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## Acclimation, duration and intensity of cold exposure determine the rate of cold stress accumulation and mortality in *Drosophila suzukii*

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### ABSTRACT

The spotted wing drosophila (SWD), *Drosophila suzukii*, is a major invasive fruit pest. There is strong consensus that low temperature is among the main drivers of SWD population distribution, and the invasion success of SWD is also linked to its thermal plasticity. Most studies on ectotherm cold tolerance focus on exposure to a single stressful temperature but here we investigated how cold stress intensity affected survival duration across a broad range of low temperatures (−7 to +3 °C). The analysis of  $Lt_{50}$  at different stressful temperatures (Thermal Death Time curve - TDT) is based on the suggestion that cold injury accumulation rate increases exponentially with the intensity of thermal stress. In accordance with the hypothesis,  $Lt_{50}$  of SWD decreased exponentially with temperature. Further, comparison of TDT curves from flies acclimated to 15, 19 and 23 °C, respectively, showed an almost full compensation with acclimation such that the temperature required to induce mortality over a fixed time decreased almost 1 °C per °C lowering of acclimation temperature. Importantly, this change in cold tolerance with acclimation was uniform across the range of moderate to intense cold stress exposures examined.

To understand if cold stress at moderate and intense exposures affects the same physiological systems we examined how physiological markers/symptoms of chill injury developed at different intensities of the cold stress. Specifically, *hsp23* expression and extracellular  $[K^+]$  were measured in flies exposed to different intensities of cold stress (−6, −2 and +2 °C) and at various time points corresponding to the same progression of injury (equivalent to 1/3, 2/3 or 3/3 of  $Lt_{50}$ ). The different cold stress intensities all triggered *hsp23* expression following 2 h of recovery, but patterns of expression differed. At the most intense cold stress (−6 and −2 °C) a gradual increase with time was found. In contrast, at +2 °C an initial increase was followed by a dissipating expression. A gradual perturbation of ion balance (hyperkalemia) was also found at all three cold stress intensities examined, with only slight dissimilarities between treatment temperatures. Despite some differences between the three cold intensities examined, the results generally support the hypothesis that intense and moderate cold stress induces the same physiological perturbation. This suggests that cold stress experienced during natural fluctuating conditions is additive and the results also illustrate that the rate of injury accumulation increases dramatically (exponentially) with decreasing temperature (increasing stress).

### 1. Introduction

The spotted wing drosophila (SWD), *Drosophila suzukii* (Matsumura) (Diptera: Drosophilidae), originates from South-East Asia and was introduced to both Europe and America in 2008 where it is now emerging as a major fruit pest (Calabria et al., 2012; Cini et al., 2012; Hauser, 2011). Various distribution models suggest that low temperature and humidity drive SWD global and local distributions, population

dynamics, as well as their seasonal phenology (de la Vega and Corley, 2019; Gutierrez et al., 2016; Langille et al., 2016; Ørsted and Ørsted, 2019; Ørsted et al., 2021; Santos et al., 2017). SWD is native to a temperate climate (Kimura, 2004, 1988) and considered to be a freeze-intolerant and chill-susceptible species (Enriquez and Colinet, 2017; Jakobs et al., 2015; Overgaard and MacMillan, 2017). Like most *Drosophila* species, the adult appears to be the overwintering life stage (Stockton et al., 2019) and seasonal dimorphism of winter and summer

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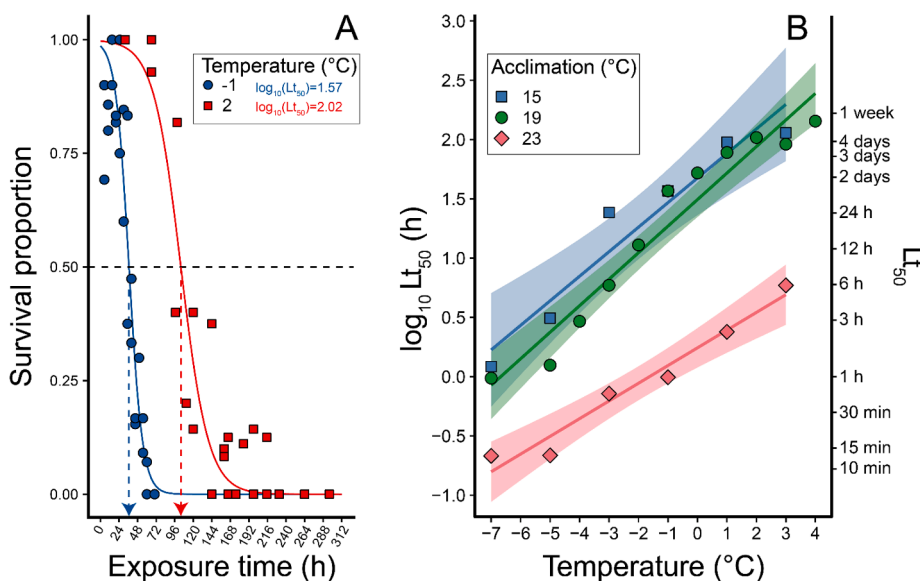
morphs has often been put forth as a strategy to survive hot summers and cold winters in SWD (Rossi-Stacconi et al., 2016; Shearer et al., 2016; Stockton et al., 2019; Zhai et al., 2016). After developmental winter acclimatization, adult flies emerge as a darker winter morph, and this seasonal transition has been reported to be accompanied by reproductive dormancy and a considerable increase in cold tolerance (Enriquez and Colinet, 2019a; Everman et al., 2018; Jakobs et al., 2015; Rossi-Stacconi et al., 2016; Shearer et al., 2016; Zhai et al., 2016). While it remains under debate whether this transition can be considered a true diapause or simply quiescence (Everman et al., 2018; Leach et al., 2019; Toxopeus et al., 2016), it is clear that knowledge of SWD cold tolerance is important. Such knowledge can for example be used to develop predictive models of the invasive capacity in un-colonized areas and for predictions of early population build-up in the growing season (Asplen et al., 2015; Hamby et al., 2016).

