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FREEZING SURVIVAL BY ISOLATED MALPIGHIAN TUBULES OF THE NEW ZEALAND ALPINE WETA *HEMIDEINA MAORI*

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Summary

The ability of isolated Malpighian tubules from a freeze-tolerant insect, the New Zealand alpine weta (*Hemideina maori*), to withstand freezing was assessed by measuring post-freeze membrane potentials and rates of fluid secretion. The hemolymph of cold-acclimated *Hemideina maori* was found to contain relatively high concentrations of the cryoprotectants trehalose (>300 mmol l^{-1}) and proline (41 mmol l^{-1}). Survival of isolated Malpighian tubules was correspondingly high when a high concentration of trehalose was present in the bathing saline. Tubules allowed to recover for 20 min from a 1 h freeze to –5 °C in saline containing 400 mmol l^{-1} trehalose had a basolateral membrane potential of –53 mV compared with a potential of –63 mV in tubules not exposed to a freeze/thaw cycle. Fluid secretion in tubules that had experienced a freeze/thaw cycle in saline containing 400 mmol l^{-1} trehalose was 9.9±2.6 nl h^{-1} compared with 18.7±5.0 nl h^{-1} (means ± S.E.M., *N*=18) in tubules that had not been frozen. Tubules frozen in saline containing a lower concentration of trehalose (200 mmol l^{-1}) or in glucose (400 mmol l^{-1}) showed a similar ability to survive freezing to –5 °C. In contrast, freezing for 1 h at –5 °C in saline containing 400 mmol l^{-1} sucrose produced a 57 % decrease in membrane potential and an 88 % decrease in secretion rate. Tubules held in saline lacking high concentrations of sugars showed no survival after freezing to –5 °C for 1 h. When frozen to –15 °C, tubules appeared to survive best in saline with the highest trehalose concentration (400 mmol l^{-1}). Freezing damage was not simply the result of exposure to cold, since tubules chilled (unfrozen) to –5 °C for 1 h were not compromised even when the bathing saline lacked a high sugar concentration. Exposure of tubules to a combination of low temperature and high osmolality mimicked damage caused by actual freezing: the membrane potential showed a 60 % recovery when the test was performed in saline containing trehalose, but showed no recovery in saline containing sucrose.

Key words: *Hemideina maori*, alpine weta, freezing tolerance, Malpighian tubules, trehalose.

Introduction

Water is a crucial element in all physiological processes, and yet a large number of ectothermic animals share the challenge of surviving environments where temperatures regularly drop below the freezing point of water. While most ectotherms under these conditions avoid freezing by supercooling their body fluids, a surprising number of organisms respond to this challenge with the remarkable ability to survive repetitive freeze/thaw cycles. Insects are, in fact, the group that show the most widespread adaptations for surviving freezing; a wide variety of insects are now known to be freeze-tolerant and spend a portion of their life-cycle in a frozen state (Zachariassen, 1985; Storey and Storey, 1988; Leather *et al.* 1993). Studies have correlated the ability of insects to survive freezing with the presence of cryoprotective agents (Zachariassen, 1985; Storey and Storey, 1988), and it is evident that the use of particular cryoprotectants such as glycerol, sorbitol and trehalose is highly conserved among insects. These compounds are also widely recognised as providing protection during freezing (and supercooling) in other animals (Ashwood-Smith, 1987).

Despite the widespread occurrence of freeze tolerance in insects, only a few studies (Bennett and Lee, 1997) have addressed the gap between the ecological and biochemical observations by studying freeze tolerance at the organ or cellular levels. Cellular studies in freeze tolerance have instead concentrated on vertebrate, and especially mammalian, cell systems because of the obvious biomedical implications (Mazur, 1984). While considerable progress has been made in correlating cellular events with freezing survival, the interactive nature of the multitude of stresses occurring during freezing has resulted in difficulty in assessing their relative importance. From the standpoint of physiological ecology, it is notable that these studies represent tissues which do not, under natural conditions, normally encounter and survive freezing conditions (aside from

the comprehensive work on the freeze-tolerant wood frog *Rana sylvatica*; e.g. Storey *et al*. 1992; Storey and Mommsen, 1994).

The New Zealand alpine weta (*Hemideina maori*) presents an ideal model for connecting the ecological context of a freeze-tolerant organism to the events occurring at the cell and organ levels during freezing. Wetas are a group of large insects (Family Stenopelmatidae) found throughout the Southern Hemisphere and include some of the largest and heaviest insects known. New Zealand has a particularly diverse fauna of wetas, which appear to occupy many of the ecological niches usually filled by rodents. *Hemideina maori*, a nocturnal species which is found only in the South Island above 1000 m, is the only species known to tolerate freezing. In addition, *H. maori* has the advantage of possessing many large Malpighian tubules. Previous studies have established Malpighian tubules as an ideal model for studies on the function of a transporting epithelium (Maddrell and Overton, 1990). Given the recognition that freezing stresses in the cellular suspensions often used in freeze-tolerance studies may not represent freezing stresses that become operative in a cellular aggregate (Pegg, 1987; Bischof *et al.* 1990; Storey *et al.* 1992) and, in particular, in a monolayer epithelium (Zachariassen, 1985), we have used the Malpighian tubules of *H. maori* as an appropriate model for observing the cellular response to a freeze/thaw cycle in the context of a simple organ.

Materials and methods

Animal collection and maintenance

We collected specimens of the New Zealand alpine weta *Hemideina maori* Hutton from the summits of the Rock and Pillar Range in Central Otago in June and September 1996. *H. maori* is a nocturnal insect and is easily located beneath slabs of schist rock above an elevation of 1200 m. Permission to collect this protected insect was given by the Department of Conservation, provided that the number of animals collected was kept to a minimum. We maintained animals in a chamber on a diet of native plants, carrots and apples. The chamber had a 12 h:12 h light:dark cycle and a temperature of between 15 °C (in the dark) and 20 °C (in the light). Wetas survived at least 6 months under these conditions.

