Surviving the Ice: A critical review of cold tolerance strategies in Antarctic nematodes

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Abstract

Antarctica presents an extreme environment. Diversity is restricted yet life thrives, particularly nematodes. Nematodes are a dominant soil invertebrate in Antarctic ecosystems and have developed a suite of cold tolerance mechanisms to cope with freezing events and desiccation. *Panagrolaimus davidi* is one of these nematode species, believed to have exceptional cold tolerance abilities, above and beyond that of its counterparts. Several years of research has produced an impressive literature base on cold tolerance mechanisms. Upon reading this literature it is immediately clear that investigators agree to the classes of cold tolerance mechanisms and the species which employ each strategy. However, there are areas requiring further research; switching between strategies is apparent in some species, which is suggestive of plasticity among mechanisms, yet a solid conclusion has not been reached. In the case of *P. davidi* there is little information as to how members of *Panagrolaimus* vary in their cold tolerance mechanisms. It is also obvious that studying cold tolerance abilities can be problematic as nematodes have to be removed from their natural habitat.

Introduction

Aristotle warned “everything in moderation” yet the Romans (well-known for being excessive) penned the term ‘extremus’, and by the fifteenth century the word ‘extreme’ had appeared. As the twenty-first century dawns, it is becoming widely accepted that on Earth there exists habitable, extreme environments inconceivable to our predecessors. ‘Extreme’ refers to physical extremes (temperature) and geochemical extremes (salinity) (Rothschild & Mancinelli 2001, Oarga 2009). Animals that inhabit these extreme environments have earned themselves a name - ‘extremophiles’ (Rothschild & Mancinelli 2001). Extremophiles are usually highly specialised organisms, which in contrast to other species, bridge different stress conditions (Oarga 2009). Temperature extremes create several challenges, ranging from structural destruction wrought by ice crystals at low temperatures to cellular denaturation at high temperatures (Rothschild & Mancinelli 2001, Oarga 2009). There are two types of responses which organisms can apply in unsuitable conditions: resistance adaptations and capacity adaptations. These responses allow the organism to resist stress, or continue to grow and reproduce through harsh environmental conditions (Wharton 2002). The ability to withstand temperatures below freezing is reliant on two strategies: protection of cells from ice formation by freezing avoidance, or if ice forms, protection from damage during thawing by freezing tolerance (Rothschild & Mancinelli 2001).

Antarctic ecosystems epitomize one extreme in a continuum of extreme environmental conditions. In Antarctica, the physical characteristics of certain environments represent the extreme ends of the range of characteristics (snow, ice, solar radiation) and in these environments, quite often the physical characteristics dominate and prevail over biological considerations (Hennion *et al*. 2006), Therefore, to an outside observer the environment appears harsh, and yet a wide range of life is present and thriving despite restricted diversity (Hennion *et al*. 2006). All but 2% of the continent is ice-free (Freckman & Virginia 1993) and therefore Antarctic organisms inhabit one of the harshest environments on Earth. Nematodes are the dominant soil animals of the ice-free regions of Antarctica (Freckman & Virginia 1993, Wharton 2003, Hennion *et al*. 2006, Adhikari *et al*. 2010) although they are known to occupy both temperate and tropical regions also (Pickup 1990a). Nematodes are also an important component of the terrestrial invertebrate communities within the Antarctic ecosystem (Wharton & Barclay 1993, Wharton & Block 1993). These terrestrial communities are also home to algae, bacteria, and many other micro-invertebrates (Freckman & Virginia 1993, Wharton 2003).

In their Antarctic habitats nematodes face a variety of environmental stresses including low temperatures and freezing events for most of the winter and occasionally during the summer (Wharton & Block 1993, Adhikari *et al*. 2010). In addition to temperature extremes nematodes must also survive desiccation brought about by humidity, wind, and low precipitation in both winter and summer (Wharton & Barclay 1993). Often these departures from optimal environmental conditions are easily predicted and as a result of this, Antarctic nematodes have developed a range of physiological mechanisms to aid in their survival (Wharton & Barclay 1993, Smith *et al*. 2008). These physiological adaptations are believed to be an important part of their life history and ecology (Wharton & Block 1993). Typically these nematodes exhibit resistance adaptations to survive freezing and desiccation in a dormant state, reproducing in the summer when conditions are more favourable for survival (Wharton 2002, Wharton 2003).

