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METABOLIC CONSEQUENCES OF RAPID CYCLES OF TEMPERATURE CHANGE FOR FREEZE-AVOIDING VS FREEZE-TOLERANT INSECTS

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Abstract—The metabolic responses to repeated cycles of temperature change, alternating 24 h at −16 and +3°C, were compared for a freeze-tolerant (*Eurosta solidaginis*) vs a freeze-avoiding (*Epiblema scudderiana*) insect. The two species differed most strongly in the response by cellular energetics. ATP content and energy charge were depressed in *E. scudderiana* larvae with each −16°C exposure but rebounded with each return to +3°C; after 12 such cycles, final energy status at +3°C was not significantly different than control values. By contrast, *E. solidaginis* larvae maintained a high energy charge (at the expense of a decrease in the total adenylate pool) over the first two cycles of freeze/thaw only. Subsequently energy stress was cumulative, with no recovery in the +3°C half of each cycle, and energy charge fell to 0.70–0.75. Both species showed a rise in glucose-6-*P* with each cold exposure, indicative of cold shock activation of glycogen phosphorylase. *E. scudderiana* showed no net increase in cryoprotectant content over the experimental course but sorbitol levels rose 4-fold in *E. solidaginis*. Alanine content increased significantly in *E. solidaginis* over the 12 cycles but no glycolytic end products accumulated in *E. scudderiana*. The data indicate that the freeze-avoiding specie is better able to maintain cellular homeostasis in the face of rapid and wide variations in environmental temperature than is the freeze-tolerant specie and that this may represent a worthwhile advantage of the freeze-avoidance strategy of cold hardiness for some species.

Key Word Index: *Epiblema scudderiana*, *Eurosta solidaginis*, insect cold-hardiness, energetics at subzero temperature

INTRODUCTION

Winter hardiness in insects takes one of two forms: freeze avoidance or freeze tolerance (Zachariassen, 1985; Storey and Storey, 1988). Freeze-avoiding insects utilize strategies that permit deep undercooling of body fluids and maintain a liquid state that for some species extends as low as −40°C. Freeze-tolerant insects, by contrast, initiate and control extracellular ice formation and survive for weeks or months with up to 65% of total body water as ice. Each strategy had its positive and negative points and the rationale for the 'choice' of strategy is not clear. Numerous factors might have influenced the development of one strategy over the other including type of hibernation site, temperature and moisture conditions of the winter microhabitat, requirement for motor activity, presence/absence of a cocoon, phylogeny, and life stage of the overwintering form. Both strategies require a range of biochemical adaptations with some elements in common (polyol cryoprotectants, thermal hysteresis factors) (Storey and Storey, 1988; Knight and Duman, 1986). In addition, the larvae of at least two species of beetles can apparently alter the strategy used from one year to the next, probably in response to some environmental cue (Duman, 1984; Horwath and Duman, 1984). What then are the critical risks and benefits that determine the choice of freeze tolerance vs freeze avoidance for any given species? Is each strategy optimal for certain types of environmental conditions or are they simply two separate, but equally good, strategies for winter survival?

Temperature is undoubtedly the most important environmental variable that must be accommodated for winter survival. At its two extremes, animals should be able to cope with both long-term exposures to deep subzero temperatures and with rapid changes in ambient temperature, that for the freeze-tolerant animal must include multiple freeze/thaw cycles. In two recent papers, we have analyzed the responses to constant low subzero temperature by goldenrod gall formers, the freeze-tolerant larvae of *E. solidaginis* (Fitch) (Diptera, Tephritidae) and the freeze-avoiding larvae of *E. scudderiana* (Clemens) (Lepidoptera, Olethreutidae) (Storey and Storey, 1985; Churchill and Storey, 1989a). The present paper examines the metabolic responses of both species to repeated cycles of rapid temperature change between +3 and −16°C.

MATERIALS AND METHOD

Animals and chemicals

All chemicals and biochemicals were purchased from Sigma Chemical Co., St Louis, Mo. or Boehringer Mannheim Corp., Montreal, PQ. Galls containing *E. scudderiana* or *E. solidaginis* were

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collected in Ottawa, Canada in mid-September, 1987. Galls were held at a constant 3°C for 5 months before use.

