J. exp. Biol. **145**, 353–369 (1989)
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COLD TOLERANCE MECHANISMS OF THE FREE-LIVING STAGES OF *TRICHOSTRONGYLUS COLUBRIFORMIS* (NEMATODA)

BY D. A. WHARTON AND G. S. ALLAN

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

Accepted 27 April 1989

Summary

1. All free-living stages of the nematode parasite of sheep, *Trichostrongylus colubriformis* Giles, survived exposure to freezing temperatures in contact with water, with the exception of the first-stage juvenile (J1). The third-stage juvenile (J3) was the most resistant stage. The order of relative survival of the different stages was different from that of the lowest F_{50} (the temperature at which 50% froze), suggesting that an ability to supercool was not the only determinant of survival.

2. The F_{50} was shown to be a good measure of the degree of supercooling and to extend greatly the lower size limit of organisms that could be measured.

3. The J3 uses a freeze-avoiding strategy by supercooling when in air or covered by liquid paraffin. In water it uses a mixture of freeze-avoiding and freeze-tolerant strategies, with a proportion of the population surviving freezing caused by exogenous ice nucleation.

4. Removal of the J3 sheath results in a shift from freeze avoidance to freeze tolerance, with an overall reduction in survival. A major function of the sheath may be to reduce the probability of exogenous ice nucleation.

Introduction

Various species of trichostrongyle nematodes cause diseases in sheep, cattle and other ruminants, resulting in considerable economic losses caused by reductions in weight gain and wool growth and the death of heavily infected animals. Despite the considerable research effort directed towards the control of these parasites, we know remarkably little about the basic biology of their free-living stages which would enable us to understand the mechanisms by which they survive on pasture and thus interpret the epidemiological patterns that have been observed. In most species of trichostrongyle the egg is passed out in the faeces of the host and hatches as a first-stage juvenile (J1) which rapidly develops to a second-stage juvenile (J2) and a third-stage, infective juvenile (J3). J1s and J2s feed on bacteria in the faeces

Key words: *Trichostrongylus colubriformis*, nematode, freezing, cold tolerance, freeze tolerance, freeze avoidance, supercooling, ice nucleation, sheath.

and soil. J3s are non-feeding and can survive on pasture for a year or more (Michel, 1976), relying on food reserves accumulated by the J1s and J2s. The J2/J3 moult is incomplete and the J3s retain the cuticle of the previous stage as a sheath, which is shed upon ingestion by the host in a process known as exsheathment (actually an ecdysis, Wharton, 1986).

In parts of their ranges, the free-living stages of trichostrongyle nematodes may be exposed to the hazard of freezing. There are many reports of J3s overwintering on pasture, which includes areas that are regularly exposed to sub-zero temperatures (Kates, 1950). They must therefore possess a degree of cold tolerance under field conditions and can survive at least brief exposure to freezing temperatures. *Trichostrongylus colubriformis* J3s have been reported to survive soil surface temperatures as low as −10°C (Anderson *et al.* 1970). In Poland, a variety of trichostrongyle J3s can survive winter temperatures down to −28°C (Wertejuk, 1959).

Cold-tolerant arthropods may use either a freeze-tolerant or a freeze-avoiding strategy (Zachariassen, 1985). Little is known about cold-tolerance mechanisms in nematodes but several species have been shown to supercool in the absence of water (Wharton, 1986). Although some species of nematode can survive periods of desiccation, for movement and growth to occur at least a film a water must be present which may result in exogenous ice nucleation during freezing. This study examines the cold tolerance of the different free-living stages in the life cycle of *T. colubriformis*, methods of measuring supercooling in nematodes, mechanisms of cold tolerance in J3s in the presence and absence of external water and the role of the sheath in preventing exogenous ice nucleation.

Materials and methods

An infection of *T. colubriformis* was maintained in sheep. Faeces were collected at intervals. Eggs were separated from the faeces by flotation in saturated sodium chloride; J1s, J2s and J3s were obtained by incubating the faeces for an appropriate period at room temperature and separating the juveniles using the Baermann funnel technique. Eggs, J1s and J2s were used immediately; J3s were stored at 20°C.

Desheathed J3s were obtained by exposing ensheathed J3s to 0·5% (v/v) sodium hypochlorite for 3 min, followed by two rinses in distilled water. Exsheathed J3s were obtained by immersing ensheathed J3s in 1% pepsin in HCl/KCl buffer, pH 1·6 at 38°C. Carbon dioxide was bubbled through the suspension for 10 min, followed by air for 60 min. The specimens were rinsed twice in distilled water. Exsheathed and desheathed specimens were used within 3 h of preparation.

A sample in which the sheath of the J3s had been cut was prepared. A suspension of J3s was pipetted onto a small square of cellulose acetate and the excess water removed with a wedge of filter paper. They were then placed in a desiccator at 76% relative humidity (over a saturated solution of NaCl; Winston &

Bates, 1960) and kept at 20°C for 24 h. The sample was then removed from the desiccator and placed in 100% relative humidity (over distilled water in a sealed container at 20°C) for 10–15 min. During the time the nematodes are in the 100% relative humidity they take up water and many contract away from their sheaths (G. S. Allan & D. A. Wharton, unpublished results). The tips of the sheaths were cut off, under a dissecting microscope, using a piece of razor blade mounted in a micromanipulator. They were then placed in water and the ones with cut sheaths were separated using a micropipette. They were then kept in water for at least 2 h before mounting on the supercooling stage in an attempt to minimize the residual effects of desiccation stress.

