CryoLetters 46(2), 67 – 73 (2025)

https://doi.org/10.54680/fr25210110112

PERSPECTIVE

STRATEGIES USED BY ORGANISMS TO SURVIVE VERY COLD CLIMATES – STUDENT'S GUIDE

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ABSTRACT

We briefly examine how cold-hardiness in general, including freeze-tolerance, freeze-avoidance and dehydration strategies allow survival in cold climates, through the eyes of some specific insects and fish. Strategies do vary with geography and latitude, even between two types of insects living in the same area. We look at ice nucleation proteins to enhance freezing and antifreeze proteins to help avoid ice formation or, in some cases, to hinder what is known as recrystallization, as a frozen organism thaws.

Keywords: cold-hardiness; freeze-tolerance; antifreeze; nucleation; supercooling.

INTRODUCTION

Polar fishes live in oceans which do not vary significantly in temperature and, in fact, are often at the freezing point of about -1.8 °C. Because of this, the need for some form of antifreeze to stop ice from growing inside the fishes is confined to a narrow temperature range and the required efficiency of the process is clear. Conversely, an insect living on, say, a rock wall in Norway cannot readily anticipate how cold it may get on a given day or night and the cold-hardiness, or cold-tolerance, must be viable over a much larger temperature range, often to well below -30 °C. There are generally two strategies (freezing-tolerance, freezing-avoidance) an insect may adopt, in order to avoid lethal freezing, and occasionally a mixture of both:

1. to tolerate the freezing process, at least extracellularly, and then to thaw out again when the weather warms. This may require the use of some form of antifreeze proteins (AFPs) to help in that thaw process and also ice nucleation proteins (INPs) to assist in deliberately freezing at high subzero temperatures. It is necessary to avoid deep supercooling and subsequent, rapid, ice growth, which may be uncontrollable. Supercooling is reducing the temperature of a liquid to below its freezing point without turning it into a solid. This occurs when there is no seed, or parent crystal, to form a crystal structure. It is common for water droplets in some types of clouds to be deeply supercooled, due to the water being very clean and there being no available nucleation site, often dust or mineral crystals.
2. to avoid freezing, by one of three methods:

a) to increase overall osmolality through loading up on, for instance, sugars or salts and thus lowering the melting point (m.p.) to below what will be encountered at that locale;

b) to use the AFP strategy to avoid ice forming and so enhance supercooling, i.e., the body fluids remain liquid below their m.p.. This is then complex, for if there is never any ice present, how do the AFPs do anything?

c) to lose much of the body water and so to dry out such there is no water to freeze, a method known as anhydrobiosis. Examples of organisms which utilize this strategy include tardigrades ('water bears'), nematodes, rotifers, springtails and some midge larvae.

This, then, is a story about Bruce the Norwegian bark beetle, Florence the Antarctic fish, Wendy the New Zealand weta and Bertie, an African midge larvae. Bruce lives on the side of a tree in Norway, at 1000 m altitude and 62° North. Wendy lives amongst a rocky outcrop on a tussocky hillside at 1000 m altitude in New Zealand at 45° South. A weta is like a big grasshopper, found only in New Zealand. Each insect must cope with the air temperature dropping to well below 0 °C for days at a time. Bertie lives in a very hot climate but is nonetheless a good example of drying out to survive. Bertie has even been on the Space Station (more later).

Figure 1. Bruce, the Norwegian bark beetle is capable of surviving cold winter conditions and can produce a remarkable, almost 8 °C, of thermal hysteresis. (Photo: Wilson)

We do not distinguish here between insect, arthropod, beetle or grasshopper. Neither Wendy nor Bruce can fly away, nor can they bury deep enough in the ground to avoid the cold. They each must cope with being isothermal with the outside world, i.e., as the air temperature changes, so too does their body temperature.

