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The role of the eggshell in the cold tolerance mechanisms of the unhatched juveniles of *Globodera rostochiensis*

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Summary – The eggshell of *Globodera rostochiensis* prevents exogenous ice nucleation during freezing and allows the eggs to supercool in contact with water. Hatched second stage juveniles (J2) are frozen by exogenous ice nucleation and are not freezing tolerant. The effect of low temperatures on the hatching of eggs indicates that the unhatched J2 cannot survive freezing. The eggs exhibit a freeze avoiding strategy by supercooling in the presence of external ice. There is no evidence for a cold-induced diapause. Exposure to high temperatures and to potato root diffusate does not destroy the ability of the eggshell to prevent exogenous ice nucleation; indicating that this is not a property of the lipid layer of the eggshell. Exposure to 10 % NaOCl, however, does destroy this ability and is accompanied by a pronounced thinning of the eggshell. This suggests that the prevention of exogenous ice nucleation is a property of the chitinous layer of the eggshell.

Résumé – ***Le rôle de la coque de l'œuf dans les mécanismes de tolérance au froid des juvéniles non éclos de* Globodera rostochiensis** – La coque des œufs de *Globodera rostochiensis* empêche la formation exogène de noyaux de glace durant la congélation et permet aux œufs d'entrer en surfusion au contact de l'eau. Les juvéniles de deuxième stade (J2) congelés par formation exogène de noyaux de glace ne sont pas tolérants à une telle congélation. L'action des basses températures sur l'éclosion des œufs prouve que les J2 non éclos peuvent survivre à la congélation. En présence de glace externe, les œufs échappent à la congélation grâce à un mécanisme de surfusion. Il n'existe pas d'évidence d'une diapause induite par le froid. L'exposition à de hautes températures, ou aux diffusats de racines de pomme de terre, ne détruit pas la capacité de la coque de l'œuf à empêcher la formation exogène de noyaux de glace, ce qui indique qu'il ne s'agit pas d'une propriété liée à la couche lipidique de la coque. En revanche le contact avec NaOCl à 10 % supprime cette propriété et provoque un amincissement prononcé de la coque. Il est donc suggéré que le phénomène empêchant la formation exogène de noyaux de glace est redevable à une caractéristique de la couche chitineuse de la coque de l'œuf.

Key-words : *Globodera rostochiensis,* nematode, cold tolerance, freezing avoidance, supercooling, exogenous ice nucleation, eggshell, hatching.

Nematodes may survive exposure to subzero temperatures either by freezing tolerance, surviving the formation of extracellular ice in their bodies, or by freeze avoidance, maintaining their body fluids in a liquid state at temperatures below their melting point and supercooling (Sayre, 1964; Wharton & Brown, 1991). A number of nematode species have been shown to supercool in the absence of surface water and where surface water has been removed and replaced with liquid paraffin to prevent desiccation (Wharton, 1986). However, nematodes are essentially aquatic organisms and are likely to be exposed to subzero temperatures in the presence of external water. When the water freezes, the nematode will either be frozen by exogenous ice nucleation via the cuticle and body orifices or it must possess the means of preventing exogenous ice nucleation (Wharton & Brown, 1991).

The unhatched second stage juvenile (J2) of *Globodera rostochiensis* survives freezing in contact with water, whereas the hatched J2 does not (Perry & Wharton, 1985). The prevention of ice nucleation by the eggshell when the medium freezes may be important for the survival of the unhatched J2 by allowing the egg to supercool. Alternatively, the egg may be frozen by exogenous ice nucleation across the eggshell but the unhatched J2 survive freezing, perhaps because it has a lower water content than hatched J2s (Perry, 1989). In this paper we examine these contrasting hypotheses and investigate the role of the different layers of the eggshell in cold tolerance.

Materials and methods

NEMATODE MATERIAL

Cysts of *G. rostochiensis* Ro1, grown on potato cv. Désirée in pots, were taken from a single generation harvested in 1986 and stored dry at 5 °C after extraction from the soil. Cysts were soaked for one week in artificial tap water (ATW; Greenaway, 1970) before experimen-

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tation. Free eggs were obtained from soaked cysts by cutting the cysts open with a scalpel. Potato root diffusate (PRD) was obtained from pot cultures of potato cv. Désirée by the method of Fenwick (1949) and diluted with glass distilled water (GDW) 1 in 4 by volume before use. Hatched J2s were obtained by immersing soaked cysts in PRD for 24 hours at 20 °C. Approximately 100-300 eggs or hatched juveniles were used for each replicate.

