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Water or ice? – the challenge for invertebrate cold survival

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The ecophysiology of cold tolerance in many terrestrial invertebrate animals is based on water and its activity at low temperatures, affecting cell, tissue and whole organism functions. The normal body water content of invertebrates varies from 40 to 90% of their live weight, which is influenced by water in their immediate environment, especially in species with a water vapour permeable cuticle. Water gain from, or loss to, the surrounding atmosphere may affect animal survival, but under sub-zero conditions body water status becomes more critical for overwinter survival in many species. Water content influences the supercooling capacity of many insects and other arthropods. Trehalose is known to maintain membrane integrity during desiccation stress in several taxa. Dehydration affects potential ice nucleators by reducing or masking their activity and a desiccation protection strategy has been detected in some species. When water crystallises to ice in an animal it greatly influences the physiology of nearby cells, even if the cells remain unfrozen. A proportion of body water remains unfrozen in many cold hardened invertebrates when they are frozen, which allows basal metabolism to continue at a low level and aids recovery to normal function when thawing occurs. About 22% of total body water remains unfrozen from calculations using differential scanning calorimetry (compared with ca 19% in food materials). The ratio of unfrozen to frozen water components in insects is 1:4 (1:6 for foods). Such unfrozen water may aid recovery of freezing tolerant species after a freezing exposure. Rapid changes in cold hardiness of some arthropods may be brought about by subtle shifts in body water management. It is recognised that cold tolerance

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strategies of many invertebrates are related to desiccation resistance, and possibly to mechanisms inherent in insect diapause, but the role of water is fundamental to them all. Detailed experimental studies are needed to provide information which will allow a more complete and coherent understanding of the behaviour of water in biological systems and aid the cryopreservation of a wide range of biological material.

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Introduction

Some insects freeze, others do not. Some insects die when frozen, others do not. This encapsulates the problem facing insects and other invertebrates living in cold environments in which there is a potential to freeze. Nature has chosen not to solve the problem in a single or simple way – a wide variety of adaptations has evolved to cope with cold stress in many different environments.[1] Insects and other invertebrates comprise some of the largest multicellular animals to tolerate cold and some of them survive freezing. Three main strategies of invertebrate survival are recognised: freeze avoidance, which relies on lowering the freezing point of the animal's body fluids in order to supercool; freeze tolerance in which extracellular fluids freeze in a controlled manner, thereby maintaining the cellular contents in an unfrozen state; and, more recently, vitrification (the formation of a glass without crystallisation) is thought to occur under certain conditions in nature. Although the physiological and biochemical make-up of a particular species and, to a lesser extent its biology and morphology, may predispose it towards one or other strategy, the variation found is huge and has attracted the attention of ecophysiologists for many years ever since Réamur[2] discovered that some insect larvae survived freezing in 1734. Other classifications have been proposed to embrace the very wide range of natural variation in cold hardiness, *e.g*. Bale (1993)[3] for insects.

Freeze avoiding species generally exhibit a low freezing or supercooling point (SCP), which is also the lethal temperature; thermal hysteresis proteins (THPs), which depress the freezing point relative to the melting point of the body fluids, may be present together with compatible solutes acting colligatively as antifreezes (polyhydric alcohols, sugars, etc); ice nucleating agents (INAs) are absent or inactivated.[4] In freeze tolerant forms, on the other hand, the SCP temperature is generally higher than that of freeze avoiding species

and the lethal temperature is much lower; THPs may be present and INAs are active in promoting extra-cellular freezing at high sub-zero temperatures; compatible solutes may act non-colligatively as cryoprotectants. The essential difference between these two strategies is that freeze avoiding species are killed at the SCP temperature (with freezing occurring throughout the tissues and the intracellular compartment), whereas in freeze tolerant species, ice is confined to the extracellular compartment, resulting in a high survival rate. To date, only one invertebrate species is known to survive extensive intracellular ice formation, the Antarctic nematode *Panagrolaimus davidi* (Figure 1).[5] It is freezing tolerant when ice inoculates the worm during freezing of the surrounding water. In biological materials in which vitrification occurs, a supercooled, concentrated solution undergoes a transition into an amorphous solid (*i.e.* glass) without crystallisation. Although such a mechanism has been postulated to occur under natural conditions, to date, few examples have been reported[6].

It has also been stated that the physiology of overwintering insects is largely the activity of water at low temperatures (Zachariassen 1985[7]), and it is clear from many studies both of insects and other terrestrial invertebrates that water is pivotal to their physiological functioning and ecology. This is especially true for those species

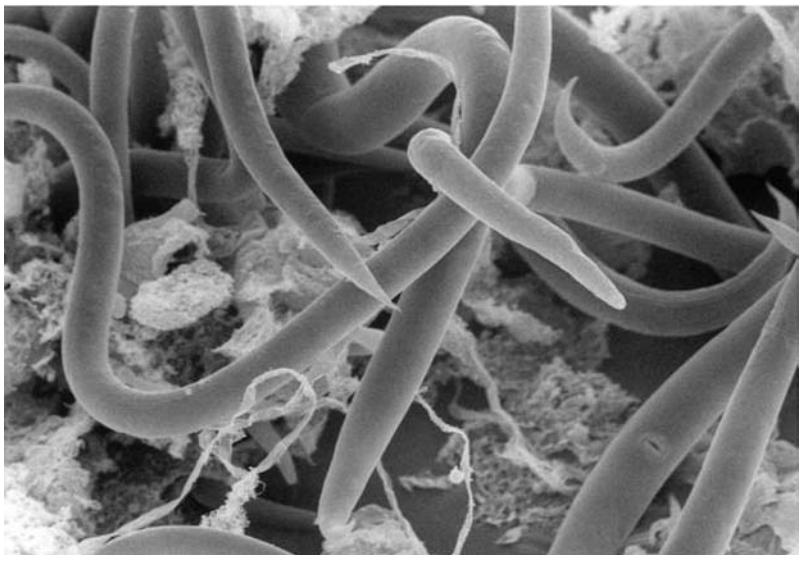


Fig. 1. *Scanning electron micrograph of an Antarctic nematode (*Panagrolaimus davidi*), which survives intracellular freezing.[5] The worms are about 1 mm in length and the width of the largest is 39 μm. Reproduced with permission from D. A. Wharton.*

which inhabit low temperature environments, where the risk of freezing is increased in particular seasons or is present throughout the year. This review will concentrate on the role of water, in its various states, on the survival and ecophysiology of cold-adapted terrestrial invertebrates, especially insects and other arthropods, which have been extensively studied in this regard. I will concentrate mainly on polar species and in reviewing recent developments in this field, I attempt to answer questions such as 'Are there limits to dehydration in terms of cold survival?', 'What proportions of frozen and unfrozen body water are attained during cold hardening?' and 'Is the desiccation protection strategy widespread in invertebrates?'.

