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# Freeze Tolerance in Hermit Flower Beetle (*Osmoderma eremicola*) Larvae

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**Larvae of *Osmoderma eremicola* (Knoch)(Coleoptera: Scarabaeidae) were found to be freeze tolerant. Early (1–2.5 cm) and late (3–5 cm) larval stages survived freezing to −8.3°C for 96 h with 64% of body water as ice. Glycogen phosphorylase activity in the fat body was increased 6–7-fold during freezing, and glycogen stores were depleted in fat body (from 237 ± 1 to 57 ± 7 μmol/g fresh weight) and body wall (89 ± 4 to 40 ± 1 μmol/g fresh weight). However, no glycogen derived cryoprotectants (glycerol, sorbitol, glucose, fructose) were found to accumulate in tissues or haemolymph during freezing or acclimation at 9, 0 and −5°C. Osmolality (~450 mOsmol) of haemolymph was constant during freezing and acclimation from 9 to −5°C, also suggesting the absence of low molecular weight cryoprotectants. Haemolymph trehalose levels never rose above 2.7 mM as detected by NMR. Total protein levels in fat body and body wall remained unchanged during acclimation. HPLC analysis showed increases in amino acid levels by 10–15 μmol/g fresh weight in the body wall of both early and late instar larvae during freezing to −8.3°C for 96 h, with consistent increases in glycine (7.4–8.7 μmol/g fresh weight), alanine (4.2–5.8 μmol/g fresh weight), glutamate (2.4 μmol/g fresh weight), and valine (0.8–1.6 μmol/g fresh weight). Smaller increases in glutamic acid (by 1 μmol/g fresh weight) and alanine (by 3.5 μmol/g fresh weight) were also seen in late instar fat body, whereas glycine levels remained unchanged and valine decreased slightly (by 1.4 μmol/g fresh weight) in this tissue. Proline levels, detected by NMR, ranged from 10 to 18 μmol/g fresh weight in fat body and 40–70 μmol/g fresh weight in haemolymph, making it the most abundant amino acid in fat body.**

*Osmoderma eremicola* Beetle Insect freeze tolerance Cold hardiness Cryoprotectants

## INTRODUCTION

Freeze tolerance is a common strategy of cold hardiness among insects that must endure temperatures below the freezing point of their body fluids while overwintering (Storey and Storey, 1989). Freeze tolerant insects initiate and control ice formation in the extracellular matrix, and have developed mechanisms by which to protect cells from extracellular ice and the osmotic stress that it causes (Storey and Storey, 1989). Ice formation is initiated in extracellular spaces, often with the help of ice nucleating agents. Water is removed from cells as a consequence of the concentration of extracellular fluids due to ice formation, thereby reducing the supercooling point within the cells. Low molecular weight carbohydrates often accumulate as colligative cryoprotectants and these help to limit the extent of ice formation and the reduction of cell volume below a critical minimum.

Many freeze-tolerant species of beetles have been described; see Zachariassen (1980) for a partial list. These can be separated into two groups, based on their lower lethal temperatures and whether they accumulate polyols. Some species tolerate freezing in the absence of any significant increase in colligative cryoprotectants, but endure freezing only at relatively high sub-zero temperatures. For example, *Eloedes blanchardi* beetles survive freezing to −8°C in the absence of colligative cryoprotectants (Zachariassen *et al.*, 1979). Other species accumulate high levels of polyols such as glycerol, sorbitol, and threitol, and endure much lower temperatures during freezing episodes. For example, *Phyllodecta laticollis* beetles accumulate 1500 mMolal glycerol and survive in the frozen state down to −42°C (van der Laak, 1982).

Larvae of the hermit flower beetle, *Osmoderma eremicola* Knoch (Coleoptera: Scarabaeidae), overwinter in the heartwood of decaying trees, usually deciduous, where they could face low, freezing temperatures in the winter. These insects have a 3-year life cycle and undergo an undetermined number of larval stages (Hoffman, 1939). The adults emerge to mate in the summer months (June–August) and live an average of 38 days (males) or 58 days (females) (Hoffman, 1939). The insects range throughout the eastern half of the U.S.A. except for the southernmost areas, and extend into the southern parts of eastern Canada (Howden, 1968).