Cold Tolerance Strategies

Animals capable of surviving low temperatures in their natural habitats are said to be cold tolerant or cold-hardy (Brown & Gaugler 1996, Smith *et al*. 2008). Cold tolerance strategies were first studied in insects and later in nematodes. Most studies with arthropods concluded that there were two mutually exclusive strategies (Pickup 1990a). Following this, research into nematode cold-tolerance began (Pickup 1990a). Like arthropods, many nematode species have developed several strategies to survive exposure to sub-zero temperatures and desiccation stress (Wharton & Block 1993). Early research with nematodes showed that environmental conditions or the organism's physiological state determines the mechanism adopted (Pickup 1990a). To cope with the transition from water to ice, Antarctic nematodes have evolved an array of adaptations to withstand environmental stresses in Antarctica (Smith *et al*. 2008). There are three main cold-tolerance strategies – cryoprotective dehydration, freeze avoidance, and freeze tolerance (Smith *et al*. 2008). This review focuses on the known cold tolerance strategies of several nematode species found in Antarctica, the complications of a freezing event (inoculative freezing), and covers anhydrobiosis, a cold tolerance strategy thought to be more widespread in arthropods. Additionally, this review reports on the various categories of ice-active proteins and an intensely studied Antarctic nematode species – *Panagrolaimus davidi*.

Inoculative Freezing

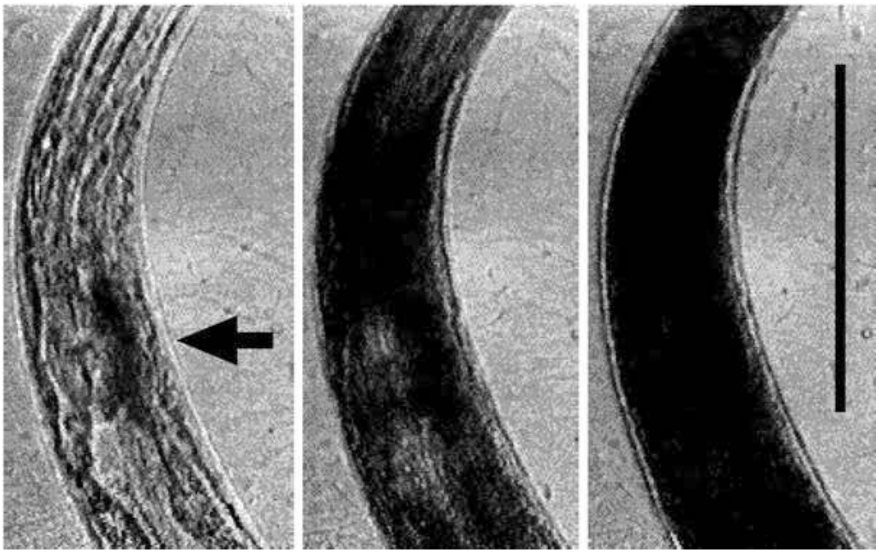

Figure 1. Inoculative freezing begins near the oesophagus (arrow) and spreads throughout the body until all compartments are frozen, including intracellular spaces (from Wharton & Ferns

Nematodes are, in essence, aquatic organisms and hence require a thin film of water in their environment for vital functions such as growth, reproduction, and movement (Wharton 2003). Due to this thin film of water, nematodes are constantly at risk of freezing and are much more likely to be exposed to inoculative freezing; the process of ice seeding across the cuticle or orifice and freezing the organism (figure 1) (Wharton 2003). Inoculative freezing is generally inhibited by slow freezing rates, enhancing survival of the organism (Wharton 2003). Alternatively, an eggshell or a sheath retained from an earlier life-stage prevents inoculative freezing and allows for supercooling (Wharton & Allan 1989, Wharton 1994).

Cryoprotective Dehydration

Traditionally cold tolerance strategies were divided into two sections: freeze avoidance and freeze tolerance. However, recently a third strategy was recognised: cryoprotective dehydration (Wharton 2003). Animals that employ cryoprotective dehydration, dehydrate due to a vapour pressure difference between the supercooled water within the animal and the surrounding medium at the same temperature (Wharton *et al*. 2005b, Smith *et al*. 2008) and have a shrunken appearance upon thawing (Wharton *et al*. 2003). First described in Earthworm cocoons (Holmstrup & Zachariassen 1996) and later in an Arctic collembolan (Holmstrup & Sømme 1998, Worland *et al*. 1998) this cold-tolerance mechanism originally fell under the label 'protective dehydration mechanism' (Holmstrup & Westh 1994) however Wharton (2003) relabelled the strategy 'cryoprotective dehydration' (Wharton *et al*. 2003). Holmstrup *et al* (2002) experiments indicated that chironomid larvae and nematodes may also employ this strategy (Wharton *et al*. 2003) but as both nematodes and chironomid larvae are freezing tolerant (Wharton 2002), the relative importance, and the link between the two strategies remains unclear in these groups (Wharton *et al*. 2003).

