

MODELING SEASONAL CHANGES IN INTRACELLULAR FREEZE-TOLERANCE OF FAT BODY CELLS OF THE GALL FLY *EUROSTA SOLIDAGINIS* (DIPTERA, TEPHRITIDAE)

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Summary

Although seasonal changes in the freeze-tolerance of third-instar larvae of *Eurosta solidaginis* have been well documented for the whole organism, the nature of this cold-hardiness at the cellular level has not been examined. Seasonal changes in the survival of fat body cells from *E. solidaginis* larvae were assessed using fluorescent vital dyes after freezing at -10 , -25 or -80 °C for 24 h both *in vivo* and *in vitro*. Cells frozen *in vitro* were frozen with glycerol, with sorbitol (both of which enhanced cell survival) or without cryoprotectants. Both cellular and organismal survival were low in August when larvae were not freeze-tolerant, then increased dramatically during September and October before leveling off from November to January. This observation for cells frozen without

cryoprotectants indicates that the cells themselves have adapted. The single most important factor influencing cell survival, as determined by logistic regression modeling, was the time of larval collection, which reflects the level of cold-hardiness achieved by field acclimation. Cells frozen *in vivo* exhibited greater survival than did those frozen *in vitro*, even with the addition of cryoprotectants. Since no differences were observed between cells frozen with glycerol or sorbitol, the role of the multi-component cryoprotectant system present in *E. solidaginis* should be investigated.

Key words: *Eurosta solidaginis*, freeze-tolerance, fat body cells, gall fly, logistic regression.

Introduction

The goldenrod gall fly *Eurosta solidaginis* (Fitch) has received much attention as a model of a naturally freeze-tolerant organism (Baust and Nishino, 1991). *E. solidaginis* develops through three larval instars. The first two instars pass rather quickly during early summer before the molt to the third instar in late July or early August. Although early third-instar larvae are not freeze-tolerant in summer and early autumn, late third instars are freeze-tolerant for the duration of the winter.

The major cryoprotectants utilized by this species are glycerol, sorbitol and, to a lesser extent, trehalose (Baust and Nishino, 1991; Morrissey and Baust, 1976; Storey and Storey, 1986). While no significant levels of cryoprotectants are found during August, glycerol levels rapidly increase between September and early October, reaching peak levels of up to 1 mol l^{-1} (Morrissey and Baust, 1976; Storey and Storey, 1986) throughout the winter. Sorbitol levels do not increase until mid-November, and trehalose levels increase even later. Glycerol synthesis is induced by larval dehydration as the goldenrod plant senesces and the gall dries (Rojas *et al.* 1986), whereas sorbitol synthesis is induced by decreasing temperature in the autumn (Baust and Lee, 1981). During this period of cryoprotectant synthesis and plant senescence, larval dry mass decreases by almost 50 %, but larval

water content remains constant owing to lipid hydrolysis (Rojas *et al.* 1986). During the development of cold-hardiness, cryoprotectants are synthesized from glycogen.

Inter-population differences exist, both in crystallization temperature (T_c , defined as the lowest temperature reached by the organism before the release of the latent heat of fusion as the body fluids spontaneously freeze) and cryoprotectant levels, indicating differing mechanisms for overwintering survival (Baust and Lee, 1981; Lee and Lewis, 1985). Fully cold-hardy larvae are freeze-tolerant, with 75 % surviving to adulthood after freezing at -25 °C, although none survives freezing at -80 °C (Lee *et al.* 1993). T_c also shows seasonal variation, averaging -17 °C in August and stabilizing at -9 °C in November where it remains for the duration of winter (Morrissey and Baust, 1976).

Fat body cells

It is generally believed that freeze-tolerant organisms survive only if ice is limited to extracellular spaces. This assumption is based primarily on investigations of mammalian cells and tissues, including those of humans (Lee *et al.* 1993; Mazur, 1984). Salt (1959, 1962) was the first to challenge this

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assumption when he demonstrated that fat body cells of *Eurosta solidaginis* survived intracellular ice formation. Lee *et al.* (1993) confirmed Salt's earlier observations using cryomicroscopy and fluorescent vital dyes.

Insect fat body tissue is a loosely organized collection of cells that are bathed in hemolymph and fill much of the coelomic cavity. Fat body tissue has frequently been equated to the vertebrate liver in terms of physiological function because of its role in carbohydrate, protein and glycogen storage, and in intermediary metabolism (Wigglesworth, 1972). Fat body cells in *E. solidaginis* larvae are the most obvious tissue present because of their distribution and their relatively large size (350–500 µm in diameter). Their cytoplasm is filled with many small lipid drops, which may coalesce into one or a few large lipid drops after intracellular freezing (Lee *et al.* 1993). Fat body cells undergo dramatic changes in morphology, organization and storage contents, especially during metamorphosis (Wigglesworth, 1972). Because the fat body of *E. solidaginis* (and other insects) has been identified as the major site of cryoprotectant synthesis (Storey *et al.* 1981; Storey and Storey, 1985, 1986), it plays an important role in cold-hardiness and its acquisition in *E. solidaginis*. Seasonal changes in larval freeze-tolerance associated with the development of cold-hardiness have been well documented (for a review, see Baust and Nishino, 1991). However, the nature of this process at the cellular level has not been examined.

