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Interactions of water, ice nucleators and desiccation in invertebrate cold survival

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Abstract. Four case studies are used to examine the relationships of water, ice nucleators and desiccation in the cold survival of invertebrates and the viability of frozen plant material: the freeze intolerant Antarctic springtail *Cryptopygus antarcticus* (Willem) (Collembola, Isotomidae), the freeze tolerant larvae of the fly *Heleomyza borealis* Boh. (Diptera: Heleomyzidae), the freeze intolerant Arctic springtail *Onychiurus arcticus* (Tullberg) (Collembola, Onychiuridae) and meristems of the currant *Ribes ciliatum* Humb. & Bonpl.(Grossulariaceae) from Mexico. Prevention of ice nucleation, lowering the water content by removal of osmotically active (freezable) water are critical features of the different cold survival strategies of the three species of invertebrates. In *C. antarcticus*, which desiccates rapidly by losing water via the cuticle to the atmosphere, the number of ice nucleators (and their activity) increases with lowered ambient temperature. During prolonged cold exposure ice nucleators are masked, but re-activated rapidly by water uptake in this species. Larval *H. borealis* do not readily desiccate and conserve their body water, 20–25% of it being bound (osmotically inactive). Experiments showed that a high proportion (c. 80%) of slowly cooled larvae survived exposure to –60°C. By comparison *O. arcticus* is able to sustain up to 40% loss of its body water and desiccation lowers its supercooling point to promote over winter survival. Dehydration leading to partial vitrification of currant (*R. ciliatum*) meristems improves their viability after cryopreservation in liquid nitrogen. From this comparison of four biological systems, it is concluded that the role of water and its activity at sub-zero temperatures are fundamental to the survival of freezing conditions by all the species studied. Although similar features exist in the four systems, no common basic mechanism was found.

INTRODUCTION

It has long been assumed that survival of sub-zero temperatures, especially in freeze intolerant organisms, largely depends on the ability of the organism to supercool below the melting point temperature of its contained fluids (Zachariassen, 1985; Block, 1995). Thus freezing may be avoided until lower temperatures are reached, at which freezing is invariably lethal. On the other hand, freeze tolerant species supercool only slightly and survive partial freezing of their (extracellular) fluids at relatively high sub-zero temperatures before much lower lethal temperatures are reached (Lee, 1991). Increasingly, recent evidence has highlighted the role of water contained within a freeze intolerant organism in determining the sub-zero temperatures which can be survived by particular taxa (eg. Holmstrup & Westh, 1994; Worland, 1996). The quantity of contained water and its dissolved solutes, often in the form of polyhydric alcohols and sugars for the promotion of supercooling or cryoprotection, and its location and form (free or bound) are important characteristics for the cold survival of an organism. Ice nucleation in such supercooled solutions is promoted by a range of particles and substances of biological or physical origin, whereas antinucleation processes include antifreeze proteins and other mechanisms to reduce the possibility of ice nuclei being formed (Zachariassen & Kristiansen, 2000). Thus, a biophysical balance exists in supercooled aqueous solutions of biological systems, although the supercooled state may be described as unstable (Zachariassen & Husby, 1982). Reduction in water content through desiccation leads to an increase in cold hardiness for many species: the resultant smaller liquid volume, increased solute concentration, and an increase in the proportion of bound water relative to the total water content may be responsible.

This paper compares what is known about the interaction of the main parameters which influence cold survival in different biological systems with the aim of seeking commonality of their features and mechanism. Specifically, four case studies are discussed to examine the interaction of water, ice nucleators and desiccation in the survival of invertebrates and the viability of plant material:

- the freeze intolerant Antarctic springtail *Cryptopygus antarcticus* (Block & Worland, 2001);
- larvae of the freeze tolerant fly *Heleomyza borealis* in Arctic habitats (Worland et al., 2000);
- the freeze intolerant springtail *Onychiurus arcticus* in the High Arctic (Worland et al., 1998);
- cryopreservation of meristems of the currant (*Ribes ciliatum*) from Mexico (Dumet et al., 2000a, b).

Three of these case studies demonstrate a range of responses and survival features by different arthropods to sub-zero temperatures, whilst the fourth is an example of the application of our knowledge of natural systems to a cryopreservation technique applied to plant material. Together they highlight the central role of water in the survival of cold stress induced by sub-zero temperatures