Methods to evaluate cold tolerance of chill susceptible insects include non-lethal assays, such as determination of chill coma temperature (often called  $CT_{min}$ ) or chill coma recovery time (CCRT), and mortality assays such as median (50%) lethal time or temperature ( $Lt_{50}$  or  $LT_{50}$ , respectively) (Andersen et al., 2014; Sinclair et al., 2015). Cold mortality is typically only assessed by exposing the animals to a single temperature for different durations, or using one exposure duration at a range of low temperatures. It is also possible to combine information of time-dependent mortality at different cold stress intensities (temperatures) using the framework of thermal death time (TDT) curves (Jørgensen et al., 2019; Rezende et al., 2014). The TDT curve approach is based on the linear relation between  $\log_{10}(Lt_{50})$  and exposure temperature (see example in Fig. 1) and for insects TDT has primarily been used to describe heat tolerance where the relation between tolerance time and temperature fits this exponential model very closely (Jørgensen et al., 2019; Rezende et al., 2014; Smith, 1957). If a similar exponential relationship can be modeled for cold tolerance it will be useful to describe cold tolerance to any temperature/time combination using the model parameters. An ecologically relevant assessment of thermal tolerance in insects should also consider the effect of prior acclimation or acclimatization as this is known to have dramatic effects cold susceptibility of insects including *D. suzukii* (Enriquez and Colinet, 2019a, 2019b; Jakobs et al., 2015; Shearer et al., 2016; Sinclair and Roberts, 2005; Sørensen et al., 2016; Toxopeus et al., 2016; Wallingford and Loeb, 2016). It is therefore important to understand how acclimation affects the relationship between duration of cold mortality and cold

stress intensity described by the TDT curve.

The physiological processes that determine chill susceptibility/tolerance in insects are not fully understood, but low temperature exposure has been suggested to involve a number of cellular processes such as membrane phase transition, ROS production, induction of heat shock proteins (Hsps) and cellular depolarization due to loss of ion balance (Hayward et al., 2014; Overgaard et al., 2021; Overgaard and MacMillan, 2017; Teets and Denlinger, 2013). Two of the most studied responses associated with the development of chill injury in insects are induction of Hsp's during and after cold stress (Colinet et al., 2010a; Hoffmann et al., 2003; Košťál and Tollarová-Borovanská, 2009; Štětina et al., 2015) and the gradual loss of ion balance, where an increase of extracellular  $[K^+]$  is tightly coupled to the development of chill injury in many insects (Bayley et al., 2018; Kostál et al., 2004; MacMillan et al., 2015a; Overgaard et al., 2021; Overgaard and MacMillan, 2017). Induction of stress proteins is a symptom of disturbed proteostasis, and together with depolarization caused by loss of ionic balance, these effects are likely to be responsible for loss of cellular homeostasis at low stressful temperatures. Yet, at present it is poorly understood whether the injuries that develop gradually at a moderately stressful temperature (i.e., chronic stress) are similar to the form of chill injury that develops quickly during intense low temperature stress (i.e., acute stress). If the same physiological perturbations occurs during acute and chronic cold stress, respectively, then it can be assumed that stress acquired at moderate and intense cold are additive. Such additivity will allow TDT parameters to be used directly to model the effects of exposure to natural fluctuations (Jørgensen et al., 2021). Conversely, if moderate and intense cold stress perturb different physiological systems, then more complicated models will be required to predict the consequences of exposure to fluctuating cold conditions.

To address these questions, the present study estimated  $Lt_{50}$  of SWD across a broad range of low temperature exposures (ranging from  $-7$  to  $+3$  °C) to test the hypothesis that an exponential relation exists between  $Lt_{50}$  and cold stress intensity (exposure temperature), i.e. forming a TDT curve. Experiments were performed on flies acclimated at three different developmental temperatures (15, 19 and 23 °C) to test the hypothesis that cold acclimation improves cold tolerance proportionally across the range of moderate to intense cold stress. Further, we examined whether similar physiological markers/symptoms of chill injury developed at different rates depending on the intensity of the thermal stress. Specifically, we sampled flies exposed to three intensities of cold stress ( $-6$ ,



**Fig. 1.** Survival curves and thermal death time curves for female *D. suzukii*. A) Example of two survival curves made for female *D. suzukii* (acclimated to 19 °C) exposed to  $-1$  °C (blue circles) or 2 °C (red squares) for different durations. Each point represents the survival proportion in a single replicate ( $N$  of female flies). From the survival curve which has been fitted to the points, the  $Lt_{50}$  can be calculated (dashed arrow protruding from the intercept between the dashed line at survival proportion 0.5 and the survival curve). Values of  $Lt_{50}$  are then  $\log_{10}$ -transformed to use in thermal death time curves (panel B). B) Thermal death time curves for female *D. suzukii* acclimated to three temperatures (15 °C: blue square, 19 °C: green circle, 23 °C: red diamond). Points represent  $\log_{10}$ -transformed  $Lt_{50}$  estimates from survival curves (panel A) made for each test temperature. Lines and shades illustrate the linear regressions and corresponding confidence intervals. The regression lines were all well-fitted ( $R^2 = 0.93, 0.94, 0.96$  for flies acclimated to 15, 19, 23 °C, respectively). Similar data for male flies are found in Fig. S2. For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

–2 and +2 °C) at time points equivalent to 1/3, 2/3 or 3/3 of the specific  $Lt_{50}$  for each of these exposure temperatures (Fig. S1) to measure markers of cold stress (*hsp23* expression and extracellular  $[K^+]$ ).

## 2. Materials and methods

### 2.1. Experimental animals

The stock population of *Drosophila suzukii* used in this study was established from field collected flies (700 pairs) caught on blueberries and raspberries in Thorigné Fouillard, France (48.1523N, –1.6216E) in September 2016. Fly stocks were maintained in 250 mL bottles containing 70 mL of Leeds medium (Recipe: 1 L water, 60 g yeast, 40 g sucrose, 30 g oatmeal, 16 g agar, 12 mL nipagin and 1.2 mL acetic acid).

Parental fly stocks were kept at  $19 \pm 1$  °C at ~70% RH using a summer light regime of 14 h light and 10 h night. Flies for experiments were produced in uncrowded conditions (200–500 flies per bottle) by allowing adults to lay eggs for 1–3 days in bottles with 70 mL fly medium (adults were removed when a moderate density of eggs was observed). Bottles with eggs were then placed at 15, 19 or 23 °C to allow for developmental acclimation in light and humidity conditions as described above. Emerging adults were used for experiments at age 5 to 8 days (for 19 and 23 °C acclimation), or at 8 to 13 days (for 15 °C acclimation). Flies were allowed to age longer at 15 °C to ensure that all experimental flies were sexually mature at the time of experiments. Although we did not quantify this, we noticed that 15 °C acclimated flies emerged darker and larger as is typical for drosophilids, but these flies were also observed to continue reproductive activity. It is therefore questionable if the 15 °C flies are representative of the “winter-morph”, also considering that light and food conditions were unaltered for the 15 °C acclimated flies.

