The Journal of Experimental Biology **198**, 1381–1387 (1995)
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SURVIVAL OF INTRACELLULAR FREEZING BY THE ANTARCTIC NEMATODE *PANAGROLAIMUS DAVIDI*

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Accepted 23 February 1995

Summary

Animals are usually thought to survive ice formation in their bodies only if the ice is confined to the body cavity and to extracellular spaces. Intracellular ice formation is believed to be fatal. This conclusion is based on studies of the cryopreservation of mammalian cells. Intracellular freezing has been observed in some living insect cells but has not been observed in intact animals. Nematodes are transparent and so the location of ice in their bodies can be observed directly using a cryomicroscope stage. We have observed freezing and melting in all body compartments, including intracellular compartments, of the Antarctic nematode *Panagrolaimus davidi*. Inoculative freezing from the surrounding water occurs *via* the body openings, rather than across the cuticle; most frequently it occurs *via* the excretory pore. Individual nematodes that have frozen intracellularly will subsequently grow and reproduce in culture. Determining the mechanisms by which this nematode survives intracellular freezing could have important applications in the cryopreservation of a variety of biological materials.

Key words: intracellular freezing, Antarctic nematode, *Panagrolaimus davidi*, cold tolerance.

Introduction

Nematodes are aquatic animals, and when ice forms in the surrounding water they are frozen by inoculative freezing, although they may be freezing-tolerant (Wharton, 1995; Wharton and Block, 1993). Alternatively, they may possess a structure such as a sheath or an eggshell which prevents inoculative freezing and allows them to supercool in the presence of external ice (Wharton, 1994; Wharton and Allan, 1989; Wharton *et al.* 1993). *Panagrolaimus davidi*, an Antarctic nematode, is frozen by inoculative freezing when cooled in water, but survives (Wharton and Brown, 1991). Whether inoculative freezing occurs across the cuticle or *via* one of the body openings is unknown.

Freezing tolerance in animals is thought to be possible only if ice is confined to the body cavity and extracellular spaces (Lee, 1991). This conclusion is based on the cryopreservation of mammalian cells and has not been tested on intact animals. Intracellular freezing is usually considered to be fatal (Karlsson *et al.* 1993; Lee, 1991). However, the survival of intracellular freezing has been reported in isolated insect fat body cells (Lee *et al.* 1993; Lozina-Lozinskii, 1967; Salt, 1959, 1962) and in other cells during warming (Karlsson *et al.* 1993; Rall *et al.* 1980). The survival of intracellular freezing in an intact animal has not been demonstrated.

Nematodes are transparent, allowing ice formation in their bodies to be observed directly during freezing on a cryomicroscope stage. We have observed ice formation in all body compartments, including intracellular compartments, during freezing and the melting of ice crystals during warming. We have also determined that inoculative freezing occurs *via* body orifices, usually the excretory pore, and have shown that nematodes that are known to have undergone intracellular freezing will subsequently recover, grow and reproduce in culture.

Materials and methods

The Antarctic nematode *Panagrolaimus davidi* (Timm), isolated from the McMurdo Sound region of the Antarctic (Wharton and Brown, 1989), was grown on agar plates at 15 °C (Wharton and Brown, 1989, 1991).

Freezing and melting of *P. davidi* was videotaped during cooling and warming on a thermoelectric cold stage mounted on a Zeiss Axiophot photomicroscope. The cold stage was similar to a published design (Wharton and Rowland, 1984). Cooling and warming rates were not controlled for most specimens but were approximately 5 °C min^{-1} during cooling and 4 °C min^{-1} during warming. A computer control system (Wharton and McCormick, 1993) was used to control the rates for some specimens (5, 2, 1, 0.1 °C min^{-1} from 2 °C to −15 °C, followed by holding at −15 °C for 1 min and then warming to 2 °C at 2 °C min^{-1}); there was no difference in the freezing pattern or survival of these specimens. Nematodes were

mounted in artificial tap water (ATW, Greenaway, 1970) between two coverslips designed to fit the specimen chamber of the cold stage, with a few silver iodide crystals to prevent undercooling.

