

# Role of Chilling in the Acquisition of Cold Tolerance and the Capacitation to Express Stress Proteins in Diapausing Pharate Larvae of the Gypsy Moth, *Lymantria dispar*

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Cold hardiness in eggs (pharate first instar larvae) of the gypsy moth is not a component of the diapause program, but is acquired only after the pharate larvae have been chilled. The supercooling points of unchilled (25°C) and chilled (5°C) eggs are nearly the same (ca. -27°C), and chilling does not further elevate concentrations of glycerol, the major cryoprotectant, yet chilling at 5°C greatly increases the pharate larva's tolerance of -20°C. One conspicuous difference between the chilled and unchilled pharate larvae is their ability to express stress proteins. The most abundantly expressed stress protein, 75,000 M<sub>r</sub>, was expressed more highly in chilled pharate larvae than in unchilled pharate larvae, both at high temperatures (> 40°C) and in response to low temperature (-15°C). This correlation suggests a link between stress protein synthesis and the acquisition of cold tolerance. © 1992 Wiley-Liss, Inc.

**Key words:** cold hardiness, diapause, heat shock proteins, supercooling point, glycerol

## INTRODUCTION

The gypsy moth, *Lymantria dispar*, is among the relatively few insects with an obligatory diapause. Though a few first instar larvae do hatch from eggs in late summer without the intervention of diapause [1,2], most halt development as pharate first instars and do not hatch until the following spring. This diapause is genetically programmed and the developmental decision to enter diapause does not appear to be influenced by photoperiod, temperature, or other factors that commonly serve as environmental regulators of diapause

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induction [1]. Environmental conditions, however, do play a critical role in its termination. A period of chilling is essential for diapause termination in this species [3,4].

Gypsy moth pharate first instars, though intolerant of freezing, are well adapted to survive the low temperatures of winter [5-7], but it is not clear whether chilling is required to elicit cold hardiness. Is a diapausing pharate larva already cold hardy, or is this attribute only acquired in response to low temperature exposure? Examples of both possibilities exist in the literature [8]. In this study, we examine the importance of chilling in the development of cold hardiness by comparing supercooling points, cryoprotectant levels, and tolerance to low temperature in unchilled and chilled samples. The recent discovery that diapausing pharate first instars of the gypsy moth can respond to both high and low temperature extremes by synthesizing heat shock proteins [9] suggests that these stress proteins may also contribute to overwintering success, and in this study we demonstrate that chilling has a role in capacitating the gypsy moth pharate larvae to synthesize stress proteins in response to temperature stress.

## MATERIALS AND METHODS

### Insects

Newly deposited gypsy moth egg masses were obtained from the Center of Biological Control, Forest Service, USDA, Hamden, CT, and stored in Petri dishes at 25°C for at least one month to permit the completion of embryogenesis. When the diapause stage (pharate first instar larva) was attained, the egg masses were either held at 25°C (unchilled) or 5°C (chilled).

### Measurement of Cold Tolerance

Groups of 25 pharate larvae were placed in individual test tubes immersed in a -20°C ethanol bath within a freezer, and test tubes were removed at various intervals. Samples that had previously been chilled at 5°C for 100 days were transferred directly from -20 to 25°C (15 h light:9 h dark cycle), but those that were previously unchilled were transferred to 5°C for 100 days of chilling before being placed at 25°C. Hatching success was recorded for each group.

### Supercooling Point Determination

SCPs\* of individual pharate larvae were determined by positioning a 30 gauge copper-constantan thermocouple in contact with the chorion. A cooling rate of ca. 1°C/min was maintained using a low temperature bath. The SCP was the lowest temperature recorded prior to the release of the latent heat of fusion.

\*Abbreviations used: SCP = supercooling point; NMR = nuclear magnetic resonance; SDS = sodium dodecyl sulfate.

