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Supercooling and Freezing Tolerant Animals

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1. Introduction

Subzero temperatures may adversely affect animals by their direct lethal effects and by the damage caused by ice formation (Ramløv, 2000). Animals deal with the latter by three basic strategies. Freeze avoiding animals prevent ice formation in their bodies and supercool, keeping their body fluids liquid at temperatures below their melting point, but die if freezing occurs. In contrast, freezing tolerant animals can survive ice forming inside their bodies (Lee, 2010). Although both these categories of cold tolerance have been further subdivided (Bale, 1993; Sinclair, 1999) freeze avoidance and freezing tolerance are still recognised as fundamental cold tolerance strategies (Wharton, 2011a). In the third mechanism, cryoprotective dehydration which is found mainly in soil-dwelling animals, the body fluids remain unfrozen whilst surrounded by frozen soil. Since ice has a lower vapour pressure than liquid water at the same temperature the animal dehydrates and lowers the melting point of its body fluids, thus preventing freezing (Lee, 2010). In this review I examine the role of ice nucleation and supercooling in the main groups of freezing tolerant animals.

2. Freezing tolerant animals

Freezing tolerance has been most extensively studied in insects. It has been demonstrated in six insect orders, in which it appears to have evolved independently (Sinclair et al., 2003). Freeze tolerance is the dominant cold tolerance strategy in Southern Hemisphere insects, being found in 77% of cold hardy Southern Hemisphere insects (Sinclair & Chown, 2005). Amongst non-insect arthropods, however, freeze avoidance is the dominant cold tolerance strategy and freezing tolerance has only been demonstrated in a single species of centipede (Tursman et al., 1994), in an aquatic subterranean crustacean (Issartel et al., 2006) and in intertidal barnacles (Storey & Storey, 1988).

In nematodes, *Panagrolaimus davidi* from Antarctica is freezing tolerant and has survived temperatures down to -80°C (Wharton, 2011a). Other species of nematodes seem to have more modest cold tolerance abilities (Smith et al., 2008), although some have a small amount of freezing tolerance (Hayashi & Wharton, 2011). Tardigrades are thought to be freezing tolerant (Hengherr et al., 2009), although the role played by inoculative freezing and whether ice formation is intracellular or extracellular is yet to be determined in this phylum.

Some rotifers can survive at very low temperatures (Newsham et al., 2006) but their cold tolerance mechanisms have not been studied.

Some intertidal molluscs have an ability to tolerate freezing, at least in a part of their tissues. This depends upon inoculative freezing from the surrounding seawater, the seasonal production of INAs and the proportion of tissue that is frozen (Ansart & Vernon, 2003). Most earthworms avoid freezing by migrating to deeper soil layers during winter and thus avoiding contact with frozen soil. However, some species permanently inhabit the surface layers of the soil and if frost occurs they must survive contact with ice. A few earthworm species are freezing tolerant and, although some can remain unfrozen at -1°C, appear to rely on inoculative freezing from the surrounding soil to ensure freezing at a high subzero temperature (Holmstrup, 2003; Holmstrup & Overgaard, 2007).

Amongst vertebrates, several species of North American and European frogs are freezing tolerant and this cold tolerance strategy appears to have evolved several times amongst anurans (Voituron et al., 2009). Freezing tolerance has also been reported in the Siberian salamander (Berman et al., 1984) and in a single species of frog from the Southern Hemisphere (Bazin et al., 2007). In reptiles, hatchling turtles that overwinter in terrestrial hibernacula (the nest in which they were born) have the ability to tolerate freezing but the role this plays under natural conditions has been the subject of debate (Costanzo et al., 2008;

