

Reprint Series
4 December 1987, Volume 238, pp. 1415-1417

SCIENCE

A Rapid Cold-Hardening Process in Insects

RICHARD E. LEE, JR., CHENG-PING CHEN, AND DAVID L. DENLINGER

A Rapid Cold-Hardening Process in Insects

RICHARD E. LEE, JR., CHENG-PING CHEN, DAVID L. DENLINGER

Traditionally studies of cold tolerance in insects have focused on seasonal adaptations related to overwintering that are observed after weeks or months of exposure to low temperature. In contrast, an extremely rapid cold-hardening response was observed in nonoverwintering stages that confers protection against injury due to cold shock at temperatures above the supercooling point. This response was observed in nondiapausing larvae and pharate adults of the flesh fly, *Sarcophaga crassipalpis*, nondiapausing adults of the elm leaf beetle, *Xanthogaleruca luteola*, and the milkweed bug, *Oncopeltus fasciatus*. The rapid hardening response is correlated with the accumulation of glycerol.

MANY INSECTS RESPOND TO THE approach of winter by entering a period of dormancy (diapause) and by making physiological adjustments that increase their ability to tolerate low temperatures. For the many species that can-

not tolerate tissue freezing, preparation for winter usually involves a gradual accumula-

R. E. Lee, Department of Zoology, Miami University, Hamilton, Ohio 45011.

C.-P. Chen and D. L. Denlinger, Department of Entomology, Ohio State University, Columbus, Ohio 43210.

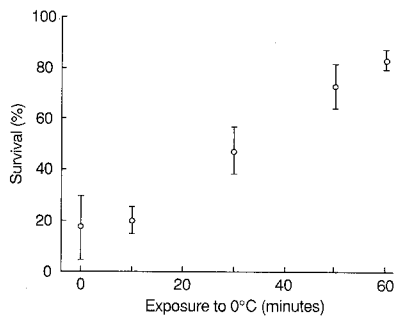


Fig. 1. Effect of the duration of chilling at 0°C before exposure to -10°C on the survival to adult eclosion in *Sarcophaga crassipalpis*. Flies were reared at LD 15:9 and 25°C and tested 12 days after pupariation as pharate adults in the red-eye stage of development. Each value represents the mean (\pm SEM) survivorship of three replicate trials of ten flies each.

tion of a cryoprotectant such as glycerol and other low molecular weight polyhydric alcohols and sugars. We report a response operating even in nondiapausing species that provides a rapid protective mechanism against cold injury that may enable insects to respond to changing environmental temperatures on a daily or even an hourly basis.

Flesh flies (*Sarcophaga crassipalpis* and *Sarcophaga bullata*) develop without diapause when reared under long day lengths [light: dark cycle (LD) 15:9] and at 25°C (1). Generation time is 1 month. In contrast, flies reared at short daylength (LD 12:12) enter an overwintering pupal diapause that may persist for more than 100 days (1). These flies are freeze susceptible and do not tolerate tissue freezing in any stage of development (2). The supercooling point refers to the temperature at which spontaneous nucleation of body water occurs. Though both diapause and nondiapause pupae can supercool to -20°C or below, the super-

cooling point is not indicative of the lower lethal temperature: both types of pupae die at temperatures far above the supercooling point (2). This mortality occurring in the absence of tissue ice formation is known as cold shock, direct chilling injury or thermal shock, and is known from a variety of biological systems including bacteria, algae, protozoa, higher plants, and mammalian embryos, but is little known among the insects (3).

We found that most nondiapausing larvae and pharate adults of *Sarcophaga* do not survive even a 2-hour exposure to -10°C (Table 1). Survival was defined as successful adult emergence. For the unchilled control groups of larvae and pupae, emergence rates were always greater than 95%. The lethal effects of chilling were not immediately evident since flies often continued development for several days, but ultimately were unable to survive to adult emergence. In contrast, most flies survive if subzero exposure is preceded by a 2-hour period of chilling at 0°C. As little as 30 minutes of chilling at 0°C before exposure to -10°C doubled the rate of survival in *S. crassipalpis* (Fig. 1), whereas an hour of chilling resulted in a fourfold increase. Therefore short-term chilling results in protection against the cold shock injury that occurs at temperatures as much as 10°C above the supercooling point. Similar responses were obtained for nondiapausing adults of the elm leaf beetle, *Xanthogaleruca luteola*, and the milkweed bug, *Oncopeltus fasciatus* (Table 1).

This response is not restricted to nondiapausing stages. During the first month of diapause, pupae of *S. crassipalpis* gradually accumulate glycerol and concomitantly enhance their tolerance to low temperature. Thus, early in diapause pupae are still vul-

nerable to cold shock injury. Again, a brief cold pulse enhances survival. Three days after pupariation, diapausing pupae of *S. crassipalpis* were exposed to -17°C for 1 day: no individual ($n = 30$) survived direct exposure, whereas a 2-hour cold pulse (0°C) before subzero exposure yielded a survival rate of 91.1% ($n = 45$).

Rapid accumulation of glycerol may provide at least a partial basis for this cryoprotective response. Larvae and pharate adult flies rapidly accumulate glycerol in response to short-term chilling (4). After 2 hours of exposure to 0°C, wandering larvae have glycerol levels 2.4 times those of prechilled values of 18.2 mM, whereas glycerol levels in pharate adults increased nearly threefold to 81.4 mM.