Invertebrate body water content

In the majority of terrestrial arthropods, 95-99% of all molecules are water and total body water contents are generally between 65 and 75% of fresh weight (or 1–3 g water per gram dry weight). The extremes of the range may be 40–90% water content.[8] The amount of water in a sample or an organism may be determined by gravimetry (fresh weight less that after drying to constant weight in air), by isotopic means (dilution of injected labelled water) and by calorimetry (using the latent heat of fusion). There are three main compartments for water in terrestrial invertebrates: the body fluids and haemolymph, the cuticle or exoskeleton and other internal tissues (*e.g.* muscle, fat deposits, alimentary system). Water is considered to exist in three main forms in biological systems, free or bulk water, structural or bound water and vicinal water. Bound water is associated with membranes, proteins, etc, whilst vicinal water, often termed interfacial water, is adjacent to solid surfaces and is thought to have properties which differ from those of bulk water especially within cells.[9] In cryobiology bound water equates with unfrozen water (on the timescale of the observation) and is osmotically inactive, whilst free water is osmotically active.[10] Bachmetjew[11] first observed in 1899 that the freezing temperatures of several insects became lower as their water contents declined.

The single main factor that influences the level of body water in terrestrial invertebrate animals is the availability of water in their immediate environment. The wide range of terrestrial habitats for invertebrates provides water directly in the form of rain, fog and dew, whilst snow, hail, frost and ice produce moisture on melting – a factor critical to the survival of several polar arthropods. In some arthropods, high atmospheric humidity may cause the hygroscopic

absorption of water molecules onto the cuticle, whilst in many species it reduces the rate of evapotranspiration via the semi-permeable cuticle of the exoskeleton.[12] Low atmospheric humidity increases water loss to the surrounding air. At sub-zero temperatures in the presence of ice, soil arthropods may lose water to their environment due to the relatively low vapour pressure of ice compared to the supercooled body fluids within the animals.

Some soil arthropods actively absorb water from unsaturated atmospheres by a combination of low cuticle permeability and low water activity in particular tissues (>60 species of insects, ticks, mites and isopods).[8] In species, where such active water uptake is impossible, the body fluids are maintained hyperosmotic to the surroundings, enabling water uptake along a gradient in water potential by passive diffusion. The increased osmotic pressure in the collembolan *Folsomia candida* occurs through the accumulation of glucose and myoinositol, which may confer protection in prolonged drought.[13] How widespread this phenomenon is in nature is not known, but its adaptive importance to soil-dwelling forms is considerable.

A study[14] of two closely-related springtail species, inhabiting a polar maritime environment, showed considerable differences in desiccation rate and pattern of water loss under identical experimental conditions (0–32°C). Further examination suggested that a small, but significant, difference in the amount of body water existed between them, which was manifested in their contrasting water balance (Table 1). In both species, the rate of body water loss increased with temperature and time, but *Isotoma (Folsomotoma) octooculata* dehydrated much faster due to its higher cuticular conductance for water vapour compared with *Cryptopygus antarcticus*. The latter species retained significantly more body water (Table 1). The cuticular conductance of *I. (F.) octooculata* increased by a Q_{10} of 2.8 over the temperature range 5–20°C, being strongly temperature dependent.[15]

Table 1 *Physiological parameters for two species of springtails from the maritime Antarctic (after Block* et al.*[14])*

Parameter	Cryptopygus antarcticus	Isotoma (F.) octooculata
Live weight ((g)	55.2	48.3
Dry weight ((g)	8.4	11.2
Weight of water ((g)	**46.8**	**37.1 (P = 0.04)**
Surface area (mm^2)	1.1	1.0
Water content (% live weight)	85.0	76.8
Water content ($g.g^{-1}$ dry weight)	1.2	1.3
Weight of water/surface area ($g\ mm^{-2}$)	43.7	37.9

These subtle physiological differences underlie the necessity for *I. (F.) octooculata* to inhabit moist habitats, owing to the greater risk of lethal dehydration when drier conditions prevail. Such desiccation susceptible forms with a relatively high surface area to live weight ratio may suffer water stress living in cold environments where liquid water may be unavailable due to freezing at any season of the year.

The water content of individuals of the same species of arthropod differs due to dehydration being temperature dependent and this also applies between species. Results of dehydration experiments undertaken on four adult beetles (Coleoptera, Perimylopidae and Carabidae) – two species of herbivores and two carnivores – on sub-Antarctic South Georgia demonstrated that the indigenous herbivores had lower water loss rates than the introduced predatory species.[16] Body water contents of both herbivore species differed when desiccated at 10°C compared with those dried between 30 and 35°C, but no such difference was found in the two carnivores. Over this latter temperature range, both herbivores survived dehydration longer than the smaller predatory carabids, which was reflected in features of their ecology. Differences in the apparent levels of body water determined after drying at low and high temperatures may be brought about either by the temperature-dependent water binding properties of cells and tissues or by body weight reduction through energy stores being utilised to maintain metabolism at low environmental temperatures.

Few studies have correlated body water content with supercooling potential, but data for adults of 15 species of mites from temperate and Antarctic coastal habitats demonstrated a general depression of the SCP with a reduction in both total live weight and body water content.[17] A comprehensive field study of monthly water contents and SCPs averaged over 4 and 8 years, respectively, in the Antarctic springtail *Cryptopygus antarcticus* showed a distinct seasonal pattern in both parameters[18]. High levels of body water (66% live weight or 2 g g^{-1} dry weight) were associated with relatively poor supercooling ability (mean SCP –5°C) in summer, whilst reduced water content (57% live weight or 1.3 g water g^{-1} dry weight) correlated with lower SCPs (–23°C) in winter[12]. Individual springtails survived the loss of 20% of their body water resulting in a significant rise in haemolymph osmolality from 285 to 397 mOsm l^{-1}.