Animal experiments

To test the effects of rapid temperature change on metabolism, larvae of *E. scudderiana* and *E. solidaginis* were subjected to alternating cycles of 24 h at −16°C followed by 24 h at 3°C. Twelve such cycles of temperature change were given in succession. Larvae were sampled in the 1st, 2nd, 6th, 9th and 12th cycles after 24 h at either temperature. In the 1st and 9th cycles, additional samples were taken after 2 and 8 h of exposure. Larvae were immediately removed from their galls and killed by immersion in liquid nitrogen. The frozen larvae were stored at −80°C until use.

Sample preparation and metabolite assay

Six larvae were pooled per sample and perchloric acid extracts were prepared as described by Storey *et al.* (1981). An aliquot of the well-mixed homogenate was removed for glycogen determination by the method of Keppler and Decker (1974). All metabolites were quantified by spectrophotometric coupled enzyme assays. Assays for adenylates were performed immediately; the remainder of the neutralized extracts were then frozen at −80°C for subsequent assays. Glycerol was determined by the method of Eggstein and Kuhlmann (1974) and sorbitol as described by Bergmeyer *et al.* (1974). All other metabolites were measured by the methods of Lowry and Passonneau (1972); arginine phosphate was determined using assay conditions described for creatine phosphate but with the substitution of arginine phosphokinase for creatine phosphokinase.

RESULTS

Freeze avoidance: Epiblema scudderiana

Prior to experimentation, larvae were held at constant 3°C for 5 months and had accumulated high levels of glycerol, 1230 ± 190 μmol/g wet wt. Cryoprotectant content was not significantly altered over the experimental course of alternating 24 h exposures to −16 and +3°C (Fig. 1). Similarly, there was no clear-cut effect of temperature cycling on glycogen reserves although values varied between 100–200 μmol/g at different sampling points.

However, the temperature cycles did have distinct effects on glucose-6-*P* levels in the larvae; these rose with each cold exposure (by about 2.5-fold) and dropped again to control levels with each 3°C exposure. Multiple sampling at 2, 8, and 24 h in the 1st and 9th cycles showed that the changes observed required at least 2 h to be initiated. Glucose followed a different pattern; exposure to −16°C had no significant effect but levels of the sugar rose over the short term (2–8 h) when larvae were transferred back to 3°C.

The lactate content of *E. scudderiana* larvae was low, ranging between 0.25 and 1.4 μmol/g and although variable, showed no distinct pattern with the cycles of −16/+3°C exposure (Fig. 2). Alanine content was much higher, 23.8 ± 1.3 μmol/g,