### 2.2. Effects of exposure duration and temperature of cold tolerance in *D. suzukii*

Cold tolerance was evaluated as lethal time to 50% mortality ( $Lt_{50}$ ) at ten different temperatures (–7, –5, –4, –3, –2, –1, 0, +1, +2 and +3 °C) for the 19 °C acclimated flies and at six temperatures (–7, –5, –3, –1, +1 and +3 °C) for the 15 and 23 °C acclimated flies. To estimate  $Lt_{50}$ , batches of approximately 25 flies of both sexes were placed in glass vials with a foam plug that allowed for gas transfer. Male and female flies were tested together to avoid unnecessary stress associated with handling or anaesthesia prior to the tests. The vials were immersed in a pre-cooled glycol–water bath and vial temperature was constantly monitored in two identical vials without flies to ensure that the flies were exposed to the desired temperature. Vials were then removed after different durations of cold exposure and the flies were quickly transferred to 25 mL plastic vials with 2 mL of Leeds medium. These vials were then returned to the flies’ acclimation temperature, and after 48 h of recovery, the flies were scored for mortality (“Alive” if they were standing up or “Dead” if they were immobile or injured to a degree where they could not stand up). The dead and surviving flies from each vial were split and frozen at –18 °C such that they could later be sexed to assess sex-specific mortality. The 48 h recovery period was chosen to allow plenty of time for chill coma recovery, while also allowing time for delayed cold mortality to manifest itself irrespective of the different recovery temperatures in the different acclimation groups. The random selection of flies for each replicate used to assess  $Lt_{50}$  resulted in variation of the sex ratios within replicates. In the few cases where a replicate contained fewer than five of a sex, the data for that sex was excluded from subsequent analyses. At each test temperature the cold stress durations were selected to cover the full range of mortality from full recovery to complete mortality (See Fig. 1A as example). At each test temperature, this resulted in at least seven different durations of cold exposure with one to four replicates for each duration. Across the many different temperature and acclimation combinations, the exposure

durations lasted from 10 min to 504 h (3 weeks) (See the number of replicates in each (temp × duration) assay in Table S1). This cold tolerance analysis resulted in a range of  $Lt_{50}$  estimates across different sexes, acclimation groups and test temperatures. These  $Lt_{50}$  estimates were then subsequently used as replicates for further analysis of TDT curves (See below).

### 2.3. *hsp23* expression

An experiment was designed to investigate whether cold stress resulted in similar induction of a heat shock protein (*hsp*) gene when flies were exposed to the same relative level of injury during either acute and intense or chronic and moderate cold stress. We selected *hsp23* for this experiment as it is among the most cold-responsive *hsp* genes in *Drosophila* (Colinet and Hoffmann, 2012). It is expressed in *D. suzukii* (Toxopeus et al., 2016) and the transcript sequence is available in SpottedWingflybase (<http://spottedwingflybase.org>). We exposed SWD female flies (19 °C acclimated, 5 to 8 days old) to three test temperatures (–6, –2 and +2 °C) and for each test temperature we sampled flies when they had experienced cold stress durations corresponding to 1/3, 2/3 or 3/3 of the  $Lt_{50}$  value (Table 1; Fig. S1). The flies were allowed to recover at 19 °C for 2 h after sampling. We also sampled a set of flies that were exposed to the 3/3 of the  $Lt_{50}$  value but these were frozen immediately after the cold treatment to examine whether *hsp* induction occurred during the cold stress or only during the subsequent recovery. Finally, we collected a control sample of flies kept at 19 °C as well as a positive heat control to verify the inducibility of *hsp23* (32.7 °C for 1 h followed by a recovery at 19 °C for 1 h). For each treatment we sampled four replicates of 10 female flies which were snap frozen in liquid N<sub>2</sub> and subsequently stored at –80 °C for later measurements of *hsp23*.

To measure *hsp23* expression, RNA was extracted using the “RNeasy Mini Kit” (Qiagen, Denmark) following the protocol for “Purification of Total RNA from Animal Tissues”. The amount of RNA was measured spectrophotometrically with the Qubit 2.0 fluorometer (Invitrogen A/S, Nærum, Denmark) and cDNA was synthesized based on 1.5 µg total RNA for all samples using the “High Capacity cDNA Reverse Transcription Kit” (Applied Biosystem, 4375575 Rev. E) with an Anchored Oligo(dT) 20 primer (Invitrogen A/S, Nærum, Denmark). The expression level of *hsp23* was measured in duplicates for each of four biological replicates per treatment on a Stratagene MX3005P using the Stratagene Brilliant II SYBR Green qPCR Mastermix (AH Diagnostics, Denmark) and the default two-step PCR conditions (Sørensen et al., 2013). Primers for *hsp23* (forward: 5'-AGAAACAGGTCGGCTCTTCG-3'; reverse: 5'-GAAGTGCACACATCCATGC-3') were ordered from Sigma-Aldrich (Denmark) and based on the transcript sequence form gene ID: DS10.00003841. These primers were expected to result in a single PCR product of 92 bp ([http://spottedwingflybase.org/gene?gid=DS10\\_00003841](http://spottedwingflybase.org/gene?gid=DS10_00003841)). Quality of the qPCR result was validated by visual inspection of the PCR and melting curves. Raw measurements were converted to  $R_0$  values (linear proportional to cDNA concentration) using Data Analysis for Real-Time PCR (DART-PCR) (Peirson et al., 2003). We standardized RNA concentration used to synthesize cDNA and randomized all experimental steps rather than using a housekeeping gene for

**Table 1**

Sampling times used for *hsp23* and ion measurements. Sampling times corresponding to 1/3, 2/3 and 3/3 were calculated from our initial assessment of the relation between time and temperature for the three test temperatures (–6, –2 and +2 °C). Subsequent experiments changed our  $Lt_{50}$  estimates slightly so in parenthesis is the precise percentage of  $Lt_{50}$  as calculated from the final tolerance data).

$Lt_{50}$ relative	–6 °C	–2 °C	+2 °C
1/3	35 min (41.4%)	4 h05 (36.8%)	28 h51 (33.1%)
2/3	70 min (82.8%)	8 h10 (73.6%)	57 h42 (66.1%)
3/3 (x 2)	105 min (124.2%)	12 h15 (110.4%)	86 h33 (99.1%)

normalization. This ensured reliable raw values as no systematic variation influenced the among treatment results, but does slightly inflate our within treatment variation making our results conservative (As discussed in Heckmann et al., 2011).

#### 2.4. Extracellular potassium measurements

To examine how different intensities of cold stress were linked to increasing hemolymph potassium concentration (hyperkalemia), we used an experimental design similar to that used for *hsp23* measurements. For this experiment we exposed flies to the same temperature and duration combinations (Table 1) and additionally we exposed flies to 4/3 of  $LT_{50}$  exposure ( $-6^{\circ}C$ : 140 min,  $-2^{\circ}C$ : 16 h,  $+2^{\circ}C$ : 115 h). Finally, we measured hemolymph  $[K^{+}]$  in control flies ( $19^{\circ}C$ ) that had not been exposed to cold stress.

