Comparative Ultrastructure of Fat Body Cells of Freeze-susceptible and Freeze-tolerant Eurosta solidaginis Larvae After Chemical Fixation and High Pressure Freezing

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In response to low environmental temperatures third instar larvae of the goldenrod gall fly, Eurosta solidaginis, undergo the process of cold-hardening in which they acquire freeze-tolerance. Cold-hardening includes the elevation of the supercooling point, the temperature at which body water spontaneously freezes, and the accumulation of the low-molecular-mass cryoprotectants, glycerol, sorbitol and trehalose. Although it is generally believed that freezing survival is only possible if the ice lattice is restricted to the extracellular space, the larval fat body cells survive intracellular ice formation. Fat body cells of freeze-susceptible (September-collected) and freeze-tolerant (January-collected) third instar larvae were prepared for transmission electron microscopic analysis using both conventional chemical fixation and high pressure freezing with freeze substitution. High pressure cryofixation procedures were superior to conventional fixation in both freeze-susceptible and freeze-tolerant cells. Endogenous cryoprotectants in freeze-tolerant larvae contributed to the superior ultrastructure of cytoplasmic organelles and nucleoplasm in high-pressure frozen fat body cells, while freeze-susceptible larvae, which lack high levels of cryoprotectants, contained fat body cells which sustained ice crystal damage following cryofixation. Mitochondria in fat body cells prepared using high pressure freezing/freeze substitution were characterized by smooth outer membranes and uniformly electron dense mitochondrial matrix. Conventionally prepared samples exhibited crenated cytoplasmic organelles with undulating membrane profiles; smooth profiles characterized high-pressure frozen structures. Lipid droplets were abundant in all fat body cells, however glycogen, the energy source of cryoprotectants, was variable in both freeze-susceptible and freeze-tolerant cells. Coalescence of lipid droplets, induced by intracellular freezing at progressively lower temperatures, caused displacement of interior cytoplasmic organelles including the nucleus to the cell cortex. This shift in cytoplasm had no affect on survival of the cells.

Freeze-tolerance Tephritidae Intracellular freezing Cryofixation Ultrastructure

INTRODUCTION

Insects use a diverse range of biochemical, physiological and ecological adaptations to survive low temperatures (see reviews in Lee and Denlinger, 1991). It is generally believed that intracellular freezing at high sub-zero temperatures is lethal to mammalian cells (Mazur, 1984). However, more than 30 years ago Salt (1959, 1962) reported survival of intracellular freezing in fat body cells of the goldenrod gall fly, Eurosta solidaginis (Diptera, Tephritidae). Using cryomicroscopy and fluorescent vital dyes Lee et al. (1993) recently confirmed the validity of Salt’s previous reports.

E. solidaginis has been studied extensively as a freeze-tolerant insect model (Storey and Storey, 1986; Baust and Nishino, 1991). Third instar larvae overwinter in a spherical gall on the stem of goldenrod (Solidago spp.) for 11 months of the year, and it is here that they undergo cold-hardening and acquire freeze-tolerance. During summer and early autumn larvae are unable to survive freezing. However, in autumn, low temperatures and other environmental cues trigger synthesis of glycerol, sorbitol and trehalose, from glycogen. After the cold-hardening process, the combined concentration of cryoprotectants in the freeze-tolerant larvae may approach 1 M (Morrissey and Baust, 1976).

The function of insect fat body is comparable to mammalian liver in terms of metabolic activity. Fat body cells are the principle site of intermediary metabolism
and nutrient storage in insects, and in winter they are the site of cryoprotectant synthesis in freeze-tolerant larvae of *E. solidaginis* (Storey and Storey, 1985, 1986). While certain physiological aspects of these cells have been studied (Salt, 1959, 1962; Lee et al., 1993), the effects of intracellular freezing on their ultrastructure have not been previously investigated.

