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Induction of rapid cold hardening by cooling at ecologically relevant rates in *Drosophila melanogaster*

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Abstract

Over a decade ago it was hypothesized that the rapid cold hardening process allows an organism's overall cold tolerance to track changes in environmental temperature, as would occur in nature during diurnal thermal cycles. Although a number of studies have since focused on characterizing the rapid cold hardening process and on elucidating the physiological mechanisms upon which it is based, the ecological relevance of this phenomenon has received little attention. We present evidence that in *Drosophila melanogaster* rapid cold hardening can be induced during cooling at rates which occur naturally, and that the protection afforded in such a manner benefits the organism at ecologically relevant temperatures. *Drosophila melanogaster* cooled at natural rates (0.05 and 0.1°C min⁻¹) exhibited significantly higher survival after one hour of exposure to -7 and -8°C than did those directly transferred to these temperatures or those cooled at 0.5, or 1.0°C min⁻¹. Protection accrued throughout the cooling process (e.g., flies cooled to 0°C were more cold tolerant than those cooled to 11°C). Whereas *D. melanogaster* cooled at 1.0°C min⁻¹ had a critical thermal minimum (i.e., the temperature at which torpor occurred) of 6.5±0.6°C, those cooled at an ecologically relevant rate of 0.1°C min⁻¹ had a significantly lower value of 3.9±0.9°C. © 1999 Elsevier Science Ltd. All rights reserved.

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1. Introduction

Aside from the relatively few species that possess the capacity to survive freezing, most insects are killed by internal ice formation and thus are referred to as freeze-intolerant. For these species, the temperature at which ice forms within their tissues (termed the temperature of crystallization, or T_c) represents the lowest temperature at which they could potentially survive. However, the utility of this value as a general measure of an insect's ability to survive chilling is limited by the fact that many species are susceptible to chilling injury or death in the absence of ice formation (Lee and Denlinger, 1985; Knight et al., 1986; Bale, 1987; Lee et al., 1987). One form of non-freezing injury, termed cold shock or direct chilling injury, is caused by a brief exposure to low tem-

peratures above an organism's T_c (Chen et al., 1987; Watson and Morris, 1987).

The mechanisms by which organisms increase their cold hardiness have long been a central theme in the field of thermal biology. Historically, most research on insect cold tolerance has focused on seasonal acclimation, especially that associated with the acquisition of overwintering cold-hardiness. These processes occur over periods of days, weeks or months and are often linked with the organism's entry into a state of quiescence or diapause (Denlinger, 1991). The mechanisms underlying cold hardening include the accumulation, to sometimes multi-molar levels, of sugars and polyhydric alcohols such as glycerol, replacement of cellular proteins with isoforms adapted to low temperature, and modification of membrane lipids (Hochachka and Somero, 1984; Lee, 1991).

In contrast to overwintering cold hardiness, and most examples of thermal acclimation, which require extended periods to fully develop, a rapid cold hardening process has been described that is induced by brief exposure to moderately low temperature (Lee et al.,

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1987). Within minutes, this process affords significant protection against subsequent exposure to otherwise injurious low temperature. For instance, although adult *Drosophila melanogaster* can be chilled to -17°C before the water in their tissues freezes, these flies are killed when exposed to -5°C for 1 h (Czajka and Lee, 1990). However, if first chilled at 0°C for 1 h, most survive this otherwise lethal cold exposure. This form of protection has been identified in a number of diverse insect orders including Coleoptera, Diptera, Hemiptera, Homoptera, and Thysanoptera (Lee et al., 1987; Pullin and Bale, 1988; Lee, 1991). As with overwintering cold hardiness, it has been hypothesized that the protection afforded by rapid cold hardening is produced by cryoprotective substances such as glycerol, the concentration of which may increase as much as 300% to 81.4 ± 3.0 mM after two hours of exposure to 0°C (Chen et al., 1987).

Although well described in the laboratory, little is known of the importance of rapid cold hardening in nature (Coulson and Bale, 1990). Lee et al. (1987) hypothesized that the rapid cold-hardening response acts to prevent cold shock injury caused by a sudden decline in temperature. In this scenario, rapid environmental cooling, as often occurs during diurnal cycles, induces a corresponding increase in the exposed insect's cold hardiness. Later, as environmental temperature rises, the insect's cold tolerance decreases as it readjusts to higher temperatures.