The present study examines the development of freeze-tolerance in fat body cells of third-instar *E. solidaginis* within the context of whole larval cold-hardiness. Parameters that have significant influences on the survival of fat body cells during the seasonal development of cold-hardiness of *Eurosta solidaginis* are identified using logistic regression models. One model compares the seasonal ontogeny of the acquisition of cold-hardiness and freeze-tolerance in fat body cells prepared *in vivo* versus *in vitro*. Using *in vivo* whole larval cell preparations as a reference group establishes a biological context for comparison with *in vitro* treatments. Other models compare the efficacy of glycerol and sorbitol on the survival of fat body cells exposed to intracellular freezing.

Materials and methods

Goldenrod galls from *Solidago* sp. containing third-instar larvae of *Eurosta solidaginis* (Fitch) were collected from fields at Miami University's Ecology Research Center once every 2 weeks from mid-August to mid-December 1994, and once at the end of January 1995, and were stored under ambient field temperatures until used for experiments. During this sampling period, weekly maximum, minimum and mean ambient field temperatures were monitored by the Miami University weather station at the field site.

At each collection, mean larval mass, water content (measured as described by Rojas *et al.* 1986) and crystallization temperature (T_c) were measured for 20 larvae. The lowest body temperature recorded before the release of the

latent heat of fusion of body water (as evidenced by an exotherm, i.e. temperature increase) was taken to be the T_c (Baust and Lee, 1982).

Larval survival of freezing was assessed at each of three temperatures: –10, –25 and –80 °C for 24 h (Lee *et al.* 1993). At each temperature, 15–20 larvae were frozen. Larvae were judged to be alive if, after 2 h of thawing, they showed signs of peristaltic muscular contractions in response to an anterior mechanical stimulus. The number of larvae that successfully pupated and the number that successfully emerged as adults following freezing at these temperatures were also recorded.

Fat body cell viability

Freeze-tolerance of larval fat body cells (FBCs) from each collection was assessed both *in vivo* and *in vitro* using the same methods as Lee *et al.* (1993), which involved fluorescent vital dye assays for cell viability. Lee *et al.* (1993) confirmed intracellular freezing using cryomicroscopy. For *in vitro* analysis, FBCs from 10 larvae were isolated and dissociated from other tissues by gently shaking the viscera for 5 min in a solution of 5 mg of collagenase and 10 mg of hyaluronidase in 10 ml of Schneider's solution, then centrifuging at 9500 revs min^{–1} for 5 min, allowing the FBCs to float to the top on the test tube (N. L. Pruitt, personal communication). Three samples of FBCs were then placed in each of three solutions: Schneider's insect cell culture medium (Sigma Chemicals), Schneider's medium plus 1 mol l^{–1} glycerol, or Schneider's medium plus 1 mol l^{–1} sorbitol. One sample of each FBC preparation was frozen for 24 h at –10, –25 or –80 °C. After thawing at room temperature, FBCs from each test temperature and preparation combination were stained with either an Acridine Orange/ethidium bromide mixture (AO/EtBr) or propidium iodide (PI). For *in vivo* analysis, three larvae were frozen at each of the three temperatures. Upon thawing, FBCs were immediately isolated using the same digestion procedure, and similarly stained slides were prepared.

Stained FBCs were viewed under a fluorescence microscope using blue/green filters (Lee *et al.* 1993). One hundred cells on each slide were scored as live or dead depending on the color of fluorescence. For cells stained with AO/EtBr, live cells and nuclei stain green whereas dead cells and nuclei stain orange. For cells stained with PI, dead cells and nuclei stain orange.

Logistic regression

Logistic regression is a statistical modeling tool that has been widely used in the field of epidemiology (Hosmer and Lemeshow, 1989). It models the likelihood of the outcome of a dichotomous variable (such as live versus dead or presence versus absence of a symptom or illness) as a function of a number of predictor variables or factors. In the field of cryobiology, defining 'freeze-tolerance' can be difficult since there are many factors, such as temperature, levels of cryoprotectants, developmental state, etc., which can affect the survival of an organism or cell. By using logistic regression to analyze cell survival as a binomial variable, details regarding

the strength of influence exerted by each of these factors can be elucidated.