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In this study we describe freeze tolerance in *O. eremicola*. Animals in two distinct larval stages were collected from decaying hardwood trees, and parameters including the capacity to survive freezing of varying lengths of times, ice content when frozen, the production of carbohydrate or amino acid cryoprotectants, and energy status were assessed.

## MATERIALS AND METHODS

### *Chemicals and larvae*

All chemicals and biochemicals were purchased from Boehringer Mannheim Corp. or Sigma Chemical Co. Animals were collected in two consecutive years from the decaying hardwood of trees on 15 September, 1990 and 7 October, 1991. The 1990 animals were kept at 5°C in a jar containing decaying matter from the source trees for 2 months before use (until 21 November, 1990). The 1991 larvae were kept at 9°C for 2 months. Larvae were kept moist by occasional watering. Insects ranged from approx. 1.5 to 5 cm in length, and were separated by size into two larval stages (clearly distinguishable between 1.5–3 cm and ~3.5–5 cm). For acclimation studies, control larvae (1991 animals) were kept at 9°C. One group of animals was acclimated to 0°C for 1 week, whereas the second group was frozen and kept at −5°C for 24 h.

### *Freezing and supercooling point determination*

Larvae were frozen in a Precision 815 low temperature incubator with a thermistor (YSI 400 series) attached to a YSI model 42SC tele-thermometer in contact with their body wall. Output was to a Kipp and Zonen BD111 recorder. Cooling rates were typically −0.1 to −0.25°C/min. The lowest temperature attained prior to the release of the latent heat of fusion was taken as the supercooling point. Immediately following the appearance of the exotherm the temperature was adjusted to −2.5°C and animals sampled at intervals up to 112 h of freezing exposure. Other larvae were frozen at −6.2 or −8.3°C.

Ice content of larvae was determined calorimetrically. The calorimetric constant ($F$), specific heat of the larval dry mass, and the weight of ice in the larvae were determined as in Lee and Lewis (1985). For the purpose of calculations the melting point (m.p.) of the larvae was taken as −0.84°C, corresponding to a 450 mOsm solution (the determined osmolality of larval haemolymph).

An examination of the effect of external water on freezing behaviour was also carried out by first wetting the larvae with water (forming droplets on the body wall) before exposure to freezing temperatures under conditions identical to "dry" larvae.

Body water content was determined from the percentage of weight lost by larvae when dried to a constant weight for 48 h at 73°C.

### *Tissue isolation*

Tissue isolation was done as quickly as possible, immediately after thawing for frozen larvae. Haemolymph was collected from the dorsal midline by puncture with a syringe tip. This was quickly followed by the separation of head, fat body, gut, and body wall (cuticle and muscle wall) tissues. These were stored at −75°C until use.

### *Glycogen phosphorylase*

Frozen fat body, gut, and body wall were homogenized 1:5 w/v in homogenization buffer (20 mM Imidazole–HCl pH 7.2, 15 mM $\beta$-mercaptoethanol, 5 mM EDTA, 5 mM EGTA, and 50 mM NaF) in the presence of a few crystals of phenylmethylsulphonyl fluoride (~1 mM). Homogenates were centrifuged for 2 min at full speed in a Brinkmann model 5412 centrifuge. Supernatant from this spin was used as the enzyme source. No activity was detected in resuspended pellets (data not shown). Assays were performed as in Storey and Storey (1981), in the presence of 2 mg/ml glycogen (oyster, dialysed), 0.2 mM NADP, 15 mM $MgSO_4$, 5 $\mu$M $G16P_2$, and 1 IU/assay of phosphoglucomutase and glucose-6-phosphate dehydrogenase for the active ($a$) form; for total phosphorylase ($a + b$) the reaction mixture also contained 2 mM AMP.

