J. exp. Biol. **155**, 629–641 (1991)
Printed in Great Britain © *The Company of Biologists Limited 1991*

COLD-TOLERANCE MECHANISMS OF THE ANTARCTIC NEMATODE *PANAGROLAIMUS DAVIDI*

BY D. A. WHARTON AND I. M. BROWN

Department of Zoology, University of Otago, PO Box 56, Dunedin, New Zealand

Accepted 6 September 1990

Summary

1. When free of surface water in air or liquid paraffin, the antarctic nematode *Panagrolaimus davidi* is freezing intolerant but avoids freezing by supercooling.

2. Survival of long-term exposure is enhanced by sub-zero temperatures compared with controls maintained at 99 % relative humidity and 15 °C.

3. In water the nematodes are seeded by exogenous ice nucleation and a proportion are freezing tolerant. Ice formation appears to be restricted to the pseudocoel.

4. The degree of freezing tolerance is dependent upon the age of the culture and its thermal history.

5. *P. davidi* is freezing tolerant when exposed to sub-zero temperatures in water and freezing intolerant when free of surface water and able to supercool. These two strategies are not mutually exclusive as they are often thought to be in arthropods.

Introduction

Cold-tolerant arthropods may either survive freezing of their extracellular fluids (freezing tolerant) or avoid freezing by supercooling (freezing intolerant or freeze avoiding) (Zachariassen, 1985). Sayre (1964) suggested that nematodes may use either of these strategies. Most studies have shown that nematodes are freezing intolerant (Wharton, 1986) but there have been reports of nematodes surviving freezing (Wharton and Allan, 1989; Asahina, 1959; Pickup, 1990*a*,*b*). The relative importance of these two cold-tolerance strategies in nematodes is unclear.

Nematodes are important constituents of the antarctic terrestrial fauna, with a high proportion of endemic species (Maslen, 1979). There have been few studies of the environmental physiology of antarctic nematodes. Studies have been conducted on nematodes from the maritime Antarctic (Pickup, 1990*a*,*b*) based at Signy Island (60°43'S). A recent survey reported seven species of nematode from the McMurdo Sound region of the continental antarctic (Wharton and Brown, 1989). This survey was based at Scott Base, Ross Island (77°51'S), and the most

Key words: nematode, antarctic, *Panagrolaimus davidi*, cold tolerance, freezing tolerance, freezing intolerance, ice nucleation.

southerly records of nematodes are from this region. At these sites nematodes are exposed to sub-zero temperatures for most of the year and to episodes of sub-zero exposure all year round. Block (1985) recorded summer temperatures in moss patches in this region down to −8.4 °C. Nematodes from this region may therefore be expected to have well-developed mechanisms for cold tolerance. The antarctic nematode *Panagrolaimus davidi* has been successfully established in laboratory culture (Wharton and Brown, 1989) and should prove a useful model for studying nematode cold-tolerance mechanisms. In this paper the relative importance of freezing-tolerant and freeze-avoiding strategies in the cold tolerance of this species is examined.

Materials and methods

The antarctic nematode *Panagrolaimus davidi* was isolated from various sites in the McMurdo Sound region (Wharton and Brown, 1989) and established in culture on agar plates, consisting of 1 % agar and 0.1 % nutrient broth. The nematodes fed upon bacteria which grew from the original isolates. Cultures were maintained at 15 °C and subcultured at intervals. Observations on freezing tolerance were made using a thermoelectric microscope stage. The design and operation of the stage has been described elsewhere (Wharton and Rowland, 1984; Wharton and Allan, 1989). Freezing and freezing survival were observed using the stage mounted on a dissecting microscope. To determine the location of ice crystals in the body, the stage was modified to fit the stage carrier of a Zeiss Axiophot photomicroscope and specimens were observed using polarising optics and photographed using a MicroFlash III unit.

Determination of freezing-tolerant and freezing-avoidance strategies

The ability of *P. davidi* to survive cooling at 1 °min^{-1} to temperatures down to −60 °C was determined using the thermoelectric stage. For nematodes in air, 50–100 mixed instars were washed off the surface of a culture and transferred to a 3 mm disc of cellulose acetate. The surface water was removed using a fine pipette and slivers of filter paper and the nematodes were immediately transferred to the specimen chamber of the thermoelectric stage and cooled at 1 °min^{-1} to various sub-zero temperatures. The specimens were observed during cooling for signs of freezing, the percentage frozen was counted at −5 °C during rewarming and the survival was determined after transfer to an artificial tap water (ATW) for 24 h at room temperature (Wharton and Allan, 1989). For nematodes in liquid paraffin, the surface water was removed as before and immediately replaced with liquid paraffin, and for nematodes in water, excess water was removed with a fine pipette so that when a second cellulose acetate disc was placed on top of the specimen the water just filled the space between the two discs. The specimen was then transferred to the thermoelectric stage, cooled to various sub-zero temperatures at 1 °min^{-1} and freezing and survival were determined as before.