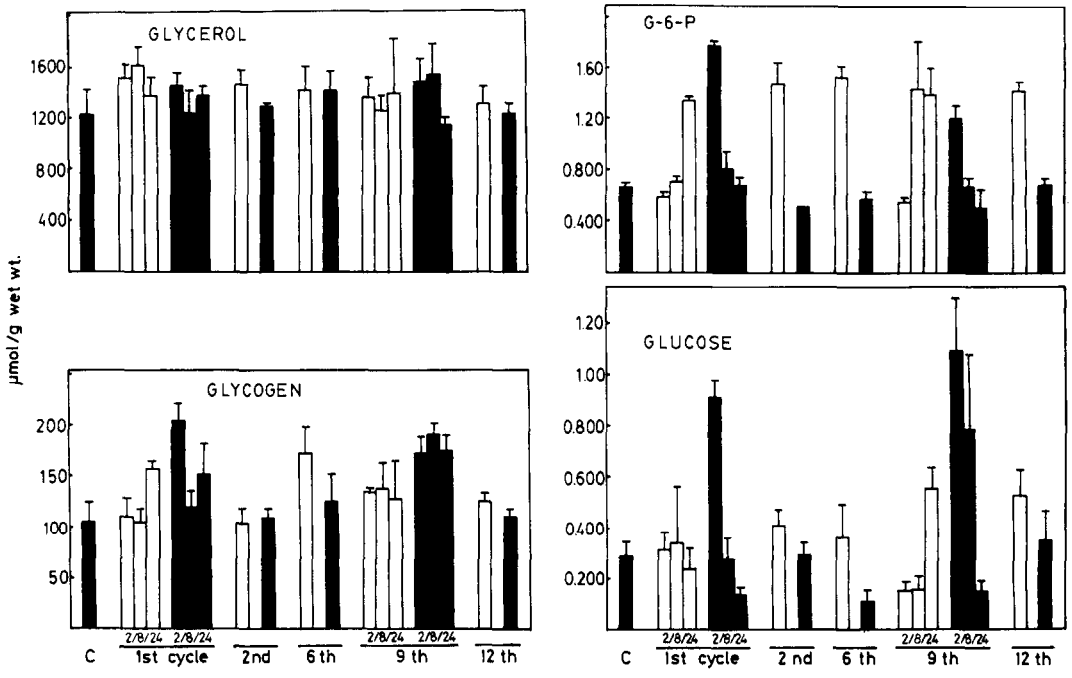

Fig. 1. Effect of alternating 24 h cycles of exposure to −16 and 3°C on glycerol, glycogen, glucose and glucose-6-P levels in the freeze-avoiding larvae, *Epiblema scudderiana*. Filled bars represent 3°C, open bars are −16°C. Larvae were sampled at the end of a 24 h exposure to either test temperature and in the 1st and 9th cycles additional samples were taken at 2 and 8 h. C—control larvae sampled at 3°C prior to initiating cycling. Data are μmol/g wet wt, means ± SEM, $n = 4$ samples with 6 larvae pooled per sample. Glycogen is expressed in glucose units.

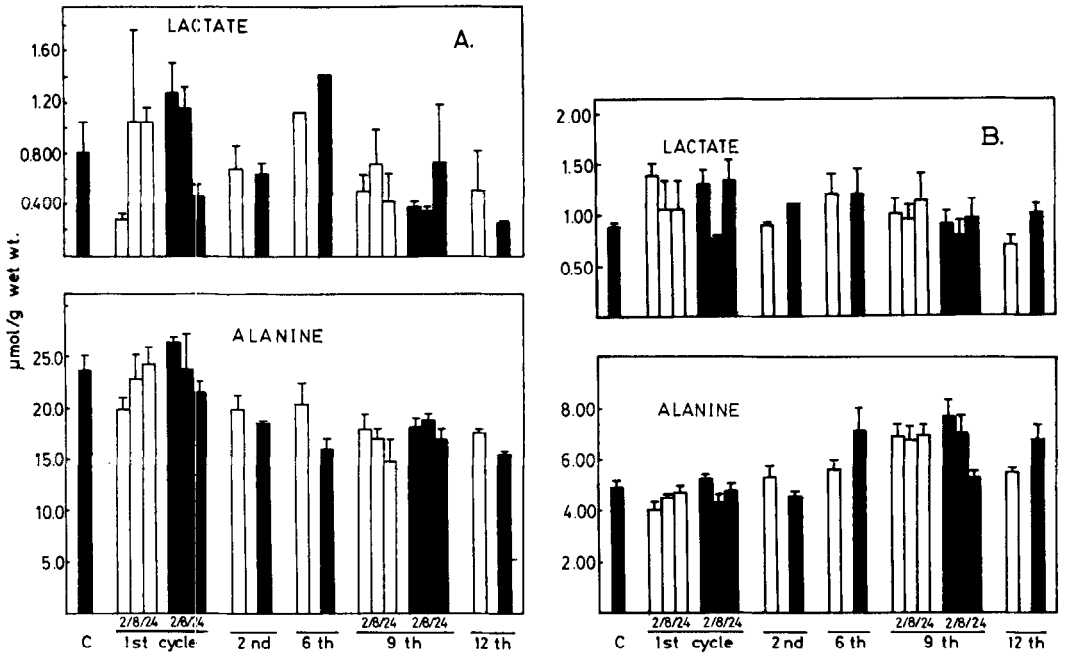

Fig. 2. Effects of alternating 24 h cycles at −16 and 3°C on *L*-lactate and *L*-alanine levels in freeze-avoiding, *Epiblema scudderiana*, (A) vs freeze-tolerant, *Eurosta solidaginis*, (B) larvae. Details are as in Fig. 1.

in control larvae and decreased overall by about 8 µmol/g over the experimental time course.

