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RESEARCH ARTICLE

The ability to survive intracellular freezing in nematodes is related to the pattern and distribution of ice formed

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ABSTRACT

A few species of nematodes can survive extensive intracellular freezing throughout all their tissues, an event that is usually thought to be fatal to cells. How are they able to survive in this remarkable way? The pattern and distribution of ice formed, after freezing at −10°C, can be observed using freeze substitution and transmission electron microscopy, which preserves the former position of ice as white spaces. We compared the pattern and distribution of ice formed in a nematode that survives intracellular freezing well (*Panagrolaimus* sp. DAW1), one that survives poorly (*Panagrellus redivivus*) and one with intermediate levels of survival (*Plectus murrayi*). We also examined *Panagrolaimus* sp. in which the survival of freezing had been compromised by starvation. Levels of survival were as expected and the use of vital dyes indicated cellular damage in those that survived poorly (starved *Panagrolaimus* sp. and *P. murrayi*). In fed *Panagrolaimus* sp. the intracellular ice spaces were small and uniform, whereas in *P. redivivus* and starved *Panagrolaimus* sp. there were some large spaces that may be causing cellular damage. The pattern and distribution of ice formed was different in *P. murrayi*, with a greater number of individuals having no ice or only small intracellular ice spaces. Control of the size of the ice formed is thus important for the survival of intracellular freezing in nematodes.

KEY WORDS: Intracellular ice, *Panagrolaimus*, Antarctic, Freeze substitution, Transmission electron microscopy

INTRODUCTION

Freezing-tolerant animals can survive ice forming in their bodies (Lee, 2010). They include some invertebrates (e.g. insects, nematodes, molluscs) and ectothermic vertebrates (e.g. frogs, hatchling turtles). Intracellular ice formation is usually thought to be fatal because mechanical disruption may be caused by the expansion of water as it freezes, the puncturing of membranes by ice crystals and the redistribution (recrystallization) of ice crystals after freezing and during thawing (Acker and McGann, 2001; Karlsson et al., 1993; Muldrew et al., 2004). There are, however, examples of particular cells and tissues of insects (e.g. fat body and midgut cells) surviving intracellular freezing, and this may be widespread in freezing-tolerant insects (Sinclair and Renault, 2010). Even some isolated mammalian cells and tissues will survive intracellular freezing (Acker and McGann, 2002; Rall et al., 1980; Sherman, 1962) and the frozen cells may even survive better than unfrozen cells (Acker and McGann, 2002).

The only animals that have been shown to survive extensive intracellular freezing throughout their tissues are a few species of nematodes, particularly *Panagrolaimus davidi* Timm 1971 (Wharton and Ferns, 1995). The species used in this study is now known as *Panagrolaimus* sp. DAW1, as rRNA sequencing shows that field-sourced *P. davidi* and the cultured strain are different species (Raymond et al., 2014). *Panagrolaimus davidi* (field strain) is found in coastal areas in the McMurdo Sound region of Antarctica (Wharton and Brown, 1989), particularly in soil supplied with liquid water close to bird colonies (Raymond and Wharton, 2013), whereas *Panagrolaimus* sp. DAW1 has been isolated from similar habitats at Cape Bird and Cape Royds, Ross Island, Antarctica (D.A.W., unpublished observations). Soil surface temperatures as low as −39.6°C have been recorded at Cape Bird (Sinclair and Sjursen, 2001). Some other species of nematodes have been shown to have a limited ability to survive intracellular freezing (Ali and Wharton, 2014; Wharton and Raymond, 2015).

