Archives of Insect Biochemistry and Physiology 48:199–205 (2001)

Intracellular Freezing, Viability, and Composition of Fat Body Cells From Freeze-Intolerant Larvae of *Sarcophaga crassipalpis*

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Although it is often assumed that survival of freezing requires that ice formation must be restricted to extracellular compartments, fat body cells from freeze-tolerant larvae of the gall fly, *Eurosta solidaginis* (Diptera, Tephritidae) survive intracellular freezing. Furthermore, these cells are highly susceptible to inoculative freezing by external ice, undergo extensive lipid coalescence upon thawing, and survive freezing better when glycerol is added to the suspension medium. To determine whether these traits are required for intracellular freeze tolerance or whether they are incidental and possessed by fat body cells in general, we investigated the capacity of fat body cells from nondiapause-destined and diapause-destined (i.e., cold-hardy) larvae of the freeze-intolerant flesh fly *Sarcophaga crassipalpis* (Diptera, Sarcophagidae) to survive intracellular freezing. Fat body cells from both types of larvae were highly susceptible to inoculative freezing; all cells froze between –3.7 to –6.2°C. The highest rates for survival of intracellular freezing occurred at –5°C. The addition of glycerol to the media markedly increased survival rates. Upon thawing, the fat body cells showed little or no lipid coalescence. Fat body cells from *E. solidaginis* had a water content of only 35% compared to cells from *S. crassipalpis* larvae that had 52–55%; cells with less water may be less likely to be damaged by mechanical forces during intracellular freezing. Arch. Insect Biochem. Physiol. 48:199–205, 2001. © 2001 Wiley-Liss, Inc.

Key words: intracellular freeze tolerance; cold-hardiness; diapause; cryoprotection; fat body

INTRODUCTION

A variety of insects and other invertebrates as well as a few lower vertebrates naturally survive freezing (see reviews Lee, 1991; Storey and Storey, 1988; Costanzo et al., 1995; Lee and Costanzo, 1998). However, it is generally assumed that survival is possible only if ice formation is restricted to the extracellular fluid compartments. Nonetheless, in 1959 and 1962 R.W. Salt demon-

Contract grant sponsor: USDA CSRS; Contract grant number: 96-35302-3419; Contract grant sponsor: NSF; Contract grant number: IBN-0090204.

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Received 25 February 2001; Accepted 4 July 2001

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strated that fat body cells of the freeze-tolerant larvae of *Eurosta solidaginis* (Diptera, Tephritidae) survive intracellular freezing (Salt, 1959, 1962). These reports are especially noteworthy since the insect fat body is a critical tissue that plays a primary role in carbohydrate, protein, and lipid metabolism similar to that of mammalian liver (Keeley, 1985).

Our research group confirmed and extended Salt's investigations of intracellular freeze tolerance in *E. solidaginis* (Lee et al., 1993). Using fluorescent vital dyes, we determined that many fat body cells survive freezing to –80°C and that survival increased when glycerol was added to the cryopreservation medium. However, we were surprised to find that internal freezing was readily initiated when fat body cells contacted external ice, even at high subzero temperatures (–4.6°C). This susceptibility to inoculative freezing differs considerably from the case with mammalian cells that generally resist inoculation by ice to –15°C or below (Mazur, 1984). We also observed extensive coalescence of lipid droplets into larger droplets or even a single large droplet occupying the center of the cell (Lee et al., 1993; Morason et al., 1994). Consequently, considering the novelty of this phenomenon, we wondered whether these distinctive characteristics are required for intracellular freeze tolerance. Or are they incidental traits also possessed by other fat body cells including ones from freeze-intolerant species?

To address these questions, we investigated the capacity of fat body cells from nondiapause-destined and diapause-destined (i.e., the type that gives rise to the cold-hardy overwintering pupae) larvae from *Sarcophaga crassipalpis* (Diptera, Sarcophagidae) to survive chilling and intracellular freezing. Diapause-destined larvae refer to ones that will become diapause pupae, the overwintering stage for this species. Diapause-destined larvae are substantially more cold tolerant than nondiapause-destined ones, although neither larval type survives freezing (Adedokun and Denlinger, 1984; Lee and Denlinger, 1985). Consequently, investigation of the fat body cells from these two types of larvae should prove useful in determining whether the traits observed in fat body cells from *E. solidaginis* are specific to freeze tolerant cells. We also determined whether these cells were susceptible to inoculative freezing and whether the addition of glycerol to the medium enhanced freezing survival. Finally, we measured the water and lipid content of fat body cells from diapause-destined and nondiapause-destined *S. crassipalpis* larvae, and from freeze-tolerant larvae of *E. solidaginis*.

