

Freezing survival and cryoprotective dehydration as cold tolerance mechanisms in the Antarctic nematode *Panagrolaimus davidi*

David A. Wharton^{1,*}, Gordon Goodall^{1,2} and Craig J. Marshall²

Departments of ¹Zoology and ²Biochemistry, University of Otago, PO Box 56, Dunedin, New Zealand

*Author for correspondence (e-mail: david.wharton@stonebow.otago.ac.nz)

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Summary

The relative importance of freezing tolerance and cryoprotective dehydration in the Antarctic nematode *Panagrolaimus davidi* has been investigated. If nucleation of the medium is initiated at a high subzero temperature (-1°C), the nematodes do not freeze but dehydrate. This effect occurs in deionised water, indicating that the loss of water is driven by the difference in vapour pressure of ice and supercooled water at the same temperature. If the nematodes are held above their nucleation temperature for a sufficient time, or are cooled slowly, enough water is

lost to prevent freezing (cryoprotective dehydration). However, if the medium is nucleated at lower temperatures or if the sample is cooled at a faster cooling rate, the nematodes freeze and can survive intracellular ice formation. *P. davidi* thus has a variety of mechanisms that ensure its survival in its harsh terrestrial Antarctic habitat.

Key words: Antarctic, nematode, *Panagrolaimus davidi*, freezing, ice, nucleation, dehydration.

Introduction

Panagrolaimus davidi is a free-living Antarctic nematode associated with ice-free terrestrial sites that receive sufficient meltwater during spring and summer to support the growth of algae, moss and cyanobacteria. It has been isolated and grown in culture (Wharton and Brown, 1989). This nematode is freezing-tolerant and can survive extensive intracellular freezing (Wharton and Ferns, 1995). During spring its habitat is often saturated with water and the nematode experiences regular cycles of freezing and thawing (Wharton, 1998). The nematode is thus at risk of inoculative freezing by ice seeding through body openings such as the excretory pore (Wharton and Ferns, 1995).

Similar hazards are faced by earthworms in cold terrestrial environments. Earthworm eggs are enclosed within a fibrous cocoon, which provides protection against inoculative freezing (Holmstrup and Zachariassen, 1996). The cocoon fluid can thus remain unfrozen even though the cocoon is in close contact with ice. This results in a vapour pressure difference between the unfrozen, supercooled cocoon fluids and the surrounding ice. Earthworm cocoons are very permeable to water molecules and thus lose water until they are in vapour pressure equilibrium with the ice. This dehydrates the cocoons, to below $0.5\text{ g water g}^{-1}$ dry mass, so that they cannot freeze. This cold tolerance mechanism has been called the 'protective dehydration mechanism' (Holmstrup and Westh, 1994), although 'cryoprotective dehydration' might be a more appropriate term (M. Holmstrup, personal communication) and will be used here. A similar phenomenon has been described in an Arctic

collembolan, *Onychiurus arcticus* (Holmstrup and Sømme, 1998; Worland et al., 1998) and in three species of adult or preadult enchytraeid oligochaetes (Sømme and Birkemoe, 1997). It has been suggested that cryoprotective dehydration occurs in nematodes and chironomid larvae (Holmstrup et al., 2002). A shrunken appearance upon thawing suggests that dehydration may be occurring (Forge and MacGuidwin, 1992; Scholander et al., 1953). Both nematodes and chironomid larvae have been shown also to tolerate freezing (Scholander et al., 1953; Wharton, 2002), so the relative importance of freezing tolerance and cryoprotective dehydration in these groups is unclear.

The rate of ice formation in the soil water surrounding the nematode is dependent upon the volume of water and the temperature at which ice nucleation occurs. In samples of *P. davidi* frozen in Eppendorf tubes in a cooling block, survival depended upon the rate of freezing, which was determined by the sample volume and the temperature at which ice nucleation was initiated (Wharton et al., 2002). If contact between the growing ice crystals in the soil water and the nematode occurs at a temperature above the melting point of the body fluids of the nematode, inoculative freezing cannot occur and the nematode may undergo cryoprotective dehydration, which prevents them from freezing even if exposed to lower temperatures. In this paper we determine how nucleation temperature, cooling rate and the medium in which freezing occurs affect the relative importance of cryoprotective dehydration and freezing survival in this nematode.