The effect of temperature cycling on adenylates and phosphagen levels of *E. scudderiana* larvae are shown in Fig. 3. Cold exposure placed an energy stress upon the larvae. In general, exposure to −16°C lowered ATP content and raised ADP and AMP contents; in the 1st and 9th cycles the temporal changes were clearly seen. The effects of temperature on energy status were most evident in the response of energy charge ([ATP + 1/2 ADP]/[ATP + ADP + AMP]); this fell below 0.90 with each −16°C exposure but quickly recovered when larvae were returned to 3°C. The effects of temperature cycling on arginine phosphate content were more variable with the net effect being an overall increase of about 2-fold in phosphagen content as a result of the temperature stress conditions.

Freeze tolerance: Eurosta solidaginis

The equivalent experiment carried out on the freeze-tolerant larvae had the added feature of freeze/thaw exposures since the supercooling point of these larvae is about −10°C (Morrissey and Baust, 1976). *E. solidaginis* has two cryoprotectants, glycerol and sorbitol (Morrissey and Baust, 1976; Storey and Storey, 1986). After 5 months at constant 3°C prior to experimentation, initial levels of these compounds in the larvae were 270 ± 54 µmol/g wet wt glycerol and 18 ± 4 µmol/g sorbitol. Subsequent exposure to the 12 cycles of −16/+3°C exposures had no significant effect on the glycerol pool but sorbitol levels progressively rose to a final content of about 75 µmol/g (Fig. 4). The first significant rise occurred in the 3°C portion of the 2nd cycle, continued through the 6th cycle, and reached a plateau level between the 9th and 12th cycles. Glycogen reserves responded oppositely, a substantial drop in content being seen by the 6th cycle with an overall decrease in glycogen of about 100 µmol/g. This was more than sufficient to account for both sorbitol and glucose accumulation.

In general, glucose-6-P content of the larvae rose with each −16°C exposure and fell with each 3°C exposure. The 1st and 9th cycle, however, show that there was also a time-dependent component. Glucose-6-P was highest at the earliest sampling point (2 h) and fell over time at either 3 or −16°C. A rise in glucose content occurred during 3°C exposure in the first cycle, following the increase in glucose-6-P content and preceding the rise in sorbitol; as has been suggested previously, this indicates the precursor–product relationship between glucose and sorbitol (Storey and Storey, 1983). Glucose remained high while sorbitol synthesis continued and then declined slowly. Glucose levels changed over time in the 3°C portion of the 1st and 9th cycles but, in contrast to glucose-6-P, were not affected over time at −16°C.

The transition to the frozen state at −16°C limits oxygen availability to the larvae and, over the long term, causes an accumulation of fermentative end products (Storey and Storey, 1985). A freezing stress of 24 h duration did not, however, produce a build-up of lactate nor did the 12 cycles of −16/+3°C exposures have a cumulative effect (Fig. 2). The same was true of *L*-alanine; levels in the larvae were somewhat variable, rising as high as 30% above control values by the 9th cycle but, overall, the net alanine accumulation was minor.

Freeze/thaw cycles had a strong cumulative effect, however, upon adenylate levels and energy charge in *E. solidaginis* larvae. With the first exposure to

