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Freezing and cellular metabolism in the gall fly larva, *Eurosta solidaginis*

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Summary. The effects of extracellular freezing on intracellular metabolism were monitored over both a short (9 h) and long (12 weeks) time course using the freeze tolerant larvae of the gall fly, *Eurosta solidaginis*.

The process of freezing, monitored over the short time course, had no effect upon cellular energy levels (adenylates, arginine phosphate) but initiated a rise in glucose-6-P and lactate levels. This suggests that freezing initiates a shift towards glycolysis as the predominant mode of energy production. The process of thawing at 3 °C (after 24 h at −16 °C) also had no effect, even transient, on cellular energy levels demonstrating that thawing and the rapid redistribution of water and solutes which must accompany it does not disrupt cellular metabolism. During thawing accumulated lactate was quickly cleared with a $t^1/_2$ of 20–30 min.

Long term freezing at −16 °C had dramatic effects on energy metabolism. Freezing for up to 1 week had minimal effects with only a small drop in arginine phosphate reserves and an increase in lactate content noted. Between 1 and 2 weeks of freezing, however, larvae showed strong signs of energy stress. The arginine phosphate pool fell from 75% to 30% of control levels, ATP content dropped by 50% and energy charge dropped to 0.75. This state, with continued lactate accumulation, was maintained through 4 weeks of freezing. Between 6 and 12 weeks of freezing energy stress became even greater. Phosphagen and ATP contents dropped to 5 and 25% of control values and energy charge decreased to about 0.50. Despite this stress, however, 94% of larvae survived 12 weeks of freezing with an 86% hatch rate of adults. The data demonstrate that the larvae can survive prolonged periods of winter freezing drawing upon glycolysis and phosphagen reserves to supply the continued basal energy demands of the cell.

Introduction

Third instar larvae of the goldenrod gall fly, *Eurosta solidaginis*, have a tolerance for extracellular freezing during the winter months. The biochemical adaptations for cold hardiness displayed by this species include: (a) synthesis of glycerol and sorbitol as cryoprotectants (Morrissey and Baust 1976; Storey et al. 1981 a), (b) changes in the activities of certain enzymes (Storey and Storey 1981), (c) alterations to the bound water content of cells (Storey et al. 1981 b), (d) synthesis of nucleating agents to control extracellular freezing (Somme 1978) and (e) changes in mitochondrial metabolism (Ballantyne and Storey, to be published).

The larvae display a supercooling point of about −8 °C; below this extracellular freezing occurs. Larvae survive a maximal ice formation of about 65% of total body water (Lee and Lewis, to be published). Metabolism and the regulation of cryoprotectant synthesis at temperatures above −8 °C have been quite well studied (Storey et al. 1981 a; Storey and Storey 1983). Information about metabolism in the frozen state is limited however. The intracellular environment remains in a liquid state (presumably) although viscosity is likely to be greatly increased with water drawn out of cells into the growing ice crystals and polyols concentrated in the intracellular fluid until an equilibrium is reached where the melting point of the intracellular fluid equals the actual temperature of the insect (Zachariassen 1980). The production of polyols ceases in the frozen state but energy

expenditure and production appear to continue at a low rate; Storey et al. (1981 a) reported a decrease in ATP and arginine phosphate content and an increase in lactate content in larvae exposed to a 1 °C per day decrease in temperature from −5 °C to −30 °C.

The present study examines in detail the effects of freezing upon cellular metabolism in *E. solidaginis* larvae. Both short (1 day or less) and long (12 weeks) term freezing were examined.

Materials and methods

Chemicals. Biochemicals were purchased from Sigma Chemical Co. or Boehringer Mannheim Corp..

Animal experiments. Galls containing third instar larvae were collected from fields around Ottawa during late September and early October. Galls were kept for 2 months in a cold room at 5 °C. Galls were then opened and larvae were placed in groups of 50 in plastic petri dishes. Animals were then transferred to a cold cabinet at 3 °C (range 1–4 °C) and were held for a further 2 weeks (for the long term freezing experiment) or 5 weeks (for the short term freeze/thaw experiment). To examine the effects of long term freezing on metabolism, animals were transferred to a freezer at −16 °C and were sampled over time from 0 to 12 weeks. For short term freezing, animals were moved to −16 °C and sampled over time from 0 to 9 h. To examine the effects of thawing, larvae which had been frozen for 24 h were transferred back to 3 °C and were sampled over time from 0 to 48 h.

