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Survival of Intracellular Freezing, Lipid Coalescence and Osmotic Fragility in Fat Body Cells of the Freeze-tolerant Gall Fly *Eurosta solidaginis*

RICHARD E. LEE JR,* JOHN J. MCGRATH,† R. TODD MORASON,* RONALD M. TADDEO*

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Although it is generally believed that under natural conditions, freeze-tolerant organisms can survive only if ice formation is restricted to the extracellular space, in 1959 R. W. Salt reported that fat body cells of the freeze-tolerant gall fly, *Eurosta solidaginis* (Diptera: Tephritidae), survive intracellular freezing. Using cryomicroscopy, intracellular freezing was observed at -4.6 ± 0.1°C for cells in Grace's media. Freezing was apparently caused by inoculative freezing from outside the cell, since fat body cells in oil cooled to below −15°C without internal ice formation. Viability of cells was assessed using fluorescent vital dyes immediately following freezing for 24 h at temperatures between −5 and −80°C. At −25°C or lower few cells survived freezing in Grace's medium alone. At −25 and −80°C, cells frozen in Grace's media supplemented with 1 M glycerol exhibited rates of survival similar to those in whole larvae. No larvae ($n = 20$) survived freezing to −80°C, but more than 60% of the fat body cells survived this treatment. Most fat body cells survived osmotic concentrations from 0.25 to 2 times that of the normal concentration of Grace's media (340 mOsm) for 24 h at 4°C. More than 60% of the cells survived 5 M glycerol in Grace's media under these conditions. An unusual response of these cells to freezing is the coalescence of intracellular lipid droplets upon thawing. The magnitude of coalescence increases with decreasing temperature and increasing duration of exposure, but decreases with the addition of glycerol to the media. Coalescence in itself is not indicative of injury, since larvae frozen under conditions that cause extensive coalescence readily survived to complete their development and emerge as adults.

Cold-hardiness Freeze tolerance Cryoprotection Glycerol

INTRODUCTION

Although it is generally believed that survival of freezing under natural conditions occurs only if ice formation is restricted to the extracellular space (Mazur, 1984), this assumption is based on an extrapolation from the cryopreservation of mammalian cells that would never *naturally* experience subzero temperatures. This premise has rarely been directly investigated in cells from naturally freeze-tolerant organisms. However, on two occasions Salt (1959, 1962) reported intracellular freeze tolerance in the fat body cells of the goldenrod gall fly, *Eurosta solidaginis*. For more than 30 yr Salt's observations on this novel phenomenon have been largely ignored.

The goldenrod gall fly, *E. solidaginis*, has received extensive study as a freeze-tolerant insect model (Storey and Storey, 1988; Baust and Nishino, 1991). In the spring, adults emerge from ball galls of goldenrod (*Solidago* spp), mate and females oviposit into the stem of young plants. Larvae pass through two instars during the summer and overwinter within the gall as a third instar. During the summer and early autumn, *E. solidaginis* is intolerant of freezing; however, in response to environmental cues, larvae acquire freeze tolerance in mid to late autumn (Morrissey and Baust, 1976). The supercooling point, sometimes referred to as the temperature of crystallization, of the freeze-tolerant larvae is approximately −8 to −10°C (Baust and Lee, 1981; Bale *et al.*, 1989). Larval exposure to low temperatures between 0 and 5°C trigger an accumulation of sorbitol (Baust and Lee, 1981, 1982; Rojas *et al.*, 1984; Storey and Storey, 1988). Desiccation may also play a role in the accumulation of cryoprotectants; glycerol accumulation in third instars is closely correlated with the drying of the surrounding gall tissues as the plant senesces (Rojas *et al.*, 1986).