The ability of *Panagrolaimus* sp. DAW1 to survive intracellular freezing is dependent upon its nutritional state (Raymond and Wharton, 2013). This provides the opportunity to make comparisons between fed cultures with a high level of survival of this stressor and starved cultures with a lower level of survival. Freeze substitution preserves the former position of ice in frozen nematodes and the pattern and distribution of ice formed can be observed by transmission electron microscopy (Wharton et al., 2005). In the present study, we have compared the pattern and distribution of ice formed in fed and starved *Panagrolaimus* sp. after freezing at a temperature (−10°C) that produces intracellular freezing (Raymond and Wharton, 2013) and used vital dyes (Lee et al., 1993) to look for cellular damage. We have also made comparisons with the oatmeal nematode, *Panagrellus redivivus* (Linnaeus 1767) Goodey 1945, whose natural habitat is thought to be fermenting tree resin, has very low survival after freezing at −10°C (Hayashi and Wharton, 2011); and with another Antarctic nematode with a similar distribution to that of *P. davidi*, *Plectus murrayi* Yeates 1970, that has intermediate levels of survival (Raymond et al., 2013; Wharton and Raymond, 2015). We used nematodes grown at 20°C to avoid any starvation effects of cold acclimation in our fed cultures (Raymond and Wharton, 2013). Our hypothesis is that the pattern and distribution of ice formed will reflect the different levels of survival of intracellular freezing between these treatments and species.

MATERIALS AND METHODS

Panagrolaimus sp. DAW1 (formerly known as *Panagrolaimus davidi* or *P. davidi* CB1) is available from the Caenorhabditis Genetics Centre (www.cbs.umn.edu/research/resources/cgc). Fed and starved cultures were generated as described by Raymond and Wharton (2013). *Plectus murrayi* were isolated from the Victoria Land coast at Terra Nova Bay Antarctica and cultured on 0.1%

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nutrient agar or sand agar plates (Wharton and Raymond, 2015), with a layer of artificial tap water (ATW; Greenaway, 1970) or balanced salt solution (BSS; Piggott et al., 2000). Oatmeal nematodes, *Panagrellus redivivus*, were sourced from a commercial supplier (as 'microworms', Biosuppliers, Auckland, New Zealand) and cultured on autoclaved rolled oats (Hayashi and Wharton, 2011). *Panagrolaimus* sp. and *P. murrayi* are parthenogenetic females, *P. redivivus* are mixed males and females. Cultures (mixed stages) were grown at 20°C for 2 weeks and nematodes were harvested using a modified Baermann funnel technique (Hooper, 1986). Samples were taken from four replicate cultures, and the nematodes were washed three times in ATW or BSS and used immediately for experiments. This work was performed in accordance with the procedures of the University of Otago's Animal Ethics Committee.

Survival of a stress that produces intracellular freezing

Survival of a freezing stress known to produce intracellular freezing in *Panagrolaimus* sp. DAW1 (Raymond and Wharton, 2013) was tested by transferring subsamples of the cultures used for freeze substitution (see below) of ~100 nematodes in 10 µl of ATW to small microcentrifuge tubes. These were then placed in a cooling block (Wharton et al., 2004), the temperature of which was controlled by a programmable refrigerated circulator (Haake Phoenix II, Thermo Fisher Scientific, Waltham, MA, USA). Sample temperatures were monitored by thermocouples immersed in the sample and interfaced to a computer via a Powerlab A/D converter (ADInstuments, Dunedin, New Zealand). The samples were cooled from +1°C to −10°C at 0.5°C min^{-1} and if freezing had not occurred spontaneously it was initiated by adding a small ice crystal. Samples were held at −10°C for 30 min, warmed to +1°C at 0.5°C min^{-1} and then transferred to a watchglass at room temperature in 1 ml of ATW. Survival was determined after 24 h by counting the proportion of nematodes moving after a physical stimulus (stirring with a pipette) and compared between groups (fed and starved *Panagrolaimus* sp., *P. murrayi* and *P. redivivus*) using one-way ANOVA after arcsin transformation of percentages in SPSS (SPSS Inc., Chicago, IL, USA). Levene's test indicated that variances were significantly different between treatments and so a *post hoc* test that does not assume equal variances was used (Tamhane's T2) to compare means.