### *Amino acid analysis*

Samples were homogenized 1:20 (w/v) in 0.5% (w/v) sulphosalicylic acid, centrifuged in an International micro-capillary centrifuge model MB at full speed for 10 min, and the supernatant neutralized with 1 M KOH. A 30 $\mu$l aliquot of 2 mM $\alpha$-amino adipic acid was added to 270 $\mu$l of sample, and samples were then analysed on a Waters HPLC using pre-column derivatization with $o$-phthaldialdehyde.

### *Metabolite analysis*

Perchloric acid extracts were prepared from frozen tissue (−80°C) as described in Storey and Storey (1984). The pellets from the PCA extracts were retained for protein determinations. All metabolites were quantified using coupled enzyme assays with spectrophotometric detection based on the enzymatic utilization of NAD(H). ATP, ADP, and AMP were assayed immediately after extraction using the method of Lowry and Passonneau (1972). Glycogen was determined using the method of Keppler and Decker (1974), glucose and lactate from Lowry and Passonneau (1972), glycerol from Eggstein and Kuhlmann (1974), and sorbitol from Bergmeyer *et al.* (1974).

### *Protein*

Pelleted protein from the PCA extracts was resuspended in 750 $\mu$l 0.2 N NaOH and stored at −75°C until assayed. Protein levels were determined by the Coomassie blue dye-binding assay of Bradford (1976)

using prepared reagents from Bio-Rad, with bovine serum albumin as a standard.

*Osmolality*

Osmolality of haemolymph samples was measured using a Wescor 5100C vapor pressure osmometer.

*NMR*

NMR analysis for determination of proline and trehalose levels was performed on the perchloric acid extracts used for metabolite assays. These were diluted with an equal volume of 10 mM potassium phosphate (pH 7.0). Samples were run on a Bruker AMX-400 NMR. Calibration of the instrument was done with standard solutions of proline and trehalose in the phosphate buffer.

## RESULTS

When cooled at a rate of 0.1–0.25°C/min on a dry substrate, *O. eremicola* larvae supercooled to $-4.1 \pm 0.2$°C ($n = 12$) before freezing. Within 15 min at $-2.5$°C ice content had risen to 20% of total body water (Fig. 1). The equilibrium ice content at $-2.5$°C, $50.2 \pm 2.7$%, was reached after 2 h of freezing and was maintained up to 112 h of freezing. Freezing at colder temperatures increased the ice content of the larvae. Larvae frozen for 1 h at $-6.2$°C had $53.5 \pm 4.9$% ice whereas freezing at $-8.3$°C for 96 h resulted in $64.2 \pm 2.5$% body water as ice. Survival at all temperatures tested was 100%, and larvae were seen to burrow into the material from the tree from which they were captured (material in which they were replaced after thawing) within 10–30 min after thawing. When larvae were first wetted prior to freezing, the supercooling point remained unchanged at $-4.2 \pm 0.2$°C ($n = 10$). The ice content formed during the first 2 h of freezing at $-2.5$°C was also not significantly different for wet and dry larvae (data not shown).

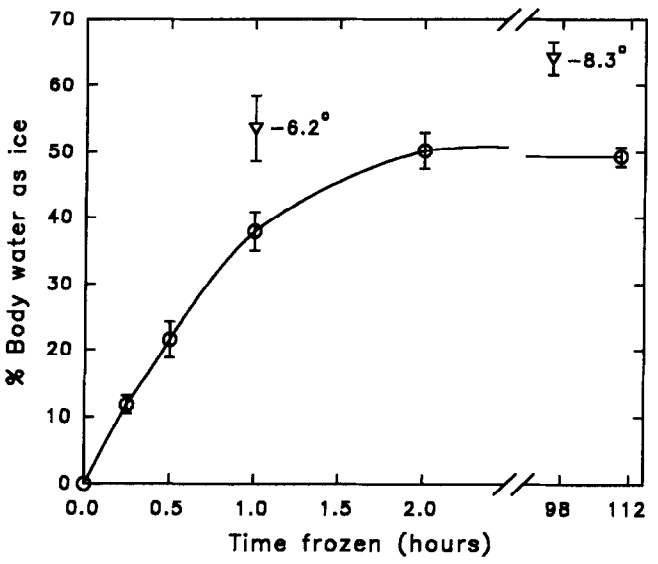


FIGURE 1. Ice formation of *O. eremicola* larvae held at $-2.5$°C (○) or other temperatures (▽). Time is measured from the appearance of the freezing exotherm. The percentage of body water as ice is shown as mean ± SEM for $n = 4$–6 at each point. Survival after thawing was 100% at all times.