Larvae were killed by immersion in liquid nitrogen and were then stored at −80 °C until analysis.

Sample preparation and metabolite analysis. Eight larvae (approximately 400 mg) were pooled and were ground to a powder under liquid nitrogen using a mortar and pestle. Perchloric acid extracts of the tissue were then prepared as described by Storey et al. (1981 a). Extracts were stored at −80 °C until analysis. Metabolites were quantitated by enzymatic assay as described previously (Storey et al. 1981 a; Storey and Storey 1983).

Results

Short term freezing and thawing

To examine short term freezing effects, larvae were moved from 3 °C to −16 °C and were sampled at 0, 5, 10, 15, 20, 30, 45, and 60 min and 2, 5 and 9 h. Preliminary observations showed that larvae were semi-solid after 15 min at −16 °C and well frozen after 45 min. Short term freezing had little effect upon larval energy metabolism. Levels of adenylates and arginine phosphate remained constant over the full time course as did glycogen, glycerol and sorbitol levels. Only glucose-6-P and lactate showed small, but significant, changes (Fig. 1 A). Glucose-6-P levels showed an increase after 5 min at −16 °C and remained elevated throughout the freezing exposure. Lactate increased gradually over the freezing exposure from 0.43 ± 0.03 to 1.08 ± 0.22 µmol/g wet weight after 9 h.

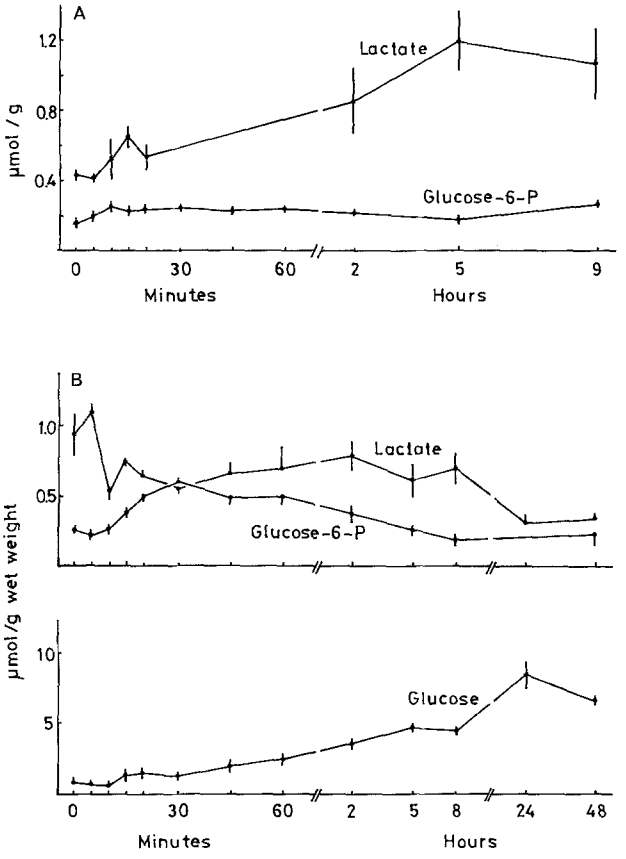

Fig. 1 A, B. Levels of some metabolites in *E. solidaginis* larvae during a short term freeze (**A**) and thaw (**B**). Larvae used to study thawing were first frozen at −16 °C for 24 h. Results are means ± SEM (indicated by vertical line) for $n = 3$ samples at each point with 8 larvae pooled per sample

To examine the effects of thawing, larvae which had been frozen for 24 h at −16 °C were transferred back to 3 °C and were sampled at 0, 5, 10, 15, 20, 30, 45, and 60 min and 2, 5, 8, 24 and 48 h. Larvae were soft to the touch after 15 min at 3 °C. As during short term freezing, thawing produced no alterations, even transient, in the levels of ATP, ADP, AMP and arginine phosphate. Glycerol and sorbitol levels were also unaltered. Lactate concentration was reduced from 1.02 ± 0.08 µmol/g at time zero to 0.34 ± 0.03 µmol/g by 24 h after thawing (Fig. 1 B). Glucose-6-P levels were 0.27 ± 0.01 µmol/g at time zero, rose to a peak of 0.60 ± 0.02 µmol/g after 30 min and then declined to 0.24 ± 0.07 µmol/g by 48 h. Following this elevation of glucose-6-P levels was a rapid increase in glucose content from 0.88 ± 0.05 µmol/g at time zero to a peak of 8.58 ± 1.04 µmol/g at 24 h. Glucose levels had begun to decline (6.73 ± 0.04 µmol/g) by 48 h after thawing (Fig. 1).

