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RAPID COLD-HARDENING OF *DROSOPHILA MELANOGASTER* (DIPTERA: DROSOPHILIDAE) DURING ECOLOGICALLY BASED THERMOPERIODIC CYCLES

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Summary

In contrast to most studies of rapid cold-hardening, in which abrupt transfers to low temperatures are used to induce an acclimatory response, the primary objectives of this study were to determine (i) whether rapid cold-hardening was induced during the cooling phase of an ecologically based thermoperiod, (ii) whether the protection afforded was lost during warming or contributed to increased cold-tolerance during subsequent cycles and (iii) whether the major thermally inducible stress protein (Hsp70) or carbohydrate cryoprotectants contributed to the protection afforded by rapid cold-hardening. During the cooling phase of a single ecologically based thermoperiod, the tolerance of *Drosophila melanogaster* to 1 h at –7 °C increased from 5±5 % survival to 62.5±7.3 % (means ± S.E.M., N=40–60), while their critical thermal minima ($CT_{\min}$) decreased by 1.9 °C. Cold hardiness increased with the number of thermoperiods to which flies were exposed; i.e. flies exposed to six thermoperiods were more cold-tolerant than those exposed to two. Endogenous levels of Hsp70 and carbohydrate cryoprotectants were unchanged in rapidly cold-hardened adults compared with controls held at a constant 23 °C. In nature, rapid cold-hardening probably affords subtle benefits during short-term cooling, such as allowing *D. melanogaster* to remain active at lower temperatures than they otherwise could.

Key words: acclimation, cold shock, cold tolerance, cryoprotectant, Hsp70, heat-shock protein, *Drosophila melanogaster*.

Introduction

In contrast to most studies of acclimation, which examine phenomena induced over prolonged exposures lasting days, weeks or even months, a number of studies during the past 15 years have begun to examine relatively rapid processes of acclimation to low and high temperature (for reviews, see Feder and Krebs, 1997; Denlinger and Lee, 1998; Denlinger and Yocum, 1998). Upon exposure to moderately low temperature, diverse insect species exhibit a rapid cold-hardening response, which within periods of minutes to hours enhances their cold-tolerance (Chen et al., 1987; Lee et al., 1987; Coulson and Bale, 1990; Coulson and Bale, 1992; Kelty and Lee, 1999). For example, prior exposure of house fly (*Musca domestica*) pupae to 0 °C for 90 min increased their survival following treatment for 2 h at –7 °C from 0 % to above 80 % (Coulson and Bale, 1990). Similarly, insects can acclimate rapidly to high temperature (Milkman, 1963; Levins, 1969; Chen et al., 1991; Krebs and Feder, 1997).

The physiological mechanisms underlying rapid acclimation to low and high temperature remain poorly understood. In preparation for winter, insects accumulate low-molecular-mass polyhydric alcohols and sugars (Denlinger and Lee, 1998). Similarly, during rapid cold-hardening in *Sarcophaga crassipalpis*, the glycerol concentration within the pupal hemolymph increases by approximately 300 % (Chen et al., 1987). On a time scale similar to that of rapid cold-hardening (i.e. within minutes), high temperatures elicit the synthesis of heat-shock proteins (Burton et al., 1988; Goto and Kimura, 1998; Feder and Hofmann, 1999). These proteins presumably protect against thermal injury by acting as molecular chaperones, minimizing the aggregation of non-native proteins and promoting the degradation and removal of both non-native and aggregated proteins from the cell (Feder and Hofmann, 1999).

Within an organism, a variety of thermal protective mechanisms may act in synchrony to prevent cellular damage (Feder and Hofmann, 1999). Substantial evidence demonstrates that sugars and polyols both serve protective roles at low temperatures and help to stabilize membrane lipids and cellular proteins at high temperature (Kim and Lee, 1993; Ramos et al., 1997). Similarly, exposure to low temperature induces the expression of stress proteins, but only upon return to higher temperatures, and high temperature shock increases the tolerance of insects to low temperatures (Burton et al., 1988; Joplin et al., 1990; Nunamaker et al., 1996; Yiangou et al., 1997; Goto and Kimura, 1998; Goto et al., 1998). Thus, common elements may underlie a tolerance of low and high temperatures.