Materials and methods

Panagrolaimus davidi Timm was grown in liquid cultures, as described by Wharton et al. (2000). Cultures were grown at 20°C for 1 week and then acclimated at 5°C for at least a further week. Nematodes were separated from cultures by allowing them to migrate through tissue paper (Hooper, 1986), washed several times in tapwater and then into an artificial tapwater (ATW; Greenaway, 1970), before storage at 5°C until use (on the same day). Nematodes were observed during freezing, under various conditions, on a thermoelectric microscope cold stage mounted on a Zeiss Axiophot Photomicroscope using a 10× objective lens. The cold stage was similar to a published design (Wharton and Rowland, 1984). The heat sink for the cold stage was fluid from a Haake F8-C35 refrigerated circulator, with fine control of temperature being provided by a thermoelectric cooling module. Nematode samples (5 µl) were mounted between two small glass coverslips in the sample chamber of the cold stage. The temperature of the sample was monitored *via* a NiCr/NiAl thermocouple and a Comark electronic thermometer. The thermometer was calibrated using the melting point of a sample of deionized water (Milli-Q), mounted in the same fashion as a nematode sample. Temperatures and cooling/warming rates were controlled using the programmer of the refrigerated circulator.

Freezing of nematodes was observed as a sudden darkening, whilst those assumed to be unfrozen tended to shrink and remain clear. After melting the sample was recovered from the cold stage and transferred to 1 ml of ATW in a watchglass. Nematode survival was determined after 24 h at room temperature, following a mechanical stimulus (expelling the medium from a glass pipette several times), by counting the number of motile and non-motile individuals (a total of at least 100 being counted).

The effect of nucleation temperature on freezing and survival

Nematodes were transferred to the cold stage and cooled at 0.5°C min⁻¹ to the test temperature (-1, -2, -3, -4, -5, -6°C). The sample was checked to ensure that freezing had not occurred and then freezing was initiated by placing a small ice crystal in contact with the edge of the coverslip. The sample was observed to ensure that freezing did occur and to qualitatively monitor the rate of freezing of the sample. The test temperature was maintained for a further 30 min and the proportion of frozen nematodes determined. The sample was warmed to 0°C at 1°C min⁻¹ and, after melting, the sample was recovered and survival determined.

The effect of cooling rate on freezing and survival

Nematodes were transferred to the cold stage, cooled rapidly to 0°C and then to -5°C at rates of 0.5, 0.2 or 0.1°C min⁻¹. Freezing was initiated with an ice crystal at -1°C and the proportion of frozen nematodes counted at 1°C intervals. The sample was then held at -5°C for 30 min, warmed to 0°C at 1°C min⁻¹, the sample recovered from the cold stage and survival determined as before.

The effect of media on freezing and survival

Nematodes were transferred to either ATW, deionized water (dH₂O) or 0.1 mol l⁻¹ NaCl dissolved in ATW. The osmolalities of these solutions, measured using a Knauer semi-micro osmometer, were: ATW, 7 mosmol l⁻¹; dH₂O, 0 mosmol l⁻¹; 0.1M NaCl dissolved in ATW, 195 mosmol l⁻¹. The sample was washed three times in the test solution, transferred to the cold stage and cooled to -1°C. Freezing of the sample was seeded with an ice crystal and the sample was held at -1°C for either a further 5 min or 30 min. The sample was then cooled to -5°C at 0.5°C min⁻¹ and the proportion of frozen nematodes determined at 1°C intervals. The sample was held at -5°C for a further 10 min, photographed, and then warmed to 0°C at 1°C min⁻¹ before being retrieved and survival determined as before.