Dissection was performed on narcotised (with carbon dioxide) animals under cold insect Ringer's saline. We first removed the dorsal portion of the abdominal cuticle and then carefully manipulated the Malpighian tubules so that their point of connection to the gut could be easily seen. Malpighian tubules were cut at this point of connection, transferred to a Petri dish containing the appropriate cold saline, and stored at 4 °C until use. Tubules were allowed to equilibrate at 4 °C for several hours before experiments were performed. Long-term survival of frozen or control tubules was investigated over a 24 h period; all measurements were therefore completed within 1 day of dissection.

Solutions

All experiments used a standard insect Ringer's saline which contained (in mmol l^{-1}): Na^+, 140; K^+, 10; Ca^{2+}, 2; Mg^{2+}, 1; Cl^-, 137; HCO_3^-, 18; $H_2PO_4^-$, 1; and glucose, 30. This saline approximates the composition of hemolymph from *Hemideina* (Leader and Bedford, 1978). The osmolality of this insect saline was approximately 300 mosmol kg^{-1}, and pH was adjusted to 7.4 using 0.1 mol l^{-1} NaOH or HCl. Salines of higher osmolality were made by adding sucrose, glucose or trehalose to this basic saline solution, bringing the final osmolality to either 500 or 700 mosmol kg^{-1}. Saline for the study of hyperosmotic effects was created in an identical manner except that the concentration of all constituents was increased proportionally so that the final osmolality was 2.69 osmol kg^{-1}.

Hemolymph measurement techniques

We collected hemolymph either from a cut in the membrane between the dorsal abdomen and the thorax, or from a cut leg or antennae. Hemolymph was centrifuged at 16 000 ***g*** for 3 min to remove cellular debris and subsequently stored at −15 °C until analysis was performed. We used a vapor pressure osmometer (Wescor 5100C) for osmolality measurements. For measurements of organic solutes, we prepared hemolymph using a perchloric acid extraction. We added 500 µl of 7 % perchloric acid to each hemolymph sample (of 50–100 µl), vortexed it, and then centrifuged it at 18 000 ***g*** for 10 min. We added 160.5 µl of 2 mol l^{-1} K_2CO_3 to neutralise the supernatant, left the sample overnight in the refrigerator, and then centrifuged the sample again at 18 000 ***g*** for 10 min. One half of the resulting sample was used for amino acid analysis, and the other half was used for the analysis of carbohydrates.

For amino acid analysis, we added 500 µl of 0.1 % trifluoroacetic acid (TFA) in water/methanol (70/30) to half of the above preparation. This sample was passed through a SepPak cartridge (Waters, Milford, MA) activated with methanol and TFA mixtures. Finally, the sample was passed through a 0.22 µm filter (Millipore). A phenylthiocarbamyl derivative of the sample was subsequently formed and separated by reverse-phase high-performance liquid chromatography (HPLC) (Waters Instruments; Hubbard, 1995). The sample was injected into the machine using sodium acetate/acetonitrile buffer at a flow rate of 0.18 ml min^{-1} and a temperature of 46 °C. Separation of the amino acids was complete in 30 min; amino acids were detected at 254 nm. Data acquisition and analysis was by a Macintosh computer and MacLab system (Analog Digital Instruments).

For carbohydrate analysis using either HPLC or nuclear magnetic resonance (NMR), we passed the other half of the perchloric-acid-extracted sample through a SepPak cartridge activated with methanol and water mixtures. Samples (1 ml) were washed through a SepPak C18 (Waters) cartridge with purified water, and then through a 0.22 µm filter (Millipore). We injected 20 µl of this sample into the HPLC column, and separated the sugars on a Sugarpak 1 column (Waters) isocratically perfused with 0.1 mol l^{-1} calcium EDTA made up with purified water. The flow rate was 0.5 ml min^{-1} and the column temperature was 75 °C. Peaks were detected by refractive index spectrophotometry (Jasco 830-RI). Standards

of trehalose and glucose (0.5 mmol l^{-1}) were run, and sample peak areas were compared with standards within a similar concentration range using a Hewlett Packard 3395 integrator.

^{1}H-NMR spectra were collected using a Varian VXRS-300 NMR spectrometer fitted with a 'Nalorac' ZSPEC 5 mm i.d. 300-5V indirect detection probe at 25 °C. Water signal suppression was achieved using a presaturation pulse sequence with an 80 % duty cycle [delay (d1)=0 s, acquisition time (at) 1.333 s, pulse width (pw)=5.6 µs, decoupler power 20 dB, number of transients 128]. To the samples was added a known quantity of sodium 3-(trimethylsilyl)propionate-2,2,3,3-d4 (TSP) as a reference (0.0 p.p.m.). Chemical shifts arising from trehalose and proline were identified by reference to published spectra (Pouchert and Behnke, 1992).

Freezing protocol

The freezing of tubules for physiological measurements of survival (fluid secretion rates and membrane potentials) was performed by controlling the position of samples over a reservoir of liquid nitrogen. At the start of an experiment, isolated tubules (stored at 4 °C) were transferred to 0.5 ml of the appropriate saline in a 3×3 well section of a 24-well culture dish. The saline volume of 0.5 ml was chosen as being sufficient to fill the bottom of the well and yet small enough to minimise vertical temperature gradients. All experiments were performed in the wells at the edge of the dish, so that conditions were nearly identical for each set of tubules in a particular trial. A thermocouple was placed into one well to monitor the temperature during the experiment. The dish was then positioned over a reservoir of liquid nitrogen and lowered at a rate that cooled the sample at approximately 1 °C min^{-1}. Freezing is a stochastic process influenced by a combination of factors; in the conditions used for these experiments, the saline undercooled to approximately –4 °C before freezing was initiated. The exposure period was timed from when the target temperature was reached. Once a position was set, the temperature were monitored over the entire test period, and short-term variations in temperature were less than 1 °C of the target temperature. For those tubules frozen for 18 h, the frozen samples were moved to a water bath at –5 °C for the duration of the experiment. Thawing was performed by simply allowing samples to warm at room temperature (20 °C) (approximately 20 min). All tubules were allowed to equilibrate at 20 °C for 20 min before measurements of secretion rates or membrane potentials were made. Measurements of physiological parameters in tubules that had been frozen were compared with those for control tubules, i.e. tubules which had not experienced a freeze/thaw cycle, but instead had been kept at 4 °C for an equivalent period.