As a first step in determining the ecological significance of the capacity for rapid acclimation to low or high temperature, we recently demonstrated that when *D. melanogaster* were cooled at slower, and thus more natural, rates they exhibited a significantly greater cold-tolerance than did flies cooled at higher rates (Kelty and Lee, 1999). When cooled at ecologically relevant rates (e.g. 0.05 °C min^{-1}), flies not only exhibited higher rates of survival at sub-zero temperatures than their more rapidly cooled counterparts, but rapid cold-hardening provided them with protection at 11 °C, a temperature that flies are more likely to encounter in nature than the near 0 °C exposures used to elicit rapid cold-hardening in most previous studies. Furthermore, the fact that *D. melanogaster* cooled at slower rates entered a state of cold torpor (i.e. reached their critical thermal minimum or CT_{min}) at lower temperatures than those cooled at higher rates provides strong evidence that rapid cold-hardening could benefit this species at the temperatures they are likely to encounter in nature.

In the present study, we investigated rapid acclimation to low temperatures in *D. melanogaster* during an ecologically based thermoperiod. The cooling phase of a single thermoperiod induced substantial protection against cold-shock injury, much of which was retained during the warming phase of the thermoperiod. The increased cold-tolerance appeared to be not only a function of the phase of the thermoperiod from which adults were removed, but was also dependent on the number of thermoperiods to which they had been exposed; flies exposed to multiple thermoperiods were substantially more tolerant of low temperatures than flies exposed to a single thermoperiod.

Materials and methods

Insect rearing

Drosophila melanogaster (Oregon-R strain) were reared under a long-day photoperiod (15 h:9 h L:D) at 23 °C in half-pint milk bottles containing *Drosophila* medium (corn meal, molasses, yeast, agar) as food and as a substratum for oviposition. Newly emerged adults were removed from bottles daily and transferred to fresh medium on which they were allowed to feed and lay eggs for 9 days. Within 8 h of eclosion, the flies were lightly anesthetized with CO_2, segregated by gender and transferred into fresh bottles containing *Drosophila* medium. This enabled us to study virgin flies and examine potential differences in the capacity of male and female *D. melanogaster* to cold-harden rapidly. Because age may affect this capacity (Czajka and Lee, 1990), all flies were 2 days old at the beginning of each experiment, except where the effect of age was examined.

Thermoperiod experiments

To determine what thermoperiod a fruit fly is likely to experience in nature, a computerized data-acquisition system (Campbell Scientific, model CR10) was used to record temperature every hour at the leaf litter/soil interface in a shaded *D. melanogaster* habitat at Miami University's Ecology Research Center (Oxford, OH, USA) during the spring, summer and autumn of 1996. These data were used to program an incubator to cycle between 9 ° and 23 °C over a period of 24 h (Fig. 1); similar thermoperiods frequently occurred during mid-spring and mid-autumn. The incubator was set to a photoperiod of 12 h:12 h L:D, with the lights turned on at 09:00 h.

