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COLD-SHOCK INJURY AND RAPID COLD HARDENING IN THE FLESH FLY *SARCOPHAGA CRASSIPALPIS*¹

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Direct exposure to -10 C , in the absence of tissue freezing, causes high mortality in *Sarcophaga crassipalpis*: this result suggests that injury is due to cold shock. However, brief acclimation at 0 C enables larvae, pupae, and pharate adults of *Sarcophaga crassipalpis* to survive -10 C . Chilling for as short a period as 10 min enabled 50% of the flies to survive a 2-h exposure to -10 C . Enhancement of cold tolerance was linear over the first hour of chilling at 0 C . The optimal temperature range eliciting the rapid acclimation response was $6-0\text{ C}$, but the effect could also be stimulated by high temperature (36 C). The rapid increase in cold tolerance correlates with concomitant increases in hemolymph osmolality and glycerol levels. This response suggests a novel role for glycerol in protecting insects against injury resulting from cold shock, although other unidentified mechanisms may be involved in this response. That both nondiapauses- and diapause-programmed flies respond to short-term chilling indicates that this rapid response is not part of the diapause syndrome but probably functions in either type of fly as an adaptation to survive brief periods of low temperature.

INTRODUCTION

Cold shock is a form of cellular injury observed immediately after rapid cooling but in the absence of ice formation in extracellular fluids (Morris et al. 1983). This phenomenon is also referred to as thermal shock or direct-chilling injury. Cold shock is distinct from indirect-chilling injury, which occurs after long-term exposure—days or weeks—to low temperatures (Levitt 1980). The extent of cold-shock injury increases with higher rates of cooling and the absolute limit of low-temperature exposure. Although not generally accepted as a widespread cellular response to chilling, recently it has been argued that cold shock may be a significant, but unrecognized, factor

causing injury in cells during freezing (McGrath 1985).

Studies of cold hardening in insects have generally focused on cold tolerance and survival above and below the temperature at which spontaneous nucleation of body water occurs, termed “the supercooling point (SCP)” (Baust and Lee 1982). Based on this criterion, a species is categorized as freeze-tolerant or freeze-intolerant. The cold-hardening process may include changes in whole-body SCPs, the accumulation of low molecular weight polyols and sugars, and the synthesis of thermal hysteresis factors and ice-nucleating agents (see reviews by Baust 1981; Duman and Horwath 1982; Zachariassen 1985). To study the dynamics of the cold-hardening process, most investigators have used relatively long periods of acclimation to low temperature lasting more than 1 day, or more commonly, weeks (Ring 1981; Lee and Baust 1985). But, in this study of *Sarcophaga crassipalpis*, we find that a very short (10 min–2 h) exposure to low temperature has a dramatic effect in allowing flies, even nondiapausing ones, to survive subzero temperatures.

Sarcophaga crassipalpis does not tolerate tissue freezing at any stage of development (Lee and Denlinger 1985). The SCP in both diapausing and nondiapausing pupae is around -23 C , but diapausing pupae are

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able to survive temperatures near the SCP only after being in diapause several weeks. Nondiapausing pupae, although they have the same low SCP, are unable to survive temperatures approaching the SCP (Lee and Denlinger 1985).

This study focuses mainly on the pharate adult stage of nondiapausing flesh flies and describes the effect of brief periods of chilling at 0 C on enhancing the fly's capacity to survive at lower temperatures (-10 C). Several other developmental stages, including flies programmed for diapause, are also examined. We test the possibility that cryoprotectant levels may rise rapidly in response to short-term, low-temperature exposure.

MATERIAL AND METHODS

INSECT REARING

A colony of the flesh fly, *Sarcophaga crassipalpis* Macquart, was maintained in the laboratory as described by Denlinger (1972). Parental adults were reared at 25 C with either a diapause-inducing photoperiod (12L:12D) or a nondiapausing photoperiod (15L:9D). Larvae and pupae were kept either at 20 or 25 C under the maternal photophase. Short-day conditions at 20 C produce a high incidence (>95%) of pupal diapause. The developmental status of each pupa was determined by removing the anterior portion of the puparium and looking for signs of antennal formation and the eye-pigmentation characteristics of pharate adult development (Fraenkel and Hsiao 1968).