Ultrastructural observations

The freezing of tubules for ultrastructural observations using light microscopy was performed on an inverted microscope (Nikon Diaphot-TMD) equipped with a long working distance condenser and a Leitz 25× objective (nominal aperture 0.75). Differential interference contrast (DIC) images were taken with a Cohu television camera equipped with an image intensifier and summed into an image board (Matrox). Tubules were held on a stage cooled by a Peltier unit. Circulation of water through the stage provided a heat sink for the Peltier unit. A Petri dish with a center hole fitted into the cooling stage, and a coverslip was sealed over the center hole using Vaseline. Tubules were secured by gently pressing the ends into small drops of Vaseline on the coverslip.

For electron microscopy, we fixed tissues with 2 % glutaraldehyde in solution containing 0.2 mol l^{-1} NaH_2PO_4 and 0.4 mol l^{-1} trehalose. The tissue was postfixed in 1 % OsO_4, rinsed in buffer solution, and then dehydrated in a series of ethanol and propylene oxide washes. After embedding in Spurr's resin, ultrathin sections were cut using an ultramicrotome (Leica/Reichert Ultracut E) and stained with uranyl acetate and lead citrate. The stained sections were viewed using an Akashi EM-002A transmission electron microscope.

Measurement of secretion rates

As a measure of the viability of organ function, we measured fluid secretion by Malpighian tubules using the standard oil drop technique (Maddrell and Overton, 1990). A single, isolated tubule was transferred to a 50 µl drop of Ringer's saline that was covered in paraffin oil. The bottom of the experimental dish was covered with a layer of cured Sylgaard resin (Dow Corning), allowing us to pull the open end of the tubule out of the drop with a fine pin, and then secure the pin into underlying Sylgaard. The closed end of the tubule (normally approximately 5 mm in length) was therefore in the bathing drop, and a droplet formed around the pin as secreted fluid exited the cut end of the tubule. At the start of the experiment, a microcapillary tube (2 µl) was used to remove any adhering fluid from the cut end of the tubule. Phenol Red was added to all bathing drops (to 0.05 % w/v) to aid in identification of damaged tubules, as well as to aid in visualisation of the secreted droplet. Since fluid secretion by unstimulated tubules is relatively slow (Neufeld and Leader, 1997), we included 0.1 mmol l^{-1} dibutyryl cyclic AMP in the bathing drop of all experiments to boost the rate of secretion. The volume of secreted fluid was calculated by collecting the droplet in a 0.5 µl microcapillary tube, and then measuring the length of the droplet within the microcapillary tube.

Membrane potential

As a second estimate of cell viability, we measured the potential difference across the basolateral membrane of Malpighian tubules. Microelectrodes were pulled to a resistance of approximately 25 MΩ from borosilicate glass tubing (Clark Electromedical Instruments) and backfilled with saturated KCl. To impale cells, we advanced the electrode with micromanipulators into a cell of the tubule wall. As the electrode advanced, the cell surface visibly deformed and then rebounded to its original tension. If the electrode potential abruptly changed to a new, stable value at this point, then the impalement was judged successful.

Statistics

Because of the non-normal distribution of some of the data, all pairwise statistical comparisons were made using the nonparametric Mann–Whitney *U*-test (Sokal and Rohlf, 1981). Comparisons were made between tubules that had experienced a freeze/thaw or chill cycle and tubules in identical saline that had been kept at 4 °C for the experimental period. All data are presented as means ± 1 S.E.M.

Results

Hemolymph composition

The hemolymph of *H. maori* collected in June, when ambient temperatures had reached a winter minimum and there was a substantial amount of snow cover at the collection site, had a significantly higher osmolality (760±57 mosmol kg^{-1}, *N*=5) than that of *H. maori* collected in September (441±17 mosmol kg^{-1}, *N*=8), when ambient temperatures had risen considerably and there was very little snow remaining. These results are consistent with a previous study showing a higher hemolymph osmolality in cold- *versus* warm-acclimated *H. maori* (Ramlov *et al.* 1992). We used NMR and HPLC to show that this difference in osmolality was mainly due to a large increase in the trehalose concentration in cold-acclimated *H. maori*. Two pooled hemolymph samples were taken from freshly collected cold-acclimated wetas (*N*=5). NMR indicated large peaks associated with trehalose and proline. The trehalose concentrations in the two pooled samples were calculated at 316 and 407 mmol l^{-1}. Hemolymph samples taken from two cold-acclimated animals were analysed with HPLC and found to have trehalose concentrations of 361 and 422 mmol l^{-1}, in the same range as the NMR measurements. In addition, amino acid analysis of cold-acclimated animals (Table 1) verified the presence of a high concentration of proline, as was observed using NMR. Warm-acclimated *H. maori* had a much lower concentration of trehalose (42.0±5.9 mmol l^{-1}; *N*=7, measured with HPLC) and proline (Table 1).

Table 1. *Concentrations of free amino acids in the hemolymph of cold- and warm-acclimated* Hemideina maori

	Cold-acclimated (mmol l^{-1})	Warm-acclimated (mmol l^{-1})
Glutamine	0.7, 0.7	0.1±0.1
Serine	3.2, 1.9	1.4±0.4
Glycine	2.5, 4.3	3.2±0.8
Histidine	5.0, 4.6	0.6±0.2
Arginine	4.0, 4.6	0.2±0.1
Threonine	0.4, 3.8	0.3±0.7
Alanine	0.5, 2.7	2.0±0.7
Proline	28.2, 52.9	6.9±2.0
Tyrosine	1.6, 0.9	0.3±0.2
Valine	1.5, 4.4	1.6±0.4
Methionine	1.1, 0.8	0.1±0.1
Isoleucine	1.3, 2.8	0.5±0.2
Leucine	0.6, 3.3	0.3±0.1
Phenylalanine	2.7, 2.7	0.2±0.1
Lysine	0.8, 1.8	0.3±0.1

Values for warm-acclimated animals are means ± S.E.M., *N*=7 individuals.

Values for cold-acclimated animals are for two individuals.

