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Freezing avoidance in the eggs of the antarctic nematode *Panagrolaimus davidi*

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Summary – The eggshell of the antarctic nematode *Panagrolaimus davidi* protects the egg from exogenous ice nucleation and allows it to supercool in contact with external ice and be freeze avoiding. The proportion of eggs frozen by exogenous ice nucleation increases with decreasing nucleation temperature but this is unlikely to have a significant effect on survival on the field. Once the eggs have survived the initial freezing event there is little further risk of exogenous ice nucleation and the supercooled state is stable until the egg freezes at its supercooling point.

Résumé – ***L'évitement du gel chez les œufs du nématode antarctique* Panagrolaimus davidi** – Grâce à sa coque, l'œuf du nématode antarctique *Panagrolaimus davidi* est protégé de la formation de noyaux de glace exogènes en lui permettant d'entrer en surfusion au contact d'un milieu extérieur glacé et d'échapper ainsi au gel. La proportion d'œufs gelés par la formation de noyaux de glace externes augmente avec la diminution de la température à laquelle sont formés ces noyaux, mais il est peu probable que ce phénomène ait un effet significatif sur la survie du nématode dans les conditions naturelles. Après que les œufs ont survécu au gel initial, le risque est faible d'une formation ultérieure de noyaux de glace exogènes, et l'état de surfusion demeure stable jusqu'à ce que les œufs gèlent, le point de surfusion étant atteint.

Key-words : *Panagrolaimus davidi,* nematode, antarctic, cold tolerance, freeze avoiding, eggshell, exogenous ice nucleation.

Nematodes are aquatic organisms and may face exposure to low temperatures whilst in contact with water. The freezing of water in the nematodes' environment might be expected to initiate the freezing of the nematode by exogenous ice nucleation via the cuticle or body orifices. The larvae and adults of the antarctic nematode *Panagrolaimus davidi* are frozen by exogenous ice nucleation but can survive freezing and are thus freezing tolerant (Wharton & Brown, 1991). The sheath of the infective larvae of *Trichostrongylus colubriformis,* however, enables a freeze-avoiding strategy by preventing exogenous ice nucleation and allowing the larvae to supercool in the presence of external ice by maintaining their body fluids in a liquid state at temperatures below their melting point (Wharton & Allan, 1989).

The nematode eggshell is a substantial structure which might be expected to restrict ice nucleation. The eggshell of *Globodera rostochiensis* has been shown to prevent exogenous ice nucleation and allow the enclosed infective larva to supercool in the presence of external ice (Perry & Wharton, 1985; Wharton *et al.*, 1993). This paper examines the capacity of the eggshell of *P. davidi* to prevent exogenous ice nucleation and the effect of nucleation temperature on this ability.

Materials and methods

Panagrolaimus davidi was isolated from various sites in the McMurdo Sound region, Antarctica (Wharton & Brown, 1989). It was cultured on agar plates, consisting of 1 % agar and 0.1 % nutrient broth, and fed on bacteria which grew from the original isolates. The cultures were maintained at 15 °C and subcultured at intervals.

To isolate eggs, a large number of nematodes were transfered to a fresh agar culture. This resulted in a burst of egg laying after one to two weeks culture. Eggs, and other stages, were washed off the surface of the agar and mixed with a saturated solution of sodium chloride. The mixture was transferred to centrifuge tubes and the tubes filled so there was a slight positive meniscus. A coverslip was placed over the top of the tube, ensuring that there were no air bubbles trapped between the coverslip and the liquid. The samples were then centrifuged at approximately 1500 rpm for 5 min. The eggs floated to the top of the tube and adhered to the coverslip. The coverslip was removed using forceps using a sharp vertical movement and the eggs washed off the coverslip using either distilled water or an artificial tap water (ATW : Greenaway, 1970). The eggs were rinsed three times in distilled water or ATW before use. This resulted in an egg sample free of larvae and adults.