### TLC Separation

Pharate larvae from a single egg mass ( $N = \text{ca. } 800$  eggs) were ground with mortar and pestle and centrifuged at  $1,000g$  for 1 min. The supernatant was spotted on precoated silica gel F<sub>254</sub> plates 0.25 mm thick (Merck, Darmstadt, Germany) using two different solvent systems: solvent A, butanol: acetone: water, 4:5:1; solvent B, isopropanol: ethyl acetate: water, 83:11:6. The plates were visualized by ultraviolet light and also by spraying with 1:1 sulfuric acid:water. The following standards were run with each analysis: alanine, glutamine, proline, ethylene glycol, glycerol, sorbitol, and trehalose.

### HPLC Separation

Concentrations of glycerol were determined as described by Lee et al. [10] using HPLC (Waters Associates, Milford, MA). For each determination, 5 egg masses were homogenized in 3 ml ethanol and centrifuged 3 times at  $2,000g$  for 5 min each. Each time, the pellet was reextracted with 3 ml methanol and pooled supernatants were passed through a Sep-Pak C<sub>18</sub> cartridge (Waters Associates) and evaporated to dryness. Samples were resuspended in 0.5 ml ethanol:water (1:1), filtered through a  $0.22 \mu\text{m}$  nylon filter (MSI, Honeoye Falls, NY) and injected into a radially compressed silica column (Waters Associates) modified with tetraethylenepentamine.

### NMR Analysis

<sup>1</sup>H-Decoupled <sup>13</sup>C NMR spectra were obtained on a Bruker AM-500 Fourier-transform NMR spectrometer equipped with a pulse programmer and quadrature phase detection. Spectra were generated with 70 pulse widths and a recycle time (acquisition + relaxation delay) of 1 s; 16–32K real points were obtained over a 0 to 190 ppm spectral width. Chemical shifts are reported in ppm (parts per million) relative to the C1 signal of deuteriochloroform.

<sup>13</sup>C NMR spectra of intact eggs (pharate larva and undigested yolk encased within the chorion) were determined by inserting ca. 20 eggs into a 5 mm i. d. NMR tube. Spectra of homogenates were obtained by analyzing the supernatant collected from a homogenate of ca. 800 eggs, as described above. All <sup>13</sup>C spectra were compared to standard chemical shifts determined previously [11] and to the standard for ethylene glycol (63.3 ppm).

### Protein Labeling and Separation

Pharate larvae were separated under saline from the rest of the egg by using a forceps to mechanically strip off the chorion and remaining yolk [12]. Five pharate larvae were bisected and cultured together in a 1.5 ml vial containing 10  $\mu\text{l}$  methionine-free Grace's culture medium [13]. Cultures to be heat shocked were placed in a water bath at the heat shock temperature and then labeled using 1  $\mu\text{l}$  (10  $\mu\text{Ci}$  [370 kBq]) <sup>35</sup>S-labeled methionine (Trans <sup>35</sup>S-label™, specific activity 1,259 Ci/mmol, ICN Radiochemicals, Costa Mesa, CA) for an additional 1 h at the same temperature. Cultures to be cold shocked were exposed to  $-15^\circ\text{C}$  in a low temperature bath for 24 h, transferred to  $4^\circ\text{C}$  for various intervals, and labeled as above for an additional 24 h at  $4^\circ\text{C}$ . The Grace's medium was then removed, the tissue homogenized with a disposable

pestle (Kontes, Vineland, NJ) in 20  $\mu$ l sample buffer (0.6% Tris, 10%  $\beta$ -mercaptoethanol, bromophenol blue), boiled for 5 min, and stored at  $-70^{\circ}\text{C}$ .

The amount of  $^{35}\text{S}$  incorporation for each sample was determined by trichloroacetic acid precipitation [14]. SDS-PAGE was run using equal amounts of radioactivity per sample on a discontinuous 10% gel [15] with a modified stacking gel [16]. Gels were run at 15 mA, fixed, and stained using a modified Coomassie blue method [17], destained with acetic acid:methanol:water (0.18:1:1), and saturated with 1 M sodium salicylate [18]. Gels were dried onto Whatman filter paper and exposed to Kodak X-Omat X-ray film (Rochester, NY) at  $-70^{\circ}\text{C}$ .