Tubules frozen at –5 °C for 1 h

As a relatively 'mild' freezing condition, we first tested the ability of *H. maori* tubules to survive freezing to –5 °C for 1 h. As judged by secretion rates and membrane potentials, tubules were obviously able to survive freezing, but survival was dependent on the specific cryoprotectant present in the saline (Figs 1, 2). Tubule survival was high in the saline that most closely approximated the hemolymph constituents, i.e. in the presence of a high concentration of trehalose. At the highest (400 mmol l^{-1}) trehalose concentration, there was little difference in either the secretion rate (Fig. 1) or membrane

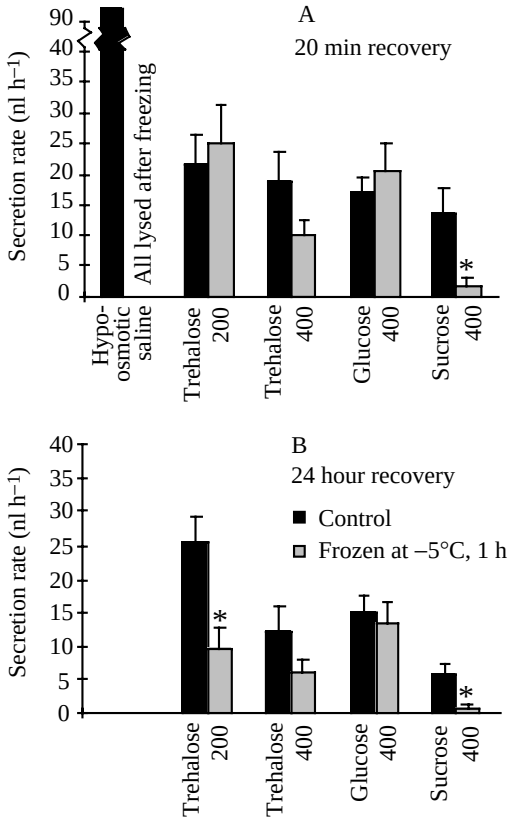

Fig. 1. Rate of fluid secretion by isolated Malpighian tubules either exposed to 1 h of freezing at –5 °C and then thawed ('frozen') or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline either lacking high sugar concentrations ('hypo-osmotic') or containing high concentrations (given in mmol l^{-1}) of trehalose, glucose or sucrose. Measurements of secretion rates were started following a 20 min recovery at room temperature (A) or following a 24 h recovery at 4 °C (B). Values are means + S.E.M. (*N*=15–30). *Secretion rate of frozen tubules significantly different from that of control tubules ($P<0.05$).

potential (Fig. 2) between control and frozen/thawed tubules, following either a 20 min or a 24 h recovery period. Light micrographs indicated that cell boundaries and ultrastructure were similar in control and frozen/thawed tubules (Fig. 3A). Similar observations were noted when cells were examined with electron microscopy: there was no obvious damage to such subcellular structures as apical microvilli, basolateral invaginations, mitochondria, intercellular junctions or nuclei (Fig. 4). At a lower trehalose concentration (200 mmol l^{-1}), recovery was generally good, although membrane potential was significantly reduced after 20 min of recovery from freezing (as it was in the presence of 400 mmol l^{-1} trehalose), and secretion rate was significantly depressed after a 24 h recovery from the freezing. Survival was also high in saline containing glucose (400 mmol l^{-1}), where membrane potential and secretion rates from frozen/thawed tubules were not significantly different from those for control tubules.

Tubules did not survive well when frozen in saline containing 400 mmol l^{-1} sucrose. The mean secretion rate was almost zero on thawing (Fig. 1), and the membrane potential was significantly reduced, although membrane potential in control tubules was also reduced after 24 h (Fig. 2). Consistent with this suggestion of cellular damage, there were large areas of the tubules held in sucrose saline in which the apical boundaries and other cellular structures were no longer visible (Fig. 3B). Tubules held in hypo-osmotic saline, in which the only sugar present was 30 mmol l^{-1} glucose, showed no tolerance of freezing (Figs 1A, 2A). All tubules lysed after thawing, characterised by an obvious, rapid bursting of cells and a flowing of cellular contents out the cut end of the tubule.

Tubules frozen at –5 °C for 18 h

Since *in vivo* freezing of tubules in *H. maori* under natural conditions would generally last for periods longer than 1 h, we tested the ability of isolated tubules to survive overnight (18 h) freezing at –5 °C. Tubules in saline containing either trehalose or glucose showed high rates of survival under these conditions: both membrane potential and secretion rates (Fig. 5) recovered fully in tubules thawed after 18 h of freezing to –5 °C. There was an unexplained low membrane potential in control tubules held in saline containing 400 mmol l^{-1} glucose.

Tubules frozen at –15 °C for 1 h

Temperatures in the natural habitat of *H. maori* (the exposed summits of central mountain ranges of southern New Zealand) extend to minimum temperatures that are below –5 °C (Bliss and Mark, 1974). As a minimum temperature encountered by wetas, we tested the ability of tubules to survive freezing at –15 °C for 1 h. Although survival was clearly lower than at –5 °C, it was notable that tubules retained some function even after freezing to –15 °C (Fig. 6). After 24 h of recovery from freezing in saline containing 400 mmol l^{-1} trehalose, membrane potential had recovered to 37 % of the control value and there was still significant fluid secretion, albeit at a much reduced rate compared with the control. Survival in lower