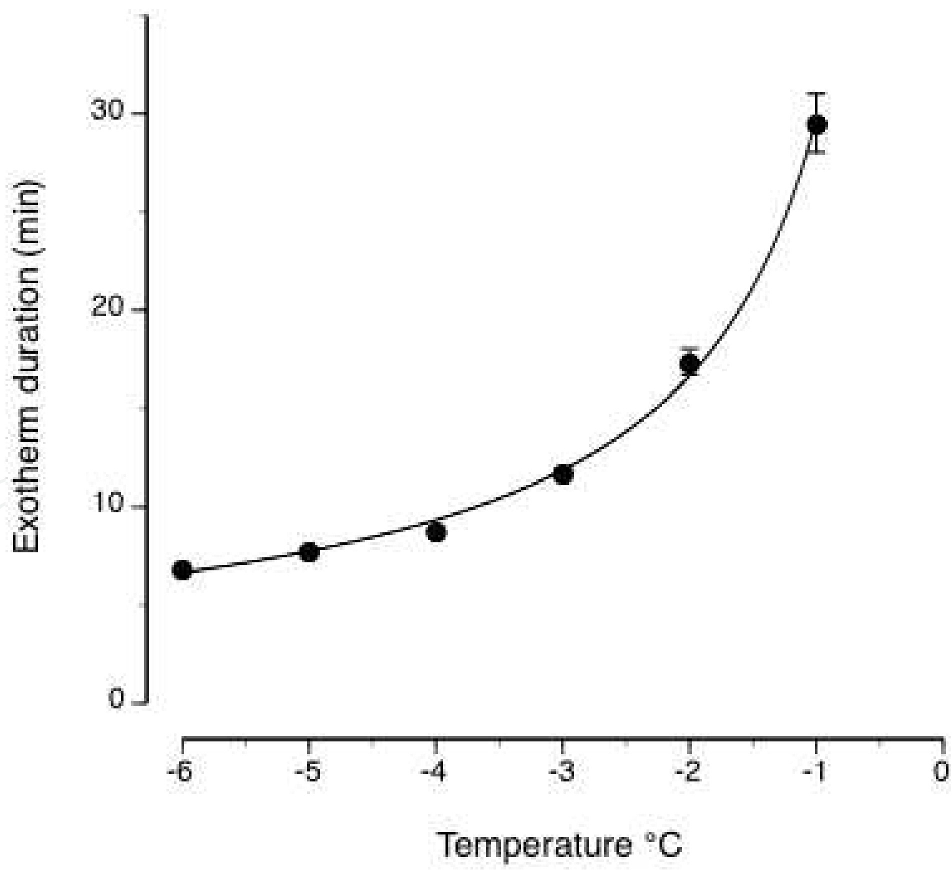

Fig. 1. The effect of temperature on the exotherm duration of 10µl suspensions of the Antarctic nematode *Panagrolaimus davidi*. N = 4, bars are standard errors. The line is the fit to the equation f(x) = exp(3.39) * ($x^{-0.83}$), R^2 = 0.996. Redrawn from (Wharton et al., 2002).

Packard & Packard, 2004). Some species of lizards and snakes can survive partial freezing of their bodies but die once a critical level of ice is exceeded (Storey, 2006). The European common lizard, *Lacerta vivipara*, can survive 50% of its body water freezing for at least 24 h, which is an ecologically-relevant level of freezing tolerance (Voituron et al., 2002). No fish, mammal or bird has been reported to survive more than a small amount of their body fluids freezing.

3. Most freezing tolerant animals limit supercooling

In contrast to freeze avoiding animals (see Ramløv, this volume), most freezing tolerant animals prevent extensive supercooling and encourage ice formation at a high subzero temperature. A clue to the reason why they do this can be seen from the relationship between the rate of ice formation and temperature. Figure 1 shows that the rate at which water freezes (or in this case a suspension of nematodes in water; with the duration of the exotherm produced during the freezing of the sample used as a measure of the freezing rate) decreases dramatically as the temperature approaches its melting point. Figure 2 compares exotherms in 50µl of nematode suspension where freezing was initiated at -1.3°C and -11.0°C. The exotherm duration is about 4.5 times longer when freezing is initiated at -1.3°C than at -11°C. Perhaps of more significance is that at -1.3°C the temperature becomes elevated to the melting point of the suspension (-0.3°C) and remains there until the freezing process is completed, indicating that the spread of ice through the sample is slow. At -11°C the temperature fails to reach the melting point of the suspension and declines rapidly from the maximum temperature reached (-0.9°C), indicating the rapid spread of ice through the sample.