Specimens were videotaped during freezing and melting and the videotape analysed frame by frame to determine the order of freezing of body compartments and whether ice melting could be observed. The time taken for the whole nematode to freeze was determined by counting the number of video frames from the initiation to the completion of the freezing process and by counting the number of video frames during a known time period. Frames were captured from the videotape using a Raster-ops video digitiser, recorded with a Quicktime movie recorder and edited using Adobe Photoshop 2.5LE on an Apple Macintosh computer. Images were compressed with JPEG compression. Some specimens were not videotaped but instead a series of microflash pictures was taken during melting.

In specimens videotaped using a low-power microscope objective lens (×2.5), the body was divided into four equal sections and the section in which ice nucleation began was noted. The nematodes were then transferred to ATW in a watchglass and their survival determined after 24 h at room temperature.

Using higher power objective lenses (×10, ×20, ×40), freezing in different body compartments could be observed in nematodes on the cold stage, mounted individually between two coverslips sealed with Vaseline. The compartments observed were: the anterior oesophagus, the posterior bulb of the oesophagus, the intestine, the pseudocoel (including the space between the oesophagus and the muscle cells, which contains the nerve ring and excretory ampulla) and the muscle cells. These are all intracellular compartments, with the exception of the pseudocoel. The order of freezing of the different body compartments was noted, as was any melting of ice during warming.

Single nematodes, which were known to have frozen intracellularly, were transferred to ATW in a watchglass and their survival was determined after 24 h at room temperature. They were then placed on a 0.1 % nutrient agar plate with a bacterial lawn (one nematode per plate), incubated at 15 °C and examined at intervals for survival and reproduction.

To confirm the presence of intracellular ice in frozen *P. davidi*, the specimens were examined under a transmission electron microscope using freeze-fracture techniques. A drop of nematode suspension in ATW was mounted on a gold disc and lowered into the cooled nitrogen gas curtain of a Reichert KF80 cryofixation unit. The specimen was observed using the microscope mounted on the KF80 and, after freezing, it was rapidly plunged into liquid propane. Control samples were plunged directly into liquid propane without prior freezing. Specimens were transferred into a Balzers BAF300 freeze-fracture unit, fractured, etched for 2 min and platinum/carbon replicas made of the surface. Replicas were cleaned in bleach, picked up on uncoated copper grids and observed using a Phillips EM410 transmission electron microscope.

Results

The water surrounding a nematode always froze first and there was a short but variable delay before ice nucleation within the nematode by inoculative freezing was observed as a marked darkening of the transparent body. If ice nucleation occurred across the cuticle there would be an equal chance of nucleation being initiated in each section of the body. This was not the case; nucleation occurred most frequently in the anterior section (chi-square test; χ^2=50, P=0.00001; 21 of 24 trials). Nucleation did not occur at the tip of the head, and sometimes two ice fronts were seen passing anteriorly and posteriorly from a region just posterior to the tip of the head. At a higher magnification, nucleation was observed to be initiated in the region just anterior to the posterior bulb of the oesophagus. Differential interference contrast optics revealed this to be the opening of the excretory pore. Inoculative freezing occurs most frequently *via* the excretory pore, but was occasionally observed to occur *via* the mouth and anus. Darkening of the whole body was very rapid (0.21±0.04 s, ± S.E.M., N=8). Recovery after 24 h in ATW in these specimens was 72.8±8.7 % (N=8).

Freezing of the different body compartments could be observed using higher-power objective lenses (Fig. 1). All compartments froze, including intracellular compartments. A selection of results are shown in Table 1. A total of 45 individual nematodes were either videotaped or photographed during freezing and melting. Intracellular freezing was observed in 44 of these specimens.

