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A RAPID COLD-HARDENING RESPONSE PROTECTING AGAINST COLD SHOCK INJURY IN *DROSOPHILA MELANOGASTER*

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Summary

In studies of insect cold-hardiness, the supercooling point (SCP) is defined as the temperature at which spontaneous nucleation of body fluids occurs. Despite having an SCP of −20°C, adults of *Drosophila melanogaster* did not survive exposure to −5°C, which suggests that cold shock causes lethal injury that is not associated with freezing. If, however, flies were chilled at 5°C, for as little as 30 min, approximately 50 % of the flies survived exposure to −5°C for 2 h. This capacity to cold-harden rapidly was greatest in 3- and 5-day-old adults. The rapid cold-hardening response was also observed in larvae and pupae: no larvae survived 2 h of exposure to −5°C, whereas 63 % pupariated if chilled at 5°C before subzero exposure. Similarly, although exposure of pupae to −8°C was lethal, if pre-chilled at 5°C 22 % eclosed. This extremely rapid cold-hardening response may function to allow insects to enhance cold-tolerance in response to diurnal or unexpected seasonal decreases in environmental temperature.

Introduction

Overwintering insects rely on a variety of physiological and biochemical adaptations to survive low temperature exposure (Baust and Rojas, 1985; Cannon and Block, 1988; Duman and Horwath, 1983; Lee, 1989; Ring, 1981; Storey and Storey, 1988; Zachariassen, 1985). These include the accumulation of glycerol and trehalose, and other low molecular weight polyols and sugars; the synthesis of antifreeze proteins and ice-nucleating agents; and changes in whole-body supercooling points (SCP), the temperature at which spontaneous nucleation of body fluids occurs (Salt, 1961). A species is described as freeze-tolerant if it survives below the SCP and freeze-intolerant if it does not survive extracellular ice formation. Although the SCP is sometimes used as a measure of the lower lethal

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temperature for freeze-intolerant species, lethal injury may occur at temperatures 10–15°C above the SCP (Ring, 1980; Lee and Denlinger, 1985; Knight *et al.* 1986; Bale, 1987).

Generally, studies of insect cold-hardiness have focused on the capacity for low-temperature survival for extended periods during the winter (Baust and Rojas, 1985; Somme, 1982; Turnock *et al.* 1983; Cannon and Block, 1988). Cold-hardening in preparation for winter is triggered by exposure to low environmental temperatures (Baust and Lee, 1982) or, sometimes, photoperiodic cues (Horwath and Duman, 1982). In contrast, recent reports describe a novel rapid cold-hardening response that occurs within minutes, even in non-overwintering insects, and protects against a form of non-freezing injury called cold shock (Chen *et al.* 1987; Lee *et al.* 1987). Cold shock, thermal shock or direct chilling injury is a form of cellular injury which occurs after rapid cooling, but in the absence of ice formation (Morris *et al.* 1983). Cold shock occurs in bacteria, protozoa, algae, fungi, higher plants, fish and mammalian somatic cells and spermatozoa (Morris and Watson, 1984).

The rapid cold-hardening response is distinctly different from winter cold-hardening (Lee, 1989). Winter cold-hardening is a seasonal phenomenon that generally occurs in an inactive or diapausing stage, while the rapid cold-hardening response occurs even in feeding and reproductively active insects throughout the year. Studies of overwintering cold-hardiness typically classify insects as being freeze-tolerant or not, based on their ability to survive cooling to below the SCP. In contrast, the rapid cold-hardening response occurs at temperatures 10–15°C above the temperature at which body fluids freeze. In preparation for winter the cold-hardening process requires days or weeks, while the rapid cold-hardening process associated with cold shock involves only minutes or hours.

We report on the presence of the rapid cold-hardening response protecting against cold shock in the fruit fly *D. melanogaster*. Understanding the underlying mechanisms of survival of this dipteran at subzero temperatures is of particular importance since, at present, there is no system for the long-term cryopreservation of individuals of this species. The ability to cryopreserve *Drosophila* would save on the high cost of maintaining the many thousands of strains of this insect, as well as reduce the chance of stock loss due to either human error or change in genetic composition.