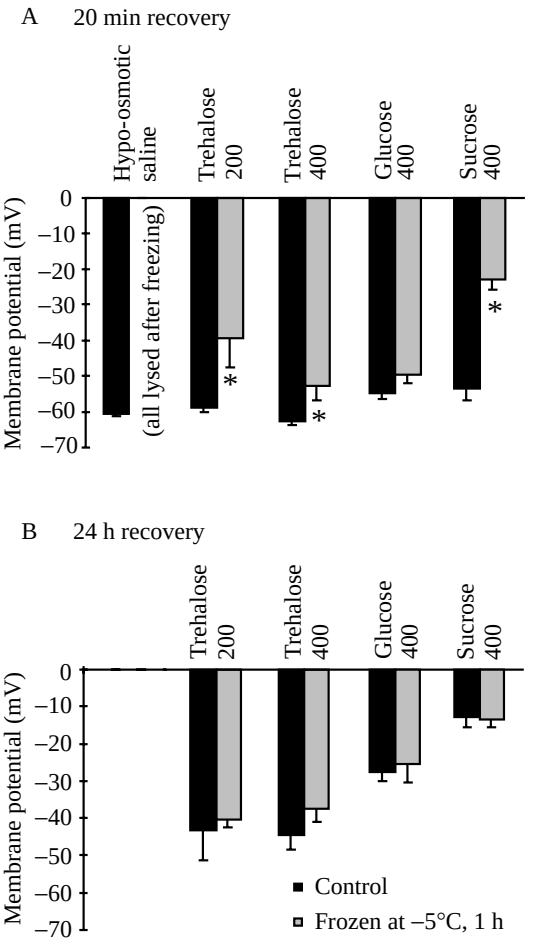

Fig. 2. Basolateral membrane potential in tubule cells either exposed to 1 h of freezing at –5 °C and then thawed ('frozen') or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline either lacking high sugar concentrations ('hypo-osmotic') or containing high concentrations (given in mmol l^{-1}) of trehalose, glucose or sucrose. Measurements of membrane potentials were taken following a 20 min recovery at room temperature (A) or following a 24 h recovery at 4 °C (B). Values are means + S.E.M. (N=7–18). *Membrane potential of frozen tubules significantly different from that of control tubules (P<0.05).

(200 mmol l^{-1}) trehalose concentrations, or with glucose as the cryoprotectant, appeared to be reduced (Fig. 6). Clearly, survival of tubules at temperatures representative of extreme winter conditions was highest in the presence of high concentrations of trehalose, which corresponded to the increase in trehalose hemolymph concentration that occurs naturally in *H. maori* during cold acclimation (see above).

Tubules chilled to –5 °C in iso-osmotic or hypo-osmotic saline for 1 h

As a test of whether temperature alone was the causative factor in the tubule damage associated with freezing, we tested the survival of tubules that had been chilled to –5 °C for 1 h. Tubules held in saline in Eppendorf tubes did not freeze when held in a water bath at –5 °C; ice nucleation was evidently less

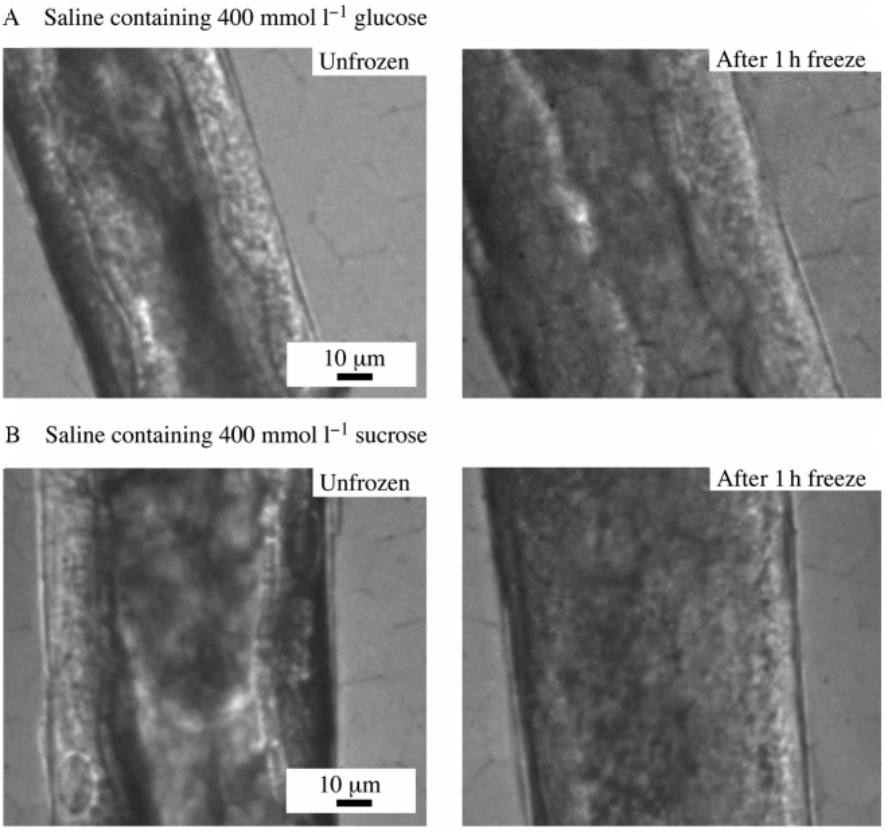

Fig. 3. Differential interference contrast micrographs of tubules before and after a freeze/thaw cycle for tubules held in saline containing 400 mmol l^{-1} trehalose and in saline containing 400 mmol l^{-1} sucrose.

likely under these conditions. In the presence of 400 mmol l^{-1} trehalose, chilling caused a significant reduction in the membrane potential, but no change in the secretion rate (Fig. 7). There was no significant difference in either membrane potential or secretion rate (Fig. 7) in tubules held in either 400 mmol l^{-1} sucrose or hypo-osmotic saline, both salines in which tubules suffer extensive damage when frozen. The temperature decrease alone, therefore, does not appear to be the causative agent in the cell damage associated with freezing.

Tubules chilled to –5 °C in hyperosmotic saline for 1 h

We tested the ability of tubules to survive chilling to –5 °C, while exposing them to an osmolality near to that encountered during freezing to –5 °C (2.69 osmol kg^{-1}, with the concentrations of all saline constituents increased proportionately). As with chilling in iso-osmotic solution, there was significant survival of tubules chilled in hyperosmotic saline containing trehalose (Fig. 8). However, while tubules survived chilling in iso-osmotic saline containing sucrose (Fig. 7), chilling in hyperosmotic saline containing sucrose caused lysis of all tubules (Fig. 8). The pattern of tubule survival in hyperosmotic, chilled conditions therefore mimics the pattern of tubule survival in frozen conditions.