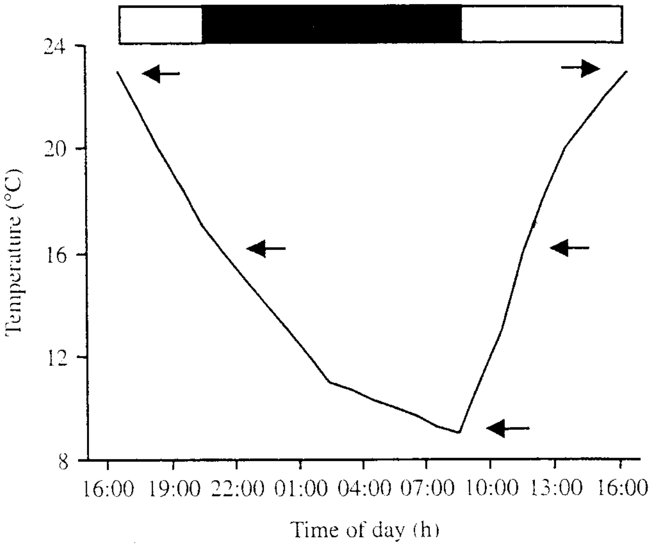

Fig. 1. The 24 h thermoperiod to which *Drosophila melanogaster* were exposed. Arrows represent the points at which flies were removed and their cold-tolerance (assessed as survival after 1 h at −7 °C and as CT_{min}) or heat-tolerance (assessed as survival after 1 h at 38 or 39 °C) determined. The upper bar shows the 12 h:12 h light:dark photoperiod, with the filled region representing the dark period. CT_{min}, critical thermal minimum.

We first determined whether *D. melanogaster* cold-hardened rapidly during the cooling phase of the programmed thermoperiod and whether hardening was lost during warming. Flies were lightly anesthetized with CO_2 and transferred in groups of 10 into glass culture tubes (12×75 mm) containing approximately 0.3 ml of *Drosophila* medium and a few grains of live yeast. These flies were then allowed to recover at 23 °C for at least 6 h. After recovery, flies were (i) kept at 23 °C for up to 6 days, (ii) transferred to the thermoperiod (beginning at 23 °C) for 0–6 days, (iii) kept at 16 °C for up to 6 days or (iv) maintained at 23 °C for one or five additional days then subjected to all or part of the thermoperiod. As outlined below, the thermal tolerances of *D. melanogaster* subjected to the thermoperiod were assessed at the times and temperatures indicated in Fig. 1. The thermal tolerances of *D. melanogaster* held at constant 16 or 23 °C were assessed every 12–24 h.

Survival at low and high temperatures

As an index of overall cold-hardiness, we assessed the capacity of *D. melanogaster* to survive 1 h of exposure to −7 °C. We chose this temperature because preliminary data indicated that, in the absence of cold-hardening, it produced nearly 100 % mortality as a result of non-freezing injury (Kelty and Lee, 1999). Tubes of flies were inverted in glass test tubes partially (>90 %) submerged in a refrigerated bath (NesLab, model RTE140) set at −7 °C. Inversion prevented entrapment of flies in the medium and the initiation of inoculative freezing by frozen medium. The temperature in each tube was monitored on a chart recorder using a copper–constantan thermocouple whose tip was positioned against the inner surface of the foam plugging the tube, which is where the flies fell when incapacitated by chilling. Exposures were timed from the point when the average temperature amongst tubes reached −6.9 °C (within 10 min of transfer to the bath). Survival was assessed 24 h after the end of the sub-zero exposure period as the percentage of flies per tube able to right themselves and walk.

Assessment of critical thermal minima

To assess $CT_{\min}$, groups of 40–60 flies were transferred into a jacketed glass column, the temperature of which was controlled by circulating fluid from a programmable refrigerated bath. After 10 min, the temperature was decreased from 23 °C at 1.0 °C min^{-1}. Flies unable to cling to surfaces in the column (i.e. those cooled to their $CT_{\min}$) fell into glass culture tubes, which were changed every 0.1 °C fall in temperature. To prevent flies from crawling to a position sufficiently close to the open end of the tube that they could fly out and escape, the inner surface of each tube was coated with Fluon. Although *D. melanogaster* of both genders exhibited negative geotropism, males exhibited greater locomotor activity and often became prematurely trapped in the collecting tubes, thereby biasing our estimation of $CT_{\min}$. For this reason, 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).