For each time  $\times$  temperature combination,  $[K^{+}]$  was measured in droplets from individual flies (using 20–25 five to eight days old females reared at  $19^{\circ}C$  acclimation for each treatment). Prior to the experiments the flies were loaded individually into 200  $\mu$ L pipette tips, closed off by parafilm. The pipettes were put into glass vials (5 to 6 pipettes per glass vial) that were placed in pre-cooled cold baths for the desired duration. After the cold exposure, a drop of hemolymph was retrieved from each fly following the protocol described by MacMillan and Hughson (2014) and the hemolymph drop was quickly placed under hydrated paraffin oil to avoid evaporation. Hemolymph potassium concentration ( $[K^{+}]$ ) was measured using ion-sensitive glass micro-electrodes and the procedure for these measurements as well as preparation of electrodes, standards, and electrode quality criteria followed the same protocol as described by MacMillan et al. (2015a) and MacMillan et al. (2015b).

#### 2.5. Data analysis

Data analysis was performed in R version 3.5.2 (R Core Team, 2018). Unless otherwise stated, the results are presented as mean  $\pm$  SEM using  $p = 0.05$  as the critical value for statistical significance in all analyses.

For each test temperature, the mortality assessment was modelled as a function of exposure time (Fig. 1A).  $LT_{50}$ , the exposure duration causing 50% mortality, was estimated using the *nls()*-function in R with the following equation for a sigmoidal curve:

$$Mortality(t) = \frac{1}{1 + \exp(-a*(t - b))}$$

where *Mortality*(*t*) is mortality proportion at the time *t*, *a* is the slope of the descending part of the sigmoidal curve and *b* is the estimate of  $LT_{50}$ .

Using the  $LT_{50}$  values, thermal death time (TDT) curves were created using linear regressions on  $\log_{10}$ -transformed values of  $LT_{50}$  against test temperature (Fig. 1B and Fig. S2). Arrhenius plots were also created by regressing the inverse of the test temperature (in Kelvin) against the natural logarithm (ln) to  $LT_{50}$  (Fig. S3). As the coefficients of determination of the Arrhenius fits were similar to those of the linear fits of  $\log_{10}$ -transformed  $LT_{50}$ , it was decided to use the “simpler” exponential relation rather than the Arrhenius fits for further analysis (see discussion in Jørgensen et al., 2021). The effect of test temperature, acclimation temperature and sex on  $LT_{50}$  was examined in a three-way ANCOVA including all interaction terms (3 two-ways and 1 three-way) with test temperature as a continuous variable and acclimation temperature and sex as categorical variables. Non-significant interaction terms were removed step-wisely, and pairwise differences in the remaining model were tested with a Tukey’s Honest Significant Differences *post hoc* test.

The effect of acclimation was further analysed by examining the TDT curves separately for each sex. Slopes of the TDT curves from the three acclimation temperatures were compared pairwise using the functions *emrends()* and *pairs()* in the R package “emmeans” (Lenth, 2019), which are based on *least-squares means*. When no significant difference between slopes was found we tested whether the intercepts differed at  $0^{\circ}C$  using

the function *emmeans()* in “emmeans” (Lenth, 2019). The effect of sex on slopes and intercepts of TDT curves was tested within each acclimation group using linear regressions. Slopes were deemed similar between sexes when there was no significant interaction between sex and test temperature, and a significant effect of sex indicated that the intercepts were different.

The *hsp23* expression results were transformed into fold change relative to the control group ( $19^{\circ}C$  flies before stress exposure). To retain the variance, all expression values were divided by the mean expression value from the control group. The values of *hsp23* expression relative to  $19^{\circ}C$  were  $\log_{10}$  transformed in all statistical analyses to reduce residuals and minimise heteroscedacity but are presented as non-transformed relative values. A group of flies were heat exposed to verify that the primers were useful for detecting thermal induction, and this group was compared to the control group using a Welch two-sample *t*-test. All cold-treated groups were compared to the control group using *least-squares means* with a Dunnett adjustment (*emmeans()* with the contrast method *trt.vs.ctrl*) and the exposure duration and temperature as categorical variables. The cold-treated flies that were allowed to recover at  $19^{\circ}C$  before sampling were compared to each other using a two-way ANOVA with Tukey’s HSD *post hoc* test with test temperature and duration as categorical variables. The effect of recovery on *hsp23* expression was tested using a pairwise test on *least-squares means* (*emmeans()*) between the recovered group and the group immediately frozen (with the same cold exposure duration as the recovered group, i. e. 3/3 of  $LT_{50}$ ).

Hemolymph  $[K^{+}]$  measurements from cold-treated flies were tested against the control group using *least square means* with a Dunnett adjustment (*emmeans()* with the contrast method *trt.vs.ctrl*) and the exposure duration and temperature as categorical variables. Groups of cold-treated flies were then compared using a two-way ANOVA with Tukey’s HSD *post hoc* tests to examine the effect of temperature and exposure duration.

### 3. Results

#### 3.1. Thermal death time curves

Flies were acclimated to 15, 19 or  $23^{\circ}C$  and then exposed to a range of test temperatures for different durations after which mortality was assessed to estimate  $LT_{50}$  (the temperature-specific exposure duration resulting in 50% mortality; Fig. 1A). For each sex and acclimation group, we performed a linear regression between  $\log_{10}$ -transformed  $LT_{50}$  against test temperature to form TDT curves (Fig. 1B and Fig. S2). Differences caused by acclimation temperature and/or sex were then tested by comparing slopes and intercepts of this relation. Generally, it was expected that flies would die after shorter exposures with decreasing temperature (i.e., lower values of  $LT_{50}$ ) and that cold acclimation would increase cold tolerance (i.e., higher values of  $LT_{50}$ ).

The TDT curves for the three acclimation groups of SWD were all well-fitted to an exponential function as seen by the high coefficients of determination ( $R^2$  ranging from 0.93 to 0.99) of the linear regression of  $\log_{10}(LT_{50})$  vs. test temperature (female SWD in Fig. 1B and Table 2, male SWD in Fig. S2 and Table 2). As expected, exposure to lower temperatures resulted in lower  $LT_{50}$  (i.e. 50% mortality was reached faster) and from the slope it is possible to assess the “ $Q_{-10}$ ” as a measure of how much the rate of injury accumulation increases with a  $10^{\circ}C$  reduction in temperature (Table 2). These  $Q_{-10}$  values ranged from 31 to 158 suggesting that cold survival duration is very sensitive to even small changes in temperature.