Bruce (Fig. 1) avoids freezing by a combination of increasing osmolality and making AFPs (also known as ice-binding proteins, IBPs, or thermal hysteresis proteins, THPs). Wendy (Fig. 2), on the other hand, tolerates being frozen solid in her extracellular spaces. In fact, she is the largest freeze-tolerant arthropod known. Each has body fluid known as hemolymph, the insect equivalent to blood. The amounts of solute, such as sugars and salts, determine the level of lowering of the freezing point below 0 °C. Actually, it is the m.p. which is lowered by having saltier, or more sugary, hemolymph. This is in the same way that icy roads in cities in high latitudes are dosed with salt, where the melting point is lowered so that, at say -4 °C, the water will remain liquid, a process known as colligative melting point depression.

Note here that polar fishes such as Florence "know" that the water temperature will not drop below -1.9 °C and so the cold-tolerance strategy can be more clear-cut than it may be for Bruce and Wendy. In fact, for much of the year, Florence will ingest and then harbor very small ice crystals which are stopped from growing further, strictly speaking giving her a mixed strategy, neither freeze-avoiding nor freeze-tolerant.

FREEZE AVOIDANCE

Freeze avoidance can be achieved in a variety of ways.

1. Bruce could load up on sugar or salt and lower his m.p. to below that which he is ever likely to be exposed to. But since the m.p. depression is, for sugars at least, 1.86 °C for every mole of solute, he may need to have hemolymph osmolality of 5 or 6 molar. This is physiologically very difficult for normal functioning of insects. This osmotic freeze avoidance is seen when an insect releases sugar alcohols (polyols) into its body fluids to lower the m.p.. Simple sugars, such as glucose, and quite commonly, trehalose, are also often accumulated as potential cryoprotectants in some arthropod species at low temperatures. Bruce could use osmotic freeze avoidance to survive extreme cold temperatures, the polyols thickening the blood and lowering the m.p..

2. Alternatively, Bruce may want to supercool, i.e., remain liquid even when the temperature is below the colligative m.p.. This would require there to be no good ice nucleating sites inside his gut (or hemolymph) and even if he could supercool to say -10 °C, it is a delicate, even dangerous, strategy, since this is known as a metastable state and he could freeze at any time, which would then be lethal. He may like to synthesis and utilize AFPs to aid supercooling by masking potential ice binding sites and so make it harder for ice to form.

FREEZE TOLERANCE

Freeze tolerance allows organisms to inhabit cold habitats, often at high elevations or high latitudes. For insects in environments with lots of ice nucleators (e.g., moist habitats) freeze-tolerance as a strategy may have the advantage over freeze avoidance (1). Kenneth Story, a specialist in the Canadian wood frog cold-hardiness, says “you can get a better niche in the world if you can freeze” (2).

Several animal species can survive up to 82% of their body water being frozen, despite the fact that ice formation can destroy tissues and kill other species readily. The most common of these freeze tolerant species are insects, but lizards, painted turtle hatchlings, garter snakes and wood frogs are also tolerant of freezing (3). Most freezing seems to be extracellular, however it is believed that a very few species freeze intracellularly, i.e., ice exists even inside the cells.

The first freeze tolerance was observed by Reaumur, in the early 1700s, in caterpillars and species from a minimum of six orders are now known to withstand freezing. Once frozen, some species are capable of surviving temperatures as cold as liquid nitrogen, -196 °C. Freeze-tolerant insects begin freezing at high sub-zero temperatures, assisted by INAs. These are often large proteins (INPs) with a repeating sequence which mimics the repeat spacing of ice and gathers many water molecules into an ice-like structure and initiates the freezing (extracellularly). Microbes in the guts of alpine insects, such as Wendy, are likely to also play a role in the ability to be freeze tolerant (4).