Results

The effect of nucleation temperature on freezing and survival

The rate of ice propagation through the samples was slow at -1 and -2°C, becoming progressively faster with decreasing test temperature until at -4°C and below, when freezing was very rapid. No nematodes were frozen after 30 min at -1°C, some nematodes froze at -2°C and the proportion of frozen nematodes increased with decreasing test temperature. All nematodes froze at -5 and -6°C. Survival declined with decreasing test temperature but some nematodes (36%) survived even after freezing at -6°C (Fig. 1). The 50% survival temperature (S_{50}), determined by probit analysis (Finney, 1952), was $-4.9 \pm 0.7^\circ\text{C}$ (mean \pm S.E.M.), whilst the temperature at which 50% of nematodes froze (F_{50}) was $-2.4 \pm 0.2^\circ\text{C}$. There was a significant difference between the S_{50} and the F_{50} (t -test: $t=3.55$, d.f.=54, $P<0.05$, $N=3$). Some nematodes that had been observed to freeze were seen to recover activity upon melting. Those nematodes that did not freeze developed a shrunken appearance.

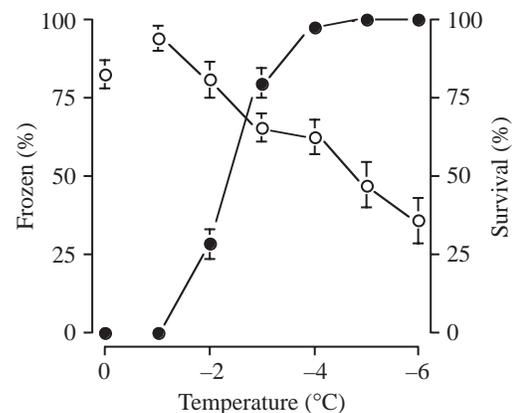


Fig. 1. The effect of temperature on the freezing (filled circles) and survival (open circles) of *P. davidi*. Samples were nucleated and held at the test temperature for 30 min. Samples at 0°C were unfrozen controls. Values are means \pm S.E.M., and where not visible error bars are contained within the data point; $N=4$.

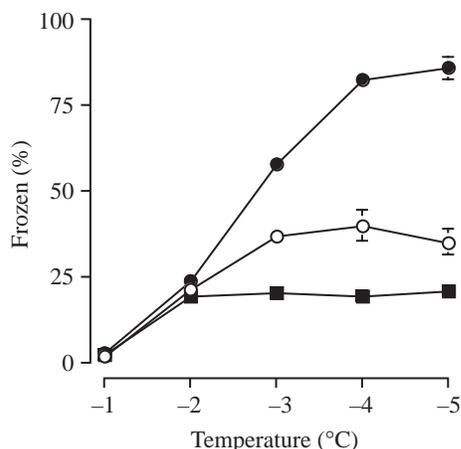


Fig. 2. The effect of cooling rate (filled circles, $0.5^{\circ}\text{C min}^{-1}$; open circles, $0.2^{\circ}\text{C min}^{-1}$; filled squares, $0.1^{\circ}\text{C min}^{-1}$) on the freezing of *P. davidi* during decreasing temperature and after nucleation at -1°C . Values are means \pm S.E.M., and where not visible error bars are contained within the data point; $N=3$.

The effect of cooling rate on freezing and survival

When cooled at $0.5^{\circ}\text{C min}^{-1}$, most nematodes froze during cooling and after nucleation at -1°C . The majority of nematode nucleation events occurred between -2 and -4°C . In samples cooled at rates of 0.2 or $0.1^{\circ}\text{C min}^{-1}$ the freezing of nematodes during cooling was much less (Fig. 2). Those nematodes that did not freeze took on a shrunken appearance, with the amount of shrinkage increasing as the temperature decreased. Cooling rate had a significant effect on the proportion frozen at -5°C (factorial analysis of variance, ANOVA, after arcsin transformation: $F_{(2,6)}=98.7$, $P<0.05$) but not on survival ($F_{(2,6)}=2.2$, $P>0.05$) (Fig. 3).

The effect of media on freezing and survival

In samples in ATW or dH₂O held for 5 min at -1°C after ice nucleation, the proportion of nematodes frozen increased during cooling to -5°C . In samples in 0.1 mol l^{-1} NaCl dissolved in ATW, the increase in freezing was much less (Fig. 4). The proportion freezing during cooling to -5°C was reduced in samples held at -1°C for 30 min after ice nucleation, compared to those held at -1°C for 5 min (Fig. 4). The time held at -1°C had a marked effect on the proportion frozen at -5°C for samples in ATW or dH₂O but not for those in 0.1 mol l^{-1} NaCl dissolved in ATW. Survival was similar in all treatments (Fig. 5). In ATW and dH₂O, those nematodes that did not freeze took on a shrunken appearance. The amount of shrinkage appeared to be greater in nematodes immersed in dH₂O than in those in ATW (Fig. 6). The freezing process was not completed in 0.1 mol l^{-1} NaCl dissolved in ATW at -1°C . No shrinkage was observed in this medium, even during cooling to -5°C (Fig. 6).

