THE ROYAL SOCIETY

Published online 19 June 2002

Physiological and ecological significance of biological ice nucleators

Rolv Lundheim

Allforsk Biology, Queen Maud College, Thonning Owesensgt 18, 7044 Trondheim, Norway (*rolv.lundheim@dmmh.no*)

When a pure water sample is cooled it can remain in the liquid state at temperatures well below its melting point (0 °C). The initiation of the transition from the liquid state to ice is called nucleation. Substances that facilitate this transition so that it takes place at a relatively high sub-zero temperature are called ice nucleators. Many living organisms produce ice nucleators. In some cases, plausible reasons for their production have been suggested. In bacteria, they could induce frost damage to their hosts, giving the bacteria access to nutrients. In freeze-tolerant animals, it has been suggested that ice nucleators help to control the ice formation so that it is tolerable to the animal. Such ice nucleators can be called adaptive ice nucleators. There are, however, also examples of ice nucleators in living organisms where the adaptive value is difficult to understand. These ice nucleators might be structures with functions other than facilitating ice formation. These structures might be called incidental ice nucleators.

Keywords: freeze tolerance; proteins; evolution; ice nucleation

1. INTRODUCTION

The term *ice nucleation* describes the initiation of the phase transition of water from a liquid to a solid state. When a water sample of moderate size is cooled, it will normally not freeze at 0 °C. If the water is pure, it can be cooled to temperatures near to −40 °C before it freezes. Liquid water at temperatures lower than 0 °C is termed supercooled water, and this supercooled state is metastable. To enable ice formation to take place, water molecules must cluster in an ice-like pattern and this cluster must reach a critical size. It is probable that it is the surface curvature of the cluster and not the size *per se* that is important. When the critical size (or curvature) is reached, ice will spread rapidly in the supercooled water. If the initial aggregation of water molecules takes place on a foreign structure, the process is termed *heterogeneous* ice nucleation. If the water molecules aggregate without the help of another structure, the nucleation is termed *homogenous* (Knight 1967). In most practical situations, it is believed that the water aggregate leading to nucleation is organized around some impurity in the sample, or around the wall of the container holding the sample. A structure that organizes water into an ice-like pattern so that nucleation takes place is called an *ice nucleator*. By this definition, any substance leading to heterogeneous nucleation is an ice nucleator. However, the term is usually not used for substances inducing ice nucleation at temperatures lower than *ca.* −10 °C.

The freezing process of an aqueous sample can be separated into two phases, (i) a rapid crystallization where the sample is heated by the released heat of fusion until the temperature reaches the melting point of the water in the sample, and (ii) following this, freezing will continue at a rate that is determined by how fast heat is removed from the sample.

2. SOURCES OF BIOLOGICAL ICE NUCLEATORS

Most attention has been given to the proteinaceous ice nucleators of bacteria. In 1970, Russell C. Schnell discovered that the very potent ice nucleators derived from decaying leaves were of microbiological origin (Upper & Vali 1995) and in 1972 *Pseudomonas syringae* was identified as the organism causing the high nucleation temperatures (Maki *et al.* 1974). Almost simultaneously, another research group interested in frost damage to crop plants realized that plants infested by microorganisms were susceptible to frost damage. They discovered that the susceptibility to frost of plants infested by *P. syringae* was caused by ice nucleation at high temperatures, rather than by a reduced resistance to stress caused by the infection (Arny *et al.* 1976). A large number of ice nucleating bacterial strains from more than 10 species have now been isolated and investigated.

In the same issue of *Nature* as Arny *et al.* published their work, Zachariassen & Hammel (1976) published their findings regarding ice nucleators in the haemolymph of a freeze-tolerant insect. This revealed the dual, and sometimes confusing, role of ice nucleators; causing injuries to plants and protecting freeze-tolerant animals.

Subsequently, potent ice nucleators have been found in a variety of organisms such as *Bivalvia* (Aunaas 1982*a*), *Gastropoda* (Hayes & Loomis 1985), *Lichenes* (Kieft 1988), *Reptilia* (Costanzo *et al.* 1988), *Amphibia* (Wolanczyk *et al.* 1990), *Tardigrada* (Westh *et al.* 1991), fungi (Pouleur *et al.* 1992) *Angiospermae* (Gross *et al.* 1988), *Chilopoda* (Tursman *et al.* 1994) and algae (Lundheim 1997*a*).

One contribution of 15 to a Discussion Meeting Issue 'Coping with the cold: the molecular and structural biology of cold stress survivors'.

Table 1. Chemical composition of some biological ice nucleators.
(If nothing else is indicated, protein molecular weights are given for SDS denatured proteins and is therefore not representative for the native size of nucleation sites. (Modified from Lundheim & Zachariassen 1999.))

organisms	protein molecular weight (kDa)	lipids	phospholipids	carbohydrates	heat sensitivity (°C)
bacteria	120–180[a]	yes[a]	PI and others[a]	yes[a]	yes, 20–30[b]
insects	74[c], 265[d], 81[d], 77[e]	yes[d], no[c,e,t]	PI[d]	yes	< 100[f,g] 70–90[h] 40–70[t]
molluscs	16,6[k] 17,7[k]	no[k]	no[k]	no[k]	< 100[i,j]
Tardigrada	native > 200[s]				60–70[s]
vertebrates	native > 300[l]				85–90[l]
vascular plants	native > 300[m], probably not protein[o]	yes[m,n]	yes[n], other than PI[m]	yes[n]	20–30[m], < 90[n], 50[o]
lichen		no[p]	no[p]	no[p]	60–70[p]
fungi (*Fusarium*)		no[r]	no[r]	no[r]	60–70[q], 40–50[r]