Although proposed over a decade ago, the adaptive hypothesis proposed by Lee and colleagues has yet to be tested. Rather, most research to characterize the rapid cold-hardening process has focused on its underlying mechanisms using protocols involving the direct transfer of organisms from their rearing temperature to protection-inducing temperatures, and then to potentially injurious temperatures. While useful in extending our understanding of the physiological basis of the protective process, these studies subjected organisms to cooling rates and thermal extremes that would rarely, if ever, occur in nature. In insects and other poikilotherms, less severe chilling (and thus more ecologically relevant) often induces more subtle, yet significant deleterious effects. For instance, when chilled to moderate temperatures, well above those which directly cause death, many organisms enter a state of cold torpor. At or below the temperature at which this occurs (the critical thermal minimum or CT_{min}), they are unable to seek refugia or food, or to avoid predation (Layne et al., 1985; Rome et al., 1992).

In this study, we determined whether rapid cold hardening could be induced in *D. melanogaster* during cooling at rates which often occur during diurnal cycles. We found that fruit flies cooled at natural rates exhibited higher survival after exposure to subzero temperatures, than did those directly transferred to these temperatures

or those cooled at higher rates. Furthermore, flies cooled at ecologically relevant rates exhibited significantly lower CT_{min} than did those cooled at higher rates.

2. Materials and methods

2.1. Insect rearing

Drosophila melanogaster (Oregon-R strain) were reared under a long-day photoperiod (LD, 15:9 h) at 23°C in half-pint milk bottles containing *Drosophila* (corn meal, molasses, yeast, agar) medium as food and as a substrate for oviposition. Newly emerged adult flies were removed from bottles daily and transferred to fresh medium on which they were allowed to feed and reproduce for 9 d, at which time they were removed and euthanized by freezing. Because of the potential effects of incubation density on cold-hardening, all bottles contained less than 200 adult flies (R. Huey, pers. comm.). Because the ability of fruit flies to rapidly cold-harden decreases with age (Czajka and Lee, 1990), only 2-d-old flies were used for experiments.

2.2. General chilling protocol

Groups of 10 adult *D. melanogaster* were chilled in glass culture tubes stoppered with foam rubber and nested in glass test tubes immersed in a refrigerated bath. To minimize desiccation stress, each tube contained c. 10 μl of *Drosophila* medium covered with a small piece of KimWipe to absorb excess moisture and prevent flies from becoming trapped in the medium after being immobilized by chilling. The air temperature in each tube was monitored on a chart recorder via a 36 ga copper-constantan thermocouple, the tip of which was suspended near the bottom of the culture tube.

2.3. Effects of the induction of rapid cold hardening during cooling on survival

To examine the induction of rapid cold hardening during cooling at different rates in *D. melanogaster*, three sets of experiments were performed in which cold hardiness was assessed as survival at low temperature. In the first experiment, eight to 21 groups of flies were either transferred directly, or steadily cooled at either 1.5, 0.5 or $0.1^{\circ}\text{C min}^{-1}$, to potentially injurious low temperatures (-6 , -7 , -8 or -9°C). These animals were held at the experimental temperature for 1 h, then returned to their rearing temperature. Survival was assessed at 24 h following chilling treatments. Flies were graded as alive if they were able to right themselves and walk. For each treatment, the number and percentage of flies surviving was recorded.

During the next survival experiment, flies ($n=4$ to 11

groups of 10 flies) were cooled at 1.0, 0.5, 0.1 and 0.05°C min⁻¹ to 0°C, directly transferred to -6, -7 or -8°C for 1 h, then returned to their rearing temperature. Survival was assessed as in the previous experiment.

In the final survival experiment, the degree of cold hardiness accrued at different stages of cooling from 23°C at 0.05 and 0.1°C min⁻¹ was assessed. Samples (n=4 to 12 groups of 10 flies) were removed at 23, 11, 4 and 0°C, then transferred directly to -6, -7 or -8°C for 1 h and their survival assessed as above.