The effect of test solution on the proportion frozen at -5°C was significant (factorial ANOVA after arcsin transformation: $F_{(2,12)}=16.4$, $P<0.05$), as was the effect of time held at -1°C ($F_{(1,12)}=57.8$, $P<0.05$) and the interaction between test

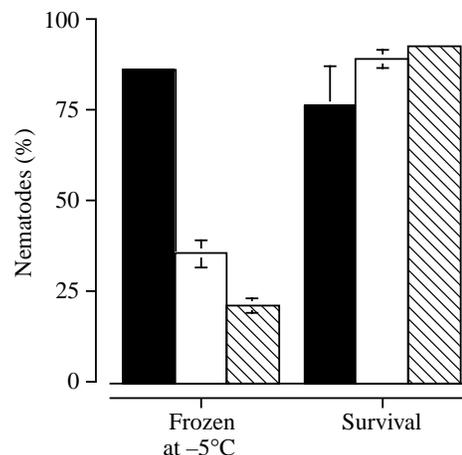


Fig. 3. The effect of cooling rate (filled bars, $0.5^{\circ}\text{C min}^{-1}$; open bars, $0.2^{\circ}\text{C min}^{-1}$; hatched bars, $0.1^{\circ}\text{C min}^{-1}$) on the freezing of *P. davidi* after nucleation at -1°C and cooling to -5°C , and on survival. Values are means \pm S.E.M., $N=3$.

solution and time held at -1°C ($F_{(2,12)}=10.6$, $P<0.05$). There were no significant differences in the proportion frozen at -5°C between ATW and dH₂O (Scheffe *post hoc* test: $P>0.05$, d.f.=12) but 0.1 mol l^{-1} NaCl dissolved in ATW produced significantly lower freezing than either ATW or dH₂O ($P<0.05$, d.f.=12). The proportion frozen at -5°C after being held for 5 and 30 min at -1°C was significantly different in ATW and dH₂O ($P<0.05$, d.f.=12) but not in 0.1 mol l^{-1} NaCl dissolved in ATW ($P>0.05$, d.f.=12). There was no significant effect on survival of either test solution ($F_{(2,12)}=0.33$, $P>0.05$) or of time held at -1°C ($F_{(1,12)}=0.04$, $P>0.05$).

The effect of freezing on survival

Using data from the previous experiments, survival was compared with that predicted if each nematode that had frozen died (Fig. 7A). Most points lie above the prediction line, indicating that nematodes are surviving freezing. There was a significant difference between survival and that predicted by freezing ($\chi^2=11561$, $P<0.0001$, d.f.=48). There was a significant negative correlation between freezing and survival ($r^2=45.2\%$, d.f.=49, $t=-6.36$, $P<0.0001$). However, if only the data for samples that froze at -1°C is considered (Fig. 7B), there is no correlation between freezing and survival ($r^2=7.3\%$, d.f.=25, $t=-1.1$, $P>0.05$).

General observations on freezing and thawing

The spread of ice through the body of nematodes was faster the lower the temperature at which inoculative freezing occurred. If inoculative freezing occurred at a high temperature (such as -1°C), ice propagation through the nematode was slow and appeared to be consistent with the ice being confined to extracellular compartments. If inoculative freezing occurred at a low temperature (such as -5°C), ice propagation was rapid and both extracellular and intracellular compartments appeared to freeze.