[a] Fall & Wolber (1995). [b] Obata *et al.* (1990). [c] Duman *et al.* (1984). [d] Neven *et al.* (1989). [e] Duman *et al.* (1995). [f] Zachariassen & Hammel (1976). [g] Duman & Patterson (1978). [h] Duman (1980). [i] Aunaas (1982*a*). [j] Hayes & Loomis (1985). [k] Madison *et al.* (1991). [l] Storey *et al.* (1992). [m] Lundheim (1997*c*). [n] Brush *et al.* (1994). [o] Gross *et al.* (1988). [p] Kieft & Ruscetti (1990). [q] Pouleur *et al.* (1992). [r] Hasegawa *et al.* (1994). [s] Westh *et al.* (1991). [t] Hirai & Tsumuki (1995).

3. CHEMICAL COMPOSITION OF ICE NUCLEATORS

Almost all ice nucleators of biological origin have a dominating proteinaceous compound that is essential for their activity.

A characteristic of bacterial ice nucleators is that they possess a section that consists of repeated sequences of amino acids. The repeats are 48 residues long. This unit can be subdivided into 16 and 8 residues with lower fidelity. It is reasonable to assume that such a repeated pattern is involved in the organization of water into a crystal-like ice pattern. Several 3D models of the ice-nucleating agent protein have been suggested (Warren *et al.* 1986; Mizuno 1989; Kajava & Lindow 1993), but experimental evidence to support any of the models is still scarce.

Another section of the protein is non-repetitive, but still conservative between species. Both of these sections are required for nucleation (Warren 1995). Other sections of the protein that are not conservative between species are not required for nucleation.

An overview (table 1) shows that the properties of biological ice nucleators are diverse even if most of them are at least partially proteinaceous. One should keep in mind that the nucleators listed in table 1 induce nucleation at temperatures ranging from −2 °C to below −7 °C.

Membrane phospholipids are often implicated in ice nucleation activity (Govindarajan & Lindow 1988). PI seems to play an important role in anchoring or positioning the protein to the cell membrane (Turner *et al.* 1991). PI is also important in the action of the lipoprotein ice nucleator from the insect *Tipula trivittata* (Neven *et al.* 1989). The ice nucleator from the vascular plant *Hippophae rhamnoides* also depends on a phospholipid, but probably not PI (Lundheim 1997*c*).

A variety of non-proteinaceous substances can induce ice nucleation. Crystals of silver iodide have been used in many studies. Monolayers of aliphatic alcohols have been shown to be very potent ice nucleators (Gavish *et al.* 1990). In the freeze-tolerant goldenrod gallfly *Eurosta solidaginis*, spherules of calcium phosphate are responsible for the relatively high nucleation temperature (Lee *et al.* 1992).

4. WHY ARE ICE NUCLEATORS PRODUCED?

The first ice-nucleating bacteria were isolated from frost-damaged plants, and it is probable that one of the adaptive advantages of producing ice nucleators for these bacteria is to induce frost damage, thereby giving the bacteria access to nutrients from the plant (Buttner & Amy 1989). In insects, the nucleators appear to play a different role, since they are mainly produced by insects that survive freezing. Zachariassen & Hammel (1976) suggested that the nucleators are produced to ensure that nucleation takes place in compartments of the body where ice formation is tolerable. This may be the reason why ice nucleators are found extracellularly. Ice formation intracellularly would probably cause both direct mechanical damage and rupture of the cell membrane and organelles in the cell, caused by the osmotic transport of water following the increased osmolality of the intercellular fluid. In the relatively open haemolymph system of insects, ice formation can take place without causing lethal damage. When ice is formed in the haemolymph, the osmolality of the haemolymph increases and water will be drained from the cells, causing them to shrink (Zachariassen 1985). Glycerol and other cryoprotectants may counteract the shrinking by reducing the amount of ice formed and by adding volume to the cells. The low molecular weight cryoprotectants, such as glycerol, might also stabilize membranes and proteins at low temperatures and the high salt concentrations caused by the ice formation (Hochachka & Somero 1984). In freeze-tolerant vertebrates, ice is formed mainly in the extracellular compartments, such as the abdominal, thoracic and coelomic cavities and subdermal lymph sacs (Lee & Costanzo 1998).

5. ADAPTIVE AND INCIDENTAL ICE NUCLEATORS

Ice nucleators are sometimes probably part of a strategy of cold hardiness that is advantageous in the evolutionary selection process. These ice nucleators could be called *adaptive ice nucleators*. However, ice nucleators can also be found in organisms where freezing accrues no advantage. They can be described as *incidental ice nucleators*. An example of this is human low-density lipoproteins that induce nucleation at temperatures between −4.9 and −9.4 °C (J. G. Duman, K. E. Zachariassen and R. Lundheim, unpublished data). These proteins play other important roles in the organisms but their ability to induce ice nucleation is incidental. It is often difficult to reveal the selective advantage of the ice nucleators. If they are produced as a response to low temperatures, this may indicate their function in enhancing the cold tolerance of the species and they are thus adaptive. However, there is still a possibility that the nucleation is incidental and reflects temperature-dependent synthesis or biochemical modification of structures that primarily have functions other than ice nucleation.