The response variable for logistic regression models is called the logit or log-odds ratio of the proportion of live cells (p) to the proportion of dead cells ($1-p$). It was modeled as a function of a constant (μ) modified by three different parameters: time of collection of larvae (α_i), temperature at which FBCs were frozen (β_j) and cell preparation, and all of their possible interactions. Time of collection and temperature were treated as continuous variables, and cell preparation was treated as a categorical variable. Since there were four cell preparations, three indicator variables (scored as 0 or 1) were used (γ , Schneider's medium; δ , Schneider's+glycerol; and ϕ , Schneider's+sorbitol) to identify the cell preparation. The reference group (i.e. when $\gamma=\delta=\phi=0$), against which all others were compared, was the *in vivo* whole larval cell preparation. This allowed us to investigate how well the *in vitro* preparations containing cryoprotectants simulated *in vivo* conditions. The fully specified model (i.e. one containing all main effects and possible interactions) follows the form:

$$\ln \left(\frac{p}{1-p} \right) = \mu + \alpha_i + \beta_j + \gamma_k + \delta_l + \phi_m + \alpha\gamma_{ik} + \alpha\delta_{il} + \alpha\phi_{im} + \beta\gamma_{jk} + \beta\delta_{jl} + \beta\phi_{jm} + \alpha\beta\gamma_{ijk} + \alpha\beta\delta_{ijl} + \alpha\beta\phi_{ijm} \quad (1)$$

An additional categorical variable for distinguishing between cells scored using AO/EtBr *versus* PI was originally included in the model. However, these replicates were almost identical (as also reported by Lee *et al.* 1993) and confirmed the results well, i.e. the models with and without the dye term were not significantly different ($G=0.04$, d.f.=1, $P \gg 0.25$). Therefore, the dye replicates were pooled for all further analyses and the dye term was eliminated from the model.

Model parameter estimates were calculated by the maximum likelihood method (SAS Institute, 1989: LOGISTIC procedure). Beginning with a fully specified model, the likelihood ratio statistic (G statistic) for goodness of fit was used to partition the factors and eliminate non-significant interactions, resulting in a reduced model that best described the influences on fat body cell survival in these experiments.

Results

During the late summer and early autumn of 1994, third-instar larval mass increased by over 450 % from August to mid-September, reaching a maximum of 53.5 ± 10.8 mg (mean \pm S.E.M.) in late September (Fig. 1A). Larval mass leveled off after this time, when mean ambient temperatures began to decrease from 22 to 12 °C, and minimum temperatures dropped below 5 °C (Fig. 2). From November to January, larval mass decreased by 22 % to 42.9 ± 2.4 mg (Fig. 1A). Water content was highest (72.1 ± 1.0 %) in mid-August, but remained constant at 59–62 % for the remainder of the study (Fig. 1B).

Crystallization temperatures (T_c) were highly variable

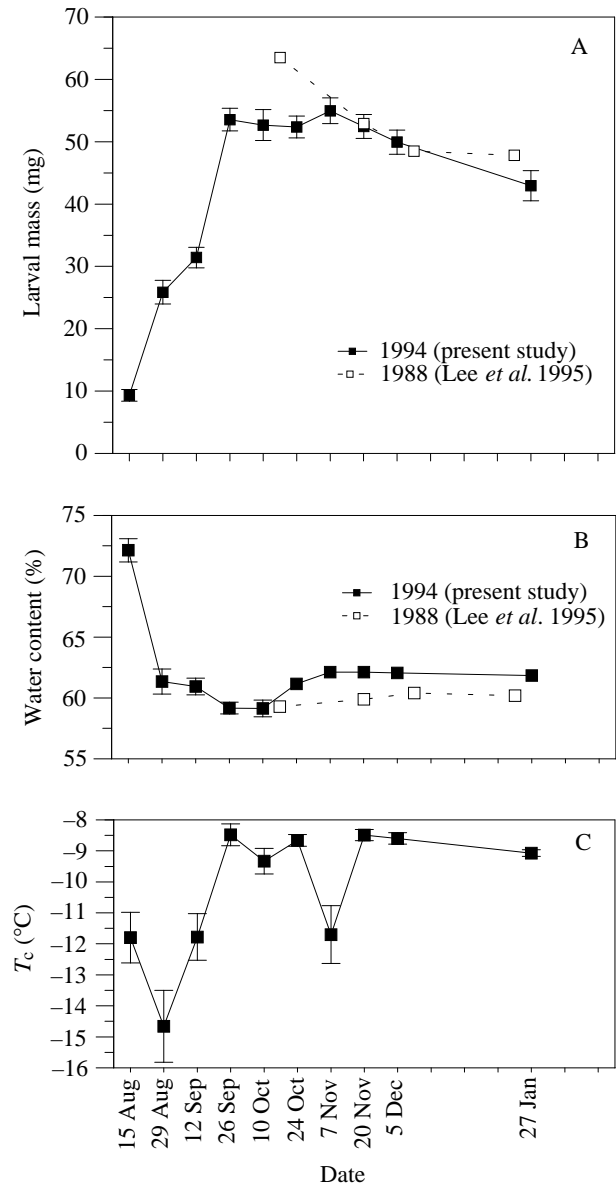


Fig. 1. Seasonal changes in mean (\pm S.E.M.) larval mass ($N=20$) (A), water content expressed as a percentage of live mass ($N=20$) (B) and crystallization temperature (T_c) ($N=15$) (C) for a southern Ohio population of field-collected *Eurosta solidaginis* third-instar larvae. Filled symbols are data from larvae collected during autumn/winter 1994/1995 (present study); open symbols are data from larvae collected during autumn/winter 1988/1989 (Lee *et al.* 1995).