Immediately upon thawing some nematodes had a shrunken

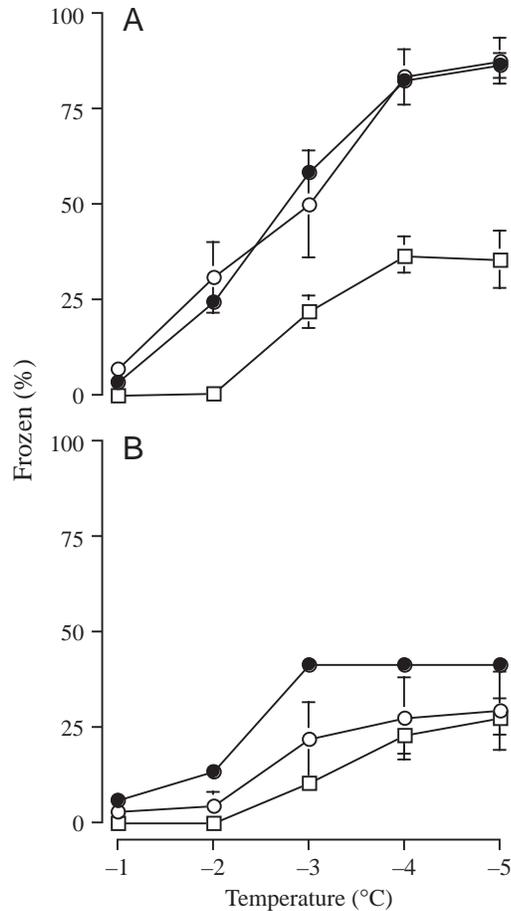


Fig. 4. The effect of different media (filled circles, ATW; open circles, dH₂O; open squares, 0.1 mol l⁻¹ NaCl dissolved in ATW) on the freezing of *P. davidi* during cooling from -1°C to -5°C at 0.5°C min⁻¹ after ice nucleation and holding at -1°C for 5 min (A) or 30 min (B). Values are means ± S.E.M., and where not visible error bars are contained within the data point; *N*=3.

appearance. Those that did not freeze but dehydrated appeared more shrunken, but shrinkage was also observed in nematodes that had frozen. Internal gas bubbles were observed in some samples. These effects were not quantified.

Discussion

P. davidi can survive freezing with an appearance that is consistent with intracellular ice formation, confirming the earlier work of Wharton and Ferns (1995). In samples nucleated at -4°C to -6°C, nearly all nematodes froze (95–100%) but many survived (20–73%). Freezing survival is also demonstrated by a greater recovery of nematodes than that predicted if each nematode that had frozen died, particularly for samples where the initial freezing event occurred at -1°C. Survival does decline with decreasing nucleation temperature and with increasing rate of freezing of the sample. This is thought to be due to physical damage resulting from the more rapid spread of ice through the medium (Wharton et al., 2002).

When freezing of the medium was completed at -1°C the

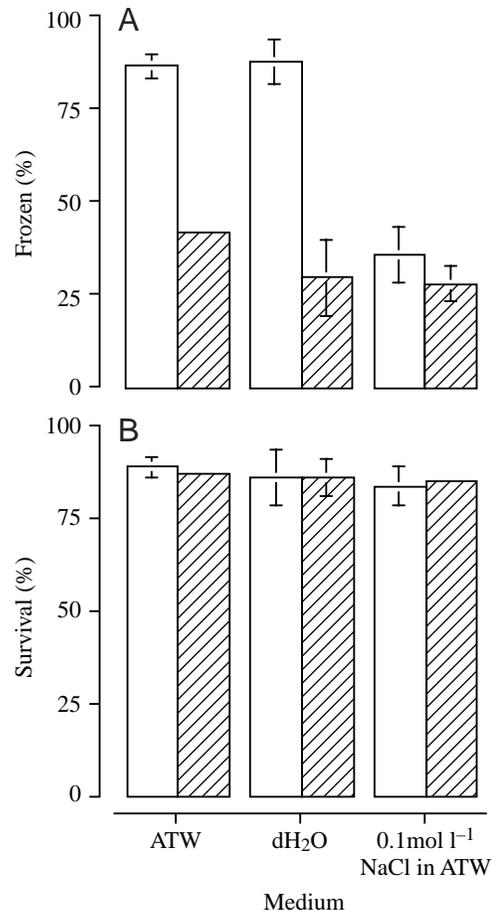


Fig. 5. The effect of different media on the freezing (A) and survival (B) of *P. davidi* after ice nucleation at -1°C and cooling to -5°C. The samples were held at -1°C for 5 min (open bars) or 30 min (hatched bars) after nucleation. Values are means ± S.E.M., *N*=3.