Long-term survival

The long-term survival at sub-zero temperatures by unembryonated eggs, embryonated eggs, J1s, J2s, ensheathed J3s and desheathed J3s was observed after exposure to −14°C in water. Ensheathed J3s were also exposed to −28°C. 100–300 J3s (1 ml of suspension) were placed in 10 ml glass vials and transferred to domestic chest freezers set at −14°C or −28°C. The temperature was monitored using a Comark 1606BP electronic thermometer with a NiCr/NiAl thermocouple by placing the thermocouple in one of the glass vials. The experiment was started when the sample temperature reached within 1·5°C of the set temperature of the freezer. One set of five vials was removed when the temperature reached the set temperature (time = 0) and further sets after various periods of exposure. 1 ml of tap water was added to each of the vials which were then incubated at 20°C for 24 h. The survival of J1s, J2s and J3s was determined by counting the proportion that moved after a mechanical stimulus, which consisted of sucking the nematode suspension into a Pasteur pipette and blowing it out again. Unembryonated eggs were considered to have survived if they had proceeded to embryonate, and embryonated eggs were considered to have survived if they hatched or if movement within the egg was observed. The time at which 50% of the nematodes were killed by freezing (S_{50}) was determined using the methods of probit analysis (Finney, 1952).

To determine the distribution of lipids in ensheathed J3s after exposure to freezing they were exposed to −14°C for 7 days and incubated at 20°C for 24 h. They were then fixed in 4% paraformaldehyde/2·5% glutaraldehyde fixative in 0·2 mol l^{-1} sodium cacodylate buffer, pH 7·2 for 10 min, rinsed twice in distilled water and cut in two. They were then immersed in Oil Red-O (a saturated solution in 70% ethanol) for 10 min and washed in distilled water before observation.

Measurement of supercooling points

The supercooling points (=temperature of crystallization) of ensheathed J3s in water or in liquid paraffin were determined using a cold microscope stage. The design and operation of the stage has been described by Wharton & Rowland (1984). Briefly, the stage consists of an aluminium bar with holes drilled for the specimen chamber and a thermocouple. The bar is cooled by a thermoelectric

cooling module (Camcool, Midland-Ross Co., 445 Concord Ave, Cambridge, MA 02238, USA), the hot face of which is mounted on a heatsink which is cooled by ethanol from a refrigerated circulator. The temperature of the specimen chamber is monitored with an electronic thermometer and a constant rate of cooling of $1\,°C\,min^{-1}$ is achieved by a control unit which compares the output from the thermometer to a voltage ramp and switches power to the module to match the two rates. The stage assembly is mounted on a microscope and the specimen can be observed directly during cooling and rewarming.

A drop of nematode suspension was transferred to a small disc of cellulose acetate. The sample was covered by a second cellulose acetate disc (nematodes in water) or the surface water was removed using filter paper and the nematodes covered with a drop of liquid paraffin and a second disc of cellulose acetate. The sample was transferred to the specimen chamber of the cold microscope stage and placed on the thermocouple. The chamber was sealed and insulated.

The specimen was cooled rapidly to 0°C and then at a constant rate of $1\,°C\,min^{-1}$. The specimens were observed as they cooled from 0°C to −40°C and the temperature at which a nematode froze (the supercooling point) determined by a marked decrease in transparency.

Attempts to measure the supercooling point of J3s by detecting the latent heat of fusion produced by the freezing of worms mounted directly on the thermocouple were not successful, even using a full-scale deflection of 10°C on the chart recorder and fine wire (75 μm diameter) thermocouples.

F_{50} *determination and survival*

The temperature at which 50% of nematodes froze (F_{50}) was determined by transferring the sample to the specimen chamber of the cold microscope stage and cooling the nematodes at $1\,°C\,min^{-1}$ to various low temperatures. The sample was then allowed to rewarm by switching off the thermoelectric cooling module and allowing the circulating fluid to warm to 20°C. The rate of rewarming was not controlled. During rewarming there is a marked decrease in transparency. Maximum opacity occurs at −5°C and the number of nematodes which had frozen was counted at this temperature. The sample was allowed to warm above 0°C, removed from the specimen chamber, immersed in tap water and incubated for 24 h at room temperature. The survival was determined by counting the proportion of nematodes that moved after a mechanical stimulus, as before. The F_{50} and the S_{50} were calculated using the methods of probit analysis (Finney, 1952). Specimens were observed in water, in liquid paraffin and with the surface water removed by filter paper spills (nematodes in air).

Ice inoculation and freezing tolerance

Nematodes in water were transferred to the specimen chamber of the cold microscope stage and cooled from 0°C at a rate of $1\,°C\,min^{-1}$. The temperature at which the water in the sample froze was detected by the latent heat of fusion which was recorded as a temperature increase on a chart recorder monitoring the output

Table 1. *Cold tolerance of the free-living stages of* Trichostrongylus colubriformis

Stage	Temperature (°C)	% Survival at time 0	S_{50} (h)	F_{50} (°C)
Unembryonated eggs	−14	91·5 ± 1·4	1·8 ± 1·1	
Embryonated eggs	−14	89·8 ± 2·5	13·4 ± 1·0	−32·0 ± 1·01
J1s	−14	0	0	−26·2 ± 1·02
J2s	−14	63·4 ± 3·2	1·1 ± 1·0	−24·9 ± 1·02
Ensheathed J3s	−14	99·0 ± 0·15	53·7 ± 1·0	−25·8 ± 1·0
Ensheathed J3s	−28	73·3 ± 4·9	0·96 ± 1·0	
Desheathed J3s	−14	82·3 ± 2·07	250 ± 1·2	
Exsheathed J3s				−26·9 ± 1·02

Values are mean ± S.E.

from the electronic thermometer. The specimen was allowed to cool further to 2°C below the initial water freezing temperature. The specimen was then allowed to warm and the number of nematodes frozen counted at −5°C. Nematode survival was determined as before.