It can be difficult to evaluate whether the presence of an ice nucleator is part of a cryoprotection strategy. Ice nucleators active at temperatures above −7 °C are found in both freeze-tolerant and freeze-susceptible organisms (Duman *et al.* 1995). There are also several examples showing that ice nucleation at high sub-zero temperatures is not a requirement for freeze tolerance (Miller 1978; Ring 1982). Further, if a relatively potent ice nucleator is present in a freeze-tolerant organism it cannot be assumed that the ice nucleator is required to maintain freeze tolerance. Organisms that tolerate freezing can have incidental ice nucleators in the body fluids, since the nucleators do not harm the organisms even if they initiate freezing. There is thus no selection pressure against them. An example of this is found in the freeze-tolerant frog *Rana sylvatica*. Lee & Costanzo (1998) argue against an adaptive significance of the ice nucleators found in the blood of this frog because

(i) nucleators with a similar activity (−7 to −8 °C) are also found in freeze-sensitive animals;
(ii) some freeze-tolerant species lack such nucleators;
(iii) the nucleators in the blood have a lower activity than some of the frog's tissues;
(iv) the nucleation temperatures are far higher in intact animals than those of the blood;
(v) the nucleation temperatures of these nucleators found in the blood are lower than the lethal temperature of the frog.

This list of arguments supports the view that freeze-tolerant vertebrates can tolerate ice nucleators in their blood but, because they are not necessarily needed for freeze tolerance, they are probably incidental nucleators.

Bacterial ice nucleators are probably adaptive, e.g. by giving the bacteria access to nutrients (Buttner & Amy 1989) and, possibly, by protecting the bacteria against freeze injuries (Lindeman & Suslow 1987). The role of ice nucleators in regulating osmotic and mechanical stress during freezing is probably very important (Zachariassen 1992). Another adaptive function of ice nucleators might be to improve the water balance of organisms, by inducing freezing of the body fluids and thereby reducing the vapour pressure so that evaporative water loss is reduced (Lundheim & Zachariassen 1993). The possible uptake of water from the air may also be enhanced by a similar mechanism in lichen (Kieft 1988). As discussed by Duman *et al.* (1995), ice formation often leads to a pronounced reduction in metabolism. By inducing freezing, ice nucleators can therefore contribute to energy saving during the winter.

Nucleators found in freeze-susceptible organisms are clearly incidental since they cause the death of the organism when they nucleate.

6. IS NUCLEATION AT AN OPTIMAL TEMPERATURE THE ONLY FUNCTION OF ICE NUCLEATORS?

Duman *et al.* (1992) emphasized that insect ice nucleators generally initiate nucleation at temperatures between −6 and −10 °C, while bacteria produce ice nucleators that initiate freezing at higher temperatures (−2 to −5 °C). They explained this by referring to Farrant (1980) who reviewed effects of slow and fast cooling on freezing tolerance of cells. Both slow and fast cooling may damage cells, whereas an intermediate cooling rate seems to be optimal. By nucleating at a moderate temperature, the organism can optimize the rate of initial freezing when the body temperature is brought to equilibrium with the melting point of the body fluids. The thermal gradient between body fluids and the environment during freezing is also dependent on nucleation temperatures. An organism nucleating when the environmental (and body) temperature reaches −3 °C will freeze at a slower rate than an organism that nucleates at −10 °C. By selecting an optimal nucleation temperature, the rate of freezing can be optimized. The question then arises: does an optimal freezing rate exist, common to freeze-tolerant organisms? If this is the case, it could explain why freeze-tolerant organisms commonly nucleate at temperatures lower than those of bacterial nucleators. Heat conductance and specific heat capacity also influence the rate of freezing. Even if the nucleation temperatures of mussels (Lundheim 1997*b*) and many freeze-tolerant beetles (Zachariassen 1980) are similar, freezing rates are probably very different in the two types of organisms. The surface to volume ratio of most mussels is much lower than that of insects. Mussels also contain mantle fluid that will freeze and increase the thermal buffer capacity. This will reduce the freezing rate. When a blue mussel freezes at an ambient temperature of −10 °C, it will take several hours for the mussel to reach the environmental temperature (Williams 1970). The small insects will often be in thermal equilibrium with the surrounding air after a shorter time. The initial freezing rate (when the water temperature is increased to the melting point) is probably relatively similar in organisms with the same degree of supercooling prior to nucleation, but as the example with mussels and insects demonstrates, during the second phase the freezing rate is dependent on other factors in addition to nucleation temperature.

The effect of ice crystal shape and size on freezing damage may be another reason for selecting nucleators active between −6 and −10 °C. Even if the relationship between

nucleation temperatures, freezing rate and solute concentration is complex, a general observation is that nucleation at relatively high temperatures produces large and sparse ice crystals. Freezing at lower temperatures gives more and smaller crystals (Knight 1967). Mechanical damage from the expanding ice may be an important mechanism of freeze injury (Mazur 1984). In such cases, the shape of crystals may be important. The shape of the ice crystals may also be important for the ability of the crystal to penetrate cell membranes or to grow through water-filled channels in the cell membrane. According to the Kelvin equation, an ice crystal with a radius of 20 Å has a melting point of −15 °C (Mazur 1984). If the pores in the cell membrane are smaller than this, ice crystals cannot penetrate the pores at this temperature.