To examine the effects of long term freezing on metabolism in *E. solidaginis* larvae, animals were

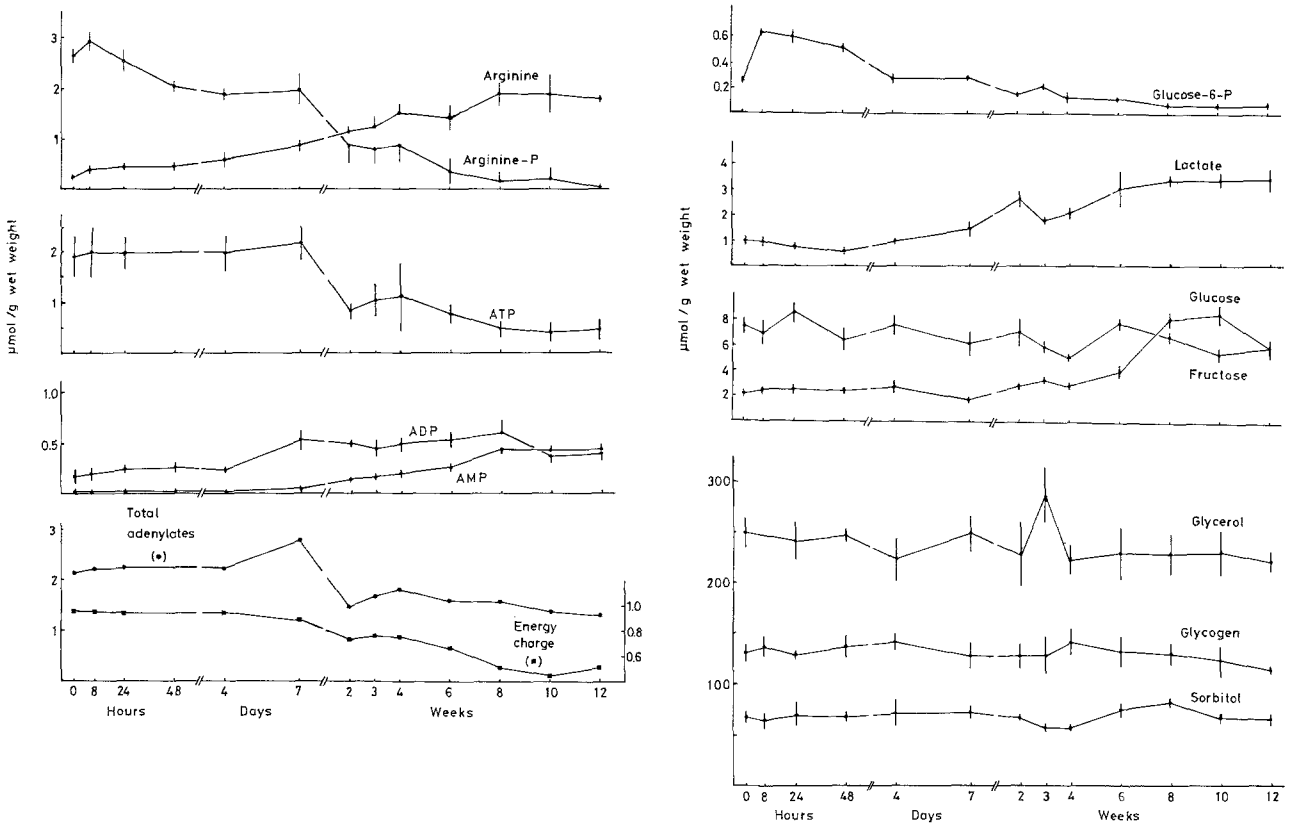

Fig. 2. Levels of some metabolites in *E. solidaginis* larvae during long term freezing at −16 °C. Results are means ± SEM for $n = 3$ samples at each point with 8 larvae pooled per sample. Glycogen is given as glucose units. Adenylate energy charge is calculated as ([ATP] + ½[ADP])/([ATP] + [ADP] + [AMP])

