Impact of fluctuating thermal regimes on \textit{Drosophila melanogaster} survival to cold stress

Marion Javal\textsuperscript{1}, David Renault\textsuperscript{2} and Hervé Colinet\textsuperscript{2,*}

\textsuperscript{1} URZF, INRA, 45075 Orléans, France
\textsuperscript{2} Université de Rennes 1, UMR CNRS 6553 ECOBIO, 263 Avenue du Général-Leclerc, 35042 Rennes, France

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Abstract

Temperature directly affects survival, development and reproduction in insects and thereby it is a key environmental driver for geographic distribution and population dynamics. This study aims at testing the survival of \textit{Drosophila melanogaster} under constant low temperatures (CLTs) (2, 3, 4, and 5°C) vs. fluctuating thermal regimes (FTRs). In the latter, the cold stress period was interrupted daily by 2 h pulses at 20°C. Since acclimation enhances cold tolerance, we tested whether benefits of acclimation can combine with those of FTRs. Since \textit{D. melanogaster} overwinters as non-reproductive adults, we tested if actively reproducing adults are more susceptible to cold stress than virgin females that have a much reduced reproductive activity. The results show that short interruptions of cold stress enhanced survival of adult flies. Survival was time- and temperature-dependent. Prior acclimation to low temperature allowed flies to better cope with cold stress under CLTs. On the other hand, acclimated flies did not profit from the benefits of FTRs and even showed lower survival under FTRs, probably because flies deacclimated during the periodic warm intervals. Gravid females were overall less cold tolerant than virgin females, and both survived better under FTRs. Cold survival at pupal stage was much lower than at adult stage, and no clear benefit of FTR was observed in this life stage. Our study highlights critical variables to take into account when designing experiments of prolonged exposure to low temperature in insects.

Keywords

Acclimation; cold stress; \textit{Drosophila}; fluctuating thermal regimes; recovery

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\textsuperscript{*} Corresponding author; e-mail: herve.colinet@univ-rennes1.fr

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Introduction

Temperature is a key environmental driver of insect geographic distribution and population dynamics, because it directly affects survival, developmental rate and ability to reproduce (Angilletta, 2009). Survival of insects exposed to low temperature has been extensively studied (e.g. Denlinger & Lee, 1998) and the underlying mechanisms allowing insects to increase their cold-hardiness have long been a central theme in the field of thermal biology (e.g. Sinclair et al., 2003; Clark & Worland, 2008; Lee, 2010; Storey & Storey, 2012; Teets & Denlinger, 2013). Behavioral avoidance, which includes search for shelter to minimize temperature stress can also be used by insects (Hawes et al., 2008). In addition, physiological changes such as synthesis of heat shock proteins (Boardman et al., 2013) and cryoprotective molecules (Clark & Worland, 2008) can contribute to thermal acclimation (Colinet et al., 2013; Teets & Denlinger, 2013). This plastic response relies on physiological adjustments that enhance tolerance to thermal stress following pre-exposure to sub-lethal thermal conditions (Rako & Hoffmann, 2006; Colinet & Hoffmann, 2012; Teets & Denlinger, 2013).

Historically, most thermobiological studies have focused on the impact of constant temperatures, both warm and cold, on the performances of ectotherms (Davidson, 1944; Maynard Smith, 1958; David et al., 1994; Ju et al., 2011; Wang et al., 2012; Dillon & Frazier, 2013; Mawela et al., 2013). However, variability of thermal regimes suggests that the sole consideration of constant temperatures is insufficient to fully understand ectotherms’ responses to stressful temperatures (Niehaus et al., 2012; Colinet et al., 2015a). Since the pioneer study of Casagrande & Hayes (1976), an increasing number of investigations have focused on the impact of thermal fluctuations during cold stress in insects. The temperature that just follows a cold exposure can have a profound effect on survival. This was emphasized by Turnock & Bodnaryk (1991), who observed that *Mamestra configurata* pupae held at 0°C following a cold exposure had very low survival, but when these were allowed to recover at 20°C for a relatively short duration before returning to 0°C, their survival was promoted. The “rescue” at 20°C could be repeated after a second cold shock and resulted again in higher survival. These authors suggested that insects possess the ability to switch very quickly between injury and non-injury state. Since then, studies repeatedly reported that exposing chill susceptible insects to low temperatures interrupted by periodic bouts of warmer temperatures (of varying durations), referred to as fluctuating thermal regimes (FTRs), significantly prolong cold survival (e.g. Renault et al., 2004; Colinet et al., 2006a; Colinet & Hance, 2009; Boardman et al., 2013; Rinehart et al., 2013). FTRs provide short periods of favorable temperature that allow insects to quickly recover from cold stress (Colinet et al., 2015a). Therefore, FTRs have direct applications in cold storage of useful species, such as predators or parasitoids used in integrated pest management programs (Colinet & Boivin, 2011) or in cold preservation of pollinators (Rinehart et al., 2011, 2013). In consequence, there is a real interest in understanding how FTRs
affect cold survival of insects, from both an applied and a fundamental point of view.