It may be concluded that measured rates of water loss are influenced by both the thermal and atmospheric humidity properties of the invertebrate habitat, that the quantity of body water is critical for survival of dehydration, especially in Antarctic species, that haemolymph osmolality rises with dehydration and that water loss is

mainly via the cuticle in cold hardy species, its permeability to water vapour being a crucial factor. Further, the supercooling ability of several micro-arthropods are known to increase not only with lowered temperature but also with reduced body water.

Dehydration

Invertebrates can withstand considerable losses of body water dependent on taxon, habitat, physiological state, etc. Arthropods may lose between 17 and 89% of their body water and survive.[8] There appear to be some similarities between arthropods living in hot, dry conditions and those inhabiting cold, dry environments in terms of desiccation resistance and water balance[12]. In polar arthropods, considerable variation may be found in resistance to water loss, even within a small area such as a maritime Antarctic island. For seven species of microarthropods (four mites and three springtails) sampled from different terrestrial habitats, rates of water loss in dry air varied from 0 to *ca* 30% of fresh weight per hour over the temperature range –10 to 45°C[19]. Figure 2 shows the variation in weight loss (= water loss) rates for the seven species, which represent three responses to desiccation: two species of Collembola being least resistant to drying, whilst two species of oribatid mites were most resistant with three other species (two mites and a springtail) showing an intermediate response. It should be noted that at sub-zero temperatures (0 to –10°C) four species lost significant amounts of body water over the timescale of the experiments; this would also represent their response under field conditions, especially during winter when dry conditions would prevail in the soil and hypolithic (under rock) habitats.

Dehydration affects many aspects of the ecophysiology of terrestrial invertebrates including individual metabolism and cold tolerance.[8] These are most marked in freeze avoiding species.[12] Partial desiccation of the High Arctic collembolan *Onychiurus arcticus* enabled individuals to survive losses of up to 40% of their total body water content (but not 50%).[20] Dehydration at –2.5°C for a long time (up to 7 months) lowered their water content from 74 to 43% of fresh weight and depressed the SCP from –6 to –15°C, but they regained their body weight within 24 h when provided with water at 0°C. Desiccation at temperatures down to –5.5°C brought about an increase in concentration of trehalose (from 0.9 to 94.7 $\mu g\ mg^{-1}$ live weight) while glycogen reserves declined.[21] In polar habitats, severe drought or desiccation stress may be experienced particularly in winter at low environmental temperatures. Trehalose has been implicated in the maintenance of the stability of phospholipid bilayers and proteins in

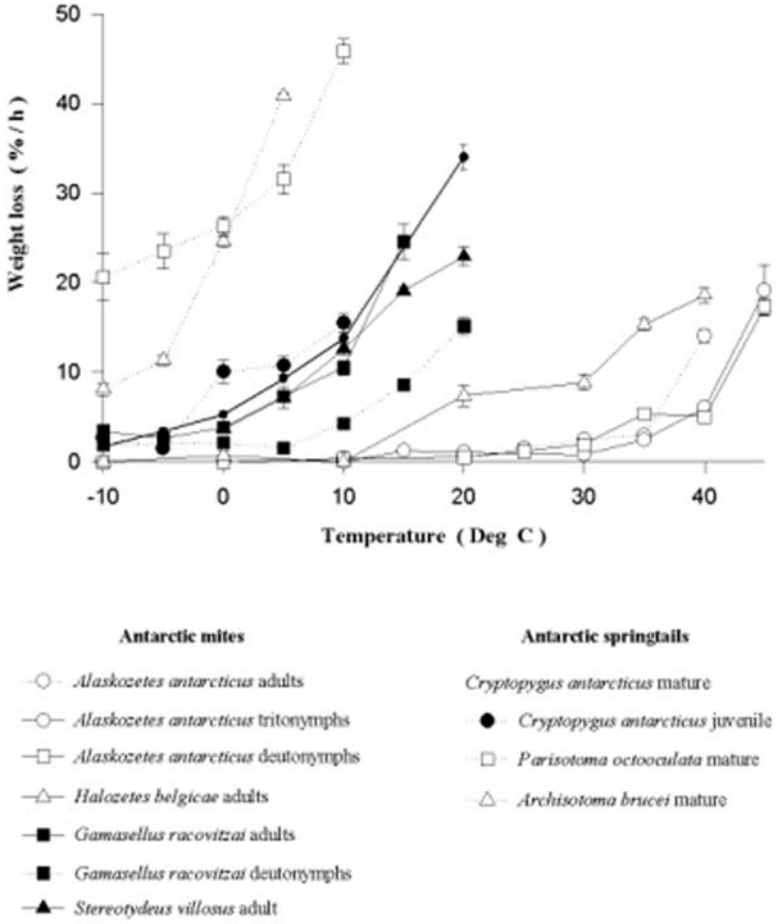


Fig. 2. *Rates of weight (= water) loss of seven species of Antarctic microarthropods under desiccating conditions (r.h. = 5 (1%) at a range of constant temperatures (reprinted from* Journal of Insect Physiology ***32****, Worland and Block, Survival and water loss in some Antarctic arthropods, pp. 579–584, © 1986, with permission from Elsevier Science).*

the absence of water and hence in the survival of intact cells.[22] The sugar has a very high hydration ability, which lowers the mobility of water molecules hydrogen bonded with saccharides, which improves the stability of the lipid bilayer.[23]

As loss of water through dehydration produces physiological stress, the recovery from dehydration is also of importance for cell and whole organism survival. Winter acclimatised, dehydrated springtails (*Cryptopygus antarcticus*) showed an immediate and rapid loss of supercooling ability when provided with distilled water in laboratory