## RESULTS

### Low Temperature Survival

To determine whether chilling enhances low temperature survival, unchilled pharate larvae and those that had been chilled at  $5^{\circ}\text{C}$  for 100 days were exposed to  $-20^{\circ}\text{C}$  challenges of various lengths (Fig. 1). The unchilled pharate larvae were held at  $25^{\circ}\text{C}$  for one month before exposure to  $-20^{\circ}\text{C}$  and then chilled for 100 days at  $5^{\circ}\text{C}$  before being returned to  $25^{\circ}\text{C}$ . In contrast, the chilled pharate larvae were held at  $25^{\circ}\text{C}$  for one month, chilled at  $5^{\circ}\text{C}$  for 100 days, then challenged with  $-20^{\circ}\text{C}$ , and thereafter placed at  $25^{\circ}\text{C}$ . Chilled pharate larvae were clearly more tolerant to  $-20^{\circ}\text{C}$  than pharate larvae that had not been chilled.

### Supercooling Points

The SCPs of newly deposited unchilled diapausing pharate larvae held at  $25^{\circ}\text{C}$  were already low ( $-27.7^{\circ}\text{C}$ ), and chilling at  $5^{\circ}\text{C}$  did not alter the SCP (Table 1). Though the SCP of the sample chilled at  $5^{\circ}\text{C}$  for 55 days was significantly different from the others, the difference was slight and is not

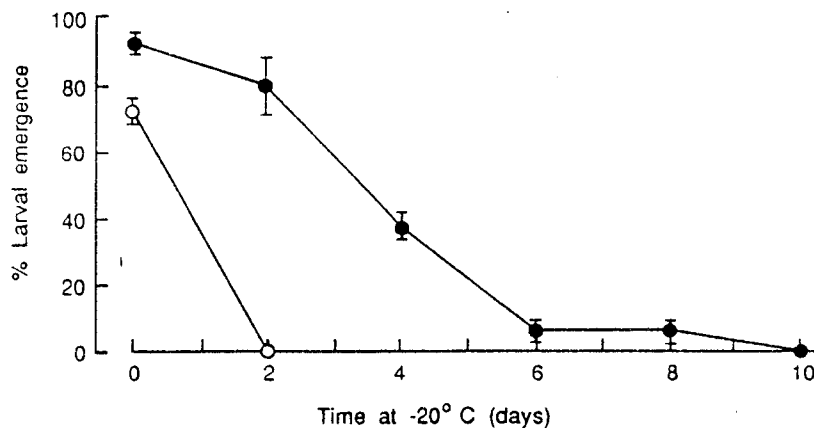


Fig. 1. Success of larval emergence (mean  $\pm$  S.E. of 3 groups of 25 eggs) from eggs that were exposed to  $-20^{\circ}\text{C}$  for various numbers of days. The egg masses were first held at  $25^{\circ}\text{C}$  for one month to permit completion of embryogenesis. One group then received the  $-20^{\circ}\text{C}$  exposure before being held at  $5^{\circ}\text{C}$  for 100 days (open circles), and the other group was chilled at  $5^{\circ}\text{C}$  for 100 days and then exposed to  $-20^{\circ}\text{C}$  (solid circles). All samples were then transferred to  $25^{\circ}\text{C}$  to monitor hatching.

**TABLE 1. Comparison of Supercooling Points and Glycerol Concentrations in *L. dispar* in One-Month-Old Unchilled Diapausing Eggs and Eggs That Were Held Unchilled for One Month and Subsequently Chilled at 5°C for Various Intervals<sup>†</sup>**

Chilling status	SCP (°C)		Glycerol (mM)	
	n	mean ± S.E.	n	mean ± S.E.
Unchilled, 25°C	9	-27.7 ± 0.1a	3	9.9 ± 1.4a
Chilled, 5°C for 55 days	15	-26.9 ± 0.2b	3	3.9 ± 1.1b
Chilled, 5°C for 100 days	10	-25.6 ± 1.0a	—	—
Chilled, 5°C for 55 days, then to 25°C for 7 days	11	-27.0 ± 0.1a	—	—

<sup>†</sup>Unchilled diapausing eggs = pharate larvae and yolk encased within the chorion. Means within each column followed by the same letter are not significantly different; ANOVA, Scheffe F-test for SCP comparisons; t-test for glycerol comparison,  $P < 0.05$ .

likely to be biologically important. Likewise, the SCP was not altered by transferring pharate larvae that had been chilled at 5°C for 55 days to 25°C for 7 days.