This study investigated the phenomenon of cold shock and rapid cold-hardening in larval, pupal and adult stages of *D. melanogaster*. Our specific objectives were: (1) to determine the lower lethal temperature and its relationship to the supercooling point, (2) to examine the effect of chilling at 5°C on survival of subzero exposure, and (3) to investigate the effect of adult age on the capacity to cold-harden rapidly.

Materials and methods

Insect rearing

Drosophila melanogaster (Oregon R strain) (Diptera: Drosophilidae) were

maintained at 23±1°C, 75 % relative humidity and L: D 12 h: 12 h in half-pint milk bottles. They were provided with *Drosophila* medium (Formula 4–24, Carolina Biological Supply) and yeast.

Supercooling point (SCP)

A 36 gauge copper–constantan thermocouple was positioned next to the insect to determine the SCP. Insects were cooled at a rate of approximately 0.1 °C min^{-1} using a refrigerated bath. The SCP was recorded as the lowest temperature reached prior to the release of the latent heat of crystallization as body water freezes (Lee and Denlinger, 1985).

The SCPs were determined for actively crawling larvae (approximately 4–5 days old) and for pupae, 2–4 days post-pupariation. To load adults into the SCP apparatus it was necessary to anesthetize them lightly for 2–10 min using a cryolizer (Bioquip Products). This alternative approach to standard etherization methods was used because ether is reported to increase the sensitivity of *D. melanogaster* to low temperature (Novitski and Rush, 1949).

Lower lethal temperature

The lower lethal temperature (LLT) for *D. melanogaster* was determined by directly transferring flies from 23 °C to zero or sub-zero test temperatures for 2 h. The highest temperature at which no fly survived 2 h of exposure was defined as the lower lethal temperature (LLT). The flies were held in plastic test tubes (1.0 cm×7.5 cm) that were subsequently placed in dry test tubes immersed in a refrigerated bath. The exposure temperature was recorded using a digital thermometer (Sensortek, Inc. model BAT-12) inserted into the plastic test tubes. All flies were returned to 23 °C and survival rates recorded 8 h later. Adult flies that were able to fly, walk or stand were scored as alive. Emergence was the survival criterion for pupae, while the incidence of pupariation was used for larvae.

Rapid cold-hardening response

The following protocol was based on the work of Chen *et al.* (1987) with *Sarcophaga crassipalpis*. Using the survival data from the LLT tests of *D. melanogaster*, we determined that −5 °C was lethal for adults, and this temperature was chosen as the basis for the cold-hardening experiment. The control group was exposed to −5 °C for 2 h. A chilled group was subjected to 5 °C for 2 h, followed by a 2-h exposure to −5 °C to determine if chilling prior to subzero exposure increased survival, compared with flies directly subjected to −5 °C. In separate experiments varying exposure periods at 5 °C were used to determine the effect of the duration of pre-chilling on survival at −5 °C.

Age factor

We investigated whether the age of the adult fruit fly affected its capacity to supercool or cold-harden rapidly. Although the adult fruit flies were the primary

focus of this study, pupae and larvae were also tested for SCP, LLT and their potential to cold-harden rapidly.

Statistical treatment

Statistical analyses were made using analysis of variance (ANOVA) (SAS, 1982). All percentage survival data were arcsin-transformed before analysis. Mean separations were determined using Duncan's multiple range test at a predetermined significance level of 0.05.

Results

The SCPs for *D. melanogaster* larvae, pupae and adults were in the range −17 to −20°C (Fig. 1). The SCPs remained relatively constant for adults aged 1–28 days. Although the larvae had the highest SCP by rank order, no obvious trend was evident with regard to developmental stage. No individual at any stage of development survived cooling to below the SCP. The use of a cryolizer as a means of cold immobilization had no statistically significant effect on the supercooling point.