Results

Cold tolerance of the free-living stages

Survival by the different free-living stages of *T. colubriformis* after long-term exposure to low temperatures in water and their F_{50} values in liquid paraffin are shown in Table 1. J1s do not survive low temperatures in contact with water, despite the observation that they supercool in liquid paraffin. All the other stages showed an ability to survive sub-zero temperatures in water. In order of greatest tolerance the stages are: desheathed J3s > ensheathed J3s > embryonated eggs > unembryonated eggs > J2s. Some J1s, J2s and desheathed J3s that did not survive freezing appeared to be physically damaged and the body contents were observed extruding through the body wall in places. Such damage did not occur in ensheathed J3s, but in non-survivors and in a small number of survivors (approximately 1%) large refringent droplets were observed within the intestinal cells. These stained with Oil Red-O, indicating that they consisted of lipid.

All stages supercooled in liquid paraffin. In order of lowest F_{50} the stages are: embryonated eggs < exsheathed J3s < J1s < ensheathed J3s < J2s. This is different from the order for freezing survival and suggests that the ability to survive low temperatures may be dependent upon factors other than the ability to supercool.

Cold tolerance of the ensheathed J3s

J3s were the most resistant of the free-living stages and the mechanisms of cold tolerance in this stage were therefore investigated further. The supercooling points of ensheathed J3s were determined in water and in liquid paraffin. The mean supercooling points were −25·5 ± 3·3°C in water (±1 S.D., $N = 30$) and

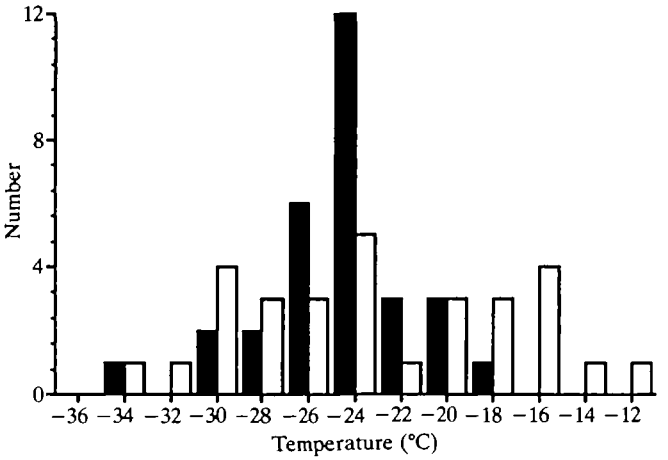
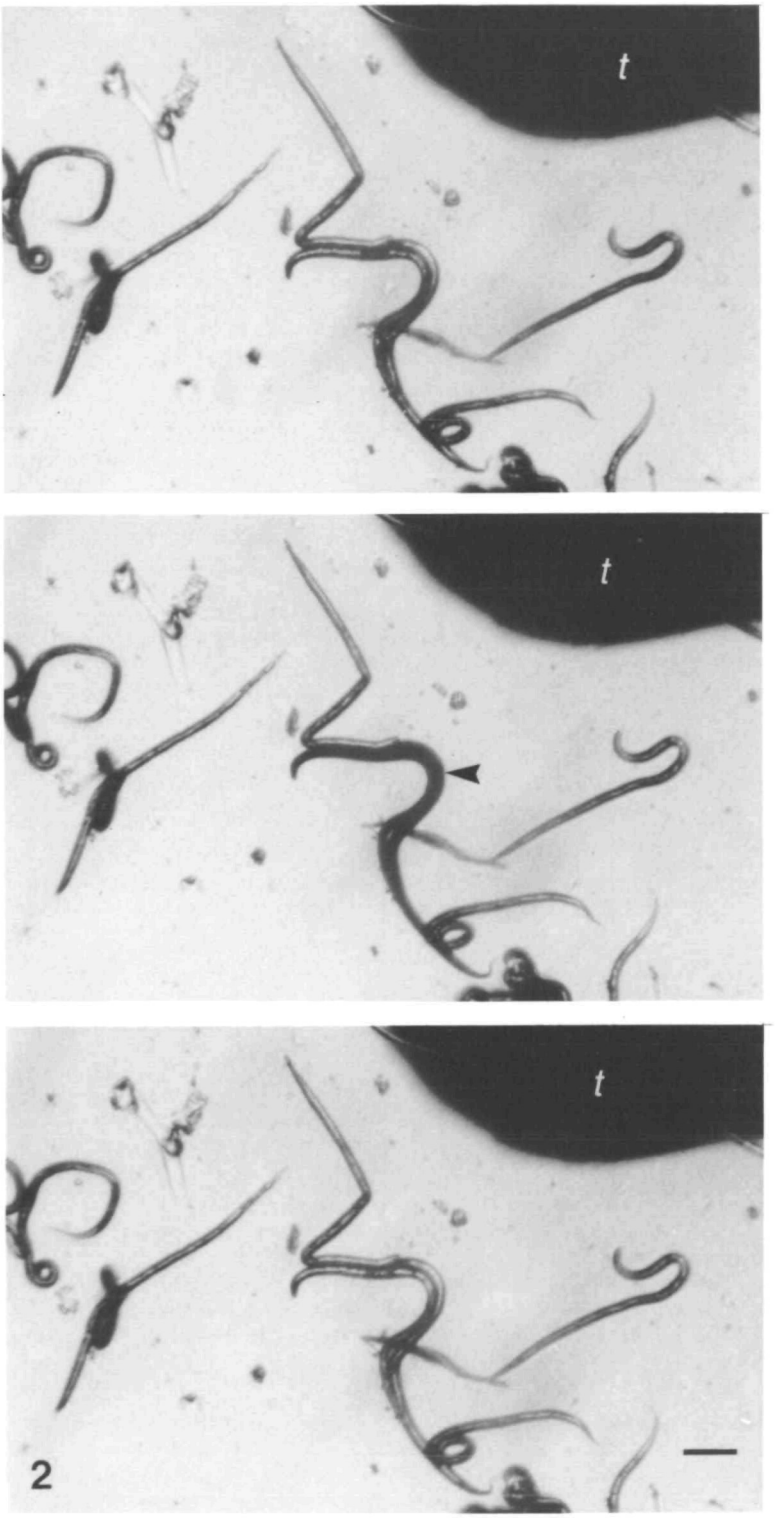


Fig. 1. Distribution of supercooling points of ensheathed J3s of *Trichostrongylus colubriformis* in water (closed histogram) and in liquid paraffin (open histogram).