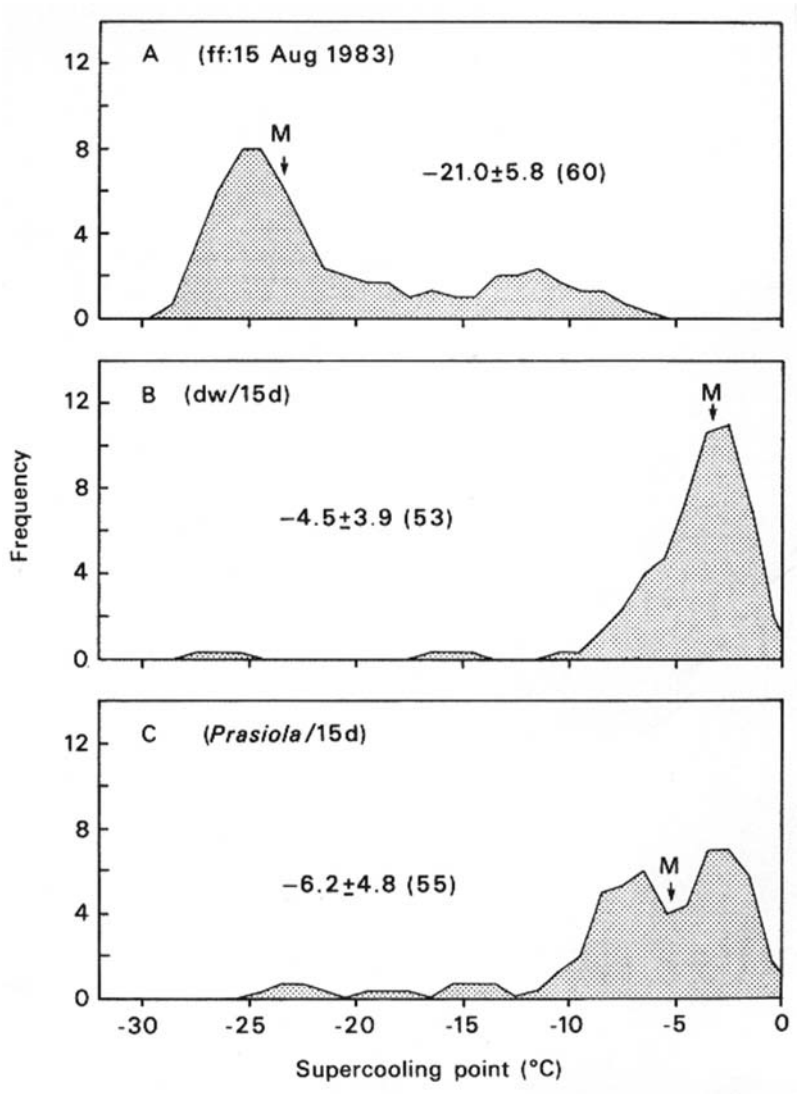


Fig. 3. *Effects of distilled water and food (the alga* Prasiola crispa*) on the supercooling point (SCP) distribution of the Antarctic collembolan* Cryptopygus antarcticus *compared with a field sample (after Cannon[25]). Three-point running means were used to smooth the frequency curves shown. A: ff – field fresh on 15 August 1983; B: dw/15d – access to double distilled water for 15 days; C:* Prasiola*/15d – access to the alga for 15 days. M: median SCP; figures are mean (± SD) SCP (*n*).*

experiments (Figure 3).[24,25] Sixty-eight per cent of field-fresh insects collected in winter (August) at Signy Island, maritime Antarctic, exhibited SCPs below –20°C with a mean of –21°C (Figure 3A), whereas after 15 days of access to distilled water the SCP distribution had markedly altered with the majority of springtails now having SCPs higher than –5°C (Figure 3B). When the alga *Prasiola crispa* was grazed (Figure 3C) the response after 15 days was similar to the distilled water treatment (mean SCP was –6.2°C). It appears that seasonal fluctuations in available water in the environment influence the freezable water component of this species resulting in the activation or deactivation of ice nucleators. In a parallel experiment, it was found that the concentrations of two out of seven potential cryoprotectants in *C. antarcticus* increased with the dry treatment compared with field samples (Table 2). Animals maintained without water for 5 days increased their concentrations of both glucose and trehalose and their total hydroxyl equivalent (or E^{OH}).[25] E^{OH} is the total number of hydroxyl groups present in a multi-component system and provides an index of its cryoprotective potential. Once again, the effect of dehydration on the level of trehalose is clear and this is reflected in the calculated E^{OH} being almost twice that of specimens given water, and both treatments having a much higher E^{OH} than the field-hydrated sample.

Dehydration influences cold hardiness in several species of arthropods whilst triggering synthesis of antifreeze cryoprotectants in others. Desiccation may deactivate or mask particular ice nucleating agents, which are reactivated with water uptake concomitant with increased concentrations of potential cryoprotectants. Cold and drought conditions need not necessarily be viewed as conflicting evils for terrestrial arthropods, as adaptations which have evolved for coping with desiccation may aid cold hardiness. Such pre-adaptations are likely to be found in invertebrates living in cold terrestrial environments.

Table 2 *Mean concentrations (μg mg^{-1} fresh sample weight) of potential cryoprotectants in the Antarctic springtail* Cryptopygus antarcticus *in a 5-day water availability experiment compared with field-fresh samples (after Cannon[25])*

$$E^{OH} = \frac{\text{cryoprotectant concentration x number of hydroxyl groups}}{\text{water content}}$$

Compound	Field-fresh	No water	Water
Glucose	1.8	3.9	1.7
Trehalose	0.4	8.1	2.3
E^{OH}	**15.4**	**89.9**	**40.9 (P<0.05)**

Water and ice

The freezing of water into ice is a first-order phase transition – a transformation of matter from one state to another which brings about a marked change in properties. The threshold to crystallisation is defined by the formation of a 'critical nucleus', which is likely to be a crystallite of ice of a sufficiently large mass that it does not re-dissolve back into a liquid, and which will grow until the entire sample is transformed into ice[26].

The transition of water to ice via crystallisation is a complex process involving three principal events:

i) supercooling (maintenance of a solution in the liquid phase below its equilibrium melting point temperature);
ii) nucleation (formation of an ice nucleus); and
iii) freezing (birth of an ice crystal and growth of an ice front).