Fat body cells with their natural, endogenous cryoprotectants for tolerance of intracellular freezing may also promote optimal cryofixation of these cells since some of the same and related compounds are used for ultrastructural preservation. Cryoimmobilization by high pressure was selected to avert potential ultrastructural differences in fat body cells which, depending on the season, may be ascribed to either freeze-susceptible (early autumn) or freeze-tolerant (late autumn or winter) physiological conditions in the third instar larvae of *E. solidaginis*. Advantages of high pressure freezing/freeze substitution (HPF/FS) compared to conventional fixation have been documented (Studer et al., 1989). Plant tissues and fungi (Kiss et al., 1990; Lichtscheidl et al., 1990; Staehelin et al., 1990) and a limited number of animal tissues (Studer et al., 1989; Allenspach, 1993) including early embryonic stages of insects have been studied (McDonald, 1990).

Upon thawing an unusual and marked consequence of intracellular freezing in fat body cells is the coalescence of large numbers of lipid droplets into several or even a single large droplet (Salt, 1959, 1962; Lee et al., 1993). The ultrastructural consequence of this dramatic change in the morphological appearance of the cell is unknown.

The objective of this study was to compare the ultrastructure of freeze-susceptible and freeze-tolerant larvae of *E. solidaginis*. Particular attention was paid to the lipid coalescence and the quality of ultrastructural preservation achieved using high pressure freezing and freeze substitution.

**MATERIALS AND METHODS**

**Larval cold-hardiness**

Goldenrod galls were collected in September 1991 and January 1992. Galls collected in September were stored at 23°C, while those collected in January were stored at −18°C until they were used for experimentation. Both groups were tested for freeze-susceptibility or freeze-tolerance. Fifteen larvae from each group were placed in a cold bath at −15°C for 24 h, after which movement of the larvae was used to assess survival. The supercooling point, the temperature to which the larvae cool before internal ice formation occurs, was determined for larvae.
CRYOFIXED EUROSTA FAT BODY CELLS

FIGURE 3. High magnification photograph of mitochondria in a freeze-tolerant cell prepared by HPF/FS. Note the smooth, rounded appearance of the organelles and the uniform distribution of internal matrix. The cytoplasm is uniformly preserved and demonstrates an excellent quality of freeze. Bar: 0.5 μm.

from both the September and January groups. Twelve larvae from each group were placed in a cold bath at 5°C and the temperature was decreased by 0.3°C/min until all larvae had frozen. The temperature of each larva was monitored using a thermocouple during cooling, and the sharp increase in temperature, signalling the release of the latent heat of fusion from the larva's body water, was used to determine the temperature at which freezing began.

To induce coalescence whole larvae of the freeze-tolerant type were removed from a cold room (4°C) to a water bath where they were cooled (0.3°C/min) to and equilibrated at -25°C, at which temperature they were held for 24 h. Larvae were then warmed passively to room temperature prior to the isolation of fat body cells for cryofixation.

FIGURE 4. High magnification view of a multi-vesicular body (upper left) and mitochondria in a freeze-tolerant cell prepared by HPF/FS. Concentric lamellae and internal products of the vesicles are densely osmiophilic. Cristae are evident in the mitochondria, whose outer membranes are smooth and osmiophilic. Bar = 0.2 μm.

FIGURE 5. Cytoplasm of a fat body cell from a freeze-susceptible larva, chemically fixed. Mitochondria contain irregular outer membranes and the cytoplasm appears empty in some places suggesting that some leaching has occurred during processing. Arrows, Glycogen granules. Bar = 0.5 μm.

Conventional chemical fixation

Freeze-susceptible and freeze-tolerant larvae were dissected in Grace's solution (at 5°C) after which fat body cells were removed and grouped into clumps of 5-10 cells each. The cells were then placed in a primary fixative of 2.0% glutaraldehyde in 0.1 M cacodylate buffer at pH 7.0 for 1.5 h. Following a brief wash in 0.1 M cacodylate buffer, also at pH 7.0, the cells were post-fixed at 5°C in Millonig's osmium tetroxide solution for 1.5 h. The cells were then washed with buffer a second time, dehydrated in a graded acetone series and embedded in Epon resin.