$-24{\cdot}0 \pm 5{\cdot}7$°C in liquid paraffin. There was no significant difference between supercooling points measured in water and in liquid paraffin (t-test: $t = 1{\cdot}22$, df = 58, $P > 0{\cdot}1$). Supercooling points were harder to observe in liquid paraffin than in water, perhaps because of the difference in refractive index. In liquid paraffin, freezing of one individual initiated the freezing of others that were in contact with it. This was counted as a single freezing event. This effect did not occur in J3s in water and perhaps accounts for the greater spread in the data for nematodes in liquid paraffin (Fig. 1).

The proportion of ensheathed J3s that had frozen after cooling to various sub-zero temperatures in air could be clearly observed after rewarming to −5°C (Fig. 2). The relationship between freezing and temperature is shown in Fig. 3. The correlation between temperature and freezing was significant ($r^2 = 0{\cdot}969$, $t = 17{\cdot}6$, df = 11, $P < 0{\cdot}001$) and the number of ensheathed J3s that froze in air was a good predictor of their subsequent survival (Fig. 4: $X^2 = 19{\cdot}57$, $P > 0{\cdot}05$). The F_{50}, calculated by probit analysis, was $-30{\cdot}15 \pm 1{\cdot}0$°C and the S_{50} was $-28{\cdot}83 \pm 1{\cdot}0$°C. There was no significant difference between the F_{50} and the S_{50} (from a comparison of the 50 % values and their standard errors: $t = 0{\cdot}93$, df = 30, $P > 0{\cdot}1$). Similar results were obtained with nematodes in liquid paraffin. The correlation between temperature and freezing was significant ($r^2 = 0{\cdot}893$, $t = 10{\cdot}82$, df = 15, $P < 0{\cdot}001$) and the number of ensheathed J3s in liquid paraffin that froze was also a good predictor of their subsequent survival ($X^2 = 21{\cdot}89$, $P > 0{\cdot}05$). The F_{50} was $-25{\cdot}8 \pm 1{\cdot}0$°C and the S_{50} was $-25{\cdot}03 \pm 1{\cdot}0$°C. There was

Fig. 2. Ensheathed J3s in air during a cooling/rewarming cycle. The specimen was cooled to −30°C (top) and then rewarmed to −5°C (middle). Nematodes which had frozen blackened during rewarming (arrowhead), unfrozen nematodes remained clear. The frozen nematodes melted and became clear again after further warming to 0°C (bottom). *t*, thermocouple. Scale bar, 100 μm.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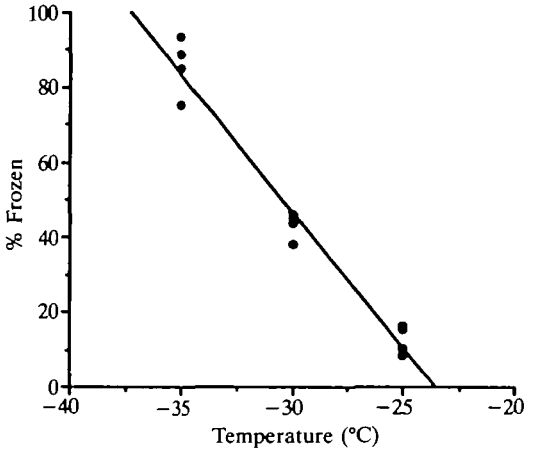

Fig. 3. Freezing of ensheathed J3s in air. Nematodes were cooled to various temperatures, allowed to rewarm to −5°C, and the proportion frozen counted.

no significant difference between the F_{50} and the S_{50} ($t = 0{\cdot}54$, df = 30, $P > 0{\cdot}1$). There was also no significant difference between the F_{50} value and the mean supercooling point of J3s measured in liquid paraffin ($t = 0{\cdot}05$, df = 44, $P > 0{\cdot}1$).

The freezing of ensheathed J3s in water is shown in Fig. 5. The increase in numbers frozen was spread across a much greater temperature range than it was in specimens in air or in liquid paraffin. The correlation between temperature and freezing was significant ($r^2 = 0{\cdot}932$, $t = 12{\cdot}31$, df = 12, $P < 0{\cdot}001$). The number that froze in water, however, was not a good predictor of subsequent survival ($X^2 = 37{\cdot}61$, $P < 0{\cdot}001$); the survival deviating from that predicted by the numbers frozen for the higher temperatures (Fig. 6). This indicates that ensheathed J3s in water can tolerate freezing if freezing occurs at a high sub-zero temperature. The

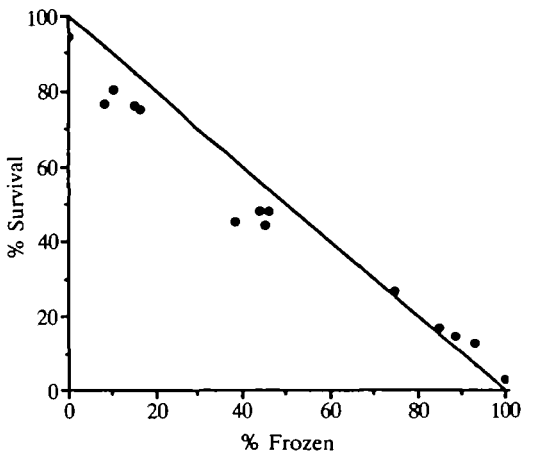

Fig. 4. Ensheathed J3s in air. The observed survival (●) compared with that predicted from the number frozen (——).