Determination of carbohydrate cryoprotectants

High-performance liquid chromatography (as described by Hendrix and Wei, 1994) was used to determine which, if any, carbohydrates were synthesized by *D. melanogaster* during rapid cold-hardening. Under light CO_2 anesthesia, groups of 90–150 (totaling 40–85 mg) 2-day-old adults were weighed and placed in glass culture tubes, which were then sealed with foam rubber. Flies were allowed to recover at 23 °C for 6 h, and were then either kept at 23 °C or transferred to 16, 9 or 1 °C for 2 h. Flies were then preserved in 1 ml of 80 % non-denatured ethanol. Carbohydrates were extracted from *D. melanogaster* samples in three steps (80 % non-denatured ethanol at 80 °C for 20 min, 50 % ethanol at 80 °C for 15 min, and 80 % ethanol at 80 °C for 15 min). Charcoal was added to each extract to remove most lipid, all amino acids and most, if not all, color (Hendrix and Peelen, 1987). The charcoal was then removed by passing the extracts through a glass-fiber filter paper (Whatman GF/A) and two layers of extra-fine glass-fiber paper (Whatman GF/F). Because ethanol interferes with the detection of potential cryoprotectants, the portion of each extract to be assayed was evaporated to dryness at 45 °C under nitrogen gas, and then resuspended in water before being injected onto a Dionex PA-1 column. Upon elution from the column, sugars and polyols were detected by pulsed amperometry.

Detection of Hsp70 by gel electrophoresis and western blotting

D. melanogaster were allocated to five groups of three. These flies were kept at 23 °C, subjected to 37 °C for 90 min, removed from the thermoperiod at the points indicated in Fig. 1, or held at 0 °C for 2 h. After each treatment, flies were immediately snap-frozen in liquid nitrogen, then stored at −80 °C until assayed for the heat-shock protein Hsp70. Soluble proteins were extracted by homogenizing each group of flies in ice-cold 2 % (w/v) complete protease inhibitor (Boehringer Mannheim 1697498) in phosphate-buffered saline. The resulting homogenate was centrifuged for 30 min at 14 000 revs min^{-1}, and the supernatant was recovered. Protein content was determined using a BCA assay (Pierce Biochemicals). Proteins were separated by electrophoresing 10 μg of protein in each lane of a 10 % Tris-HCl sodium dodecyl sulfate polyacrylamide (SDS–PAGE) gel. Following their separation by SDS–PAGE, proteins were transferred electrophoretically onto a PVDF membrane that was then blocked with phosphate-buffered saline containing 10 % non-fat powdered milk. The membrane was washed successively in solutions containing primary antibody specific for inducible *Drosophila* Hsp70 (7.FB, a gift from Susan Linquist, University of Chicago), then peroxidase-conjugated goat anti-rat IgG secondary antibody (Jackson ImmunoResearch Laboratories, no. 112-035-003). Bound antigen was detected by chemiluminescence using the SuperSignal CL-HRP substrate system according to the manufacturer's instructions (Pierce Biochemical no. 34080).

Data analysis

Data were analyzed using analysis of variance (ANOVA). 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 $P<0.05$. Values are reported as means ± S.E.M.

Results

Rapid cold-hardening during an ecologically based thermoperiod

We first determined whether rapid cold-hardening was induced during the cooling phase of an ecologically based thermoperiod and whether the protection it provided was lost during warming. To do so, *D. melanogaster* were subjected to all or part of such a thermoperiod, and their capacity to survive 1 h of exposure to −7 °C was then assessed (Table 1). As flies cooled from 23 to 16 °C at an average rate of 1.4 °C h^{-1}, their survival after sub-zero treatment increased significantly from 5±5 % to 29.2±6.3 % (P=0.0022). Then, as they cooled from

Table 1. *Effects of repeated thermal cycling on the* CT_{min} *of* Drosophila melanogaster *and on their ability to survive 1 h exposure to −7 °C*

Day of adult life	Time (h)	Temperature (°C)	Survival (%)	CT_{min} (°C)
2	17:00	23	5.0±5.0	7.9±0.2
2	22:00	16	29.2±6.3	6.1±0.1
3	09:00	9	62.5±7.3	6.0±0.1
3	12:30	16	62.5±9.6	6.1±0.1
3	17:00	23	48.3±8.9	6.9±0.2
3	22:00	16	60.0±5.8	6.7±0.1
4	09:00	9	66.7±9.2	5.8±0.2
4	12:30	16	78.3±6.0	6.2±0.1
4	17:00	23	70.0±5.8	5.6±0.1
7	17:00	23	83.3±9.2	6.8±0.2
7	22:00	16	90.0±2.6	6.5±0.2
8	09:00	9	86.7±6.1	5.8±0.1
8	12:30	16	93.3±4.9	NA
8	17:00	23	93.3±3.3	6.9±0.1

CT_{min}, critical thermal minimum.