Long-term exposure to sub-zero temperatures

Specimens were prepared as for 'nematodes in air' above on glass coverslips and transferred to humidity chambers containing potassium sulphate sludge to maintain a relative humidity of 99% (Winston and Bates, 1960). The chambers were placed in deep freezes at −80°C and −20°C and a control maintained at 15°C. At intervals, six replicates were removed and survival was assessed after 24 h in ATW at room temperature.

The effects of culture age and acclimation on freezing tolerance

To determine the effects of culture age and acclimation on cold tolerance, cultures were standardised to minimise the effects of inter-culture variation. Nematodes were separated from 30–50 culture plates using the Baermann funnel technique (Hooper, 1986) and mixed thoroughly. Fresh culture plates were inoculated with a standard concentration of nematodes and spread using a glass spreader to ensure an even distribution over the plate. The cultures were incubated for 14 days at 15°C. One set of plates was transferred to 0°C and one set maintained at 15°C. At intervals, freezing tolerance was examined by cooling a sample of nematodes in water to −20°C at $1°min^{-1}$ on the thermoelectric microscope stage and the survival determined after 24 h at room temperature in ATW. Six replicates were used for each time interval and a fresh plate was used for each replicate.

In a further experiment, cultures were standardised and incubated at 15°C. On day 30, the freezing tolerance of six replicates was tested as before. One set was replated and maintained at 15°C, one set was transferred to 0°C without replating and a control set was maintained at 15°C without replating. Freezing tolerance was tested after a further 15 and 25 days of culture.

The effect of replating on freezing tolerance

To determine the effects of replating on freezing tolerance, bacterial lawns were established on agar plates and incubated for 14 days at 15°C. Nematodes from standardised cultures, which had been incubated for 50 days at 15°C, were transferred to fresh bacterial lawns. A control set was not replated. The cultures were maintained at 15°C and their freezing tolerance was tested at intervals as before.

The distribution of lipids in nematodes from fresh and old cultures was compared by staining with Oil Red-O (Wharton and Allan, 1989).

The effect of acclimation on freezing tolerance

Standardised cultures were incubated for 30 days at 15°C. They were then replated onto 14-day-old bacterial lawns and incubated for a further 15 days at 15°C. The cultures were then transferred to 0°C for 15 days. The ability to survive cooling at $1°min^{-1}$ to various sub-zero temperatures was determined as for nematodes in water (above).

Results

Freezing-avoidance and freezing-tolerant strategies

After the removal of surface water, nematodes in air supercooled and none froze at temperatures down to −60°C. Some were killed by desiccation effects (Fig. 1). The effect of sub-zero temperatures on survival was not significant (r^2=0.065, t=1.66, P>0.1). In liquid paraffin, added after the removal of surface water, nematodes froze at −15 to −30°C (Fig. 2). Survival was divided into three phases: from 0 to −15°C mortality was due to desiccation effects, from −15 to −25°C there was additional mortality associated with freezing and from −30 to −40°C there was a slight improvement in survival. Although at −30°C and below all nematodes froze, some survived, indicating a degree of freezing tolerance.

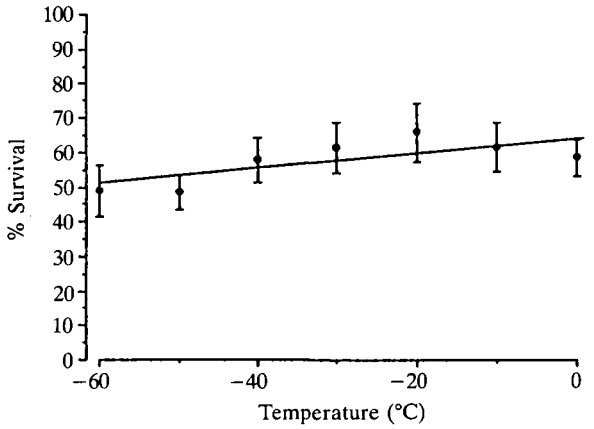

Fig. 1. The survival of *Panagrolaimus davidi* after cooling to sub-zero temperatures at 1°min^{-1}. The nematodes were exposed to air with surface water removed. Vertical lines represent the standard error of the mean, N=6.