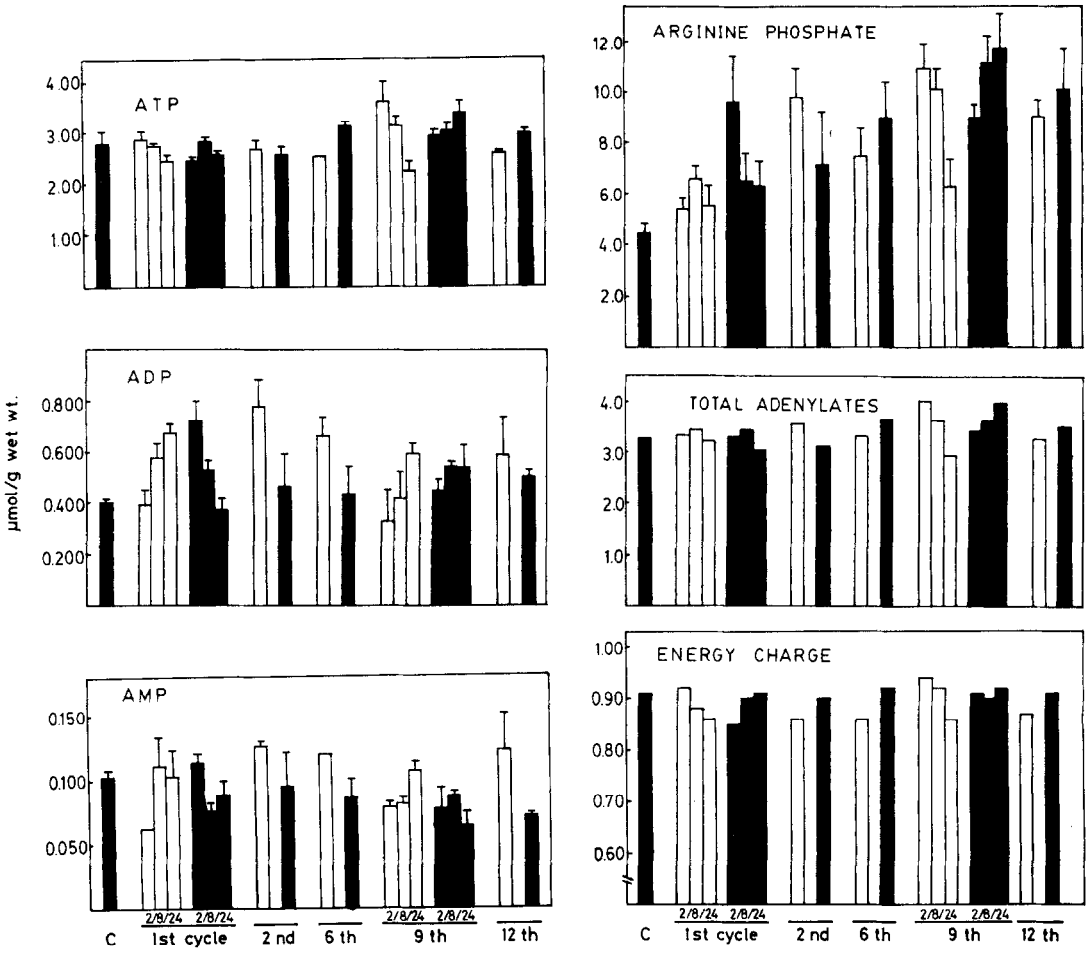

Fig. 3. Effect of alternating 24 h cycles at −16 and 3°C on adenylates and phosphagen in the freeze-avoiding larvae, *Epiblema scudderiana*. Details are as in Fig. 1.

−16°C, ATP levels dropped to an average of 56% of control levels (Fig. 5). During the first 2 cycles levels recovered somewhat when larvae were returned to 3°C but in later cycles, 24 h at 3°C had no significant affect on ATP content. Surprisingly, arginine phosphate content remained constant throughout, and obviously did not play a role in buffering ATP levels during freezing stress. Despite the drop in ATP content with the first freezing exposure, ADP and AMP levels were not affected. Instead, the total adenylate content of the larvae dropped, a response that kept energy charge high over the first two cycles. By the 6th cycle and subsequent cycles, however, ADP and AMP levels were elevated and energy charge had dropped to 0.70–0.75.

DISCUSSION

E. scudderiana and *E. solidaginis* both overwinter in galls on the stems of goldenrod; quite often these occur in close proximity to each other on the same plant. The galls have virtually no insulation value and the two species effectively experience the same ambient thermal environment over the winter months. Therefore, they are excellent model animals with which to compare and contrast the adaptations supporting freeze avoidance vs freeze tolerance and to explore the metabolic consequences of each strategy. The literature on cold hardiness in *E. solidaginis* is extensive (e.g. Baust *et al.*, 1985; Storey and Storey, 1988 for reviews) and is rapidly accumulating for *E. scudderiana* (Rickards *et al.*, 1987; Kelleher *et al.*, 1987; Churchill and Storey, 1989a–c).