7. BIOLOGICAL ICE NUCLEATION AND ENVIRONMENTAL TEMPERATURE

Among adult insects from interior Alaska, the strategy of freeze tolerance (as opposed to the freeze-avoiding strategy) is most frequently found in species hibernating whilst exposed to extreme cold. Among beetles, the freeze-tolerance is normally linked to high nucleation temperatures, indicating potent ice nucleators. Insects from other orders can be freeze-tolerant even with nucleation temperatures lower than −20 °C (Miller 1982). Only in a few studies have freeze-tolerant beetles with extremely low nucleation temperatures been found (Ring 1982). The general impression is that ice nucleators are probably enhancing freeze tolerance. In spite of this, seasonal variation in nucleation temperatures of freeze-tolerant insects is often low. Data from Gehrken (1988) show no pronounced effects of acclimation temperature on nucleation temperatures of the freeze-tolerant beetle *Mellasoma collaris* when the data are corrected for haemolymph osmolality. The freeze-tolerant beetle *Upis ceramboides* has relatively stable nucleation temperatures during the year (Miller 1978), while the freeze-tolerant beetle *Pterosticus brevicornis* has lower nucleation temperatures during the winter than during the summer. This reduction can only partially be accounted for by changes in haemolymph osmolality (Baust & Miller 1972). However, there are also reports on nucleator production, prior to, or during, winter. Duman (1980) found that the supercooling capacity (melting point minus organismal nucleation temperature) was smaller during the winter than during the rest of the year in the beetle *Dendroides canadensis*. This indicates a production of novel nucleators during the winter. A similar pattern is seen in a study by van der Laak (1982) when data are corrected for haemolymph osmolality. Zachariassen *et al.* (1982) acclimated larvae of the gallfly *Eurosta solidaginis* to different temperatures in the laboratory and found the highest concentrations of nucleators at +5 °C.

In vertebrate ectotherms, seasonal conditioning has little influence on supercooling (Costanzo & Lee 1995). However, Churchill & Storey (1992) reported an increase in nucleation temperatures of the garter snake *Thamnopis sirtalis* when acclimated to winter conditions.

Theede & Stein (1989) found that mussels (*Mytilus edulis*) acclimated to short-day and low temperatures during the summer produced nucleators, while mussels acclimated to long-day and high temperatures during the winter lost their haemolymph ice nucleators.

The haemolymph of the mussel, *M. edulis*, nucleates at higher temperatures in winter than in summer (Aunaas 1982*a*). Aunaas (1982*b*) also found higher nucleation temperatures of the haemolymph in the gastropod *Littorina littorea* during the winter than during the summer. The haemolymph nucleation temperatures of the intertidal gastropod *Melampus bidentatus* are *ca.* −7.5 °C in January and February, but below −11 °C in the summer months, indicating the seasonal occurrence of ice nucleators (Loomis 1985). The production of these nucleators was induced in the laboratory by low temperature. The photoperiod did not seem to be important for the production of the nucleator. The results indicated that another factor besides environmental temperature was influencing the nucleation temperature of animals in the field (Loomis & Hayes 1987).

The ice nucleators of bacteria are influenced by temperature. A shift from high (more than 30 °C) to low (e.g. 5 °C) incubation temperatures induces the appearance of active (type 1) ice nucleators. After transfer from low to high temperatures, the nucleators disappear (Rogers *et al.* 1987; Nemecek-Marshall *et al.* 1993).

The nucleation temperatures of leaves of different species of the rosette plant *Espeletia* in the Andes mountains are correlated with altitude (and environmental temperature) (Goldstein *et al.* 1985; Rada *et al.* 1987). The nucleation temperatures are reduced with altitude and the lowest tolerable temperatures of the leaves are close to the nucleation temperature, indicating a removal of nucleators to promote survival by supercooling. A similar pattern has been found in seaweed from the littoral zone, where the species living highest on the shore (most exposed to low air temperatures during winter) have lower nucleation temperatures than those living low on the shore protected by the relatively warm seawater (Lundheim 1997*a*).

In controlled laboratory studies, Brush *et al.* (1994) compared winter rye (*Secale sereale*) grown under short-day and long-day conditions. The threshold nucleation temperatures of leaf extracts remained relatively constant, while the number of nucleators per cell was higher in short-day plants. No effect of growth temperature was found on threshold ice-nucleation temperatures, but the number of ice nucleators increased at low temperature.

The apparent surplus of nucleators with virtually identical nucleation temperatures in freeze-tolerant animals is remarkable. The haemolymph of larvae of the beetle *Pytho depressus* can be diluted by a factor of 10^4 before the nucleation temperatures are significantly lower than that of the 10% haemolymph. It must be diluted by a factor of 10^8 before the nucleation temperature is approaching that of a 0.9% NaCl solution (figure 1). To induce ice formation at an optimal temperature, only one active nucleation site would be sufficient. Why are so many nucleators produced? One explanation may be that a relatively high concentration of individual nucleator molecules is needed to form an aggregate of the size needed for nucleation at the optimal temperature.