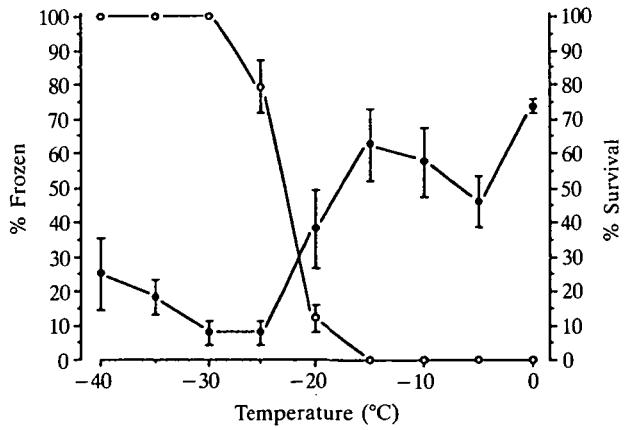

Fig. 2. The survival (●) and freezing (○) of *Panagrolaimus davidi* after cooling to sub-zero temperatures at 1°min^{-1}. The nematodes were in liquid paraffin after the removal of surface water. Vertical lines represent the standard error of the mean, N=6.

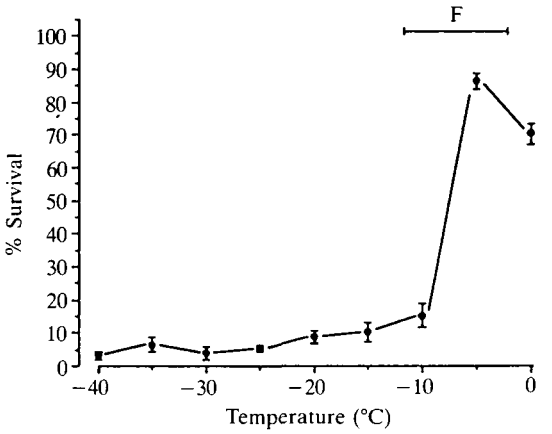

Fig. 3. The survival of *Panagrolaimus davidi* after cooling to sub-zero temperatures at $1°\,min^{-1}$. The nematodes were in water (F shows the range of sample freezing points). Vertical lines represent the standard error of the mean, $N=6$.

All nematodes exposed to sub-zero temperatures in water froze when the water froze. In samples where the water did not freeze (0°C and −5°C) there were high levels of survival (Fig. 3). Freezing resulted in a marked decline in survival, although some survived freezing down to −40°C, indicating a degree of freezing tolerance. A modification of the thermoelectric stage was used to observe freezing and thawing of *P. davidi* using polarising optics under a Zeiss Axiophot photomicroscope (Fig. 4). The freezing of body contents by exogenous ice nucleation was confirmed using this technique and the distribution of ice crystals during melting indicated that freezing is confined to the extracellular compartment.

Long-term freezing avoidance

The effect of temperature on the long-term survival of sub-zero temperatures by nematodes in air is shown in Fig. 5. Initial mortality was due to the effect of desiccation during sample preparation. The decline in survival with time was significant for all three treatments (control: $r^2=0.36$, $t=5.31$, $P<0.001$; −20°C: $r^2=0.47$, $t=6.77$, $P<0.001$; −80°C: $r^2=0.21$, $t=3.94$, $P<0.001$). Analysis of covariance, after arcsin transformation, showed a significant difference between treatments ($F=6.51$, $P<0.01$). Three comparison reports (Duncan's range test, Newman–Keul's range test and Fisher's LSD: $\mu=0.05$) showed that there were no significant differences in the slopes of the regressions between the control and the −80°C treatments or between the −20°C and −80°C treatments. There was a significant difference between the control and the −20°C treatment. Survival after 28 days' exposure was: control, 1.2±1.1%; −20°C, 10.2±2%; −80°C, 23.8±3.8%. The effect of treatment on survival after 28 days' exposure was significant (analysis of variance after arcsin transformation: $F=14.44$, $P<0.01$).