The compartments in which freezing was initiated were: anterior oesophagus (11.8 %), posterior bulb (5.9 %), intestine (17.6 %), pseudocoel (52.9 %), muscle cells (11.8 %) (N=26). These observations include those where the freezing of two compartments was simultaneous. Freezing was only clearly initiated in the pseudocoel, anterior oesophagus and the intestine. Initiation of freezing in the pseudocoel was significantly higher than would be expected if compartments froze at random (χ^2=24.2, P=0.0001). Inoculative freezing occurs mainly *via* the excretory pore but can also occur *via* the mouth and the anus.

Melting of ice crystals was observed in all body compartments (Table 1; Fig. 2). Melting tended to occur at the periphery of the nematode before the centre, but there was little difference in the melting temperature of the different body compartments.

The presence of intracellular ice in all body compartments was confirmed using freeze-fracture techniques (Fig. 3). Ice crystals were observed in all parts of the body of frozen specimens (Fig. 3A,B) but not in non-frozen controls (Fig. 3C). The viability of specimens frozen in the gas curtain of the cryofixation apparatus but not plunged into liquid propane was 49.6±11 % (±1 S.E.M., N=4).

Our strain of *P. davidi* is parthenogenetic and the reproduction of single nematodes can be followed on agar plates. Of 33 nematodes that were known to have frozen intracellularly and whose survival on agar cultures was followed, 23 (70 %) laid eggs and 22 (67 %) produced the next generation of larvae (Table 1).