Vital staining

Cell survival was tested using vital dyes on nematodes frozen at −10°C by the regime above after 24 h recovery and on unfrozen controls. Whole nematodes were stained with propidium iodide and Acridine Orange using previously described protocols (Lee et al., 1993; Sinclair and Wharton, 1997), observed by epifluorescence on a Zeiss Axiophot microscope, and the proportion containing all green nuclei (alive), all red nuclei (dead) and a mixture of red and green nuclei was counted.

Freeze substitution and transmission electron microscopy

Concentrated samples of nematodes (100–200 in 10 µl of ATW, four replicates with each replicate being from a separate culture) were frozen by the regime described above and were plunged into liquid nitrogen after completing 30 min at −10°C. They were then transferred to a freeze substitution mixture (3% glutaraldehyde, 1% osmium tetroxide and 0.5% uranyl acetate in methanol) at −90°C in a Reichert automatic freeze substitution apparatus. They were processed for transmission electron microscopy as described by Wharton et al. (2005) into Spurr resin in a single conical beam capsule for each replicate. Sections were cut on a Reichert ultramicrotome, picked up on Formvar-coated copper grids, stained with uranyl acetate and lead citrate and observed on a Philips 410 transmission electron microscope operated at 80 kV. Unfrozen control samples were processed by conventional chemical fixation (Wharton et al., 2005).

The highly concentrated nature of the sample (100–200 nematodes) ensured that a single section of each replicate provided enough individuals for analysis. The section was selected randomly as the first suitable section to be seen under the microscope (no tears, not too thick). The nematodes within that section were selected randomly by systematically searching the section starting at its top left corner, to ensure that no individual was missed or counted/measured twice. All the nematodes that were transversely sectioned (indicated by a round rather than an oblique profile) through the intestinal region were classified as containing no ice (these often had a shrunken appearance), extracellular ice only or intracellular ice (these often also had extracellular ice), as described by Wharton et al. (2005). Differences between species or treatment in the proportion containing intracellular ice were analysed using one-way ANOVA as for survival. Levene's test indicated that variances were not significantly different between treatments and so a Scheffe *post hoc* test was used to compare means.

The section was searched again as before and the first 10 nematode transverse sections seen from each of the four replicates for each group were photographed for further analysis. The total body area and the area of the five largest ice-filled spaces were measured using Image J (Rasband, 1997-2005). The proportional body area occupied by the five largest ice-filled spaces was compared using univariate GLM after square root transformation of percentages. Levene's test indicated that variances were not significantly different between treatments and so a Scheffe *post hoc* test was used to compare means. There were no significant differences between replicate cultures and so the data from these were combined. The proportion of the body area occupied by the largest ice-filled space and the proportion of nematodes that contained at least one large ice-filled space (defined as being >10% of the total body area) were also calculated.

RESULTS

Survival and vital staining

Fed *Panagrolaimus* sp. DAW1 survived freezing at −10°C best with 79% surviving, followed by starved *Panagrolaimus* sp. DAW1 and then *P. murrayi*; survival was very low (<3%) in *P. redivivus* (Fig. 1). Survival was significantly different between the four groups ($F_{3,12}$=134.5, P≤0.0001).

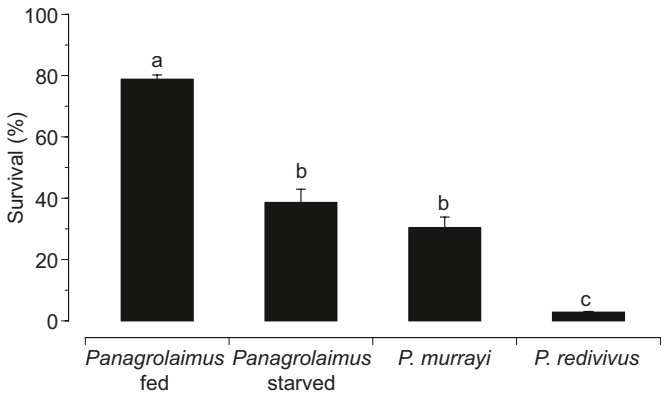

Fig. 1. Survival of fed and starved *Panagrolaimus* sp. DAW1, *Plectus murrayi* and *Panagrellus redivivus* after freezing at −10°C for 30 min. Data are means ±s.e.m. (N=4, replicate cultures). Different lowercase letters indicate significant differences between means (P<0.05).