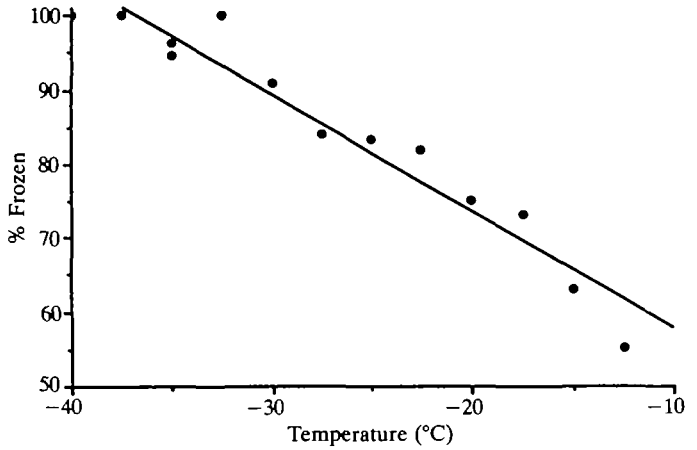

Fig. 5. Freezing of ensheathed J3s in water. Nematodes were cooled to various temperatures, allowed to rewarm to −5°C, and the proportion frozen counted.

F_{50} was $-12{\cdot}3 \pm 1{\cdot}1$°C and the S_{50} was $-15{\cdot}86 \pm 1{\cdot}0$°C. The difference between the F_{50} and the S_{50} was significant ($t = 7{\cdot}59$, df = 24, $P < 0{\cdot}001$).

There were significant differences between the F_{50} values measured in air and in liquid paraffin ($t = 3{\cdot}08$, df = 30, $P < 0{\cdot}001$), air and water ($t = 12{\cdot}01$, df = 27, $P < 0{\cdot}001$) and liquid paraffin and water ($t = 9{\cdot}08$, df = 27, $P < 0{\cdot}001$).

Freezing tolerance and ice nucleation in the ensheathed J3s

To determine the ability of the ensheathed J3s to prevent ice nucleation or tolerate freezing in the presence of external ice they were cooled to 2°C below the temperature at which the water in the sample froze. The proportion that had

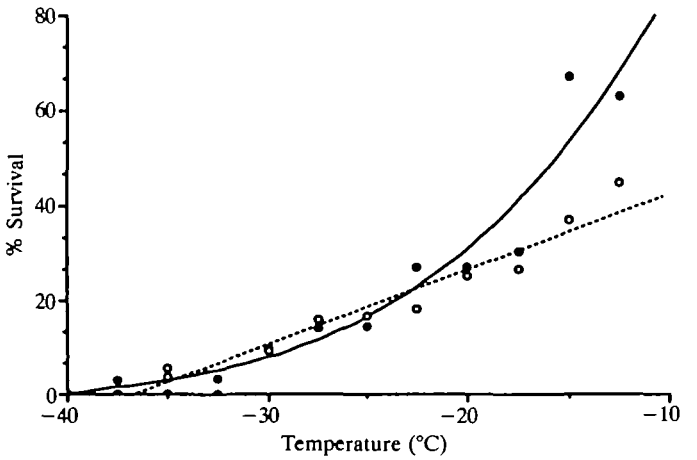

Fig. 6. Ensheathed J3s in water. The observed survival (●) compared with that predicted from the number frozen (○).