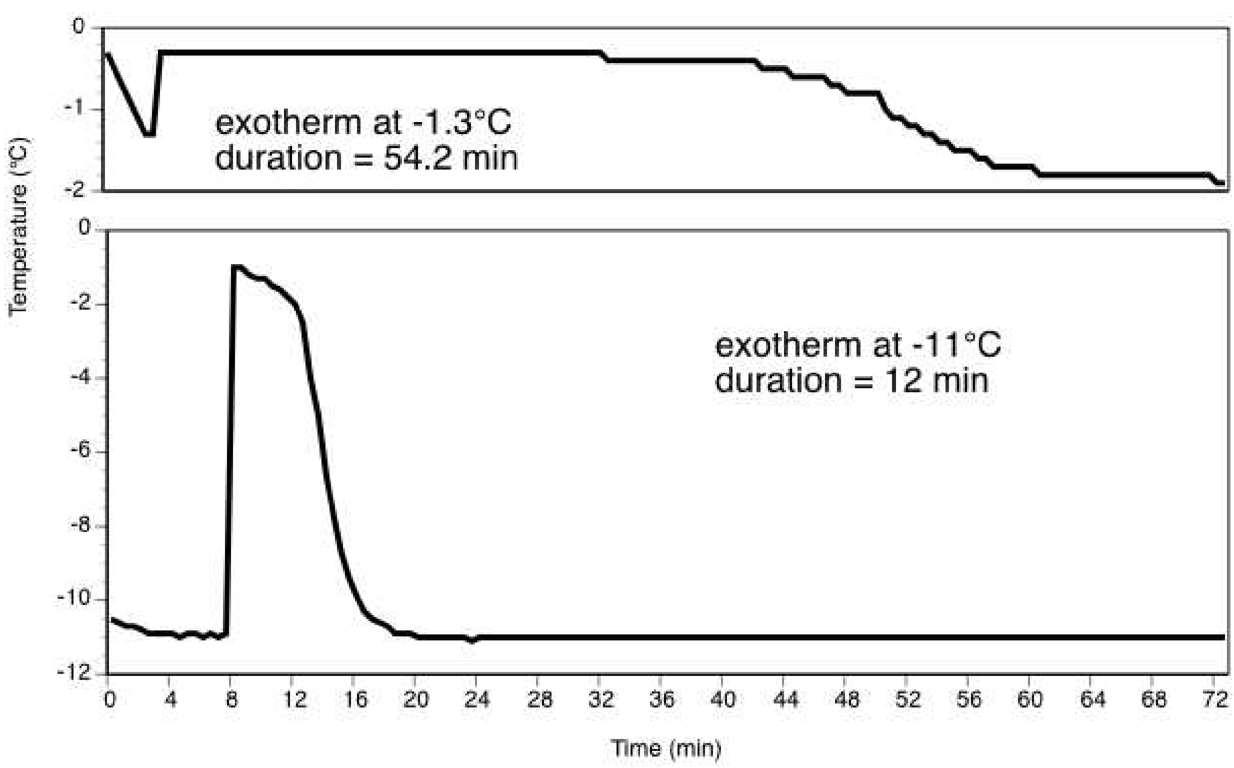

Fig. 2. Exotherms from 50µl suspensions of the Antarctic nematode *Panagrolaimus davidi*, with freezing initiated at -1.3°C (top) and -11.0°C (bottom) (data from Wharton et al., 2002).