Cryoprotective dehydration was originally thought to be caused by one of two mechanisms; as the animal freezes salts are excluded from growing ice crystals and concentrate in the surrounding medium, causing animals to dehydrate due to osmotic stress, or the animal may dehydrate due to an interaction between a freeze-concentration affect and a vapour pressure difference (Wharton & To 1996). However, Wharton *et al* (2003) determined that in nematodes, cryoprotective dehydration is caused solely by vapour pressure differences between the supercooled water within the nematode and the surrounding medium. Wharton *et al* (2003) hypothesised that in nematodes with a sheath or cuticle, the cuticle not only acts as a barrier against ice nucleation but also separates the supercooled liquid within a nematode from the adjacent ice, and thus may mediate the transport of water between the two environments (Wharton *et al*. 2003).

Anhydrobiosis

Clegg (1978) described anhydrobiosis as the ability to survive water loss and direct exposure to 0% relative humidity (Clegg 1978 in Wharton & Barclay 1993). However, anhydrobiosis is now defined as an organism which loses all its body water and survives in a dormant, ametabolic state until environmental conditions improve (Wharton 2003). In 1987, Womersely identified two broad categories of anhydrobiotic nematodes – fast-dehydration strategists and slow-dehydration strategists (Womersely 1987). Wharton (2002) went on to identify several nematode species capable of anhydrobiosis and further defined the two anhydrobiotic categories. Fast-dehydration strategists have adaptations that slow the rate of water loss from their bodies while slow-dehydration strategists do not have this adaptation and instead rely on their environment slowly drying to provide a slow rate of water loss (Wharton 2002, Wharton 2003). A 'lag phase' follows rehydration in a previous anhydrobiotic organism. Post-rehydration, metabolic activity returns immediately, however, activity isn't resumed for another 2-3 hours. Anhydrobiosis wasn't described in an Antarctic nematode species until 1991 when Pickup & Rothery investigated the survival abilities of *Teratocephalus tilbrooki* and an organism of the *Ditylenchus* species (Wharton & Barclay 1993). This became the first evidence that anhydrobiosis was more widespread in nematodes than originally thought (Wharton & Barclay 1993).

Freeze avoidance

Animals that employ freeze avoidance as their cold tolerance strategy maintain their body fluids in a supercooled state at temperatures below their melting point – i.e. freeze avoiding animals supercool to avoid the lethal process of inoculative freezing (Wharton & Block 1993, Wharton 2003, Sinclair & Rinault 2010). Unfortunately if freezing occurs at the supercooling point the organism will die (Brown & Gaugler 1996). Additionally for supercooling to occur, nematodes must be free of surface water (Wharton & Block 1993). If freeze avoiding nematodes are in water, it is vital to prevent inoculative freezing. Therefore freeze avoiding animals retain their sheath or eggshell as a protector against exogenous ice nucleation (Wharton & Brown 1991). It is also possible for supercooling points to fluctuate with season and feeding activity (Wharton 2003) yet Wharton & Block (1993) illustrated that ice nucleators, such as food in the gut or bacteria, are of little significance.

Pickup (1990a) investigated the cold tolerance abilities of three species of free-living Antarctic nematodes *Eudorylaimus coniceps, E. spaulIi*, and *E. Pseudocarteri* and discovered that each species exhibites differing degrees of both freeze avoidance and freeze tolerance. Supercooling points fluctuated considerably in *E. coniceps,* and *E. spaulIi,* while *E. pseudocarteri* showed the most consistent supercooling ability of all three species (Pickup 1990a). Additionally, cold-tolerance abilities of *E. coniceps,* and *E. spaulIi* varied seasonally, although this was consistent with another Antarctic nematode species *Plectus antarcticus* (Pickup 1990b). Overall, variation in cold-hardiness of three closely related species suggests plasticity in cold-tolerance mechanisms implemented by nematodes (Pickup 1990a).