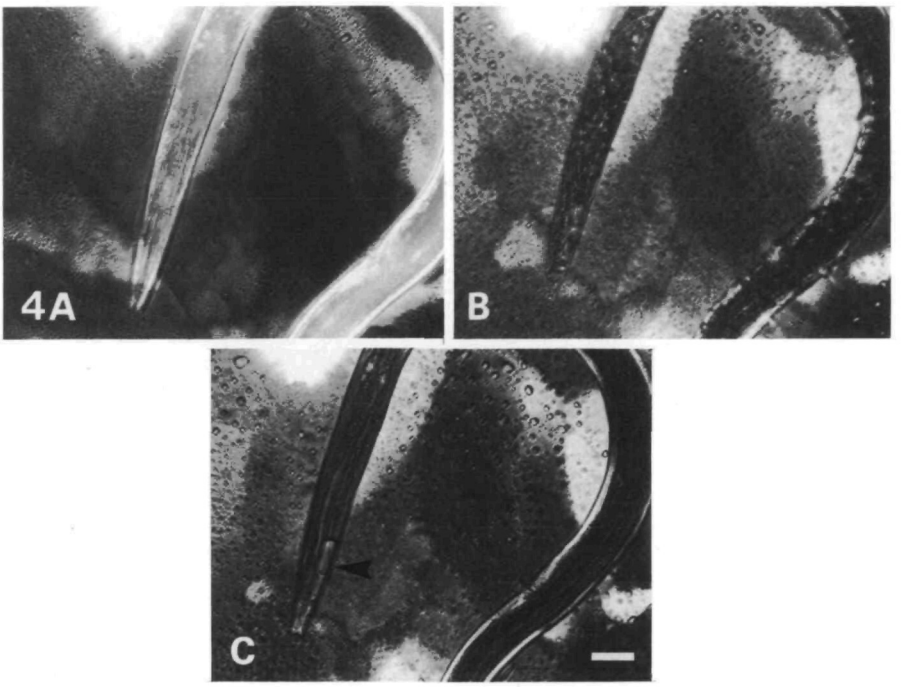

Fig. 4. Photomicrographs of *Panagrolaimus davidi* during a freezing and warming cycle, taken using a thermoelectric stage modified to fit a Zeiss Axiophot photomicroscope with polarising optics and a microflash unit. Accurate temperature measurement was not possible using our present equipment, but events during freezing and melting could be clearly observed. (A) Frozen at approximately −20°C. Bright areas in the nematode and the background are due to ice crystals. (B) During the initial stages of melting. (C) Just before the disappearance of the last ice crystals during melting. The ice appears to be confined to the pseudocoel (arrowhead). Scale bar, 20 μm.

Exposure to sub-zero temperatures thus enhances survival compared with nematodes exposed to desiccation at 15°C.

The effect of culture age and acclimation

For nematodes maintained at 15°C, there was an increase in freezing tolerance up to a culture age of 29 days, followed by a rapid decline (Fig. 6). For nematodes transferred to 0°C, freezing tolerance increased up to 27 days after transfer and then declined (Fig. 7), indicating some ability to acclimate in response to low temperatures.

Further experiments confirmed the effect of culture age and acclimation on freezing tolerance by comparing the survival of nematodes from replated cultures, non-replated cultures and cultures transferred to 0°C (Fig. 8). The survival of the non-replated group declined between day 30 and day 45 but there was an increase in the survival of the replated group (Fisher PLSD: $P<0.05$). This confirms that culture age affects the ability to tolerate freezing. The survival of the replated

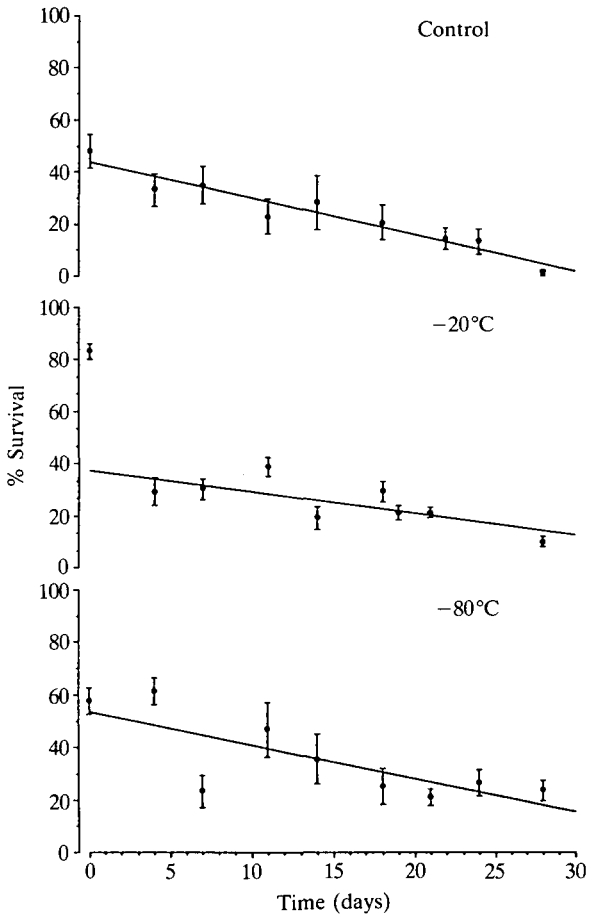

Fig. 5. The effect of long-term exposure to sub-zero temperatures on the survival of *Panagrolaimus davidi*. (A) Control at 15°C and 99% relative humidity. (B) At −20°C over a potassium sulphate sludge. (C) At −80°C over a potassium sulphate sludge. Vertical lines represent the standard error of the mean, $N=6$.

group declined between days 45 and 55 ($P<0.05$). Cultures transferred to 0°C showed no significant changes in survival between days 30, 45 and 55 ($P>0.05$). The decrease in survival observed in non-replated cultures at 15°C did not occur in non-replated cultures transferred to 0°C.