In the moderately freezing tolerant insect *Celatoblatta quinquemaculata* cooled to different temperatures (Fig. 3), exotherms are similar since freezing is initiated at a relatively high subzero temperature by ice nucleators in the gut or haemolymph of the cockroach (Worland et al., 1997). The temperature at which the insect freezes spontaneously (the whole body supercooling point, SCP or temperature of crystallization, T_c) is not significantly different between animals frozen to -5, -8 or -12°C, with a mean SCP of -4.0±0.2°C (mean±se, N = 12) and the temperature becomes elevated to the melting point of the animals' body fluids in each case (-0.5 to -1.4°C) (Worland et al., 2004).

Across a range of freezing tolerant animals freezing tends to occur at high subzero temperature, the rate of ice formation is slow and it takes a long time for the exotherm associated with the freezing event to be completed (Table 1). Perhaps not surprisingly these parameters are broadly correlated with the size of the animal.

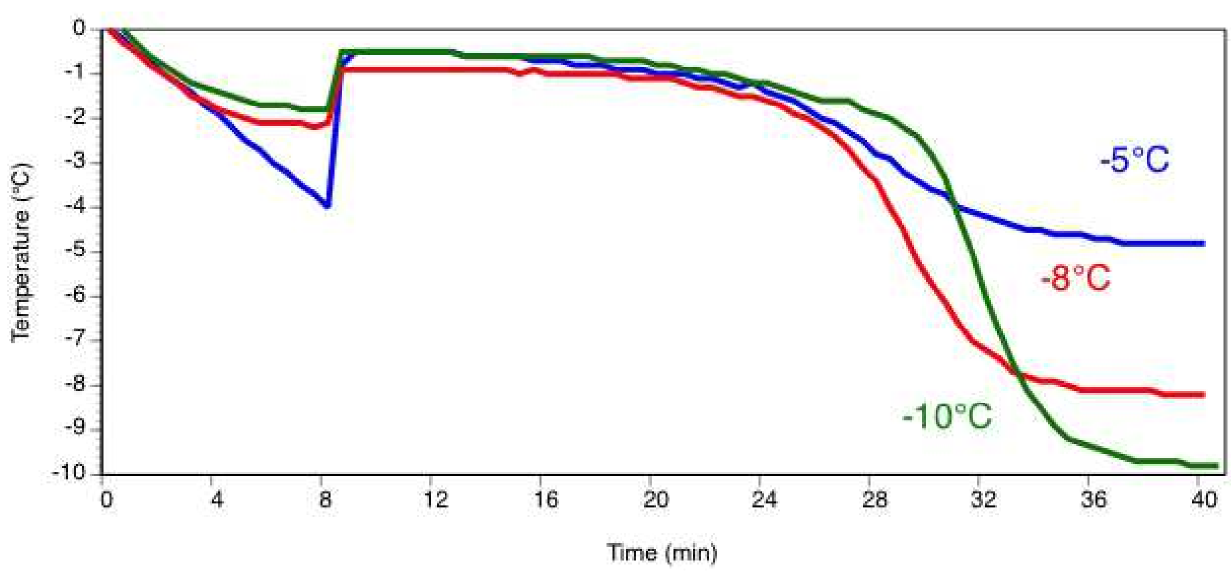

Fig. 3. Exotherms from the New Zealand alpine cockroach cooled to -5, -8 or -10°C at 0.5°C min^{-1} (data from Worland et al., 2004).

4. Freezing tolerance types in insects

Freezing tolerant insects vary in the relationship between the SCP and their lower lethal temperature (LLT). In order to be categorized as freezing tolerant the LLT of the insect must be lower than its SCP, and the insect survives ice formation in its body proceeding to completeness. In some species the LLT is only a few degrees below the SCP, which occurs at a high subzero temperature. These have been called 'moderately freezing tolerant' insects (Sinclair, 1999), although it would be more correct to call them freezing tolerant but moderately cold tolerant. In other freezing tolerant insects the LLT is many degrees below the SCP, which occurs at a high subzero temperature. These have been called (Sinclair, 1999) 'strongly freezing tolerant' (freezing tolerant and strongly cold tolerant). A final type has a low SCP but the LLT is a few degrees lower still: 'freezing tolerant with a low SCP' (Sinclair, 1999). 'Partial freezing tolerance' has also been proposed as a category, for those insects that will survive some ice forming in their bodies but die if the exotherm is completed (Sinclair, 1999). These insects are not freezing tolerant (since they do not survive completion of the freezing process) but they could represent a stage in the evolution of freezing tolerance (Hawes & Wharton, 2010; Sinclair, 1999).