In this report we have confirmed and extended the initial observations of Salt (1959, 1962) on survival of

*Department of Zoology, Miami University, Oxford, OH 45056, U.S.A.
†Department of Mechanical Engineering, Michigan State University, East Lansing, MI 48823, U.S.A.

intracellular freezing in *E. solidaginis*. Specifically we used a combination of *in vivo* and *in vitro* approaches to determine the following: the temperature at which intracellular freezing occurs, survival of the fat body cells frozen with and without cryoprotectant, factors influencing the coalescence of cytoplasmic lipids during freezing and osmotic fragility of fat body cells.

MATERIALS AND METHODS

Goldenrod galls were collected in St Paul, Minnesota on February 16, 1991. For the first 20 days they were held at 4°C before transfer to −15°C where they were kept until they were used for experimentation. Supercooling points and glycerol determinations were determined as described previously (Baust and Lee, 1981).

Cryomicroscopy was preformed using a conduction type cryomicroscope system as described by McGrath (1987). Fat body cells were frozen in Grace's insect media (340 mOsm) unless otherwise indicated. To prevent inoculation of cells by extracellular ice cells were frozen in a light paraffinic oil called American White Oil (Standard Oil Company).

Following various treatments cell survival was assessed using fluorescent vital dyes as follows (Haugland, 1992). A mixture of acridine orange and ethidium bromide stained live cells and nuclei green, while dead cells and nuclei appeared orange. Propidium iodide stained the nuclei of dead cells orange. Stained cells were viewed using an Olympus BH-2 microscope equipped with a reflected light fluorescence attachment.

Coalescence was scored on a relative scale from 0 to 4. A score of 0 was assigned to unfrozen cells that