Discussion

While freeze tolerance is a strategy adopted by many other insects in a freezing environment (Zachariassen, 1985; Storey and Storey, 1988), freeze tolerance in *H. maori* is unique in several respects. (1) As it is the largest insect known to survive freezing (adults length reaches 55 mm), temperature scaling could give rise to higher internal temperature gradients than in other freeze-tolerant insects (Ramlov *et al.* 1992). (2) Freeze tolerance was thought to be a characteristic of more advanced insects (Block, 1991), yet the orthopterans are considered to be relatively primitive (MacKerras, 1970). (3) Observations on the metabolic consequences of freeze avoidance *versus* freeze tolerance led Churchill and Storey (1989) to suggest that a strategy of freeze avoidance is the more advantageous in environments with large and rapid fluctuations in temperature. *H. maori* inhabits such an environment (temperatures on the mountain summits of the central south island of New Zealand are extremely variable and can fall below freezing at any time of the year; Bliss and Mark, 1974), and yet this insect follows a strategy of freeze-tolerance.

We found that this ability to survive freezing extends to individual Malpighian tubules that have been removed from the animal, provided that high concentrations of either trehalose or glucose were present in the saline. Malpighian tubules recovered despite the fact that susceptibility to freezing is usually much greater in cell systems integrated into an organ than in suspensions of single cells (Pegg, 1987; Bischof *et al.* 1990; Storey *et al.* 1992). This susceptibility ought to be particularly acute in a monolayer epithelium such as the Malpighian tubule, where limited cell damage can easily have an effect on the organ as a whole (Zachariassen, 1985).

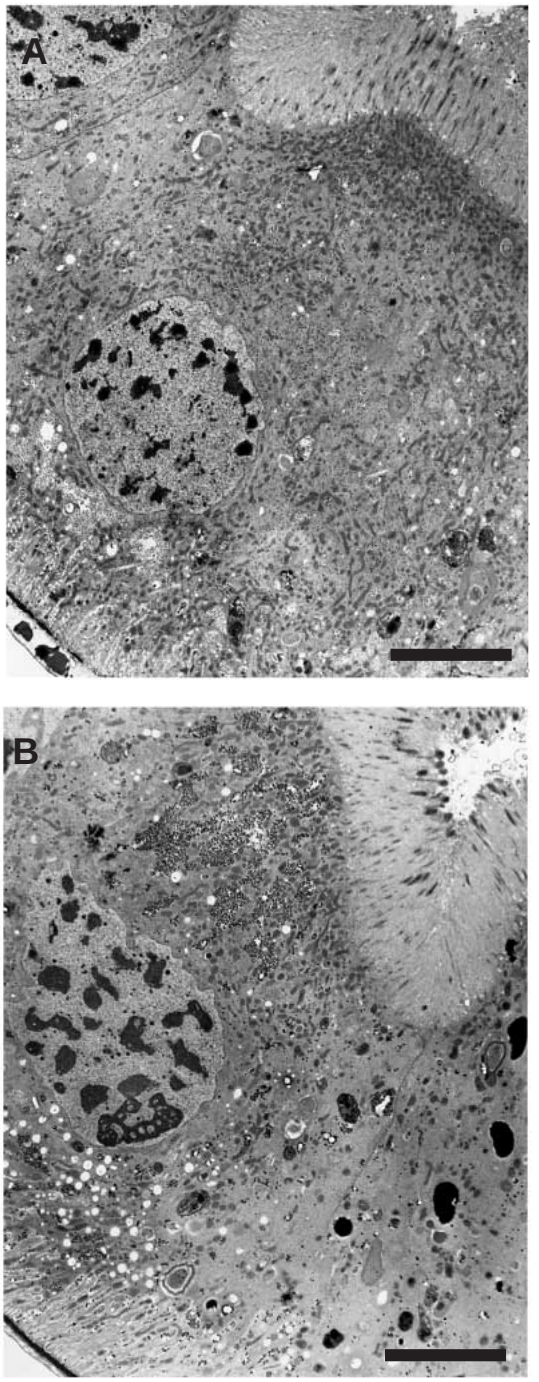

Fig. 4. Electron micrographs of tubules held in saline containing 400 mmol l^{-1} trehalose before (A) and after (B) a freeze/thaw event. Scale bar, 5 μm.

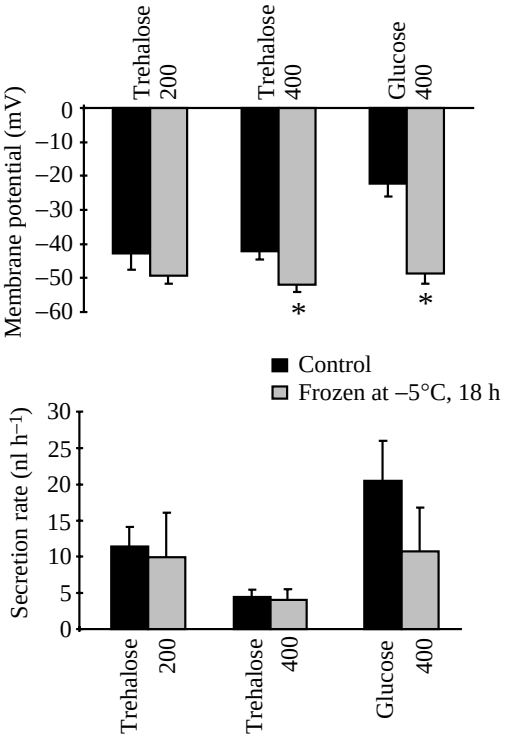

Fig. 5. Secretion rate and basolateral membrane potential in tubule cells either exposed to 18 h of freezing at −5 °C and then thawed ('frozen') or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline containing high concentrations (given in mmol l^{-1}) of trehalose or glucose. Measurements were taken following a 20 min recovery at room temperature. Values are means + S.E.M. (N=7–8 for secretion rates, N=14–20 for membrane potentials). *Value for frozen tubules significantly different from that for control tubules (P<0.05).

However, both the basolateral membrane potential and fluid secretion were re-established after freezing to −5 °C for up to 18 h (Fig. 5). This was confirmed by ultrastructural observations in which no apparent cellular damage was visible (Figs 3, 4).