### Cryoprotectants

Two potential cryoprotectants, glycerol and trehalose, were detected by TLC analysis of eggs (pharate larvae and yolk) that had been chilled at 5°C for 100 days. <sup>13</sup>C NMR spectra of intact, chilled eggs (Fig. 2A) indicated the presence of saturated and unsaturated lipids (resonances at 30, 130, and 175 ppm) and phospholipids (55–75 ppm), and the presence of glycerol (resonances of 65 and 75 ppm), trehalose (62, 71, 73, 74, and 95 ppm), and glucose (10 peaks of a- and b-pyranose ranging between 62.2 and 97.4 ppm) could be verified by the <sup>13</sup>C NMR spectra of the supernatant of egg homogenates (Fig. 2B).

Glycerol was the only potential cryoprotectant identified by HPLC in unchilled eggs and eggs that had been chilled at 5°C for 55 days. Concentrations in both unchilled and chilled eggs were low (Table 1), and there is no evidence that glycerol concentrations increase in response to chilling.

### Stress Proteins

The capacity of chilled and unchilled pharate larvae to synthesize stress proteins was evaluated by comparing the responses of these two types of pharate larvae to heat shock and low temperature. At high temperatures, little difference between the two types of pharate larvae was apparent at temperatures below 40°C (data not shown), but at both 41 and 43°C, the 75,000 M<sub>r</sub> protein, a protein immunologically related to the human 70,000 M<sub>r</sub> heat shock protein [9], was more strongly expressed in the chilled pharate larvae (Fig. 3A, compare lanes 2 and 4 with lanes 1 and 3 from unchilled pharate larvae). Among the 7 gels of this type that were run, the chilled pharate larvae consistently expressed the 75,000 M<sub>r</sub> protein more highly than unchilled pharate larvae at high temperature, and in all cases the differences were at least as pronounced or much more pronounced than in the example shown in Figure 3A.