TABLE 1. Changes in glycogen phosphorylase activity in tissues of *O. eremicola* larvae after freezing at $-8.3$°C for 96 h

| Tissue | Activity | Control | Frozen |
|---|---|---|---|
| Fat body | Total | $0.11 \pm 0.01$ | $0.74 \pm 0.10$* |
| | %*a* | <2% | $20.3 \pm 2.2$% |
| Body wall | Total | $0.81 \pm 0.05$ | $0.49 \pm 0.01$* |
| | %*a* | $23.5 \pm 0.7$% | $20.7 \pm 1.6$% |

Values are U/g fresh weight, mean ± SEM for $n = 4$.
*Significantly different from the corresponding control (5°C larvae) value by the Student's *t*-test at $P < 0.01$; †$P < 0.05$ from control.

The water content of the larvae was found to vary considerably in the two different larval stages. The later instar animals (3–5 cm in length) contained $67.4 \pm 3.5$% ($n = 4$) of their total body weight as water, whereas earlier instar larvae (1.5–2.5 cm) contained $87.6 \pm 0.2$% ($n = 4$). This, however, did not affect the freeze tolerance of animals: the two larval stages showed no difference in ice content, metabolite levels, osmolality or survival. Thus, all subsequent data presented represents pooled values for both larval stages.

As a result of freezing total ($a + b$) glycogen phosphorylase activity increased 7-fold in fat body during freezing, from 0.11 to 0.74 U/g fresh weight, with a concomitant increase in the active form ($a$) from <2% to >20% (Table 1). This change did not occur in the body wall, total activity decreasing from 0.81 to 0.49 U/g fresh weight, and the percentage of the active form remaining unchanged at about 20%. Both tissues showed a significant decrease in glycogen content during freezing (Table 2), suggesting that carbohydrate products may be produced during freezing.

However, several tests for the presence of low molecular weight cryoprotectants proved uniformly negative. The osmolality of haemolymph remained constant throughout acclimation and freezing at $451 \pm 22$, $454 \pm 34$, and $469 \pm 10$ mOsmol/l for 9, 0 and $-5$°C larvae respectively, indicating that there was no net accumulation of low molecular weight cryoprotectants as the result of cold or freezing exposure. In agreement, metabolite analyses failed to show any large increase in the concentration of typical colligative cryoprotectants

TABLE 2. Sugar, glycogen and polyol levels after freezing at $-8.3$°C for 96 h in body wall and fat body of *O. eremicola* larvae

| | Fat body | | Body wall | |
|---|---|---|---|---|
| Compound | Control | Frozen | Control | Frozen |
| Glucose | $0.12 \pm 0.07$ | $0.29 \pm 0.20$ | $0.09 \pm 0.04$ | $0.79 \pm 0.53$ |
| Glycogen | $237 \pm 1$ | $57 \pm 7$* | $89 \pm 4$ | $40 \pm 1$* |
| Fructose | <0.5 | <0.5 | <0.5 | <0.5 |
| Glycerol | $0.41 \pm 0.01$ | $0.44 \pm 0.29$ | $0.34 \pm 0.24$ | $0.64 \pm 0.05$ |

Values are μmol/g fresh weight, mean ± SEM for $n = 3$ or 4.
*Significantly different from the corresponding control (5°C larvae) value by the Student's *t*-test at $P < 0.01$.