In the Southern Hemisphere, there is a larger proportion of freeze-tolerant species than the Northern Hemisphere. This may be due to unexpected cold snaps in summer. If they are freeze-tolerant, they do not have to clear ice nucleators and food from the gut to survive the cold snaps. In New Zealand, many insects survive freezing all year round, with 85% of the mountain invertebrates able to freeze when faced with temperatures below 0 °C. With these temperatures occurring potentially at any time of year, these insects are capable of coping with freezing. When the area warms up, they thaw and become mobile again (5).

Figure 2. Wendy, sitting atop a willing participant’s head (Photo: Wilson)

While ice formation can be manipulated through production of INAs and retention of gut contents by many freeze-tolerant insects, other species can only survive internal freezing if the ice is introduced by surrounding external ice. These species are limited to overwintering in moist habitats (6). One well known host for INAs is the bacterium *Pseudomonas syringae*, often used in the proprietary product Snowmax, used to seed snow on ski fields.

Some freeze tolerant species also make use of ice recrystallization-inhibiting proteins, which are often in fact AFPs which bind to ice and inhibit the migration of water from the melting smaller crystals to the larger growing ones.

Freeze tolerant organisms utilise INAs with the capability of initiating nucleation between -2°C - and -5°C, thus limiting supercooling. These higher temperatures create slow ice growth, limiting stress to tissues and forcing the ice formation to be restricted to the exterior of the cells (7). Ice particles have many sources; some are formed by the insects (endogenous ice) and others are in the environment (exogenous). The animals that use endogenous ice tend to freeze at temperatures lower than -6 °C, the ones that freeze between -2 °C and -6 °C tend to use exogenous ice from their environment. Bacteria and fungi are common sources of ice nucleation mechanisms and some species rely on these for a high freezing temperature. Ice microbes are common environmentally, causing ice formation in clouds and even on the surface of leaves. In most insects, the freezing is usually originated in the gut where the highest levels of ice activity is found. Symbiotic relationships are common between insects and the bacteria that inhabits them.

Figure 3. Wendy, having successfully thawed after a week spent frozen "solid" (Photo: Wilson)

Wendy, *Hemideina maori*, lives in environments that can fluctuate by 30 °C in a single day. Even in the middle of summer, hard frost is possible, meaning that the ability to tolerate freezing and thawing is essential. The region Wendy inhabits is inhospitable in winter, with winds blowing the snow off the land and icicles growing horizontally and temperatures falling below freezing. Wendy survives with almost all her body fluids frozen and in a state of semi-animation (8). Due to having ice nucleation activity in its gut all year round, Wendy is freeze tolerant all year round (Fig. 3). Not only are they the heaviest insects to survive that climate but they are the largest freeze tolerant insects.

Brent Sinclair states about the alpine weta, "You can freeze it and it thaws out fast – it takes about an hour, then they are up and running again after 30 to 60 min. They are often thirsty afterward". As water forms ice in the cavities between cells, solute concentration rises and cell water is drawn out. In general, then, animals that are freeze-tolerant also must tolerate some level of dehydration. Freezing at high temperatures assists in the survival of these animals as cells are pushed aside rather than pierced, due to slower ice formation. High amounts of trehalose are produced which in some way helps with shielding and preventing distortion and destruction of proteins. The tissues of the weta are resilient to cope with the freezing, thawing and refreezing process, with a lethal temperature of around -10°C. Ice formation begins in the hindgut and then spreads through the gut cells until it reaches the haemolymph. Osmotic dehydration may occur, but is not at sufficient levels to prevent freezing, and up to 82% of water is converted to ice when Wendy is held at a constant -5 °C.