MATERIALS AND METHODS

Insect Rearing

Sarcophaga crassipalpis were reared according to the methods described by Lee and Denlinger (1985). During the first 6 days post-emergence, flies were provided with beef liver as a protein source to promote normal oogenesis and embryonic development. Eleven days after the adult emergence, a 50-g packet of liver was provided as a substrate for larviposition. On the following day, approximately 50 larvae were transferred to fresh packets of liver. These packets were placed in plastic tubs lined with a 2-cm layer of sawdust into which the larvae were allowed to wander and pupariate. Following pupariation, puparia were sifted from the sawdust and stored in Petri dishes. To avoid diapause, animals were maintained at 25°C under a long day photoperiod (LD, 15:9 h), while diapause was induced by rearing flies at LD 12:12 h and 20°C. Freeze-tolerant larvae of *E. solidaginis* were field-collected near Oxford, Ohio, in mid-January 1996 and stored frozen at –22°C until used.

Intracellular Freezing

Fat body cells from *S. crassipalpis* nondiapause-destined and diapause-destined larvae were placed in 360 or 1,000 mosmol Schneider's media, respectively. A small amount of a killed preparation of ice nucleating active *Pseudomonas syringae* (provided by Genencor International, Rochester, NY) was added to the media to prevent supercooling and insure that ice formed at temperatures slightly below 0°C. Cells were cooled at a rate of 2°C/min. Freezing of the cells, identified as an abrupt darkening of the cytoplasm, was visualized by cryomicroscopy using a Linkham computer-controlled conduction cryostage after Lee et al. (1993).

Viability of Frozen Fat Body Cells

Whole nondiapause-destined and diapause-destined larvae were placed in plastic 1.5-ml microcentrifuge tubes and held for 24 h at –5, –10, –22, or –83°C. Larvae were transferred directly to room temperature and the fat body cells were dis-

sected from these larvae and placed into either 360 mosmol Schneider's media (nondiapause-destined) or 1,000 mosmol Schneider's media (diapause-destined) and stained with a 1:50 mixture of 0.02% acridine orange and 0.01% ethidium bromide after Lee et al. (1993). Cells were examined with fluorescence microscopy (Olympus BH-2), and live and dead cells were counted. Cells with bright green nuclei were scored as live, and those with bright orange nuclei were scored as dead. In a second experiment, fat body cells from 5 individuals were removed, pooled, and placed in 1 ml of media in microcentrifuge tubes before placing them at the treatment temperatures.

Fat body cells from five nondiapause-destined larvae were pooled and half were placed in 1 ml 360 mosmol Schneider's insect media and half into 1 ml 360 mosmol Schneider's insect media with 1 M glycerol. Aliquots of 200 µl were incubated for 24 h at –5, –10, –22, or –83°C. Fat body cells from diapause-destined larvae were treated in the same way but were placed in 1,000 mosmol Schneider's insect media. After incubation, the survival status of these cells was assessed by acridine orange/ethidium bromide staining as described above. We compared survival among the various treatments using multiple linear regression using SAS version 6.12.

Composition of Fat Body Cells

Groups of fat body cells were harvested from larvae, lightly blotted, weighed immediately to 0.01 mg, and placed in a desiccator over P_2O_5 (Sigma Chemical Co.) until dried to a constant weight. To determine lipid content, the lipids were extracted from dried fat body cells according to the method of Folch et al. (1957).