DEVELOPMENTAL STAGES

At 20 C, larvae feed for about 7 days and then leave the food as third instar larvae and enter a wandering phase that lasts 4 days for nondiapausing larvae and 6 days for diapause-destined larvae. Pupation occurs 4 days after pupariation, and, if diapause intercedes, development is halted at the stage of the phanerocephalic pupa. In nondiapausing pupae, pigmentation is visible 14 days after pupariation, and adults emerge around day 21. At 25, pupariation occurs 4 days earlier, and the time from pupariation to adult eclosion is 9 days less. Diapause can be maintained at 20 C for more than 120 days, but some individuals start breaking diapause after 60 days.

LOW-TEMPERATURE EXPOSURE

Pupae used for low-temperature exposure were placed in test tubes (10 × 1.5 cm). Each treatment consisted of three replicates of 15–20 pupae each. After exposures of various durations to chilling temperatures (0 and/or -5 ± 1 C), pupae were exposed to temperatures of -10 C or below using a Lauda RMT-20 (Brinkmann) low-temperature bath filled with water and ethylene glycol (1:1). All pupae were then returned to 26 C until adult emergence.

CRYOPROTECTANT DETERMINATION

Low molecular weight polyols were analyzed by high performance liquid chromatography (Waters Associates) as described by Lee et al. (1983). Samples were stored in a freezer at -40 C before analysis. For each extraction, two specimens were weighed and homogenized in 3 ml of methanol in a Teflon-glass tissue homogenizer for two 20-s intervals. The homogenizer was rinsed with 2 ml of methanol and the sample centrifuged at 2,000 g for 5 min. The supernatant was transferred to a clean sample tube, and the pellet was reextracted two more times with 3 ml of methanol. The pooled supernatant was forced through a prewashed (2 ml methanol and 2 ml distilled water) Sep-Pak C₁₈ cartridge and evaporated to dryness using a Reacti-Vap evaporator (Price Chemical Co.) with low heat and compressed air for 3 h. The sample was then resuspended in a 0.5-ml ethanol:water mixture (1:1) and filtered through a 0.22-μm filter and analyzed. Glycerol concentrations were expressed in mM units based on water-content data (Adedokun and Denlinger 1985) reported for corresponding developmental stages of the same species.

MELTING POINT DETERMINATION

Hemolymph melting points were measured with a nanoliter osmometer (Clifton Technical Physics) using the method described by Frick and Sauer (1973). Standard osmolar concentrations and distilled water were used with each sample platform. During the melting process, temperature was slowly increased until only a single crystal was visible. Readings in mosmolars were transformed to melting points using a

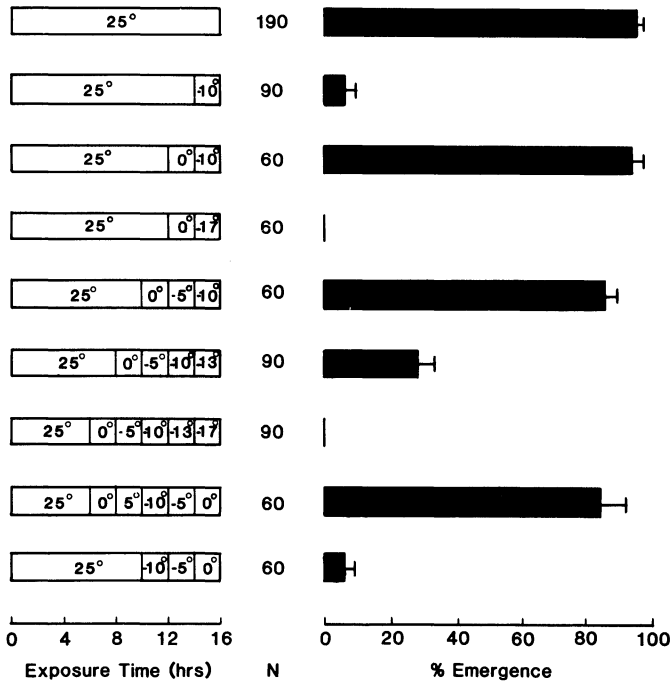


FIG. 1.—Effect of short-term chilling on cold tolerance of *Sarcophaga crassipalpis*. All samples were reared under the nondiapause program at 15L:9D and 25 C and tested as pharate adults (red-eye stage). After exposure to -10 C for 2 h, survivorship to the adult stage is expressed as mean ± SE of at least three replicates consisting of 20 flies each.

melting point depression of 1.86 C per 1.0 osmole.