Effect of low temperature on freezing of eggs in contact with water

The freezing of eggs of *G. rostochiensis* was observed using a cold microscope stage similar to that described by Wharton and Rowland (1984). This is based on a thermoelectric cooling module, the hot face of which is cooled by circulating fluid from a refrigerated circulator. The control unit for the stage was replaced by a BBC model B microcomputer. The design of the computer control system will be described in detail elsewhere. Briefly, the signal from the stage's monitoring thermocouple is fed after amplification into the analogue input (analogue to digital converter) of the microcomputer. The programme calculates the actual temperature and the required temperature, from the designated 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 control programme was calibrated using a Comark thermocouple simulator kit (TCH 3000 - Comark Electronics Ltd., U.K.) and checked against a platinum resistance temperature probe. The cold stage was mounted on a Wild M32 dissecting microscope with a MPS 51S camera system.

Approximately 100 eggs in ATW were transferred to a small circular coverslip designed to fit the cold stage. They were then covered with a second coverslip and transferred to the specimen chamber of the cold stage. They were cooled rapidly to 2 °C and then at 0.5 °C/min to various subzero temperatures down to – 42 °C. The eggs were held at the minimum temperature for 1 min and then warmed at 2 °C/min. Frozen eggs darkened during rewarming and the proportion of eggs frozen were counted between – 10 °C and – 5 °C. Four replicates were used for each minimum temperature, the eggs for each replicate being from a separate cyst. Photographs were taken of eggs at different points of the cooling/warming cycle.

Supercooling points of eggs and J2s in water

A computer control programme was also 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 and J2s in water.

50-100 eggs or hatched J2s were transferred to the cold stage and cooled rapidly to 2 °C and then at 0.5 °C/min until all the specimens had frozen. The specimens were observed during cooling and the supercooling points were recorded when the freezing of the eggs/J2s were observed. Three runs were completed for both eggs and hatched J2s.

Effect of high temperature on eggshell permeability and ice nucleation

Cysts in ATW were exposed to high temperatures (40-80 °C) in a water bath for 1 h. They were allowed to cool to room temperature (1 h at approximately 20 °C) and eggshell permeability tested by dissecting the cysts and exposing the eggs to 1 % (w/v) acid fuschin in 0.05 M sulphuric acid (1 h at 15 °C). The percentage of stained eggs were counted.

The ability of the eggshell to prevent ice nucleation was tested by transferring approximately 100 eggs after high temperature treatment to the cold stage as before. They were cooled from 2 °C to – 10 °C at 1 °C/min, held at – 10 °C for 1 min, warmed to 2 °C at 1 °C/min and the proportion frozen counted at – 5 °C during rewarming.

Effect of potato root diffusate on ice nucleation

Cysts were exposed to PRD at 20 °C for 24 h or to ATW as a control (six replicates). The cysts were cut open and free eggs were transferred to the cold stage as before and cooled from 2 °C to – 10 °C at 1 °C/min, held at – 10 °C for 1 min, warmed to 2 °C at 1 °C/min and the proportion frozen counted at – 5 °C during rewarming.

Effect of sodium hypochlorite treatment on ice nucleation

Batches of four cysts were exposed to 10 % (v/v) NaOCl at room temperature for various times. They were washed three times in distilled water and free eggs transferred to the cold stage as before. They were then cooled from 2 °C to – 15 °C at 1 °C/min and the proportion frozen counted at – 15 °C. Only intact eggs containing juveniles were counted to exclude partially-hatched eggs. Photographs of NaOCl-treated eggs and untreated eggs were taken using differential interference contrast optics on a Zeiss photomicroscope.

Effect of low temperature on cyst hatching

Batches of 20 cysts (with three replicates for each temperature) were soaked for 1 week in ATW at 20 °C in excavated glass blocks and were then 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 electronic control to give the required cooling/warming rate. The sample was cooled rapidly to 2 °C and then to various temperatures (– 25 °C to – 45 °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. A control batch of cysts was maintained at 20 °C.

For hatching tests, each replicate batch of twenty cysts was transferred to an excavated glass block containing 2 ml of PRD. At weekly intervals for 8 weeks, hatched juveniles were removed and counted; fresh PRD was added to the cysts. At the end of the hatching tests, cysts were broken open and the number of unhatched juveniles was counted and the percentage hatch determined.