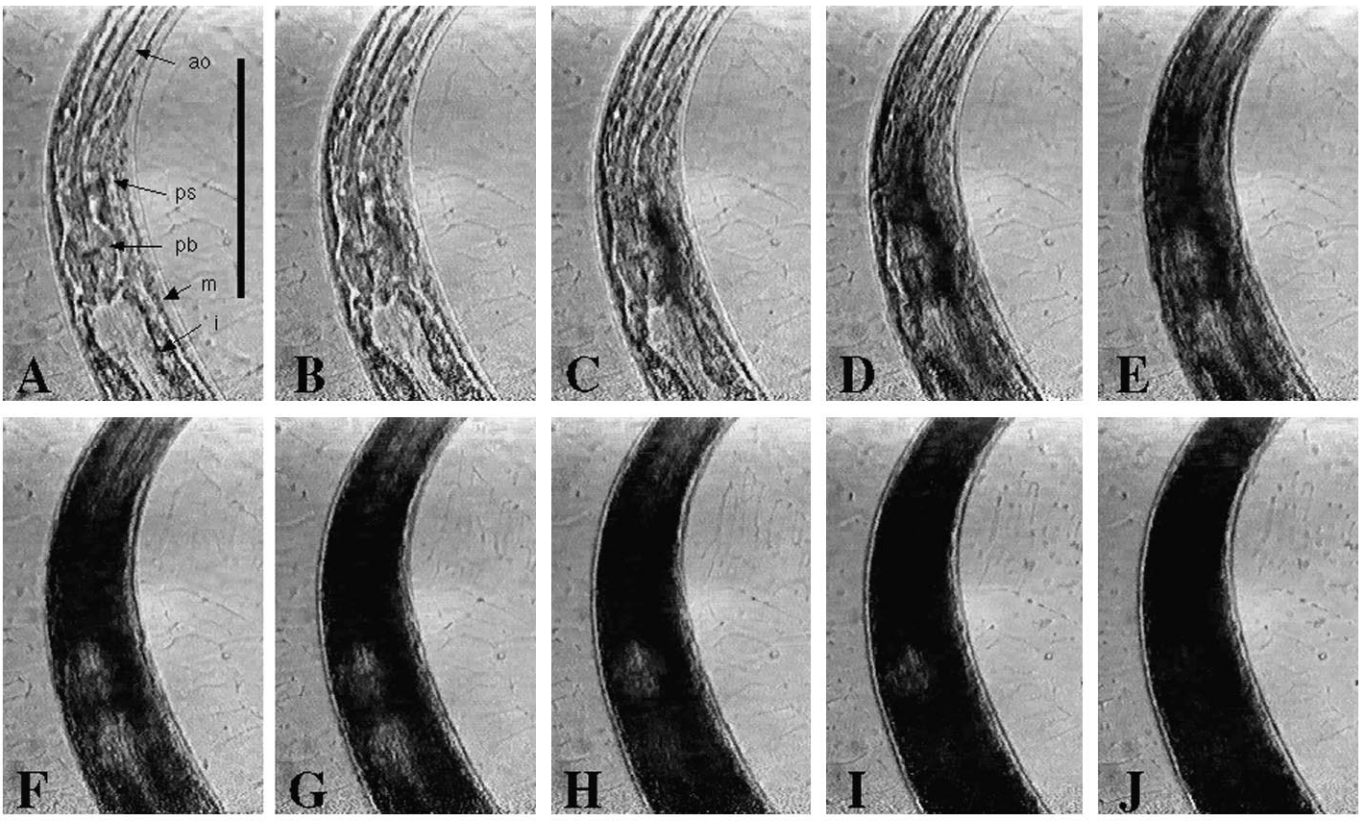

Fig. 1. The freezing of *P. davidi* videotaped during cooling on a cold stage mounted on a Zeiss Axiophot photomicroscope. Freezing was initiated in the space between the oesophagus and the body wall in the region just anterior to the posterior bulb of the oesophagus (A,B). Freezing spread through the pseudocoel (C–G) and then to the intestine (H,I) and the oesophagus (I,J). ao, anterior oesophagus; pb, posterior bulb of oesophagus; i, intestine; ps, pseudocoel (including the space between the oesophagus and the muscle cells which includes the nerve ring); m, muscle cells. Scale bar, 100 μm.

Discussion

Panagrolaimus davidi survives intracellular freezing that is initiated by inoculative freezing from the surrounding medium. We have demonstrated intracellular freezing by observing freezing and melting in intracellular compartments using a cold microscope stage and the presence of intracellular ice has been confirmed at the electron microscope level using freeze-fracture techniques. The profiles of ice crystals in our

Table 1. *Freezing and melting events during cooling and warming of* Panagrolaimus davidi

		Freezing					Melting					
Specimen number	Magnif-ication	Anterior oesophagus	Posterior bulb	Intestine	Pseudo-coel	Muscle cells	Anterior oesophagus	Posterior bulb	Intestine	Pseudo-coel	Muscle cells	Survival
3	10	1	2	1	1	?	+	+	+	+	?	2
9	20	3	3	2	1	?	–	–	+	+	?	3
11	20	–	3	2	1	?	–	+	+	+	?	3
12	20	1	2	3	2	?	+	+	+	+	?	3
14	40	–	–	3	1	2	–	–	+	+	+	3
17	40	–	3	1	2	2	–	+	+	?	+	3
18	40	–	–	1	2	2	–	–	+	+	+	3