Survival data are means ± S.E.M. survival of 6–18 tubes each containing 10 flies.

Critical thermal minimum values are means ± S.E.M. for 40–60 flies.

NA, not sampled.

16 to 9 °C (average rate 0.6 °C h^{-1}), their capacity to survive sub-zero treatment again increased significantly (to 62.5±7.3 %; $P<0.0001$ when compared with either the 23 °C or 16 °C groups). Although their cold-hardiness decreased significantly as they were warmed at an average rate of 1.75 °C h^{-1} from 9 to 23 °C ($P=0.026$), they retained much of the cold-hardiness that had accrued during the thermoperiod (48.3±8.9 %).

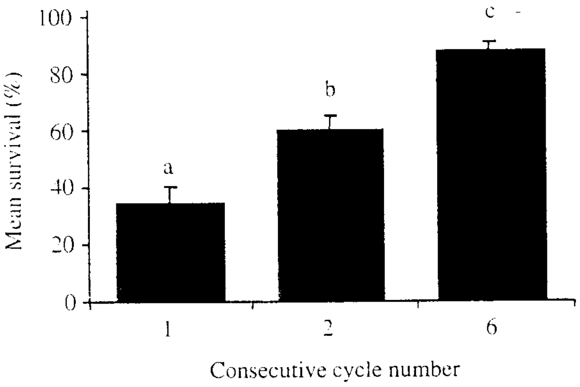

Fig. 2. Effects of repeated thermoperiodic cycling, for up to 7 days, on the cold-tolerance of *Drosophila melanogaster* assessed as survival after 1 h at −7 °C. Each column represents the mean percentage survival + S.E.M. of 45–60 tubes of 10 flies averaged over an entire thermoperiodic cycle. Different letters above the columns indicate that survival levels are significantly different from each other at $P<0.0002$.

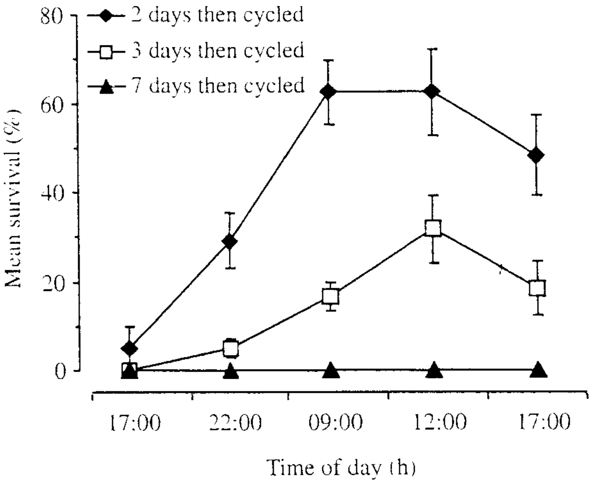

Fig. 3. Effects of age on the capacity of *Drosophila melanogaster* to cold-harden rapidly during an ecologically based thermoperiod. Overall, flies cycled from the beginning of the second day of adult life were significantly better able to survive 1 h of exposure to −7 °C than were flies that began cycling at an older age ($P<0.0001$). Each point represents the mean percentage survival ± S.E.M. of 6–18 tubes each containing 10 adult flies.

Cold-tolerance continued to increase during subsequent thermoperiods over the next 6 days (Fig. 2). By the time *D. melanogaster* had cooled to 16 °C during the cooling phase of the second thermoperiod, their survival after 1 h at −7 °C (60.0±5.8 %) was significantly greater than that of flies cooled to 16 °C during the first thermoperiod (Table 1, $P=0.0033$). Furthermore, the average survival of flies tested during all or part of 2 days of cycling (60.3±4.7 %) was significantly greater than that of flies tested during all or part of the first thermoperiod (35.0±5.0 %, $P=0.0003$, Fig. 2). Overall, flies subjected to all or part of a sixth thermoperiod were significantly more cold-tolerant than those cycled for 1 ($P<0.0001$) or 2 days ($P=0.0001$), exhibiting an average survival rate of 88.3±3.0 % following 1 h of exposure to −7 °C (Fig. 2).