Group	Animal	mass	T_{min} °C	T_c °C	Exotherm duration	Reference
Vertebrates	*Rana sylvatica*	~14g	-2	-2.2 ±0.1	14 to 20+ h	Layne & Lee, 1987
	Litoria ewingii	~1g	-2	-1.7 ±0.3[1]	1.7 ±0.3 h	Bazin et al., 2007
	Chrysemys picta	3-6g	-2.5	-2.5 ±0.46	~12 h[2]	Churchill & Storey, 1992
Insects	*Celatoblatta quinquemaculata*	~0.1g	-5	-4.6 ±0.1	15.1 ±1.2 min	Worland et al., 2004
	Hemideina maori	~5g	-5	-0.8 to -2.5	10 h[2]	Ramløv & Westh, 1993
	Eurosta solidaginis	~0.05g	-23	-10.6	48 h[2]	Lee & Lewis, 1985
Nematode	*Panagrolaimus davidi*[3]	<1µg	T_c	-26.5 ±0.9	13.0 ±2.0 s	Wharton & Block, 1997

[1]cooled on a dry substrate at 1°C h^{-1} until freezing occurred
[2]time to maximum ice formation, [3]for single nematodes free of surface water in liquid paraffin
T_{min} minimum temperature, T_c temperature of crystallization.

Table 1. The freezing characteristics of some freezing tolerant animals

Moderately freezing tolerant insects predominate in the Southern Hemisphere, where they are associated with climates that have mild winters but where temperatures are unpredictable and can fall below 0°C at any time of the year (Chown & Sinclair, 2010; Sinclair, et al., 2003). These insects have relatively high SCPs and a large proportion of their body water is converted into ice. In *C. quinquemaculata* 74% of their water freezes (Block et al., 1998) and in *H. maori* 82% freezes (Ramløv & Westh, 1993). However, in both of these insects their LLT is only about 6°C below their SCP (Wharton, 2011b). There must be further lethal events at lower temperatures after ice formation has been completed. Given the high proportion of ice that is formed initially it seems unlikely that this is due to further compartments freezing; as has been observed in an Alaskan fungus gnat (Sformo et al., 2009).

Using a live/dead cell stain Worland et al. (Worland, et al., 2004) showed that a high proportion of cells in the midgut, fat body and Malpighian tubules of *C. quinquemaculata* survived freezing at -5°C. At lower temperatures (-8°C, -12°C) the proportion of dead cells increases, with the fat body cells being the most sensitive tissue of those tested. In *H. maori* the Malpighian tubule cells are more sensitive to low temperatures than are the fat body cells but the survival of both declines with temperature (Sinclair & Wharton, 1997). These results suggest that the LLT of these insects reflects the accumulation of damage in a critical tissue. In the strongly freezing tolerant insect *E. solidaginis* tissues vary in the ability of the cells to survive freezing, with the cells of the alimentary system being the most resistant (Yi & Lee, 2003). Again it is the most sensitive tissue that will set the lower limit on the survival of the organism.