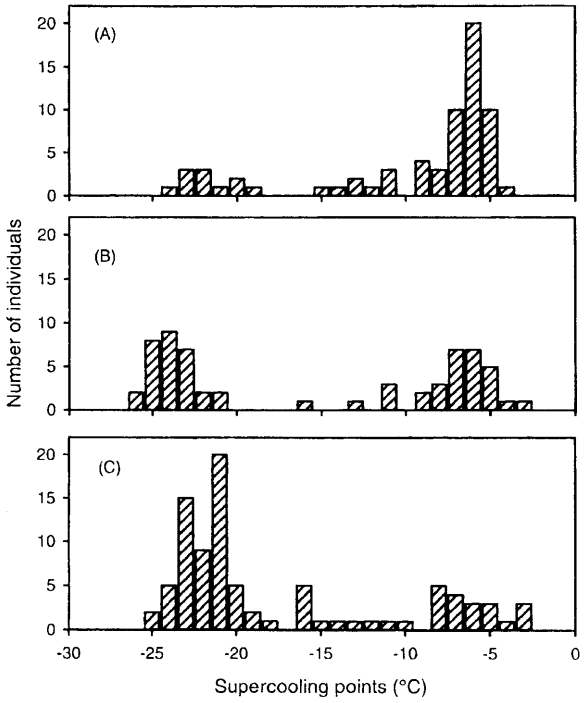

Fig. 1. Supercooling point profiles of samples of *Cryptopygus antarcticus*: A – field sample [*N* = 67 individuals] on 30 December 1992; B – sample acclimated at 4°C for 11 days without food [N = 61]; C – sample acclimated at 4°C for 18 days without food [*N* = 89]. Data from Block & Worland (2001).

by arthropods and emphasise how an understanding of such natural biological systems can contribute to the development of successful cryopreservation protocols for biological materials.

***Cryptopygus antarcticus* (Willem)**

Experiments conducted on the freeze intolerant Antarctic springtail *Cryptopygus antarcticus* at Rothera Research Station, Adelaide Island, Antarctica investigated the relationship between the mean supercooling point (SCP) and ice nucleating activity (INA) of field-collected and temperature-acclimated samples in conjunction with water content and polyol assays (Block & Worland, 2001).

SCPs of individual collembolans were measured by attaching them by a thin film of grease to fine cu-con thermocouples connected to a 6-channel recorder, enclosed in a vial and cooled at 1°C min^{-1} from 5°C using Peltier heat-transfer modules. The SCP was read as the temperature at which the freezing exotherm was initiated. Samples of > 60 individual were used to produce SCP profiles (Fig. 1). The ice nucleating activity of pooled samples of *C. antarcticus* was determined with an INA spectrometer similar to that of Vali (1971). Stock suspensions (10 gL^{-1}) were prepared by grinding weighed quantities of fresh material in a ground glass homogenizer and making up to the required volume with double-distilled sterile water. A series of 10-fold dilutions (1, 0.1, 0.01 gL^{-1}) was prepared and sub-samples of each dilution were tested on the INA spectrometer. A matrix of 20 × 5 µl droplets, together with 5 × 5 µl droplets of double-distilled sterile water as controls, were placed on a polished brass plate, coated with a thin film of liquid paraffin oil. The plate was covered to prevent condensation, evaporation, contamination and to provide insulation. The plate was cooled at 1°C min^{-1} from 5°C until all the droplets had frozen. By observation during cooling the freezing temperature for each droplet was recorded, thus allowing the proportion of droplets frozen at 0.5°C intervals to be calculated. A minimum of 40 sample and 10 control droplets was tested for each dilution. The number (*N*) of active INAs present in each solution at 0.5°C intervals was calculated by the formula

$$N(T) = (-\ln f) / V$$

where *f* is the proportion of unfrozen droplets at temperature *T*, and *V* is the volume of the droplets in litres. The data obtained from the serial dilutions of each sample were pooled by dividing *N*(*T*) by the density of the suspension (gL^{-1}) to give the number of ice nucleators per gram. Nucleation spectra (Fig. 2) were derived, which related the number of active INAs (*N*) at 0.5°C intervals to temperature. Plots of $\log_{10} N$ on $\log_{10} (-T)$ were also made (Fig. 3). These exhibit a linear relationship, which allows the calculation of the number of active INAs at any temperature from the fitted regression line (Table 1) and establishes a useful means for comparison of different samples.

Polyol assays of parallel samples of *C. antarcticus* were undertaken using a gas chromatograph (Hewlett-Packard 5890A) with a Chrompack (CP-Sil5) capillary column after derivatisation in trimethylsilyl with pyridine (Sigma Sil-A) and dulcitol (10 µg) as an internal standard. The water content of samples was obtained from 6 replicates of c. 50 individuals each, weighed on a microbalance (Sartorius MP3) to ± 1 µg, immediately after sampling and after oven drying at 60°C to constant weight.

The SCP distribution of individuals in the field population was bimodal (Fig. 1A) and comprised a high group (HG) and a low group (LG) with mean SCPs of –8 to –10°C and –23 to –25°C, respectively. Although acclimation in moist conditions at 4°C for up to 18 days without food or access to water altered the SCP distributions (Figs 1B, C) by increasing the proportion of individuals in the LG, it did not significantly alter the mean SCP of either group.