The effect of replating on freezing tolerance

The freezing tolerance of nematodes from 50-day-old cultures increased after replating to fresh bacterial lawns (Fig. 9). Non-replated controls showed a continuing decline in their ability to tolerate freezing, with no nematodes from 64-day-old cultures surviving exposure to −20°C.

Nematodes from fresh cultures contained numerous granules within their intestinal cells and other sites which stained intensely for lipid with Oil Red-O.

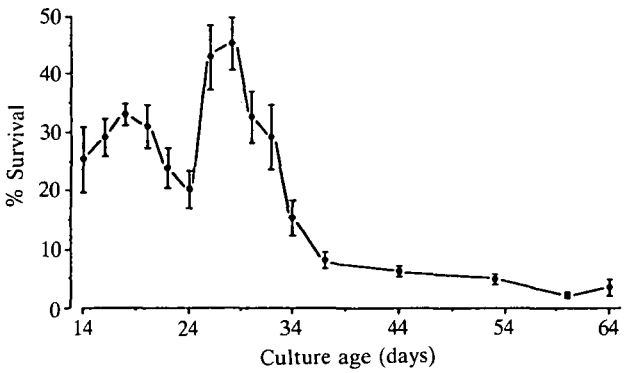

Fig. 6. The effect of culture age on the freezing tolerance of *Panagrolaimus davidi*. Cultures were grown at 15°C for 14 days and then maintained at 15°C. At intervals, freezing tolerance was examined by cooling a sample of nematodes in water to −20°C at 1°min^{-1} on the thermoelectric microscope stage and determining the survival after 24 h at room temperature. Six replicates were used for each time interval and a fresh plate for each replicate. Vertical lines represent the standard error of the mean.

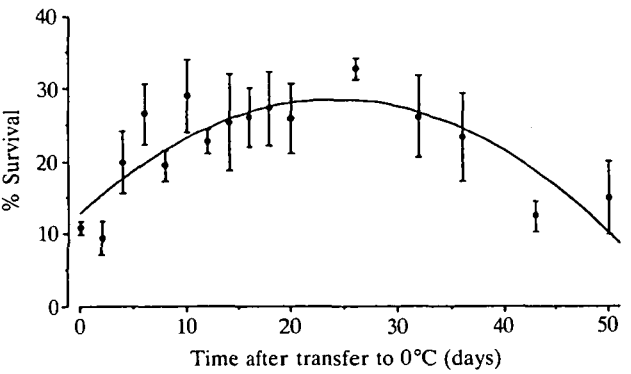

Fig. 7. The effect of acclimation on the freezing tolerance of *Panagrolaimus davidi*. Cultures were grown at 15°C for 14 days and then transferred to 0°C. At intervals, freezing tolerance was examined by cooling a sample of nematodes in water to −20°C at 1°min^{-1} on the thermoelectric microscope stage and determining the survival after 24 h at room temperature. Six replicates were used for each time interval and a fresh plate for each replicate. Vertical lines represent the standard error of the mean.

These granules were absent from nematodes from old cultures, indicating the depletion of food stores.

The effect of acclimation on freezing tolerance

The freezing tolerance of cultures acclimated for 15 days at 0°C, following incubation at 15°C for 30 days, replating onto fresh bacterial lawns and incubating for a further 15 days at 15°C, is shown in Fig. 10. Replicates exposed to −5°C were

divided into those in which the water froze and those in which the water did not freeze. A marked fall in survival was associated with the freezing event. A proportion of nematodes survived freezing down to −40°C. The proportion that were freezing tolerant was much higher than in non-acclimated nematodes (Fig. 3).

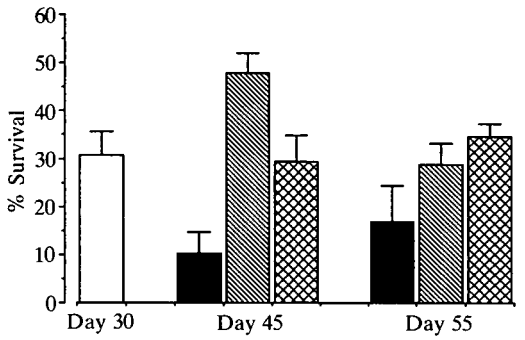

Fig. 8. The effect of culture age and acclimation on the freezing tolerance of *Panagrolaimus davidi*. 50-day-old stock cultures were transferred to fresh plates and incubated at 15°C. On day 30, the freezing tolerance of six replicates was tested by cooling a sample of nematodes in water to −20°C at 1°min^{-1} on the thermoelectric microscope stage and the survival determined after 24 h at room temperature (□). One set was replated (▨) and maintained at 15°C, one set was transferred to 0°C without replating (▩) and a control set was maintained at 15°C (■). Freezing tolerance was tested after a further 15 and 25 days of culture. Vertical lines represent the standard error of the mean, N=6.