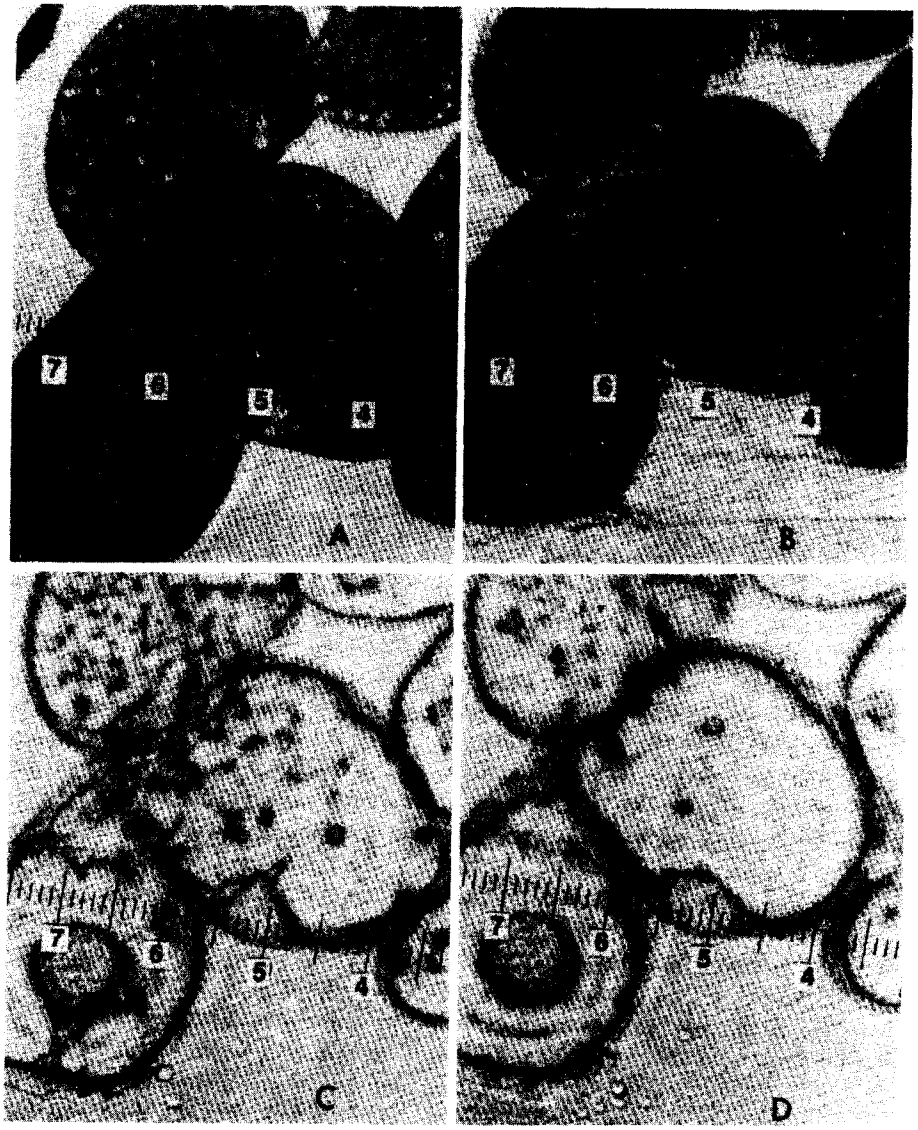

FIGURE 1. Representative cryomicroscopic photographs of freezing and thawing in fat body cells of the freeze-tolerant gall fly, *Eurosta solidaginis*, in Grace's medium. (A) Unfrozen fat body cells at 5°C. (B) Intracellular freezing of fat body cells. (C) Beginning of lipid coalescence immediately after thawing. (D) Extensive lipid coalescence within 1 min after thawing. Each minor scale division = 10 μm.

TABLE 1. Survival of third-instar *Eurosta solidaginis* following 24 h of freezing

Temperature	Adults fully formed	Adult emergence
−25°C	85% (17/20)	75% (15/20)
−80°C	0% (0/20)	0% (0/20)

contained a large number of small lipid droplets dispersed evenly throughout the cytoplasm [see Fig. 1(A)]. Cells scored as 1 contained in excess of 15 medium-sized droplets. As coalescence progressed the number of droplets decreased, but droplets became larger. Cells with 2–15 large droplets were scored a 2. Cells scored as 3 or 4 contained a single large lipid droplet which occupied the majority of the cell volume. In cells scored as 3, the single droplet only partially occupied the cell, and those scored as 4 contained the single lipid droplet occupied most of the center of the cell and approached the cell membrane [see Fig. 1(D)].

RESULTS

Cold-hardiness of gall fly larvae

The mean supercooling point of larvae was −10.1 ± 0.8°C ($n = 14$). Glycerol titers were 32.3 μg/mg live weight. The limit of freezing tolerance was assessed by freezing larvae for 24 h at either −25 or −80°C and then holding them at 22–24°C until they emerged from the galls (Table 1). Of the larvae frozen at −25°C, 85% successfully metamorphosed from larvae to adults, and 75% successfully emerged from the gall. In contrast, no adults formed from larvae frozen to −80°C.

Intracellular freezing

Cryomicroscopy was used to directly observe the effects of freezing and thawing on fat body cells in Grace's insect media. Intracellular freezing, visualized as flashing, was identified as an abrupt darkening of the cytoplasm [cf. Fig. 1(A) vs (B)] (McGrath, 1987). Cells were cooled at 2°C/min. The mean onset temperature of intracellular freezing was −4.6 ± 0.1°C (mean ± SEM), $n = 68$ (Fig. 2). Some cells froze at temperatures as high −3°C, while a few cells did not freeze until −8°C. Ice always formed in the surrounding media before intracellular flashing was observed. Freezing was apparently

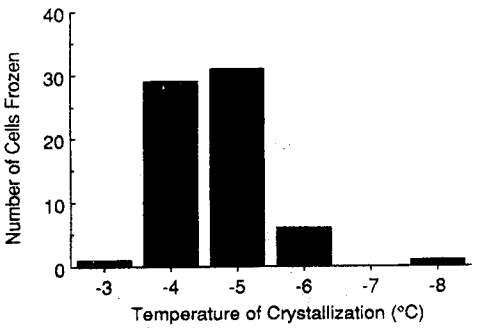

FIGURE 2. Temperature at which intracellular freezing occurred in fat body cells ($n = 68$) frozen in Grace's insect media. Cooling rate was 2°C/min.