transferred from 3 °C to −16 °C and sampled after 8 h, 1, 2, 4, and 7 days and 2, 3, 4, 6, 8, 10, and 12 weeks. Survivorship of freezing for 12 weeks was tested by allowing 50 larvae to thaw, pupate and hatch into adults. Ninety-four percent of larvae survived the 12 weeks of freezing, 88% pupated and 86% hatched as adults. The effects of long term freezing on intermediary metabolism are shown in Fig. 2. ATP content of the larvae remained constant at about 2 µmol/g over the first 7 days of freezing but after 2 weeks had declined by 50% and after 8 weeks had declined to 25% of control values. ADP and AMP showed opposite changes. Both were elevated after 7 days of freezing. ADP content remained relatively constant between 0.4 and 0.6 µmol/g between 1 and 12 weeks but AMP content showed a continual rise with a plateau of about 0.45 µmol/g reached after 8 weeks of freezing. Total adenylates remained constant over the first week of freezing and then decreased gradually with only 60% of the control pool size remaining after 12 weeks. Energy charge was high (0.95) during the early days of freezing, decreased to 0.73–0.76 between 2 and 4 weeks and to 0.44–0.51 between 8 and 12 weeks. Arginine phosphate content of the larvae remained constant only for the first 24 h of freezing and then began to decline. The arginine phosphate pool decreased to 75% of control levels by 7 days and then to 29–33% of control between 2 and 4 weeks. After 12 weeks of freezing only 5% of phosphagen reserves remained. Arginine content of the larvae increased in an opposite pattern to the decline in arginine phosphate.

Freezing initiated a rise in glucose-6-P levels as was noted in the short term freezing experiment. Glucose-6-P rose from control levels of 0.25 ± 0.01 µmol/g to a maximum of 0.63 ± 0.02 µmol/g after 8 h and then declined throughout the remainder of the freezing exposure reaching levels of 0.07–0.08 µmol/g between 8 and 12 weeks of freezing. Lactate content of the larvae remained relatively constant at about 1 µmol/g over the first 4 days of freezing exposure and showed the first significant rise to 1.45 ± 0.30 µmol/g after 7 days. Lactate content then rose gradually to 3.39 ± 0.18 µmol/g after 8 weeks and remained constant at this level between 8 and 12 weeks.

Polyols in the larvae were not affected by freezing (Fig. 2); the overall average levels of glycerol and sorbitol were 244 ± 7.8 and 68 ± 1.8 µmol/g

wet weight, respectively. Glycogen content of the larvae was not significantly altered over 10 weeks of freezing but showed a small decline at 12 weeks. Fructose content remained constant at an overall average of 6.8 ± 0.27 μmol/g but glucose content increased after 6 weeks of freezing with a peak content at 8.7 μmol/g (at 10 weeks) of about 3.5 times greater than control. Glycerol-3-P, a product of anaerobic glycolysis in insects, was not altered during freezing.

Discussion

The process of ice formation in *E. solidaginis* has recently been examined by Lee and Lewis (to be published). Freezing is not a rapid process in intact larvae as is the almost instantaneous freezing of isolated hemolymph when the supercooling point is exceeded. Although ice nucleation begins within 1.5 minutes at −23 °C, Lee and Lewis found that only 7% of total body water is frozen after 5 minutes of exposure. This rises to 20% after 1 h, 47% after 6 h and then slowly increases to reach a plateau at about 64% by 48 h at −23 °C. The higher initial rate of freezing probably represents freezing of bulk extracellular water; freezing rate then declines as cells dehydrate and an osmotic equilibrium is approached between the growing extracellular ice crystals and the increasingly concentrated intracellular fluid. With respect to the present experiments, then, the short term freezing experiment covers the period of active freezing when ice content of the larvae is continuously increasing. The long term freezing experiment covers the period of relatively constant (beyond 48 h) ice content in the larvae and monitors metabolism in the stable frozen state. Parenthetically it can be noted that the data of Lee and Lewis would predict a final maximal ice content of between 56 and 60% of total body water in larvae at −16 °C.