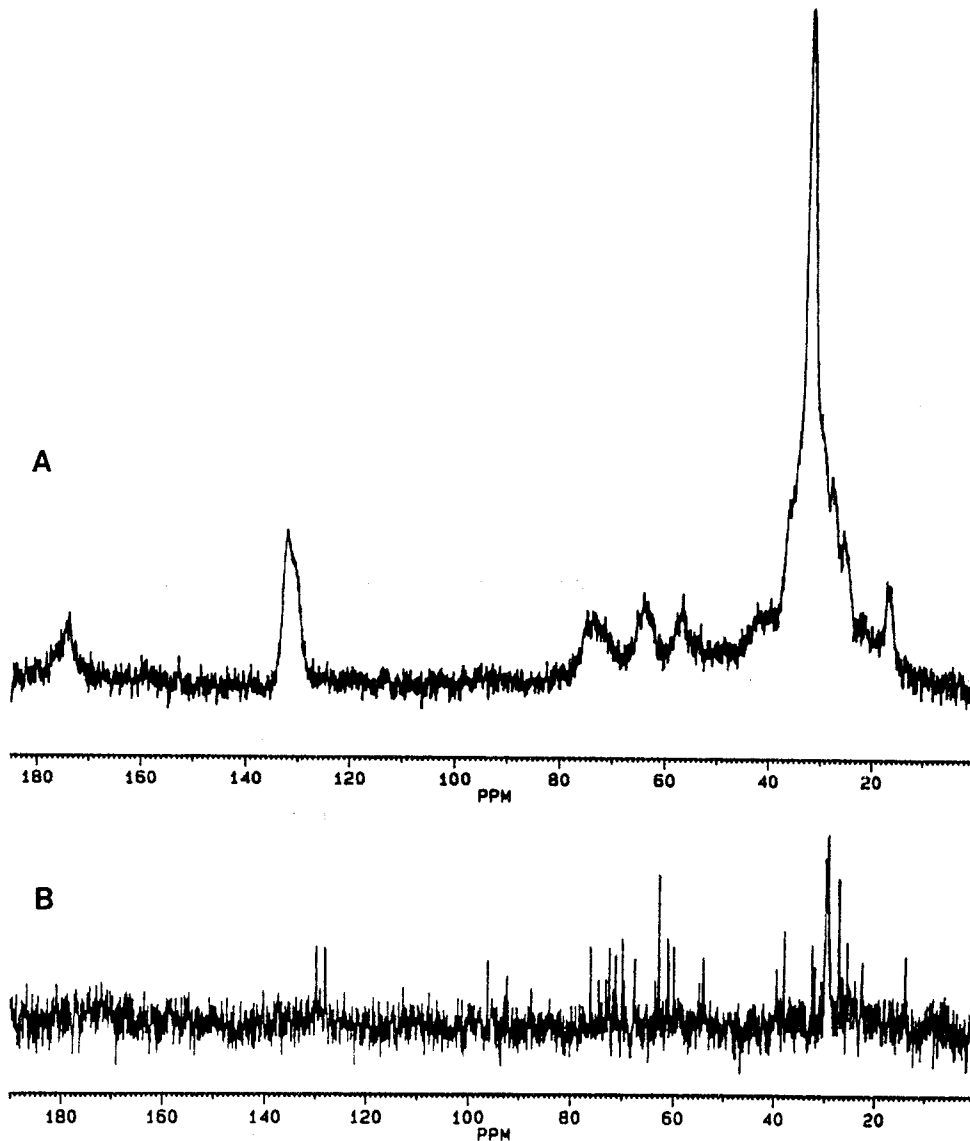


Fig. 2.  $^{13}\text{C}$  NMR spectra of (A) intact eggs (i.e., diapausing pharate larvae and yolk enclosed within the chorion) and (B) centrifuged egg homogenates of the gypsy moth. The diapausing samples were chilled at  $5^\circ\text{C}$  for 2 months and spectra were recorded at  $25^\circ\text{C}$ . Spectra of intact eggs show prominent broad resonances at ca. 30, 130, and 175 ppm which correspond to lipids, and 55–70 ppm corresponding to phospholipids. Spectra of homogenates indicate the presence of trehalose represented by 6 carbon resonances between 62–95 ppm, glucose (10 peaks of  $\alpha$ - and  $\beta$ -pyranose ranging between 97.4 and 62.2) and glycerol (65 and 75 ppm). Peaks arising from resonances around the 30 and 130 ppm regions show contamination of the centrifuged sample with saturated and unsaturated lipids, respectively.

Likewise, pharate larvae that had been chilled at  $5^\circ\text{C}$  for 65 days were more responsive to a  $-15^\circ\text{C}$  exposure than unchilled pharate larvae. After a 24 h exposure to  $-15^\circ\text{C}$ , chilled pharate larvae expressed the 75,000  $M_r$  stress protein to a greater extent than unchilled pharate larvae (Fig. 3B). This difference was apparent within 48 h after  $-15^\circ\text{C}$  exposure (compare lanes 1 and 2), and the difference persisted for at least 144 h (compare lanes 5 and 6).