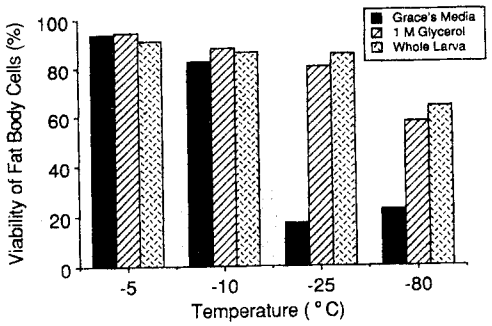

FIGURE 3. Effect of subzero temperature on the viability of fat body cells after a 24 h freeze. Cells were either frozen in Grace's insect media with or without the addition of 1 M glycerol, or following low temperature exposure they were dissected from larvae and tested for viability. Bars represent the average cell survival as determined using acridine orange/ethidium bromide and propidium iodide fluorescence (50 cells were used for each assay).

caused by inoculative freezing from outside the cell, since fat body cells in oil could be cooled to below −15°C without internal ice formation.

Viability of fat body cells vs larvae

The viability of fat body cells dissected from whole larvae or isolated cells frozen in Grace's media with or without the addition of 1 M glycerol was determined after a 24-h freeze at various temperatures (Fig. 3). Under all treatments cells frozen to −5 or −10°C had high survival rates based upon our criteria for survival. Furthermore, survival of the fat body cells frozen *in vitro* at these high subzero temperatures correlated closely with the survival of intact larvae frozen under these conditions. At −25°C or lower, few cells survived freezing in Grace's medium alone. At −25 and −80°C, cells frozen in Grace's plus glycerol exhibited rates of survival similar to those from whole larvae. No larvae (Table 1) survived freezing at −80°C, but more than 60% of the fat body cells dissected from larvae that had been frozen survived this temperature (Fig. 3).

Lipid coalescence within fat body cells

Unfrozen cells were filled with a large number of small lipid droplets dispersed evenly throughout the cytoplasm

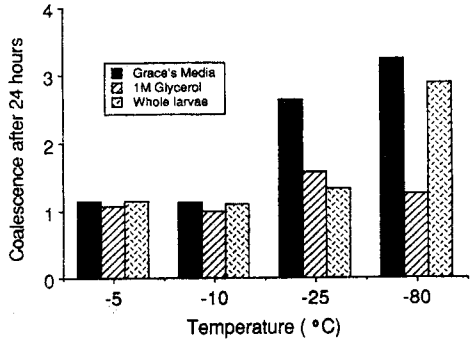

FIGURE 4. Relative coalescence of intracellular lipids after freezing for 24 h at various temperatures. Cells were either frozen in Grace's insect media with or without the addition of 1 M glycerol or they were dissected from larvae and tested for viability following low temperature exposure.

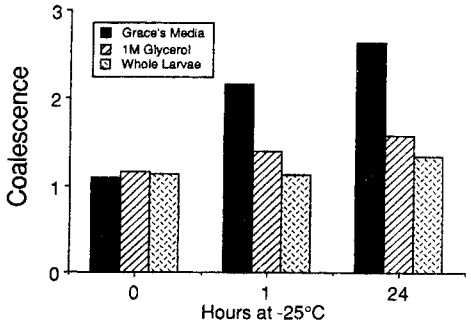

FIGURE 5. Relative coalescence of intracellular lipids during 24 h of freezing at −25°C. Cells were either frozen in Grace's insect media with or without the addition of 1 M glycerol or they were dissected from larvae and tested for viability following low temperature exposure.