The effect of test temperature, acclimation temperature and sex on  $LT_{50}$  was examined in a three-way ANCOVA including all interaction terms. After stepwise removal of non-significant interactions this analysis found that acclimation temperature interacted with both sex and test temperature (Table S2). Accordingly, to test the effect of acclimation temperature combined with test temperature, we split the dataset

**Table 2**

Parameters of thermal death time curves. Acclimation, sex and species for each tested group. Coefficients of determination ( $R^2$ ) from the linear regressions, and the regression parameters:  $\log_{10}(Lt_{50})$  at 0 °C (also called the intercept), and the slope. The coefficient of thermal sensitivity,  $z'$  and  $Q_{-10}$  are derived from the slope. Superscript letters in  $\log_{10}(Lt_{50})$  at 0 °C mark significant differences in intercept between acclimation groups *within* a sex, while asterisks mark a significant difference *between* sexes within an acclimation group.

Acclimation	$R^2$	$\log_{10}(Lt_{50})$ at 0 °C (h)	Slope (h °C <sup>-1</sup> )	$z'$	$Q_{-10}$
<b>Female <i>D. suzukii</i></b>					
15 °C	0.93	1.675 <sup>a</sup>	0.207	4.83	118
19 °C	0.94	1.493 <sup>a</sup>	0.224	4.46	175
23 °C	0.96	0.243 <sup>b,*</sup>	0.149	6.71	31
<b>Male <i>D. suzukii</i></b>					
15 °C	0.97	1.781 <sup>a</sup>	0.220	4.55	158
19 °C	0.96	1.581 <sup>a</sup>	0.211	4.74	129
23 °C	0.99	0.729 <sup>b,*</sup>	0.164	6.1	44

according to sex so that slopes and intercepts from different acclimation groups were compared within each sex. Neither of the sexes displayed slopes that differed significantly with acclimation temperature ( $p$ -values with Tukey adjustment ranging from 0.057 to 0.904, for female 19–23 °C and male 15–19 °C, respectively). Since the slopes (thermal sensitivity) were not different, we tested if the intercept ( $Lt_{50}$  at 0 °C) were different between acclimation groups. For both sexes we found that flies acclimated to 23 °C had a  $\log_{10}(Lt_{50})$  at 0 °C that was significantly lower than that of flies acclimated to 15 and 19 °C ( $p < 0.001$  for both comparisons in the two sexes, Table 2).

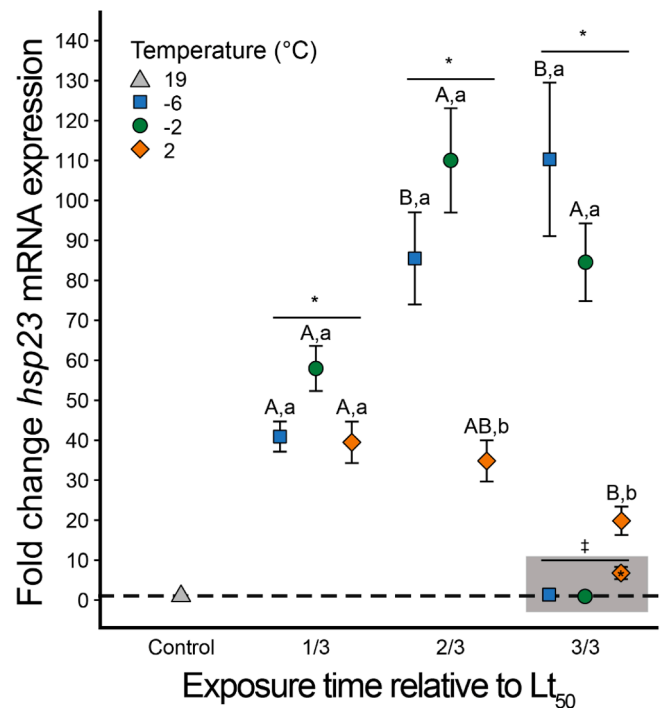
The effect of sex on cold tolerance was examined within each acclimation group. Linear regressions using test temperature and sex as independent variables against  $\log_{10}(Lt_{50})$  revealed that within an acclimation group sex did not affect the slope, and the intercept ( $\log_{10}(Lt_{50})$ ) at 0 °C was only significantly affected by sex in flies acclimated to 23 °C ( $df = 2, 9$ ,  $F = 221.9$ ,  $p < 0.001$ ), with males being slightly more cold-tolerant than females in this acclimation group (Table 2).

### 3.2. *hsp23* expression

We first verified the temperature induction of *hsp23* in samples from flies that had been heat exposed (32.7 °C for 1 h). This heat exposure resulted in a 1700-fold increase in *hsp23* expression relative to control flies (Welch two-sample  $t$ -test;  $df = 34.7$ ,  $t = -24.4$ ,  $p < 0.001$ ). We also used pairwise comparison of flies exposed to 3/3 of  $Lt_{50}$  with and without subsequent recovery to examine whether *hsp23* expression took place during cold exposure or during the subsequent two hours of recovery and found that recovery significantly increased *hsp23* expression following all cold exposures ( $p < 0.001$ ).

When *hsp23* levels of cold-treated groups were compared to that of the control group we found significantly higher *hsp23* expression levels for all three temperatures irrespective of exposure duration (Fig. 2, Table S3).

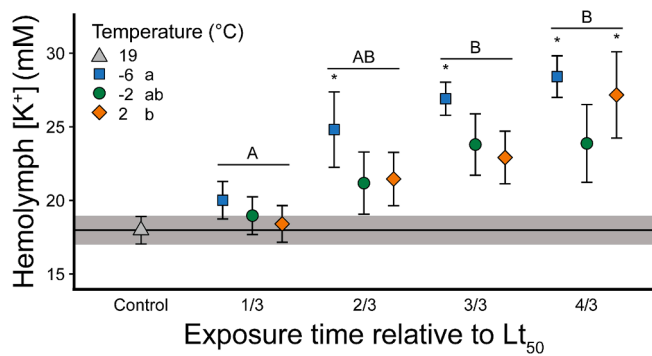
Comparison of *hsp23* expression across exposure durations within a temperature showed different temporal patterns between the temperature treatments, as attested by the interaction between temperature and exposure time (two-way ANOVA;  $df = 4$ ,  $F = 10.13$ ,  $p < 0.001$ ). Generally, the sub-zero treatments (−6 and −2 °C) were characterised by a gradual increase in *hsp23* expression with increasing duration of cold exposure, while there was a tendency for the +2 °C flies to gradually decrease *hsp23* after the initial peak at 1/3 of  $Lt_{50}$  (Fig. 2, Table S4). Within a relative exposure duration, there was no significant difference between the temperatures after a 1/3 exposure (Fig. 2, Table S4). However, after 2/3 and 3/3 of  $Lt_{50}$  the two lowest temperatures (i.e. −6 and −2 °C) had significantly higher *hsp23* expression than the flies exposed to +2 °C.