among individual larvae throughout November, ranging from -5.3 to -23.0 °C, after which they were elevated and mean values stabilized at approximately -8.5 °C (Fig. 1C). This is a typical seasonal pattern for this species, and the increase in T_c is associated with the acquisition of freeze-tolerance (Morrissey and Baust, 1976).

The progressive development of freeze-tolerance at the level of the whole organism is shown in Fig. 3. Most larvae were capable of movement after 2 h of thawing following being frozen for 24 h at -10 °C throughout the study period. Of those

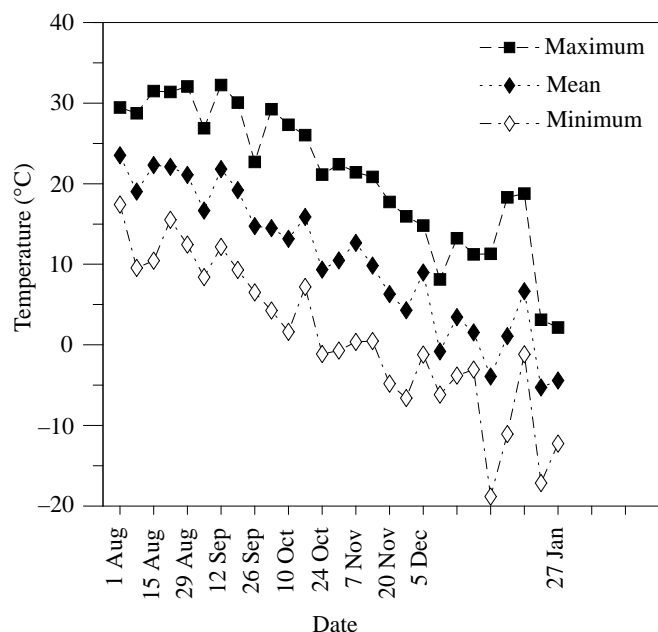


Fig. 2. Weekly maximum, minimum and mean ambient temperatures (°C) at the Miami University Ecology Research Center during autumn/winter 1994/1995.

collected in August, 75 % were able to tolerate this exposure, increasing to over 90 % in September and October, and 100 % by November. Most larvae died when frozen at -25°C early in the study; however, freeze-tolerance increased dramatically in October and, by late November, all larvae were able to respond to a stimulus after 2 h of thawing following this freezing treatment. All larvae collected before October died after 24 h at -80°C , and even in October only 30 % were capable of responding to a stimulus after thawing. Only for samples collected in late January were all larvae able to respond (Fig. 3). No larvae collected earlier than November pupated, regardless of temperature exposure. A small proportion of the December larvae and 80 % of the January larvae frozen at -10 or -25°C successfully emerged as adults. No larvae frozen at -80°C survived to adulthood.

Fat body cell survival

In general, cell survival was low in August, increased through October, and then leveled off for the remainder of the study (Fig. 4). Overall, cell survival was reduced with decreasing freezing temperature. Cells frozen *in vivo* showed the best survival rates throughout the study, while those frozen *in vitro* in Schneider's medium alone had the lowest proportion of viable cells.

Modeling seasonal changes in FBC freeze-tolerance

The first model analyzed the primary factors influencing seasonal changes in freeze-tolerance of FBCs. The best-fitting model of FBC survival was obtained by logistic regression using the *in vivo* (whole larval) treatment as the reference group to assess the efficacy of the *in vitro* preparations to

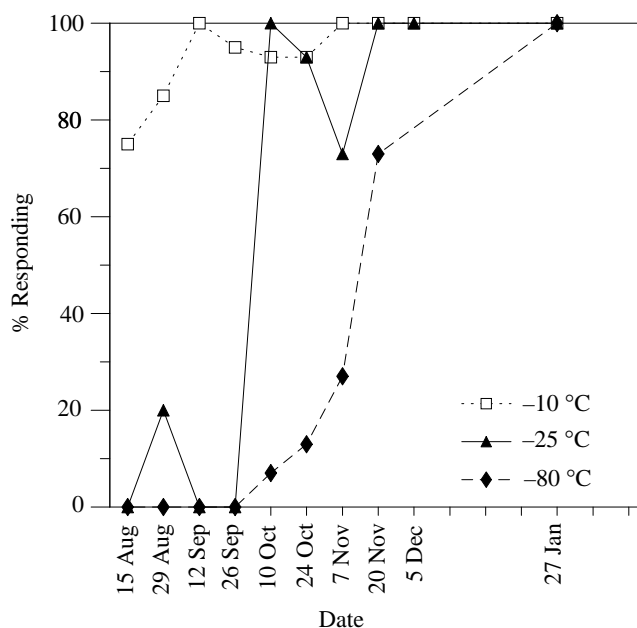


Fig. 3. Percentage ($N=15$) of *Eurosta solidaginis* third-instar larvae responding to a physical stimulus after 2 h of thawing following 24 h of freezing at -10 , -25 or -80°C .