RESULTS

EFFECTS OF SHORT-TIME CHILLING ON SURVIVAL

When nondiapause pharate adults (red-eye stage) were transferred directly from 25 to -10 C for 2 h, very few survived until adult emergence (fig. 1). In contrast, flies were highly tolerant of a 2-h exposure to -10 C if they first experienced a 2-h acclimation period of 0 C. Some flies (38%) could even tolerate a 2-h exposure to -13 C if they were first acclimated by 2-h exposure to 0, -5, and -10 C. However, short periods of chilling did not enable the flies to survive a temperature of -17 C.

The data of figure 1 indicate that survival at -10 C is enhanced only by short-term chilling that precedes exposure to -10 C, rather than exposure to -5 or 0 C, which occurs after the -10 C treatment. Thus, a short-term warm-up following exposure to -10 C cannot reduce cold injury.

MINIMUM EFFECTIVE DURATION OF CHILLING

To determine the minimum duration of chilling required to enhance survival at -10

C, pharate adults were exposed to 0 C for different intervals and then transferred to -10 C for 2 h. A 10-min exposure to 0 C was sufficient to permit 50% of the flies to survive -10 C, and exposure of 1 h or more at 0 C permitted nearly all flies to survive -10 C (fig. 2). The increase in survivorship was nearly linear for the first 30 min of exposure to 0 C. The relationship between chilling time and log increase in survival over the first 60 min is defined by the

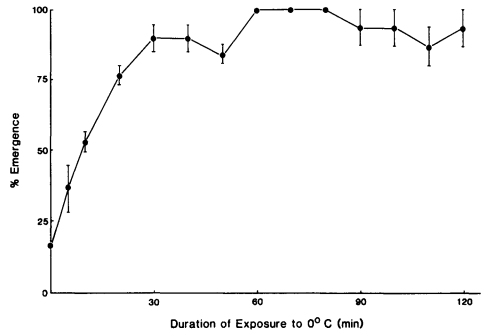


FIG. 2.—The relationship between cold tolerance for 2 h at -10 C and duration of prior exposure to 0 C. Larvae and pupae were maintained at 20 C and tested as pharate adults (red-eye stage). $\bar{X} \pm SE$, survivorship of three replicates containing 20 flies each.

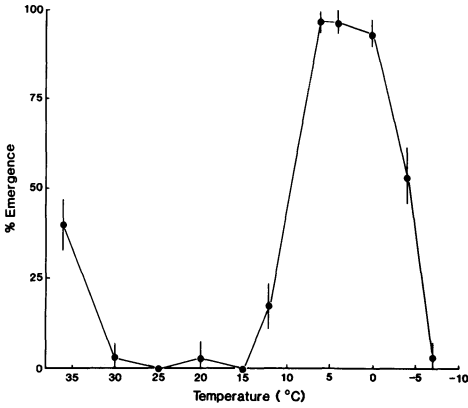


FIG. 3.—Effects of different temperatures (2-h exposure) on the capacity of pharate adults (red-eye stage) of *Sarcophaga crassipalpis* to survive a 2-h exposure to -10°C . Flies were reared under the nondiapaue program at 15L:9D, 25 C prior to the experiment. $\bar{X} \pm \text{SE}$, survivorship of three replicates containing 15 flies each.

regression equation $y = 0.654 - 0.009x$; $r = -0.978$.

OPTIMAL CHILLING TEMPERATURE

The above experiment used 0°C as the stimulant for enhancing survival at -10°C . In this experiment, a range of temperatures

was tested: pharate adults (red-eye stage) reared at 25 C were exposed for 2 h to temperatures from 36 to -7°C and then transferred to -10°C for 2 h. A temperature range between 6 and 0°C was most effective (fig. 3). Temperatures ranging from 12 to 30 C had little or no effect in enhancing survival at -10°C , but, surprisingly, a 2-h exposure to 36 C enabled 40% of the flies to survive a 2-h exposure at -10°C .

EFFECT OF GRADUAL COOLING

To test the effect of gradual cooling versus an abrupt temperature drop, flies were cooled from 25 to -10°C gradually (0.54 C/min) or in abrupt steps (fig. 4). Only 4.5% of the flies survived a 2-h exposure to -10°C following a gradual temperature decrease from 25 to -10°C . Survival at -10°C was directly related to the absolute amount of previous exposure to 0°C and was not further enhanced by a gradual transition.

