Published in final edited form as:

Can Entomol. 2008 ; 140(3): 306–311.

Slow and stepped re-warming after acute low temperature exposure do not improve survival of *Drosophila melanogaster* larvae

Brent J. Sinclair* and Arun Rajamohan

Department of Biology, The University of Western Ontario, London, ON N6A 5B7, Canada

Abstract

We tested that hypothesis that slow re-warming rates would improve the ability of *Drosophila melanogaster* Meigen larvae to survive acute low temperature exposure. Four larval stages (1st, 2nd, 3rd instars and wandering stage 3rd instars) of four wild-type strains were exposed to -7°C for periods of time expected to result in 90 % mortality. Larvae were then either directly transferred to their rearing temperature (21°C), or returned to this temperature in a stepwise fashion (pausing at 0 and 15°C) or by slow warming at 1 or $0.1^{\circ}\text{C}/\text{min}$. We observed a reduced rapid cold-hardening effect and no general increase in survival of acute chilling in larvae re-warmed in a stepwise or slow fashion, and hypothesise that slow re-warming may result in accumulation of further chill injuries.

Introduction

The ability of insects to tolerate low temperatures can depend not only on the temperature to which they are cooled, but also the conditions under which that cooling occurs. For example, Miller (1978) showed that survival in adults of *Upis ceramoides* Linnaeus (Coleoptera: Tenebrionidae) exposed to -50°C was best at a cooling rate of $0.28^{\circ}\text{C}/\text{min}$, and that increasing or decreasing that cooling rate reduced survival. Re-warming rates did not affect survival in this case (Miller 1978), but are a critical component of survival in some cryopreservation applications (Rall and Polge 1984; Mazur *et al.* 1993). Surprisingly, the effects of re-warming conditions after cold exposure have not been widely-investigated in insects.

Drosophila melanogaster Meigen (Diptera: Drosophilidae) is increasingly being used as a model insect for studies of thermal biology, and it is a useful model for cold tolerance in species that are killed by cold at temperatures above those at which ice formation occurs (non-freezing cold injury) (Hoffmann *et al.* 2003). *Drosophila melanogaster* larvae and adults die at temperatures above -10°C (depending on time exposed), but freeze at much lower temperatures. In addition, their cold tolerance may change in a matter of hours through the rapid cold-hardening (RCH) response (Czajka and Lee 1990). Mortality from acute chilling is thought to be associated with phase transitions in cell membranes and apoptosis, and the mechanisms of RCH seem to include membrane remodelling, blocking of apoptosis and glucose accumulation (Overgaard *et al.* 2005; Yi *et al.* 2007; Overgaard *et al.* in press). However, cold exposure studies of *D. melanogaster* usually pay close attention to exposure conditions (standardising cooling rate or method, and time spent at a low temperature), but seldom control recovery conditions (for an example from our own work, see Sinclair *et al.* 2007b). Expression of genes associated with stress tolerance in adult *D. melanogaster* increases markedly during recovery from, but not exposure to, low temperatures (Sinclair *et al.* 2007a),

*Corresponding author: tel. 519-661-2111 ext 83138; fax 519-661-3935, email bsincl7@uwo.ca.

and this, in combination with observations of the importance of re-warming conditions in cryopreservation, led us to hypothesise that recovery conditions may be an important aspect of cold tolerance in insects. Here we test this hypothesis in four larval developmental stages of four wild-type strains of *Drosophila melanogaster*, in which we compare cold tolerance under different recovery conditions with and without a pre-treatment that induces RCH. We use larvae because the optimisation of chilling survival in these life stages is part of a wider cryopreservation goal. We predicted that slower or stepwise rewarming would increase survival, and that this effect would not mask RCH, such that an RCH effect would also be evident.

Methods

Drosophila melanogaster wild-type strains Oregon-R-S, Canton S and Berlin K were obtained from the Bloomington Drosophila Stock Center, while a recently-collected (2005) line (W5N, from Washington State, U.S.A.) was provided by Michael Dillon, University of Washington. Flies were reared on a standard cornmeal-agar medium in a combination of vials and population cages as described by Sinclair et al. (2007a). Eggs were collected on Petri plates of food and incubated at 21 °C; larvae were picked off with a fine brush after various periods of time and a subset decapitated and checked for developmental stage using mouthpart characteristics (Ashburner *et al.* 2005). Only samples for which the entire sub-sample was the appropriate developmental stage were used in experiments. Wandering third instar larvae were collected once they had left their food.