5. Ice nucleation in freezing tolerant animals

In contrast to freeze avoiding animals, that eliminate or mask sources of ice nucleation, freezing tolerant animals allow and encourage ice nucleation. Some freezing tolerant insects produce proteins or lipoproteins that have ice nucleating activity. These ensure that freezing occurs at a relatively high subzero temperature. They may also control the site of ice formation so that it occurs in the haemocoel, preventing potentially fatal intracellular freezing (Duman et al., 2010). The freezing tolerant Southern Hemisphere frog *Litoria ewingi* has ice nucleators in its skin secretions (Rexer-Huber et al., 2011), which ensure that this winter-active and largely terrestrial frog will freeze at a very high subzero temperature (-1.7°C) even on a dry substrate (Bazin, et al., 2007).

Moderately freezing tolerant insects continue to feed during the winter, ensuring the year-round presence of food and microorganisms in their gut that could act as ice nucleators. *Celatoblatta quinquemaculata* and *H. maori* have ice nucleators in their haemolymph, gut contents and faeces (Wilson & Ramløv, 1995; Worland, et al., 1997). The nucleating activity of the faeces is greater than that of the haemolymph (Sinclair et al., 1999; Worland, et al., 1997). This suggests that the gut is the primary site of ice nucleation, with nucleators in the haemolymph providing a back-up system if the gut is empty (Worland, et al., 1997).

The strongly freezing tolerant insect *E. solidaginis* forms a non-feeding dormant larval stage overwinter, surviving within the gall it induces in the stem of its host plant (Baust & Nishino, 1991). When the water content of the gall is high the larvae freeze by ice inoculation from the surrounding plant tissue (Lee & Hankison, 2003). As the autumn and winter progresses the galls dry out, inoculative freezing decreases and the insects rely on endogenous nucleators. These include calcium phosphate spherules that accumulate in the Malpighian tubules of overwintering larvae. These spherules, and the insect's fat body cells, have ice nucleating activity that ensure that the larvae freeze at -8°C to -10°C (Mugnano et al., 1996).

Although some nematodes can survive desiccation, for growth and reproduction to occur at least a film of water must be present. Nematodes, and animals that live in similar habitats (such as tardigrades and rotifers), are likely to be faced with the risk of inoculative freezing from ice in their surroundings. Few species have been examined in this respect, but in those that have (*Panagrolaimus davidi* and *Panagrellus redivivus*) they have little ability to resist inoculative freezing (Hayashi & Wharton, 2011; Wharton & Ferns, 1995; Wharton et al., 2003). This also is the case in the infective larvae of the insect parasitic nematode *Steinernema feltiae* (Farman & Wharton, unpublished results) and the free-living Antarctic nematode *Plectus murrayi* (Raymond, 2010). In *P. davidi* inoculative freezing occurs via body openings, especially the excretory pore (Wharton & Ferns, 1995) and endogenous ice nucleators are absent (Wharton & Worland, 1998). However, if freezing of the media occurs at a high subzero temperature (-1°C) inoculative freezing does not occur in *P. davidi* and the nematode can survive by cryoprotective dehydration (Wharton, et al., 2003). In *P. redivivus*, however, inoculative freezing occurs in some individuals even at -1°C and the small amount of cold tolerance that this species possesses is largely due to freezing tolerance, although those few nematodes that remain unfrozen survive by cryoprotective dehydration (Hayashi & Wharton, 2011).

Other freezing tolerant animals can use cryoprotective dehydration as an alternative strategy to freezing tolerance, especially under conditions where the chances of inoculative freezing is reduced (such as in soil of low water content). This has been reported in freezing-tolerant earthworms (Pedersen & Holmstrup, 2003) and in the Antarctic midge, *Belgica antarctica* (Elnitsky et al., 2008).

Some freezing tolerant arthropods appear to rely on inoculative freezing for survival. The centipede *Lithobius forficatus* freezes at a temperature just below the melting point of their haemolymph (about -1°C) by inoculative freezing when in contact with ice and survives, but if it supercools to -7°C and below it dies when it freezes (Tursman, et al., 1994). Caterpillars of the moth *Cisseps fulvicolis* also require inoculative freezing at a high subzero temperature to tolerate freezing (Fields & McNeil, 1986). Diapausing larvae of the fly *Chymomyza costata* can survive to low temperatures better if freezing is initiated by inoculative freezing at -2°C, than if they are allowed to supercool (Shimada & Riihimaa, 1988).