RESULTS

Intracellular Freezing

As groups of fat body cells were cooled on the cryostage, ice began to form in the surrounding media at approximately –3.3°C (Fig. 1). However, fat body cells only began to freeze after the growing ice lattice in the surrounding medium contacted the cells. Intracellular freezing was clearly indicated by an abrupt darkening of the cytoplasm due to the rapid growth of ice. Within the larvae, the fat body cells are arrayed in chains with close contact between adjacent cells. Nonetheless, cells froze independently of each other (e.g., the time at which a particular cell froze did not influence when nearby or touching cells froze), suggesting that ice within one cell could not propagate to the next cell in the chain. The temperature of crystallization was determined by observing when the cells froze and recording the temperature indicated by a thermocouple that monitored the sample. The temperature of crystallization (–4.61 ± 0.06°C, mean ± SEM) for the nondiapause-destined larvae was significantly different from the diapause-destined larvae, –4.11 ± 0.04°C ($P < 0.001$) (Fig. 1). However, the overall range of values for individual cells, –3.7 to –6.2°C, was nearly identical for the two types of larvae.

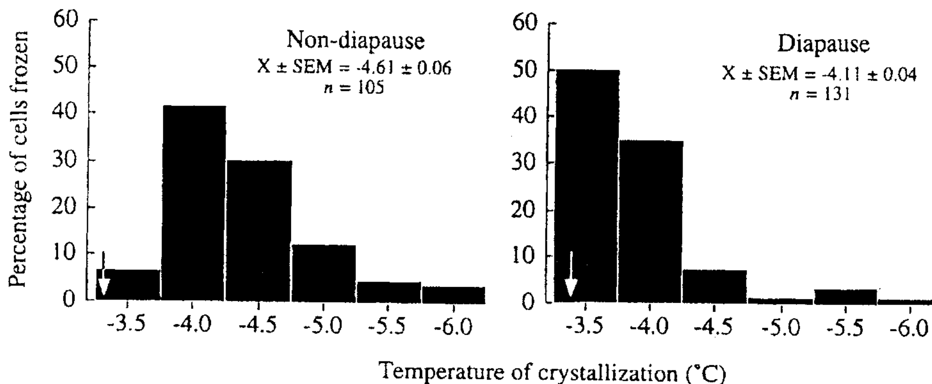

Fig. 1. Temperature at which intracellular freezing occurred in isolated fat body cells from nondiapause-destined larvae (n = 105) and diapause-destined larvae (n = 131) of *Sarcophaga crassipalpis* in Schneider's insect media. Cooling rate was 2°C/min. Arrows indicate the approximate temperature (–3.3°C) at which the media froze.

Viability of Frozen Fat Body Cells

The survival curves were similar for fat body cells dissected from intact non-diapause and diapause-destined whole larvae of *S. crassipalpis* that were exposed to various subzero temperatures (Fig. 2). The highest rates of fat body cell survival occurred at –5°C for both larval types. Survival rates decreased abruptly and few cells survived when they were exposed to –10°C or lower ($F_{23,103} = 19.94$, $P = 0.0001$).

A very similar pattern of survival was evident for the treatment in which the fat body cells first were dissected from the two types of larvae, and then held in the Schneider's insect media during subzero exposures. Once again the highest rates of survival occurred at –5°C with few cells surviving exposure to temperatures between –10 and –83°C. No difference was detected between the overall survival rates of cells from diapause-destined larvae and nondiapause-destined larvae.

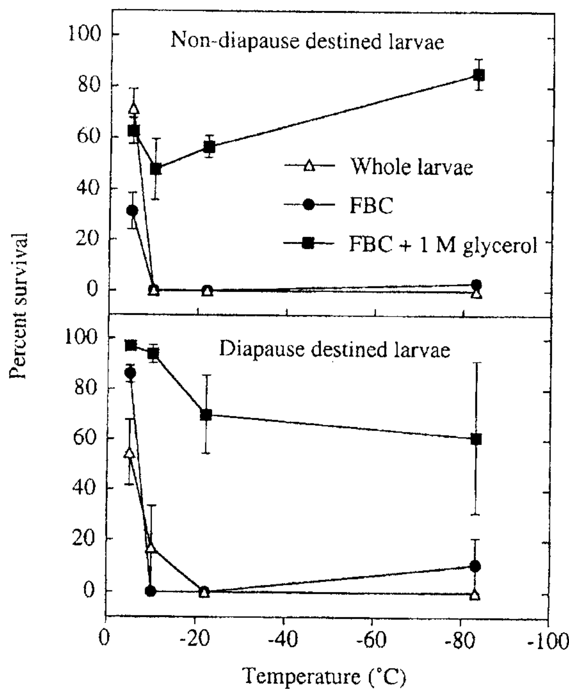

Fig. 2. Effect of 24 h of exposure to various subzero temperatures on the survival of fat body cells from nondiapause-destined (**top**) and diapause-destined (**bottom**) larvae of *Sarcophaga crassipalpis*. The whole larvae treatment group refers to intact larvae that were first exposed to subzero temperature before removing their fat body cells and testing them for survival. The two additional treatment groups used isolated fat body cells held in Schneider's insect media or Schneider's insect media supplemented with 1 M glycerol prior to subzero exposure.