2.4. Effect of cooling rate on critical thermal minimum

We assessed CT_{min} in a manner similar to that employed by Webber (1988) and by Huey et al. (1992). Briefly, two days following their eclosion from the puparium, *D. melanogaster* were introduced into a jacketed glass column, the temperature of which could be controlled by circulating fluid from a programmable refrigerated bath. After allowing the flies 5 min to adjust to the environment of the column, its temperature was decreased from 23°C at either 1.0, 0.5 or 0.1°C min⁻¹. At their CT_{min}, flies were unable to cling to surfaces in the column and fell into glass culture tubes which were changed every 0.1°C using a fraction collector. To prevent escape, the inner surface of each tube was coated with fluon which kept flies from crawling up its inner surface or perching sufficiently near its opening to fly out. Although both males and females exhibited negative geotropism, males tended to walk out of the column's open lower end during cooling and were often trapped in the collecting tubes, artificially increasing our estimation of CT_{min}. Therefore, only females were used for this determination. Because of the small size of *D. melanogaster*, body temperature was approximated as the air temperature within the column (Huey et al., 1992).

2.5. Assessment of fecundity

To assess the effects of chilling on fecundity, flies were either directly transferred, or chilled at 2°C h⁻¹, to -1, or -3°C at which they were held for 1 h. These temperatures were chosen because they are near, but are more severe, than those which induce the rapid cold-hardening process in *D. melanogaster*. Furthermore, these temperatures are well above those which induce obvious signs of cold shock injury such as death or severe impairment of motor control. Fecundity was assessed using the method of Huey et al. (1995). Briefly, trios (n=6 to 15, each containing 1 female and 2 males) of *D. melanogaster* were transferred to 8 dram shell vials containing c. 3 ml of cornmeal-molasses medium and a few grains of live yeast. Fecundity was then indexed as the sum of the eggs deposited over the first 5 d following chilling. During this period, trios were transferred daily

to fresh vials and the number of eggs they deposited on the medium counted.

2.6. Glycerol assays

Whole-body glycerol content was determined in *D. melanogaster* from (i) controls, (ii) groups cooled from 23 to 0°C at 1.0 and 0.1°C min⁻¹, and (iii) groups held at 0°C for 2 h, using an enzymatic assay (Sigma Chemical Co. #337-40A). Samples of 10 *D. melanogaster* were ground in perchloric acid which was then neutralized with sodium bicarbonate to pH 7.0. Glycerol levels were determined spectrophotometrically by measuring sample absorbance of light ($\lambda=520$ nm).

2.7. Data analysis

Parametric data were analyzed using analysis of variance (ANOVA). Non-parametric data were analyzed using the Kruskal-Wallis test. When comparing survival rates, data were first transformed by taking the arcsine and square root of the observed survival proportions. Treatment differences were considered significant at $P<0.05$. Data are reported as mean \pm SEM.

3. Results

3.1. Effects of the induction of rapid cold hardening during cooling on survival

During our initial survival experiment, we determined whether cooling at constant slow rates protected *D. melanogaster* at subzero temperatures that would otherwise be injurious. Flies cooled at 0.1, 0.5 and 1.5°C min⁻¹ exhibited significantly greater survival than those directly transferred (i.e., cooled at c. 11.25°C min⁻¹) to -7, -8, or -9°C (Fig. 1). Although no flies directly transferred to -8°C survived after 1 h at this temperature, more than 63% of those cooled at rates between 0.1 and 1.5°C min⁻¹ survived. Similarly, whereas none of the flies directly transferred or cooled at 1.5°C min⁻¹ survived 1 h at -9°C, cooling at 0.1 and 0.5°C min⁻¹ allowed >15% survival.

Flies chilled at 0.1°C min⁻¹, a rate measured in nature (J.T. Irwin, R.E. Lee and J.P. Costanzo, unpublished data), exhibited significantly lower survival at -7°C than did flies cooled at 0.5°C min⁻¹ (Fig. 1). Although not statistically significant, this trend was also seen in flies chilled to -8 and -9°C. Rather than reflecting a lesser degree of cold hardiness in more slowly cooled flies, this trend likely reflects the more substantial cold stress that these groups experienced as a result of the greater time they spent at potentially injurious subzero temperatures.