Of the conditions tested, we found that higher concentrations of glucose and trehalose provided better protection against freezing for Malpighian tubules, a result which correlates well with the seasonal presence of trehalose in the hemolymph of *H. maori*. Hemolymph trehalose levels reached 300–400 mmol l^{-1} in the winter, when freezing temperatures occur daily (Bliss and Mark, 1974). Survival in isolated tubules tested at −15 °C was obviously reduced; however, there was significant recovery of function that was greater with 400 mmol l^{-1} than with 200 mmol l^{-1} trehalose in the bathing medium. At temperatures representative of the minimum encountered in the summer (aproximately −5 °C), 200 mmol l^{-1} trehalose provided sufficient protection for tubules to regain function. This correlates with a summer drop in the hemolymph trehalose concentration to 42 mmol l^{-1}. Thus, the characteristics of *in vitro* tubule survival correlated well with the seasonal biochemical composition of the hemolymph and the climatic conditions encountered by the animal.

Trehalose serves as a metabolic intermediate in insects (Friedman, 1985) and is a common constituent of insect hemolymph (Mullins, 1985), although it is generally at much lower concentrations than that found in cold-acclimated *H. maori*. Higher trehalose concentrations are associated with the high energetic demands of flying insects in particular (Bedford, 1977; Friedman, 1985); however, our demonstration of

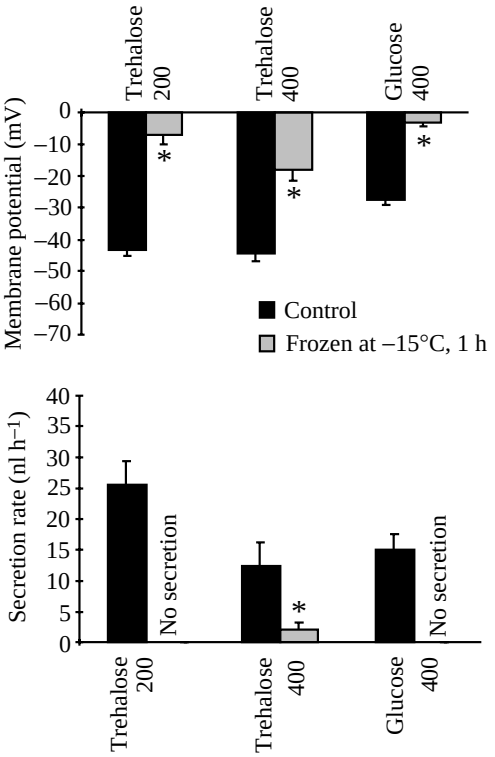

Fig. 6. Secretion rate and basolateral membrane potential in tubule cells either exposed to 1 h of freezing at −15 °C and then thawed ('frozen') or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline containing high concentrations (given in mmol l^{-1}) of trehalose or glucose. Measurements were made following a 24 h recovery at 4 °C. Values are means + S.E.M. (N=12 for secretion rates, N=20 for membrane potentials). *Value for frozen tubules significantly different from that for control tubules (P<0.05).

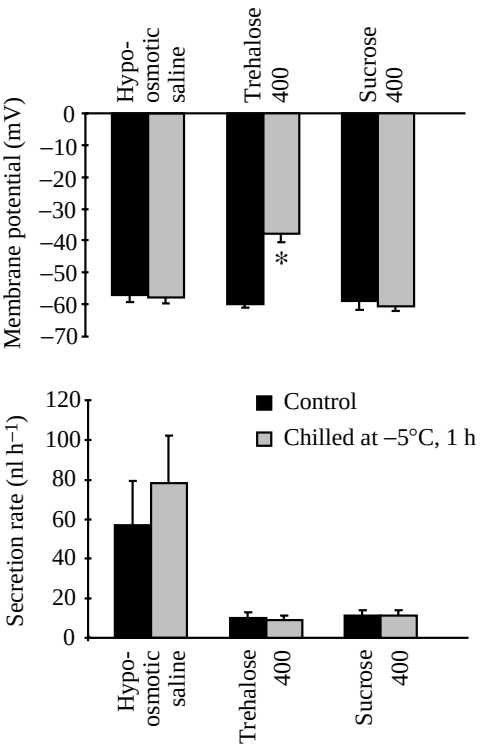

Fig. 7. Secretion rate and basolateral membrane potential in tubule cells either chilled at −5 °C ('unfrozen') for 1 h or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline either lacking high sugar concentrations ('hypo-osmotic') or containing high concentrations (400 mmol l^{-1}) of trehalose or sucrose. Measurements were taken following a 20 min recovery at room temperature. Values are means + S.E.M. (N=7–12 for secretion rates, N=14–20 for membrane potentials). *Value for frozen tubules significantly different from that for control tubules (P<0.05).

extremely high concentrations in cold-acclimated *H. maori* (which is flightless) is almost certainly reflective of a cryoprotective rather than a metabolic role. The seasonal dependence of trehalose concentrations in *H. maori* hemolymph is similar to that reported in a number of other insects where trehalose acts as a cryoprotectant (e.g. Rains and Dimock, 1978; Storey *et al.* 1981; Baust and Lee, 1982). At the molecular level, trehalose has been shown to stabilise both membranes (Bakaltchava *et al.* 1994) and proteins (Carpenter *et al.* 1986) during a freeze/thaw cycle. The presence of trehalose in *H. maori* hemolymph and its protective effects on Malpighian tubules therefore concur with many studies demonstrating both its role in cold-hardening of insects and its molecular-level protection against freezing.

The concentration of proline in *H. maori* hemolymph also increases during the winter, a result consistent with seasonally dependent high concentrations of proline in other freeze-tolerant insects (e.g. Ring and Tesar, 1980; Storey *et al.* 1981). As with trehalose, proline also appears to stabilise membranes and proteins during a freeze/thaw cycle (Carpenter *et al.* 1986; Anchordoguy *et al.* 1988). Proline is also a metabolic intermediate (Friedman, 1985), however, and it is sometimes difficult to dissociate the role of a compound in metabolism from its role in cryoprotection (Pullins *et al.* 1991).