An explanation of why nucleators are present in surplus may be that, instead of creating a new high-molecular-weight ice nucleator protein, the structure of

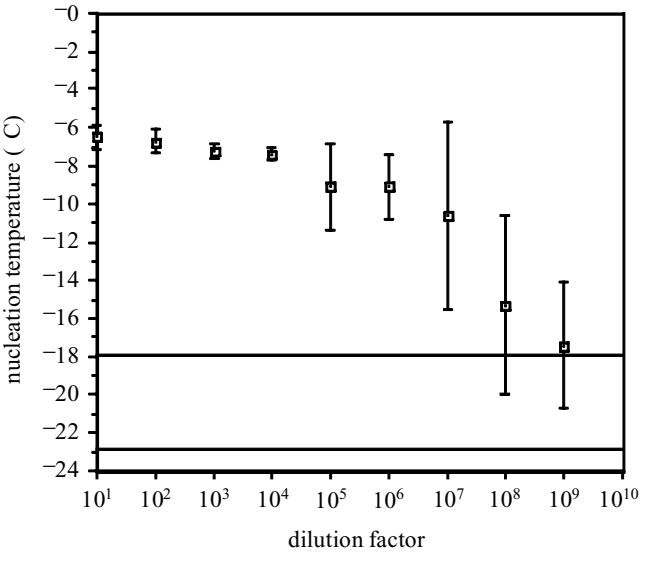

Figure 1. Nucleation temperatures of tenfold dilutions of hemolymph from *Pytho depressus* larvae. Each data point represents 10 parallel 5 μl samples. The samples were treated as described by Zachariassen *et al.* (1982). The vertical error bars indicate standard deviation. The horizontal lines indicate the 95% confidence interval of the nucleation temperatures of the 0.9% NaCl solution used for the dilution.

existing molecules has been evolutionarily adjusted to act as a nucleator, together with other functions. This may be the case for the *Tipula* lipoprotein ice nucleator that may have a function as a lipid shuttle in addition to its function as a nucleator (Duman *et al.* 1985). Substances that have a nucleating capacity will not have to be removed by freeze-tolerant species and they may be used as adaptive nucleators if they are produced extracellularly. Blood plasma ice nucleators with nucleation temperatures higher than −7 °C have been reported for freeze-susceptible ectotherms. Even the blood plasma of the endothermic *Mus musculus* contains ice nucleators capable of nucleating at −8.1 °C (Costanzo & Lee 1995). This shows that relatively potent ice nucleators may be present in organisms where nucleation has no adaptive function. Biomolecules that are convenient for use as ice nucleators may therefore often exist before an evolutionary development towards freeze tolerance. As mentioned previously, human low-density lipoproteins and lipoproteins from the cranefly *Tipula trivittata* act as nucleators. Lipoproteins from the freeze-sensitive beetle *Ceruchus piceus* also act as ice nucleators and are removed before winter (Neven *et al.* 1986; Xu *et al.* 1990). A structure frequently found in lipoproteins may possibly induce ice nucleation. However, not all lipoproteins act as ice nucleators; lipoproteins from the hawk moth *Manduca sexta* do not show ice-nucleating activity (Duman *et al.* 1992).

We have demonstrated that ice nucleators can be found in species that never freeze. In these species, the incidental nucleators probably have other important functions not related to freezing. Such incidental nucleators could have been the basis for the development of the potent ice nucleators that are often seen in freeze-tolerant organisms. It is difficult to reveal whether an ice nucleator is incidental or adaptive. In freeze-avoiding organisms, there is a strong selection pressure against nucleators, while nucleators can be tolerated in freeze-tolerant species. However, for reasons that have been mentioned in § 4, ice nucleation is probably not only tolerated but also often a requirement for the freeze tolerance of many organisms.

REFERENCES

Arny, D. C., Lindow, S. E. & Upper, C. D. 1976 Frost sensitivity of corn increased by application of *Pseudomonas syringae*. *Nature* **262**, 282–284.

Aunaas, T. 1982*a* Nucleating agents in the haemolymph of an intertidal mollusc tolerant to freezing. *Experientia* **38**, 1456–1457.

Aunaas, T. 1982*b* Nucleating agents in the haemolymph of intertidal invertebrates tolerant to freezing. *Cryo. Lett.* **3**, 287.

Baust, J. G. & Miller, L. K. 1972 Variations in glycerol content and its influence on cold hardiness in the Alaskan carabidae beetle, *Pterosticus brevicornis*. *J. Insect Physiol.* **16**, 979–990.

Brush, R. A., Griffith, M. & Mlynarz, A. 1994 Characterization and quantification of intrinsic ice nucleators in winter rye (*Secale cereale*) leaves. *Plant Physiol.* **104**, 725–735.

Buttner, M. P. & Amy, P. S. 1989 Survival of ice nucleating-active and genetically engineered non-ice nucleating *Pseudomonas syringae* strains after freezing. *Appl. Environ. Microbiol.* **55**, 1690–1694.

Churchill, T. A. & Storey, K. B. 1992 Freezing survival of the garter snake *Thamnopis sirtalis parietalis*. *Can. J. Zool.* **70**, 99–105.

Costanzo, J. P. & Lee, R. E. 1995 Supercooling and ice nucleation in vertebrate ectotherms. In *Biological ice nucleation and its applications*. (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 221–237. St Paul, MN: APS Press.

Costanzo, J. P., Claussen, D. L. & Lee, R. E. 1988 Natural freeze tolerance in a reptile. *Cryo. Lett.* **9**, 380–385.

Duman, J. G. 1980 Factors involved in the overwintering survival of the freeze tolerant beetle, *Dendroides canadensis*. *J. Comp. Physiol.* **136**, 53–59.

Duman, J. G. & Patterson, J. L. 1978 The role of ice nucleators in the frost tolerance of overwintering queens of the bald faced hornet. *Comp. Biochem. Physiol.* A **59**, 69–72.