The fruit fly (*Drosophila melanogaster*) is used worldwide and reared in laboratories and stock centers. Maintaining *Drosophila* stocks of genetic lines and mutant strains for biomedical research costs millions of dollars every year. So far, cryopreservation has not proven to be practical in fruit flies, therefore prolonging generation time and diminishing the load of stock maintenance is highly desirable (Mockett & Matsumoto, 2014). Hence, any advance in cold storage protocols, even on the short term, may facilitate the rearing practices. Recently, the use of FTRs applied during larval development has proved to be a valuable method for prolonging larval cold survival (Koštál et al., 2016). In addition, FTRs is also beneficial to adults (Colinet et al., 2016). In the present work, we focused on adult and pupal stages of *D. melanogaster* exposed to constant low temperatures (CLTs) and FTRs. Since acclimation enhances cold tolerance, we assumed that benefits of acclimation may combine with those of FTRs in adults which may further increase cold tolerance. Since *D. melanogaster* overwinters as non-reproductive adults (Williams & Sokolowski, 1993), we also speculated that actively reproducing adults would be more susceptible to cold stress than virgin females that have a reduced reproductive activity. Thus, we tested cold survival abilities of gravid vs. virgin females under both FTR and CLT conditions. Finally, we tested whether the application of FTRs is beneficial to pupae as observed in larvae and adults.

**Material and methods**

**Biological material and fly culture**

The fly rearing was established in 2010 from a large collection of flies obtained from three different locations in Brittany (Rennes, France). These three wild lines were then mixed before they were continuously reared in laboratory (about three generations every 2 months). The continuous rearing in laboratory consisted of 8 to 12 bottles representing more than 1000 individuals. Flies were kept in a breeding room in 200 ml bottles at 25 ± 1°C (12L:12D) on standard fly medium consisting of brewer yeast (80 g/l), sucrose (50 g/l), agar (15 g/l), and Nipagin® (8 ml/l). To generate flies for the experiments, groups of 15 mated females were allowed to lay eggs in 200 ml rearing bottles during a restricted period of 6 h under laboratory conditions. This controlled procedure allowed larvae to develop under uncrowded conditions. At emergence, adults were sexed visually (with an aspirator) without CO₂ to avoid stress due to anesthesia. Emergences were synchronized, and all individuals used in experiments were between 5 and 7-day-old to avoid effects of maturation at young age on stress tolerance (Colinet et al., 2015b). Preliminary experiments revealed that females showed a marked response to FTRs, therefore, we decided to use only females in the experiment with adults.
Experiment 1: acclimation
The aim of this experiment was to test whether FTRs and cold acclimation may act synergistically on cold survival. Two phenotypic groups of flies were created to compare survival of females exposed to CLTs or FTRs: 1) acclimated individuals: 5- to 7-d-old females that were exposed at 15°C for five days, and 2) control adults: 5- to 7-d-old females maintained at 25°C for 5 consecutive days. These flies were then exposed either to CLTs or FTRs. For CLTs, four different temperatures were used: 2, 3, 4, and 5°C (preliminary experiments revealed that flies eventually fall into chill-coma at all of these temperatures). FTRs consisted of exposure to the same cold temperatures (i.e. 2, 3, 4, and 5°C) but interrupted daily by short recovery periods of 2 h at 20°C. These short warm intervals are known to promote cold survival in several other insect species (Colinet et al., 2015a). During thermal fluctuations, the temperature did not change abruptly inside the incubators: it increased to 20°C and then decreased back to low temperatures at a rate of about 1.2°C/min. Therefore, in reality flies remained a little less than 2 h at 20°C. For each experimental condition, flies were randomly taken from the rearing stock and placed into independent vials by groups of 15 females. Pure agar diet was used in order to avoid any confounding effect due to re-nutrition of flies during warming intervals, which would have been possible under FTR only. For each experimental condition, ten vials of 15 females (N = 150) were placed inside programmed incubators (Model SANYO MIR-153). Temperature was checked using automatic recorders (Hobo® data logger, model U12-012, Onset Computer Corporation, accuracy ±0.35°C). The experiment lasted 10 days. Each day, a vial from each experimental condition was removed from cold incubator and transferred to the laboratory conditions (20°C) to score the survival after 4 h (T4) and 24 h (T24) of recovery. Survival was scored as the number of flies that could stand on their legs.