Even vertebrates like frogs in the Arctic Circle can tolerate freezing. Wood frogs can survive as cold as -20 °C and being frozen for several months at a time. And yet other species of wood frog from North Carolina supercool to -13 °C. In the Canadian wood frog, *Rana sylvatica*, and other freeze-tolerant frogs, freezing begins with ice formation on the skin (10). When freezing is initiated, the heart rate of the frog increases and the liver initiates glycogenolysis, this liberates the stored glucose. The glucose is circulated as a cryoprotectant, osmotically dehydrating organs to confine most of the ice. Most of the glucose is reconstituted back into glycogen in the liver, some may be passed into urine and reabsorbed in the bladder. Urea can also be used as a cryoprotectant in some frogs. The skin of the frog gets frozen, then the ice enters the frog through its veins and arteries. After that, its brain freezes, and all its blood is pushed to the heart before that finally is frozen solid. MicroRNA rearranges the cells to prevent and limit damage from ice formation. The ice forms on the outside of cells and organs. The interior of cells even receives this glucose to prevent shrinking and bursting.

One of the few species to show the ability to survive intracellular freezing is the Antarctic nematode *Panagrolaimus davidi*. The formation of ice inside the cells seems to be limited to the cytosol, with ice formation in the other organelles avoided by a process much like osmotic dehydration. Conversely, *P. davidi* is freezing intolerant when they are free of surface water in air or liquid paraffin, and instead they supercool (11). If the ice it is in contact with is formed at a higher temperature than the freezing point of its internal liquids, it will choose to dehydrate over freezing. When they are frozen at high sub-zero temperatures, they freeze extracellularly (8). Dehydration is preferred when the medium they are in is freezing slowly. When the freezing is rapid, they survive by freezing including intracellular freezing.

THAWING

Surviving the thaw for Wendy is the reverse of freezing, beginning with the vital organs. The heart resumes beating before the animal has completely thawed. It is still unknown how the heart performs under hypoxia. Respiration and tissue perfusion return, then motor faculties and nerve excitability return after. In most freeze-tolerant organisms, faculties are restored within 24 h although it may take longer after more severe freezing (12). Freeze-tolerant species must face ice recrystallization, the process of small ice crystals regrouping to form larger crystals over time. Without attention, these crystals could become so large that they cause fatality of the organism they are in (13). Various freeze tolerant species have been discovered to have antifreeze proteins (AFPs). Their use of AFPs is to manage crystal size therefore preventing ice recrystallization during thawing.

ANHYDROBIOSIS

Anhydrobiosis is the ability some insects have to lose almost all water in their bodies. The insect is then in a state of equilibrium with air. The sleeping chironomid, *Polypedilum vanderplanki* (Bertie), is one insect that can survive such severe desiccation (Fig. 4). When anhydrobiosis occurs, trehalose is thought to replace (almost) all the water in the animal's tissues (2). Late embryogenesis abundant (LEA) proteins are then expressed to provide a molecular shielding for biological molecules. This protects against a collection of a mass of particles in the body or the breakdown of proteins in the body. However, Bertie doesn't utilise the strategy to prevent freezing, instead he uses it to survive extreme drought. Once the drought is over and the rain or water begins to return, the larvae rehydrate and revive. Thus, Bertie can survive extreme desiccation, but also, he can survive liquid helium temperatures, -262 ºC and as hot as +102 ºC.

Figure 4. Bertie, the African sleeping chironomid midge larvae has the ability to survive long periods dried out, and even to survive in space. (Photo: Wilson)

Tardigrades (water bears) also utilise anhydrobiosis and, like Bertie, can survive vacuum, radiation, anoxia, high temperatures (140ºC+) and high pressure (6000 atmospheres). In fact, tardigrades and Bertie can last in this dried-out form for up to 17 years, with rejuvenation taking as little as 1 h. Bertie can even survive in space! A joint Russian-Japan experiment had some Berties at the International Space Station (actually, outside it) for 18 months before returning them to Earth and reviving them in a lab in Tsukuba, Japan (14).