nematodes did not freeze, even though they were surrounded by ice, and instead appeared to dehydrate. There are two possible mechanisms for the loss of water from the nematodes during and after the freezing of the surrounding water. As water freezes salts are excluded from the growing ice crystals and are concentrated in the remaining unfrozen medium (Shepard et al., 1976). This freeze concentration effect could dehydrate the nematodes *via* the resulting osmotic stress. Wharton and To (1996), however, found that inoculative freezing of *P. davidi* occurred in solutions of osmolalities up to 1130 mosmol l⁻¹ and that inoculative freezing would still occur even if salt concentrations were raised by a factor of 120, which is much higher than concentrations likely to be experienced in nature. In the present experiments we found that dehydration occurs upon freezing in deionized water, in which a freeze concentration effect is not possible. It thus seems likely that dehydration is driven by the vapour pressure difference between ice and supercooled water at the same temperature, i.e. the cryoprotective dehydration mechanism (Holmstrup and Westh, 1994). There could, however, be some interaction between a freeze-concentration effect and

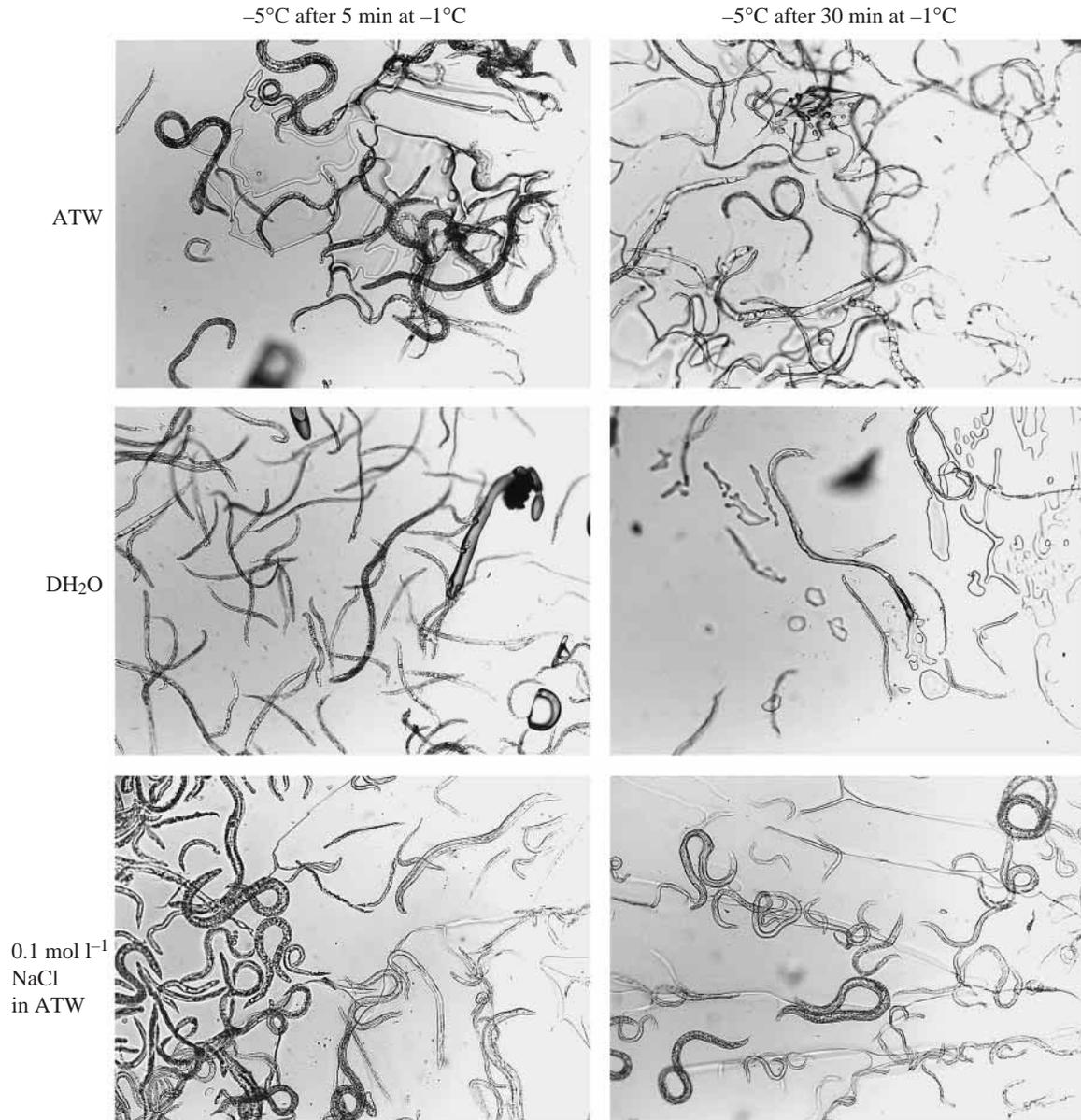


Fig. 6. Photomicrographs of *P. davidi* after cooling to -5°C . Samples were in artificial tapwater (ATW), dH_2O or 0.1 mol l^{-1} NaCl dissolved in ATW. Freezing of the medium was seeded at -1°C and the sample held for 5 min or 30 min before cooling to -5°C at $0.5^{\circ}\text{C min}^{-1}$. In ATW and dH_2O , nematodes held at -1°C for 5 min tended to freeze