To control for the effect of time spent at subzero tem-

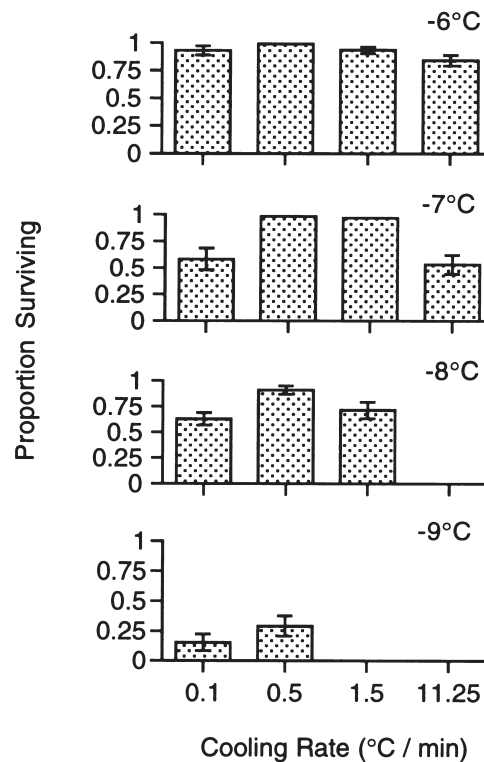


Fig. 1. Survival of *Drosophila melanogaster* directly transferred (i.e., cooled at $11.25^{\circ}\text{C min}^{-1}$) or cooled at one of three rates (0.1, 0.5, or $1.5^{\circ}\text{C min}^{-1}$) to -8 , -7 , or -6°C . Columns represent the mean survival \pm SEM of 8–21 samples, each containing 10 flies. Decreasing cooling rate significantly influenced survival at subzero temperatures (Kruskal-Wallis, $P < 0.0001$).

peratures, a second experiment was performed in which *D. melanogaster* were cooled at 1.0, 0.5, 0.1 and $0.05^{\circ}\text{C min}^{-1}$ to 0°C , then directly transferred to -6 , -7 or -8°C for 1 h, followed by return to their rearing temperature and their survival assessed 24 h later. In contrast to the conditions of the first experiment, flies treated in this manner were exposed to subzero temperatures for similar amounts of time. For example, during the first experiment a fly chilled to -7°C at $0.1^{\circ}\text{C min}^{-1}$ spent 130 min below 0°C (of which 60 min was at -7°C), whereas a fly cooled at $0.5^{\circ}\text{C min}^{-1}$ was only exposed to subzero temperatures for 74 min. When the time spent at subzero temperatures was standardized, survival at -7 and -8°C significantly increased with cooling at each progressively slower rate (Fig. 2). These results contrast with those from the first experiment (Fig. 1) in which flies cooled at $0.1^{\circ}\text{C min}^{-1}$ to subzero temperatures had lower rates of survival than did those cooled at $0.5^{\circ}\text{C min}^{-1}$.

To determine the degree of cold hardiness accrued at various stages during cooling at ecologically relevant rates, *D. melanogaster* were cooled from 23°C at 0.05 or $0.1^{\circ}\text{C min}^{-1}$ to 0, 4, or 11°C . Upon reaching these temperatures, or from groups kept at 23°C , flies were directly transferred to -6 , -7 or -8°C for 1 h, then returned to 23°C , and their survival assessed 24 h later (Fig. 3). The cold tolerance of *D. melanogaster* signifi-

cantly increased over the course of each cooling regimen; flies cooled to 0°C exhibited significantly higher rates of survival at -7 and -8°C than did those cooled to 4°C before transfer to subzero temperatures. Likewise, those cooled to 4°C exhibited significantly higher survival than those cooled to 11°C . At both rates, cooling to 11, 4 and 0°C significantly increased survival relative to flies which were directly transferred to subzero temperatures (Fig. 3).