Low temperature exposure was conducted at a discriminating temperature of -7°C for a period of time previously determined to result in c. 90% mortality for each stage-strain combination (Rajamohan and Sinclair in press). Increased survival of exposure to the discriminating temperature indicates a cryoprotective effect of a change in pre-treatment or recovery conditions. Groups of 10 to 25 larvae (3–5 replicates per stage/strain/treatment combination) were placed in foil packets and placed in a $25 \times 10 \times 2$ mm (length $\times$ depth $\times$ width) slot in an aluminium block cooled to -7°C by a propylene glycol solution circulated from a refrigerated circulator (VWR 1157p, VWR, Mississauga, Canada). Flies given a RCH pre-exposure were first placed (in their foil packets) in an identical aluminium block cooled to 0°C for one hour before being immediately transferred to the -7°C block – previous work (Rajamohan and Sinclair in press) has shown that this usually elicits the rapid cold-hardening response, resulting in improved survival of -7°C . Four different re-warming regimes were utilised (summarised in Table 1): Acute Transfer from the test temperature to 21°C ; stepwise re-warming to 21°C via pauses at 0 and 15°C ; and slow re-warming to 15°C at either $1^{\circ}\text{C}/\text{min}$ or $0.1^{\circ}\text{C}/\text{min}$. Once returned to 21°C , larvae were transferred to a fresh food vial, and the number that pupariated normally recorded as survival. Warming at $0.1^{\circ}\text{C}/\text{min}$ was only performed for Berlin K and Canton-S strains.

Comparison of the effects of recovery conditions on survival was made with generalized linear modelling with a binomial error distribution and logit link using Stata (Stata/IC 10.0, StataCorp, College Station, TX). For these analyses, a replicate is a single foil packet with 10–25 larvae. This approach takes account of the binomial error distribution of survival data, and is inherently more conservative than traditional approaches that assume a normal distribution (Quinn and Keogh 2002).

Results

The rapid cold-hardening pre-treatment resulted in significant increase in acute cold tolerance for many (Fig. 1, 2), with trends for increase in others. RCH pre-treated larvae occasionally had significantly greater survival than non-pre-treated larvae when subjected to stepwise or

slow re-warming (Berlin K 1st and 3rd instars, Fig. 1, 2), but there were many instances when the RCH pre-treatment did not appear to increase cold tolerance in these groups. Slow or stepwise re-warming without RCH resulted in a significant increase in cold survival when compared to the control only in 1st instar larvae of Berlin K (Fig. 2), and in non-significant cases there was a near-uniform trend of decreased survival, suggesting that the lack of significant increases is not an issue of statistical power. Conversely, a number of stage/strain combinations showed a significant decrease in survival with stepped or slow rewarming when compared to the controls (Figs. 1, 2).

Discussion

Slow or stepped re-warming did not provide a general increase in cold tolerance in all but one case, and in several cases resulted in a significant decrease in survival. While this is evidence that recovery conditions may influence cold tolerance, it does not support our hypothesis that slow or stepped re-warming would increase survival. A possible explanation for our results is that the accumulation of chilling injury is time-dependent (Nedved 1998), and that slow re-warming is resulting in a longer period of accumulation of chilling damage. Another possibility is that slower re-warming means that the instigation of pathways associated with recovery from injuries caused by the cold shock is retarded, meaning that the cold injuries are greater, or that downstream effects like apoptosis (see Yi et al. 2007) have more time to develop. Sinclair et al. (2007a) found that changes in gene expression only occurred during recovery from 0 °C in adult *D. melanogaster*, which suggests that our stepwise recovery treatment, at least, was at a temperature where some molecular responses may be inhibited. Almost all (67 of 78) slow or stepped re-warming treatments resulted in a significant or trend for a decrease in survival. Any increase in statistical power would therefore allow us to discriminate between a decrease or no change for the non-significant trends, but is extremely unlikely to indicate a change in direction of the response (Quinn and Keogh 2002). Berlin K 1st and 3rd instar larvae did not follow this trend, and slow/stepped re-warming significantly increased survival on three occasions (1 °C/min with and without RCH and RCH Step). This strain shows considerable tolerance to environmental stresses in both larval and adult stages (e.g., Vogel 1980; Magnusson and Ramel 1986). We are unable to conclude from our data whether the increased survival with modified recovery conditions in this case is a result of superior stress tolerance or some form of plasticity in Berlin K larvae.