Ice nucleator activity of the HG (Figs 2A, C) of *C. antarcticus* was much higher than the LG (Figs 2B, D) as indicated by the INA spectra. Further, the spectra showed that the numbers of active INAs in field and acclimated samples were related to temperature (Fig. 3). Thus the SCP profile was directly related to the number and activity of INAs present at the experimental temperatures. Acclimation reduced the INA of the LG further although the total water content increased significantly from 62 to 69% of fresh weight. The numbers of active INAs calculated at the nucleation temperature (= SCP) were higher in the LG than the HG (Table 1). Also, INAs in the HG of field samples increased by between 23 and 100 times over

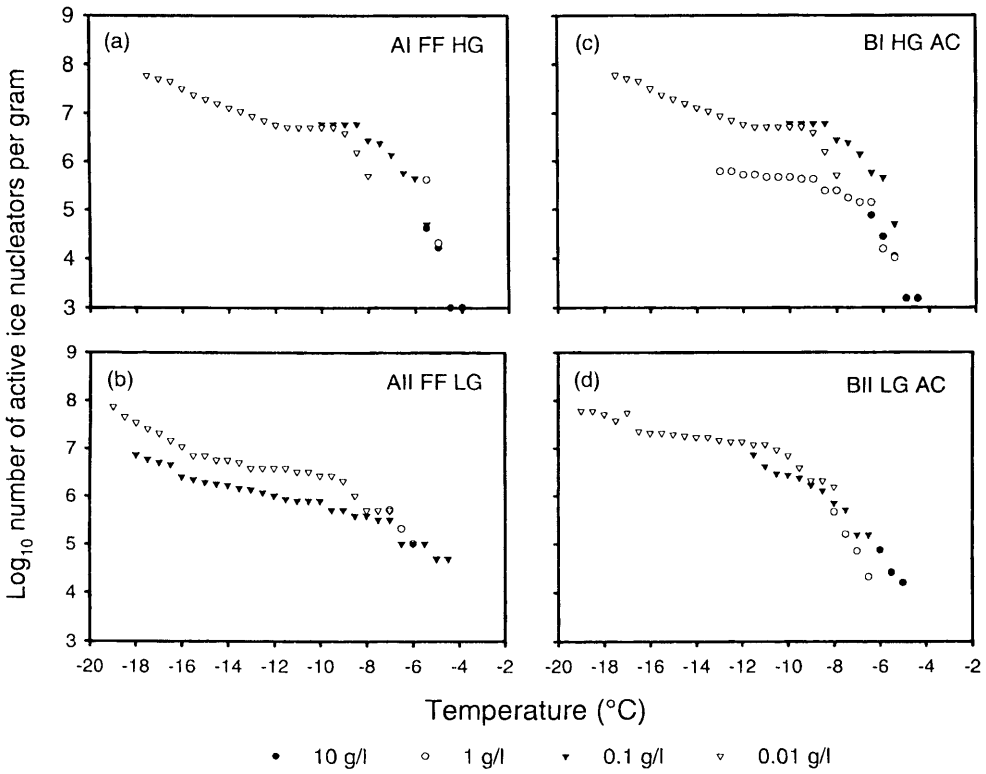

Fig. 2. Ice nucleation spectra for samples of *Cryptopygus antarcticus* for a – field-fresh [FF] HG individuals; b – field-fresh LG individuals; c – HG individuals acclimated (4°C for 11 days without food) [AC]; d – LG acclimated individuals (Block & Worland (2001).

times for the LG over the same temperature span. Glucose and galactose were the principal carbohydrates in this species, with higher concentrations of the latter compound in the LG compared with the HG; both compounds were in lower concentrations in acclimated specimens. It is feasible that during acclimation (starvation) at 4°C the springtails may have continued to digest food within their gut systems, which would produce more LG individuals with a concomitant reduction in INA content of the LG. During periods of low environmental temperatures, INAs may be masked or inactivated (see Cannon et al., 1985) and the amount and distribution of body water is crucial to these processes and hence to their survival over winter in Antarctic habitats.

TABLE 1. Numbers of active ice nucleators (N_T) at the nucleation temperatures calculated for samples of field-fresh and acclimated *Cryptopygus antarcticus* at Rothera Point, Alexander Island, Antarctica. AI, AII, etc are experiments; FF: field-fresh; AC: acclimated (4°C/11 days); HG: high group (0 to –18°C); LG: low group (> –18°C) (after Block & Worland 2001).

Sample	Temperature of nucleation (°C)	N_T
AI FF HG	–3.0	1.3×10^3
AII FF LG	–5.5	9.9×10^4
BI AC HG	–4.5	4.3×10^3
BII AC HG	–4.5	1.6×10^4
CI FF HG	–5.5	6.8×10^3
CII FF LG	–6.0	7.6×10^3
CIII FF HG+LG	–2.5	9.5×10^3
CIV AC HG	–3.0	3.8×10^3
CV AC LG	–5.5	5.4×10^3
CVI AC HG+LG	–2.5	2.3×10^4

Heleomyza borealis Boh.