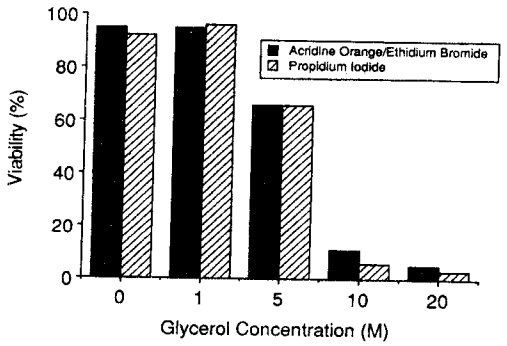

FIGURE 7. Osmotic fragility of fat body cells ($n = 50$) of third instar larvae of *Eurosta solidaginis* exposed to various concentrations of glycerol in Grace's insect media, pH 7.0 for 24 h at 4°C.

[Fig. 1(A)]. Galls containing larvae were held at −15°C until they were used in this study; these storage conditions caused partial coalescence (index score of 1) of fat body cells. When cells from these previously frozen larvae were frozen in Grace's media with or without glycerol at −5 or −10°C, index scores for coalescence remained near 1 (Fig. 4). After a 24 h freeze to −25°C fat body cells frozen in Grace's media showed a marked increase in coalescence compared to ones frozen at warmer temperatures. In contrast, cells frozen in Grace's media plus glycerol or cells from larvae frozen to −25°C exhibited only slightly greater coalescence. After the lowest temperature freeze to −80°C, nearly all cytoplasmic lipids were contained in a single droplet that occupied the majority of the cell volume [Fig. 1(D)]. At this temperature, cells dissected from whole larvae had a greater degree of coalescence than cells frozen in Grace's media containing 1 M glycerol (Fig. 4).

We examined the time–course of coalescence during a 24 h freeze at −25°C (Fig. 5). During the first hour of freezing the index of coalescence increased approximately 2-fold in fat body cells frozen in Grace's media alone with a lesser increase during the next 24 h of freezing. Slight increases in coalescence occurred for cells in Grace's media with glycerol or in cells from intact larvae during the 24 h exposure.

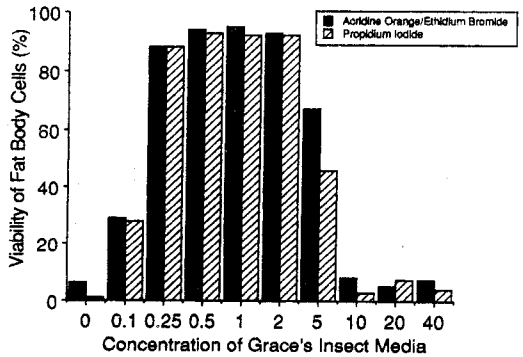

FIGURE 6. Osmotic fragility of fat body cells ($n = 50$) of third instar larvae of *Eurosta solidaginis* exposed to various concentrations of Grace's insect media, pH 7.0 for 24 h at 4°C.

Osmotic fragility of fat body cells

Since under natural conditions freezing is inextricably tied to solute concentration in the unfrozen body fluids, we also examined the tolerance of isolated fat body cells to osmotic stress at 4°C for 24 h (Fig. 6). As described earlier cell survival was based the response of cells to treatment with fluorescent vital dyes. Even in the absence of cryoprotectant these cells survived osmotic concentrations that ranged from 0.25 to 2 times that of Grace's media (340 mOsm). The independent estimates of cell viability using the two sets of vital dyes gave similar results.

Fat body cell viability was also tested in Grace's media supplemented with several concentrations of glycerol for 24 h at 4°C (Fig. 7). Slightly less than 70% of the cells survived 5 M glycerol, while few cells survived higher concentrations.

DISCUSSION

Few investigators have examined cellular mechanisms of chilling and freezing injury in insects. Two forms of low temperature injury are evident: (1) injury resulting from freezing and (2) cold shock associated with chilling, but without ice formation (Lee, 1991). We selected fat body cells because of their central role in intermediary metabolism, particularly with respect to the synthesis of cryoprotectants. Fat body functions are comparable to those of mammalian liver, serving as a primary site of protein synthesis and secretion, and the synthesis and storage of lipids and carbohydrates (Locke, 1984).