**Fig. 2.** *hsp23* mRNA expression following cold stress in *D. suzukii*. Fold change of *hsp23* mRNA expression in cold treated female *D. suzukii* relative to control flies (19 °C: grey triangle). Flies were exposed to three cold treatments (−6 °C: blue square, −2 °C: green circle, 2 °C: orange diamond) for durations that corresponded to 1/3, 2/3 or 3/3 of the estimated  $Lt_{50}$  for any given test temperature. Following cold exposure flies were allowed a brief recovery, after which they were sampled and *hsp23* mRNA expression was measured. For a set of flies that received the 3/3 of  $Lt_{50}$  cold treatment, they were sampled immediately following the cold exposure without recovery (marked with grey box). All values are shown as mean  $\pm$  SEM fold change relative to the control group (which has a mean of 1 but retains an SEM, both shown within the dashed line),  $N = 4 \times 10$  female flies (acclimated to 19 °C) for all groups, and the error bars may be hidden behind the points. An asterisk represents a significant change in a treatment relative to the control group (*least-squares means* of  $\log_{10}$ -transformed values with a Dunnett adjustment), note that in the 3/3 group that were not allowed to recover none of the temperatures caused significant changes in *hsp23* compared to the control. For groups that were allowed to recover before sampling, uppercase letters denote significant differences between exposure durations *within* a temperature, while lowercase letters mark significant differences across temperatures *within* an exposure duration (two-way ANOVA with a *Tukey post hoc* test, see Table S4). The double dagger marks a significant effect of recovery when comparing within a temperature (at 3/3  $Lt_{50}$ ) (pairwise comparison on *least-squares means* of  $\log_{10}$ -transformed values). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

### 3.3. Hemolymph hyperkalemia

Cold-treated flies were exposed to one of three low temperatures (−6, −2, +2 °C) for a duration that corresponded to 1/3, 2/3, 3/3 or 4/3 of the estimated  $Lt_{50}$  of each temperature, which allowed for comparison of 1) temperature effects on the progression hyperkalemia and 2) effect of relative cold exposure duration on the degree of hyperkalemia. When comparing hemolymph  $[K^+]$  of the cold-treated flies to the control, we found evidence for increasing  $[K^+]$  with relative exposure time but this increase was only significant for some time-points/treatment combinations (Fig. 3, Table S5). After exposure to 1/3 of the  $Lt_{50}$  none of the temperatures showed significantly increased  $[K^+]$ . Longer exposures resulted in hyperkalemia in the −6 °C flies relative to control values and in the +2 °C this was significant after exposure to 4/3 of the  $Lt_{50}$  while −2 °C never suffered a significant increase in  $[K^+]$  (Fig. 3, Table S5). Comparison of the hemolymph  $[K^+]$  measurements between groups of



**Fig. 3.** Hemolymph  $K^+$  concentration following cold stress in *D. sukukii*. Hemolymph  $K^+$  concentration in control (19 °C: grey triangle) and cold-treated female *D. sukukii*. Flies were exposed to three cold treatments (−6 °C: blue square, −2 °C: green circle, +2 °C: orange diamond) for durations that corresponded to 1/3, 2/3, 3/3 or 4/3 of the estimated  $Lt_{50}$  for the test temperature. Values are shown as mean  $\pm$  SEM, details on sample size can be found in Table S5. Asterisks denote  $K^+$  concentrations that were significantly different from control values (*least-squares means* with a Dunnett adjustment). Different uppercase letters mark significant differences between relative exposure durations, while lowercase letters in the legend denote temperature effects (two-way ANOVA with a Tukey *post hoc* test, Table S6). For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.

cold-treated flies did not reveal any interaction between exposure duration and temperature, meaning that across temperatures the exposure duration had a similar relation to the accumulation of hemolymph  $K^+$  (two-way ANOVA;  $df = 6$ ,  $F = 0.28$ ,  $p = 0.944$ ). Removing the interaction term showed that temperature ( $df = 2$ ,  $F = 3.73$ ,  $p = 0.027$ ) and exposure duration ( $df = 3$ ,  $F = 7.2$ ,  $p < 0.001$ ) affected hemolymph  $K^+$  concentration additively. The effect of temperature was greatest comparing −6 °C and +2 °C, where flies subjected to −6 °C showed a larger increase in hemolymph  $K^+$  than those at +2 °C (Tukey *post hoc*;  $p = 0.039$ ), and −2 °C had an intermediate effect (Fig. 3, Table S6). Flies subjected to 3/3 and 4/3 of  $Lt_{50}$  across temperatures had significantly higher concentrations of  $K^+$  in their hemolymph than those exposed to 1/3 of  $Lt_{50}$  (Tukey *post hoc*;  $p = 0.004$  and  $p < 0.001$ , respectively; Fig. 3, Table S6).

#### 4. Discussion

Environmental temperature has long been recognized as one of the main factors determining the geographic distribution of insect species (Addo-Bediako et al., 2000; Bennett et al., 2021; Sunday et al., 2010). This also includes *Drosophila* where heat and cold tolerance vary predictably with geographical distribution (Andersen et al., 2014; Ayrinhac et al., 2004; Gibert and Huey, 2001; Hoffmann et al., 2002; Jørgensen et al., 2019; Kellermann et al., 2012b, 2012a; Kimura, 2004). In the present study of SWD we show that the rate of injury during cold stress increases exponentially with decreasing temperature and that cold acclimation improves cold resistance equally across the range of moderate to intense cold stress exposures. Furthermore, we present some evidence that similar physiological perturbations (protein denaturation and hyperkalemia) are involved during both high and low intensity cold stress. Thus, the proportional accumulation of injury, as indicated by the proportional increase in *hsp23* and hemolymph  $[K^+]$ , were somewhat similar at the three different stress intensities examined. However, for injury related to induction of *hsp23* we did find a different pattern in flies exposed to long term moderate cold stress for several days.

##### 4.1. Thermal death time curves and cold tolerance

A primary objective of the present study was to examine how cold

survival duration of SWD is related to the intensity of the cold stress, and as predicted, we found survival duration to decrease exponentially with decreasing temperature (Fig. 1 and Fig. S2). Similar exponential relations can also be found by analysing previous data of cold survival across different low temperature exposures in *D. sukukii* (Enriquez and Colinet, 2017) or in *D. melanogaster* (Chen and Walker, 1994). Indeed, a strong linear fit between temperature and log-transformed survival duration is the pattern generally found when investigating survival across stressful high or low temperatures in ectotherms (Jørgensen et al., 2019; Kilgour and McCauley, 1986; Rezende et al., 2014).