Augmenting the Schneider's insect media with the cryoprotectant glycerol significantly increased the survival rates for isolated fat body cells from both larval types compared to the other two treatments (Fig. 2). At –5°C, cells from diapause-destined larvae had a survival rate of 97.3 ± 0.8 % (mean ± SEM), while 85 ± 5.8% survived 24 h of exposure to –83°C. Fat body cells from nondiapause-destined larvae also exhibited high rates of survival when the media was supplemented with glycerol.

For all treatments in which cells were judged to be dead based on the vital dye criteria, the cell membranes appeared to be intact and unbroken. Also it appeared that few, if any, lipid droplets within the cytoplasm had coalesced into larger droplets following intracellular freezing as was reported previously in fat body cells from larvae of *E. solidaginis* (Lee et al., 1993).

Composition of Fat Body Cells

Fat body cells of nondiapause-destined larvae of *S. crassipalpis* contained slightly, but significantly ($P < 0.001$), more lipids and less water than diapause-destined larvae (Table 1). For comparative purposes, we also determined the lipid and water contents for fat body cells from freeze-tolerant *E. solidaginis* larvae. Fat body cells of *E. solidaginis* larvae had only 34.6% water, significantly less ($P < 0.001$) than the 52–55% water content of either nondiapause- or diapause-destined larvae. The lipid content (47.3%) for these freeze-tolerant larvae was approximately twice that of cells from either type of *S. crassipalpis* larvae.

DISCUSSION

Intracellular Freezing

Our previous study of intracellular freeze tolerance in *E. solidaginis* larvae demonstrated that their fat body cells were susceptible to inoculative freezing at high subzero temperatures, –4.6°C (Lee et al., 1993). At that time, we speculated that susceptibility to inoculative freezing might be an adaptation for survival of intracellular freezing. Here, fat body cells from both types of *S. crassipalpis* larvae were also highly suscep-

TABLE 1. Relative Water and Lipid Composition (Mean ± SEM) of Fat Body Cells From Larvae of *Sarcophaga crassipalpis* and *Eurosta solidaginis*

	Relative composition of fat body cells (%)			
Species and diapause status	Water content	n	Lipid content	n
S. crassipalpis (nondiapause-destined larvae)	52.4 ± 0.3	15	23.9 ± 0.8	8
S. crassipalpis (diapause-destined larvae)	55.2 ± 0.4	24	20.3 ± 0.7	8
E. solidiginis (freeze-tolerant, diapausing larvae)	34.6 ± 0.8	30	47.3 ± 1.8	9

tible to inoculative freezing at temperatures as high as –3.5°C (Fig. 1). Cells from both nondiapause- and diapause-destined larvae froze on average between –4.1 and –4.6°C suggesting that their membranes did not differ markedly in their susceptibility to inoculative freezing, and that ice nucleating agents with activity above –4°C were not present in their cells.

The susceptibility of fat body cell membranes to ice penetration contrasts markedly with the behavior of mammalian cell membranes, which prevent inoculative freezing at these high subzero temperatures (Mazur, 1984). When mammalian cells are cooled slowly in the presence of ice in the surrounding medium, inoculative freezing of their cytoplasmic water typically does not occur until temperatures decrease to at least –10°C and often to considerably lower temperatures. This response is particularly true for cells that have not been produced in tissue culture or isolated by enzymatic digestion of their extracellular matrix (Karlsson et al., 1993). The fact that the fat body cells *E. solidaginis* and *S. crassipalpis* did not begin to freeze until external ice had contacted the cells indicates that ice nucleation was initiated by an inoculative process. The only other animal known to survive intracellular freezing is the Antarctic nematode *Panagrolaimus davidi* (Wharton and Ferns, 1995). The cells of this species are also highly susceptible to inoculative freezing.