Experiment 2: gravidity
For this experiment, we created two groups of flies to compare: 1) virgin individuals: females that were separated from the males as soon as possible at emergence (maximum 8 h after emergence), before they acquire sexual maturity, and 2) mated females that stayed with males for four days after emergence to allow mating. To separate females from males, individuals were sexed visually with an aspirator. After sexing, virgin females laid very few non-fecundated eggs over the next few days, while mated females laid many eggs, thus confirming the virgin vs. mated status. As described before, 5- to 7-d-old mature females were placed into agar vials (15 females/vial), and for each experimental condition, ten vials of 15 females (N = 150) were placed inside programmed incubators (Model SANYO MIR-153) set at the requested temperature and thermal regime (CLT or FTR). Here, we selected only one cold temperature (5°C) because the previous experiment revealed a marked difference between CLTs and FTRs at this thermal condition. For FTRs, cold exposure was interrupted daily by short recovery periods (2 h at 20°C) as described before. The experiment lasted 10 days: each day, a vial was removed from each experimental condition (incubator) to score survival after 4 h (T4) and 24 h (T24).
Experiment 3: developmental stages

For this experiment, cold survival of flies that had pupated since maximum 24 h was assessed (i.e. corresponding to 6 days after egg laying at 25°C). Pupae randomly taken from the rearing stock were placed into independent vials by groups of 20 pupae. Preliminary experiment revealed that mortality increased rapidly with pupae (about 100% mortality after 5 to 6 days); therefore, here the experiment lasted only 6 days. To get a complete coverage of survival (spanning from low to high mortality) over this short experimental period, we doubled the number of sampling times. Each day, we monitored cold survival of different and independent samples in the morning (at 10 am) and in the evening (at 7 pm). Cold exposure durations were computed in hours. Vials with pupae contained neither food nor agar in order to avoid fungal infection that can develop on pupae due to agar humidity. The same temperatures and thermal regimes as in the experiment 1 were applied (2, 3, 4 and 5°C, under CLTs vs. FTRs). For each experimental condition, 12 vials of 20 pupae (N = 240) were placed inside programmed incubators (Model SANYO MIR-153); at each sampling time a vial was removed and replaced at 25°C. Emergence rate of pupae was then measured by considering the proportion of adults able to fully extract from their puparium.