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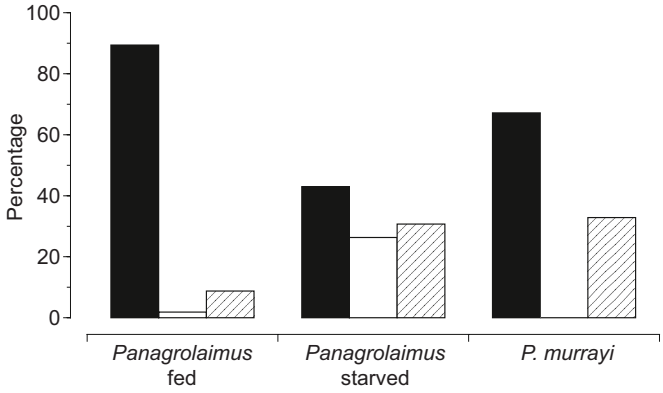

Fig. 2. Percentage of fed and starved *Panagrolaimus* sp. DAW1 and *Plectus murrayi* that had all green-stained (alive, filled bars), all red-stained (dead, open bars) or a mixture of red- and green-stained nuclei (hatched bars) after freezing at −10°C for 30 min and vital staining. Bars indicate the mean of two technical replicates.

In unfrozen controls of fed and starved *Panagrolaimus* sp. DAW1 and of *P. murrayi*, >90% of nematodes contained all live (green-stained) cell nuclei after vital staining. In *P. redivivus* unfrozen controls there was no staining of cell nuclei, just green staining of the lumen of the oesophagus.

Most fed *Panagrolaimus* sp. DAW1 contained all live (green-stained) cell nuclei after freezing and vital staining (89.4%). In contrast, only 43% of starved *Panagrolaimus* sp. DAW1 contained all live cells after freezing and vital staining; the remainder contained all dead (red-stained) cells or a mixture of red- and green-stained cells (Fig. 2). In those containing both red- and green-stained cells, dead cells were found most frequently in the head and middle regions of the body and less frequently in the tail region. All tissue types (oesophagus, muscle, intestine) contained dead cells, with damage usually occurring in several tissues.

In *P. murrayi* after freezing and vital staining, 67.2% of nematodes contained all live cells, the remainder containing a mixture of green- and red-stained cells. In *P. redivivus* after freezing and vital staining there was too much structural disruption to distinguish nuclei, but both red- and green-stained material was seen.

Pattern and distribution of ice formed

Structural differences can be seen between unfrozen chemically fixed controls of fed and starved *Panagrolaimus* sp. DAW1 (Fig. 3A,B). Most noticeable are large spaces filled with lightly stained granular material that appear in the cytoplasm of