The process of extracellular freezing itself does not disrupt cellular energy reserves in the larvae suggesting that freezing does not exact a high energetic cost from cells. Thus adenylate and phosphagen levels remained constant throughout the short term freezing experiment and only phosphagen content had decreased slightly by 48 h of freezing exposure. Freezing does however alter the mechanism of cellular energy production. Metabolism at 3 °C, prior to freezing, would likely be based upon the aerobic oxidation of proline and pyruvate; Ballantyne and Storey (to be published) found that mitochondria from cold acclimated larvae did not oxidize lipid at low temperature. Freezing appears to shift metabolism to a dependence upon glycolytic energy production. This is supported by an observed transient elevation of glucose-6-P levels, suggesting a mobilization of glycogen as fuel, in the early hours after freezing and by continuous accumulation of lactate throughout the long term freezing exposure. Freezing also probably initiates the shutdown of all but maintenance cellular functions. Thus our previous studies showed that cryoprotectant synthesis ceased below −5 °C (Storey et al. 1981 a). Glycolytic ATP synthesis and the mobilization of phosphagen reserves supply the cellular requirements for energy in the frozen state. However even though Q_{10} effects would strongly depress metabolic rate at −16 °C, energy demand in the long term (greater than 1 week of freezing) must outstrip energy production as both arginine phosphate and ATP content are depleted and energy charge falls.

Metabolism in the frozen state shows distinct stages. Freezing for periods of up to 1 week has minimal effects upon cellular metabolism. ATP and total adenylate content are maintained and energy charge remains high. This 'normal' metabolic state is maintained by the utilization of arginine phosphate reserves and the generation of energy via anaerobic glycolysis; in ATP equivalents these two sources provide approximately 0.7 and 1.0 μmol/g of ATP, respectively over the first week. During the second week of freezing metabolism becomes stressed. Energy expenditure appears to be high, 1.8, 1.0 and 1.0 μmol/g of ATP equivalents are generated from arginine phosphate, ATP and lactate production, respectively. Possibly the larvae can maintain some aerobic metabolism throughout the first week of freezing but available oxygen may run out, with no replenishment, during a second week of freezing. Between 2 and 4 weeks of freezing the larvae show a fairly stable but stressed metabolic state. Energy charge remains constant at about 0.75. This state may be the typical dormant state of the larvae in the natural environment; in most instances outdoor temperatures would probably not remain constant below −8 °C for periods of more than 4 weeks. Beyond 4 weeks of freezing energy stress becomes progressively greater. Remaining arginine phosphate reserves are utilized and lactate continues to accumulate. ATP levels fall further and a sharp rise in AMP content indicates an attempt to generate more ATP via the adenylate kinase reaction. Between 8 and 12 weeks of freezing the larvae are in an extreme energy starved state. The rise in glucose levels over this time may suggest that while glycogen breakdown can continue, glycolysis and lactate production are inhibited at some level.

However, despite the extreme energy depletion observed in the larvae after 12 weeks of freezing, the larvae survived this stress and a high percentage pupated and hatched into adults. Indeed casual observation indicated that the survival rate was much higher for larvae which had been frozen than for those which had been held at 3 °C over the 12 weeks. These larvae were much smaller than those which had been frozen probably due to the catabolism of endogenous reserves over time at 3 °C. Thus optimal conditions for survival and adult emergence may depend upon spending a considerable portion of the winter in the frozen state, at least for Canadian populations. Periods of freezing spare endogenous fuel reserves by decreasing metabolic rate and place the larvae under only limited metabolic stress.

The process of thawing had no detectable energetic cost in terms of transient alterations in adenylate or arginine phosphate levels. (Fig. 1 B). Accumulated lactate was rapidly cleared with a $t^1/_2$ of 20 – 30 min suggesting that aerobic metabolism is immediately restarted as the larva thaws. The process of recovery from the stress of a long term freeze would probably require a longer time; however this was not examined in the present study. During the thawing experiment a transient rise in glucose-6-P level was noted followed by an elevation of glucose content. The pattern and time frame of these changes are similar to those seen when sorbitol synthesis is stimulated (Storey and Storey 1983). It is possible that freezing exposure might stimulate a further synthesis of sorbitol as the sorbitol content of the larvae used in the present study (about 75 μmol/g) was fairly low.

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