High pressure freezing and freeze substitution (HPF/FS)

Freeze-susceptible and freeze-tolerant larvae were dissected in 1-hexadecene (to prevent desiccation), fat body cells were isolated and grouped into small clusters of 6-8 cells, and placed into specimen chambers (Dahl and Staehelin, 1989) which previously had been coated with a 0.1% solution of soybean lecithin in chloroform.

Freezing of the samples was accomplished in a Balzers HPM 010 high pressure freezing machine as described by
FIGURE 6. Freeze-susceptible larva fat body cell prepared by HPF/FS to show the general distribution of lipid. The interior cytoplasm is segregated into dense zones, which contain rough endoplasmic reticulum and other organelles, less dense areas of cytoplasm and lipid vacuoles. Glycogen is sparsely deposited in this cell. Bar = 4 μm.

Moor (1987). Immediately after the freezing event specimens were removed from the chambers and placed into liquid nitrogen (LN₂).

Frozen samples were freeze-substituted for 5 days at -78°C in acetone containing 1.0% osmium tetroxide in a customized aluminium chamber (Allenspach, 1993). Solutions were replaced daily. The samples were then transferred to cold, dry absolute acetone and the aluminium chamber was allowed to passively warm to room temperature. The samples were then embedded in Epon resin.

Microscopy

Thin sections were prepared from both conventionally fixed and high pressure frozen samples using a Reichert Ultracut E microtome equipped with diamond knife. Sections were stained with uranyl acetate and lead citrate, following which they were viewed and photographed on JEOL 9S or Zeiss 10C transmission electron microscopes.

RESULTS

The September larvae had not undergone winter cold-hardening and were freeze-susceptible, while the larvae collected in January proved to be freeze-tolerant. The mean values (+SEM) of the whole-larvae supercooling point were -11.8 ± 1.5°C and -9.1 ± 0.7°C for the September and January larvae, respectively. After freezing in a cold bath at -15°C for 24 h, all September larvae (n = 15) died, indicating that they were freeze-susceptible. In contrast, all 15 January larvae moved soon after thawing, indicating that they were freeze-tolerant. The elevated supercooling point and reduced variability of values among individuals of the January larvae is consistent with the onset of freeze-tolerance (Morrissey and Baust, 1976; Lee et al., 1993).

FIGURES 7 and 8. Photographs of freeze-susceptible cells preserved by HPF/FS showing concentrated ribosomes, rough endoplasmic reticulum and other organelles in the dense cytoplasm. The white halos surrounding the organelles (Fig. 7) are artifacts restricted to freeze-susceptible cells, as are the ice "ghosts" (solid stars) in the less dense cytoplasm (Fig. 8). Glycogen deposits are absent from the lighter cytoplasm of this particular cell. m, Mitochondrion; mt, microtubule. Fig. 7, bar = 1 μm; Fig. 8, bar = 0.5 μm.
Striking differences were evident in the ultrastructure of fat body cells preserved by HPF/FS and those chemically fixed (Figs 1–5). Ultrastructure of chemically fixed fat body cells whether from freeze-susceptible or freeze-tolerant larvae, showed no significant differences. The large diameter (0.27 ± 0.04 mm; n = 35) of the fat body cells undoubtedly affected the quality of preservation. Plasmalemmae of fat body cells generally were smoother in HPF/FS cells and contained an extracellular coat of variable thickness (up to 0.25 μm) which is visualized as a combination of amorphous matrix and short thin filaments. Mitochondria in fat body cells prepared using HPF/FS were characterized by smooth outer membranes and uniformly electron dense mitochondrial matrix (Figs 3 and 4). Mitochondrial cristae and lamellae of Golgi bodies and multivesicular bodies were extremely osmiophilic (Fig. 4). Chemically fixed cells exhibited mitochondria with irregular outer membranes and regions of cytoplasm in which leaching of ground substance had occurred (Fig. 5).