Statistical analysis

For experiment 1, data were expressed as proportions of flies able to survive (based on different and independent trials of 15 flies) over a period of 10 days with daily samplings. We modeled the survival data in R (version 3.0.3) by specifying a generalized linear model (GLM) with logistic link function for binary outcome (i.e. dead/alive). For each specific experimental condition, the model was based on 10 different proportions (one proportion for each sampling day) spanning from low to high mortality. The response variable was dependent on stress duration (0 to 10 days), temperature (2, 3, 4, and 5°C), thermal treatment (CLTs vs. FTRs), acclimation (acclimated vs. control) and all the interactions. We used a full factorial model and ran the Anova function in the “car” package to analyze the effect of each variable in the GLM model through the table of deviance. To help interpreting all the interaction terms, we used effect plots function in the package “effects”. These effect plots show the conditional coefficients (“marginal effects”) for all variables and interaction terms. All the effect plots are available in the online supplementary materials for each experiment separately. For experiment 2, the same GLM procedure was applied. Here, the variables were stress duration (0 to 10 days), thermal treatment (CLTs vs. FTRs), gravidity (virgin vs. gravid) and all the interactions. In the experiment 3, the results were expressed a proportion of flies able to extract from their puparium and the variables were stress duration (1 to 5 days), temperature (2, 3, 4, and 5°C), thermal treatment (CLTs vs. FTRs) and all the interactions. Probability and estimated S.E. for survival were obtained from fitted GLMs and were used to draw survival lines that are presented in figures. Lethal time 50% (LT50)
corresponding to the estimated duration of cold exposure to kill 50% of the tested population were also computed from logistic regression model.

Results

Experiment 1: acclimation

Survival probabilities of acclimated vs. control females exposed to CLTs vs. FTRs are illustrated in the multiple panels of fig. 1. Survival data were analyzed and are presented separately for T4 (fig. 1A-C) and T24 (fig. 1D-F). The models showed that all the main effects were significant at T4 and T24 (table 1). A general decrease in the probability of survival was observed with increasing duration of cold stress and with lowering temperature (table 1; see figs. S1A and S1B in the online supplementary materials). Overall, the acclimation and FTR treatments promoted cold survival (table 1, figs. S1A and S1B). The significant interaction between the

![Graphs showing survival probabilities and LT50 values](image)

Figure 1. Probability of survival (±S.E.) of *D. melanogaster* females as a function of cold exposure duration at 2, 3, 4, and 5°C (symbols ▲, ●, △, and ◆, respectively) under either constant low temperatures (CLTs, straight line, black code) or fluctuating thermal regimes (FTRs, dotted line, grey code), in acclimated (A, D) and non-acclimated (B, E) flies. Under FTRs, the cold exposure was interrupted daily by a 2 h break at 20°C. Survival was scored after 4 h (T4: A-C) or 24 h recovery post stress (T24: D-F). Probability lines and estimated S.E. were obtained from fitted generalized linear models (GLMs) with binomial logit link function. LT50 values (estimated from GLMs) are presented together with their 95% confidence intervals (CI) for acclimated (left) and non-acclimated (right) flies (C, F). LT50 values can be considered as significantly different if their CI do not overlap.
Table 1.
Table of deviance showing the effect of acclimation (females exposed at 15 vs. 25°C for 5 days), temperature (2, 3, 4 and 5°C), treatment (CLTs vs. FTRs), duration of exposure (from 0 to 10 days), and all the interactions, on the survival of *D. melanogaster* females. For each experimental condition, survival was scored after 4 and 24 h of recovery (T4 and T24, respectively). Significant values (*P* < 0.001) are highlighted in bold.