3.2. Effect of cooling rate on critical thermal minima

Our determinations of the effects of cooling rate on survival at subzero temperatures provide a useful index of the extent of protection afforded by rapid cold hardening during the cooling process. However, the thermal extremes experienced by flies subjected to the subzero temperatures used in survival experiments exceeded those which a fly would be likely to experience naturally. Consequently, we determined whether the rapid cold-hardening process could produce an ecologically relevant effect at temperatures which fruit flies would likely experience during natural cycles. To do so we assessed the effects of cooling at different rates on the CT_{min} of *D. melanogaster* by measuring the temperature at which they were incapacitated by chilling and thus were no

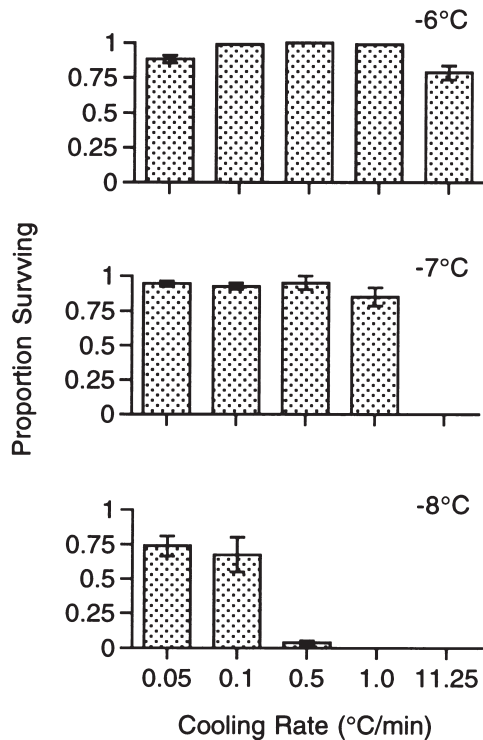


Fig. 2. Survival of *Drosophila melanogaster* after exposure to subzero temperatures when time at these temperatures was kept constant. Rather than cooling at constant rates to subzero temperatures, as in the first survival experiment (Fig. 1), fruit flies were cooled at 0.05, 0.1, 0.5, or 1.0°C min⁻¹ to 0°C, then transferred directly to -8, -7, or -6°C for 1 h. Columns represent the mean survival \pm SEM of 8–14 samples, each containing 10 flies. Cooling rate significantly affected survival at each subzero temperature (Kruskal-Wallis, $P < 0.0001$).

longer able to cling to surfaces in a temperature-controlled glass column.

As found in the survival experiments, cooling rate significantly affected the cold hardiness of *D. melanogaster* (Fig. 4). Whereas flies cooled at 1.0°C min⁻¹ exhibited a CT_{min} of 6.5 \pm 0.6°C (n = 111), those cooled at 0.5 and 0.1°C min⁻¹ exhibited significantly lower CT_{min} of 5.5 \pm 0.6° (n = 116) and 3.9 \pm 0.9°C (n = 87), respectively ($P < 0.0001$ for each comparison).

3.3. Effect of cooling on early fecundity

To determine whether or not the rapid cold-hardening response affected reproductive output, we tested the effects of protective cooling on the early fecundity (i.e., the number of eggs produced during the first five days following chilling) of female fruit flies. No significant differences were found in egg production between (i) control females, (ii) those transferred from 23 directly to -1°C and held at this temperature for 3 h, (iii) those cooled from 23 to -1°C at 2°C h⁻¹ and returned immediately to 23°C, or (iv) those cooled from 23 to -1°C at 2°C h⁻¹, held at this temperature for 1 h, then returned to 23°C (Table 1). Similar results were obtained

for flies chilled in analogous manners to -3°C. The *D. melanogaster* in this group also exhibited a high degree of variability in their egg production (Table 1). For instance, control females produced from 18 to 237 eggs over the first five days following chilling and those directly transferred to -3°C for 3 h produced from 6 to 220 eggs over the same period.