The lower lethal temperature (LLT) was defined as the temperature at which no individual survived a 2-h exposure. No adult survived exposure to −5°C (Fig. 2). Surprisingly, however, raising the temperature to −4°C resulted in nearly 100% survival. The lower lethal temperature of pupae was −8°C, while no larvae survived 2 h at −5°C (Fig. 2).

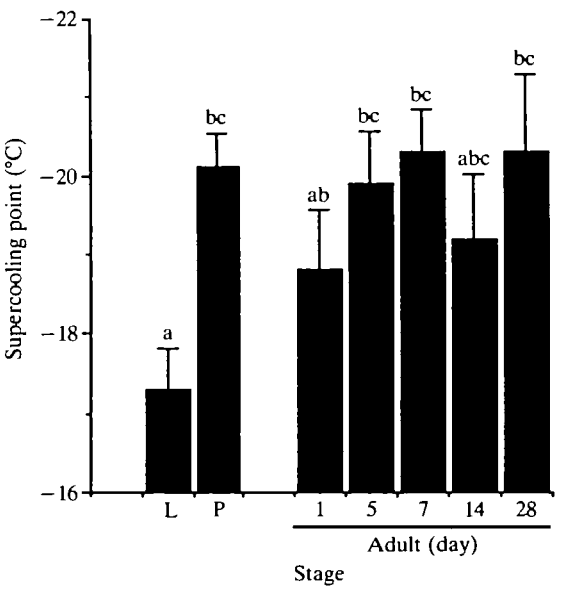

Fig. 1. Supercooling points for larvae (L), pupae (P) and adults (1–28 days old) of *Drosophila melanogaster*. Each column represents the mean±S.E.M. for 10–14 flies. No individual survived below the SCP. SCPs with the same letter are not significantly different at $a=0.5$ (Duncan's multiple range test).

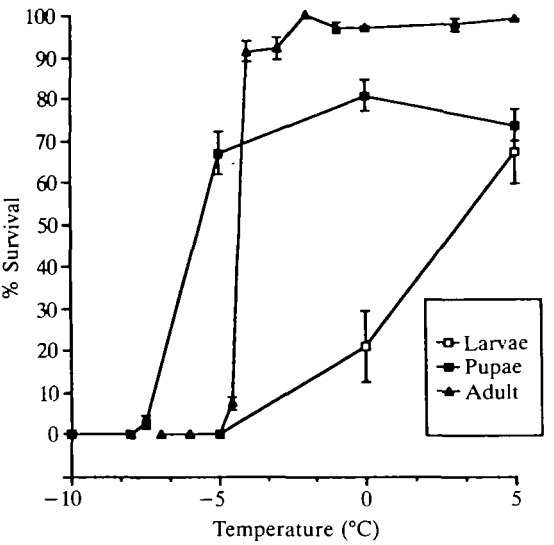

Fig. 2. The low-temperature tolerance profile of *Drosophila melanogaster*. Flies were transferred directly from 23°C to a given temperature for 2 h. Adults able to stand, walk or fly 8 h later were scored as alive. Survival was based on the incidence of emergence for pupae and the incidence of pupariation for larvae. Each point represents the mean±S.E.M. survivorship of 10 replicates of 10–25 flies each.

Although 5-day-old adults did not survive exposure to −5°C, if 2 h of chilling at 5°C preceded the −5°C exposure, the rate of survival increased dramatically to approximately 70% (Fig. 3). One hour of chilling at 5°C prior to exposure to −5°C markedly increased survival to about 90%, while as little as 30 min of chilling increased the rate of survival to more than 50% (Fig. 4).

The capacity to cold-harden rapidly varied with the age of the adult (Fig. 5). Flies ranging in age from 1–30 days old were tested. Over 70% of 3- to 5-day-old flies survived 2 h at −5°C when they had previously been chilled at 5°C (Fig. 5). One-day-old flies were less able to cold-harden rapidly than 3- to 5-day-old adults. The capacity to cold-harden rapidly was variable and survival rates were lower among older individuals.