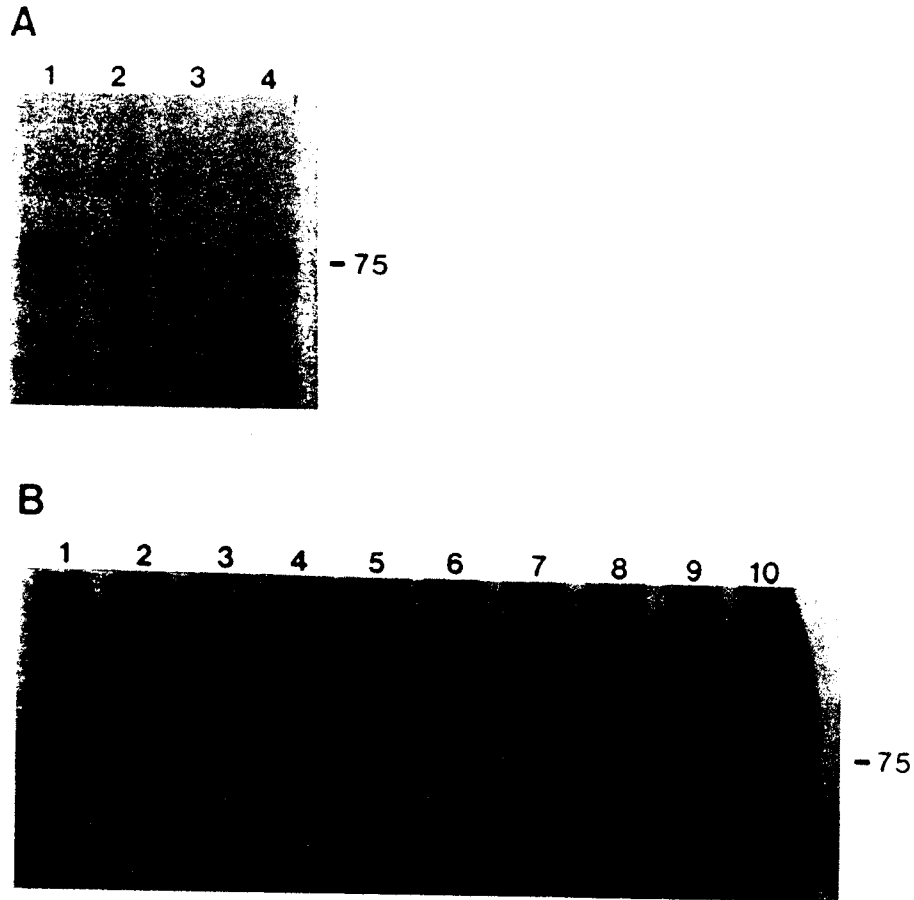


Fig. 3. Stress protein expression by diapausing pharate larvae in response to (A) elevated temperatures or (B) during recovery from cold shock. Pharate larvae were either unchilled (35 days at 25°C) (A: lanes 1,3; B: lanes 1,3,5,7,9) or chilled at 4°C for 65 days (A: lanes 2,4; B: lanes 2,4,6,8,10) before exposure to the experimental temperatures. For the high temperature experiments (A), pharate larvae were dissected from the chorions and exposed to 41 (lanes 1,2) or 43°C (lanes 3,4) for 1 h, then labeled for 1 additional hour at the same temperature. For the low temperature experiments (B), chorionated pharate larvae were exposed to -15°C for 24 h and then after 48 h (lanes 1,2), 96 h (lanes 3,4) or 144 h (lanes 5,6) at 4°C, the pharate larvae were dissected from their chorions and labeled 24 h at 4°C. Controls were labeled at 40°C (lanes 7,8) and 25°C (lanes 9,10). Proteins were separated on 10% SDS-PAGE gels.  $M_r$  of the major stress protein is expressed in kilodaltons.

## DISCUSSION

In some insects, including the silkworm *Bombyx mori* [19], *Hyalophora cecropia* [20], and the flesh fly *Sarcophaga crassipalpis* [21], cold hardiness is firmly linked to the expression of diapause. The two events cannot be separated and apparently share a common genetic basis for induction. This does not appear to be true for the gypsy moth. Our experiments demonstrated that cold hardiness, measured as tolerance to -20°C, is not inherent to the diapause program. Rather, it is acquired in response to chilling. This implies that eggs, laid during the summer, lack cold hardiness at first and only become cold hardy in response to the low temperatures of autumn. Though diapause and cold hardiness clearly coincide during the winter months, these two events

do not share a common inductive pathway. This, of course, does not mean that the diapause program is not involved in preparing the pharate larva to respond to chilling, and this is indeed a likely scenario [8], but from this set of experiments it is clear that the induction of diapause, by itself, does not cause the gypsy moth to become cold hardy. In this respect, the gypsy moth is similar to the parasitoid *Bracon cephi* [22], the European corn borer *Ostrinia nubilalis* [23], and the Viceroy butterfly *Limenitis archippus* [24].