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<td>1</td>
</tr>
<tr>
<td>Treatment × temperature</td>
<td>2.58</td>
<td>1</td>
</tr>
<tr>
<td>Duration × acclimation</td>
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<td>1</td>
</tr>
<tr>
<td>Treatment × acclimation</td>
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<td>1</td>
</tr>
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<tr>
<td>Duration × treatment × temperature × acclimation</td>
<td>20.95</td>
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duration and treatment (observed at T4 and T24) indicates that the temporal decrease in survival was dependent on whether flies were exposed to CLTs or FTRs; this was clearly mitigated when FTRs were applied (table 1, figs. S1A and S1B). In addition, the interaction duration × treatment × acclimation at T24 suggests that this temporal process also varied with the acclimation status (table 1, figs. S1B). In acclimated flies, a difference between CLTs and FTRs appeared from the first days of exposure (fig. 1D), while in non-acclimated flies, it required several days before observing the effect of FTRs (fig. 1E). FTRs promoted cold survival regardless of the temperature (insignificant treatment × temperature interaction, table 1); however, the significant interaction term treatment × temperature × acclimation suggests that this pattern also depended on whether the flies were acclimated or not. Indeed, at T4, survival of acclimated flies (fig. 1A) was little affected by temperature and FTRs, while survival of non-acclimated flies (fig. 1D) was superior at the greatest temperatures and under FTRs (fig. S1A). The effect of FTRs was manifested only in non-acclimated flies and in this group, survival under FTRs was clearly further improved at the highest tested temperatures (see fig. S1A). At T4 and T24, thermal treatment significantly interacted with acclimation (table 1) but in opposite directions, suggesting an antagonistic relation (figs. S1A and S1B). Indeed, the effect of FTRs was beneficial to non-acclimated flies (fig. 1B, E), while
it appeared as neutral or even slightly detrimental to acclimated flies (fig. 1A,D) (see also figs. S1A and S1B). At T4 and T24, the temperature also interacted with acclimation (table 1). Non-acclimated flies (fig. 1B, E) generally survived better as temperature increased, while acclimated flies (fig. 1A,D) were poorly responsive to temperature change (figs. S1A and S1B). LT50 values for each temperature (2, 3, 4 and 5°C) under CLTs and FTRs are provided in fig. 1C (for T4) and fig. 1F (for T24). In most of the cases, a beneficial effect of fluctuations was observable, with females subjected to FTRs exhibiting higher LT50 than those exposed to CLTs, except in acclimated flies where the trend was opposite.

Experiment 2: gravidity

Survival probabilities of gravid vs. control females exposed to CLTs or FTRs are illustrated in the multiple panels of fig. 2. Survival varied significantly with thermal treatment (CLTs vs. FTRs), gravidity (virgin vs. fertile flies) and duration of the experiment (table 2), both at T4 (fig. 2A) and T24 (fig. 2C). Again, applying FTRs to females generally promoted cold survival (see figs. S2A and S2B in the online supplementary materials). Virgin females had higher survival probabilities than mated ones (figs. S2A and S2B). Survival probability diminished as duration of exposure increased (figs. S2A and S2B). The effect of gravidity was non-significant when crossed with the thermal treatment, meaning that virgin and mated females were positively and equally affected by FTRs (table 2; figs. S2A and S2B). The interaction between treatment and duration (table 2) indicates that the temporal decrease in survival was mitigated when females were exposed to FTRs (figs. S2A and S2B). Finally, the effect of gravidity crossing with the duration of the experiment was not significant (table 2), showing that survival decreased with time regardless of the mating status (figs. S2A and S2B). The LT50 values at T4 (fig. 2B) and T24 (fig. 2C) illustrate that survival probabilities of virgin flies were higher than those of gravid females, and that the LT50 values were further improved when flies received FTRs (especially at T4).

Experiment 3: developmental stages

The results are expressed as a percentage of emergence, considered here as a proxy of survival. Flies were considered as alive when the adult had totally emerged from the puparium. Adults that could not fully extract from the puparium were considered as dead. The probabilities of survival were similar regardless of the experimental temperature (fig. 3A, table 3). The statistical analysis revealed an effect of treatment (CLTs vs. FTRs) and duration on the proportion of emergence (table 3). In pupae, the overall effect of FTRs appeared weakly detrimental (slightly lower emergence; see figs. S3 in the online supplementary materials). The survival decreased with duration of exposure regardless of treatment (insignificant duration × treatment interaction) (table 3). However, temporal decrease of survival was dependent on the temperature (significant duration × treatment interaction) and was
Figure 2. Probability of survival (±S.E.) of *D. melanogaster* as function of cold exposure duration at 5°C under constant low temperatures (CLTs, straight line, black code) or fluctuating thermal regimes (FTRs, dotted line, grey code), in virgin (symbol ▲) and gravid (symbol ▼) females. Under the FTRs, the cold exposure was interrupted daily by a 2 h pulse at 20°C. Survival was scored after 4 h (T4: A, B) or 24 h of recovery post stress (T24: C, D). Probability lines and estimated S.E. were obtained from fitted generalized linear models (GLMs) with binomial logit link function. LT50 values (estimated from GLMs) are presented together with their 95% confidence intervals (B, D). LT50 values can be considered as significantly different if their CI do not overlap.