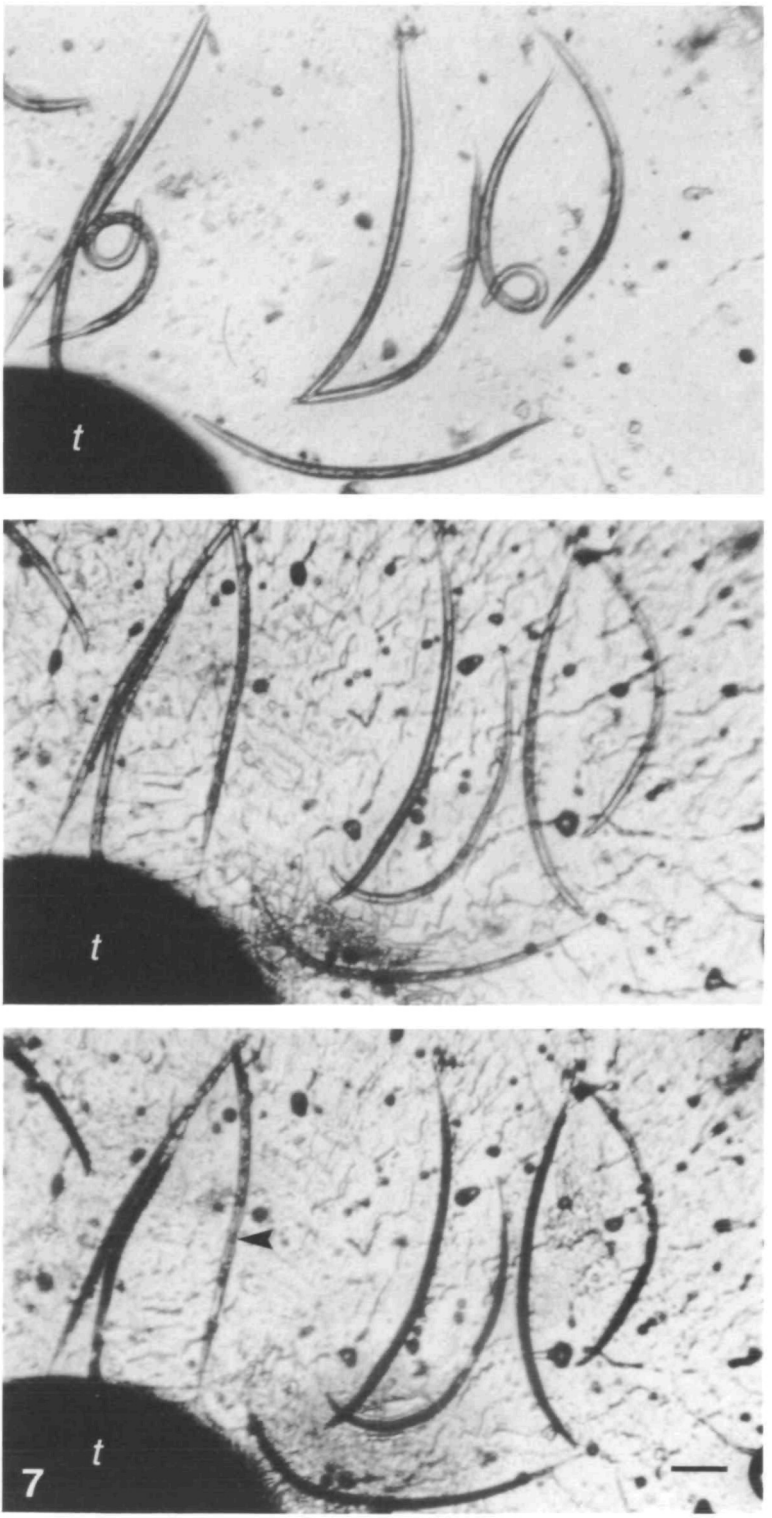
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Fig. 7. Ensheathed J3s in water during a cooling/rewarming cycle. The specimen was cooled to 0°C (top); both the nematodes and the water were unfrozen. During further cooling the water froze at −10°C (middle). The specimen was cooled to −30°C and then rewarmed to −5°C (bottom). Nematodes which had frozen blackened during rewarming, an unfrozen nematode remained clear (arrowhead). *t*, thermocouple. Scale bar, 100 μm.

frozen could be clearly observed (Fig. 7). 64·5 ± 3·8 % (±1 S.E., $N = 20$) of ensheathed J3s did not freeze when the water surrounding them froze. Of the remainder which did freeze, 24·9 ± 13·9 % subsequently recovered and could thus tolerate ice formation within their tissues. There was no apparent relationship between the temperature at which the water froze and the freezing tolerance or exogenous ice nucleation of the ensheathed J3s. The survival of ensheathed J3s deviated markedly from that predicted by the numbers which froze (Fig. 8). The percentage frozen was not a good predictor of subsequent survival ($X^2 = 147·4$, df = 19, $P < 0·001$). The numbers of freezing-tolerant nematodes could be determined by subtracting the predicted survival from the observed survival. The proportion of nematodes that were freezing tolerant increased with the percentage that were nucleated (Fig. 9: $r^2 = 0·553$, $t = 4·525$, df = 19, $P < 0·001$).

The effect of sheath removal on ice nucleation and freeze tolerance

The ability to prevent ice nucleation or to survive freezing in ensheathed J3s, desheathed J3s, exsheathed J3s and J3s in which the sheath had been cut was compared. The proportion which supercooled (did not freeze when the water in the sample froze), were freezing tolerant (froze but recovered) or were freezing intolerant (froze and did not recover) is shown in Fig. 10. Removal of the sheath reduced the proportion of nematodes which could prevent ice nucleation when the

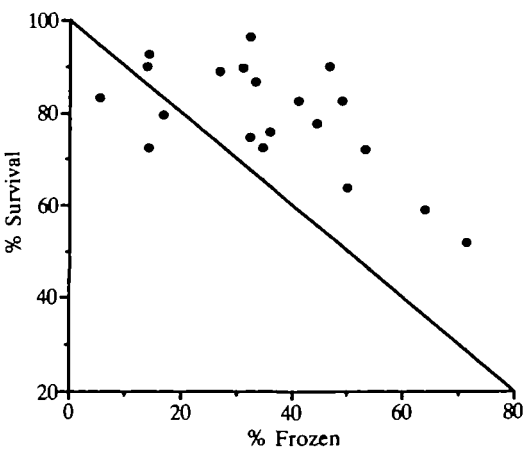

Fig. 8. Ensheathed J3s in water, cooled to 2°C below the temperature at which the water froze. The observed survival (●) compared with that predicted from the number frozen (——).

external water froze. Differences between treatments for percentage supercooling were significant (ANOVAR on arcsin-transformed data: $F = 63{\cdot}02$, df = 3/57, $P < 0{\cdot}001$). Cutting the sheath had less effect than removing the sheath. Differences between exsheathed and desheathed J3s were not significant, whereas

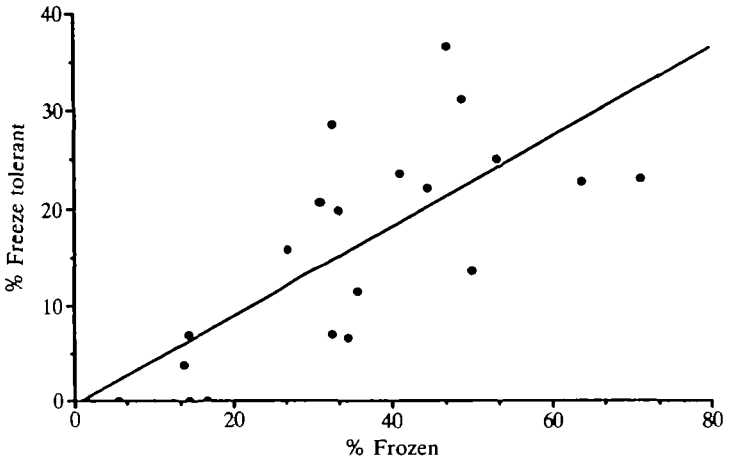

Fig. 9. The proportion of ensheathed J3s in water which survived freezing after ice nucleation in water and exposure to a temperature 2°C below the temperature at which the water froze. Calculated from the difference between the observed survival and the survival predicted if all the nematodes that froze were killed.