Overwintering larvae of the freeze tolerant fly *Heleomyza borealis* in Arctic habitats beneath sea bird cliffs close to Ny-Alesund, Spitsbergen freeze at c. –7°C, and temperature acclimation experiments were conducted on samples: larvae were slowly cooled in damp moss from 5 to –60°C over 6 weeks (Worland et al., 2000). Ten samples of larvae, each containing 100 specimens, were acclimated at 5°C for a week. Then the first sample was removed and the larvae assessed for survival (motility was checked weekly for 4 months), freezing point temperature and water content (by differential scanning calorimetry, DSC), soluble carbohydrates (high performance liquid chromatography, HPLC) and lipid content (gravimetric method). The temperature of the remaining samples was reduced to 0°C at 1°C h^{-1}, held for 3 days and the second sample was removed. This procedure was repeated, reducing the temperature in steps of ≅2°C from 0 to –20°C at intervals of c. 3 days. Finally, the temperature of the remaining samples was lowered from –20 to –60°C.

At –20°C, 44% of the larvae had pupated (Fig. 4). 80% of larvae survived exposure to –60°C, of which 11% subsequently pupated. Larvae maintained at 5°C for > 18

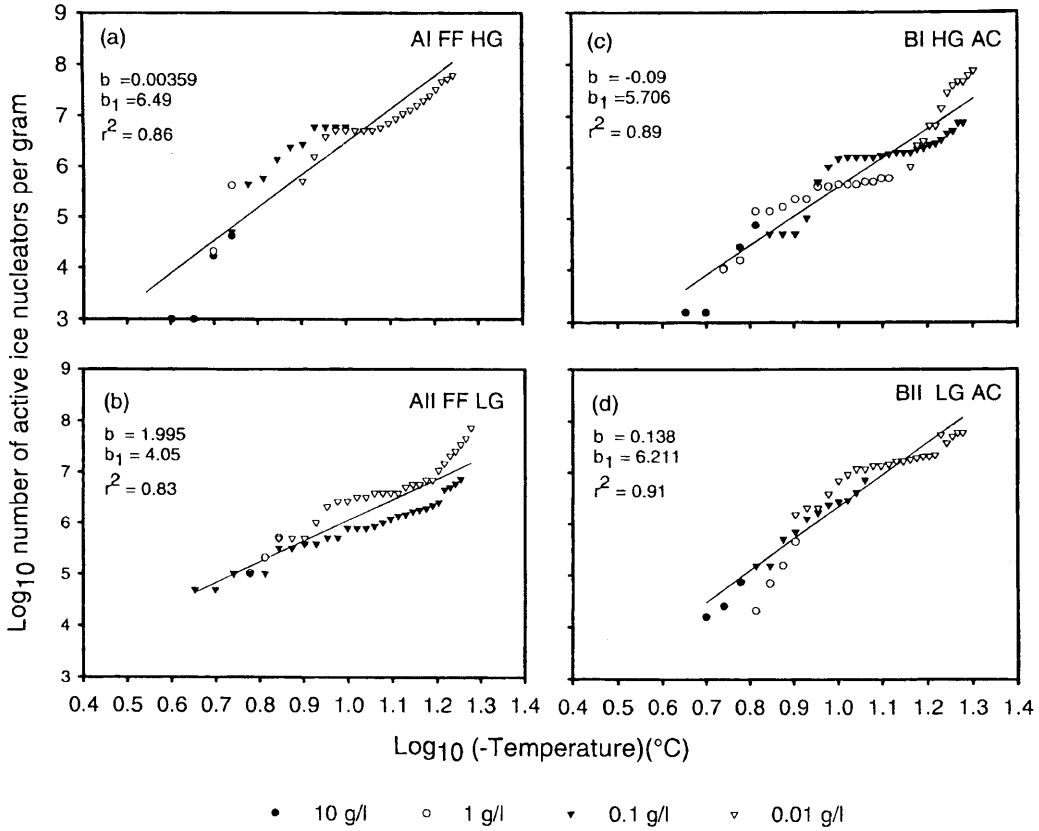

Fig. 3. Regressions of $\log_{10}$ number of nucleators on $\log_{10}$ –(minus) temperature for samples of *Cryptopygus antarcticus* from a – field-fresh [FF] HG individuals; b – field-fresh LG individuals; c – HG individuals acclimated (4°C for 11 days without food) [AC]; d – LG acclimated individuals (Block & Worland, 2001).

months showed minimal loss in body weight (mean 7.5 ± 1.2 mg to 7.0 ± 1.2 mg fresh weight), but none pupated. Individual larvae became dormant after reaching a live weight of c. 7.5 mg and did not feed or grow. Thereafter they required a low temperature stimulus (below –15°C) followed by a warmer period (5°C), when pupation and adult emergence followed. During slow cooling from 5 to –20°C over 6 weeks, *H. borealis* larvae showed little