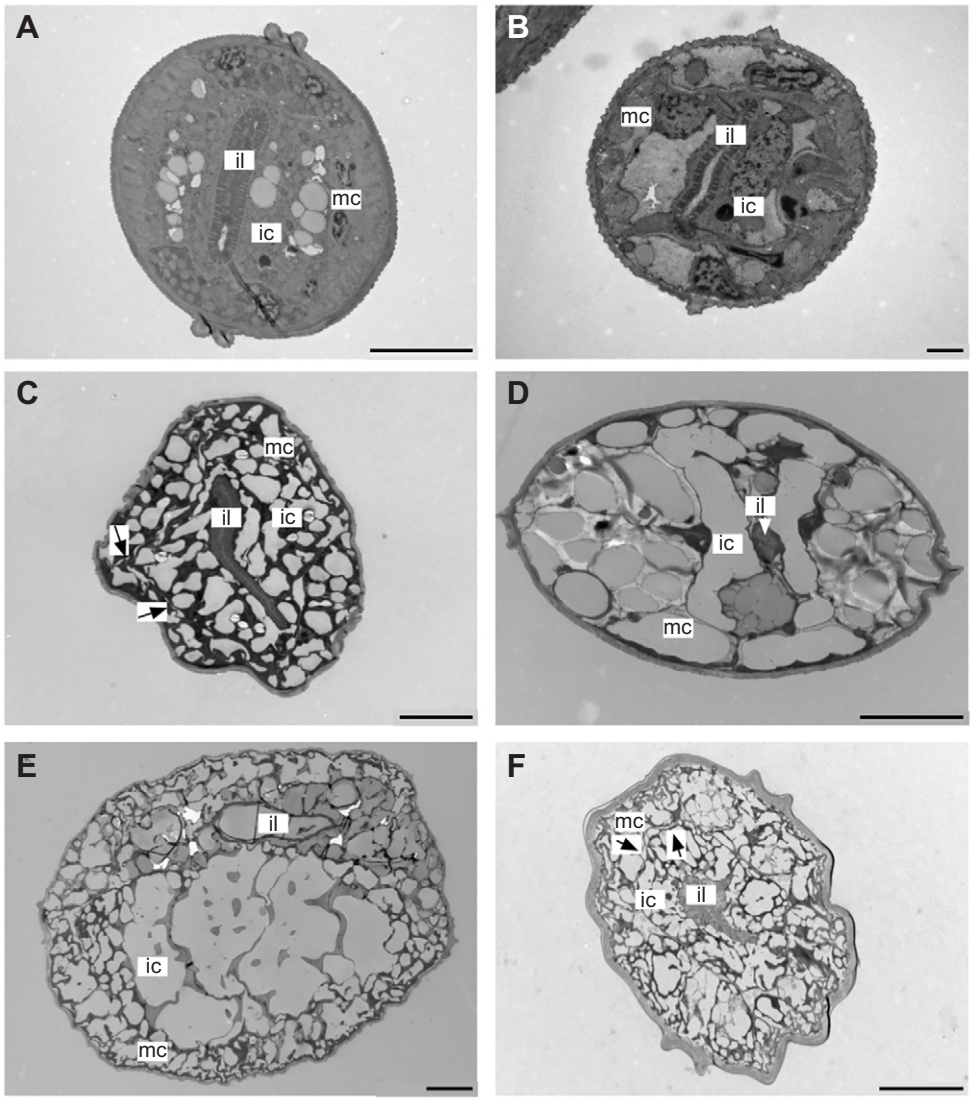

Fig. 3. Transmission electron micrographs of *Panagrolaimus* sp. and *Panagrellus redivivus*. (A) *Panagrolaimus* sp. DAW1 fed, unfrozen, conventional fixation; (B) *Panagrolaimus* sp. DAW1 starved, unfrozen, conventional fixation; (C) *Panagrolaimus* sp. DAW1 fed, frozen, freeze substitution; (D) *Panagrolaimus* sp. DAW1 starved, frozen, freeze substitution; (E) *P. redivivus* fed, frozen, freeze substitution; (F) *P. murrayi* fed, frozen, freeze substitution. ic, intestinal cell cytoplasm; il, intestinal lumen; mc, muscle cell. Scale bars=5 µm. Most of the ice is intracellular but some areas of extracellular ice are indicated in C and F (arrows).

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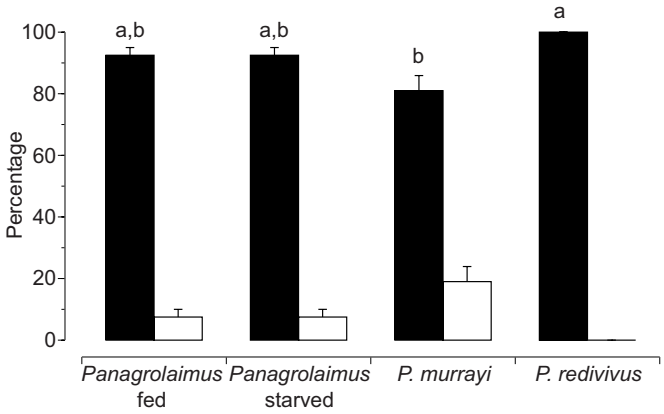

Fig. 4. Percentage of fed and starved *Panagrolaimus* sp. DAW1, *Plectus murrayi* and *Panagrellus redivivus* that had intracellular ice (filled bars) or no ice (open bars) after freezing at −10°C for 30 min. There were no nematodes that had extracellular ice only. Data are means±s.e.m. (N=4, replicate cultures). Different lowercase letters indicate significant differences between means (P<0.05).