Differences between the two species in the effect of multiple cycles of rapid temperature change were most obvious when examining cellular energetics. Over the first 2 cycles of −16/+3°C exposure the two species responded similarly with a drop in energy charge (ATP decreased, ADP and AMP increased) upon exposure to −16°C and recovery upon rewarming. This behaviour continued for *E. scudderiana* throughout the 12 cycles; energy status at the final 3°C sampling was equivalent to that of the initial controls. Indeed, in another study we showed that the perturbation of energetics at low subzero temperature was only a temporary 'cold shock' response and recovered after several days at constant low temperature (Churchill and Storey, 1989a). The situation for *E. solidaginis* was different, however. Freeze/thaw cycling between −16 and 3°C had a cumulative detrimental effect on cellular energetics. A drop in total adenylates (probably due to deamination of AMP) was used initially to help maintain a high energy charge but by the 6th cycle, energy charge was

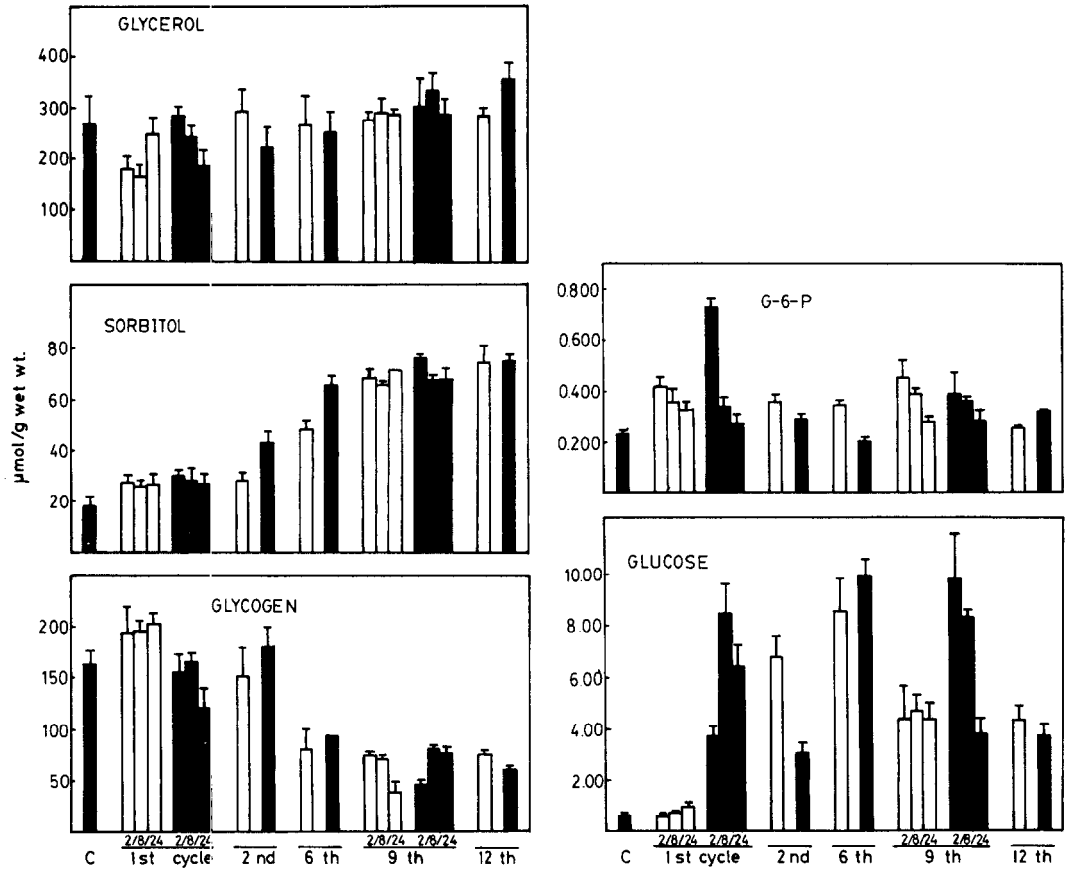

Fig. 4. Effect of alternating 24 h cycles at −16 and 3°C on glycerol, sorbitol, glycogen, glucose and glucose-6-P levels in the freeze-tolerant larvae, *Eurosta solidaginis*. Details are as in Fig. 1.

sharply reduced. In addition, in later cycles there was no trend towards recovery in the 3°C portion of each cycle. The stresses of freezing and thawing on *E. solidaginis* cells are undoubtedly energy expensive since they require extensive redistribution of ions and other solutes across the plasma membrane, processes that often rely upon active transport. Multiple rapid freeze/thaw transitions apparently overburden the ATP-producing capacity of the larvae, particularly during the freezing process when oxygen supply to cells becomes limiting. Indeed, in a previous study we found that long-term freezing (beyond the first week) caused a severe stress on energetics in *E. solidaginis*; after 12 weeks at constant −16°C, only 5% of phosphagen reserves remained, total adenylates were reduced by 40% and energy charge was about 0.5 (Storey and Storey, 1985). However, the energy stress experienced by *E. solidaginis* was not lethal; the larvae survived, and continued development to pupate and hatch, after either rapid freeze/thaw (this study) or long-term freezing exposures (Storey and Storey, 1985). This indicates that the adaptive strategies of freeze tolerance must include mechanisms that sustain cell survival even when energetics are severely compromised.