Ice nucleation and supercooling of eggs in water

To determine the ability of eggs to prevent exogenous ice nucleation and supercool in the presence of water, 100-200 eggs in distilled water were transferred to a thermoelectric microscope stage. This is based on a thermoelectric cooling module, the hot face of which is cooled by circulating fluid from a refrigerated circulator. The design and operation of the stage was similar to that described by Wharton and Rowland (1984) and Wharton and Allan (1989). The control unit for the stage has been replaced by a computer control system, this will be described in detail elsewhere. Briefly the signal from the monitoring thermocouple on the cold stage is fed after amplification into the analogue input (analogue to digital converter) of a microcomputer (BBC model B). The programme calculates the actual temperature and the required temperature, from the required cooling rate, and switches power to the thermoelectric cooling module by switching the power supply via the cassette relay to match actual to required temperature. The programme was calibrated using a Comark thermocouple simultator kit (TCH 3000) and checked against a Pt resistance temperature probe. The cold stage was mounted on a Wild M 32 dissecting microscope.

The eggs were mounted in distilled water on a small coverslip, designed to fit the specimen chamber of the cold stage. A second coverslip was placed on top of the sample to produce an optically flat surface which allowed the eggs to be easily observed during cooling. The sample was transferred to the cold stage, cooled rapidly to 2 °C and then to successively lower temperatures down to – 35 °C at 0.5 °C/min. They were held at the test temperature for 1 min and then warmed to 2 °C at 2 °C/min. The temperature at which the water froze was recorded and the proportion of eggs which froze by exogenous ice nucleation at this temperature counted (as indicated by the sudden darkening of the egg contents). After cooling to the test temperature the proportion of eggs which had frozen were counted at – 10 °C during rewarming.

Measurement of supercooling points

A computer control programme was developed to allow temperatures to be recorded when the spacebar of the computer is pressed. The data were accumulated and a histogram of temperature events generated at the end of the run. This was used to record the supercooling points of eggs in distilled water, ATW and liquid paraffin. For samples in ATW or distilled water 100-400 eggs were prepared as described above. For samples in liquid paraffin, the eggs were transferred to the coverslip in a drop of distilled water. The water was then removed using a fine pipette and filter paper spills. As soon as the eggs were free of surface water they were covered with a drop of liquid paraffin and a second coverslip.

The sample was transferred to the specimen chamber of the cold stage, cooled rapidly to 2 °C and then at 0.5 °C/min until all the eggs in the sample had frozen. The temperature at which the water in the sample (if present) froze was noted and the freezing points of eggs recorded. Two runs were completed for each medium (ATW, distilled water and liquid paraffin), resulting in 200-300 supercooling point measurements for each.

The ability of the eggshell to prevent exogenous ice nucleation at constant temperature

A further computer programme was written which produces a temperature hold when the spacebar of the computer is pressed. A sample of eggs in ATW was prepared as before. The sample was transferred to the specimen chamber of the cold stage, cooled rapidly to 2 °C and then at 0.5 °C/min until the water froze. When the water froze the temperature was held constant and the proportion of eggs which had frozen counted at intervals.

The survival of eggs after exposure to low temperatures

Egg samples were prepared as before and transferred in eppendorf tubes to a controlled rate cooling block in ATW. The controlled rate cooler is similar in design to the cold microscope stage and is based on a thermoelectric cooling module, cooled by a refrigerated circulator with the power supplied to the cooling module under computer control to give the required cooling/warming rate. The sample was cooled rapidly to 2 °C and then to various temperatures (0 °C to – 40 °C) at 0.5 °C/min. The sample was maintained at the test temperature for 1 min and then warmed to 2 °C at 2 °C/min. The sample was inoculated with an ice crystal to initiate freezing at – 5°C during cooling.

To assess survival the eggs were transferred to microtitre wells containing a layer of 1 % agar, enclosed in an open plastic bag to reduce evaporation, incubated at 20 °C for 48 hours and the number of hatched and unhatched eggs counted.

Results

Ice nucleation and supercooling of eggs in water

In distilled water a proportion of the eggs are seeded by exogenous ice nucleation (mean ± se = 44.6 ± 5.6 %, n = 18) and freeze shortly after the water surrounding them freezes (Fig. 1). Of the remaining eggs, an increasing proportion are frozen as the temperature is lowered (Fig. 1). The increase in the proportion frozen after cooling to the test temperature is correlated with the test temperature (Fig. 2: $r^2 = 0.599$, $df = 16$, $p < 0.01$). A proportion of the eggs can prevent exogenous ice nucleation and supercool in the presence of external ice but as the temperature decreases an inceasing proportion reach their supercooling point.