Survival of intracellular freezing

It is generally believed that freeze tolerance at high subzero temperatures with slow cooling rates is possible only if ice formation is restricted to the extracellular space (Mazur, 1984). In contrast, our present study confirmed the previous reports of Salt (1959, 1962) that fat body cells of the freeze-tolerant gall fly larvae survive intracellular freezing.

A cryomicroscope was used to directly observe the effects of freezing and thawing on fat body cells. Using this instrument we observed intracellular freezing of individual cells at -4.6 ± 0.1°C. In every instance ice

had already formed in the surrounding media before intracellular freezing occurred. Furthermore, when cells were cooled to −15°C in oil, to prevent inoculative freezing, no flashing was observed. These results indicate that efficient heterogeneous ice nucleators are absent in fat body cells and that intracellular freezing resulted from inoculative freezing from ice in the surrounding media.

In mammalian cells that are rapidly cooled, inoculative freezing of the intracellular fluid occurs between −5 and −30°C depending on the cell type, however nucleation is typically blocked by the cell membrane at temperatures above −15°C (Mazur, 1984). In comparison the fat body cells were frozen at the high end of this temperature range. The relative susceptibility of the fat body cell to inoculative freezing may represent an adaptation promoting intracellular freeze tolerance. Since so few cells have been examined for intracellular freeze-tolerance it is possible that this trait will be found in other freeze tolerant organisms.

Another objective of our study was to compare the low temperature limit of freeze tolerance for whole larvae vs their fat body cells. Although greater than 60% of the fat body cells survived freezing to −80°C, no larvae survived freezing to this temperature. These results suggest that fat body cells are not the cell type within the larvae that is most susceptible to freezing injury.

Coalescence of lipid droplets within fat body cells

Asahina (1969) reported lipid coalescence in fat body cells of insects that did not survive freezing. However, Salt (1959) described coalescence of intracellular lipid droplets in fat body cells that survived intracellular freezing. Prior to freezing, each cell contained many tens of lipid droplets; however, intracellular freezing resulted in the coalescence of these into fewer, larger droplets. At relatively low freezing temperatures the cytoplasm appeared to be filled with a single large lipid unit following thawing. The magnitude of coalescence is related to the duration of exposure to subzero temperatures: longer exposures produced increased levels of coalescence. We also observed that the addition of glycerol to the Grace's media greatly decreased the amount of coalescence in cells frozen to −80°C. Coalescence in itself was not indicative of injury, since larvae frozen under conditions that cause extensive coalescence of lipid droplets within their fat body cells readily survived to complete their development and emerge as adults. It appears that the presence of coalescence may be useful as a marker indicating that fat body cells have experienced intracellular freezing.

During freezing only water molecules join the growing crystal, rejected solute becomes concentrated in the remaining unfrozen body fluids. The resulting osmotic gradient removes water from the cells. This process may play a role in promoting lipid coalescence. If water is removed from the cell during freezing it should bring the lipid droplets closer together, and thereby facilitate their fusion with each other. It is also possible that the lipid droplets may fuse due to their compression between growing arms of the ice lattice within the fat body cell. Mazur (1984) suggested a similar process to explain mechanical injury to cells within narrow channels of unfrozen fluid during freezing. Lipid coalescence may also be caused by a combination of freezing-induced cell dehydration and compression of lipid droplets between arms of the ice lattice.

Osmotic fragility of fat body cells

Freezing-induced cellular dehydration is an important mechanism of freezing injury (Mazur, 1984). The cell membrane is commonly identified as the primary site of freezing injury due to the action and interaction of hypertonicity and/or the direct effect of low temperature. Hypertonic stress may result in the actual loss of membrane material predisposing the cell to lethal injury during thawing (Steponkus, 1988). Our preliminary cryomicroscopoic observations did not suggest that this happened in the fat body cells that we examined. Our study demonstrated that fat body cells are resistant to a range of osmotic stresses (Figs 6 and 7), a trait that is not surprising in a cell that survives both intra- and extracellular ice formation.

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