TABLE 3. Sugar and polyol levels during acclimation and freezing in tissues of *O. eremicola* larvae

| | Fat body | | | Body wall | | |
|---|---|---|---|---|---|---|
| Compound | 9°C | 0°C | −5°C | 9°C | 0°C | −5°C |
| Glucose | 0.14 ± 0.02 | 0.20 ± 0.03 | 0.26 ± 0.05 | 0.12 ± 0.04 | 0.31 ± 0.09 | 0.16 ± 0.05 |
| Sorbitol | 1.45 ± 0.19 | 1.05 ± 0.18 | 0.78 ± 0.04 | 1.50 ± 0.19 | 1.07 ± 0.11 | 0.37 ± 0.20 |
| Fructose | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |
| Glycerol | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 | <0.5 |

Values are μmol/g fresh weight, mean ± SEM for $n = 3$ or 4.

and other molecules in the larvae. Glucose, glycerol, sorbitol, and fructose levels remained low and essentially unchanged during acclimation at 0°C and freezing at −5°C (Table 3). Similar results were obtained in larvae frozen at −8.3°C for 96 h (Table 2). Trehalose levels, measured by NMR, ranged from approx. 0.62 to 2.7 mM in haemolymph from control and frozen larvae, respectively, and 0.27–0.78 mM in control and frozen fat body. NMR also showed that trehalose was the major low molecular weight carbohydrate in these larvae.

Amino acid analysis by HPLC showed that total amino acid (minus proline) levels increased during freezing by 10–15 μmol/g fresh weight in the body wall of both early and late instar larvae (Table 4). In body wall the greatest accumulations occurred (in decreasing order) for glycine, alanine, glutamic acid, and valine. Although total amino acid levels remained constant in fat body (Table 4), alanine and glutamic acid concentrations increased by about 2-fold each, possibly reflecting the decrease in glutamine levels. Unlike the body wall, fat body valine levels decreased and those of glycine remained constant during freezing. Both tissues showed significant net decreases in glutamine (and/or histidine) levels of 4–7 μmol/g fresh weight. NMR analyses showed proline levels of 40.2 and 69.5 μmol/g fresh weight for haemolymph from control and frozen larvae respectively. Fat body proline levels ranged from 10.6 in control to 18.0 μmol/g fresh weight in frozen larvae. The total protein content of body wall and fat body remained essentially constant during acclimation and freezing (Table 5). Changes in total protein content do not then account for the observed amino acid changes.

Freezing for 96 h at −8.3°C failed to result in any changes in adenylate levels, arginine phosphate levels, or energy charge in any of the tissues tested (Table 6).

## DISCUSSION

*Osmoderma eremicola* is clearly a freeze tolerant species; the larva are capable of at least 4 days of continuous freezing at −8.3°C. However, the absence of any increase in osmolality during freezing episodes indicates that this species is able to tolerate freezing without the need to accumulate high concentrations of low molecular weight colligative cryoprotectants in haemolymph or tissues. The possibility that novel low molecular weight cryoprotectants (other than polyols or amino acids) might be present is eliminated by the haemolymph osmolality studies, for the osmolality of *O. eremicola* haemolymph was essentially equivalent to that reported for summer beetles or polyol-free beetles tolerant to freezing (around 500 mOsm) (Zachariassen, 1980). Polyol accumulating beetles, such as *Phyllodecta laticollis*, which accumulate glycerol, have winter osmolalities of about 2500 mOsm (van der Laak, 1982). The reported osmolality values also argue against the larvae having accumulated cryoprotectants prior to experimentation.

The absence of polyol accumulation in freeze-tolerant beetles is not novel, and appears to relate to the lower lethal temperature of these animals (van der Laak, 1982; Zachariassen *et al.*, 1979; Somme and Conradi-Larsen, 1979; Duman, 1979). Polyol-free freeze-tolerant beetles, such as *Eloedes blanchardi*, will survive freezing at temperatures down to −8°C (Zachariassen *et al.*, 1979). Increasing the osmolality with the accumulation of