Freezing tolerance

Contrastingly, freezing tolerant animals show little or no supercooling ability but instead withstand the formation of extracellular ice within their bodies (Brown & Gaugler 1996, Wharton & Block 1993, Wharton 2003, Hennion *et al*. 2006). At high sub-zero temperatures animals that employ this strategy can 'seed' ice formation within their bodies with the use of ice nucleators or bacteria. Controlling the process of ice formation appears to be vital to an organism's survival in the Antarctic environment. As temperatures decrease following the initial freezing event there is a great deal of variation among taxa in the degree of survival and protection this cold tolerance strategy provides (Willmer *et al*. 2005, Hennion *et al*. 2006). Freezing tolerance is believed to be widespread among Antarctic nematode species, occurring in both larvae and adults (Wharton & Block 1993). Several studies have shown that most nematode species fall into this category (Wharton & Allan 1989, Wharton & Brown 1991, Wharton & Block 1993); Wharton & Block (1993) study showed that of nine

Antarctic nematode species isolated from moss and algae sites in Antarctica, 7 species were freeze tolerant. Freezing tolerance in nematodes has also been reported in *Panagrolaimus davidi* (Wharton & Brown 1991) and several *Trichostrongylus* species (Wharton & Allen 1989, Pickup 1990b).

Ice-active Proteins (IAPs)

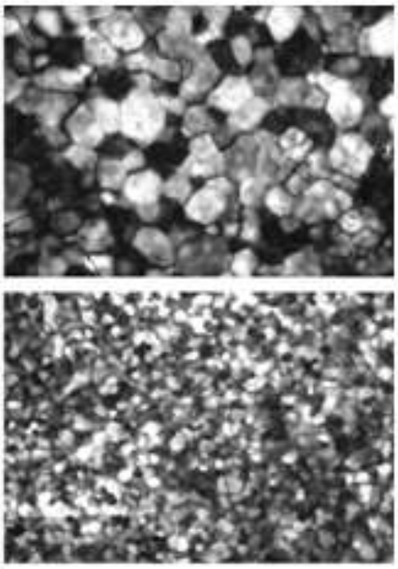

Figure 2. Frozen samples of pH 7.8 control (top) and *P. davidi* extract (bottom) subsequent to annealing at -8°C for 20 min (from Wharton *et al*. 2005a).

Several studies have described a protein component to cold tolerance strategies (Wharton *et al*. 2005a, Smith *et al*. 2008). However, it appears that the terminology for these proteins is in a constant state of flux with confusion arising from the use of the term 'antifreeze' proteins (Wharton *et al*. 2005a). Wharton *et al* (2005a) argues that many proteins show little or no antifreeze activity and proposes the term 'ice-active proteins'. Ice-active proteins (IAPs) interact with ice in some way to assist in survival and can be divided into three classes (Wharton 2003, Wharton *et al*. 2005a). Ice-nucleating proteins (INPs) trigger ice formation, anti-freeze proteins (AFPs) prevent ice nucleation, and recently a third class was described – recrystallisation-inhibition proteins (RIPs). Recrystallisation results in the growth of large ice crystals at the expensive of smaller ones so that fewer but larger ice crystals form. However, this can be particularly damaging to frozen animals as ice crystals grind against membranes. Therefore an organism will produce proteins that inhibit the process of recrystallisation (figure 2) (Wharton 2003, Wharton *et al*. 2005a, Smith *et al*. 2008).

Panagrolaimus davidi

Panagrolaimus davidi deserves special mention here. *P. davidi* is a free-living terrestrial Antarctic nematode, endemic to continental Antarctica (Smith *et al*. 2008). This species occupies coastal areas, including the McMurdo Sound region and penguin colonies (Wharton & Brown 1989, Wharton & Ferns 1995, Wharton *et al*. 2003). As Antarctica is such a harsh and variable environment it is possible that physiological responses may not be typical of nematodes that are faced with less extreme and stressful conditions (Smith *et al*. 2008). Therefore, Antarctic nematodes have been studied extensively. Of the known species of Antarctic nematodes, both larvae and adults of *P. davidi* have become the focal point of several studies investigating cold tolerance (Wharton 1994, Wharton & Ferns 1995, Ramløv *et al*. 1996, Wharton *et al*. 2003, Wharton *et al*. 2005a, Wharton *et al*. 2005b, Smith *et al*. 2008). As the years have progressed a greater understanding of the cold tolerance strategies implemented by *P. davidi* has come about. The larvae of *P. davidi* are protected by an eggshell which allows larvae to supercool in the presence of external ice and larvae are said to be freezing tolerant (Wharton & Brown 1991, Wharton 1994). This differs from other species of nematodes; Wharton & Allen (1989) demonstrated that the sheath surrounding *Trichostrongylus colubriformis* larvae prevents exogenous ice nucleation, allowing this species to be freeze avoiding (Wharton & Allen 1989, Wharton 1994).