The exponential relation between cold survival duration and temperature is easily analysed in a thermal death time (TDT) plot where  $\log_{10}$  (survival time) is plotted against temperature (Fig. 1). Using the slope and intercept from this analysis it is possible to model how exposure to a specific stressful temperature will affect survival after a given duration (Rezende et al., 2014), but it is important that such TDT model predictions are not extrapolated beyond the boundaries of the data used to generate the TDT (Jørgensen et al., 2021; Jørgensen et al., 2019). Data from the present study are, for example, useful to predict survival durations in the temperature span from +7 to −3 °C and, depending on acclimation status, this model can predict survival durations spanning from <1 h to >1 week (Fig. 1). The exponential nature of the TDT curves could potentially also be used to model the impact of fluctuating temperatures on cold survival duration. However, such models need experimental validation, particularly regarding the assumption that cold stress acquired at a relatively high and mild cold stress is additive to that acquired at a lower and more severe cold stress temperature (See also discussion below). When modelling cold exposure during natural thermal fluctuations it may also be important to consider whether fluctuations include periods at benign temperatures that allow for recovery of injury (Jørgensen et al., 2021). For example it has been shown in many insects, including *D. sukukii*, that fluctuating thermal regimes can improve cold survival substantially (Colinet et al., 2014; El-Saadi et al., 2020; Enriquez et al., 2020; Grumiaux et al., 2019; Lalouette et al., 2007; Wallingford and Loeb, 2016). Future studies should therefore consider investigating the additivity of cold stress and repair across temperatures, as necessary steps that would allow for direct modelling of temperature impacts in natural fluctuating environments of this invasive pest species.

##### 4.2. Acclimation

A second objective of this study was to examine how thermal acclimation affects cold tolerance across different intensities of cold stress in SWD. Numerous studies have shown how acclimation to low temperature increases different measures of cold tolerance in SWD and in *Drosophila* in general (Enriquez and Colinet, 2019a, 2019b; Jakobs et al., 2015; Shearer et al., 2016; Sinclair and Roberts, 2005; Sørensen et al., 2016; Toxopeus et al., 2016; Wallingford and Loeb, 2016). The use of TDT analysis enables a more direct quantification of the acclimation effects as they can be analysed directly from the horizontal displacement of TDT curves in Fig. 1B (Particularly in situations where the thermal sensitivity factor (the slope expressed by  $z$ ) is similar between acclimation groups). Here we found that acclimation effects were noticeable when flies were moved from 23 °C to 19 °C, while smaller effect was found when flies were moved from 19 °C to 15 °C. Thus, cold acclimation increased cold tolerance by almost 1 °C per °C of decreased acclimation temperature across the 8 °C temperature span examined for acclimation (e.g. the temperature that could be survived for 3 h was 1.6 °C, −4.5 °C and −5.8 °C for the 23 °C, 19 °C and 15 °C acclimated flies, respectively)(Fig. 1B and Table 2). This represents a potent acclimation response and reaffirms that for insects in general (Lee and Denlinger, 1991; Zachariassen, 1985), and for *Drosophila* specifically (Overgaard et al., 2011; Sinclair and Roberts, 2005; Sørensen et al., 2016), cold acclimation has the potential to improve cold tolerance dramatically. A comparative meta-analysis of acclimation responses in

*Drosophila* found  $CT_{min}$  to decrease approximately 0.35 °C per °C of acclimation (Sørensen et al., 2016) and the effect of cold acclimation on  $Lt_{50}$  in SWD is therefore even larger than that observed for neuromuscular limits ( $CT_{min}$ ). If the effects of cold acclimation were more profound at high or low intensity cold stress, then we should have observed a significant change in the slope of the TDT curve. Although there was a trend for steeper slopes in the TDT curve of 15 and 19 °C acclimated flies, this was not significant (Table 2) and cold acclimation therefore improves cold tolerance proportionally irrespective of the stress intensity. This observation supports (but is not proof) that similar mechanisms of cold injury are involved at the different stress intensities examined. Thus, a working hypothesis of the present study proposes that the exposure temperature determines the intensity of the cold stress, but the nature of the cold stress perturbations is similar irrespective of the intensity. According to this hypothesis, different intensities of cold stress (different temperatures) are simply resulting in injury accumulating at different rates.

#### 4.3. Physiological perturbations at high and low intensity cold stress

The linear relationship between  $\log_{10}(Lt_{50})$  and exposure temperature shows that cold injury increases exponentially with decreasing temperature across the range of stressful temperatures examined. Classically, such data are analyzed using Arrhenius plots to look for breakpoints that could indicate shifts between different physiological processes in different temperature intervals (Angilletta, 2009; Cossins and Bowler, 1987; Schmidt-Nielsen, 1997). However, when  $Lt_{50}$  data was analyzed in Arrhenius plots (Fig. S3) we could not identify obvious breakpoints to indicate that fundamentally different processes of thermal injury are in play within the range of temperatures tested. The absence of breakpoints is further supporting the suggestion that similar underlying physiological perturbations are at play at the different cold stress intensities (but it is not direct proof of this suggestion).

To examine the physiological perturbations occurring at different stress intensities further, we investigated two of the classical physiological markers of cold stress in *Drosophila*: Induction of a heat shock protein (*hsp23*) as a response to protein denaturation (Colinet et al., 2010a; Goto and Kimura, 1998; Sørensen et al., 2003; Štětina et al., 2015) and loss of ion balance (increase in extracellular  $[K^+]_e$ ) as a consequence of failing osmoregulatory capacity (MacMillan et al., 2015a; Overgaard et al., 2021). Specifically, we hypothesized that signs of physiological perturbations (*hsp* induction or hyperkalemia) would develop similarly at different cold stress intensities if we considered the proportional progression of lethal injury. To examine this we sampled flies exposed to 1/3, 2/3, 3/3 and 4/3 of the  $Lt_{50}$  at three different temperatures representing intense, moderate and slow progression of cold mortality (−6 °C, −2 °C and +2 °C, for 19 °C acclimated *D. suzukii*) (Fig. S1 and Table 1).

#### 4.4. Induction of *hsp*

Heat shock proteins (Hsps) are universally expressed in response to environmental stressors, typically signaled by increased protein denaturation (Feder and Hofmann, 1999; Sørensen et al., 2003), and several *hsp* genes are expressed during the recovery from cold stress in *Drosophila*. Genetic manipulations with RNAi or mutant insects have demonstrated that expression of *hsp* genes is important for cold tolerance and recovery from cold stress in insects (Colinet et al., 2010b; Košťál and Tollarová-Borovanská, 2009; Rinehart et al., 2007; Štětina et al., 2015). This is also true for the *hsp23* which has repeatedly been implicated in cold stress in *Drosophila*, including *D. suzukii* (Colinet et al., 2010b; Qin et al., 2005; Toxopeus et al., 2016). In the present study we found *hsp23* expression to increase (>20 fold) in response to all three cold stress intensities, but this induction was only found in flies that were allowed a recovery period after the cold stress (however, note a small elevation in expression level during cold exposure in the +2 °C