TABLE 4. Changes in amino acid composition in tissues of *O. eremicola* larvae after a freezing episode at −8.3°C for 96 h as measured by HPLC

| | Early instar body wall | | Late instar body wall | | Late instar fat body | |
|---|---|---|---|---|---|---|
| Amino acid | Control | Frozen | Control | Frozen | Control | Frozen |
| Glycine | 19.3 ± 0.6 | 26.7 ± 0.3* | 11.1 ± 2.6 | 19.8 ± 0.2† | 9.48 ± 1.5 | 9.48 ± 0.1 |
| Alanine | 3.84 ± 0.14 | 8.04 ± 0.18* | 2.41 ± 0.31 | 8.21 ± 0.25 | 2.7 ± 0.3 | 6.2 ± 0.1* |
| Glutamic acid | 1.55 ± 0.05 | 3.88 ± 0.34† | 1.01 ± 0.11 | 2.42 ± 1.00 | 1.97 ± 0.13 | 2.74 ± 0.11 |
| Valine | 2.24 ± 0.01 | 3.02 ± 0.06* | 2.43 ± 0.13 | 4.03 ± 0.04* | 3.54 ± 0.26 | 2.15 ± 0.10 |
| Gln(+His) | 24.2 ± 0.8 | 17.6 ± 0.4* | 9.36 ± 0.7 | 5.62 ± 0.85† | 10.7 ± 1.5 | 3.19 ± 0.01 |
| Total | 57.5 ± 0.2 | 67.8 ± 0.3† | 32.6 ± 4.1 | 47.1 ± 0.1† | 35.7 ± 4.2 | 31.1 ± 0.1 |

Values are μmol/g fresh weight mean ± SEM for $n = 2$.
*Significantly different from the corresponding control (5°C larvae) value by the Student's $t$-test at $P < 0.01$; †different at $P < 0.05$ from control.

TABLE 5. Protein levels in tissues of *O. eremicola* larvae following acclimation to different temperatures

| Tissue | Acclimation temperature | | |
|---|---|---|---|
| | 9°C | 0°C | −5°C |
| Fat body | 15.7 ± 1.1 (3) | 24.9 ± 5.2 (2) | 21.0 ± 7.5 (3) |
| Body wall | 99.9 ± 2.3 (3) | 81.5 ± 7.4 (3)* | 85.7 ± 3.9 (3) |

Animals at −5°C were frozen. Values are mg protein/g fresh weight and given as mean ± SEM with *n* in parentheses.
*Significantly different from the corresponding 9°C value by the Student's *t*-test at $P < 0.05$.

cryoprotectants, usually polyols, decreases the lower lethal temperature (van der Laak, 1982; Zachariassen, 1985). Even beetles which synthesize cryoprotectants in the winter may survive freezing to higher subzero temperatures in their absence. For example, summer *P. laticollis* are freeze tolerant, surviving to −9°C with no increase in osmolality (van der Laak, 1982).

Species of beetles overwintering in protected sites apparently show lower (or no) increases in haemolymph osmolality during the winter (Zachariassen, 1980). As such, *O. eremicola* larvae are not unusual. These same insects generally have fairly high lethal freezing temperatures, ranging to about −8 to −10°C. We could then predict that whereas *O. eremicola* larvae readily endured freezing at −8.3°C, their lower lethal temperature would be only slightly colder. Although we could predict that the natural habitat of these larvae, within the trunk of a decaying tree, offers some protection from the cold, little is known about the insulatory effects of the tree wood or of the microbial activity within the decaying matter. It is possible that even on the coldest day temperatures never dip below the −10°C range in the natural microenvironment of the species.

Maximal ice formation in *O. eremicola* larvae rose to approx. 65% of total body water under the conditions used (Fig. 1). This amount is commonly the limit for survival of freezing as at this level cell volume approaches the critical minimum due to dehydration (Storey, 1989). Cells of *O. eremicola* then face a stress equivalent to the maximum endured by other freeze tolerant animals, but are successful in surviving this stress without the help of high cryoprotectant concentrations. The presence of such a high ice content at relatively high subzero temperatures (65% at −8.3°C) further suggests that the lower lethal temperature of these larvae is not much lower. Unfortunately, the lower lethal temperature could not be determined due to a limited supply of larvae.