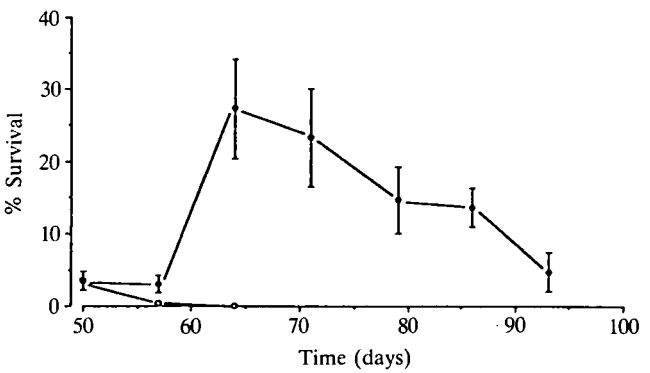

Fig. 9. The effect of culture age on the freezing tolerance of *Panagrolaimus davidi*. Nematodes from standardised cultures which had been incubated for 50 days at 15°C were transferred to fresh bacterial lawns (●). A control set were not replated (○). The cultures were maintained at 15°C and their freezing tolerance tested at intervals by cooling a sample of nematodes in water to −20°C at 1°min^{-1} on the thermoelectric microscope stage and determining the survival after 24 h at room temperature. Six replicates were used for each time interval and a fresh plate for each replicate. Vertical lines represent the standard error of the mean.

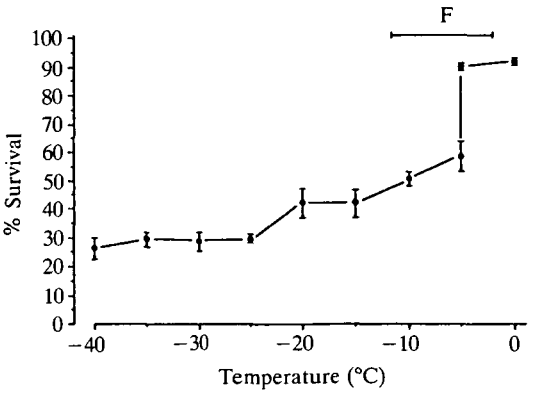

Fig. 10. The survival of *Panagrolaimus davidi* after cooling to sub-zero temperatures at $1°min^{-1}$. The nematodes were in water and were acclimated by incubating standardised cultures for 30 days at 15°C, replating onto 14-day-old bacterial lawns and incubating for a further 15 days at 15°C. The cultures were then transferred to 0°C for 15 days. F shows the range of sample freezing points, vertical lines represent the standard error of the mean, $N=6$.

Discussion

Most studies on nematode cold tolerance have measured the supercooling points of specimens from which the surface water had been removed and replaced with liquid paraffin to prevent desiccation (Wharton *et al.* 1984; Perry and Wharton, 1985; Ash and Atkinson, 1986; Mabbett and Wharton, 1986; Pickup, 1990*a*,*b*). It is difficult to envisage an ecological situation in which nematodes are free of surface water and yet not exposed to desiccation. Our results with *P. davidi* indicate that the freezing and survival of nematodes in liquid paraffin is different from that of nematodes exposed to sub-zero temperatures in water or in air with the surface water removed. Measurements in liquid paraffin may therefore be of limited use in studying the environmental physiology of nematodes.

The antarctic nematode *P. davidi* avoids freezing by supercooling when free of surface water, but when in contact with water the freezing of the body is seeded by exogenous ice nucleation across the cuticle or *via* body orifices. The degree of freezing tolerance is dependent upon the age of the culture and its thermal history. Although many species of nematode can survive exposure to desiccation, they are essentially aquatic organisms and at least a film of water must be present for feeding, growth and reproduction to occur (Wharton, 1986). The probability of exogenous ice nucleation during freezing must therefore be high, although dependent upon the microenvironmental conditions to which the nematodes are exposed during a sub-zero temperature event. Freezing tolerance in nematodes has also been reported in *Trichostrongylus colubriformis* (Wharton and Allan, 1989), *Aphelenchoides ritzemabosi* (Asahina, 1959), *Coomanus gerlachei* (Pickup, 1990*a*) and *Tetracephalus tilbrooki* (Pickup, 1990*b*).