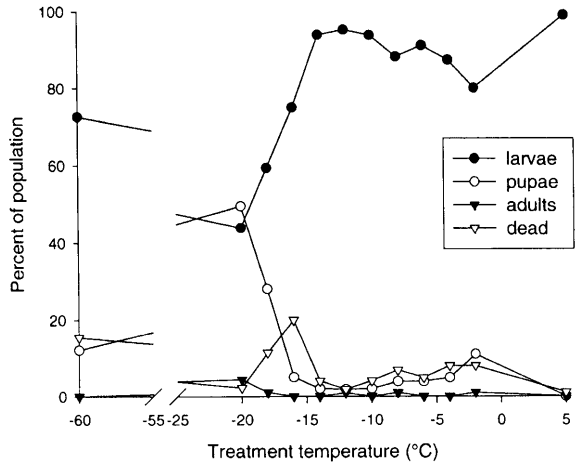

Fig. 4. The effect of treatment temperature on survival of *Heleomyza borealis* larvae. Final assessment was after 120 days post-removal from the low temperature exposure (n = 100 individuals at each temperature). Data from Worland et al. (2000).

deviation from the field SCP with a mean of –6.9 ± 0.4°C. Larvae did not synthesise high concentrations of cryoprotectants at low temperatures (only the fructose content increased from 6.1 μg mg^{-1} fresh weight to 17.0 μg mg^{-1} fresh weight when exposed to –2°C for 7 days). Lipid content of larvae remained stable at 0.22 ± 0.002 μg.g^{-1} fresh weight throughout. Larvae conserved their body water, even at subzero temperatures, with a mean of 2.2 ± 0.1 g.g^{-1} dry weight over the 6 weeks exposure (Fig. 5). The ratio of osmotically active (frozen) water (OA) to osmotically inactive (unfrozen/bound) water (OI) remained stable at 4:1 during exposure to temperatures declining from 5 to –60°C in the experiment. At –60°C, 81% of the total body water of larvae was frozen, which may have been a reflection of low levels of cryoprotectants. The body fluids of such freeze tolerant invertebrates are in vapour pressure equilibrium with ice in their habitats, which minimises desiccation, whereas many freeze intolerant forms lose water to ice in their surroundings.

Onychiurus arcticus (Tullberg)

Partial desiccation, induced by sub-zero temperatures, has been reported as a vital component of the survival strategy of the Arctic springtail *Onychiurus arcticus* in Svalbard (Worland, 1996; Holmstrup & Sømme, 1998; Worland et al., 1998). The species is found in large aggregations under stones and in moist, nutrient-enriched, soils beneath sea bird cliffs close to Ny-Alesund, West Spitsbergen (Block et al., 1994). Experiments were con-

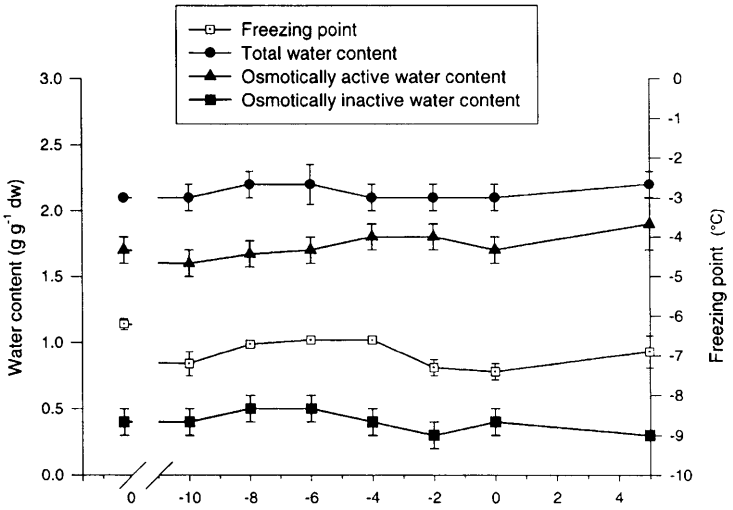

Fig. 5. Changes in water status and SCP of *Heleomyza borealis* larvae during long-term exposure to sub-zero temperatures determined by Differential Scanning Calorimetry (mean ± SE, n = 10). Freezing point = supercooling point temperatures (Worland et al., 2000).

ducted to determine the effects of desiccation in both the short and long term in this species.

Short term desiccation effects were measured by monitoring the change in live weight of individual, field-fresh, collembolans using a recording microbalance (accuracy of ± 1 mg), whilst the sample was held at a constant temperature in a dry atmosphere (< 5% RH) (Worland, 1996). Rates of water loss of live individuals at 0°C and < 5% relative humidity were 5% h^{-1} of the initial total water content (Fig. 6), similar to those measured for Antarctic Collembola. Under these experimental conditions, springtails survived up to 40% loss of their original field water content, but not a 50% loss. Mean SCP in summer was –6.1 ± 2.1°C, and there was no clear relationship of this parameter to field water content (c. 68% of fresh weight). This suggested that INAs were not affected by variation in body water status of springtails in the field.