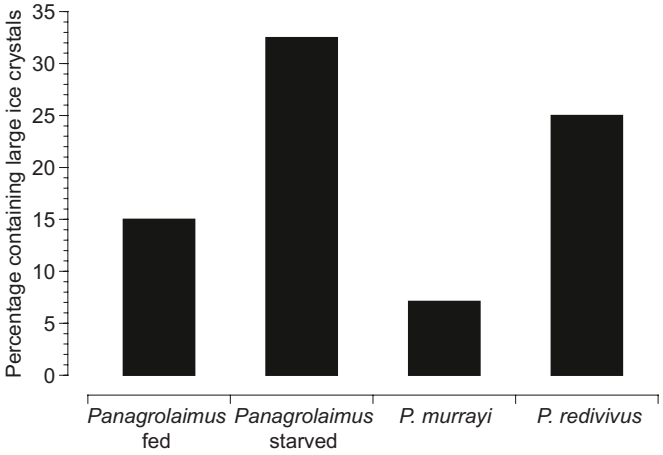

Fig. 6. Percentage of *Panagrolaimus* sp. DAW1, *Plectus murrayi* and *Panagrellus redivivus* that contained at least one large ice-filled space (defined as being >10% of the total body area). The number of nematodes measured is shown in Fig. 5.

intestinal, muscle and epidermal cord cells of starved nematodes (Fig. 3B). In contrast, the cytoplasm of fed nematodes is filled with densely stained granular material, organelles and lipid droplets (Fig. 3A).

In nematodes frozen at −10°C, processed by freeze substitution, the former positions of ice are retained as lightly stained empty spaces (Fig. 3C–F). The ice spaces were larger, fewer and less regular in frozen starved *Panagrolaimus* sp. DAW1 (Fig. 3D) than they were in frozen fed nematodes (Fig. 3C), and cellular structures were much more disrupted in starved than in fed nematodes. Ice spaces in *P. redivivus* were even larger and more irregular (Fig. 3E), although these nematodes often also contained many small ice spaces. The pattern and distribution of ice formed in *P. murrayi* was quite variable but appeared intermediate between the pattern and distribution in fed *Panagrolaimus* sp. and that seen in *P. redivivus* and in starved *Panagrolaimus* sp. (Fig. 3F). Enlarged versions of Fig. 3C–F are provided as Figs S1–S4.

In all three species, and in both fed and starved *Panagrolaimus* sp. DAW1, most individuals contained intracellular ice (Fig. 4).

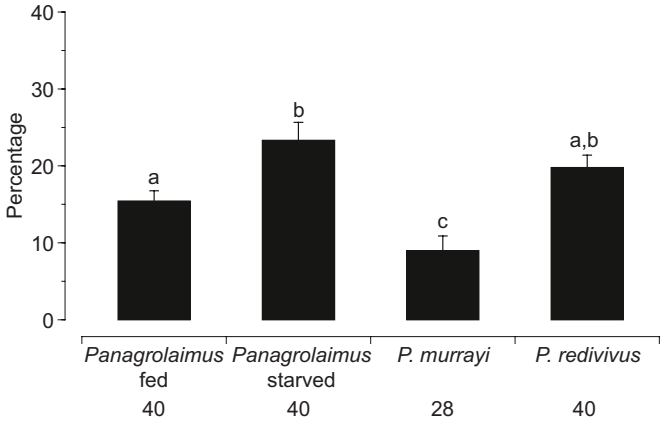

Fig. 5. Percentage of *Panagrolaimus* sp. DAW1, *Plectus murrayi* and *Panagrellus redivivus* cross-sectional area occupied by the five largest ice-filled spaces. The number shown below the *x*-axis labels is the number of nematodes measured, after excluding those without ice. Data are means± s.e.m. Different lowercase letters indicate significant differences between means (P<0.05).