Antarctic nematodes inhabiting moss are in an environment that is often saturated with water (Pickup, 1990*a*,*b*). Cold tolerance must therefore involve preventing exogenous ice nucleation or surviving freezing as a result of exogenous ice nucleation. The sheath of the infective juveniles of *T. colubriformis* prevents exogenous ice nucleation in a proportion of specimens but this species can also survive freezing (Wharton and Allan, 1989). *Ditylenchus dipsaci* and the eggs of *Globodera rostochiensis* can survive freezing in contact with water (Wharton *et al.* 1984; Perry and Wharton, 1985) but the techniques used in these studies could not distinguish between survival and prevention of exogenous ice nucleation. The freezing of *P. davidi* is seeded by exogenous ice nucleation and this species is freezing tolerant. Freezing-tolerant insects and frogs may also be seeded by exogenous ice nucleation and this may be an important site of nucleation in the field under suitable environmental conditions (Layne *et al.* 1990; Bale *et al.* 1989).

Freezing tolerance in arthropods involves freezing at high sub-zero temperatures (Zachariassen, 1985). Nematodes freezing by endogenous ice nucleation must also have relatively high supercooling points to survive freezing (Pickup, 1990*a*,*b*). In arthropods this is a result of the seasonal synthesis of ice nucleating agents. Nematodes may largely rely on exogenous ice nucleation to ensure freezing at high sub-zero temperatures.

Nematodes are able to acclimate to lowered temperatures by depressing their supercooling points (Mabbett and Wharton, 1986; Ash and Atkinson, 1986). Supercooling points of nematodes in the field can also closely track the seasonal variation in temperature (Pickup, 1990*a*,*b*). *P. davidi* can acclimate in response to lowered temperatures by increasing its freezing tolerance. The freezing tolerance of *P. davidi* is also dependent upon the age of the culture. The decrease in lipid content of the nematodes and the increased freezing survival after transfer to fresh culture plates indicate that the decline in survival in old cultures is due to nutrient depletion. There may, however, be shifts in population structure or the accumulation of waste products in old cultures, which may adversely affect the ability to survive freezing. High levels of nutrient availability may allow the excess to be converted into cryoprotectants. In contrast, the ability of nematodes to supercool is adversely affected by recent feeding (Pickup, 1990*a*,*b*).

When free of surface water *P. davidi* can supercool to very low temperatures. Preparation of specimens may have resulted in rapid desiccation and some nematodes were killed by this desiccation stress. In those that survived, exposure to sub-zero temperatures did not result in any additional mortality and in long-term exposures survival at −20 or −80 °C was enhanced compared with nematodes maintained at 15 °C and 99 % relative humidity. Desiccation stress may increase cold tolerance by reducing the proportion of freezable water in the body and by increasing the concentration of cryoprotectants (Zachariassen, 1985). Some species of nematode require that their environment loses water slowly before they are able to enter into a state of anhydrobiosis and survive desiccation (Womersley and Ching, 1989). Nematodes inhabiting moss may be expected to experience low rates of water loss from their environment and there may be a significant

interaction between desiccation and cold tolerance under some microenvironmental conditions.

Freezing intolerance, together with the avoidance of freezing by supercooling, appears to be the commonest cold-tolerance strategy in arthropods. Freezing tolerance is largely limited to higher insect orders (Block, 1990) but the strategy may vary within an insect order and even between different stages of the same species (Block, 1982). These strategies have been thought to be mutually exclusive (Zachariassen, 1985) but there have been reports that suggest that some insects can switch strategies in successive seasons (Duman, 1984; Horwarth and Duman, 1984). The rate of cooling and rewarming may be critical for the cold tolerance of insects and the strategy that they appear to employ (Baust and Rojas, 1985). Few studies, however, have investigated optimal rates of cooling and rewarming. In this study we have used the standard cooling rate ($1°\,min^{-1}$) used in cold-tolerance studies (Baust and Rojas, 1985) and did not control the rate of rewarming. These rates do not reflect cooling and rewarming rates likely to be experienced in the antarctic environment and it is likely that freezing survival is influenced by rate effects.

Nematodes appear to be able to use a freezing-tolerant strategy when exposed to sub-zero temperatures in water when they are seeded by exogenous ice nucleation, but a freezing-intolerant strategy when they are free of surface water and can supercool. These options are continually available and do not represent a seasonal shift in strategy as observed in arthropods (Pickup, 1990*a*). These observations suggest that freezing-tolerant and freezing-intolerant cold-tolerance mechanisms in nematodes are not mutually exclusive and that nematodes possess adaptations that allow them to employ either strategy as dictated by microenvironmental conditions.

We would like to thank DSIR Antarctic Division for their support during the field work, the NZ University Grants Committee for financial support, Todd Crowl for advice on statistics and Claudine Tyrrell and Murray McKenzie for technical assistance.