simulate *in vivo* conditions. The model included all the factors and interactions listed in Table 1. The likelihood ratio (G) statistic (χ^2 distribution, d.f.=1) is an indicator of the relative importance of each term affecting the probability of FBC survival.

By far the single most important factor influencing cell survival was the main effect of the time of collection ($G=387.7$, $P<0.0001$), a trend consistent with the increase in larval cold-hardiness including the acquisition of freeze-tolerance during the autumn (Fig. 3). The second most important term was the Schneider's medium treatment ($G=65.4$, $P<0.0001$), in which FBCs showed considerably lower survival than did those frozen *in vivo*. The main effect of temperature also had a significant effect of reduced cell survival with decreasing temperature ($G=32.0$, $P<0.0001$). However, the main effects of the cryoprotectants glycerol ($G=2.1$, $P>0.1$) and sorbitol ($G=0.03$, $P>0.8$) did not significantly alter FBC survival compared with the *in vivo* whole larval preparations (Fig. 4A).

A number of important two-way interactions were found among the treatment variables tested. First, both glycerol and sorbitol showed two-way interactions with time, which suggests that the effects of both cryoprotectants changed over time compared with the *in vivo* whole larval preparation, i.e. survival was greater *in vitro* with cryoprotectants in August and early September, but greater in whole larval preparations for the remainder of the season (Fig. 4A). FBCs were more susceptible to freezing injury early in the season, because larvae had not yet developed cold-hardiness or produced an endogenous supply of cryoprotectants.

The second two-way interaction suggested that both cryoprotectants tested, glycerol and sorbitol, interacted