What do these results suggest about the relative merits of freeze avoidance vs freeze tolerance as insect strategies of winter hardiness? Obviously, the two strategies differ in the ability to maintain cellular energetics over widely varying winter temperatures. The freeze avoidance strategy appears to be the less stressful, at least for the two goldenrod gall formers. For some species, then, this may have been one positive pressure towards the development of a freeze-avoidance strategy. The ability to maintain cellular energetics at a near constant level (and/or recover rapidly after a temperature shock) probably means that all normal cellular functions can continue even at low temperatures near the supercooling point of *E. scudderiana*. This may represent a worthwhile advantage of the freeze-avoiding strategy, compared to the freeze-tolerance strategy, for some species. If one excludes, momentarily, the risks of spontaneous freezing (either below the supercooling point or due to inoculation) that accompany this strategy, then the freeze-avoiding insect probably requires many fewer adaptations by cellular metabolism to survive at subzero temperatures. Temperature change (cooling or warming) probably elicits only minor metabolic adjustments that compensate for the differential effects of temperature on metabolic processes. The freeze-tolerant insect, however, faces much greater metabolic consequences when temperature change causes a freeze/thaw transition including water and solute redistribution between intra- and extracellular spaces and an aerobic–anoxic transition. Additional adaptations that permit anoxia tolerance, survival of low energy stress, cell volume regulation, and membrane stabilization had to be developed. Obviously, this package has been developed by many

