and for experiments 5 and 6: CTRL vs. FTR and CLT). The symbol "/" indicate parameters not considered in the experiments and the sign "–" is used for data not included in the statistical analysis. TRT: treatment (CLT: constant low temperature; FTR: fluctuating thermal regime; CTRL: control).