Results

When the surrounding water freezes the intact eggs of *G. rostochiensis* do not freeze but supercool (Fig. 1). The effect of low temperatures in the range – 25 °C to – 42 °C on the freezing of eggs in water is shown in Fig. 2. The increase in egg freezing occurred between – 35 °C and – 40 °C. The supercooling points of most eggs also fell in this range with a mean supercooling point of – 38.2 ± 0.1 °C (Fig. 3). A few eggs (2.1 %)

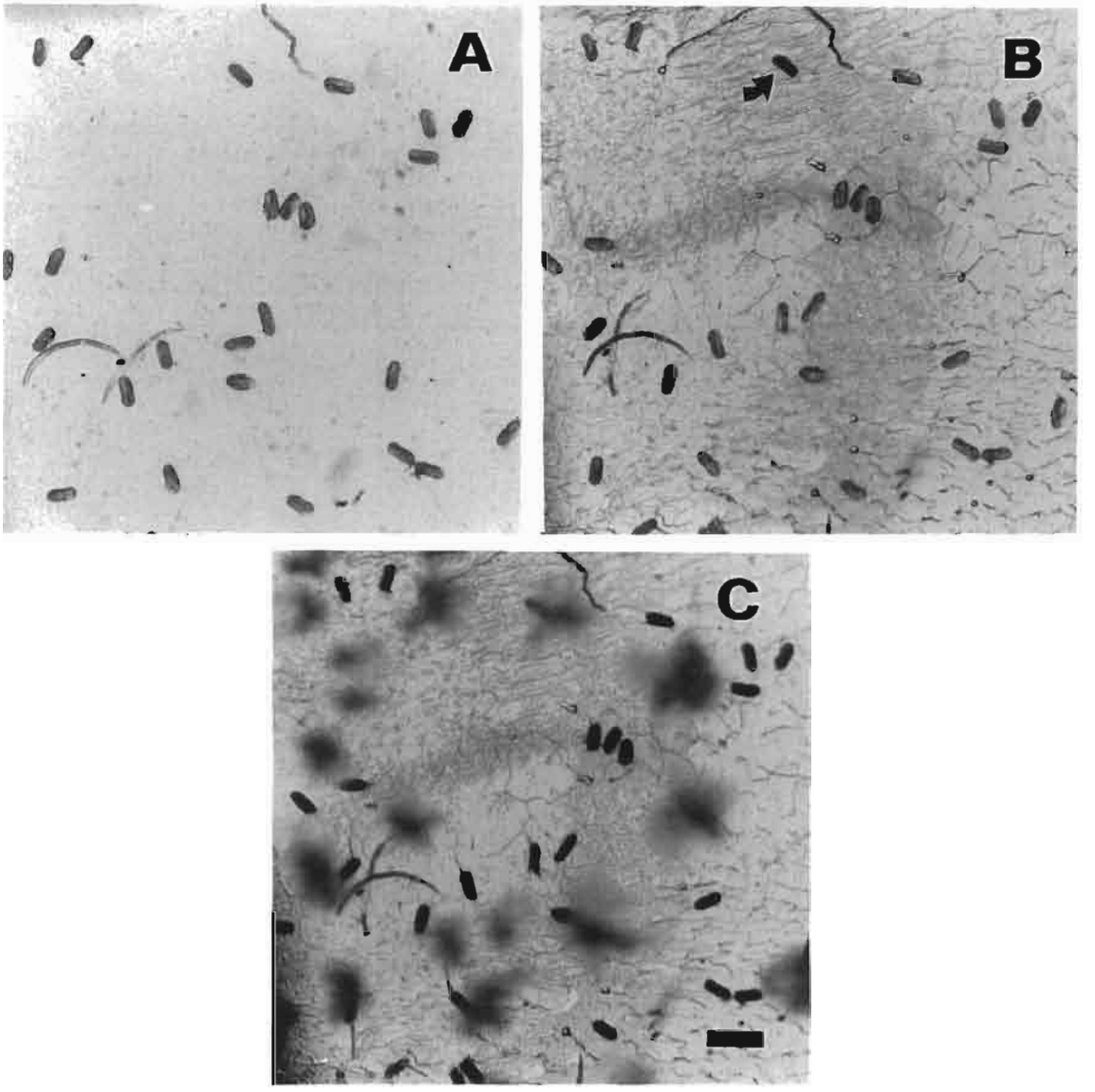

Fig. 1. Photomicrographs of eggs of *Globodera rostochiensis* and J2s during a freezing/warming cycle on the microscope cold stage. A : At 2 °C before the freezing of the medium; B : At – 3 °C the medium has frozen, all J2s have frozen due to exogenous ice nucleation, all the eggs remain unfrozen (with the exception of one frozen egg, indicated by an arrow); C : During rewarming at – 10 °C after cooling to – 35 °C, all the eggs have frozen. *(Scale bar = 200 μm.)*

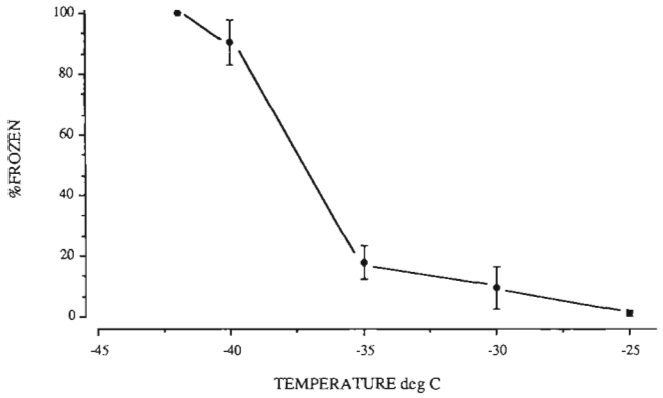

Fig. 2. Effect of exposure to subzero temperatures on the freezing of the eggs of *Globodera rostochiensis* in ATW. (Vertical lines are standard errors; four replicates, approximately 100 eggs/replicate.)