Solutions supercool to varying degrees before spontaneously freezing, the termination of supercooling being termed the supercooling point (SCP) temperature. In the supercooled state liquid water is less stable, thermodynamically, than ice but it will remain in the liquid phase unless perturbed beyond a certain threshold. Supercooling is enhanced in the absence of potential ice nucleating agents (INAs) or when INA activity is reduced or masked. The presence of polyols and sugars in the fluids of cold hardy organisms also aid supercooling. Antifreeze proteins (AFPs) or ice structuring proteins[27] may further reduce the potential for freezing in haemolymph and other invertebrate body fluids. Such proteins control the size, shape and aggregation of ice crystals by binding onto their surfaces. Finally, the water content of organisms has a direct influence on their supercooling capacity through solution concentration effects and other, more subtle, processes.

Nucleation in aqueous systems and in whole animals is generally heterogeneous, involving an ice nucleator (*e.g.* food or mineral particles, micro-organisms especially bacteria, proteins and lipoproteins, intracellular supramolecules, *etc*). The SCP temperature of a whole animal or a fluid sample is its heterogeneous nucleation temperature. The phenomenon is widespread in natural systems, whereas homogeneous nucleation, the formation of a critically sized nucleus by spontaneous aggregation of water molecules, is unknown. The critical size for such a nucleus is c. 45,000 water molecules at –5°C and only *ca* 70 molecules at –40°C.[28] Recently, Matsumoto *et al.*[29] have successfully simulated freezing by developing a molecular

dynamics trajectory that captures the molecular processes involved in the freezing of pure and spatially unconfined water. It was found that ice nucleation occurs once a sufficient number of relatively long-lived hydrogen bonds develop spontaneously at the same location to form a compact initial nucleus. The nucleus slowly changes shape and size until it reaches a stage allowing rapid expansion, resulting in crystallisation of the entire system.

Both nucleation and ice crystal growth are time- and temperature-dependent processes, which gives rise to an enormous variation in natural systems, especially in whole animal field studies. "Anti-nucleation" is a collective term which has been applied to the various processes that inhibit freezing,[30] *e.g.* the removal or inactivation of INAs, the accumulation of sugar alcohols (polyols), so that the SCP temperature is lowered as a consequence. Polyols may depress the SCP temperature sometimes by up to twice as much as the corresponding melting point (MP) depression. If antifreeze proteins (AFPs) are present, by stabilising the supercooled state in a non-colligative fashion, they may reduce the potential for nucleation. Furthermore, AFPs may inhibit inoculation of external ice into organisms, prevent spontaneous nucleation in supercooled material and inhibit the recrystallisation of ice in freeze tolerant species.[30]

The mechanism by which AFPs lower the temperature of ice crystal growth below the melting point has been studied for many years. It has been postulated that AFPs adsorb onto the surface of potential seed ice crystals, thus inhibiting water molecules from joining the ice lattice and preventing growth: the adsorption – inhibition model.[31] Insect AFPs are much more effective than those from fish (3–4 times) and plants at depressing solution freezing points by the inhibition of ice growth. Recent studies[32] of insect AFPs suggest that in the small (8.4 kDa) protein from the beetle *Tenebrio molitor* the surface hydoxyl (hydrophilic) groups closely mimic the surface ice structure, whilst the spruce budworm (*Choristoneura fumiferana*) AFP has been shown to match the ice lattice on both prism and basal planes.[33] Both these AFPs are β-helical molecular structures.

During freezing of water and aqueous solutions, the heat of crystallisation (latent heat of fusion) is released exothermically; 1 g of water freezing to 1 g of ice at 0°C producing 80 calories of heat energy; this fact is used extensively to determine the temperature at which organisms nucleate and freeze in ecophysiological experiments by detection of the heat increase of the sample. Thus differential scanning calorimetry provides a wealth of techniques for the quantitative examination of the freezing process in aqueous solutions.

Calorimetric determination of water freezing

The most frequently used technique to determine the amount of water freezing in a sample is by differential scanning calorimetry (DSC), now widely used in ecophysiological and cryobiological studies.[34] Its applications in cold tolerance research are numerous and include:

a) the study of pre-freeze thermal events which influence organism survival (*e.g.* Knight, *et al.*[35]);
b) the determination of the activity of thermal hysteresis/antifreeze proteins (*e.g.* Hansen and Baust[36]);
c) the study of supercooling and crystallisation in natural systems (*e.g.* Block[37]);
d) the identification and quantification of ice nucleator activity in natural and simulated biological samples (*e.g.* Worland and Lukesova[38]);
e) the detection and evaluation of glass transitions (vitrification) (*e.g.* Dumet *et al.*[39]); and
f) the characterisation of the processes of recrystallisation and melting in samples (*e.g.* McAllen and Block[40]).

In total, the various techniques employed in DSC studies enable us to examine the activity of water and solutions at sub-zero temperatures in considerable detail, and provide a greater understanding of how invertebrates, and other biological samples, respond to cold and drought through the management of water within their tissues.

In current DSC work on the freezing of invertebrates and other biological samples several types of instrument are employed, and the DSC820 system (Mettler–Toledo) which incorporates a heat flux module, is typical. The system determines the temperature difference between a pan containing the sample and a reference pan, recording the temperature difference as 'heat flow', whilst both are subject to heating or cooling at a constant rate. Figure 4 shows the furnace arrangement. Calibration uses indium (melting point temperature 156.6°C, enthalpy 28.71 J g^{-1}) as the upper temperature and enthalpy standard, whilst a substance such as dodecane (melting point –9.65°C) may be utilised as the lower temperature standard. The DSC needs to be calibrated both for temperature and enthalpy over the temperature range to be used in the experiments. The melting point of HPLC-grade water can be used as a calibration check, but not as a calibrant. The sample, hermetically sealed in a sample pan, is subjected to a defined programme of cooling and warming, which is computer con-