DEVELOPMENTAL EFFECTS

The above experiments focused on pharate adults (red-eye stage) reared under nondiapaue conditions (25 C; 15L:9D). In this experiment, we evaluated the effect of short-

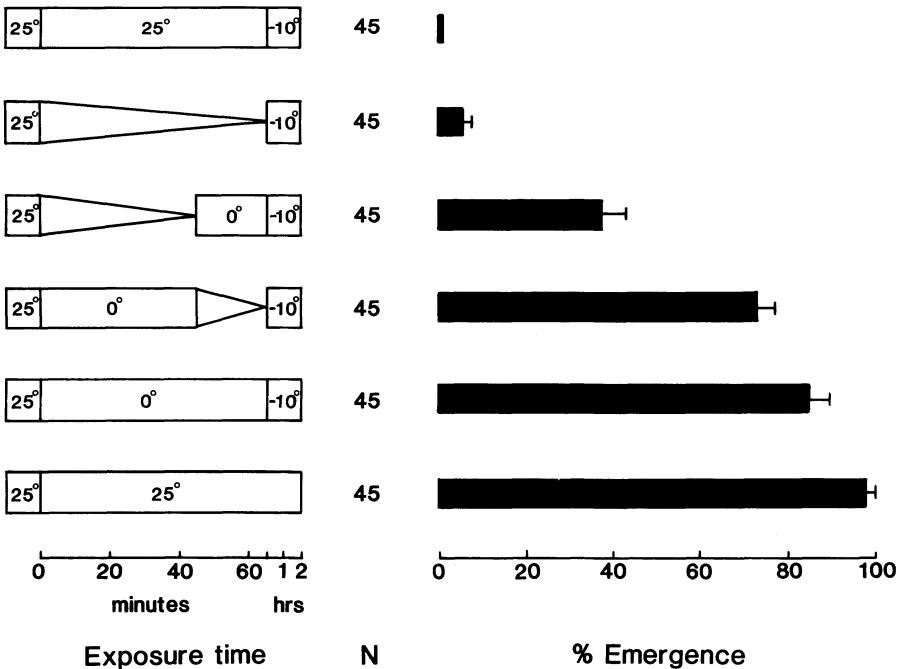


FIG. 4.—Effects of abrupt and gradual chilling on cold tolerance (2-h exposure to -10°C) in pharate adults (red-eye stage) reared at 15L:9D, 25 C. $\bar{X} \pm \text{SE}$, survivorship of three replicates. Gradual chilling represented by converging lines.

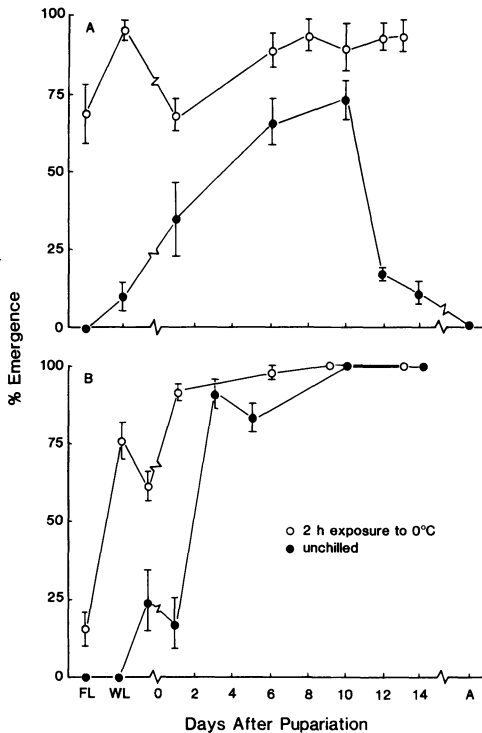


FIG. 5.—Developmental changes in the ability of *A*, nondiapause- and *B*, diapause-programmed flies to respond to chilling for 2 h at 0 C. Prior to chilling, flies were maintained at 20 C. Following chilling, flies were exposed to -10 C for 2 h and $\bar{X} \pm SE$ survivorship was recorded for three replicates of 15–20 flies each. FL = feeding larvae, WL = wandering larvae.

term chilling on other developmental stages of nondiapause flies and also on flies programmed for diapause. A 2-h period of

chilling at 0 C consistently enhanced the ability of nondiapause flies to survive a 2-h exposure to -10 C (fig. 5A). The effect of chilling was most dramatic in increasing survivorship of third instar larvae and advanced stages of pharate adult development. The pupal stage (about 2 days after pupariation) and the early phase of pharate adult development are more tolerant of direct exposure to -10 C, but, even during these stages, survivorship is greatly enhanced by a 2-h pretreatment at 0 C.