However, long term desiccation (exposure to a constant sub-zero temperature –2.5°C for 7 months) of *O. arcticus* produced different effects (Worland et al., 1998); reducing the mean water content from 74 ± 10.1 to 43 ± 7.2% of fresh weight and depressing the mean SCP from –6.1 ± 2.1 to –15.5 ± 2.3°C. Such desiccated specimens re-gained their body weight within 24 h when provided with water. During periods of desiccation, water losses were attributed to loss of freezable water, whereas the unfreezable (chemically/physically bound) water remained constant at 16.5 ± 2.0% of the total body water content. During a 3-week exposure to temperatures decreasing from 0 to –5.5°C, a similar reduction in body water (70 to 40% of fresh weight) occurred with a slight loss in the OI component; the mean SCP was also depressed from –7 to –17°C (Fig. 7). Trehalose levels during desiccation rose from 0.9 to 94.7 μg mg^{-1} fresh weight, while glycogen declined. Analysis suggested that if the species experienced –7°C for an extended time period in the presence of ice, most of the OA water would be lost and the SCP would reach a minimum of c. –27°C. In this dehydrated state, individuals of *O. arcticus* probably would not freeze, and could survive extended cold exposures, which may help to explain its ability to colonise and maintain populations in high Arctic habitats.

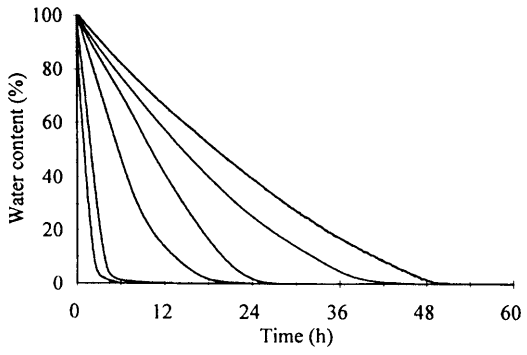

Fig. 6. Short term drying curves for individual *Onychiurus arcticus* obtained with a recording microbalance at a range of constant temperatures and R.H. < 5%. Curves are from right to left: –10, –5, 0, 5, 10, 15 and 20°C. Data from Worland (1996).

Ribes ciliatum Humb. & Bonpl.

Water content has been the focus of research into the cryopreservation of various soft fruit germplasm and encapsulation in alginate beads followed by dehydration is commonly used in the process (eg. Benson, 1999). An ongoing study (Dumet et al., 2000a) of the currant *Ribes ciliatum*, originating from a cold site at 3960 m a.s.l. on north face of Popocatapetal volcano (Tlalacas, Puebla State) in Mexico utilises meristems excised from tissue cultured shoots. Meristems were encapsulated in alginate beads, subsequently dehydrated in a sucrose solution (0.75M), air dried and plunged into liquid nitrogen resulting in > 45% survival on thawing. Thermal analysis by Differential Scanning Calorimetry (DSC) revealed glass transitions at –71 to –81°C during cooling at 10°C min^{-1} from 25 to –150°C with no crystallisation, event,