Inoculative freezing may also be an important factor in the cold tolerance mechanisms of vertebrate ectotherms. The skin of frogs has a high permeability to water and if they are cooled in contact with a moist natural substrate (such as soil or leaf mould) they freeze by inoculative freezing when ice forms in the substrate. Soil contains abundant ice nucleators that initiate freezing at a high subzero temperature (Costanzo et al., 1999). The skin of hatchling turtles is much less permeable to water than that of frogs but inoculative freezing can still occur via body openings, such as the eyes, ears, nose, cloaca, umbilicus, mouth and anus. Given the high levels of ice nucleators in their natural substrate this necessitates a level of freezing tolerance (Costanzo, et al., 2008) and inoculative freezing may be required to ensure survival over winter (Baker et al., 2006).

6. Intracellular freezing

The use of ice nucleators to induce freezing at high subzero temperature in the body fluids of many freezing tolerant animals is thought to ensure that freezing occurs extracellularly (Duman, et al., 2010). Intracellular freezing is thought to be fatal due to the mechanical disruption of cells by the expansion of water as it freezes, the puncturing of membranes by ice crystals or the redistribution of ice crystals (recrystallization) after freezing and during thawing (Acker & McGann, 2001; Muldrew et al., 2004). However, some examples of survival of intracellular freezing have been discovered in particular cells and tissues of some freezing tolerant animals (Sinclair & Renault, 2010). The only animal shown to survive extensive intracellular freezing throughout its body is the Antarctic nematode *P. davidi* (Wharton & Ferns, 1995). Some other nematodes have now been shown to have at least some ability to survive intracellular freezing, including *Steinernema feltiae* (Farman & Wharton, unpublished results) and *Plectus murrayi* (Raymond, 2010).

7. Conclusions

An animal is said to be freezing tolerant if it can survive the freezing and thawing of a biologically significant amount of its body water under thermal and temporal conditions that reflect its exposure to low temperatures in nature (Baust, 1991). In the laboratory this is demonstrated by the ability to survive ice formation, or the exotherm associated with freezing, going to completion but is harder to demonstrate in a natural situation (Costanzo, et al., 2008). Animals from many different phyla are freezing tolerant, including both invertebrate and vertebrate ectotherms. Most freezing tolerant animals, but not all, freeze at a high subzero temperature; with the SCP being controlled by the production of ice nucleators, the retention of food and bacteria in the gut or by allowing inoculative freezing. This ensures that the freezing process is slow and gentle, allowing the animal to adjust to the changing physiological conditions. Ice formation is usually thought to be extracellular but this has rarely been examined and survival of intracellular freezing may be much more widespread amongst freezing tolerant animals than we currently realise.

8. References

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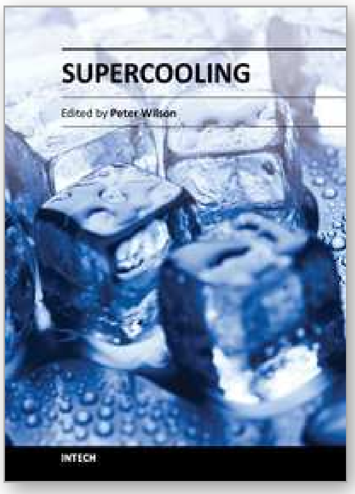

Supercooling
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Supercooled liquids are found in the atmosphere, in cold hardy organisms, in metallurgy, and in many industrial systems today. Stabilizing the metastable, supercooled state, or encouraging the associated process of nucleation have both been the subject of scientific interest for several hundred years. This book is an invaluable starting point for researchers interested in the supercooling of water and aqueous solutions in biology and industry. The book also deals with modeling and the formation subsequent dendritic growth of supercooled solutions, as well as glass transitions and interface stability.

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