22	20	4	5	2	1	3	–	–	+	+	+	3
25	20	3	3	1	2	2	+	+	+	+	+	1
37	20	–	nf	3	1	2	–	–	+	+	+	3

Freezing: 1–5, order of freezing; nf, not frozen; –, not determined (obscured); ?, unsure.
Melting: +, ice melting observed; –, ice melting not observed (obscured or not frozen); ?, unsure.
Survival: 1, feeding; 2, egg laying; 3, hatched first stage larvae.

specimens were similar to those observed in frozen yeast cells (Bank and Mazur, 1973). Freezing tolerance has been demonstrated in only a few species of nematodes. All freezing-tolerant nematodes that have been observed using cryomicroscopy darken after freezing (Asahina, 1959; Sayre, 1964; Wharton and Allan, 1989; Wharton and Block, 1993). The degree of darkening indicates that it is unlikely that ice is confined to the body cavity and therefore that intracellular freezing probably also occurs in these nematodes.

Many freezing-tolerant animals are relatively large and opaque, so it may be difficult to determine whether they freeze intracellularly. A freezing-tolerant weta (a large orthopteran insect) was dissected in a walk-in freezer. Ice was observed in all body cavities, and most of the tissues, with the exception of the ventral nerve cord, appeared to be frozen (Ramløv and Westh, 1993). In frozen frogs, ice crystals are found under the skin, between the skeletal muscles and in the abdominal cavity (Storey and Storey, 1992). In the insect *Eurosta solidaginis*, only the fat body and labial gland cells freeze intracellularly (Salt, 1962). In contrast, intracellular freezing was observed in all parts of the body of *P. davidi*.