Table 2.
Table of deviance showing the effect of gravidity (virgin vs. mated females), treatment (CLTs vs. FTRs), duration of exposure (from 0 to 10 days), and all interactions, on the survival of *D. melanogaster* females. For each experimental condition, survival was scored after 4 and 24 h or recovery (T4 and T24, respectively). Significant values (*P* < 0.001) are highlighted in bold.

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Figure 3. Probability of survival (± S.E.) of *D. melanogaster* pupae as function of cold exposure duration at 2, 3, 4, and 5°C (symbols ■, ●, ▲, and ◇, respectively) under constant low temperatures (CLTs, straight line, black code) or fluctuating thermal regimes (FTRs, dotted line, grey code) (A). Under FTRs, the cold exposure was interrupted daily by a 2 h break at 20°C. Probability lines and estimated S.E. were obtained from fitted generalized linear models (GLMs) with binomial logit link function. LT50 values (estimated from GLMs) are presented together with 95% confidence intervals for the pupae (B). LT50 values can be considered as significantly different if their CI do not overlap.

mitigated when temperature was higher (table 3 and figs. S3). LT50 values suggest that survival was overall lower under FTRs (fig. 3B). It is worth noting that LT50 values of pupae were much lower than those observed for adults exposed to the same experimental conditions.

**Discussion**

As previously reported, insect cold tolerance is a highly plastic trait that can vary with a range of factors (Colinet & Boivin, 2011; Overgaard et al., 2011; Foray et al., 2013). In this study, we specifically tested the effects of extrinsic (temperature, duration of exposure, acclimation, thermal fluctuations) and intrinsic (gravidity, stage of development) factors on the fruit fly’s ability to tolerate a chronic cold stress (without freezing).

**Table 3.**

Table of deviance showing the effect of temperature (2, 3, 4 and 5°C), treatment (CLTs vs. FTRs), duration of exposure (from 0 to 10 days), and all interactions, on the survival of *D. melanogaster* pupae. Survival of pupa was estimated as the proportion of adults that were able to fully extract from the puparium. Significant values (*P* < 0.001) are highlighted in bold.

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Cold tolerance under FTRs vs. CLTs

As observed in other insect species (e.g. Yocum et al., 1994; Renault et al., 2004; Colinet et al., 2006a; Fischer et al., 2011), fluctuations extended the duration of cold survival in *Drosophila*. Fluctuations likely allowed flies to recover from chill coma, and females seem to have used the 2 h-pulses at an optimum temperature for repairing cold-related injuries. Low temperatures can cause a range of physiological damages, such as accumulation of metabolic waste products (lactic acid, nitrogen-containing molecules), cellular damages, inactivation of enzymes or loss of ion and water homeostasis (reviewed by Teets & Denlinger, 2013). When temperature fluctuates and becomes more favorable, the metabolism is galvanized, as suggested by metabolic rates (Lalouette et al., 2011), and damages can be repaired during warm episodes. This has been observed in many other insect species (reviewed by Colinet et al., 2015a), and only recently observed in *Drosophila* larvae and adults (Colinet et al., 2016; Koštál et al., 2016).

Acclimation promotes cold performance and interacts with FTRs and temperature

Most insect species have the ability to modify their thermotolerance to cope with environmental variations. Pre-exposure to sub-lethal temperatures triggers biochemical and physiological adjustments that usually promote subsequent thermal tolerance, a phenomenon referred to as thermal acclimation (Angilletta, 2009; Teets & Denlinger, 2013). Like many species, *D. melanogaster* increases thermotolerance in response to acclimation, and this plastic response has been described for both heat and cold (Gibert & Huey, 2001; Rako & Hoffmann, 2006; Colinet & Hoffmann, 2012; Colinet et al., 2013). Several forms of acclimation exist (rapid, gradual, or developmental), and differ according to the timing and length of the pre-exposure (Colinet & Hoffmann, 2012; Teets & Denlinger, 2013). In this study, the flies were gradually acclimated before they were exposed to low temperature under FTRs vs. CLTs. The present results show that the cold acclimation increased the probability of survival to cold stress overall.