The effect of gender on the capacity to cold-harden rapidly was also examined. In general, males and females were equally capable of surviving 2 h at −5°C when pre-chilled at 0°C (Student's *t*-test, $P>0.05$). Although no quantitative studies were made to evaluate reproductive function in the rapidly cold-hardened flies, these individuals produced offspring following treatment with no evidence of impaired reproductive function.

Pupae and larvae were also able to cold-harden rapidly (Fig. 3). Chilling at 5°C for 2 h prior to exposure to subzero temperatures significantly increased survival in these developmental stages. Survival was defined as the ability to reach the subsequent stage of development. Larvae did not survive a 2-h exposure to −5°C, yet they were able to pupariate 63% of the time if chilled at 5°C (for 2 h) before

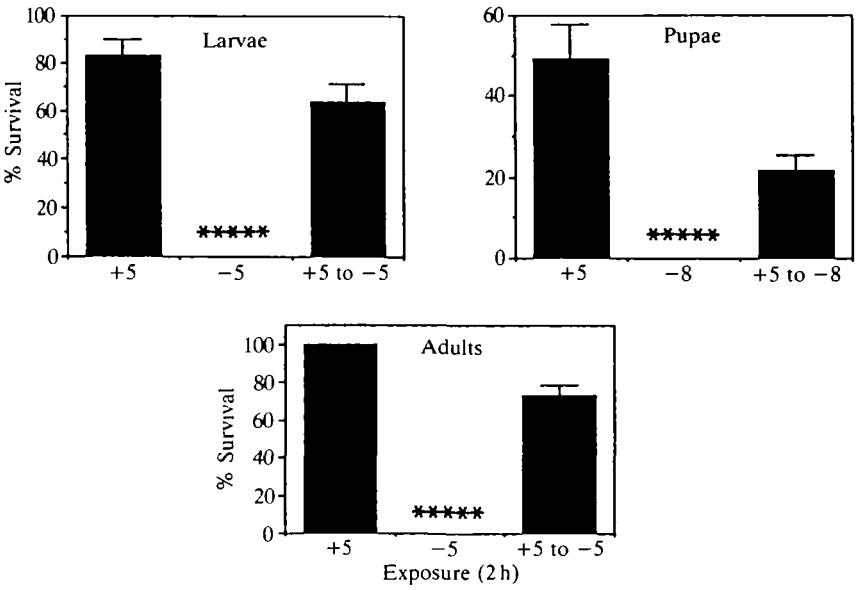

Fig. 3. The effect of rapid cold-hardening on survival at subzero temperatures for *Drosophila melanogaster*. Control treatments included those flies subjected to a lethal temperature (−5 or −8°C) or the pre-chilling temperature (+5°C) for 2 h. The experimentally treated groups (+5 to −5, or +5 to −8) were subjected to both the chilling and lethal temperatures for 2 h each. Survival for larvae and pupae was based on their ability to develop into the succeeding stage. Adults able to stand, walk or fly 8 h later were scored as alive. Adults are represented by 5-day-old flies. Each bar represents the mean±S.E.M. for 3–8 replicates of 10 flies each. Zero percent survival is indicated by *****.

the subzero exposure. This result was a substantial increase, considering that 83 % pupariated when held at 5°C for only 2 h (Fig. 5). Similarly, all pupae died after 2 h at −8°C, but 22 % eclosed if pre-chilled at 5°C.

Discussion

In some insects the SCP is a measure of the lower lethal temperature, but in *D. melanogaster* we found significant mortality at temperatures far above the SCP (Fig. 2). The SCPs of larvae, pupae and adults of *D. melanogaster* were in the range −17 to −20°C (Fig. 1). These results are consistent with the data of previous studies of cold tolerance in drosophilid species (Tucic, 1979; Krunic *et al.* 1980; Enomoto, 1981). However, in adults and larvae a 2-h exposure to −5°C was lethal, and −8°C was the lower lethal temperature for pupae. These data indicate that mortality was due to cold shock, since the insects were cooled rapidly and spontaneous ice formation in extracellular fluids did not occur until approximately 15°C lower (Chen *et al.* 1987; Lee *et al.* 1987).