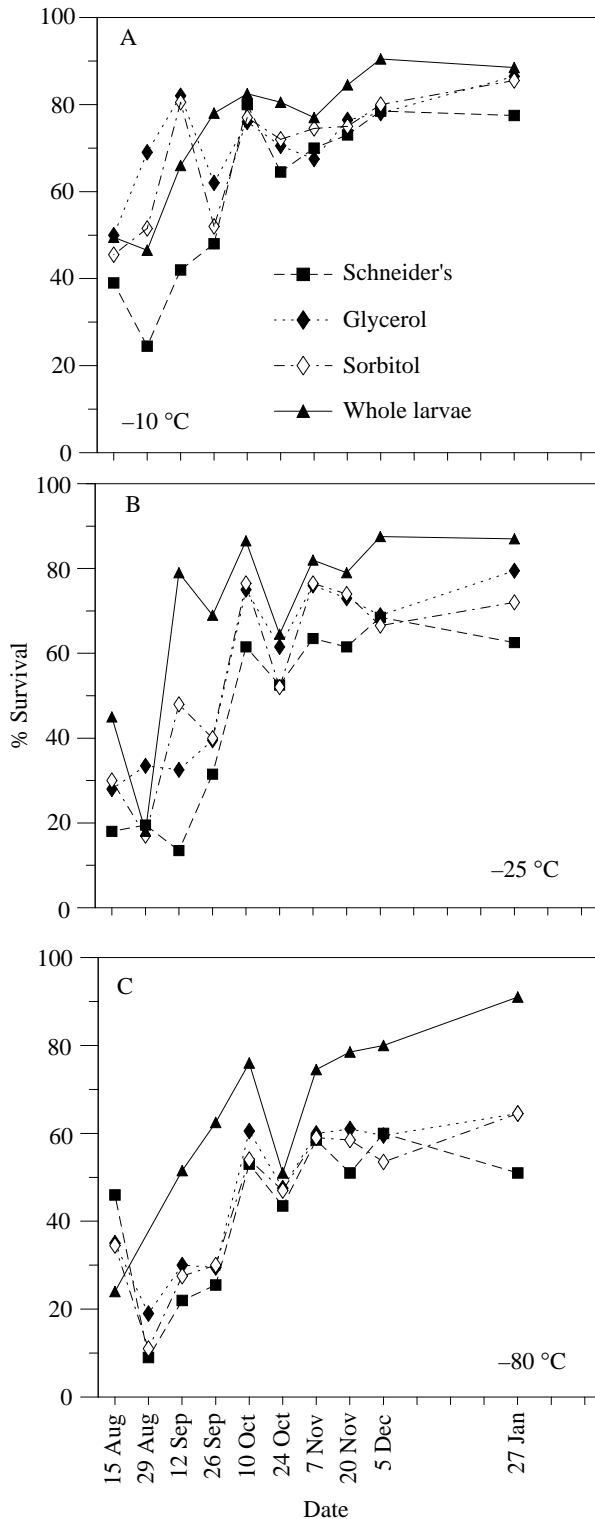


Fig. 4. Seasonal changes in *Eurosta solidaginis* larval fat body cell viability ($N=200$ cells) for four different treatments (cells frozen *in vivo*, or *in vitro* in Schneider's insect medium, Schneider's + 1 mol l^{-1} glycerol or Schneider's + 1 mol l^{-1} sorbitol) at (A) -10°C , (B) -25°C and (C) -80°C .

strongly with temperature ($G=28.0$ and $G=32.4$, respectively; $P<0.0001$). There was a greater difference between whole

Table 1. Likelihood ratio (G) statistics for main effects and interactions influencing fat body cell survival in third-instar *Eurosta solidaginis* larvae as determined by the maximum likelihood method of logistic regression

Parameter	G	P
Time	387.7	<0.0001
Temperature	32.0	<0.0001
Schneider's	65.4	<0.0001
Glycerol	2.1	0.1509
Sorbitol	0.03	0.8580
Time \times Schneider's	0.2	0.6766
Time \times Glycerol	36.4	<0.0001
Time \times Sorbitol	29.2	<0.0001
Temperature \times Schneider's	6.6	0.0099
Temperature \times Glycerol	28.0	<0.0001
Temperature \times Sorbitol	32.4	<0.0001
Time \times Temperature	2.9	0.0909
Time \times Temperature \times Schneider's	22.7	<0.0001

Schneider's, Schneider's insect medium; Glycerol, Schneider's medium + 1 mol l^{-1} glycerol; Sorbitol, Schneider's medium + 1 mol l^{-1} sorbitol.

Time and temperature were treated as continuous variables; three indicator variables were used to compare the *in vitro* treatments with the *in vivo* whole larval reference group.

larval preparations and glycerol or sorbitol preparations at the more extreme temperature of -80°C , where cell survival was closer to that observed for cells frozen in Schneider's medium alone (Fig. 4C), than at higher temperatures such as -10°C , where cell preparations with 1 mol l^{-1} glycerol or sorbitol showed similar survival to those from whole larval *in vivo* preparations (Fig. 4A).

Although FBC survival was much lower in Schneider's medium than in whole larval preparations, the temporal patterns of cell survival across temperatures essentially paralleled each other, as indicated by the lack of strong interactions between Schneider's preparation and time, and Schneider's preparation and temperature (Table 1). Although these two-way interactions did not strongly influence FBC survival in Schneider's medium, a three-way interaction was found among Schneider's medium, time and temperature ($G=22.7$, $P<0.0001$; Table 1), indicating that the pattern of FBC survival for the Schneider's medium and whole larval preparations depended on the temperature treatment. Therefore, these two-way interaction terms were retained in the overall model.

Comparison of the cryoprotective actions of glycerol versus sorbitol

Additional logistic regression models were formulated to compare the *in vitro* treatments in a pairwise fashion and to investigate the independent effects of cryoprotectants on cell survival. Three comparisons were made by analyzing subsets of data pertaining to glycerol *versus* sorbitol, Schneider's medium *versus* glycerol, or Schneider's medium *versus*

sorbitol. The following two models were fitted to all three subsets of data:

full model

$$\ln \left(\frac{p}{1-p} \right) = \mu + \alpha_i + \beta_j + \gamma_k + \alpha\beta_{ij} + \alpha\gamma_{ik} + \beta\gamma_{jk} + \alpha\beta\gamma_{ijk}, \quad (2)$$

reduced model

$$\ln \left(\frac{p}{1-p} \right) = \mu + \alpha_i + \beta_j + \alpha\beta_{ij}, \quad (3)$$

where p is the proportion of cells that survived. The fully specified model (equation 2) contained terms for time (α_i) and temperature (β_j) and an indicator variable to distinguish between the two preparations being compared ($\gamma_j=1$ for one preparation, and $\gamma_j=0$ for the other). The reduced model (equation 3) was fitted to the same data set but without any indicator variable for cell preparation type.

As before, the likelihood ratio statistic (G statistic) was used to compare these models (equations 2 and 3) to determine whether the reduced model without a term for cryoprotectant type (equation 3) would fit the data as well as the fully specified model (equation 2). No significant differences were found between models with and without indicators for the type of cryoprotectant (glycerol *versus* sorbitol) ($G=6.4$, $P>0.25$), suggesting that glycerol and sorbitol did not have differential effects on FBC survival.