Cryopreservation protocols generally seek to avoid intracellular ice formation, while cryosurgical procedures are designed to promote its occurrence in, for example, tumors (Karlsson et al., 1993). Despite its fundamental importance in cryobiology, the mechanism(s) by which extracellular ice initiates intracellular freezing remains largely unknown, but may involve growth of the ice lattice through membrane pores, membrane failure, or surface-catalyzed nucleation in which external ice changes the nature of the plasma membrane such that it induces ice nucleation within the cytoplasm (Karlsson et al., 1993). The exceptionally high susceptibility of these insects to inoculative freezing suggests that they may be valuable models for future study of this important cryobiological phenomenon.

These results for fat body cells from *S. crassipalpis* and *E. solidaginis* differ notably from those for the weta, *Hemideina maori* (Sinclair et al., 1999). Cells from the fat body and the Malpighian tubule of this species resisted inoculative freezing during cooling to –15°C, while assuming a shrunken and dehydrated appearance as ice formed extracellularly. Although this species tolerates the freezing of a remarkably large amount of its body water (82%), this response implies that its survival of freezing depends on its capacity to restrict ice formation to extracellular compartments (Sinclair and Wharton, 1997).

Viability of Frozen Fat Body Cells

Although tolerance of intracellular freezing may be promoted by susceptibility to inoculative freezing at high subzero temperatures, this trait was not sufficient to confer high levels of freeze tolerance on the fat body cells of *S. crassipalpis* larvae. In the absence of glycerol, only at the highest test temperature of –5°C were significant numbers of cells from nondiapause- and diapause-destined larvae able to survive freezing (Fig. 2). When the treatment temperature was decreased to –10°C few, if any, cells survived. This response is consistent with the fact that fewer than 10% of nondiapause- and diapause-destined larvae can survive 2 h at –10°C (Lee et al., 1987a). In contrast, fat body cells from *E. solidaginis* exhibit rates of survival greater than 80% at this temperature (Lee et al., 1993; Bennett and Lee, 1997).

The addition of 1 M glycerol, a common cryoprotective compound in cold-hardy insects, to the medium greatly enhanced fat body cell survival for both types of *S. crassipalpis* larvae with many

cells surviving exposure to –83°C (Fig. 2). Changes in glycerol levels are associated with a rapid cold-hardening response, low temperature acclimation, and developmental patterns in this species (Chen et al., 1987; Lee et al., 1987a,b). The addition of glycerol also enhances freezing tolerance in a similar fashion in fat body cells of *E. solidaginis* (Lee et al., 1993).

Other freeze tolerant plants and animals avoid the damaging effects of excessive ice formation by dehydrating sensitive tissues (Lee and Costanzo, 1998). For example, in the wood frog (*Rana sylvatica*), water is translocated from body organs to subdermal lymph sacs and the coelomic cavity where it freezes; some organs lose more than 60% of their initial water mass (Lee et al., 1992). Another advantage of dehydration is that removal of water effectively increases the concentration, and therefore the potential benefit of cryoprotectants (Costanzo et al., 1992). A number of freeze tolerant insects markedly decrease their total body water content (Ring, 1982). Presumably, both internal translocation of water and overall reduction in total body water would promote freeze tolerance by reducing cellular water content.

Consequently, another possible reason why fat body cells from *E. solidaginis* larvae survive intracellular freezing is their intrinsically low water content (Table 1). Although it is well documented in many organisms that intracellular freezing is damaging, as with the mechanism of inoculative freezing of cells, the specific nature of injury caused by freezing remains largely unknown (see review by Karlsson et al., 1993). Nonetheless, several lines of evidence suggest that mechanical damage caused by growing ice crystals may injure cells in several ways. Since fat body cells from *E. solidaginis* had a water content of only 35% while cells from *S. crassipalpis* larvae had 52–55% (Table 1), cells with less water may be less likely to be damaged by mechanical forces during intracellular freezing.

ACKNOWLEDGMENTS

We thank Jason Irwin, John Mugnano, Jon Kelty, and Shala Hankison for technical and statistical assistance, and Jon Costanzo for reviewing an earlier version of the manuscript.

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