The onset of mortality due to low temperature exposure in the adult fruit fly wa

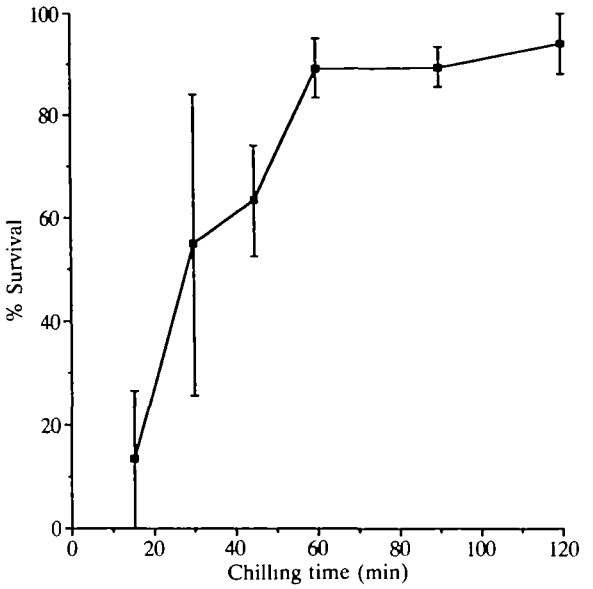

Fig. 4. The effect of variable chilling time at 5°C on rapid cold-hardening in adult *Drosophila melanogaster*. Flies were subjected to 5°C for a given time and then directly transferred to −5°C for an additional 2 h. Each point represents the mean±S.E.M. survivorship of three replicates of 10 adult flies each (age 1–5 days).

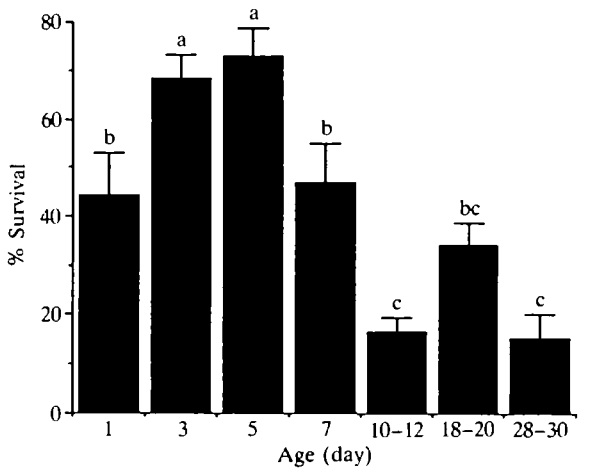

Fig. 5. The effect of the age of adult *Drosophila melanogaster* on their capacity to cold-harden rapidly. Adult flies were held at 5°C for 2 h then directly transferred to −5°C for an additional 2 h. Each bar represents the mean±S.E.M. survivorship of 16 replicates of 10 flies each. Means with the same letter are not significantly different at a=0.05 (Duncan's multiple range test).

particularly abrupt. Although more then 90 % of the flies survived 2 h of exposure to −4°C, all died when exposed to −5°C for the same interval. The rapid decline in survivorship within a 1°C increment demonstrates how suddenly pre-freeze mortality can occur. Previous studies describe similar ranges of lower lethal temperatures for *Drosophila* (Novitski and Rush, 1949; Chiang *et al.* 1962).