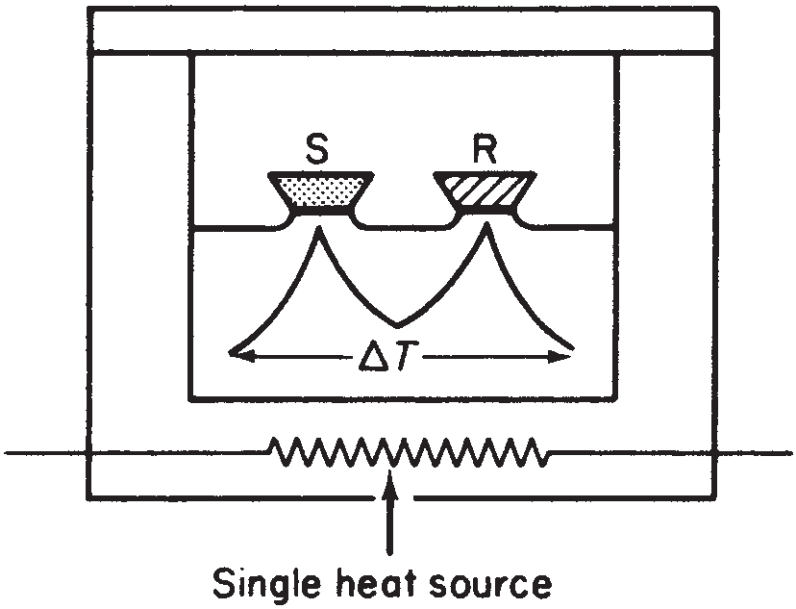
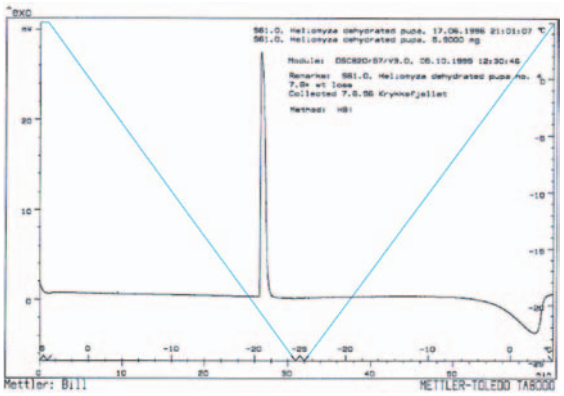


Fig. 4. *Diagram to show the arrangement of the sample (S) and reference (R) pans on the heat flux plate of a differential scanning calorimeter. ΔT: temperature difference detected by sensors under the S and R pans.*

trolled. Liquid nitrogen cooling allows minimal temperatures approaching –180°C to be achieved routinely, whilst sample temperatures to –60°C may be attained using a mechanical intracooler system. Maximum cooling and warming rates are *ca* 10°C min^{-1} whilst a minimum rate of 0.00001°C min^{-1} is possible. Samples are loaded into the calorimeter in aluminium pans, usually 40 to 150 μl in volume, and dry nitrogen gas is used to flush the furnace chamber to eliminate condensation during cooling. With certain samples, *e.g.* live nematode[41] and enchytraeid[42] worms, precautions must be taken to reduce evaporative water loss from the sample in the pan during measurement, in which case liquid paraffin and immersion oil, respectively, have proved useful. Distilled water may be used to examine inoculative freezing of small arthropods. The thermogram resulting from a typical freezing and melting experiment of a small insect is shown in Figure 5. Evaluation of the thermal characteristics of such results may be undertaken using Mettler – Toledo Star^{e} software (version 6.2 for Windows 2000) to provide freezing and melting point temperatures, the enthalpies of the freeze exotherm and the melt endotherm, glass transition and relaxation temperatures, *etc*. In addition to freezing and thawing events and vitrification, the action

(a)



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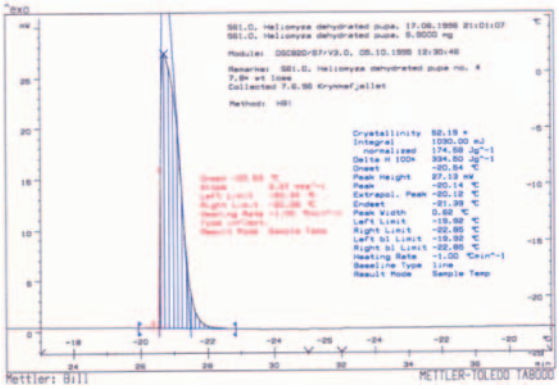

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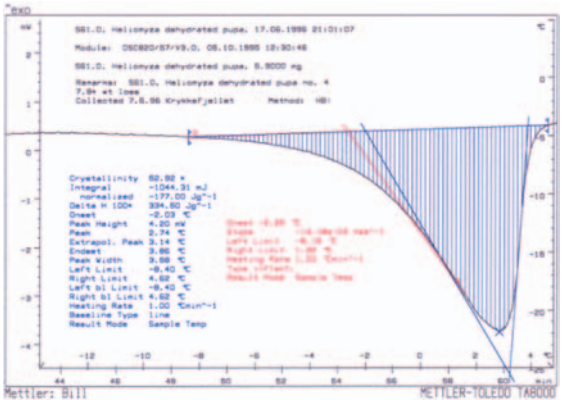

Fig. 5. *(A) DSC thermogram from a freezing and thawing experiment on a single pupa of the fly* Heleomyza borealis *from Spitsbergen, Svalbard; cooling and warming rate was 1°C min^{-1}; (B) evaluations of the freeze exotherm and (C) melt endotherm are shown with the calculated enthalpies, etc.*

of cryoprotectants, ice nucleators and thermal hysteresis proteins have been examined using DSC techniques.