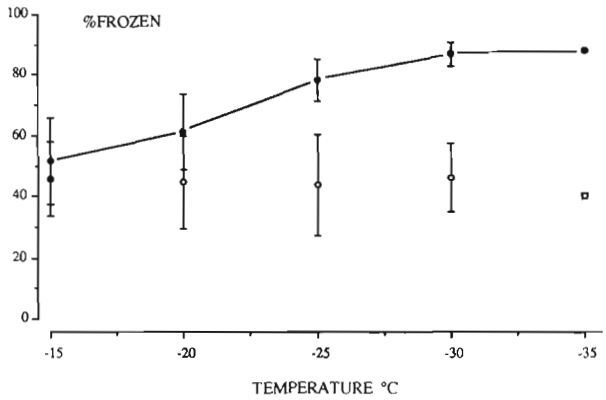

Fig. 1. *The effect of temperature on the proportion of the eggs of* P. davidi *that are frozen when the water in the sample freezes* (○) *and upon rewarming to – 10 °C after cooling to the test temperature* (●) *Vertical lines represent the standard error of the mean (n = 4).*

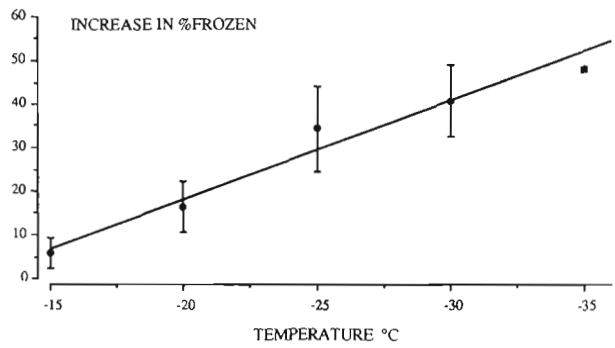

Fig. 2. *The effect of temperature on the increase in the proportion of the eggs of* P. davidi *that are frozen after cooling to the test temperature and rewarming to – 10 °C. Vertical lines represent the standard error of the mean (n = 4).*

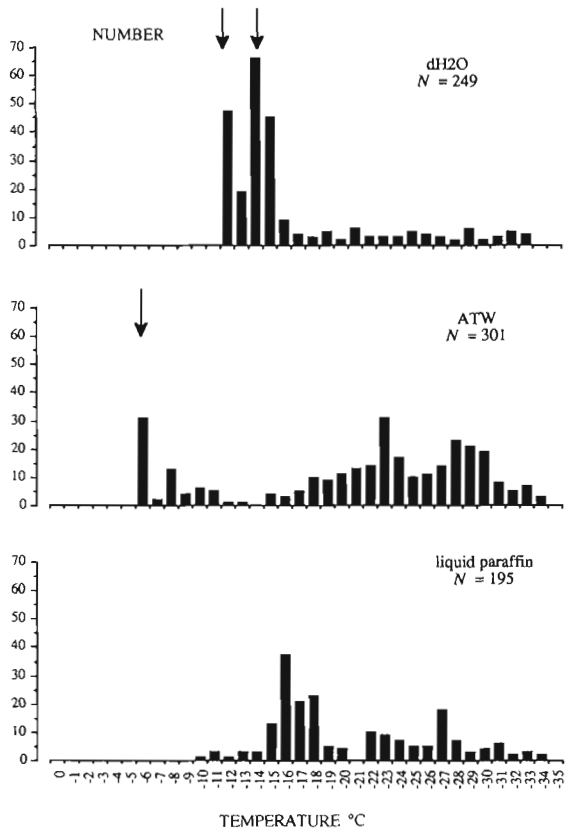

Fig. 3. *The freezing or supercooling points of the eggs of* P. davidi *during cooling at 0.5 °C in distilled water (top), ATW (middle) and liquid paraffin. Combined data from two runs for each medium. The arrows indicate the freezing of the medium.*

Supercooling points in various media

Fig. 3 shows the supercooling points of *P. davidi* eggs in distilled water (top), ATW (middle) and liquid paraffin (bottom). In distilled water the samples undercooled to – 12 °C and – 14 °C. The majority of eggs froze by exogenous ice nucleation, shortly after the freezing of the water in the sample (67.1 % froze within 1 °C of the freezing point of the medium). In ATW each of the two samples undercooled to – 6 °C. Far fewer eggs froze due to exogenous ice nucleation compared with distilled water (88.7 % supercooled in the presence of external ice to greater than 1 °C lower than the freezing point of the medium). In liquid paraffin there was no freezing of the medium and hence no exogenous ice nucleation.