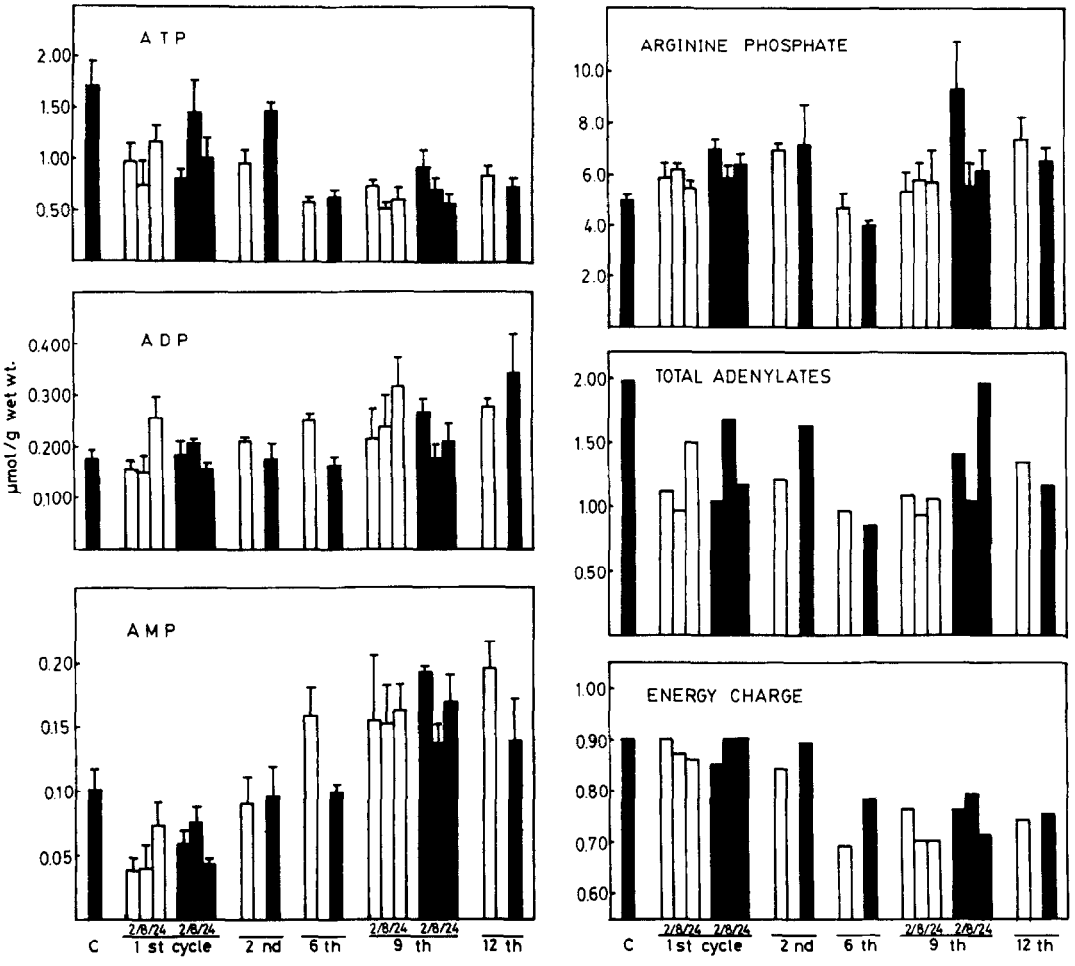

Fig. 5. Effect of alternating 24 h cycles at −16 and 3°C on adenylates and phosphagen in the freeze-tolerant larvae, *Eurosta solidaginis*. Details are as in Fig. 1.

species but one of the permissive factors that facilitated the development of freeze tolerance may have been a pre-existing ability to deal with energy stress such as a good anoxia tolerance or a capacity for metabolic rate depression (Storey, 1988).

The involvement of fermentative glycolysis in sustaining energy metabolism in *E. solidaginis* over the −16/3°C cycles is indicated by the cumulative increase in L-alanine content of the larvae. However, neither alanine nor lactate accumulated in *E. scudderiana*, suggesting that normal aerobic metabolism continues at subzero temperatures for the freeze-avoiding species.

Both species responded to the −16/3°C temperature cycles with fluctuations in glucose-6-P levels, increasing at −16°C and decreasing at 3°C. This is undoubtedly caused by a cold activation of glycogen phosphorylase as has been well documented for both these species and many other cold-hardy insects (Storey and Storey, 1988; Churchill and Storey, 1989b). The mechanism of phosphorylase activation involves differential effects of temperature on the activities of phosphorylase kinase vs phosphorylase phosphatase with low temperature favouring the kinase reaction (Hayakawa, 1985). However, although phosphorylase was apparently activated at −16°C, there was no increase in the glycerol pool of *E. scudderiana* over the 12 cycles. The reasons for this probably include: (a) maximal glycerol levels had been reached during the prior acclimation to 3°C, (b) glycogen content remaining in these February larvae was low, and (c) time at −16°C was short. In another study we found that net accumulation of glycerol by *E. scudderiana* at −20°C occurred only after several weeks (Churchill and Storey, 1989a). Glycerol content of *E. solidaginis* larvae was similarly unaffected by the temperature cycling but this was an expected result. Glycerol levels in this freeze-tolerant species are modulated at warmer temperatures but not over the subzero range (Storey *et al.*, 1981; Storey and Storey, 1983). The larvae did, however, increase their content of sorbitol by 4-fold in response to the −16/3°C cycles. Previous studies of this species have shown that sorbitol is readily interconvertible with glycogen throughout the winter (Storey and Storey, 1983, 1986). Production is triggered at temperatures of 3–5°C and rates are maximal between 0 and −5°C (Storey *et al.*, 1981; Rojas *et al.*, 1983). Apparently the equilibrium content of sorbitol for *E. solidaginis* at 3°C is 18 μmol/g, considerably lower than the average mid-winter levels of 120 μmol/g in nature (Storey and Storey, 1986). Under stimulation by −16°C exposure, this was rapidly raised over 9 days with a concomitant drop in glycogen reserves. Given

the cumulative energy stress imposed on *E. solidaginis* by freeze/thaw exposures, the differential synthesis of glycerol at high temperatures vs sorbitol at low temperatures is probably advantageous to the larvae. Glycerol synthesis is ATP-dependent and best supported by an aerobic metabolism whereas sorbitol production does not require ATP input and can be carried out under energy-limited conditions.

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