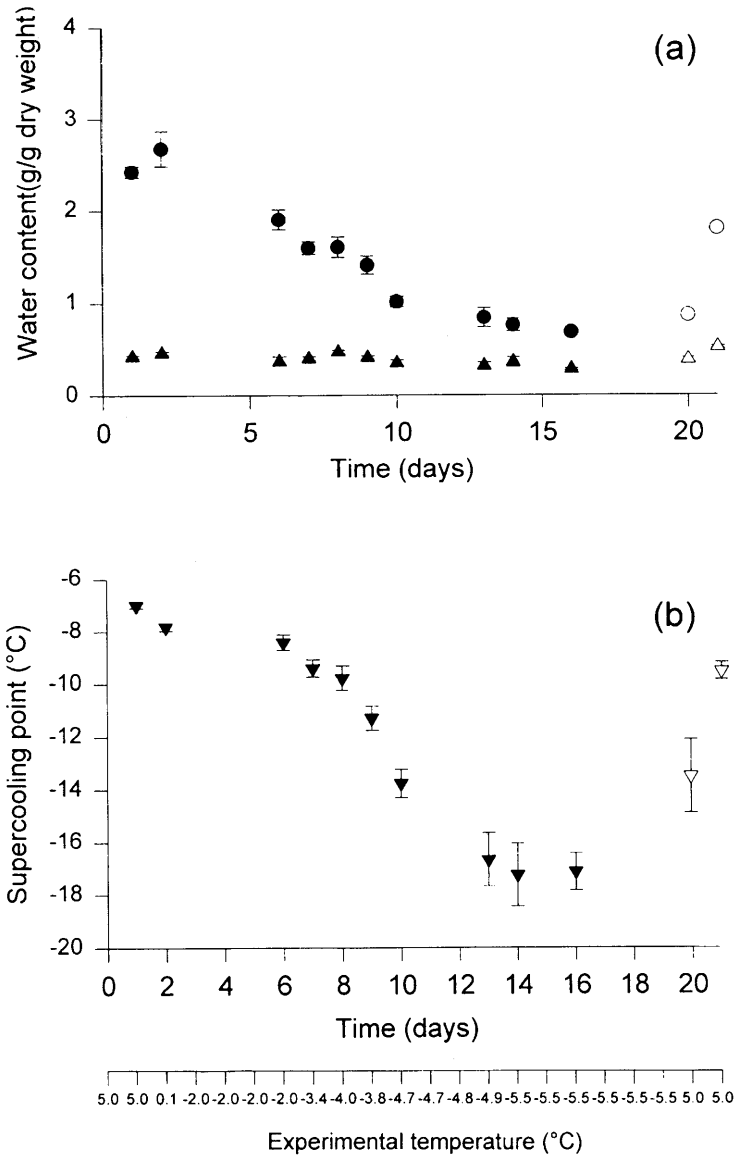

Fig. 7. Effects of reducing ambient temperature over 20 days on (a) the water content and (b) the supercooling point of *Onychiurus arcticus*. ● ○ – total water content; ▲ Δ – osmotically inactive water (mean ± SE, n = 9); open symbols are individuals allowed to recover for 24–48 h at 5°C with access to water. The lower *x* axis shows the mean experimental temperature over the previous 24 h. Data from Worland et al. (1998).

confirming that vitrification had occurred. However, on re-warming a small endothermic event invariably occurred between –30 and –26°C. This represented a very small fraction (< 1%) of the total water content (2.2 ± 0.2 $g.g^{-1}$ dry weight) of the samples and may reflect the instability of the glass formed under these conditions. Clearly, the dynamics of water play a crucial role in cryopreservation tolerance in such material. Alginate encapsulation facilitates both meristem handling and advantageously reduces the rates of desiccation (evaporative and osmotic) and subsequent re-hydration. It has been shown that sucrose pre-treatment can replace cold acclimation of this material before cryopreservation (Dumet et al., 2000b) and, together with air drying, reduces the total water content of the meristems substantially. This effectively eliminates damaging ice crystallisation within the sample and promotes ideal conditions for vitrification. Sucrose pre-treatment with high solute concentrations may also protect against further osmotic stress in this species (Dumet et al., 2000a).

DISCUSSION

Three of these case studies show a range of responses by species of invertebrates to sub-zero temperatures. At one end of the spectrum of response is the dependence of the freeze intolerant Antarctic springtail *Cryptopygus antarcticus* for its survival on the elimination, masking or sequestration of potential INAs to avoid lethal freezing within the body. The dynamic nature of its bimodal SCP profile (mean SCPs of c. –9 and –24°C) reflects a high level of individual variation in the field population. The numbers of active INAs at the SCP temperature are

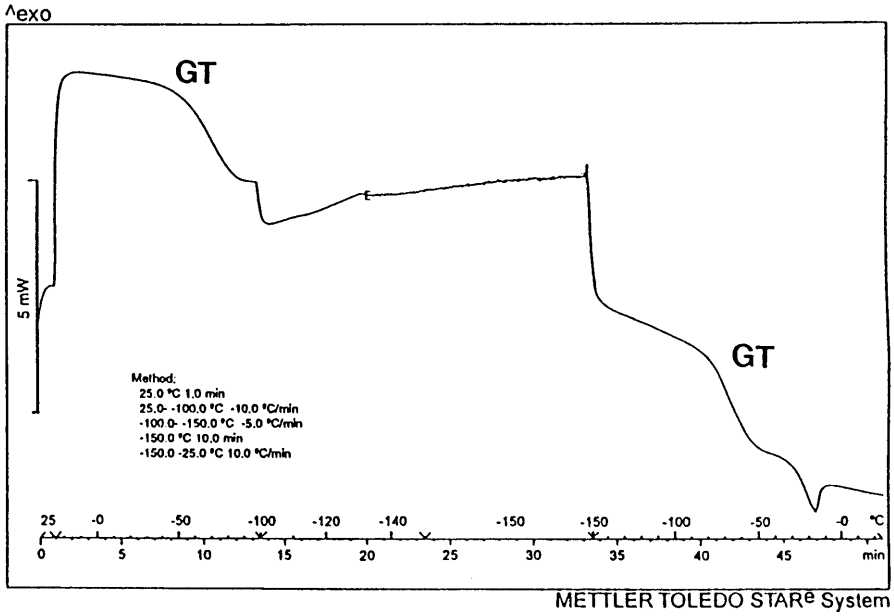

Fig. 8. DSC thermogram showing the effects of cooling and re-warming on an alginate encapsulated meristem of the currant *Ribes ciliatum* (excised from a shoot, osmotically dehydrated for 17 h in 0.75M sucrose, air dried for 4 h). DSC programme is shown as "Method", GT = glass transition. Data from Dumet et al. (2000a).

greater in the upper group (HG) than in the lower one (LG), as would be expected. The mechanism underlying the survival of this species is based almost solely on the reduction of ice nucleator activity during extended periods of sub-zero temperatures. This is aided by a low level production of antifreeze compounds and variation in body water content via environmental cues (see Block & Harrisson, 1995). Partial desiccation occurs in both summer and winter seasons which, in turn, influences its ability to supercool and avoid freezing. During winter, INAs are masked or inactivated, especially when the springtails are dehydrated, but they are rapidly re-activated when liquid water is absorbed or imbibed (Cannon et al., 1985).