An increasing range of invertebrates and other biological samples have been examined using DSC techniques for cold tolerance and water balance studies. Freezing exotherms have been detected from single nematodes; Figure 6 shows the thermogram of freezing in two individuals of the Antarctic *P. davidi* mounted under liquid paraffin. Desiccation resistance and anhydrobiosis phenomena of arthropods,[20] earthworm cocoons[43] and tardigrades[44] have been investigated experimentally using DSC. Plants have been studied rather less using DSC, but recent studies on desiccation of algae from extreme environments have used it.[38] The development of successful plant cryopreservation protocols has benefited from thermal analysis assays undertaken using DSC (*e.g*. Dumet *et al.*[39]). The critical importance of controlling the water content of plant meristems, seeds or other plant material for cryopreservation cannot be over-emphasised[45] especially if vitrification is the only potentially suitable option in freezing recalcitrant materials.[46,47]

The freezing of water and the melting of ice in scanning calorimeters have been intensively studied (e.g. Williams and Hirsch[48]). The proportion of sample water unfrozen at a particular temperature may be calculated from the total water content, determined gravi-

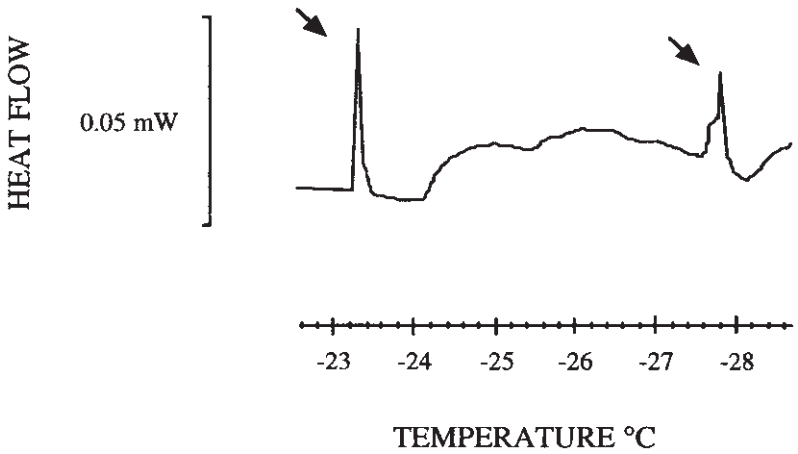

Fig. 6. *DSC thermogram showing freezing exotherms (arrowed) from single nematodes (*Panogralaimus davidi*) mounted in liquid paraffin and cooled at 1°C min^{-1} (reprinted from* Cryobiology ***34****, Wharton and Block, Differential scanning calorimetry studies on an Antarctic nematode (Panagrolaimus davidi) which survives intracellular freezing, pp. 114–121, © 1997, with permission from Elsevier Science).*

metrically from initial fresh weight and drying to constant weight at 60ºC, and the quantity of water frozen at the experimental temperature, usually calculated from the enthalpy of the melt endotherm. An alternative method estimates the quantity of frozen water from an Arrhenius plot, where unfrozen water fractions are plotted as a function of the reciprocal osmolality of the fluid fraction.[10]

Frozen and unfrozen water

In the literature, bound water is often equated with unfreezable water, but this is misleading as water only remains unfrozen over the timescale of the observation; water is not unfreezable.[49] For example, pure water in the absence of a nucleator will freeze extremely slowly (0.3 μm per year) at –150°C due to its viscosity at that temperature limiting the rate of diffusion of a water molecule through a given distance (*e.g.* 1 μm). The caveat of Franks[49] should be taken seriously "Unfrozen water, yes; unfreezable water, hardly; bound water, certainly not." It is recommended that the term unfrozen water be used, particularly for biological systems. The existence of unfrozen water at freezing temperatures in the presence of nucleators or ice itself, may be due to three main effects: (a) the depression of the freezing point by solutes; (b) the depression of the freezing point by membranes, macromolecules and other hydrophilic ultrastructures; and (c) the effects of viscosity.[50] Analyses of quantitative measurements of the hydration interaction among macromolecules or membranes suggest that during dehydration of such systems, especially at temperatures below 0°C, equilibrium is rarely achieved over experimental timescales, so the amount of unfrozen water exceeds that expected at equilibrium and that calculated for a single hydration shell (a few water molecules thick layer). During dehydration by freezing, the concentrated solutions at low temperatures have viscosities that slow both the equilibration of water in confined spaces and the growth of the ice interface to an extent that effectively stops freezing.[50] For ecophysiological purposes it is appropriate to describe water in two main classes – frozen and unfrozen water, osmotically active (OA) and osmotically inactive (OI), respectively.

Food industry research has contributed basic data on the quantities of water frozen in various food types using DSC techniques. Samples of beef semi-membranous muscle (20–25 mg fresh weight; water content 76% of fresh weight) were frozen at eight temperatures in the range from –5 to –65°C[51]. More water froze in the samples at lower temperatures (from 50 to 70%) and the latent heat of melting increased as the freezing temperature was lowered (from 135 kJ kg^{-1}

at –5°C to 185 kJ kg^{-1} at –65°C). It was calculated that between 1–2% of water that was freezable remained in the liquid phase between –30 and –65ºC. Table 3 provides water contents for four food materials obtained by DSC techniques; it can be seen that both the amount of OI water and the ratio of unfrozen to frozen (OI:OA) water varies considerably with a mean of 1:5.7 over all the foods studied.

Data for OI and OA water in insects contrasts with that from food materials (Table 4). From the limited information available it seems that the amounts of OI and OA water (and hence their ratio) are much less variable. Frozen water has a mean of 78% for the five species listed, whereas the unfrozen fraction comprises *ca* 22% (mean of 19% for four foods, Table 3) of the total water content, the OI:OA ratio averaging 1:3.6. Dehydration changes these figures reducing the total body water with concomitant alterations in the OI:OA ratio as demonstrated for the collembolan *O. arcticus*.[20] It was predicted for this species that at –7°C under the experimental conditions that all OA water would be lost and the springtail would be almost anhydrobiotic. How far these results are applicable to other invertebrates, or indeed other biological samples, remains to be seen. It is estimated[55] that between 10 and 30% of water is unfrozen in various biological systems and specifically in freeze-tolerant animals it is reported to be 20–25%. Several factors will influence the amount of OA water in biological samples including the total amount of water contained in the sample, its hydration state, the cooling and warming rates in the DSC and the temperature of freezing. There are other factors, which may be important depending upon the material being investigated. Experimental studies[56] of cold survival of cocoons of enchytraeid worms, showed that they desiccate rapidly at room temperature (39 and 57% of body water was lost after 2 and 3 min, respectively, in *Enchytreaus crypticus*), which was mainly attributed to loss of OA water only, but there was no significant increase in supercooling ability.