upon further cooling (as indicated by darkening and no shrinkage), whilst those held at -1°C for 30 min dehydrated (indicated by them shrinking and remaining clear). In 0.1 mol l^{-1} NaCl dissolved in ATW, unfrozen nematodes do not show obvious shrinkage. Scale bar, $20 \mu\text{m}$.

dehydration due to vapour pressure differences. Inoculative freezing was inhibited in 0.1 mol l^{-1} NaCl dissolved in ATW even after freezing at -1°C for 5 min. Survival was greater in this solution than in those of higher or lower osmolality during freezing to -15°C at $1^{\circ}\text{C min}^{-1}$ (Wharton and To, 1996).

The ability to resist inoculative freezing is limited, with nucleation of the nematodes' body fluids occurring at -2 to -4°C , depending upon the time held at a temperature higher than the nucleation temperature and the rate of cooling.

Shrinkage of nematodes whilst being held at -1°C and in unfrozen nematodes during cooling from this temperature indicates that they are losing water to the surrounding medium. If sufficient water is lost this prevents freezing and the nematodes survive through cryoprotective dehydration, the loss of water being driven by the vapour pressure difference between the surrounding ice and the supercooled solution within the nematode. These are separated by the cuticle of the nematode. The cuticle provides a barrier to ice nucleation

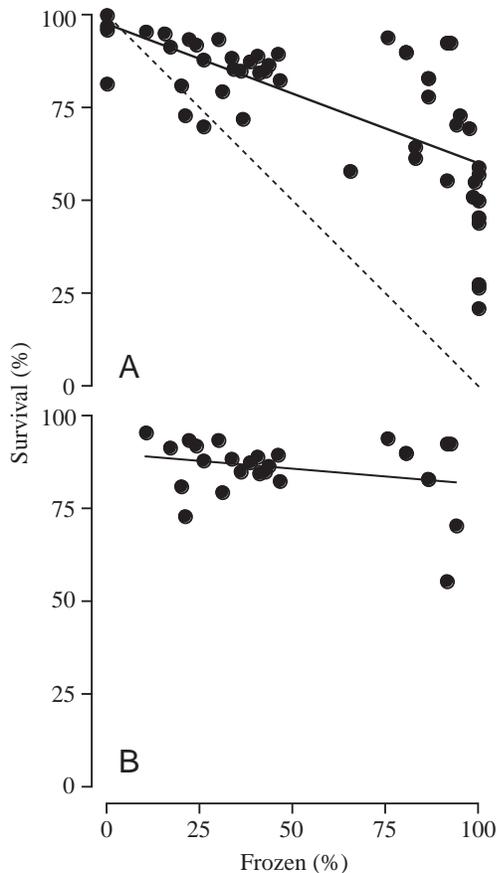


Fig. 7. The effect of freezing on survival in all samples (nucleation at -1°C to -6°C ; A) and on those where nucleation occurred at -1°C (B). The dotted line is the predicted survival if each nematode that had frozen died.