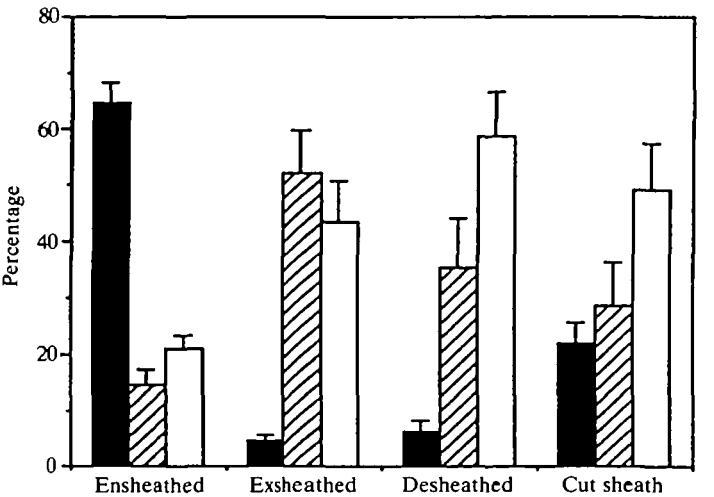

Fig. 10. The ability of ensheathed J3s, desheathed J3s, exsheathed J3s and J3s in which the sheath had been cut to prevent ice nucleation or survive freezing after exposure to a temperature 2°C below the temperature at which the water froze. The histogram shows the proportion that supercooled (did not freeze when the water in the sample froze – closed histograms), were freezing tolerant (froze but recovered – hatched histograms) or freezing intolerant (froze but did not recover – open histograms).

Table 2. *Cold tolerance of the infective juveniles of trichostrongyle nematodes*

Species	Temperature (°C)	S_{50} (h)	Reference
Trichostrongylus colubriformis	−14	53·7 ± 1·0*	Present work
Trichostrongylus vitrinus	−2	2·2 ± 1·2*	Rose & Small (1984)
Ostertagia ostertagi	−4 to −6	25†	Rose (1961)
Haemonchus contortus	−10	1·2 ± 0·3*	Todd *et al.* (1976)
Nematodirus spathiger	−2 to −1	210†	Turner (1953)
Nematodirus helvetianus	−3 to −4	21†	Rose (1966)

Values are mean ± S.E.
* From a probit analysis of published data.
† Estimated from published graphs.

differences between cut-sheath and exsheathed and between cut-sheath and desheathed J3s were significant (least significant difference: $P < 0{\cdot}05$). Differences between treatments for percentage freezing tolerant were significant ($F = 6{\cdot}97$, df = 3/53, $P < 0{\cdot}001$).

Discussion

Laboratory studies on the cold tolerance of the free-living stages of trichostrongyle nematodes have used different storage conditions prior to exposure, different conditions and periods of exposure to sub-zero temperatures and different methods of assessing recovery. It is, therefore, difficult to compare the survival abilities of different species and stages from published work. These studies do, however, indicate that a number of species can survive for considerable periods (Table 2). *T. colubriformis* appears to be one of the most resistant species. The J3 was the most resistant stage of the life cycle, as it is for other species of trichostrongyle nematodes (for references, see Table 2).

Cold-tolerant nematodes have been assumed to be freezing susceptible but to avoid freezing by supercooling (Wharton, 1986). Measurements of nematode supercooling points have, however, been made with the worms exposed to air (Ash & Atkinson, 1986) or covered by liquid paraffin to prevent water loss (Wharton *et al.* 1984; Perry & Wharton, 1985; Mabbett & Wharton, 1986). A proportion of infective juveniles of *T. colubriformis* can survive the formation of ice within their body caused by the seeding of external ice across the cuticle. The only previous report of freeze tolerance in nematodes is for *Aphelenchoides ritzemabosi* (Asahina, 1959).

Many nematodes are too small for their supercooling points to be determined by detecting the latent heat of fusion. Supercooling points can be measured by observing the freezing of the nematode directly during cooling on a cold microscope stage. However, the smaller the nematode and the lower the

supercooling point, the smaller is the change in optical density which accompanies freezing. In the present study, supercooling points were easy to observe in nematodes in water, more difficult in nematodes in liquid paraffin, and could not be observed in nematodes in air. This may have been because of differences in the refractive index of the immersion medium. During rewarming there is a marked darkening of the specimens. The cause of this darkening is uncertain but it may be due to a change in the size of ice crystals or an increase in the proportion of ice in the body. This phenomenon enables the proportion of nematodes that had frozen to be easily counted at the point of maximum darkening, −5°C. The use of the F_{50} as a measure of the mean supercooling point thus greatly increases the lower size limit of specimens that can be measured. The cooling rate of 1°C min^{-1} used in this study has been used as standard in studies on arthropod cold tolerance (Salt, 1961). However, this may not be the optimum cooling rate for survival and slower rates of cooling may more closely approximate conditions in the field.