Duman, J. G., Morris, J. P. & Castellino, F. J. 1984 Purification and composition of an ice nucleating protein from queens of the hornet *Vespula maculata*. *J. Comp. Physiol.* B **154**, 79–83.

Duman, J. G., Neven, L. G., Beals, J. M., Olson, K. O. & Castellino, F. J. 1985 Freeze tolerance adaptations, including hemolymph protein and lipoprotein ice nucleators, in larvae of the cranefly *Tipula trivittata*. *J. Insect Physiol.* **31**, 1–9.

Duman, J. G., Wu, D. W., Yeung, K. L. & Wolf, E. E. 1992 Hemolymph proteins involved in the cold tolerance of terrestrial arthropods: antifreeze and ice nucleator proteins. In *Water and life. Comparative analysis of water relationships at the organismic, cellular and molecular level* (ed. G. N. Somero, C. B. Osmond & C. L. Bolis), pp. 282–300. Berlin: Springer.

Duman, J. G., Olsen, T. M., Yeung, K. L. & Jerva, F. 1995 The role of ice nucleators in cold tolerant invertebrates. In *Biological ice nucleation and its applications* (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 201–219. St Paul, MN: APS Press.

Fall, R. & Wolber, P. K. 1995 Biochemistry of bacterial ice nuclei. In *Biological ice nucleation and its applications* (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 63–83. St Paul, MN: APS Press.

Farrant, J. 1980 General observations on cell preservation. In *Low temperature preservation in biology and medicine* (ed. M.

J. Ashwood-Smith & J. Farrant), pp. 1–8. Baltimore, OH: University Park Press.

Gavish, M., Popoviz-Biro, R., Lahav, M. & Leiserowiz, L. 1990 Ice nucleation of alcohols arranged in monolayers at the surface of water drops. *Science* **250**, 973–975.

Gehrken, U. 1988 Mechanisms involved in insect cold tolerance. PhD thesis, Department of Biology, University of Oslo, Norway.

Goldstein, G., Rada, F. & Azocar, A. 1985 Cold hardiness and supercooling along an altitudinal gradient in Andean giant rosette species. *Oecologia* **68**, 147–152.

Govindarajan, A. G. & Lindow, S. E. 1988 Size of bacterial ice-nucleation sites measured *in situ* by radiation inactivation analyses. *Proc. Natl Acad. Sci. USA* **85**, 1334–1338.

Gross, D. C., Proebsting Jr, E. L. & MacCrindle-Zimmerman, H. 1988 Development, distribution, and characteristics of intrinsic, nonbacterial ice nuclei in *Prunus* wood. *Plant Physiol.* **88**, 915–922.

Hasegawa, Y., Ishihara, Y. & Tokuyama, T. 1994 Characteristics of ice-nucleating activity in *Fusarium avenaceum* IFO-7158. *Biosci. Biotech. Biochem.* **58**, 2273–2274.

Hayes, D. R. & Loomis, S. H. 1985 Evidence for a proteinaceous ice nucleator in the hemolymph of the pulmonate gastropod, *Melampus bidentatus*. *Cryo. Lett.* **6**, 418–421.

Hirai, M. & Tsumuki, H. 1995 Characterization and localization of the ice-nucleating active agents in larvae of the rice stem borer *Chilo suppressalis*. *Cryobiology* **32**, 209–214.

Hochachka, P. V. & Somero, G. N. 1984 *Biochemical adaptation*. Princeton University Press.

Kajava, A. V. & Lindow, S. E. 1993 A model of the three-dimensional structure of ice nucleation proteins. *J. Mol. Biol.* **232**, 709–717.

Kieft, T. L. 1988 Ice nucleation activity in lichens. *Appl. Environ. Microbiol.* **54**, 1678–1681.

Kieft, T. L. & Ruscetti, T. 1990 Characterization of biological ice nuclei from a lichen. *J. Bacteriol.* **172**, 3519–3523.

Knight, C. A. 1967 *The freezing of supercooled liquids*. Princeton, NJ: D. Van Nostrand Company Inc.

Lee, R. E. & Costanzo, J. P. 1998 Biological ice nucleation and ice distribution in cold-hardy ectothermic animals. *A. Rev. Physiol.* **60**, 55–72.

Lee, R. E., Mugnano, J. A. & Taylor, R. T. 1992 Endogenous crystalloid spheres regulate supercooling point of the gall fly *Eurosta solidaginis*. *Cryobiology* **29**, 750–751.

Lindeman, J. & Suslow, T. V. 1987 Competition between ice nucleation-active wild type and ice nucleation-deficient deletion mutant strains of *Pseudomonas syringae* and *P. fluorescens* Biovar I and biological control of frost injury on strawberry blossoms. *Phytopathology* **77**, 882–886.

Loomis, S. H. 1985 Seasonal changes in the freezing tolerance of the intertidal pulmonate gastropod *Melampus bidentatus*. *Can. J. Zool.* **63**, 2021–2025.

Loomis, S. H. & Hayes, D. R. 1987 Effects of temperature, food deprivation and photoperiod on the cold tolerance of the intertidal pulmonate gastropod, *Melampus bidentatus*. *Cryo. Lett.* **8**, 25–34.

Lundheim, R. 1997*a* Ice nucleation in seaweeds in relation to vertical zonation. *J. Phycol.* **33**, 739–742.