The morphology of lipid droplets in fat body cells varied depending on the type of fixation. Lipid droplets fixed by high pressure freezing were typically spherical, whereas they were often non-spherical in chemically fixed cells (Figs 1 and 2). Lipids in high pressure frozen cells were completely extracted from the droplets by acetone during freeze substitution (Fig. 1), whereas in chemically fixed fat body cells lipids were only partially removed during processing (Fig. 2). Lipid droplets with smaller diameters appeared to be more abundant in cells preserved by HPF/FS than in chemically fixed cells, although a quantitative study was not undertaken. Small lipid droplets in HPF/FS cells were consistently 0.1 μm or less in diameter, and often separated from lipid droplets 3–4 × their size by a very thin thread of cytoplasm. The smallest lipid droplets in conventionally fixed fat body cells measured 0.15 μm across their diameter, but most were typically larger (0.16–4.0 μm dia).

The amount of glycogen apparent in micrographs of fat body cells of freeze-susceptible larvae was highly variable. Typically, cells from freeze-susceptible larvae have a cytoplasm rich in ribosomes (Figs 6–8) and relatively sparse in glycogen granules, although Fig. 2 illustrates a moderate concentration of glycogen. The

Typically freeze-tolerant fat body cells were better preserved by HPF/FS than were freeze-susceptible cells (Figs 6 and 9). The cytoplasm of fat body cells is segregated into dense and less-dense regions (Figs 6 and 8), the dense zones being heavily populated with ribosomes and organelles. The less-dense cytoplasm on the interior of freeze-susceptible cells did not freeze as well as the interior of freeze-tolerant cells (Figs 9-11). Thus, ice crystals that formed in the cytoplasm, when they were removed by freeze-substitution, left electron transparent “ghosts” in the cytoplasm (Fig. 8). The lack of ice crystal “ghosts” in the deep cytoplasm of freeze-tolerant cells indicates an optimal quality of freeze (Fig. 10). These cells were also richly laden with glycogen (Figs 10 and 11). Electron translucent halos, indicating a sub-optimal freeze, surrounding mitochondria were observed in freeze-susceptible cells (Fig. 8), but absent in freeze-tolerant cells. Ice crystal “ghosts” became evident in the mitochondrial matrix of freeze-tolerant cells only when viewed at high magnifications (100,000×).

Cytoskeletal microtubules were seen abundantly in both chemically preserved cells and in high pressure frozen fat body cells of both types.

Nuclear chromatin of freeze-tolerant and freeze-susceptible fat cells is visualized in Figs 12 and 14, respectively. The nucleoplasm, particularly the disperse euchromatin, in freeze-susceptible cells exhibited modest segregation patterns because of small ice crystal formation (Fig. 16). When viewed at comparable magnifications (>70K), nucleoplasm of freeze-tolerant cells was finely granular and free of ice crystal “ghosts” (Figs 13 and 15).

Induced coalescence of numerous cytoplasmic lipid droplets, whose normal distribution is illustrated in Fig. 2, resulted in formation of a single large lipid droplet (Fig. 17). A small arc of peripheral cytoplasm interface between densely packed ground substance and less dense glycogen-free cytoplasm (see Fig. 6) is shown in Fig. 8. Generally, fat body cells of freeze-tolerant larvae exhibited significant amounts of glycogen (Figs 9 and 10).

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surrounding a very large lipid droplet is shown in Fig. 18. This cytoplasmic zone, that contains the organelles, varies in thickness from a minimum of about 3 μm to approx. 30 μm in the region containing the nucleus. A small remaining population of lipid droplets (2 μm or less in diameter) that did not participate in coalescence are evident in the marginal cytoplasm (Fig. 18).