The cold-tolerance mechanisms of the ensheathed J3s depended upon the medium in which they were exposed to sub-zero temperatures. Nematodes in air or in liquid paraffin supercooled and the numbers that subsequently recovered could be predicted by the numbers which froze, indicating a freeze-avoiding strategy. In water, however, a proportion of the worms froze when the water froze. The number that subsequently recovered was greater than that predicted by the number frozen, indicating that a proportion of the population could tolerate freezing. In water, the nematodes appear to use a mixture of freeze-avoiding and freeze-tolerant strategies. These measurements, however, were made on worms maintained at 20°C. Acclimation at low temperatures may result in a shift in strategy. *T. colubriformis* J3s stored at 3°C had a mean supercooling point of −30·0 ± 0·7°C (Wharton *et al.* 1984) and an F_{50} of −29·0 ± 1·02°C in liquid paraffin (G. S. Allan, unpublished results), which is lower than for nematodes stored at 20°C. A depression of supercooling points in response to acclimation has been reported in the infective eggs of *Nematodirus battus* (Ash & Atkinson, 1986) and in adult *Panagrellus redivivus* (Mabbett & Wharton, 1986). It is not known, however, whether acclimation may result in a shift from a freeze-tolerant to a freeze-avoiding strategy or *vice versa* by the synthesis or removal of ice-nucleating agents or by the synthesis of cryoprotectant compounds or thermal hysteresis factors. Glycerol levels in *T. colubriformis*, however, are thought to be too low to have a significant antifreeze effect (Wharton *et al.* 1984).

There were significant differences between the F_{50} values of J3s in air, liquid paraffin and water. When exposed to air, nematodes lose water rapidly and this may affect supercooling by concentrating solutes within the body fluids. In the presence of cryoprotectants, dehydration results in a significant reduction in supercooling points (Zachariassen, 1985). Addition of liquid paraffin prevents further water loss and the water content of nematodes remains constant for several days in this medium (Perry, 1977). Some water may, however, be lost between the removal of surface water and the addition of liquid paraffin. For nematodes in water the F_{50} value is affected by exogenous ice nucleation, although a proportion

of the population does supercool. F_{50} values in water are different from S_{50} values, reflecting a degree of freezing tolerance.

Anderson & Levine (1968) have shown that desiccation, consisting of exposure to 65–75% relative humidity at 30°C for 20 h, enhances the ability of *T. colubriformis* J3s to survive exposure to low temperatures, compared to controls in water. *T. colubriformis* J3s are very resistant to desiccation (Wharton, 1982) and may enter a state of anhydrobiosis in which metabolism comes reversibly to a standstill and the animal is resistant to environmental extremes which would be lethal when hydrated. The relatively mild desiccation used by Anderson & Levine (1968) may not have been sufficient to induce anhydrobiosis but may have ensured that there was no external water, causing a shift from a freeze-tolerant to a freeze-avoiding strategy. This would indicate that freeze avoidance is the more favourable strategy under these conditions. If this were the case, the J3s would be expected to migrate from the soil onto the herbage, thus reducing the risk of exogenous ice nucleation, although perhaps increasing exposure to low temperatures. A number of species of trichostrongyle J3s have been shown to migrate from the soil onto the herbage (Anderson *et al.* 1970; Rose & Small, 1985; Krecek & Murrell, 1988). Callinan & Wescott (1986) found that the migration of a variety of trichostrongyle J3s was dependent upon temperature and moisture, with more J3s being found on the herbage at low temperatures. The majority of J3s were, however, found in the surface layers of the soil.

Removal of the sheath of the J3s, either chemically by exposure to sodium hypochlorite or in response to an exsheathment stimulus, markedly reduces the nematode's ability to prevent exogenous ice nucleation and results in a shift from a freeze-avoiding to a freeze-tolerant strategy and an overall reduction in cold tolerance. This suggests that a major function of the sheath is to prevent nucleation of the body fluids by external ice. J3s in which the sheath had been cut did not show such a marked reduction in the numbers able to prevent ice nucleation, suggesting that contact between the sheath and the cuticle is important and the greater the surface area of the cuticle directly exposed to ice, the greater the chances of nucleation occurring. Desheathed J3s which did not survive freezing were sometimes physically disrupted, suggesting that the sheath may prevent physical damage by the advancing ice front.

Although removal of the sheath results in a decrease in the proportion of J3s supercooling, the survival of desheathed J3s after exposure to −14°C in water was much greater than that of ensheathed J3s. Survival was also greater than in desheathed J3s exposed to low temperatures on the cold microscope stage. A degree of supercooling occurred before the water in the sample froze on the cold microscope stage; this may be detrimental to freeze tolerance. Freeze-tolerant arthropods secrete ice-nucleating agents which ensure that supercooling of the body contents does not occur (Zachariassen, 1985). Exsheathed J3s also survive freezing over liquid nitrogen better than ensheathed J3s (Campbell & Thomson, 1973). Rapid freezing, however, results in the formation of small ice crystals and is a different process from freezing at a slow rate of cooling. Removal of the sheath

results in a marked decrease in the water content of the J3s (Davey & Rogers, 1982). The consequent increase in the concentration of the body contents may result in an increase in freeze tolerance.

Ensheathed J3s rely on a freeze-avoiding strategy when free of surface water and a mixture of freeze-avoiding and freeze-tolerant strategies when in contact with water. In arthropods, these strategies have often been considered to be distinct (Zachariassen, 1985). The classification of a species as freeze-avoiding or freeze-tolerant may, however, be dependent upon the precise environmental conditions to which it has been exposed. The ability of some species to tolerate freezing is critically dependent upon rates of cooling and rewarming (Baust & Rojas, 1985) and a species which can supercool may experience non-freezing-related lethal effects at temperatures above the supercooling point (Bale, 1987). Horwarth & Duman (1984) have described beetle larvae which shifted their cold-tolerance strategy in successive seasons. Nematodes may have a similar plasticity and be able to optimize their strategy in response to desiccation or hydration and other changes in their environment.

We would like to thank Brian Niven for advice on statistics and Alex Vlassoff for the supply of juveniles for the initial infection.

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