When kept at a constant 23 °C, the capacity of *D. melanogaster* to cold-harden decreased rapidly with age (Fig. 3). Flies cycled beginning when they were 3 days old were significantly less able to survive 1 h at −7 °C than were their counterparts that entered the thermoperiod when 2 days old ($P<0.0001$). If kept at 23° for 7 days before cycling, all adults were killed by 1 h of exposure to −7 °C, regardless of the point at which they had been transferred from the thermoperiod (Fig. 3).

To determine whether gender affected the capacity of *D. melanogaster* to cold-harden rapidly, we compared the survival of males and females transferred at various times during two consecutive thermoperiods to −7 °C for 1 h (Fig. 4). As with the experimental population as a whole, the capacity of each gender to survive a 1 h exposure to −7 °C increased significantly between the first and the second thermoperiod ($P<0.0001$, for both genders). However, males were

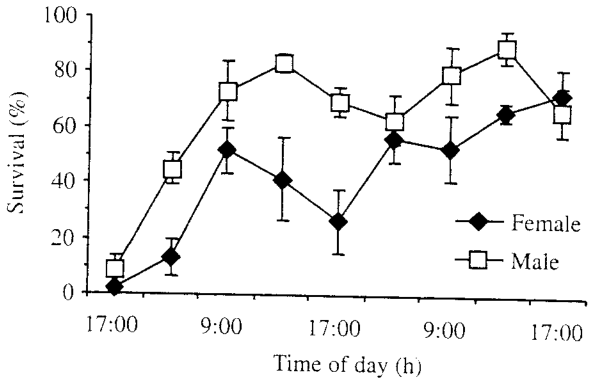

Fig. 4. Effects of gender on the capacity of *Drosophila melanogaster* to cold-harden rapidly during two successive ecologically based thermoperiods. Overall, males were better able to cold-harden rapidly, as reflected in their capacity to survive 1 h at −7 °C (F=22.74, P<0.0001). Each point represents the mean percentage survival ± S.E.M. of 3–9 tubes each containing 10 adult flies.

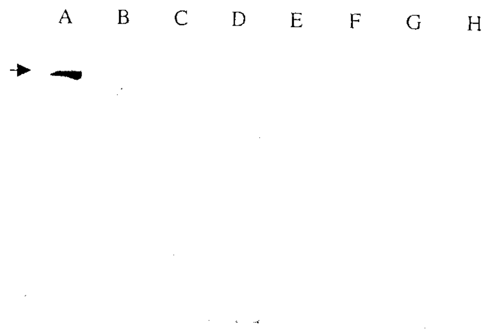

Fig. 5. A representative western blot showing the expression of the inducible 70 kDa heat-shock protein (Hsp70, indicated by the arrow) in heat-stressed *Drosophila melanogaster* (adults were exposed to 37 °C for 90 min; lane A). Hsp70 was not detected in soluble protein extracts from *D. melanogaster* kept at 0 °C for 2 h (lane C) or from those of adults sampled at any time point in the thermoperiod shown in Fig. 1 (lanes D–H). No protein was loaded into lane B.

significantly more tolerant of sub-zero exposure than were females (P<0.0001).

Effect of an ecologically based thermoperiod on CT$_{min}$

The temperature at which an organism enters a state of torpor (i.e. its $CT_{\min}$) is often used as an index of the effect of thermal acclimation on behavioral function (David et al., 1998; Hori and Kimura, 1998; Kelty and Lee, 1999). To determine whether rapid cold-hardening could benefit *D. melanogaster* at temperatures that they would be likely to encounter in nature, we determined whether their $CT_{\min}$ changed during the thermoperiod. During the first thermoperiod, as *D. melanogaster* cooled from 23 to 9 °C, their $CT_{\min}$ decreased significantly from 7.9±0.2 to 6.0±0.1 °C (P<0.0001). However, in contrast to survival after 1 h at −7 °C, $CT_{\min}$ remained unchanged during subsequent thermoperiods (Table 1).