When each cryoprotectant was separately compared with the Schneider's preparation, it was necessary to include the effects of cell treatment in modeling FBC survival. The best-fitting models, in both cases, were the fully specified models containing the terms listed in Table 2. In these cases, the reference group was Schneider's medium without cryoprotectants. The main effect of the time of larval collection had the greatest influence on cell survival for both cryoprotectants ($G_{\text{glycerol}}=253.6$, $G_{\text{sorbitol}}=293.6$; $P<0.0001$). The second greatest influence on cell survival was the main

effect of the cryoprotectant in enhancing FBC survival over levels observed by freezing in Schneider's medium without cryoprotectants.

The main effect of temperature was not significant for either model of glycerol *versus* Schneider's or sorbitol *versus* Schneider's ($G=1.2$, $P=0.27$ in both cases). However, the interaction between temperature and cryoprotectants was found to be significant in both models ($G_{\text{glycerol}}=29.3$, $G_{\text{sorbitol}}=26.8$; $P<0.0001$). The greatest differences in FBC survival between preparations with and without either cryoprotectant were found at -10°C (Fig. 4A). Cell survival was moderately higher with the addition of either cryoprotectant at -25°C (Fig. 4B). At -80°C , FBC survival was only slightly elevated over cell survival after freezing in Schneider's medium alone with the addition of either cryoprotectant (Fig. 4C).

As in the comprehensive model (Table 1), a significant interaction between time and temperature was also observed in both models ($G_{\text{glycerol}}=21.0$, $G_{\text{sorbitol}}=21.0$; $P<0.0001$). Weak two-way interactions between time and cryoprotectant, as well as a weak three-way interaction, were also present in both models (Table 2).

Seasonal changes

The week of 26 September was the first time that minimum ambient temperature fell below 5°C (Fig. 2), coinciding with the point that larval mass and T_c reached their peak values and stabilized (Fig. 1A,C). Interestingly, the greatest increases in whole larval survival at -25°C (Fig. 3), and the greatest increase in FBC survival for all temperatures and preparations during a 2 week interval (Fig. 4), occurred for samples collected between 26 September and 10 October. This is the time when larvae of *E. solidaginis* would first have been exposed to environmental temperatures below 5°C , a condition which is known to induce sorbitol synthesis in this species (Baust and Lee, 1981; Storey and Storey, 1986). Between 26 September and 10 October, whole larval survival increased dramatically at -25°C (from 0 to 100%) and rose above 0% for the first time to 7% at -80°C (Fig. 3), the greatest increase

Table 2. Likelihood ratio (G) statistics for main effects and interactions influencing *in vitro* viability of fat body cells from third-instar *Eurosta solidaginis* larvae as determined by the maximum likelihood method of logistic regression

Parameter	Glycerol		Sorbitol	
	G	P	G	P
Time	253.570	<0.0001	293.6	<0.0001
Temperature	1.2	0.2672	1.2	0.2672
Cryoprotectant	75.2	<0.0001	52.8	<0.0001
Time \times Cryoprotectant	13.9	0.0002	8.2	0.0042
Temperature \times Cryoprotectant	29.3	<0.0001	26.8	<0.0001
Time \times Temperature	21.0	<0.0001	21.0	<0.0001
Time \times Temperature \times Cryoprotectant	14.9	0.0001	11.0	0.0009

Cryoprotectant, indicator variable for appropriate cryoprotectant (glycerol or sorbitol).

Two models were developed to compare Schneider's medium *versus* Schneider's + 1 mol l^{-1} glycerol (Glycerol columns) and Schneider's medium *versus* Schneider's + 1 mol l^{-1} sorbitol (Sorbitol columns).

in FBC survival for all *in vitro* treatments at all temperatures was observed (Fig. 4), and FBC survival also stabilized at peak levels.

Discussion

Seasonal changes in cold-hardiness of *E. solidaginis*, related to the development of freeze-tolerance, occur at both the cellular and organismal level. The key time for significant increases in cold-hardiness for this species, as reflected by characteristic increases in larval mass and T_c (Morrissey and Baust, 1976; Layne, 1991; Lee *et al.* 1995), occurred during the 2 weeks from the end of September to the beginning of October. Changes in cellular freeze-tolerance temporally coincide with these organismal changes.

The pattern of T_c elevation during the autumn is consistent with that observed in previous studies (Morrissey and Baust, 1976; Baust and Lee, 1981; Bale *et al.* 1989). The period during which T_c becomes elevated also corresponds to the period when glycerol accumulation occurs (Morrissey and Baust, 1976) in response to gall dehydration (Rojas *et al.* 1986). Sorbitol synthesis is initiated slightly later in response to ambient temperatures below 5 °C (Baust and Lee, 1982; Storey and Storey, 1986; Pio and Baust, 1988). Morrissey and Baust (1976) report 'freeze-tolerance' (based on 90 % survival) of larvae collected from November to mid-March, the period during which T_c was elevated and constant in the present study. On the basis of larval survival data and using the same survival criteria as in the present study, *E. solidaginis* showed a progressive development of increasing cold-tolerance during the course of this study similar to that observed by Layne (1991) for a western New York population of *E. solidaginis*.