Lundheim, R. 1997*b* Ice nucleation in the blue mussel (*Mytilus edulis*). *Mar. Biol.* **128**, 267–271.

Lundheim, R. 1997*c* Adaptive and incidental biological ice nucleators. PhD thesis, The Norwegian University of Science and Technology, Trondheim.

Lundheim, R. & Zachariassen, K. E. 1993 Water balance of over-wintering beetles in relation to strategies for cold tolerance. *J. Comp. Physiol.* B **163**, 1–4.

Lundheim, R. & Zachariassen, K. E. 1999 Applications of biological ice nucleators. In *Biotechnological applications of cold-adapted organisms* (ed. R. Margesin & F. Schinner), pp. 309–318. Berlin: Springer.

Madison, D. L., Scrofano, M. M., Ireland, R. C. & Loomis, S. H. 1991 Purification and partial characterization of an ice nucleator protein from the intertidal gastropod *Melampus bidentatus*. *Cryobiology* **28**, 483–490.

Maki, L. R., Gaylan, E. L., Chang-Chien, M. & Caldwell, D. R. 1974 Ice nucleation induced by *Pseudomonas syringae*. *Appl. Microbiol.* **28**, 456–459.

Mazur, P. 1984 Freezing of living cells: mechanisms and implications. *Am. J. Physiol.* C **247**, 125–142.

Miller, L. K. 1978 Physical and chemical changes associated with seasonal alterations in freezing tolerance in the adult northern tenebrionid *Upis ceramboides*. *J. Insect Physiol.* **24**, 791–796.

Miller, L. K. 1982 Cold hardiness strategies of some adult and immature insects overwintering in interior Alaska. *Comp. Biochem. Physiol.* A **73**, 595–604.

Mizuno, H. 1989 Prediction of the conformation of the ice nucleation protein by conformational energy calculation. *Proteins* **5**, 47–65.

Nemecek-Marshall, M., LaDuca, R. & Fall, R. 1993 High level expression of ice nuclei in a *Pseudomonas syringae* strain is induced by nutrient limitation and low temperature. *J. Bacteriol.* **175**, 4062–4070.

Neven, L. G., Duman, J. G., Beals, J. M. & Castellino, F. J. 1986 Overwintering adaptations of the stag beetle, *Ceruchus piceus*: removal of ice nucleators in winter to promote supercooling. *J. Comp. Physiol.* **156**, 707–716.

Neven, L. G., Duman, J. G., Low, M. G., Sehl, L. C. & Castellino, F. J. 1989 Purification and characterization of an insect hemolymph lipoprotein ice nucleator: evidence for the importance of phosphatidylinositol and apolipoprotein in the ice nucleator activity. *J. Comp. Physiol.* **159**, 71–82.

Obata, H., Takinami, K., Tanishita, K., Hasegawa, Y., Kawate, S., Tokuyama, T. & Ueno, T. 1990 Identification of a new ice-nucleating bacterium and its ice nucleating properties. *Agric. Biol. Chem.* **54**, 725–730.

Pouleur, S., Richard, C., Martin, J. G. & Antoun, H. 1992 Ice nucleation activity in *Fusarium acuminatum* and *Fusarium avenaceum*. *Appl. Environ. Microbiol.* **58**, 2960–2964.

Rada, F., Goldstein, G., Azocar, A. & Torres, F. 1987 Supercooling along an altitudinal gradient in *Espeletia schultzii*, a caulescent giant rosette species. *J. Exp. Bot.* **38**, 491–497.

Ring, R. A. 1982 Freezing tolerant insects with low supercooling points. *Comp. Biochem. Physiol.* A **73**, 605–612.

Rogers, J. S., Stall, R. E. & Burke, J. M. 1987 Low temperature conditioning of the ice nucleation active bacterium, *Erwinia herbicola*. *Cryobiology* **24**, 270–279.

Storey, K. B., Baust, J. G. & Wolanczyk, J. P. 1992 Biochemical modification of plasma ice nucleation activity in a freeze-tolerant frog. *Cryobiology* **29**, 374–384.

Theede, H. & Stein, U. 1989 Further studies on frost protection in *Mytilus edulis* from the western Baltic Sea. In *Proc. 21st EMBS Gdansk 14-19 September 1986*, pp. 173–181. Gdansk: Polish Academy of Sciences—Institute of Oceanology.

Turner, M. A., Arellano, F. & Kozloff, L. M. 1991 Components of ice nucleation structures in bacteria. *J. Bacteriol.* **173**, 6515–6527.

Tursman, D., Duman, J. G. & Knight, C. A. 1994 Freeze tolerance adaptations in the centipede *Lithobius forficatus*. *J. Exp. Zool.* **268**, 347–353.