treatment). It is difficult to assign *hsp23* expression to a specific cellular process as it is likely to represent both an important repair function (subsequent to the cold stress), but also a correlate of the damage accumulated at the protein level. A functional explanation for the delayed expression is still unknown, but it may reflect a strong repression of metabolic/transcriptional activity at low temperature that prevents *hsp* genes to be expressed during the cold stress period. Irrespective of the functional role of *hsp23* expression, we find that the cold stress intensities give rise to different patterns of expression during the progression of cold stress. At the two highest intensities of cold stress (−6 and −2 °C) the pattern is a gradual increase as stress is accumulating with time. In contrast, the moderate and long-term cold stress at +2 °C is characterized by an initial increase followed by a dissipating expression. The +2 °C treatment is also the only treatment where we observe a mild increase in expression in flies that are not allowed recovery. It is therefore possible that the moderate cold stress causes a different progression of protein damage/unfolding and/or that this temperature allows for a small level of constitutive repair that suppress a gradual accumulation of damage. Future studies are needed to investigate this dynamic further, and at present we can simply conclude that all three cold intensities activate a marked expression of *hsp23* indicative of protein denaturation and unfolding, but that the pattern of this expression differs between intense brief cold stress and mild chronic cold stress.

#### 4.5. Loss of ion balance

Cold stress in chill-susceptible insects is closely correlated with a loss of hemolymph ion homeostasis which is mechanistically linked to cell depolarization, paralysis, injury and cold mortality (Andersen and Overgaard, 2019; Bayley et al., 2018; Kostál et al., 2004; Overgaard et al., 2021; Overgaard and MacMillan, 2017). This loss of ion balance is typically characterized by a marked increase of hemolymph potassium concentration ( $[K^+]_e$ ), referred to as hyperkalemia (Andersen et al., 2017). As seen in Fig. 3 all three intensities of cold stress cause a gradual increase in  $[K^+]_e$ , however this increase never reaches the level of statistical significance for the −2 °C treatment and the rate of increase is moderately faster during the progression of cold injury in the −6 °C treatment. Nevertheless, the similarities in this response are generally indicative of similar homeostatic perturbations occurring at the three different cold stress intensities.

The physiological causes and consequences of hyperkalemia in cold stressed insects are now understood at a basic level. Thus,  $[K^+]_e$  starts to increase when the active transport of osmoregulatory organs becomes insufficient to balance passive transport of ions and fluids (MacMillan et al., 2015a; Overgaard et al., 2021). This results in a reduction of hemolymph volume and a gradual increase in  $[K^+]_e$  which depolarizes excitable cells (MacMillan et al., 2015a; MacMillan and Sinclair, 2011). This chronic depolarization has been shown to activate apoptotic and necrotic signaling, presumably through voltage-dependent  $Ca^{2+}$  channels (Bayley et al., 2018), and from the data in the present study it seems that ionic imbalance occurs more gradually at mild stress such that injury also develops more slowly. Hyperkalemia could also arise as a consequence rather than a cause of cell death, as gradual development of cellular injury could cause cell rupture and therefore release of intracellular  $K^+$ . Most studies, however, find intracellular  $K^+$  concentration to be rather constant during the initial progression of chill injury (Andersen et al., 2017; Kostál et al., 2004; MacMillan and Sinclair, 2011) which would not support this suggestion. As seen in Fig. 1 acclimation changes the cold tolerance of SWD considerably and it has been shown repeatedly that cold acclimation also has profound and positive effects on the ability of insects to maintain ion balance at low temperature (Andersen et al., 2017; Kostál et al., 2004; MacMillan et al., 2015b; Overgaard et al., 2021). Together these observations are consistent with the suggestion that gradual loss of ion balance is a hallmark of chill susceptibility in insects and consistent with earlier



studies we show that this loss of homeostasis progresses at different rates depending on the intensity of the cold stress.

#### 4.6. Conclusion and perspectives

SWD is an invasive pest species of increasing concern for producers of soft-skinned fruit in both native (Asia) and invaded continents (North and South America, Europe and Africa). SWD possesses a sclerotized serrated ovipositor that enables the females to deposit eggs in healthy ripening fruits, including many wild and commercial fruits (Cini et al., 2012; Goodhue et al., 2011; Hauser, 2011). The consumption of fruit tissue by SWD larval feeding makes fruits unmarketable, causing crop losses as high as 80 % (Bolda et al., 2010). SWD has spread through human activities but the ability of SWD to establish in new environments is closely associated with environmental conditions such as humidity and low temperature (de la Vega and Corley, 2019; Gutierrez et al., 2016; Langille et al., 2016; Ørsted and Ørsted, 2019; Ørsted et al., 2021; Santos et al., 2017). How the SWD responds to cold stress is therefore of potential applied interest as species cold tolerance is likely to influence where, when and to what degree this invasive pest will pose a problem in existing and potential new environments.

In the present study, we show that TDT curves are useful to describe the relation between survival, intensity and duration of cold stress. This analysis also proved useful to analyse the importance of cold acclimation revealing a very potent acclimation response across all intensities of cold stress examined. A structured analysis such as TDT curves can potentially improve mechanistic modelling to understand whether and how environmental cold will be limiting for this pest species. Using a similar analytical approach for heat tolerance we have recently shown that analysis of TDT curves can directly estimate the impact of fluctuating stressful exposures (see discussion in Jørgensen et al., 2021) and we speculate that this will also be applicable for cold stress in SWD. We therefore conclude that a TDT analysis of cold tolerance allows for a better and more detailed assessment of the consequence of (fluctuating) low temperature exposure in this pest species and that this analytical approach has a clear potential to inform and improve mechanistic modeling of SWD distribution.

The physiological consequences of cold stress in SWD involve both protein denaturation (indicated by *Hsp23* induction) and loss of ion balance (indicated from increase in hemolymph ( $K^+$ )), and these perturbations were generally across different cold stress intensities. Further studies are still required to understand the differences in dynamics of these responses, but the present study supports the hypothesis that moderate and intense cold stress share many physiological underpinnings. If similar physiological perturbations are involved at high and low intensity cold stress then it is appropriate to assume additivity of cold injury during temperature fluctuations which gives further support for the use of TDT in modelling of thermal stress (Jørgensen et al., 2021).

#### CRedit authorship contribution statement

**Pénélope Tarapacki:** Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Lisa Bjerregaard Jørgensen:** Validation, Formal analysis, Investigation, Data curation, Writing – review & editing, Visualization. **Jesper Givskov Sørensen:** Methodology, Validation, Formal analysis, Resources, Writing – review & editing, Supervision. **Mads Kuhlmann Andersen:** Methodology, Validation, Investigation. **Hervé Colinet:** Methodology, Resources, Writing – review & editing, Supervision. **Johannes Overgaard:** Conceptualization, Methodology, Resources, Writing – original draft, Supervision, Project administration, Funding acquisition.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial

interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

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