A rapid cold-hardening pre-treatment still offered protection against cold in stepwise or slowly re-warmed larvae. This suggests that RCH prevents some general damage induced by cold. There has been a recent proliferation of candidate mechanisms of RCH in *Drosophila* and other insects (e.g. Overgaard et al. 2005; Fujiwara and Denlinger 2007; Yi et al. 2007; Overgaard et al. in press), and also evidence that RCH may act independently of longer term acclimation (Powell and Bale 2006). RCH serves to protect against both acute and longer-term cold injury (Sinclair and Rajamohan, submitted ms), so the presence of an RCH effect in slowly or stepwise re-warmed larvae does not negate the argument that additional injury caused by longer-term chilling may be the cause of the observed decreases in survival.

Discrepancies between laboratory cooling rates (typically 0.1 or 1 °C/min, Lee 1991) and field cooling rates (typically much slower, Sinclair 2001), coupled with evidence that cooling rate affects low temperature survival (Miller 1978) have led to concerns that laboratory studies of insect cold tolerance may misrepresent survival of field conditions (Baust and Rojas 1985). In particular, slow cooling elicits the RCH response, suggesting that the RCH response may have evolved as a mechanism to allow insects to tolerate diurnal thermal cycles (Kelty and Lee 1999). Here we find that slow re-warming, which more closely mimics field conditions than the acute transfer often used in the laboratory, more often than not results in a decrease in survival, and that there is some stage- and strain-specificity to the magnitude of the response.

This suggests that – contrary to the results from slow cooling studies – laboratory studies that use acute transfer may overestimate survival of low temperatures. Clearly this assertion requires validation across a range of taxa, and we suggest beginning with adults of *D. melanogaster* to determine whether our observations hold across all life stages.

Acknowledgements

We are grateful to Heather Tarnowski and Crystal Bross for their assistance in maintaining fly cultures, to Michael Dillon for providing the W5N flies and to the Bloomington *Drosophila* Stock Center for providing flies. This research was supported by grant R21-RR022885-02 the National Center for Research Resources (NCRR), a component of the National Institutes of Health (NIH). Its contents are solely the responsibility of the authors and do not necessarily represent the official views of NCRR or NIH.

References

- Ashburner, M.; Golic, K., et al. *Drosophila: A Laboratory Handbook*. Cold Spring Harbor Laboratory Press; New York: 2005.
- Baust JG, Rojas RR. Insect cold hardiness: Facts and fancy. *Journal of Insect Physiology* 1985;31:755–759.
- Czajka MC, Lee RE Jr. A rapid cold-hardening response protecting against cold shock injury in *Drosophila melanogaster*. *Journal of Experimental Biology* 1990;148:245–254. [PubMed: 2106564]
- Fujiwara Y, Denlinger DL. p38 MAPK is a likely component of the signal transduction pathway triggering rapid cold hardening in the flesh fly *Sarcophaga crassipalpis*. *Journal of Experimental Biology* 2007;219:3295–3300. [PubMed: 17766307]
- Hoffmann AA, Sorensen JG, et al. Adaptation of *Drosophila* to temperature extremes: bringing together quantitative and molecular approaches. *Journal of Thermal Biology* 2003;28:175–216.
- Kelty JD, Lee RE Jr. Induction of rapid cold hardening by ecologically relevant cooling rates in *Drosophila melanogaster*. *Journal of Insect Physiology* 1999;45:719–726. [PubMed: 12770302]
- Lee, RE, Jr. Principles of insect low temperature tolerance. In: Lee, RE., Jr; Denlinger, DL., editors. *Insects at Low Temperature*. Chapman and Hall; New York: 1991. p. 17-46.
- Magnusson J, Ramel C. Genetic variation in the susceptibility to mercury and other metal compounds in *Drosophila melanogaster*. *Teratogenesis, Carcinogenesis and Mutagenesis* 1986;6:289–305.
- Mazur P, Cole KW, et al. Contributions of cooling and warming rate and developmental stage to the survival of *Drosophila* embryos cooled to –205 degrees C. *Cryobiology* 1993;30:45–73. [PubMed: 8440129]
- Miller LK. Freezing tolerance in relation to cooling rate in an adult insect. *Cryobiology* 1978;15:345–349. [PubMed: 710160]
- Nedved O. Modelling the relationship between cold injury and accumulated degree days in terrestrial arthropods. *Cryo-Letters* 1998;19:267–274.
- Overgaard J, Malmendal A, et al. Metabolomic profiling of rapid cold hardening and heat shock in *Drosophila melanogaster*. *Journal of Insect Physiology*. in press
- Overgaard J, Sorensen JG, et al. Changes in membrane lipid composition following rapid cold hardening in *Drosophila melanogaster*. *Journal of Insect Physiology* 2005;51:1173–1182. [PubMed: 16112133]
- Powell SJ, Bale JS. Effect of long-term and rapid cold hardening on the cold torpor temperature of an aphid. *Physiological Entomology* 2006;31:348–352.
- Quinn, GP.; Keogh, MJ. *Experimental design and data analysis for biologists*. Cambridge University Press; Cambridge: 2002.
- Rajamohan A, Sinclair BJ. Short term hardening effects on survival of acute and chronic cold exposure by *Drosophila melanogaster* larvae. *Journal of Insect Physiology*. in press
- Rall WF, Polge C. Effect of Warming Rate on Mouse Embryos Frozen and Thawed in Glycerol. *Journal of Reproduction and Fertility* 1984;70:285. [PubMed: 6363690]