POLAR FISHES

Florence (Fig. 5), the Antarctic toothfish (*Dissostichus mawsoni*) is a large fish found in the freezing waters surrounding Antarctica, waters with a freezing point of -1.8 ° C. She lives her life isothermal with the water. Generally speaking, though, all fishes have a melting point of their body fluids of about -1.2 °C (15). So, her blood should freeze. Her species (and of course, other polar fish species) has adapted to these conditions and produce AFP's (in her case, they are actually antifreeze glycoproteins, AFGPs). These polypeptides are released from her pancreas and travel throughout her body, inhibiting the growth of ice, by a process known as thermal hysteresis (16). Basically, by changing the surface structure of the ice crystals and consequently lowering the local freezing point, but not by changing the colligative m.p., ice is stopped from growing (Figs. 6, 7). This prevents the ice from causing damage.

Cousins of Florence, in this case a naked dragonfish, *Gymnodraco acuticeps*, even use ice crystals on the ocean floor as shelter (Fig. 8)

Figure 5. Florence, the Antarctic cod, or toothfish can survive in ice-laden oceans. (Photo: Wilson).

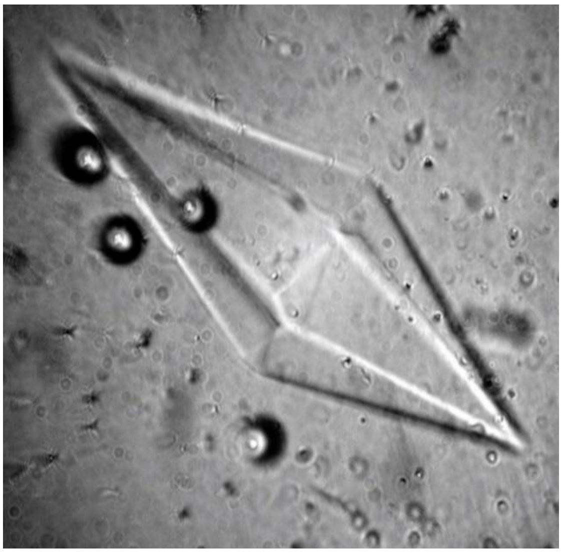

Figure 6. Florence turns ingested ice crystals into this hexagonal bipyramidal shape, making them stable and stopping further growth, preventing damage. Image taken on an Otago Osmometer nanoliter osmometer. (Photo: Wilson)

Figure 7. Insects which utilize AFPs (usually known as thermal hysteresis proteins [THP] when discussing insects) turn ice crystals into a lemon shape and growth then stops. This is then “more efficient” than fishes because the temperature will be colder and ice must be stopped from growing more effectively. (Photo: Wilson).

Figure 8. The naked dragonfish, *Gymnodraco acuticeps*, even use ice crystals on the ocean floor as shelter (Photo: Wilson).

CONCLUSIONS AND FUTURE DIRECTIONS

The ice-inhibition process is interesting to scientists on many fronts because it could have significant uses in our everyday lives. An early example of use is the adding of ice-structuring proteins to ice cream to keep it smooth when frozen, that is to avoid recrystallisation, as seen in Figure 9. Recrystallisation causes much damage to frozen foods during thawing and can be stopped by these proteins.

Recrystallisation also prevents successful thawing of frozen organs for storage. If we can learn how to replicate Florence’s survival strategies, we could apply them, for instance, to lifesaving organ transportation, impacting the lives of millions. Other applications might include stopping ice growth where it is unwanted. The many examples of this include ice build-up on power lines, on ship superstructure and on wind turbines.

For many years the use of antifreeze proteins has been mooted for a huge variety of uses. Examples include stopping ice forming on airplane wings, genetically modifying salmon so they can be farmed in colder, ice-laden waters and allowing the more successful freezing and thawing of bread.

In conclusion, we can expect many innovative industrial and medical applications of cold tolerance mechanisms in the future based on a greater understanding of nature.

Figure 9. Some ice creams today use what are now called “ice-structuring proteins” to keep the ice crystals from recrystallising and so keep the ice cream smooth. *Note that the authors are not supported by this proprietary brand and are not necessarily recommending it.*

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