The mechanism(s) used by gypsy moth eggs to achieve cold hardiness is still unclear. There is no change in the SCP associated with the cold hardiness induced by chilling. Both unchilled and chilled eggs in our study had nearly identical SCPs (ca.  $-27^{\circ}\text{C}$ ), and these values are quite similar to SCPs previously reported for overwintering gypsy moth eggs [5-7,25]. The well developed capacity of the eggs to supercool indicates the absence of efficient internal heterogeneous ice-nucleating agents [26]. Since pharate larvae of the gypsy moth do not tolerate freezing, the SCP indicates the minimum temperature above which the pharate larva might be expected to survive, but it is clear from this study and others [e.g., 27,28] that the SCP is not a good indicator of cold hardiness and many insects succumb at temperatures far above their SCP. Unlike other species, cold hardening is not associated with the accumulation of high concentrations of cryoprotectants. Although glycerol was identified as the major cryoprotectant in eggs, its concentration was low ( $< 10$  mM) as compared to levels reported in other overwintering eggs [29].

Stress proteins (heat shock proteins) are among the possible factors that may contribute to overwintering success of the gypsy moth [9]. A subset of the stress proteins expressed during heat shock, including the 75,000  $M_r$  protein observed in this study, is also expressed at low temperatures, and the expression persists for at least 6 days at  $4^{\circ}\text{C}$ . This sustained expression of a stress protein at low temperatures is especially remarkable because stress protein expression usually ceases within a few hours after a high temperature challenge [30,31]. A common mechanism may contribute to the insect's response to both high and low temperature extremes. Low temperature stimulates stress protein synthesis not only in the gypsy moth [9], but also in *Drosophila melanogaster* [32] and *Sarcophaga crassipalpis* [33]. We now report that chilling plays a critical role in capacitating diapausing pharate larvae of the gypsy moth to respond to temperature stress. In our experiments, stress protein synthesis was much more pronounced in pharate larvae that had experienced a period of chilling. This observation lends credence to the idea that stress proteins contribute to cold hardiness.

#### LITERATURE CITED

1. Leonard DE: Diapause in the gypsy moth. *J Econ Ent* 61, 596 (1968).
2. Hoy MA: Rapid response to selection for a nondiapausing gypsy moth. *Science* 196, 1462 (1977).
3. Masaki S: The effect of temperature on the termination of diapause in the eggs of *Lymantria dispar* Linne (Lepidoptera: Lymantriidae). *Jpn J Appl Zool* 21, 148 (1956).