Fat body cells of *E. solidaginis* undergo a dramatic increase in freeze-tolerance during the autumn, coinciding with increases in organismal freeze-tolerance. Interestingly, the addition of cryoprotectants appeared to enhance FBC survival compared with that of preparations frozen in Schneider's medium to a greater extent early in the season (August, September) than later in the study, when FBC survival with cryoprotectants was not much higher than without them because larvae were more cold-hardy. Since FBCs frozen *in vivo* and *in vitro* in Schneider's medium alone exhibit parallel patterns of increasing survival over time, it is apparent that other physiological changes, independent of the addition of cryoprotectants, are occurring that might account for the increase in the basal level of FBC survival *in vitro*. Seasonal increases in survival of FBCs frozen *in vitro* in Schneider's medium alone indicate that the cells themselves have adapted in some way, perhaps in terms of membrane structure and function. Thus, it appears that the freeze-tolerance of *E. solidaginis* involves not only the accumulation of hemolymph cryoprotectants but also physiological adaptations at the cellular level, such as alterations in lipid composition. Because the addition of 1 mol l⁻¹ glycerol or sorbitol does not elevate FBC survival to levels observed *in vivo*, and because of the number of interactions among cryoprotectants and other

factors influencing survival, a more complex system must be in operation to ensure the survival of FBCs.

Since *E. solidaginis* employ a multi-component system of cryoprotectants, including glycerol, sorbitol and, to a lesser extent, trehalose (Baust and Lee, 1982; Storey and Storey, 1986; Pio and Baust, 1988), an attempt to freeze FBCs *in vitro* with a mixture of these cryoprotectants should be carried out; although we suspect that *in vivo* levels of FBC survival still may not fully match *in vitro* levels because of other physiological influences on freeze-tolerance, such as changes in membrane lipid composition (V. A. Bennett, N. L. Pruitt and R. E. Lee, unpublished data). It is curious that no significant differences between the two cryoprotectants, glycerol and sorbitol, were detected in terms of their effects on FBC survival. The time×glycerol and time×sorbitol interactions (Tables 1, 2) reveal that the role played by cryoprotectants in the seasonal acquisition of freeze-tolerance is important, although complex. The artificial addition of cryoprotectants *in vitro* enhanced FBC survival above levels observed *in vivo* in August-collected animals. Later in the season, when larvae were more cold-hardy, *in vitro* preparations with a single cryoprotectant were not as effective as the natural *in vivo* multi-cryoprotectant system characteristic of freeze-tolerant *E. solidaginis*, suggesting possible synergistic effects (Fig. 4A).

Thus, the question arises as to what the adaptive significance or value of having a multi-component cryoprotectant system might be, if both glycerol and sorbitol have the same effects. It has been shown that high concentrations of cryoprotectants can have toxic effects at relatively high temperatures (Mazur, 1984; Karow, 1991). Perhaps having lower concentrations of several different cryoprotectants alleviates this effect. Cryoprotectants do not appear to be as effective at obviously lethal temperatures such as -80 °C (Lee *et al.* 1995). Instead, they seem to be more effective at enhancing cell survival at temperatures more biologically relevant to *E. solidaginis*, such as -10 and -25 °C.

Statistical tools such as logistic regression would be extremely useful for analyzing experiments in which concentrations of three different cryoprotectants (e.g. glycerol, sorbitol and trehalose) are varied *in vitro*. The tool of logistic regression in the present study has made it possible to see, for the first time, the complexity of factors involved in the freezing process and how strongly they affect survival at the cellular level. Obviously the 'formula' for survival and freeze-tolerance is not a simple one. The synergistic effects (i.e. time×cryoprotectants, temperature×cryoprotectants, etc.) were extensive, as shown by the number of interactions between terms in the models. This technique would be especially valuable when applied at the level of the whole organism. There has been much debate over labeling different species as 'freeze-tolerant', when their ability to survive a freezing episode is highly dependent on the conditions under which they were frozen and the criteria for assessing survival (Bale, 1987; Bale *et al.* 1989). In the future, it may be more productive to model the probability of survival (to production

of viable offspring) across a wide range of conditions as a function of factors such as cooling rate, temperature, duration of freezing, cryoprotectant levels, etc., which could vary under field conditions. Logistic regression would help to identify those factors that have the greatest influence on survival and to define a range of conditions that are favorable for the survival and reproduction of cold-hardy insects. The power of this tool for future studies in cryobiology, as well as other fields, is that it will allow the researcher to model a complex process in which the outcome (a binomial response variable) can be affected by a number of factors and to determine the relative importance or influence of those factors on the outcome being modeled.

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