3.4. Effect of rapid cold hardening on glycerol synthesis

Although glycerol production has been correlated with the rapid cold-hardening process in other insects, we found no evidence for similar changes in whole body glycerol levels. Control *D. melanogaster* (n = 8 groups of 20 flies) contained 0.0041 \pm 0.004 mg glycerol per mg of fresh tissue and those chilled at 0°C for 2 h (n = 8 groups of 20 flies) contained 0.0040 \pm 0.005 mg glycerol per mg. *Drosophila melanogaster* cooled from 23 to 0°C at 1.0°C min⁻¹ contained 0.0037 \pm 0.005 mg glycerol per mg of fresh tissue and those cooled at 0.1°C min⁻¹ contained 0.0038 \pm 0.008 mg glycerol per mg.

4. Discussion

Over a decade ago Lee et al. (1987) hypothesized that the rapid cold-hardening process allows an organism's overall cold tolerance to track changes in environmental temperature, such as would occur in nature during diurnal thermal cycles. Although a number of studies (e.g., Coulson and Bale, 1990, 1992; Czajka and Lee, 1990) have since focused on characterizing the rapid cold hardening process and on elucidating the physiological mechanisms upon which it is based, the ecological relevance of this phenomenon has largely been ignored. In this study we addressed this question by (i) testing the hypothesis that rapid cold hardening could be induced during cooling at rates reflecting those naturally occurring on a diurnal basis and (ii) testing the hypothesis that the protection afforded in such a manner could benefit the organism at ecologically relevant temperatures.

In our first two survival experiments we found that cooling at ecologically relevant rates (0.05 and 0.1°C min⁻¹) significantly increased cold hardiness relative to cooling at higher rates (0.5, 1.0, 1.5°C min⁻¹ and direct transfer) (Figs. 1 and 2). The increased cold tolerance of *D. melanogaster* cooled at slower rates is presumably due to the additional time these flies spent at protection-inducing temperatures. Our results correspond well with those of Coulson and Bale (1990) who found that survival of house fly (*Musca domestica*) pupae cooled at constant rates from 27 to -7°C increased with decreasing cooling rate. More importantly, our data support the hypothesis that rapid cold hardening can be induced during the cooling phase of natural diurnal thermal cycles.

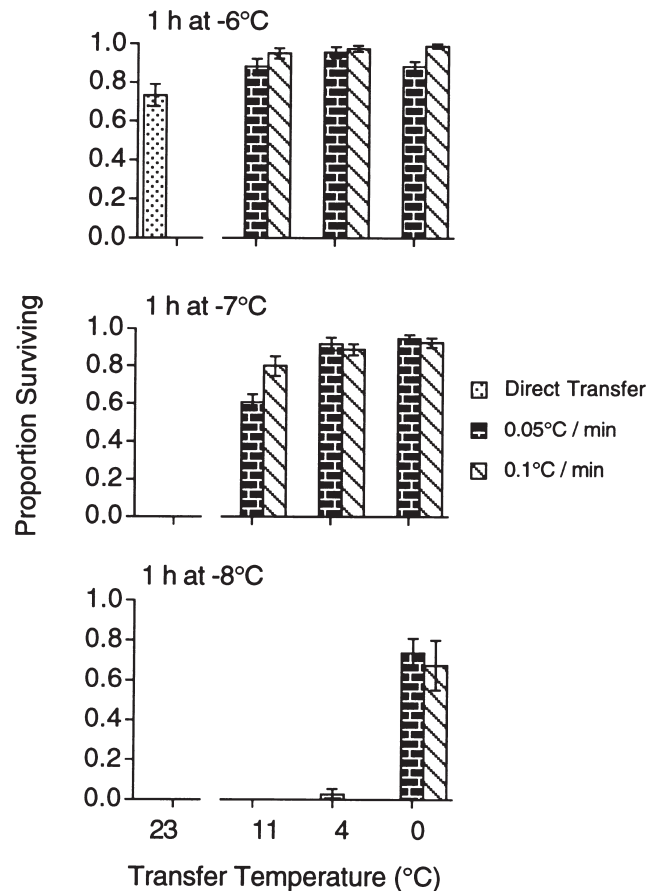


Fig. 3. Development of rapid cold hardening during cooling at 0.05 and 0.1°C min⁻¹. *Drosophila melanogaster* were cooled from 23 to 11, 4, or 0°C, then transferred directly to -8, -7 or -6°C for 1 h and their survival assessed 24 h later. At each rate, cold hardiness was significantly increased by cooling to 11, 4 and 0°C, with those flies cooled to the lower of these temperatures exhibiting significantly higher survival than those cooled to 11°C (Kruskal–Wallis, $P < 0.0001$). Columns represent the mean survival \pm SEM of 8–14 samples, each containing 10 flies.