The survival of individual nematodes after freezing intracellularly was followed in culture. The nematodes laid eggs and produced the next generation of larvae; survival after freezing is thus biologically significant. There is no difference in population size of cultures established from *P. davidi* frozen

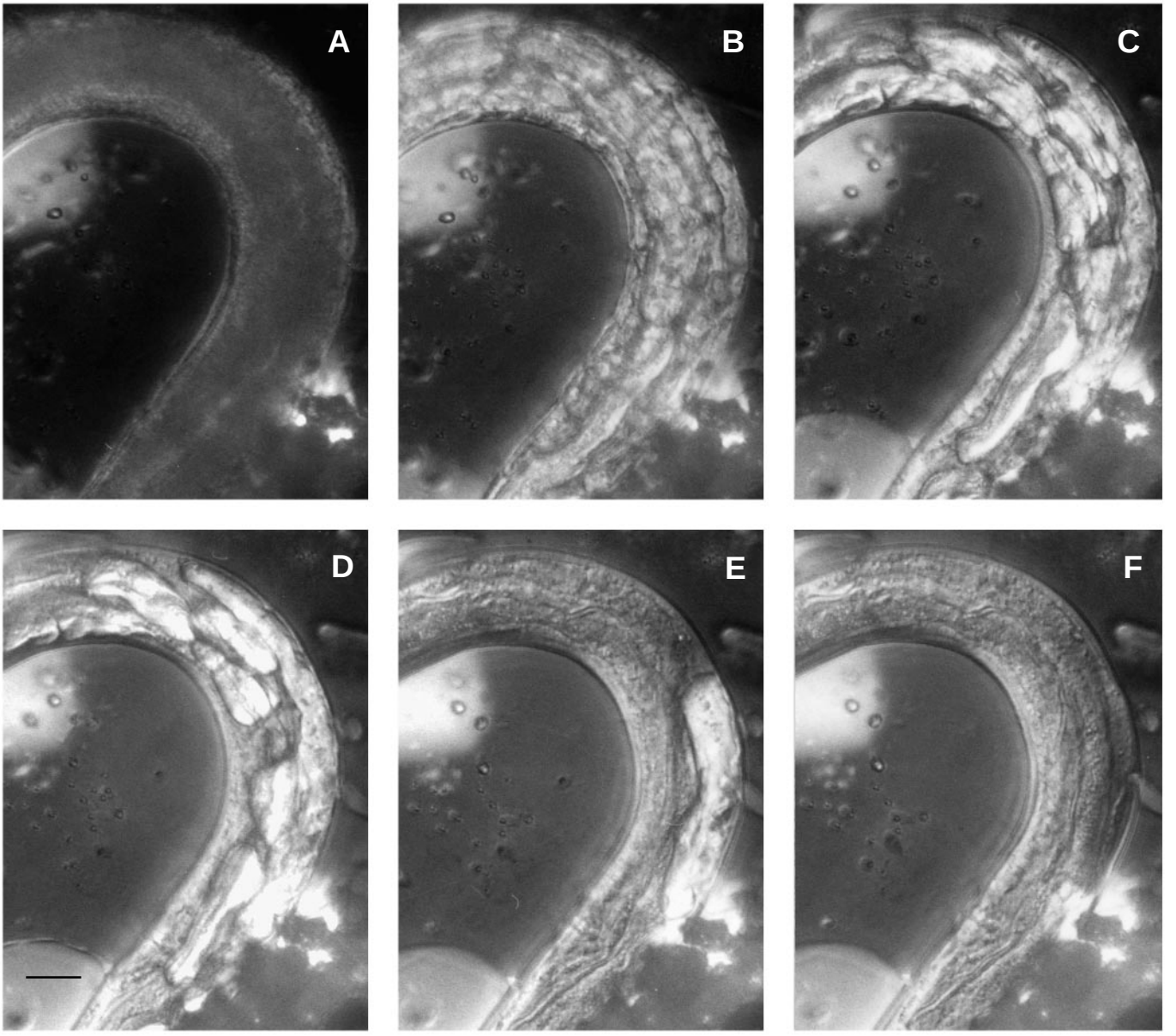

Fig. 2. Melting of ice in *P. davidi* (B–F) after freezing to −20 °C (A) on a cold stage mounted on a Zeiss Axiophot photomicroscope. The photographs were taken using a microflash and differential interference contrast optics. Ice melting can be seen in all parts of the body. Scale bar, 20 μm.

to −20 °C and those established from non-frozen controls (Brown, 1993).

The cuticle acts as a barrier to ice nucleation, which occurs *via* the body orifices. Nucleation usually occurred *via* the excretory pore, although it could also occur *via* the mouth and the anus. Body openings, such as the anus and excretory pore, possess only dilator muscles and are closed by the action of the high internal turgor pressure when these muscles relax (Wharton, 1986). The excretory pore is one of the smallest body orifices and may be less able to resist the entry of ice during inoculative freezing. In nematodes that retain their cuticle from the previous stage in their life cycle as a sheath, inoculative freezing is prevented (Wharton and Allan, 1989; Wharton and Surrey, 1994). Presumably, those parts of the sheath that had openings when it was the cuticle of the previous stage are sealed when it forms the sheath.

The plasma membrane is thought to prevent ice nucleation (Grout and Morris, 1987). However, ice rapidly spreads throughout the body of *P. davidi*. This may indicate a specialisation of the membranes of this nematode which allows ice nucleation across the plasma membrane. Intracellular freezing occurs in cell suspensions during rapid freezing and is thought to be responsible for cell death (Grout and Morris, 1987). There are a number of theories as to how intracellular ice formation causes cell death (Karlsson *et al.* 1993). Damage may result from the mechanical forces developed during ice formation, or ice may rupture the membranes of organelles.