(Wharton and Ferns, 1995). The distribution of ice within a frozen nematode can be visualised using transmission electron microscopy and freeze-substitution techniques. Although ice is formed throughout the cells of the nematode, there is no ice in the cuticle (D. A. Wharton, unpublished observations). The cuticle may thus form a vapour-filled space that separates the supercooled solution within the nematode from the surrounding ice, mediating the transport of water between them.

The relative importance of freezing survival and cryoprotective dehydration in *P. davidi* depends upon the nucleation temperature, the cooling rate and the freezing rate of the surrounding medium. If nucleation occurs at a high subzero temperature (-1°C) the nematode does not freeze. If the temperature is then held above the nucleation temperature for a sufficient time, or if the sample is cooled sufficiently slowly, enough water is lost by cryoprotective dehydration to prevent freezing during further cooling. It should be noted, however, that in all regimes tested some freezing occurred. The minimum freezing in samples at -5°C recorded in any experiment was $21 \pm 2\%$ frozen (in nematodes in ATW cooled to -5°C at $0.1^{\circ}\text{C min}^{-1}$, freezing seeded at -1°C). Nematodes

were recorded as frozen if obvious ice crystals were observed. Some nematodes that had a shrunken appearance nevertheless froze, so shrinkage is not a reliable criterion for the presence or absence of freezing. A relatively low magnification was used for these observations ($100\times$) and the amount of freezing may thus be underestimated. The pattern of ice formation varied, with those freezing at a high subzero temperature having a pattern that might be consistent with extracellular, rather than intracellular, ice formation. The use of an ultrastructural technique, such as freeze substitution (Wharton, 2002), would confirm the presence or absence and the location of ice in specimens.

There are some interesting parallels between our experiments and observations on the freezing of living, mainly mammalian, cells for the development of cryopreservation protocols. During slow freezing mammalian cells dehydrate, but survive if they can withstand the resulting dehydration, or are protected from its effects by cryoprotectants. During rapid freezing, intracellular ice formation occurs and in the cryopreservation literature this is considered always to be fatal (Pegg, 2001). In *P. davidi*, dehydration is favoured during slow freezing of the surrounding medium, whilst during rapid freezing the nematodes freeze but survive, including surviving intracellular freezing (Wharton and Ferns, 1995; present study). A cooling rate of $1^{\circ}\text{C min}^{-1}$ is considered a slow rate by cryobiologists, but in our study this would be considered to be a fast cooling rate, which results in nematodes freezing. Dehydration during slow freezing of mammalian cells is interpreted as being due to the freeze concentration effect, as salts are progressively excluded from the growing ice crystals (Pegg, 2001). Some of the earlier cryopreservation literature, however, considered dehydration of cells during slow freezing to be the result of vapour pressure differences between ice and supercooled water (Mazur, 1966).

The freezing and thawing of soil is a complex phenomenon, influenced by a variety of processes, including snow cover, thermal conductivity and diffusivity, water content, soil water and salt migration and freezing point depression (Eitzinger et al., 2000). Soil moisture content in coastal sites of the McMurdo Sound area of Antarctica varied from 15% to $<1\%$ (w/w), with appreciable differences over short distances (Campbell et al., 1997). Water content at the point across a transect that had the greatest abundance of *P. davidi* at Cape Bird, Antarctica decreased from 57.1% to 14.8% in just 3 days due to the freezing of the adjacent snow bank, which had been providing liquid water to the site (Wharton, 1998). Environmental cooling rates are probably slow, with a maximum rate of $0.021^{\circ}\text{C min}^{-1}$ recorded at Keble Valley, Cape Bird (Sinclair and Sjursen, 2001) but with frequent freeze-thaw cycles during late spring and summer (Sjursen and Sinclair, 2002). Conditions are thus variable with respect to both temperature and water content. Given the likely presence of ice nucleators and the bulk of the soil, the freezing of soil water is likely to be slow and to thus favour cryoprotective dehydration. However, with their limited ability to resist inoculative freezing and variable environment, *P. davidi* must

also have the ability to tolerate freezing. This nematode is also capable of anhydrobiosis (Wharton and Barclay, 1993), and thus has a variety of mechanisms for surviving the harsh conditions of its terrestrial Antarctic habitat.

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