Mechanisms of rapid cold-hardening: role of carbohydrate cryoprotectants

In a number of insects, increased cold-tolerance, including that afforded by rapid cold-hardening, is correlated with the production of cryoprotective substances such as polyhydric alcohols and sugars (Chen et al., 1987; Lee, 1991). High-performance liquid chromatography was therefore used to determine whether carbohydrate cryoprotectants were synthesized during the rapid cold-hardening process in *D. melanogaster*. A number of carbohydrates were found in this species, including glycerol, trehalose, fructose and other presumed, but undetermined, carbohydrates. However, the concentration of each remained approximately the same in flies kept at 23 °C or cooled to 9, 4 or 1 °C for 2 h.

Mechanisms of rapid cold-hardening: Hsp70

Although the induction of stress proteins at high temperature has been extensively studied, little is known of whether they are involved in low temperature tolerance (for a review, see Denlinger and Lee, 1998). Although Hsp70 was expressed in positive controls (flies exposed to 37 °C for 90 min), none was detected by western blot analysis of protein extracts from flies kept at 23 °C, those sampled at each point labeled in Fig. 1 or those exposed to 0 °C (Fig. 5).

Discussion

Lee et al. (Lee et al., 1987) found that rapid cold-hardening, induced by brief exposure to 0 °C, allowed a variety of insects to survive exposures to otherwise lethal sub-zero temperatures. Similarly, Meats (Meats, 1973) demonstrated that when cooled gradually (as quickly as 1 °C min^{-1}), a species of tephritid fruit fly (*Dacus tyroni*) exhibited a lower cold torpor temperature than when cooled abruptly. Both Lee et al. (Lee et al., 1987) and Meats (Meats, 1973) hypothesized that the capacity to acclimate rapidly to low temperature (i.e. to cold-harden rapidly) allows the cold-tolerance of an organism to track short-term changes in environmental temperature, such as those occurring during natural diurnal cooling. Although the most recent of these studies was published over a decade ago, little is known about the ecological relevance of rapid cold-hardening. In the present study, our primary objectives were to determine whether exposure to the cooling phase of an ecologically based thermoperiod would induce rapid cold-hardening in *D. melanogaster* and whether the protection afforded would be retained or lost during the warming phase.

We found that the cold-tolerance of *D. melanogaster* increased significantly during the cooling phase of a single thermoperiod begun on the second day of adult life. During this phase of the cycle, the capacity of *D. melanogaster* to survive exposure to a sub-zero temperature increased significantly, while their $CT_{\min}$ decreased significantly. The decrease in $CT_{\min}$ that we observed over six thermoperiodic cycles (2.1 °C) was similar to that observed previously (Hori and Kimura, 1998) in *Drosophila trapezifrons* acclimated to

15 °C for 16 days (2.3 °C) and to our previous finding that the CT_{min} of *D. melanogaster* cooled at 0.1 °C min^{-1} was 2.6 °C lower than that of flies cooled at 1.0 °C min^{-1} (Kelty and Lee, 1999). Taken together, the survival and CT_{min} data suggest that rapid cold-hardening occurs during thermoperiodic cycling in nature.

The increases in cold hardiness that we observed probably represent a conservative estimate of the capacity of *D. melanogaster* to cold-harden rapidly in nature. Populations of *D. melanogaster* kept in constant conditions exhibit quantitative genetically based differences, such as thermal range, body size and fecundity, from those kept under other conditions (Cavicchi et al., 1985; Morin et al., 1997). The line we used in this study (Oregon-R) has been held in a thermally constant environment (approximately 23 °C) for nearly a century and probably represents a more stenothermal population than those found in nature. As such, the range of temperatures to which this population is able to acclimate is probably narrower than that of natural temperate-zone populations (Somero et al., 1996).