Upper, C. D. & Vali, G. 1995 The discovery of bacterial ice nucleation and its role in the injury of plants by frost. In *Biological ice nucleation and its applications* (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 29–39. St Paul, MN: APS Press.

van der Laak, S. 1982 Physiological adaptation to low tempera-

ture in freeze-tolerant *Phyllodecta laticollis* beetles. *Comp. Biochem. Physiol.* A **73**, 613–620.
Warren, G. 1995 Identification and analysis of *ina* genes and proteins. In *Biological ice nucleation and its applications* (ed. R. E. Lee Jr, G. J. Warren & L. V. Gusta), pp. 85–99. St Paul, MN: APS Press.
Warren, G., Corotto, L. & Wolber, P. 1986 Conserved repeats in diverged ice nucleation structural genes from two species of *Pseudomonas*. *Nucleic Acids Res.* **14**, 8047–8060.
Westh, P., Kristiansen, J. & Hvidt, A. 1991 Ice nucleating activity in the freeze tolerant tardigrade *Adorybiotus conifer*. *Comp. Biochem. Physiol.* A **99**, 401–404.
Williams, R. J. 1970 Freezing tolerance in *Mytilus edulis*. *Comp. Biochem. Physiol.* **35**, 145–161.
Wolanczyk, J. P., Storey, K. B. & Baust, J. G. 1990 Ice nucleating activity in the blood of the freeze-tolerant frog, *Rana sylvatica*. *Cryobiology* **27**, 328–335.
Xu, L., Neven, L. G. & Duman, J. G. 1990 Hormonal control of hemolymph lipoprotein ice nucleators in overwintering freeze susceptible larvae of the stag beetle, *Ceruchus piceus*: adipokinetic hormone and juvenile hormone. *J. Comp. Physiol.* **160**, 51–59.
Zachariassen, K. E. 1980 The role of polyols and nucleating agents in cold-hardy beetles. *J. Comp. Physiol.* **140**, 227–234.
Zachariassen, K. E. 1985 Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799–832.
Zachariassen, K. E. 1992 Ice nucleating agents in cold hardy insects. In *Water and life. Comparative analysis of water relationships at the organismic, cellular and molecular level* (ed. G. Somero, C. B. Osmond & C. L. Bolis), pp. 262–281. Berlin: Springer.
Zachariassen, K. E. & Hammel, H. T. 1976 Nucleating agents in the haemolymph of insects tolerant to freezing. *Nature* **262**, 285–287.
Zachariassen, K. E., Baust, J. G. & Lee Jr, R. E. 1982 A method for quantitative determination of ice nucleating agents in insect hemolymph. *Cryobiology* **19**, 180–184.

Discussion

J. Laybourn-Parry (*School of Life and Environmental Sciences, University of Nottingham, Nottingham, UK*). These bacteria, that produce these ice nucleators, that then damage the tissue of the plant: it looked as if the ice nucleator was on the surface of the bacteria but do they actually secrete them into the plant?

R. Lundheim. No. As a normal rule these ice nucleators are only active when they are attached to the surface or membrane. So they need to be at the surface, in close contact with the water either inside or on the surface of the plant and induce the freezing, and when the freezing starts it will spread over a large area.

J. Laybourn-Parry. At which point it starts producing its enzymes for breaking down the carbon and taking it up, presumably, so it is all part of a mechanism of feeding.

R. Lundheim. That is one possible explanation for it.

D. Hall (*Multi-Disciplininary Research and Innovation Centre, North East Wales Institute, Wrexham, UK*). It seems to me that when you are talking about nucleation-controlled systems, the number of nuclei is going to govern, to some extent, the size of the crystals you actually end up with. In other words, the more nuclei you create the smaller the crystals. Now, I would imagine, rightly or wrongly, that cells would prefer small crystals to avoid damage than big ones. I do not know whether that is true or not but I would imagine that would be the case. Have you any information on the rate of nucleation or the numbers of nuclei formed? It seems to me to be quite an important parameter in this business.

R. Lundheim. I do not know whether this is an answer to your question, but what we see is that bacteria, which possibly try to induce damage to their host, produce very potent ice nucleators. Insects, however, which produce ice nucleators as a part of their cryoprotection, produce ice nucleators of medium activity. Maybe insects are balancing different effects, smaller ice crystals but still not the vigorous osmotic rearrangement that you would get if you waited even longer before you froze.

Anon. In the case of this frog, the nucleation starts as a very different position and it seems to me that the nucleation material which is inducing these phenomena is equally distributed, so there is maybe a specific expression mechanism for these materials, for these molecules and these substances. Do you know something about that?

R. Lundheim. No, I do not know if the structure responsible for the nucleation of the intact frog has been identified. If the only point with the ice nucleators is to ensure extracellular freezing, one nucleator would be sufficient. If this nucleator was situated intracellularly the cell would probably be destroyed. However, the loss of one cell could probably be tolerated. It is still puzzling that organisms produce so many nucleators. I did not show this, but you can dilute the haemolymph from insects by at least 1 : 10 000, and we have done this with several species before there is a marked effect on the nucleation temperature. Even 1 : 1 000 000 dilutions still have fairly good ice-nucleation activity. In theory, one nucleator should be enough to ensure extracellular freezing. So I think either these nucleators are just proteins that are doing something else and have been slightly modified to act as nucleators, or there is another reason for having all these ice active molecules in their blood. Maybe ice nucleators can also affect the way ice grows after nucleation, this is speculative but that could be a reason for having all these nucleators.

C. Gerday (*Laboratory of Biochemistry, University of Liège, Liège, Belgium*). Have you got any idea about the way the frog evenly distributes its thawing?

R. Lundheim. For thawing to take place there must be a temperature gradient in the frozen animal. Since the thawing seems to take place evenly throughout the body, one possibility is that there is also an osmotic gradient in the frozen animal. In this way thawing could take place evenly throughout the body and there would still be a thermal gradient to remove heat. This is, however, a speculative explanation. I have not worked with this frog myself. Much of the work on the freezing and thawing of this species has been done by Ken Storey and Boris Rubinsky.

GLOSSARY

PI: phosphatidyl inositol