Table 3 *Quantities of frozen and unfrozen water in four food materials measured by DSC techniques (after Aktas, et al. [51] and Roos[52])*

Material	Water content (%)			Reference
	OI	OA	OI:OA	
Beef	30	70	1 : 2.3	51
Carrot	8	92	1 : 11.5	52
Reindeer	15	85	1 : 5.7	52
White bread	22	78	1 : 3.5	52

Table 4 *Quantities of frozen (OA) and unfrozen (OI) water in five species of insects. f.w. = fresh weight; Ratio = OI : OA water*

Taxon	Water (%)				Reference
	Total (f.w.)	OI	OA	Ratio	
Eleodes blanchardi (Coleoptera)	56	25	75	1 : 3.0	10
Onychiurus arcticus[a] (Collembola)	40	22	78	1 : 3.5	21
Celatoblatta quinquemaculata (Dictyoptera)	67	26	74	1 : 2.8	53
Ceratophysella sigillata (Collembola)	72	23	77	1 : 3.3	Block and Zettel (unpublished)
Heliomyza borealis (Diptera)	69	22	78	1 : 3.5	54

[a]After dehydration for 7 months at –2.5°C the mean body water content was reduced from 74 to 43% (fresh weight) and the ration OI : OA was 1:5.3[20]

In terms of invertebrate cold survival an increase in the proportion of unfrozen water in the individual will slow the rate of freeze concentration of fluids in cells and tissues. This suggests an important difference between freeze avoiding and freeze tolerant species and raises the question as to whether the proportion of unfrozen water can be increased, especially in freeze tolerant forms, whereas many freeze avoiding insects will dehydrate to improve supercooling and hence lower their SCP. It has been reported[57] that the unfrozen water content of the freeze-tolerant larvae of the golden rod gall fly (*Eurosta solidaginis*) increases with cold acclimation, much of the increase arising from dialyzable components (mainly polyols), the remainder represented water attached to high molecular weight soluble compounds (protein, glycogen). It has been suggested[58] that a major proportion of unfrozen water is replaced by trehalose in yeast cells subjected to water stress which increases their viability.

Conclusions

In respect of those invertebrates that have been studied, their cold hardiness and survival of dry conditions are greatly influenced by their water status. It would seem that a consistent basal quantity of OI water (20–26% of total body water) is required to maintain their physiological integrity, but whether this proportion can be increased, especially in freezing tolerant forms, remains to be elucidated. The evolutionary basis of freeze avoidance and freeze tolerance has been

discussed in respect of the ability to lose or conserve water in insects and other arthropods.[59] Which arose first – desiccation resistance or cold tolerance – or did they evolve in concert? Freeze avoiders are, in the main, species which readily lose water via a permeable cuticle, whereas ice tolerators are mostly species that can conserve their body water to a greater extent or live in protected microhabitats (*e.g.* larvae of *E. solidaginis*, living in galls on golden rod). Alternatively, there may be a co-evolutionary relationship between insect diapause and cold tolerance.[60]

For soil invertebrates with high cuticular permeability to water and poor supercooling ability such as nematodes, certain mites, many springtails, enchytraeid worms and some earthworm cocoons they may possess a protective dehydration mechanism.[43] In the soil habitat, dehydration of these forms occurs rapidly under freezing conditions until the vapour pressure of their unfrozen body fluids balances that of the surrounding ice. Such animals are considered to be no longer supercooled, because the melting and freezing points of their body fluids equal the ambient temperature.[61] Under these conditions, tissue ice formation is much less a risk and inoculation of ice from the surroundings is eliminated; survival at sub-zero temperatures is therefore ensured. The High Arctic collembolan *Onychiurus arcticus* has been shown to rely on supercooling to avoid freezing,[21,62] especially during cold periods in summer and autumn, until water vapour equilibrium has been reached during winter in its microhabitat.[63] A dehydration protective mechanism would appear to be ultised by this species as it survives winter temperatures approaching –23°C[64] although measured SCPs are rarely found below –6°C.

The weakly freeze-tolerant larvae of the sub-Antarctic beetle *Hydromedion sparsutum* have relatively high SCPs (*ca* –4°C) during summer, but when subjected experimentally to repeated exposures to –6.5°C or to the previous SCP temperature did not always freeze at that temperature and some individuals survived exposure to –12°C without freezing[65]. It is unlikely that these changes were brought about by clearance of ice nucleators contained in food from the alimentary canal of the larvae, but there must be some physiological change brought about within a shorter time frame, which may be related to water balance and its distribution within the insect's body. It is interesting to note also that rapid changes in SCP temperature have been reported in the freeze-avoiding Antarctic collembolan *Cryptopygus antarcticus*.[66] Using DSC techniques and times of between 3 and 30 h, the SCP distributions of field samples generally tracked variations in microhabitat temperature. Gradual cooling of

springtails from +5 to –5°C over 20 h caused a significant increase in their supercooling ability, which was reversed on warming. The greatest response occurred between +3 and +1°C and although maximum faecal production occurred over this period, this was not sufficient to produce the measured increase in cold hardiness. It is unlikely that the increase in cold hardiness was brought about by the concentration of body fluids via water loss over this time period. The physiological mechanism underlying such a rapid cold hardening response may well be based on more subtle changes in water management by this species.

A recent publication[67] on the molecular and structural biology of cold stress survivors attempted to draw together the disparate information on low temperature detection and signalling, the molecular basis of cold adaptation and the natural processes that regulate ice formation in a range of organisms. It demonstrated many gaps in our knowledge of the processes by which cold stress is resisted, emphasised the value of comparative studies and the technological potential of natural systems. Water and ice play a fundamental role in almost all of these systems. For some species of invertebrates the limit to dehydration in terms of cold survival is reached at the point of their entering an anhydrobiotic state, whilst in others between 20 and 26% of total body water must remain unfrozen to ensure viability. The strategy of desiccation protection for cold tolerance may be much more widespread than previously envisaged, adding to the wide spectrum of variation observed in nature.

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