The rapid cold-hardening response observed in *D. melanogaster* occurred within minutes. As little as 30 min at 5°C was sufficient to stimulate the rapid physiological adjustment that enabled adult flies to survive subzero exposure. After 90 min of chilling nearly all flies recovered from the −5°C treatment (Fig. 4). Adults aged 3–5 days old exhibited the greatest capacity for rapid cold-hardening. Pupae and larvae, as well as adults, were able to cold-harden rapidly and completed the subsequent developmental stage. Two hours of chilling enabled approximately 63 % of larvae to pupariate following exposure to subzero temperatures. Similarly, pupae did not survive 2 h at −8°C, but when pre-chilled (2 h at 5°C) 22 % were able to develop into adults (Fig. 4).

The capacity for rapid cold-hardening appears to be widespread among insects as a means of protection against cold shock. Chen *et al.* (1987) first described this response in larvae, pupae and pharate adults of both diapause and nondiapause flesh flies, *Sarcophaga crassipalpis*. This extremely rapid cold-hardening response occurs in non-overwintering stages of a beetle, *Xanthogaleruca luteola*, a true bug, *Oncopeltus fasciatus,* and another fly, *Sarcophaga bullata* (Lee *et al.* 1987). Meats (1973), in a study of a tephritid fruit fly, examined temperature acclimitization as it pertains to thresholds for torpor and flight. He reported that *Dacus tryone* was capable of rapid acclimation, even at cooling rates as fast as $1\,^{\circ}C\,min^{-1}$.

D. melanogaster is a cosmopolitan species which inhabits all biogeographic realms (David *et al.* 1983). This species is reported to be the most resistant to the environmental stresses of high-temperature desiccation and low temperature among six species of the *melanogaster* subgroup (Stanley *et al.* 1980). *D. melanogaster*, the most common fruit fly, is widely used as an experimental organism for the study of genetics: yet, it is not known how this organism overwinters under natural conditions in temperate regions (David *et al.* 1983).

Our project focused on a relatively short time in which *D. melanogaster* was exposed to subzero temperatures. Yet, the fact that all stages tested could cold-harden rapidly may offer insight into the overwintering biology of this species. From the data presented here, it appears that larvae, pupae and adult fruit flies are all likely candidates for the overwintering stage. Tucic (1979) found the adult fly to be most cold resistant, but added that the other stages (including the egg) are potentially capable of surviving moderately severe winter conditions, as all stages have the genetic capacity for cold resistance adaptation.

Lakovaara *et al.* (1972) report that three species of *Drosophila* in northern Scandinavia overwinter in an adult diapause. Reproductive diapause is characteristic of four strains of fruit flies in the *D. auratia* complex which inhabit the main islands of Japan (Kimura, 1984). *D. melanogaster*, in contrast, does not appear to overwinter in diapause. Rather, it is suggested that this fruit fly passes the winter

months in a state of quiescence (McKenzie, 1975). The rapid cold-hardening response described here would enhance low-temperature tolerance in overwintering *D. melanogaster* and would also minimize mortality during unseasonably low temperatures in the autumn and spring.

In an ecological context the capacity to cold-harden rapidly, and thereby avoid injury due to cold shock, may be important for survival at any time of the year. In north temperate regions even non-overwintering developmental stages may be exposed to diurnal fluctuations in temperature of 20 to 30°C. The capacity to cold-harden rapidly may be particularly important to minimize mortality during periods of unseasonably low temperature during the spring and autumn. These data suggest that this extremely rapid acclimation response may allow insects to enhance cold tolerance as they track decreases in their environmental temperatures (Lee *et al.* 1987). It would be useful in future studies to determine whether slower cooling rates, which often occur under natural conditions, also enhance cold-hardening in *Drosophila*.

Freeze tolerance in diapausing larvae of another member of the Drosophilidae, *Chymomyza costata*, has recently been reported (Ṣhimada and Riihimaa, 1988). When acclimated for a month at 0–10°C, freeze-inoculated and cooled slowly, this fly survives freezing to approximately −80°C. Findings such as this, in addition to those reported here on *D. melanogaster*, make it necessary to re-evaluate the potential of 'freeze-intolerant' species to survive subzero exposure. In addition, information such as this may be applicable to the development of methods for the cryopreservation of this species.

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