- Sinclair BJ. Field ecology of freeze tolerance: interannual variation in cooling rates, freeze-thaw and thermal stress in the microhabitat of the alpine cockroach *Celatoblatta quinque maculata*. *Oikos* 2001;93:286–293.
- Sinclair BJ, Gibbs AG, et al. Gene transcription during exposure to, and recovery from, cold and desiccation stress in *Drosophila melanogaster*. *Insect Molecular Biology* 2007a;16:435–443. [PubMed: 17506850]
- Sinclair BJ, Nelson S, et al. The effect of selection for dessication resistance on cold tolerance of *Drosophila melanogaster*. *Physiological Entomology* 2007b;32:322–327.
- Vogel E. Genetical relationship between resistance to insecticides and procarcinogens in two *Drosophila* populations. *Archives of Toxicology* 1980;43:201–211. [PubMed: 7369868]
- Yi SX, Moore CW, et al. Rapid cold-hardening protects *Drosophila melanogaster* from cold-induced apoptosis. *Apoptosis* 2007;12:1183–1193. [PubMed: 17245639]

Figure 1.

Effects of stepwise re-warming on survival of acute cold exposure by *Drosophila melanogaster* larvae. Mean $\pm$ standard error survival of a -7°C cold exposure at a pre-determined LT_{90} by larvae of four strains of *D. melanogaster* at four developmental stages (1st and 2nd instars, I and II, respectively, and feeding and wandering 3rd instars, III and W, respectively). Acute transfer refers to larvae removed immediately from the cold exposure to room temperature, whereas step larvae were re-warmed in a stepwise fashion; RCH larvae were pre-treated at 0°C before cold exposure (see Table 1 for details of treatments). Statistical comparisons were made within stage-strain combinations using generalized linear models: + indicates a significant difference between a RCH and non-RCH pair; * indicates a difference in survival between a treatment and Acute Transfer for that stage/strain combination. Where survival is zero, no bar appears on the graph.

Figure 2.

Effects of slow re-warming at two different rates on survival of acute cold exposure by *Drosophila melanogaster* larvae. Mean $\pm$ standard error survival of a -7°C cold exposure at a pre-determined LT_{90} by larvae of four strains of *D. melanogaster* at four developmental stages (1st and 2nd instars, I and II, respectively, and feeding and wandering 3rd instars, III and W, respectively). Acute transfer refers to larvae removed immediately from the cold exposure to room temperature, whereas 1 deg/min and 0.1 deg/min larvae were re-warmed at those rates; RCH indicates larvae that were pre-treated at 0°C for 1 h prior to cold exposure (see Table 1 for details of treatments). Statistical comparisons were made within stage-strain combinations using generalised linear models: + indicates a significant difference between a RCH and non-RCH pair; * indicates a difference in survival between a treatment and Acute Transfer for that stage/strain combination. Where survival is zero, no bar appears on the graph.

Table 1

Summary of pre-treatments and re-warming conditions to which *D. melanogaster* larvae were subjected in conjunction with acute cold exposure at -7°C in this study. Combinations of RCH and recovery conditions are referred to in the text in the form “RCH Step”.

Pretreatments	
None	Larvae transferred directly from rearing conditions to test temperature.
Rapid cold-hardening (RCH)	Larvae pre-treated for 1 h at 0°C before transfer from test temperature to induce a rapid cold-hardening response.
Recovery conditions	
Acute Transfer (AT)	Larvae removed immediately to room temperature (21°C) from test temperature. Warming rate c. $23^{\circ}\text{C}/\text{min}$.
Step	Larvae removed from test temperature to 0°C for 20 min, then to 15°C for 20 min, then to 21°C .
1 deg/min	Larvae warmed from test temperature to 15°C at $1^{\circ}\text{C}/\text{min}$, then transferred to 21°C .
0.1 deg/min	Larvae warmed from test temperature to 15°C at $0.1^{\circ}\text{C}/\text{min}$, then transferred to 21°C .