Our finding that significant cold hardening could be induced by cooling to moderate temperatures (11°C), with even greater protection afforded to flies chilled to 4 and 0°C, indicates that tolerance accrued throughout much, if not all, of the cooling regimen (Fig. 3). Although a number of studies have demonstrated rapid cold hardening at temperatures between 0 and 6°C, this is the first report that this process can afford protection by chilling to temperatures as high as 11°C (Chen et al., 1987; Coulson and Bale, 1990; Czajka and Lee, 1990). This finding indicates that the induction of rapid cold hardening is not limited to a narrow range of temperatures near 0°C but that cooling over an extensive thermal range is effective. Therefore, these data suggest that rapid cold hardening could provide protection during the relatively mild thermal changes that commonly occur during natural daily cooling throughout the year.

In nature, *D. melanogaster* would rarely, if ever, encounter low temperatures severe enough to cause mortality as a direct result of cold shock injury on a diurnal basis (Izquierdo, 1991; Junge-Berberovic, 1996). If the rapid cold-hardening process has ecological relevance,

then it must be to prevent more subtle deleterious effects of chilling. Our finding that CT_{min} decreases with decreasing cooling rate (Fig. 4) evidences such an effect. As with longer term acclimation to low temperature, our data suggest that the rapid cold-hardening process could benefit an organism by lowering its CT_{min}, thus prolonging its ability to seek out thermally buffered microhabitats, or evade predators (Layne et al., 1985).

Although the capacity of *D. melanogaster* to rapidly cold harden has been documented in this and another study (Czajka and Lee, 1990), the physiological mechanism by which this species is protected remains elusive. Even though rapid cold hardening in the flesh fly (*Sarcophaga crassipalpis*) is correlated with the production of glycerol (Lee et al., 1987), we were unable to detect any change in whole body glycerol content in rapidly cold hardened *D. melanogaster*. However, while glycerol may be the most commonly observed cryoprotectant, a variety of other alcohols and sugars have been correlated with cold hardening in a number of species and may be present in *D. melanogaster* (Lee, 1991).