There were no individuals that contained only extracellular ice, although many of those that had intracellular ice also had extracellular ice. In fed and starved *Panagrolaimus* sp., a small percentage (7.5%) contained no ice and appeared dehydrated, whereas in *P. murrayi* 19% contained no ice and 29% appeared dehydrated. The dehydrated individuals contained either no ice or a small percentage of ice (<10%). In *P. redivivus* there were no individuals that did not contain ice and none appeared dehydrated (Fig. 4). Overall, the proportion that contained intracellular ice was significantly different between the four groups ($F_{3,14}$=6.99, P=0.007).

The five largest ice-filled spaces occupied the greatest proportion of the body area in starved *Panagrolaimus* sp. and the smallest proportion in *P. murrayi* (Fig. 5). Proportions between groups were significantly different ($F_{3,147}$=14.083, P<0.0001). The proportion of nematodes that contained large ice-filled spaces (at least one that was >10% of the body area) was greatest in starved *Panagrolaimus* sp. and smallest in *P. murrayi* (Fig. 6). The largest ice-filled space occupied 62.3% of the body area in starved *Panagrolaimus* sp., 15.4% in fed *Panagrolaimus* sp., 26.4% in *P. murrayi* and 15.9% in *P. redivivus*.

DISCUSSION

The survival of the nematode species and treatments tested after exposure to freezing at −10°C is in line with that observed in previous studies (Hayashi and Wharton, 2011; Raymond and Wharton, 2013; Wharton and Raymond, 2015). This freezing exposure is survived best by fed *Panagrolaimus* sp. DAW1. Survival is lower in starved *Panagrolaimus* sp. and in *P. murrayi*, and there is very low survival in *P. redivivus*. A further comparison of the cold tolerance of these species is provided by calculations of the 50% survival temperatures (S_{50}) of nematodes exposed to a series of subzero temperatures under the same conditions (not acclimated): *Panagrolaimus* sp., −25.4°C (Smith et al., 2008); *P. murrayi*, −3.9°C (Wharton and Raymond, 2015); and *P. redivivus*, −1.3°C (Smith et al., 2008). The S_{50} of starved *Panagrolaimus* sp. is −6.3°C (calculated from data from Raymond and Wharton, 2013).

Freezing injuries may occur as a result of the disruption of cell membranes. Vital dyes that assess cell viability based on membrane integrity are often used to test the viability of spermatozoa and oocytes preserved by freezing (Garner and Johnson, 1995). Vital

dyes are useful for studying freezing injuries at the cellular level by staining dissected tissues after cold exposure of insects (Yi and Lee, 2003). The use of vital dyes on whole nematodes enabled cell damage after freezing to be visualised, and may be useful for future studies on cellular injuries in nematodes, and similar animals, generally. However, the technique is not applicable to all species. Live *P. redivivus* showed limited staining, indicating that the dyes were not entering the cells, and after freezing structural damage was too extensive to distinguish stained nuclei. There is little cellular damage after freezing in fed *Panagrolaimus* sp. More cellular damage after freezing is apparent in *P. murrayi* and in starved *Panagrolaimus* sp. This may be responsible for the lower survival of these two groups of nematodes.

Freeze substitution preserves the location of ice as white spaces (Ali and Wharton, 2014; Salinas-Flores et al., 2008; Wharton et al., 2005), and confirms that for most of these nematodes freezing at −10°C produces intracellular freezing. The pattern and distribution of ice formed can thus be compared between a species that survives intracellular freezing well (*Panagrolaimus* sp.), one that survives poorly (*P. redivivus*) and one intermediate between these two (*P. murrayi*). For *Panagrolaimus* sp., the pattern and distribution of ice formed can also be seen when survival is compromised by starvation. Starvation produces structural changes in unfrozen controls, compared with that of fed *Panagrolaimus* sp., but it also results in clear changes in the pattern and distribution of ice formed.