Interestingly, non-acclimated flies clearly profited from FTRs, whereas FTRs were neutral or even noxious to acclimated flies. This could be due to a deacclimation phenomenon, defined by Angilletta (2009) as a return to a phenotype anterior to acclimation. Acclimated flies usually recover from cold stress much faster than non-acclimated counterparts (Colinet et al., 2013). Therefore, acclimated flies may also reset normal physiology and metabolism much faster during warm intervals, while control flies might still be in a ‘cold-state mode’. Acclimated flies may lose the benefits of acclimation when their metabolism is reactivated at warm temperature, and their physiological state may no longer fit with conditions when temperature drops again. If a deacclimation happened, it should have occurred from the first cycles at warm temperature. Indeed, in acclimated flies, we observed that FTRs became detrimental very early (from the first cycles of FTRs), while in non-acclimated flies,
it required several days before observing the benefits of FTRs (daily amount of accumulated injuries being progressively reduced). It has been suggested that benefits of acclimation depend on what thermal condition is to come (Salt, 1961). When temperature fluctuates, organisms acclimated to cold or hot conditions can potentially suffer a decrease in fitness as temperature moves to the opposite extreme. Costs and benefits associated with cold acclimation have been nicely demonstrated in *D. melanogaster* (Kristensen et al., 2008). We can conclude that benefits of cold acclimation do not act synergistically with those of FTRs. Thermal fluctuations that encompass high temperature pulses seem not appropriate (even detrimental) to phenotypes that are “programmed” to stay at cold.

Acclimation also interacted with temperature. Overall, non-acclimated flies survived better as temperature increased, while acclimated flies were poorly responsive to temperature changes. Acclimation is beneficial and probably offsets the detrimental effects to some extent, while in the absence of acclimation, the detrimental effects are more dependent on the intensity of stress. The benefits of FTRs manifested only in non-acclimated flies and in this group the effect was greatest at the higher temperatures. We can assume that at the lowest temperatures (e.g. 2°C), the physiological perturbations are more severe, and therefore it may not be possible to recover or this recovery may require longer than 2 h.

**Impact of cold dose and recovery period post-stress**

Rezende et al. (2014) indicated that the cold dose (i.e., combining the intensity and duration of stress), determines the level of thermal tolerance of insects. In our work, the significant effects of both the duration of exposure and the temperature are consistent with this notion. Our results corroborate an accumulation of cold-related damages: the lower the temperature and the longer the stress, the lower the likelihood of survival (Koštál et al., 2006). Post-stress recovery time also appeared to play an important role. As a general observation, LT50s were lower when flies were scored for survival after 24 h (T24) than after 4 h (T4). These two durations of recovery do not necessarily have the same ecological sense: at T4, LT50 reflects the proportion of flies unable to get out of coma; at T24, LT50 indicates the proportion of dead flies, which is directly related to the expression of latent cold damages (Turnock & Bodnaryk, 1991).

**Cold tolerance depends on egg-production activity**

The comparison of cold survival of gravid vs. virgin females was based on the general assumption that reduced reproductive activity (i.e., egg production) is linked to winter (cold) survival in fruit flies (Mitrovski & Hoffmann, 2001). Indeed, *Drosophila* flies overwinter as non-reproductive adults (Williams & Sokolowski, 1993). Therefore, we speculated that actively reproducing females would be more susceptible to cold stress than their virgin counterparts, whose egg laying activity is much reduced (Hanson & Ferris, 1929). Our results show that virgin females
were more resistant to cold than gravid ones. It is possible that higher egg production of mated females consumed energy stores required for tolerance to cold stress. Metabolic reserves of insects are consumed during prolonged cold stress (Renault et al., 2002; Colinet et al., 2006b), and management of energy reserves is a determinant of cold tolerance in *D. melanogaster* (Chen & Walker, 1994; Klepsatel et al., 2016). Therefore, energy savings resulting from lower egg production of virgin females might favor cold tolerance. It is also conceivable that high activity of ovaries makes these reproductive organs (and therefore the whole organism) more susceptible to cold injuries. Finally, it is well known that mating elicits major physiological changes in *Drosophila* females. For instance, it alters expression of many genes among which immune-related genes are overrepresented (Lawniczak & Begun, 2004). This may contribute to indirect modification of cold tolerance as low temperature and immune responses are linked (cross-talk; Sinclair et al., 2013). Mating also triggers major hormonal changes in females (Moshitzky et al., 1996), and in turn, this may modify basal cold tolerance. Hormones have indirect effects on stress tolerance (Peric-Mataruga et al., 2006). Some processes related to reproduction (e.g. vitellogenesis) are controlled by hormones and the same hormones also control elements of cold hardiness (Danks, 1996). Whatever the cause, virgin females are undoubtedly in a different physiological state than mated/gravid counterparts, and this appears to bolster (directly or indirectly) tolerance to low temperature.