The mean supercooling points, omitting events within 1 °C of the freezing of the medium to exclude exogenous ice nucleation, were : distilled water, – 22.7 ± 6.1 °C (± 1 SD, N = 82): ATW, – 23.2 ± 6.5 °C (N = 268); liquid paraffin, – 20.6 ± 5.7 °C (N = 195). The effect of the medium on supercooling points was significant (fac-

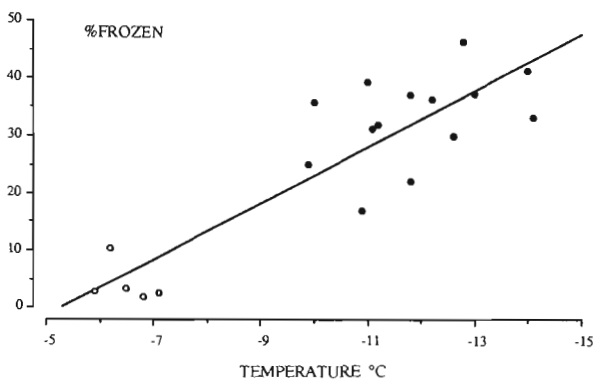

Fig. 4. *The effect of the freezing temperature of the medium on the proportion of the eggs of* P. davidi *that were frozen by exogenous ice nucleation. The eggs were frozen in ATW* (●) *or distilled water* (○) *The regression line is calculated from the data for the two media combined.*

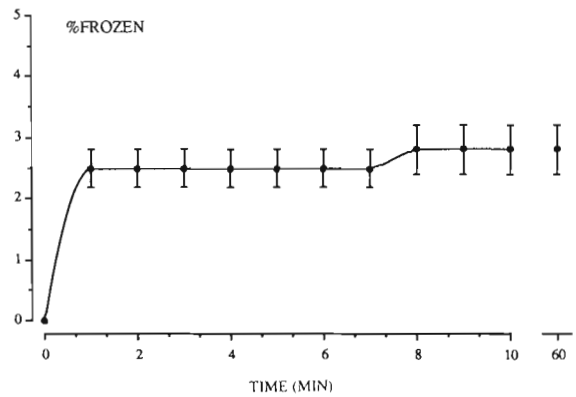

Fig. 5. *The freezing of the eggs of* P. davidi *in ATW placed on temperature hold when the water in the sample froze. Vertical lines represent the standard error of the mean (n = 4).*

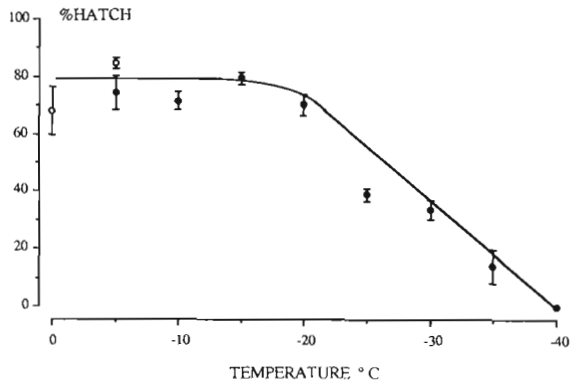

Fig. 6. *Hatching of eggs of* P. davidi *in ATW following exposure to various low temperatures (●) - frozen samples (○) - unfrozen controls. Vertical lines represent the standard error of the mean (n = 5).*

torial ANOVA, after arcsin transformation : $F = 10.2$, $p < 0.001$). The differences in supercooling points between liquid paraffin and the other two media were significant (t tests : $p < 0.005$) but not between distilled water and ATW ($p > 0.005$).

ICE NUCLEATION TEMPERATURE AND EXOGENOUS ICE NUCLEATION

The effect of nucleation temperature of the medium on the percentage of eggs that froze by exogenous ice nucleation within 1 °C of the freezing point of the medium is shown in Fig. 4. Combining the data for eggs in distilled water and ATW shows that exogenous ice nucleation increases with decreasing nucleation temperature ($r^2 = 0.795$).

THE ABILITY OF THE EGGSHELL TO PREVENT EXOGENOUS ICE NUCLEATION AT CONSTANT TEMPERATURE

The freezing of eggs placed on temperature hold when the medium froze is shown in Fig. 5. The water in the samples froze in the range – 5.9 °C to – 7.1 °C. There was little increase in freezing during one hour following the freezing of the medium. The experiment was not continued for longer than one hour as the frozen eggs gradually became lighter with time, making is difficult to distinguish between frozen and unfrozen eggs.