References

ASAHINA, E. (1959). Frost-resistance in a nematode, *Aphelenchoides ritzemabosi*. *Low Temp. Sci.* B **17**, 51–62.

ASH, C. P. J. AND ATKINSON, H. J. (1986). *Nematodirus battus*: development of cold hardiness in dormant eggs. *Exp. Parasitol.* **62**, 24–28.

BALE, J. S., HANSEN, T. N. AND BAUST, J. G. (1989). Nucleators and sites of nucleation in the freeze tolerant larvae of the gallfly *Eurosta solidaginis* (Fitch). *J. Insect Physiol.* **35**, 291–298.

BAUST, J. G. AND ROJAS, R. R. (1985). Review – insect cold hardiness: facts and fancy. *J. Insect Physiol.* **31**, 755–759.

BLOCK, W. (1982). Cold hardiness in invertebrate poikilotherms. *Comp. Biochem. Physiol.* **73A**, 581–593.

BLOCK, W. (1985). Ecological and physiological studies of terrestrial arthropods in the Ross Dependency 1984–85. *Br. Antarc. Surv. Bull.* **68**, 115–122.

BLOCK, W. (1990). Cold tolerance of insects and other arthropods. *Phil. Trans. R. Soc. Ser.* B **326**, 613–633.

DUMAN, J. G. (1984). Changes in overwintering mechanism of the cucujid beetle *Cucujus clavipes*. *J. Insect Physiol.* **30**, 235–239.

HOOPER, D. J. (1986). Extraction of free-living stages from soil. In *Laboratory Methods for Work with Plant and Soil Nematodes* (ed. J. F. Southey). London: HMSO.

HORWARTH, K. L. AND DUMAN, J. G. (1984). Yearly variations in the overwintering mechanisms of the cold-hardy beetle, *Dendroides canadensis*. *Physiol. Zool.* **57**, 40–45.

LAYNE, J. R., LEE, R. E. AND HUANG, J. L. (1990). Inoculation triggers freezing at high subzero temperatures in a freeze-tolerant frog (*Rana sylvatica*), and insect (*Eurosta solidaginis*). *Can. J. Zool.* **68**, 506–510.

MABBETT, K. AND WHARTON, D. A. (1986). Cold tolerance and acclimation in the free-living nematode, *Panagrellus redivivus*. *Rev. Nématol.* **9**, 167–170.

MASLEN, N. R. (1979). Additions to the nematode fauna of the antarctic region with keys to taxa. *Br. Antarc. Surv. Bull.* **49**, 207–229.

PERRY, R. N. AND WHARTON, D. A. (1985). Cold tolerance of hatched and unhatched second stage juveniles of *Globodera rostochiensis*. *Int. J. Parasitol.* **15**, 441–445.

PICKUP, J. (1990*a*). Seasonal variation in the cold-hardiness of a free-living predatory antarctic nematode, *Coomanus gerlachei* (Mononchidae). *Polar Biol.* **10**, 307–315.

PICKUP, J. (1990*b*). Seasonal variation in the cold hardiness of three species of free-living antarctic nematodes. *Func. Ecol.* **4**, 257–264.

SAYRE, R. M. (1964). Cold-hardiness of nematodes. I. Effects of rapid freezing on the eggs and larvae of *Meloidogyne incognita* and *M. hapla*. *Nematologica* **10**, 168–179.

WHARTON, D. A. (1986). *A Functional Biology of Nematodes*. London, Sydney: Croom Helm.

WHARTON, D. A. AND ALLAN, G. S. (1989). Cold tolerance mechanisms of the free-living stages of *Trichostrongylus colubriformis* (Nematoda: Trichostrongylidae). *J. exp. Biol.* **45**, 353–370.

WHARTON, D. A. AND BROWN, I. M. (1989). A survey of terrestrial nematodes from the McMurdo Sound region, Antarctica. *N.Z. J. Zool.* **16**, 467–470.

WHARTON, D. A. AND ROWLAND, J. J. (1984). A thermoelectric microscope stage for the measurement of the supercooling points of microscopic organisms. *J. Microsc.* **134**, 299–305.

WHARTON, D. A., YOUNG, S. R. AND BARRETT, J. (1984). Cold tolerance in nematodes. *J. comp. Physiol.* B **154**, 73–77.

WINSTON, P. W. AND BATES, D. H. (1960). Saturated solutions for the control of humidity in biological research. *Ecology* **41**, 232–237.

WOMERSLEY, C. AND CHING, C. (1989). Natural dehydration regimes as a prerequisite for the successful induction of anhydrobiosis in the nematode *Rotylenchus reniformis*. *J. exp. Biol.* **143**, 359–372.

ZACHARIASSEN, K. E. (1985). Physiology of cold tolerance in insects. *Physiol. Rev.* **65**, 799–832.