Among flies programmed for diapause (fig. 5B), a 2-h exposure to 0 C again enhanced survival at -10 C. The effect was most apparent prior to and shortly after pupariation. Within 4–5 days after pupariation, the fly is in the phanerocephalic pupal stage characteristic of diapause, and, from this time onward, diapausing pupae were already quite cold tolerant and fully capable of surviving at -10 C without pretreatment at 0 C (Lee and Denlinger 1985).

CHANGES IN GLYCEROL CONCENTRATIONS AND HEMOLYMPH OSMOLALITY

Glycerol appears to be the major low molecular weight cryoprotectant used by *Sarcophaga* (R. E. Lee and D. L. Denlinger, unpublished data). A 2-h exposure to 0 C was adequate to significantly elevate glycerol levels 2–3-fold compared to unchilled controls for all developmental stages tested (table 1).

TABLE 1
EFFECT OF SHORT-TERM CHILLING FOR 2 h at 0 C ON TISSUE CONCENTRATIONS OF GLYCEROL IN *Sarcophaga crassipalpis*

DEVELOPMENTAL STAGES	REPLICATES (N)	GLYCEROL CONCENTRATIONS	
		$\mu\text{g}/\text{mg}$ Wet Weight ($\bar{X} \pm SE$)	mM ($\bar{X} \pm SE$)
Wandering larvae (long day):			
No chilling	3	1.2 \pm .4	18.2 \pm 3.7
2 h at 0 C	3	2.4 \pm .2	43.0 \pm .2
Wandering larvae (short day):			
No chilling	6	.8 \pm .2	13.7 \pm 4.9
2 h at 0 C	3	2.4 \pm .4	42.1 \pm 6.4
Pharate adults (long day):			
No chilling	9	1.7 \pm .1	28.2 \pm 2.7
2 h at 0 C	9	3.4 \pm .1	81.4 \pm 3.0

NOTE.—Larvae were reared at 20 C at either long-day condition (15L:9D, nondiapause program) or short-day condition (12L:12D, diapause program). Differences within each couplet are significant (Student's *t*-test, $P < .05$). Two flies in each replicate.

A 2- or 3-h exposure to 0 C also resulted in a significant increase of hemolymph osmolality in pharate adults (table 2). A comparison of tables 1 and 2 suggests that the increase in hemolymph osmolality may be accounted for by the increase in glycerol concentrations for chilled pharate adults.

DISCUSSION

Short-term exposure to 0 C stimulates *Sarcophaga crassipalpis* to undergo a rapid physiological adjustment that enables the fly to survive short-term exposure to -10 C. A 10-min exposure to 0 C was adequate to allow about 50% of the flies to survive -10 C for 2 h. We have demonstrated that this mechanism can operate in larvae, pupae, and pharate adults of both nondiapauses- and diapause-programmed flies. Although nondiapauses flies cannot achieve the same level of cold tolerance as diapausing pupae, it is clear from this study that, if properly acclimated, they too can tolerate subzero temperatures.

Rapid cooling to -10 C in *S. crassipalpis* results in high mortality (fig. 1), despite the fact that tissue freezing does not occur until -23 C (Lee and Denlinger 1985). Injury after rapid cooling, but without tissue freezing, indicates that mortality was due to cold shock. In an extensive bibliography, Morris and Watson (1984) document cold shock in a wide range of organisms, including bacteria, yeast, algae, fungi, protozoa, higher plants, fish, spermatozoa, and mammalian somatic cells and embryos. Although no specific references to cold shock in insects were cited, it is well known that some insects die at temperatures above their supercooling points (Ring 1980).

In a discussion of Ushatinskaya's work, Solomon and Adamson (1955) describe an

apparent instance of cold shock in the grain weevil, *Calandra granaria*. Additional study is required to determine how common cold shock is among insects.

The generally accepted hypothesis for the mechanism of injury resulting from cold shock is the induction of phase transitions in membrane lipids and the subsequent loss of membrane permeability (Morris et al. 1983; Quinn 1985). Cryoprotective agents such as glycerol may alter the nature of these phase transitions, stabilize relationships between bilayer and nonbilayer forming lipids, and thus prevent the redistribution and segregation of membrane components upon thawing (Quinn 1985). Injury may also be a result of membrane failure owing to thermoelastic stress (McGrath 1984).