Occupying a different position in the spectrum of cold response is the freeze tolerant larva of *Heleomyza borealis* in Arctic habitats, which can survive short exposures at –60°C, its water content (2.2 $g.g^{-1}$ dry weight) and level of supercooling (c. –7°C) remaining almost constant over a wide thermal range. The unfrozen (OI) water component is stable at c. 20% of its total water content. Larvae appear to lose little moisture to their surroundings in the soil microhabitat even during winter. Individual larvae, being freeze tolerant, maintain their body water content at a high level and do not easily dehydrate (Block, unpublished), thereby being able to tolerate freezing for extended periods of time even when there are fluctuations in sub-zero temperatures (Coulson et al., 1995). The over winter and long term survival of populations of this species may be regulated more by the duration rather than the severity of cold exposure in its Arctic habitats, which directly influences its growth and developmental cycle.

Another type of response is shown by the freeze intolerant collembolan *Onychiurus arcticus* which, under Arctic conditions, can survive up to 40% loss of its body water when rapidly desiccated. Over longer time scales, desiccation depressed its SCP from c. –7 to –17°C to a point where largely only OI (16% of total body water) remained. In this desiccated state, the melting point of the small quantity of OA water remaining will be in equilibration with the ambient temperature. Therefore, even though the SCP is lowered substantially by desiccation, supercooling may not occur. Trehalose probably plays an important role in the dehydrated state by maintenance of cell integrity via membrane fluidity, etc. Driven to extremes, *O. arcticus* is unlikely to freeze in nature as during extended cold exposures it will lose almost all of its OA water component and become freeze dried (Worland, 1996).

Finally, dehydration is now known to be an essential pre-requisite to the successful cryopreservation of many biological materials using liquid nitrogen protocols, especially using the alginate encapsulation - dehydration technique. Recent studies on the currant (*Ribes ciliatum*) using Differential Scanning Calorimetry have demonstrated that reduction of meristem water content from c. 2.2 to 0.1 $g.g^{-1}$ dry weight results in no detectable crystallisation (ice formation) and glass transitions being observed between –71 and –81°C, when cooling at 10°C min^{-1} from 10 to –150°C. Although this is a much slower rate of cooling than plunging capsules directly into liquid nitrogen (cooling rate of c. 160°C min^{-1}), it is clear that high survival rates of such meristems are achieved after the sample water content is reduced to a level where there is little or no OA water present. This is achieved in the alginate capsules by immersion in sucrose (0.75M) solution and air drying. Vitrification, promoted by reducing the OA water in the sample, achieves high post-freeze viability for such material, but not for all species. Although chemical vitrification of *in vitro* shoots of *R.*

ciliatum has been shown to improve post-cryopreservation survival (Luo & Reed, 1997), it involves the application of a highly concentrated cryoprotectant solution, PVS2 to promote glass formation. The encapsulation-dehydration technique appears to be less severe and more controllable for delicate meristematic material, but the resultant glasses formed may be prone to destabilisation during warming (Dumet et al., 2000a, b).

Although there are some features which are similar in at least three of these four organisms, there is no general, common mechanism by which they cope with sub-zero temperatures and the possibility of tissue freezing. The invertebrate survival strategies differ markedly and, within themselves, may be regarded as variations on a theme with water occupying a central position. A protective dehydration mechanism has been proposed by Holmstrup & Westh (1994) for cocoons of the earthworm *Dendrobaena octaedra*, and such a strategy may be more widespread in terrestrial organisms than thought hitherto. The Arctic collembolan, *Onychiurus arcticus*, provides another example of this mechanism. The *Ribes ciliatum* study emphasises the importance of a low level of freezable (osmotically active) water for successful cryopreservation of such plant meristems. The biophysical properties of these four biological systems are likely to be very different. The water status of individual samples, the presence or absence of ice nucleating agents and the time scales of the desiccation process are the main components of their survival of cold and freezing. These parameters interact differently according to environmental conditions and the physiological state of the organism. It is concluded that the form and activity of water itself is fundamental to the responses of poikilothermic organisms to freezing temperatures. This has been highlighted in respect of cold hardy insects by Zachariassen (1985). In the wider ecological context, water has been acknowledged as playing a pivotal role in polar ecosystem functioning and response to disturbance over a wide range of spatial and temporal scales (e.g. Kennedy, 1993; Hodkinson et al., 1999).

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