Thermal hysteresis or antifreeze proteins are found in

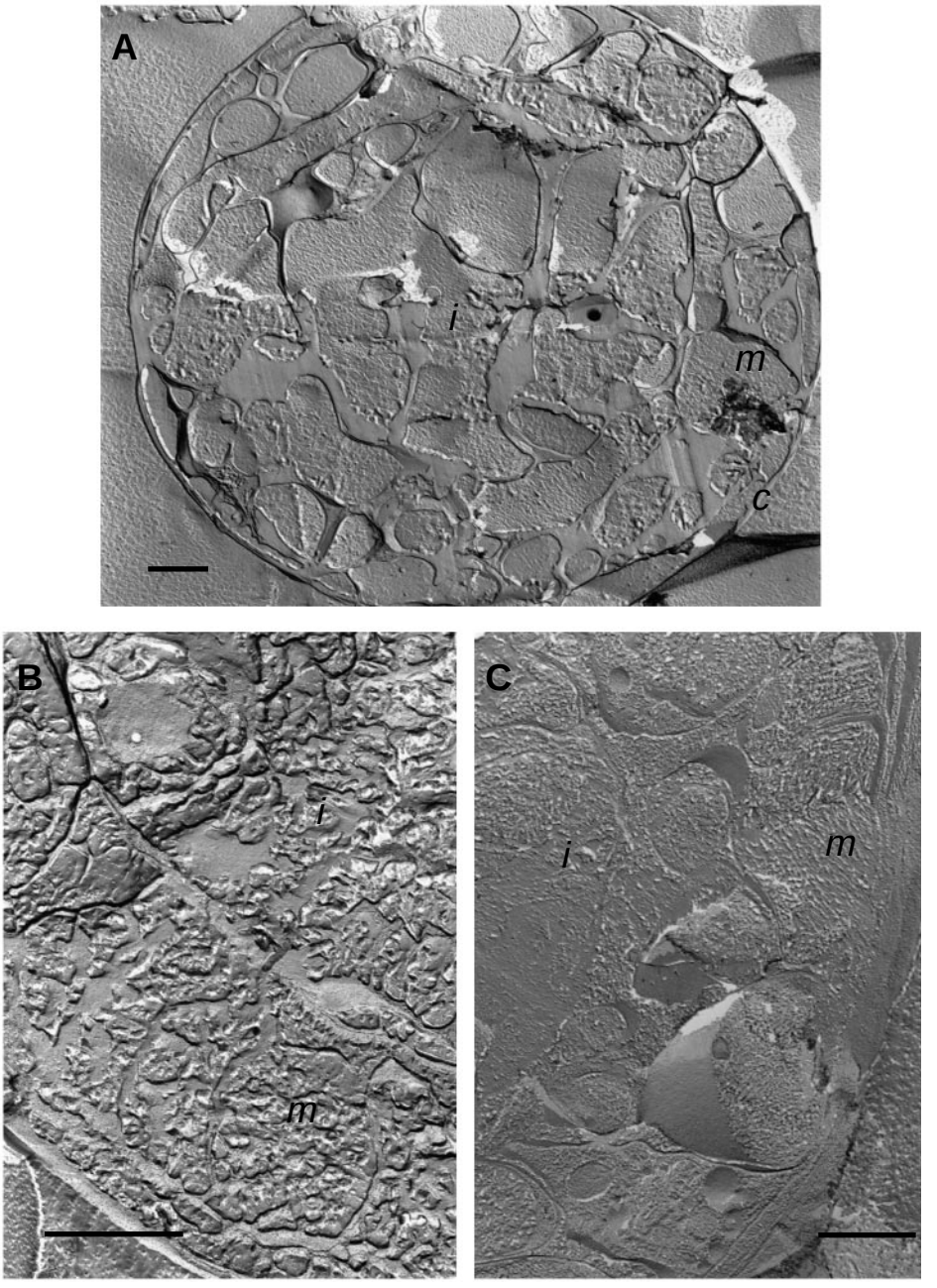

Fig. 3. Freeze-fracture replicas of *P. davidi*. Ice crystals could be observed in all parts of the body of frozen specimens (A and B) but not in non-frozen controls (C). Scale bars, 1 μm. *c*, cuticle; *i*, intestine; *m*, muscle cells.

arthropods and polar fishes. They are mainly found in freeze-avoiding species and stabilise the supercooled state by lowering the temperature at which a seed ice crystal will grow (Duman *et al.* 1991). However, thermal hysteresis proteins have also been found in a few freezing-tolerant insects and a freezing-tolerant centipede (Duman *et al.* 1991), where they inhibit recrystallization (Knight and Duman, 1986). Freezing-tolerant nematodes might produce similar substances that affect the way in which ice forms in their bodies and thus prevent mechanical damage from intracellular freezing. In some systems intracellular ice formation results in the formation of gas bubbles during warming, which may be responsible for cell death (Ashwood-Smith *et al.* 1988; Morris and McGrath, 1981). Gas-bubble formation was not observed in our system.

Ice formation results in the concentration of solutes remaining in the non-frozen portion of a solution, causing dehydration by osmosis in adjacent non-frozen cells or organelles (Karlsson *et al.* 1993). The extremely rapid freezing of all body compartments observed in *P. davidi* may prevent the osmotic stresses that would result if different compartments froze at different times.

The freezing tolerance ability of *P. davidi* appears to be related to culture age and thermal history (Wharton and Brown, 1991). We are investigating the potential role of cryoprotectants in this phenomenon. Understanding how *P. davidi* survives intracellular freezing may improve techniques for the storage of biological material for a variety of medical and industrial purposes.

We would like to acknowledge Karen Judge, Mark Gould and Richard Träbing for technical assistance, Graham Young and Alison Mercer for their comments on the manuscript, and the financial support of a Summer Vacation Research Bursary from the University of Otago.

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