**DISCUSSION**

Significant ultrastructural differences have been observed in the fat body cells of freeze-tolerant and freeze-susceptible larvae of *E. solidaginis*. Fat body cells of freeze-tolerant larvae contain endogenous cryoprotectants, while freeze-susceptible larvae do not, and are able to survive intracellular freezing (Lee et al., 1993). Cryoimmobilization by high pressure freezing successfully preserved the ultrastructure of these bulky fat cells.

The goal of any tissue fixation process is to preserve the cellular ultrastructure as it appears in its natural state. The difficulty of chemical fixatives to penetrate and preserve adequately fat body cells was noted earlier by Locke (1984) in his comprehensive study of fat body cell ultrastructure. Chemical fixation was effective in stabilizing the cytoskeletal microtubules and cytoplasmic organelles, but not without noticeable artifacts.

A major drawback of chemical fixation is its ineffectiveness in capturing rapid cellular dynamics. Chemical fixatives diffuse slowly through the cell membrane to reach the interior of cells; the rate of penetration of fixatives into cells is on the order of 1–2 μm per second (Mersey and McCully, 1978). Once sufficient concentrations have penetrated, additional time is necessary for crosslinking reactions to occur; glutaraldehyde and osmium tetroxide are not able to crosslink all types of molecules found in living cells (Bullock, 1984). These delays allow for shifts in ultrastructure and the unnatural fusion of membranes, events which can be avoided by cryoimmobilization (Wagner and Andrews, 1985).

High pressure freezing/freeze substitution appears uniquely applicable for preserving bulky fat body cells. Of the available freezing techniques, high pressure freezing is the only one that can produce a quality freeze to considerable depths (500 μm) (Studer et al., 1989). The large diameter of fat body cells (determined with light microscopy to be 0.27 ± 0.04 mm), makes HPF the best choice for preservation of this tissue. The optimal freeze quality depends on requirements of the investigator for ultrastructural investigation. In this study, trapping potentially rapid metabolic events (e.g., lipid droplet fusion), preservation of fat body cytoskeleton and immobilization of chromatin were the criteria. The removal of tissue ice by freeze substitution leaves “ghosts” in the tissue; if large enough, ice crystals can artificially displace cytoplasmic structures. Tissues free of visible ice crystals at high magnifications (>50K) were considered optimally preserved. Combining HPF with FS yields excellent preservation of tissues (Studer et al., 1989; Allenspach, 1993).
As expected, based on observations reported previously (Gilkey and Staehelin, 1986), chemical fixation resulted in crenation of organelle membranes in fat body cells. Fat body cells have an elaborate membranous system which is particularly vulnerable to distortion after chemical fixation (Locke, 1984). Membranes of the secretory system, lysosomal system, lamellar bodies and mitochondrial membranes demonstrate undulations after chemical fixation (Locke, 1984). In addition, leaching occurs in the mitochondrial matrix and some regions of cytoplasm. By contrast, fat body cells prepared by HPF/FS exhibited mitochondria with smooth outer membranes, smooth cristae and uniformly electron dense matrix, an observation reported in plants by Kiss et al. (1990). Membranes of multi-vesicular bodies and the secretory vesicles of the Golgi complex are also smooth. Because the fat body develops an extensive autophagic system in preparation for pupal transformation, we recommend that future ultrastructural studies on the fat body, or any cells with extensive lysosomal systems (Dahl and Staehelin, 1989) or inducible enzyme systems (Kellenberger et al., 1992), be carried out by cryofixation to minimize autolytic damage.