Although the synthesis of cyoprotectants is integral to

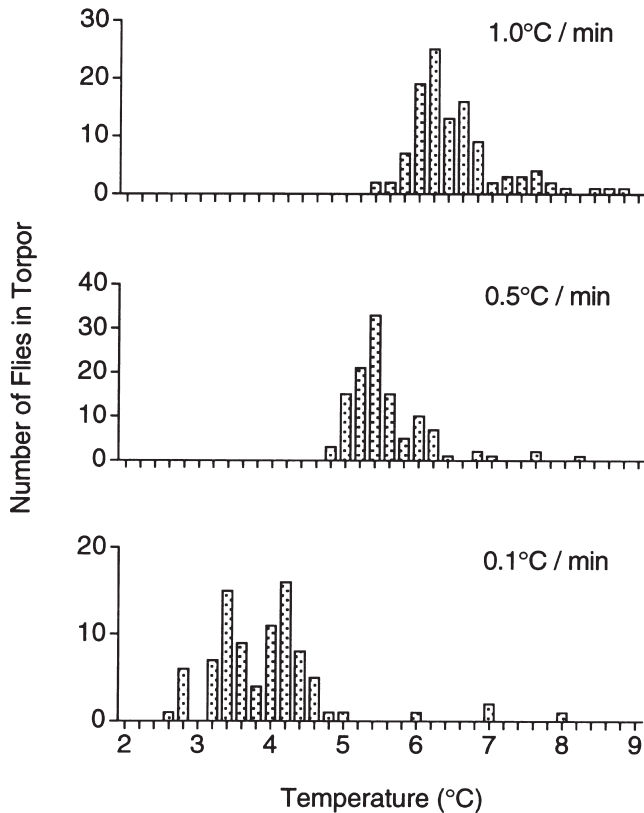


Fig. 4. Effect of cooling rate on the critical thermal minimum (CT_{min}) of *Drosophila melanogaster*. When cooled at $1.0^{\circ}\text{C min}^{-1}$, flies were incapacitated at $7.0 \pm 0.6^{\circ}\text{C}$ ($n = 111$). Decreasing the cooling rate to $0.5^{\circ}\text{C min}^{-1}$ and $0.1^{\circ}\text{C min}^{-1}$, each significantly decreased the CT_{min} to $4.7 \pm 0.6^{\circ}$ ($P < 0.0001$, $n = 116$) and $3.6 \pm 0.9^{\circ}\text{C}$ ($P < 0.0001$, $n = 87$), respectively.

the development of cold tolerance in a number of species, other physiological mechanisms are known which affect an organism's tolerance to thermal stress. Stress proteins have been implicated in the heat tolerance of *D. melanogaster* (Feder et al., 1996, 1997; Krebs and Feder, 1997). However, it is unlikely that they function as agents of rapid cold hardening; in another dipteran (*S. crassipalpis*), expression of stress proteins requires exposure to temperatures much lower than those needed to induce rapid cold hardening and does not begin until after return to higher temperature (Joplin et al., 1990). Another potential mechanism of rapid cold hardening is found in fish which rapidly undergo dramatic changes in the lipid composition of their cell membranes allowing these structures to maintain their fluidity and integrity during cooling and warming (Hazel and Carpenter, 1985; Hazel and Landrey, 1988; Carey and Hazel, 1989; Hazel, 1995).

We found no change in the early fecundity of *D. melanogaster* as a result of rapid cold hardening. In *M. domestica*, the rapid cold-hardening process caused a decrease in lifetime reproductive capacity, but mean daily oviposition per female was not significantly affected

Table 1
Early fecundity of *Drosophila melanogaster*^a

Cooling regimen	Number of trios	Mean number of eggs \pm SEM	Range
Control (no cooling) for samples cooled to -1°C	16	156 ± 16	18–237
Direct transfer from 23 to -1°C , held for 3 h at -1°C , then returned to 23°C	15	163 ± 14	8–225
Cooled at 2°C/h to -1°C , immediately returned to 23°C	15	177 ± 16	117–238
Cooled at 2°C/h to -1°C , held for 3 h at -1°C , then returned to 23°C	8	198 ± 19	159–324
Control (no cooling) for samples cooled to -3°C	7	124 ± 27	23–191
Direct transfer from 23 to -3°C , held for 3 h at -3°C , then returned to 23°C	7	113 ± 25	6–220
Cooled at 2°C/h to -3°C , immediately returned to 23°C	6	131 ± 27	12–199
Cooled at 2°C/h to -3°C , held for 3 h at -3°C , then returned to 23°C	8	129 ± 23	12–210

^a Values represent the mean number of eggs produced during the first five days following chilling. Each trio contained one female and two males. No significant differences were found between treatments.

ted (Coulson and Bale, 1990). Rather, the decreased fecundity of rapidly cold hardened flies was due to a decrease in average lifespan. We only measured the number of eggs produced by female *D. melanogaster* over the first 5 d following chilling. Perhaps, an examination of post-chilling lifespan, the number of eggs produced throughout adult life, and the success with which larvae emerge from these eggs would reveal more subtle costs of rapid cold hardening.

In contrast to most studies of thermal acclimation which have focused on acclimatory processes over periods lasting from days to weeks, or even months, we examined a rapid cold hardening process which affords significant protection within minutes. We demonstrated that rapid cold-hardening can be induced in *D. melanogaster* by cooling at rates that naturally occur during diurnal thermal cycles. Furthermore, our data indicate

that significant protection can be induced by rapid cold-hardening at higher temperatures than previously reported (i.e., 11°C), and that the protection afforded accrues throughout much, if not all, of the cooling process. Our finding that rapid cold hardening also decreases the CT_{min}, provides evidence that the protection afforded benefits the organism at temperatures they would encounter in nature.

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