Currently it is known that adults of this species can survive to temperatures as low as -80^{o}C (Wharton & Brown 1991), can completely freeze in 0.21s (Wharton & Ferns 1995), and survive up to 82% of its body water freezing (Storey & Storey 1988), a feat greater than any other freeze-tolerant species (Smith *et al*. 2008). Additionally, it has been shown that *P. davidi* is freezing tolerant and is the only animal known to survive extensive intracellular freezing, as determined by Wharton & Ferns (1995). Prior to Wharton & Ferns (1995) study it was thought that no intact animal was capable of surviving intracellular freezing (Wharton & Ferns 1995), and such survival had only been demonstrated in insect fat body cells (Smith *et al*. 2008). Largely, the cold tolerance strategy used by *P. davidi* is determined by external conditions: when freezing rates are slow, supercooled water within the animal is lost to the surrounding ice, and *P. davidi* survives via cryoprotective dehydration (Holmstrup & Westh 1994, Wharton *et al*. 2003, Wharton *et al*. 2005b, Smith *et al*. 2008). However, at fast cooling rates inoculative freezing takes place and *P. davidi* is freezing tolerant (Wharton & Ferns 1995, Wharton 2003, Wharton *et al*. 2005b). Smith *et al* (2008) compared the cold tolerance abilities of *P. davidi* with that of five other nematode species and produced interesting results; *P. davidi* had a greater survival rate than any other nematode species tested, indicating atypical survival abilities in this nematode species (Smith *et al*. 2008). *P. davidi* is also known to produce trehalose and an ice-active protein that inhibits recrystallisation, enabling the animal to enter an anhydrobiotic state (Wharton 2003, Wharton *et al*. 2005a). These strategies combined provide *P. davidi* with exceptional cold tolerance abilities in both adults and eggs (Lewis et al. 2009). Hence *P. davidi* possesses a variety of mechanisms for coping with the harsh conditions of its terrestrial Antarctic habitat (Wharton *et al*. 2005a).

Conclusions

At the dawning of the 21st century, environments as harsh and extreme as Antarctica are being recognised as habitable to some (Rothschild & Mancinelli 2001). While overall diversity is restricted locally, life is thriving (Hennion *et al*. 2006). Nematodes have become the dominant soil invertebrate in Antarctica and are an important component of the ecosystem (Wharton & Barclay 1993, Wharton & Block 1993). There exists a suite of cold tolerance strategies and mechanisms utilised by Antarctic nematode species to withstand the environmental extremes they are regularly exposed to. Largely the freezing conditions determine the cold tolerance strategy employed by the organism and the organism themselves, have developed different mechanisms for surviving in Antarctica (Pickup 1990a, Wharton & Block 1993). To date there are three strategies – cryoprotective dehydration, freeze avoidance, and freeze tolerance (Wharton 2003). Inoculative freezing is a common complication of freezing events, solved with the use of an eggshell or sheath (Wharton & Allan 1989, Wharton 1994). In recent years, several classes of ice-active proteins have been described in Antarctic nematodes. These IAPs interact with ice in some way to aid in survival (Wharton *et al*. 2005a, Smith *et al*. 2008). Anhydrobiosis is also slowly becoming accepted as another cold tolerance mechanism, believed to be widespread among nematodes (Wharton & Barclay 1993).

Additionally, the endemic Antarctic nematode *P. davidi* has, over the years, become recognised for its exceptional cold tolerance abilities. It appears that *P. davidi* utilises all described cold tolerance mechanisms, produces trehalose and a recrystallisation-inhibiting protein, and is the only animal known to survive extensive intracellular freezing. Other *Panagrolaimus* species do not share such unique cold-tolerance abilities (Smith *et al*. 2008), an area which calls for further study.

On the whole, studies generally agree on the definition of each strategy and the strategies implemented by each nematode species in Antarctica. As the years have progressed, and techniques have improved, a greater understanding of each strategy has come about. Perhaps the most recognisable issue in studying nematode cold-tolerance abilities is that to effectively study the cold-tolerance abilities of an Antarctic nematode species, organisms must first be removed from their natural habitat and transported to a laboratory, thus changing their physiological state (Wharton 2003) and impinging on any results and data collected. It is recognised that due to the harsh environment of Antarctica, responses to cold in nematode species may not be atypical of other nematodes from more tropical regions. This is also an area requiring future research.

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