Glucose was nearly as effective as trehalose in providing protection of isolated tubules against freezing (Figs 1, 2), consistent with its ability to act as a cryoprotectant in other systems (e.g. Storey and Mommsen, 1994). Curiously, while sucrose also demonstrates many cryoprotective characteristics at the molecular (Carpenter *et al.* 1986; Bakaltchava *et al.* 1994) and cellular (Mazur and Miller, 1976) levels, we found it to be a poor cryoprotectant in the present study. The discrepancy between the cryoprotective effectiveness of the two disaccharides sucrose and trehalose in *H. maori* could lie in a differential permeability of the cells to these compounds. Cell permeability is generally important for cryoprotective effectiveness (Ashwood-Smith, 1987), much as the presence of a trehalose transporter is important for protection against desiccation in yeast (Eleutherio *et al.* 1993). Malpighian tubules of *H. maori* are more likely to have a trehalose transporter than a sucrose transporter, given the high concentration of trehalose in *H. maori* and the normally low concentration of sucrose in insects (Ring and Tesar, 1980). In one of only a few studies on the renal handling of organic

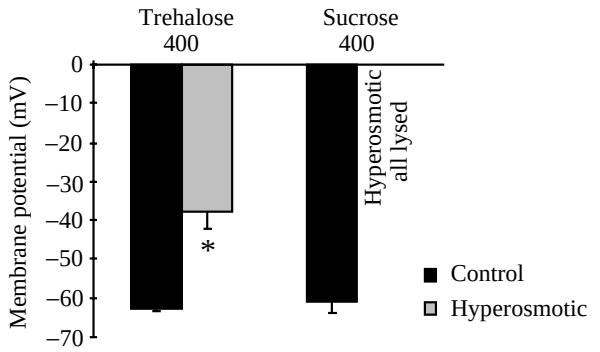

Fig. 8. Basolateral membrane potential in tubule cells either chilled at −5 °C ('unfrozen') for 1 h in hyperosmotic saline (2.69 osmol kg^{-1}, equivalent to the osmolality in the unfrozen fraction of saline frozen at −5 °C) or held at 4 °C for the duration of the experiment ('control'). Tubules were tested in saline either lacking high sugar concentrations ('hypo-osmotic') or containing high concentrations (400 mmol l^{-1}) of trehalose or sucrose. Measurements were taken following a 20 min recovery at room temperature. Values are means + S.E.M. (N=20). *Membrane potential for frozen tubules significantly different from that for control tubules (P<0.05).

compounds by insects, Knowles (1975) concluded that Malpighian tubules of the blowfly *Calliphora vomitoria* reabsorb a much larger quantity of trehalose than sucrose from the tubule fluid, consistent with the presence of a greater transport capacity for trehalose than for sucrose.

While the presence of cryoprotectants in supercooling insects may simply serve in a colligative fashion to lower the freezing point (Zachariassen, 1985; Storey and Storey, 1988), these compounds could also serve to protect both freeze-tolerant and freeze-intolerant insects against damage associated with the cold itself. Indeed, exposure to low temperature (in the absence of freezing) is often a sufficient physiological stress to cause cellular damage, a phenomenon known as cold-shock damage (Morris, 1987). As Bale (1987) has emphasised, cold exposure alone causes significant mortality well above the freezing point of some insect species; the threat of actual freezing is obviously not the only potentially lethal stress at low temperature. However, exposure of Malpighian tubules to cold temperatures did not elicit the pattern of damage caused by actual freezing. Malpighian tubules regained full functionality when chilled to −5 °C regardless of whether high concentrations of sugars were present in the bathing solution. Freezing damage (and the corresponding protection by trehalose or glucose) in *H. maori* was not, therefore, simply due to cold-shock injury.

Perturbations caused by low temperature are only one of the multitude of stresses that face cells during a freezing event and that have been postulated to be critical for cell survival. These stresses include changes in cell volume (Liebo, 1971; Meryman, 1971; Storey *et al.* 1992), changes in solute concentrations (Pegg and Diaper, 1991), physical disruption (Mazur, 1984), membrane stress due to water fluxes (Muldrew and McGann, 1994) and oxidative damage associated with ischemia (Ashwood-Smith, 1987). Most of these factors are, unfortunately, interactive and it has been difficult to test experimentally the effect of a single stress factor. Despite the continuing uncertainty about the relative importance of various stress factors, there is little doubt that the mitigation of osmotic effects is important for freezing survival (Storey and Storey, 1988; Storey *et al.* 1992; Gao *et al.* 1993). Such osmotic effects arise as the unfrozen portion of fluid, in which both solutes and cells become excluded, rises in osmolality. Tissue become excluded into these unfrozen spaces where the high solute concentration prevents actual freezing of the cells; intracellular freezing itself would be lethal (Storey and Storey, 1988). The osmolality of the unfrozen fluid is easily calculated (Mazur, 1984); in Malpighian tubules frozen at −5 °C, cells are exposed to fluid of 2.69 osmol kg^{-1}. When we exposed cells to 2.69 osmol kg^{-1} and −5 °C in the absence of freezing, Malpighian tubules survived exposure in the presence of trehalose, but not in the presence of sucrose. A similar correlation between survival during freezing and survival during exposure to low temperature/high osmolality has been noted in other systems and is taken as evidence that osmotic effects are important mediators of freezing damage (Liebo, 1971; Daw *et al.* 1973; Pegg and Diaper, 1991). While we cannot determine the specific mechanism by which osmotic stress mediates cellular damage (e.g. cell volume changes, solute concentration, membrane stress) or the manner in which trehalose mitigates such damage, it is obvious that the osmotic changes associated with freezing present a significant stress to cells.

In conclusion, the present study shows that Malpighian tubules from *Hemideina maori* survive freezing *in vitro*, with the particular freezing characteristics (cryoprotectant requirements and temperature limits) correlating well with those naturally encountered by the organ. Malpighian tubules also have a proven utility in functional studies of transport epithelia (Maddrell and Overton, 1990), indicating that tubules from *H. maori* provide a particularly good model for the study of cellular responses to a freeze/thaw cycle. In addition, the cellular damage that occurs during freezing is at least partially mimicked by exposing tubules to a combination of low temperature and high osmolality, suggesting that further investigation into the osmotic stress associated with freezing in this tissue is warranted.

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