In insects, the process of cold hardening is often associated with the accumulation of glycerol (Zachariassen 1985). Although a variety of mechanisms of protective action have been suggested for glycerol, it is generally agreed that it primarily functions to either: (1) depress the temperature of heterogeneous ice nucleation (i.e., the SCP) in body tissues or (2) prevent injury to cells after the formation of ice in extracellular spaces. In the former category, high concentrations of glycerol and, sometimes, of other polyhydric alcohols and sugars appear to function as low molecular weight antifreeze compounds that increase the capacity for the supercooling of body water and, thereby, decrease the likelihood of tissue freezing. Alternatively, in freeze-tolerant species, these compounds serve as cryoprotective agents protecting cells as ice forms in extracellular spaces.

Rapid cooling from 25 to -10 C resulted in >90% mortality in pharate adults of *S.*

TABLE 2

CHANGES IN OSMOLALITY AND HEMOLYMPH MELTING POINTS IN PHARATE ADULTS (RED-EYE STAGE) OF *Sarcophaga crassipalpis* IN RESPONSE TO CHILLING AT 0 C

Treatment	Hemolymph Osmolality mOSM ($\bar{X} \pm SE$)	Hemolymph Melting Point °C ($\bar{X} \pm SE$)
No chilling . . .	308.7 \pm 6.6	-.574 \pm .012
2 h at 0 C	346.3 \pm 4.5	-.644 \pm .008
3 h at 0 C	344.3 \pm 3.5	-.641 \pm .007

NOTE.—Larvae and pupae were reared at 15L:9D; 20 C. Differences between chilled and unchilled flies are significant (Student's *t*-test, $P < .05$). Each $N = 3$.

crassipalpis, while an interval of only 2 h at 0 C prior to exposure to -10 C yielded >90% survival (fig. 1). During the same 2-h interval at 0 C, glycerol levels increased more than 2.5 times relative to controls (table 1). Most experiments report a significant accumulation of glycerol and polyols after several days or weeks of acclimation (Baust 1982; Rojas et al. 1983; Nordin, Cui, and Yin 1984), but our results demonstrate that glycerol synthesis is a very rapid response to low temperature in flesh flies. The concomitant increase of glycerol with the rapid enhancement of cold tolerance at -10 C suggests the possible existence of a third category of protective action for glycerol: glycerol protects insects against injury caused by cold shock. But, additional cellular events such as membrane reorganization or the production of specific proteins, comparable to those associated with the heat-shock response, may be involved.

Fly survival data suggest that the optimal temperature for this short-term acclimation ranges from 6 to 0 C. This is similar to the optimal range for the induction of the synthesis of cryoprotective compounds in other insects (Baust 1982). One of the key enzymes, glycogen phosphorylase has its highest activity between 4 and 0 C in pupae of the silk moth, *Hyalophora cecropia* (Ziegler and Wyatt 1975; Ziegler et al. 1979). However, two enzyme systems are involved in regulating the response: phosphorylase kinase and phosphorylase phosphatase (Hayakawa and Chino 1983; Hayakawa 1985). Activity of phosphorylase kinase remains high at low temperature, while phosphorylase phosphatase shows very little activity near 0 C. Thus, in silk moths, the

temperature-dependent properties of the two enzymes result in a slow but continuous accumulation of glycerol at low temperature.

Interestingly, the survivorship data show that a 2-h exposure to 36 C also can enhance survival at -10 C. It thus appears that high-temperature exposure can elicit the same biological effect as temperatures around 0 C, although the mechanism is not necessarily the same. High temperature is known to stimulate polyol formation in larvae of another Dipteran, *Callitroga macellaria* (Meyer 1978).

Acclimation to low temperature generally requires exposure periods lasting days or weeks (Colhoun 1960). However, Meats (1973) reported rapid acclimation with respect to thresholds for torpor and flight in the fly, *Dacus tryoni*. Maximal levels of acclimation were observed even at cooling rates of 1 C/min.

This short-term acclimation mechanism may be of critical ecological significance. It implies that stages other than diapausing pupae can rapidly enhance low-temperature tolerance. Although only diapausing pupae can tolerate the prolonged periods of low temperature characteristic of winter (Adedokun and Denlinger 1984; Lee and Denlinger 1985), nondiapausing individuals are not likely to be killed by an occasional night in autumn or early spring when temperatures drop to an unseasonal low. In fact, our data suggest that the extremely rapid acclimation observed in *S. crassipalpis* may allow this species to "instantaneously" enhance cold tolerance as it tracks decreases in its environmental temperatures.

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