Although prolonged exposure to moderately low or high temperature often increases the cold- or heat-tolerance an organism, respectively (e.g. Levins, 1969; Hori and Kimura, 1998), animals in nature are exposed to temperatures that fluctuate on both a diurnal and a seasonal basis. We found that much of the cold-tolerance gained by *D. melanogaster* during an initial thermoperiod remained during the warming phase of the cycle. Furthermore, the level of protection increased with the number of cycles experienced. Exposure to fluctuating temperatures is an effective means of acclimating insects to low temperature (e.g. Hanec and Beck, 1960; Anderson and Harwood, 1966; Horwath and Duman, 1986). For instance, Hanec and Beck (Hanec and Beck, 1960) found that larvae of the European corn borer (*Ostrinia nubilalis*) subjected to a thermoperiod (12 h at 5 °C alternated with 12 h at 15 °C) for 3 weeks were as tolerant of a 48 h exposure to −20 °C as those acclimated at a constant 5 °C for the same period.

As in the present study, the capacity to acclimate to low or high temperatures often decreases with age (Rose, 1991). Czajka and Lee (Czajka and Lee, 1990) found that, after the fifth day of adult life, the capacity of *D. melanogaster* to cold-harden rapidly at 0 °C decreased significantly. Dahlgaard et al. (Dahlgaard et al., 1995) demonstrated that a 75 min exposure to 36.5 °C increased the capacity of young *D. melanogaster* to survive exposure to 40.7 °C much more than it did that of older flies. Although frequently observed, it remains unclear why the capacity of *D. melanogaster* to acclimate to low or high temperature decreases with age.

Neither Hsp70 nor carbohydrate cryoprotectants contributed to the capacity of *D. melanogaster* to cold-harden rapidly. The absence of Hsp70 expression during cooling is consistent with the finding that neither the mRNA coding for *Drosophila* Hsp70 nor that coding for a 23 kDa stress protein expressed by *Sarcophaga crassipalpis* in response to chilling is expressed by *D. melanogaster* at any time during the thermoperiod tested (J. Rinehart, personal communication). The absence of discernible carbohydrate cryoprotectant synthesis by *D. melanogaster* in the present study extends our previous finding that glycerol was not synthesized by this species during rapid cold-hardening (Kelty and Lee, 1999). However, this finding contrasts with data from *S. crassipalpis*, in which rapid cold-hardening is correlated with a threefold increase in hemolymph glycerol concentration (Chen et al., 1987; Lee, 1991).

Additional mechanisms may participate in rapid cold-hardening, including other stress proteins. For instance, in plants, a group of small heat-shock proteins is correlated with the acquisition of cold-tolerance (Sabehat et al., 1998). Another possibility is that, during rapid cold-hardening, the composition of lipid membranes (e.g. cell membranes, endoplasmic reticulum) is altered such that these membranes maintain their fluidity over a wider range of temperatures than would otherwise be the case. Such homeoviscous adaptation has been described in a variety of invertebrates and vertebrates during both long- and short-term acclimation (Carey and Hazel, 1989; Hazel, 1995; Ohtsu et al., 1998).

Much remains to be learned about the mechanistic basis and ecological relevance of rapid cold-hardening. Nonetheless, our previous report (Kelty and Lee, 1999) and data presented here indicate that, in nature, short-term cooling such as occurs during natural thermoperiods induces rapid increases in cold-tolerance. The importance of this phenomenon in nature is probably not to increase the capacity of *D. melanogaster* to survive exposure to sub-zero temperatures on a diurnal basis since rarely, if ever, would a temperate-zone fly encounter temperatures ranging from 23 to below 0 °C over the course of a single day. Rather, this process may benefit the organism by producing more subtle effects during less severe cooling to temperatures that this species frequently encounters. A good example of such an effect is the rapid increase in CT_{min} that occurs when *D. melanogaster* are cooled gradually rather than abruptly (Meats, 1973; Kelty and Lee, 1999). If future studies employ gradual cooling at ecologically relevant rates, then even more subtle effects at multiple physiological levels may be identified in flies cooled to even less severe low temperatures.

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