Accumulation of cryoprotective compounds, particularly glycerol, is well known to be associated with cold-hardening in insects intolerant of freezing. These compounds are believed to confer protection by a number of colligative and noncolligative mechanisms including the depression of whole body supercooling points and hemolymph melting points, the stabilization of enzyme function at low temperature and protection against desiccation during winter (5). On the basis of the rapid acclimation response that we observed, we propose that glycerol may provide cryoprotection against injury due to cold shock in nonoverwintering insects, although this suggestion does not rule out the existence of other additional mechanisms. The observed increase in glycerol was not of a magnitude to change significantly colligative properties, however recent evidence (6) suggests that specific interactions between glycerol and other cellular components may be of critical importance. For example, glycerol may alter the nature of phase transitions of membrane lipids during cooling and thereby protect against cold shock (6).

The apparently widespread, but generally unrecognized, capacity for rapid accumulation of glycerol in response to low temperature exposure in a number of phylogenetically diverse insects lends additional support to this hypothesis. Exposure of insect fat body either in vivo or in vitro to cold causes the activation of phosphorylase to the *a* form which, in turn, catalyzes the rapid breakdown of glycogen and the accumulation of glycerol (7). Within 10 minutes after the transfer of silkworm pupal fat body to 0° the percentage of phosphorylase *a* increases 2.3 times over initial levels to 30% (7). The rapidity of this response is consistent with that observed for the rate of hardening observed in this study (Fig. 1). Acclimation to warm temperatures reverses this process. Rapid cold activation of phosphorylase, 2.5

Table 1. Effect of short-term chilling at 0°C before exposure to subzero temperatures on the survival of various nondiapausing stages of insects. One group of flies (*Sarcophaga*) was transferred directly from 25°C to -10°C for an exposure period of 2 hours and returned to 25°C until adult emergence. A second group was chilled for 2 hours at 0°C immediately before exposure to -10°C. A similar protocol was used for the other species except that elm leaf beetles, *Xanthogaleruca luteola*, were chilled for 4 hours at 0°C followed by exposure to -7°C for 1 hour and milkweed bugs, *Oncopeltus fasciatus*, were chilled for 2 hours at 0°C followed by exposure to -10°C for 3 hours. All paired values show statistically significant differences ($P < 0.001$, *z*-test for two binomial proportions). Data represent mean values \pm SEM.

Stage	Survival (%)	
	No chilling	Chilling
Pupae	<i>Sarcophaga bullata</i>	
	51.1 \pm 7.5 (45)*	84.1 \pm 5.5 (44)
Pharate adult, red-eye stage	0 (45)	91.1 \pm 4.2 (45)
	<i>Sarcophaga crassipalpis</i>	
Third instar larvae	0 (45)	88.9 \pm 4.6 (45)
Pharate adults, red-eye stage	5.0 \pm 2.8 (60)	98.9 \pm 1.3 (60)
	<i>Xanthogaleruca luteola</i>	
Adults, 2 days old	15.0 \pm 7.9 (20)	90.0 \pm 6.7 (20)
Adults, mixed ages	<i>Oncopeltus fasciatus</i>	
	33.3 \pm 8.6 (30)	93.3 \pm 4.5 (30)

*Numbers in parentheses are sample sizes.

to 7.5 times that of control values within 2 hours of transfer, has been reported for other active nonoverwintering insects including crickets and locusts (7) and for diapausing silkworm pupae (8). Thus, the cold activation of phosphorylase and the ensuing rapid accumulation of glycerol may be a general adaptation of insects for protection against cold shock injury.

The rapid cold-hardening capacity we describe may be of considerable ecological importance in early spring and late autumn. Our results suggest that many insects, even those in nondiapausing stages, have the

ability to quickly enhance their cold tolerance in response to a rapid temperature drop. Though such a mechanism, in the absence of diapause, may not enable an insect to survive the prolonged cold exposure characteristic of winter, it should permit the insect to adapt to diurnal changes in temperature and enable the insect to survive brief periods of exposure to low temperature. Our observation that cold shock injury can be reduced by brief exposure to 0°C suggests that methods can be developed for long-term cryopreservation and storage of *Drosophila* and other nondiapausing insects.

REFERENCES AND NOTES

1. D. L. Denlinger, *Biol. Bull. (Woods Hole)* **142**, 11 (1972).
2. R. E. Lee and D. L. Denlinger, *Physiol. Entomol.* **10**, 309 (1985).
3. G. J. Morris & P. F. Watson, *Cryo-Lett.* **5**, 352 (1984).
4. C.-P. Chen *et al.*, *Physiol. Zool.* **60**, 297 (1987).
5. K. E. Zachariassen, *Physiol. Rev.* **65**, 799 (1985); P. W. Hochachka, G. N. Somero, *Biochemical Adaptation* (Princeton Univ. Press, Princeton, NJ, 1984).
6. P. J. Quinn, *Cryobiology* **22**, 128 (1985).
7. R. Ziegler *et al.*, *J. Comp. Physiol.* **131**, 321 (1979).
8. Y. Hayakawa and H. Chino, *Insect Biochem.* **12**, 361 (1982).
9. Supported by NSF grant DCB-8517875 to R.E.L. and by USDA-CRGO 8600186 to D.L.D.

25 June 1987; accepted 27 October 1987