**Cold tolerance varies with stage**

Temperature sensitivity can vary according to the developmental stage (Jensen et al., 2007; Bowler & Terblanche, 2008). Species often have complex life cycles and the different stages may face potentially very different environmental conditions (Kingsolver et al., 2011). Marais & Chown (2008) assumed that *D. melanogaster* adults could benefit from mobility to avoid thermal stress, while pupae and larvae were forced to endure the temperature changes of their microenvironment. These authors hypothesized a wider range of thermal tolerance for pupae than for adults, for which behavior and mobility would reduce the opportunity for adaptation. In other words, behavioral thermoregulation would be unfavorable to the emergence of physiological adaptations in response to thermal stress (Bogert, 1949). Adults would therefore be more sensitive to cold than pupae. However, in *D. melanogaster*, the overwintering stage is the adult stage (Hoffmann et al., 2003), and it seems unlikely that mobility of adults allows them to behaviorally avoid the cold during winter. In addition, the thermal environment of pupae is largely determined by the site of oviposition, most often in rotten fruit (Feder et al., 1997). Jensen et al. (2007) found large variations in cold tolerance among developmental stages of *D. melanogaster* and reported that the most tolerant stage was eggs, followed by adults, then pupae and larvae. Here, we found that cold survival of pupae was lower than that of adults which is in agreement with these observations. In addition, Mockett & Matsumoto (2014) showed that adults and pupae survived similarly in the cold,
and that the larval and prepupal stages were much less resistant. In our experiment, the individuals were tested 6 days after egg laying at 25°C, which corresponds to a prepupal stage according to the definition of Mockett & Matsumoto (2014). The greater sensitivity of this specific stage to thermal stress may be explained by high developmental activity at metamorphosis (Bainbridge & Bowness, 1981; Bowler & Terblanche, 2008). It seems that within pupal stage, the more the metamorphosis advances, the less the insect is sensitive to cold (Jensen et al., 2007).

Our results showed consistent temporal effects for adults and pupae (i.e., survival decrease with duration); however, the effect of temperature was more complex, being apparent only in adults. In addition, non-acclimated adults clearly profited from FTRs, whereas pupae did not (or were even negatively affected by FTRs). These inconsistent patterns may result from thermal effects acting on performance curves of different shapes in adults and pupae. Indeed, performance curves are not fixed, and can be modified depending on physiological parameters and individual stages (Kingsolver et al., 2011). Gravid females for example, have their amounts of resources likely reduced through investment into reproduction. It is the same for individuals at prepupal stage, for which the metamorphosis requires a considerable amount of resources (Merkey et al., 2011). At the pupal stage, relatively lower LT50 values and the low impact of FTRs suggests that temperatures used in our experiments were below the threshold of reversibility of cold-related damages.

Conclusion

As observed in previous studies (e.g., Overgaard et al., 2011; Colinet et al., 2013; Foray et al., 2013; Kobey & Montooth, 2013), the present work shows that cold tolerance is a highly plastic trait affected by many factors, such as thermal treatment, acclimation, gravidity and life stage. This study highlights the importance of taking these factors into account when designing experiments for short-term cold preservation of chill susceptible insects. Maintaining collections of Drosophila lines could be greatly facilitated by keeping the flies under low temperatures without significant impact on survival (Mockett & Matsumoto, 2014). Our data show that application of FTRs can significantly prolong adult cold survival, especially at 4 and 5°C where LT50 nearly doubled. Our results could be complemented by the study of other parameters, such as humidity, photoperiod, food, age, or microbiota.

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