The natural habitat of these larvae is damp, and therefore there exists the possibility that ice nucleation could occur externally, with ice crystals propagating across the cuticle. However, the absence of any difference in the supercooling point of dry and wet larvae indicates that these animals initiate ice nucleation internally, possibly resulting from the activity of haemolymph ice nucleating agents. In those freeze-tolerant beetle species that have been investigated, haemolymph ice nucleating agents have been found (Zachariassen, 1980, 1985). *O. eremicola* larvae also contained large deposits of material within their gut, presumably the decomposing material from the three they inhabit and feed upon. This material was not evacuated by the larvae during cold exposure and freezing. It is possible that the site of nucleation could be within this material. We also cannot rule out the presence of ice nucleating bacteria within the gut.

The disappearance of glycogen and activation of glycogen phosphorylase, common events seen in polyol accumulating insects, apparently does not relate to cryoprotectant synthesis in *O. eremicola*. The activity of glycogen phosphorylase (*a*) increased significantly in fat body during freezing exposure, indicating a much greater potential for glycogen breakdown. This, combined with the drastic decrease in glycogen in fat body (from 237 to 57 μmol/g fresh weight) is typical behaviour in cryoprotectant synthesizing insects. Although total glycogen phosphorylase decreased during freezing in the body wall, the percentage of the active form remained high, and glycogen breakdown was also seen in this tissue. The absence of cryoprotectant accumulation suggests that glycogen depletion must play another role. Glycogen depletion may partially be accounted for in energy production by fermentative processes, for energy charge remained constant after 96 h at −8.3°C with no changes in adenylates or aginine phosphate. End-products may be produced which could account for some or all of the depleted glycogen. For example, proline (apparently increasing by 30 μmol/g fresh weight in haemolymph) and alanine (which increases by 4–6 μmol/g fresh weight) may be such end-products. However, the contribution of these end-products to glycogen depletion cannot be determined without knowing the absolute changes of the levels of these metabolites in the whole animal.

TABLE 6. Adenylates in tissues of *O. eremicola* larvae

| Tissue | Arginine-P | ATP | ADP | AMP | Total adenylates | Energy charge |
|---|---|---|---|---|---|---|
| Fat body | 1.81 ± 0.22 | 0.65 ± 0.08 | 0.25 ± 0.05 | 0.080 ± 0.025 | 0.97 ± 0.09 | 0.79 ± 0.03 |
| Body wall | 3.49 ± 0.57 | 1.81 ± 0.14 | 0.39 ± 0.03 | 0.086 ± 0.022 | 2.29 ± 0.12 | 0.88 ± 0.02 |
| Gut | 3.17 ± 0.42 | 1.59 ± 0.06 | 0.28 ± 0.05 | 0.13 ± 0.05 | 2.01 ± 0.09 | 0.87 ± 0.02 |
| Head | 2.07 ± 0.35 | 2.11 ± 0.33 | 0.38 ± 0.02 | 0.073 ± 0.017 | 2.56 ± 0.34 | 0.89 ± 0.01 |

Values are μmol/g fresh weight, mean ± SEM for $n = 4$ (combining values for 2 control and 2 animals frozen for 96 h at −8.3°C)

The increase in levels of selected amino acids in tissues of *O. eremicola* during freezing was consistent in both larval stages, even though the total levels of the amino acids were different. The accumulation of free amino acids during cold acclimation has previously been shown in insects (Storey *et al.*, 1981; Zachariassen, 1985), and more specifically in beetles (Rains and Dimock, 1978; Ring and Tesar, 1980; van der Laak, 1982), and is believed to play a role in cold hardening. Interestingly, of the four amino acids which increase most during freezing, three are aliphatic (glycine, alanine, and valine). Proline is an effective membrane stabilizing cryoprotectant (Rudolph and Crowe, 1985), and high levels of this amino acid are common in many freeze tolerant insects (Storey and Storey, 1988). This amino acid may then play the same role in *O. eremicola* larvae.

In summary, *O. eremicola* larvae show freeze tolerance in the absence of low molecular weight cryoprotectant accumulation, allowing survival of low temperatures which they may face in their chosen hibernacula. Based on previously characterized beetles, the characteristics of *O. eremicola* larvae freeze tolerance suggest that these larvae will also have a relatively high lower lethal temperature (around $-10°C$).

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