THE SURVIVAL OF EGGS AFTER EXPOSURE TO LOW TEMPERATURES

The hatching of eggs after exposure to low temperatures is shown in Fig. 6. The % hatch after exposure to temperatures down to – 20 °C was fairly constant with a marked decline in survival following exposure to temperatures in the range – 25 °C to – 40 °C. The effect of temperature on survival in the range 0 °C to – 20 °C was not significant (factorial ANOVA, after arcsin transformation : $F = 68.5$, $df = 4$, $p < 0.05$) but in the range – 20 °C to – 40 °C the effect of temperature was significant ($F = 1.67$, $df = 5$, $p > 0.05$). Unhatched eggs showed clear signs of freezing damage.

Discussion

The eggshell of *P. davidi* can prevent exogenous ice nucleation and allow the enclosed embryo or larva to supercool in the presence of external ice. The decline in survival of eggs exposed to low temperatures in ATW occurs within the temperature range where the eggs are reaching their supercooling points. This indicates that in water the eggs of *P. davidi* adopt a freeze-avoiding strategy : supercooling in the presence of external ice but dying once freezing does occur. This is in contrast to the larval and adult stages of *P. davidi* which are freezing tolerant with freezing initiated by exogenous ice nucleation (Warton & Brown, 1991). In *Globodera rostochiensis* the ability to prevent exogenous ice nucleation is thought to be a property of the chitinous layer of the eggshell (Wharton *et al.*, 1993).

Although the nematode eggshell may have a very restricted permeability (Wharton, 1980), some of the variability in the results may be explained by their exposure to saturated sodium chloride solution during isolation. This would have exposed the eggs to hyperosmotic stress and individual eggs may vary in their recovery during subsequent washing in ATW or distilled water, affecting their supercooling points.

The cold tolerance of the eggs of *P. davidi* is not as great as that of the adult and larval stages which can survive exposure to – 80 °C (Wharton & Brown, 1991; Brown, 1993). *P. davidi,* however, does not lay eggs below 7 °C (Brown, 1993), suggesting that the egg stage will only be present during the Antarctic summer. The cold tolerance of the egg stage would be sufficient to survive the occasional freezing events which may occur during the summer, where temperatures in moss may go as low as – 8.4 °C (Block, 1985) but the nematode is likely to overwinter as adults and larvae.

The ability of the eggshell to prevent exogenous ice nucleation declines with decreasing nucleation temper-

ature, with a greater proportion of eggs seeded by exogenous ice nucleation if there is a substantial degree of undercooling before the freezing of the medium. For freezing tolerant insects the initiation of ice nucleation at relatively high subzero temperatures by the synthesis of potent ice nucleating agents is thought to be important in preventing lethal intracellular freezing by confining ice formation to the haemocoel (Duman *et al.,* 1991). The ability of the eggs of *P. davidi* to avoid freezing is partly dependant upon the water in the environment freezing at relatively high subzero temperatures. Whilst antarctic mosses can undercool several degrees below 0 °C without freezing (Davey *et al.,* 1992) this would not be sufficient to have an adverse effect on exogenous ice nucleation of the eggs.

Although significant differences were found between the mean supercooling points of eggs in ATW, distilled water and liquid paraffin, the means were within 3 °C of each other. Eggs in liquid paraffin are free of surface water and are thus not subject to exogenous ice nucleation. Eggs in ATW had slightly lower supercooling points than those in liquid paraffin. This suggests that once the egg survives the initial freezing of the medium, there is no further risk of exogenous ice nucleation and the egg will supercool until it freezes at its supercooling point. The absence of an increase in egg freezing in samples placed on temperature hold for one hour after the freezing of the medium also suggests that the supercooled state is stable once the egg has survived the initial risk of exogenous ice nucleation.

Nematodes thus employ two different strategies to survive subzero temperatures in contact with water. They are either freezing tolerant and freeze by exogenous ice nucleation from the surrounding medium (Wharton & Brown, 1991) or they possess a structure such as a sheath or an eggshell which prevents exogenous ice nucleation; allowing them to supercool in the presence of external ice and survive by freeze avoidance (Wharton & Allan, 1989; Wharton *et al.,* 1993; present work).

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