4. Tauber MJ, Tauber CA, Ruberson JR, Tauber AJ, Abrahamson LP: Dormancy in *Lymantria dispar* (Lepidoptera: Lymantriidae): Analysis of photoperiodic and thermal responses. **Ann Entomol Soc Am** 83, 494 (1990).
5. Leonard DE: Ecology and control of the gypsy moth. **Annu Rev Entomol** 19, 197 (1974).
6. Madrid FJ, Stewart RK: Ecological significance of cold hardiness and winter mortality of eggs of the gypsy moth *Lymantria dispar* L., in Quebec. **Environ Entomol** 10, 586 (1981).
7. Waggoner PE: How gypsy moth eggs freeze. **Agric Forest Meteorol** 36, 4 (1985).
8. Denlinger DL: Relationship between cold hardiness and diapause. In: Insects at Low Temperature. Lee RE, Denlinger DL, eds. Chapman and Hall, New York, pp 174-198 (1991).
9. Yocum GD, Joplin KH, Denlinger DL: Expression of heat shock proteins in response to high and low temperature extremes in diapausing pharate larvae of the gypsy moth, *Lymantria dispar*. **Arch Insect Biochem Physiol** 18, 239 (1991).
10. Lee RE, Friday D, Rojas R, James H, Baust JG: An evaluation of eluent recycling and column life for HPLC analysis of carbohydrates. **J Chromat** 6, 1139 (1983).
11. Kukal O, Serianni AS, Duman JG: Glycerol metabolism in a freeze-tolerant arctic insect. An in vivo <sup>13</sup>C NMR study. **J Comp Physiol B** 158, 175 (1988).
12. Ephrussi B, Beadle GW: A technique for transplantation in *Drosophila*. **Am Nat** 70, 218 (1936).
13. Grace TDC: Establishment of four strains of cells from insect tissues grown *in vitro*. **Nature** 195, 788 (1962).
14. Mans RJ, Novelli GD: Measurement of the incorporation of radioactive amino acids into protein by a filter-paper disk method. **Arch Biochem Biophys** 94, 28 (1961).
15. Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature** 227, 680 (1970).
16. Neuhoff V, Cheong-Kim K-S, Altland K: Improved design of one-dimensional vertical polyacrylamide slab gel electrophoresis avoids lateral streaking. **Electrophoresis** 7, 56 (1986).
17. Zehr BD, Savin TJ, Hall RE: A one-step, low background Coomassie staining procedure for polyacrylamide gels. **Anal Biochem** 182, 157 (1989).
18. Chamberlain JP: Fluorographic detection of radioactivity in polyacrylamide gels with the water-soluble fluor, sodium salicylate. **Anal Biochem** 98, 132 (1979).
19. Chino H: Carbohydrate metabolism in the diapausing egg of the silkworm, *Bombyx mori*-I. Diapause and the change in glycogen content. **Embryologia** 3, 295 (1957).
20. Wyatt GR, Meyer WL: The chemistry of insect haemolymph. III. Glycerol. **J Gen Physiol** 42, 1005 (1959).
21. Adedokun TA, Denlinger DL: Cold-hardiness: A component of the diapause syndrome in pupae of the flesh flies, *Sarcophaga crassipalpis* and *S. bullata*. **Physiol Ent** 9, 361 (1984).
22. Salt RW: Role of glycerol in the cold-hardening of *Bracon cephi* (Gahan). **Can J Zool** 37, 59 (1959).

23. Hanec W, Beck SD: Cold hardiness in the European corn borer, *Pyrausta nubilalis* (Hubn.). **J Insect Physiol** 5, 169 (1960).
24. Frankos VH, Platt AP: Glycerol accumulation and water content in larvae of *Limnitis archippus*: Their importance to winter survival. **J Insect Physiol** 22, 623 (1976).
25. Sullivan CR, Wallace DR: The potential northern dispersal of the gypsy moth, *Porthetria dispar* (Lepidoptera: Lymantriidae). **Can Entomol** 104, 1349 (1972).
26. Lee RE: Principles of insect low temperature tolerance. In: *Insects at Low Temperature*. Lee RE, Denlinger DL, eds. Chapman and Hall, New York, pp 17-46 (1991).
27. Lee RE, Denlinger DL: Cold tolerance in diapausing and non-diapausing stages of the flesh fly, *Sarcophaga crassipalpis*. **Physiol Ent** 10, 309 (1985).
28. Knight JD, Bale JS, Franks F, Mathias SF, Baust JG: Insect cold hardiness: Supercooling points and pre-freeze mortality. **Cryo-Lett** 7, 194 (1986).
29. Sømme L: Effects of glycerol on cold-hardiness in insects. **Can J Zool** 42, 87 (1964).
30. Lindquist S: The heat-shock response. **Annu Rev Biochem** 55, 1151 (1986).
31. Yocum GD, Denlinger DL: Prolonged thermotolerance in the flesh fly, *Sarcophaga crassipalpis* does not require continuous expression or persistence of the 72 kDa heat-shock protein. **J Insect Physiol** 38, 603 (1992).
32. Burton V, Mitchell HK, Young P, Petersen NS: Heat shock protection against cold stress of *Drosophila melanogaster*. **Mol Cell Biol** 8, 3550 (1988).
33. Joplin KH, Yocum GD, Denlinger DL: Cold shock elicits expression of heat shock proteins in the flesh fly, *Sarcophaga crassipalpis*. **J Insect Physiol** 36, 825 (1990).