The pattern of ice formed in nematodes that survive intracellular freezing at −10°C well (fed *Panagrolaimus* sp.) is that the ice spaces are small and regular in size. In nematodes that show lower levels of survival after intracellular freezing (starved *Panagrolaimus* sp., *P. murrayi* and *P. redivivus*), at least some of the ice spaces are much larger in size and less regular in shape. These are likely to have caused the structural damage to cells, as seen in starved *Panagrolaimus* sp. by staining with vital dyes.

Plectus murrayi shows a different pattern and distribution of ice formed. There were more individuals with no ice formed at −10°C and with a shrunken appearance than was observed in the other species. Those with no ice are surviving by cryoprotective dehydration; this is also indicated by the shrinkage observed. The majority of *P. murrayi*, however, do freeze, and this is the case even if freezing occurs at −1°C (Wharton and Raymond, 2015). Many of those that freeze at −10°C are killed, but a proportion survives. Frozen *P. murrayi* contain on average smaller ice spaces than do *P. redivivus* and starved *Panagrolaimus* sp.; those individuals with smaller ice crystals and a smaller proportion of ice are likely to be the ones that survive. Oyster oocytes (*Crassostrea gigas*) can survive intracellular freezing if the ice spaces are small (Salinas-Flores et al., 2008). Survival of intracellular freezing could be more common amongst cells and tissues frozen for cryopreservation than is currently realised, as the formation of small ice crystals is not observed using optical cryomicroscopy (Salinas-Flores et al., 2008).

Ice has also been observed in the entomopathogenic nematode *Steinernema feltiae* (Ali and Wharton, 2014). This nematode survives intracellular freezing but only if freezing occurs at a relatively high subzero temperature (down to −3°C). Small ice spaces are found in *S. feltiae* frozen at −1°C or −3°C and the nematodes survive, but large ice spaces are found in those frozen at −10°C and the nematodes do not survive.

Control of the size of the ice spaces thus appears to be important for the survival of intracellular freezing in nematodes. What could be controlling this? The trehalose concentration of *Panagrolaimus* sp. increases upon acclimation at low temperatures (Wharton et al., 2000); genes involved in trehalose synthesis have been identified in the transcriptome of this nematode (Thorne et al., 2014) and are upregulated during cold acclimation (A. Seybold, Molecular adaptation mechanisms in the Antarctic nematode *Panagrolaimus davidi*, PhD thesis, University of Otago, 2015). However, trehalose concentrations are still relatively low compared with those found in other cold-tolerant organisms.

Panagrolaimus sp. produces an ice active substance with strong recrystallization inhibition activity (Ramløv et al., 1996; Smith et al., 2008). In a study comparing the freezing survival and recrystallization inhibition of six nematode species (Smith et al., 2008), *P. davidi* (=*Panagrolaimus* sp. DAW1) had the highest rates of survival and levels of recrystallization inhibition activity, whilst *P. redivivus* had lower survival and the lowest level of recrystallization inhibition activity. The freezing survival of *P. murrayi* is intermediate between these two species and has only weak recrystallization inhibition activity (Wharton and Raymond, 2015). Several other *Panagrolaimus* species and strains can survive freezing, and extracts of these also inhibit recrystallization in a splat freezing assay and the growth of single crystals in a nanolitre osmometer (McGill et al., 2015). It seems likely that it is an ice active substance with recrystallization inhibition activity that is responsible for controlling the size of the ice spaces and that this is an important adaptation for the ability of some nematodes to survive intracellular freezing.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
M.R.R. and D.A.W. designed the study, M.R.R. conducted the experiments, M.R.R. and D.A.W. analysed the data, and D.A.W. and M.R.R. wrote the paper.

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Supplementary information
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