The advantage of high pressure freezing is to limit ice crystal growth to less than macromolecular dimensions so that the ultrastructure is not altered. The turgid appearance of organelles, particularly in freeze-tolerant larvae, indicated an excellent freeze quality and suggested that ice crystal formation within the matrix was minimal. As reported by Gilkey and Staehelin (1986), high concentrations of cryoprotectants increase viscosity and thereby limit ice crystal growth. Fat body cells have increased concentrations of endogenous cryoprotectants. As reported by Waslylyk et al. (1988), multi-component cryoprotectant systems, including a simulated overwintering hemolymph of E. solidaginis, promote glass transitions at higher temperature than occurs in single-component systems. Since overwintering larvae elaborate a range of cryoprotectants including glycerol, sorbitol, trehalose and fructose, this natural multi-component system may have been particularly efficient in promoting vitrification in the fat body cells.

The apparent paucity of small lipid droplets in chemically fixed cells, when compared to their relative abundance in HPF/FS cells, suggests that fusion of droplets (coalescence) must have occurred during the fixation process. This apparent fusion is similar to the fusion of labile minivesicles in capillaries (Wagner and Andrews, 1985) and the fusion of transient, labile membranes of endoplasmic reticulum (Nicolas, 1991). Cryofixation reflects more accurately the size and distribution of lipid droplets as they naturally occur in fat body cells.

Extraction of lipid by organic solvents is a well-known artifact of chemical fixation (Morgan and Huber, 1967; Stratton, 1975). Extraction of osmiophilic lipids during conventional preparation of fat body cells was reflected in electron micrographs as either partial or complete removal of lipid from the lipid droplets. In HPF/FS cells, extraction of lipid from fat body cells was complete because of the prolonged soak in the freeze substitution solution. The lipids are evidently not conjugated to proteins because the lipid vacuoles were completely electron translucent.
The accumulation of cryoprotectants in cold-hardened larvae is probably the reason why freeze-tolerant fat body cells are suitable for high pressure freezing. Endogenous cryoprotectants confer protection on cell structure during the freezing process (Gilkey and Staehelin, 1986). Freeze-tolerant larvae synthesize and accumulate cryoprotectants to concentrations of 1 M (Morrissey and Baust, 1976). By contrast, freeze-susceptible cells from September larvae that have not yet accumulated cryoprotectants contain quantities of cryoprotectants insufficient to prevent ice-crystal-induced segregation patterns. The difference in freeze quality was most evident in the less dense interior cytoplasm of freeze-susceptible cells and in cytoplasm surrounding mitochondria. Halos around organelles are artifacts that indicate sub-optimal freeze quality (Kiss et al., 1990); they were absent in cryoprotected fat body cells. In addition, chromatin in fat body cells of freeze-tolerant larvae appears better cryopreserved than chromatin in freeze-susceptible cells, which suggests that cryoprotectants may also protect chromatin under natural conditions.

Glycogen levels peak in late September, after which they decline throughout the winter as cryoprotectants are synthesized; glycogen reaches its lowest concentrations in mid-February (Storey and Storey, 1986). The variability of glycogen observed in fat body cells probably reflects the sampling technique for study and the degree to which glycogen is accumulated in freeze-susceptible larvae or utilized for cryoprotectant synthesis and basal metabolism in freeze-tolerant larvae during the winter (Storey and Storey, 1986).

Coalescence of lipid droplets is an unusual response of fat body cells to freezing (Salt, 1959; Lee et al., 1993). Cells which have never been frozen contain a large number of small lipid droplets spread evenly throughout the cytoplasm. Intracellular freezing at low sub-zero temperatures or freezing for extended periods at high sub-zero temperatures results in the formation of progressively larger lipid droplets (Lee et al., 1993). Extreme low temperature or extended sub-zero exposure times result in the coalescence of nearly all cytoplasmic lipids to form a single, central droplet which occupies much of the cell volume. Upon thawing, organelles and cytoskeleton in fully coalesced cells underwent major rearrangement and relocation into a narrow band of cytoplasm at the periphery of the cell. Considering the rapid and dramatic reorganization of the cytoplasmic components it is remarkable that larvae frozen under conditions which cause extensive coalescence of lipid droplets in fat body cells readily survive to emerge as adults (Salt, 1959, 1962; Lee et al., 1993).

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