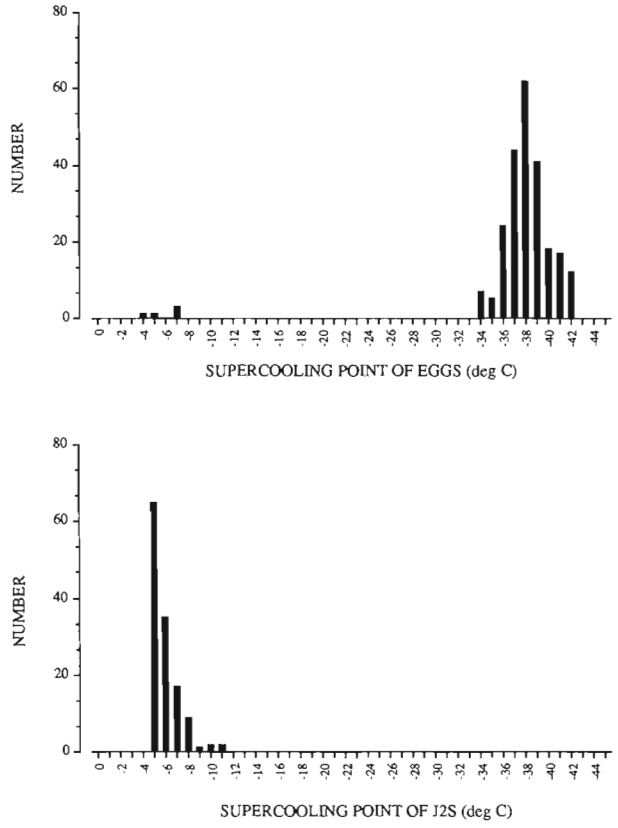

Fig. 3. Distribution of supercooling points of eggs (top: N = 235, $\bar{X} = -38.2 \pm 0.1$) and hatched J2s (bottom: N = 131, $\bar{X} = -5.9 \pm 0.1$) of *Globodera rostochiensis* in ATW.

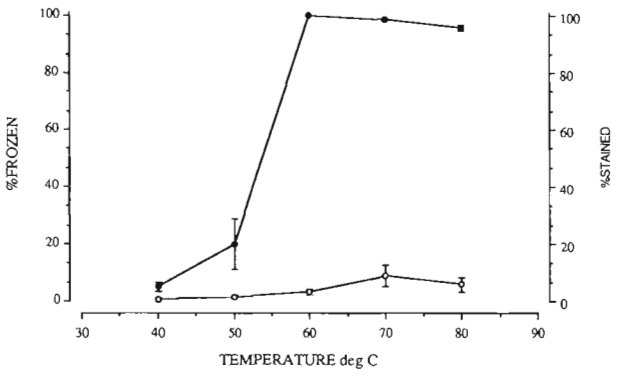

Fig. 4. Effect of exposure to high temperature on the subsequent staining of eggs of *Globodera rostochiensis* with 1 % acid fuchsin (●) and on the subsequent freezing of eggs in ATW at – 10 °C (○). (Vertical lines are standard errors; four replicates, approximately 100 eggs/replicate.)

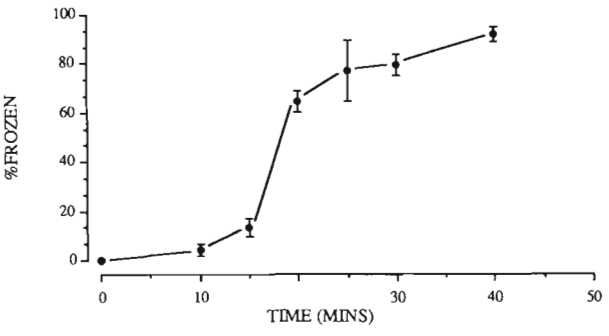

Fig. 5. Effect of exposure to 10 % NaOCl on the subsequent freezing of eggs of *Globodera rostochiensis* in ATW at – 15 °C. (Vertical lines are standard errors; four replicates, approximately 100 eggs/replicate.)

froze soon after the freezing of the water in the sample and exhibit high supercooling points. The distribution of supercooling points is different in hatched J2s (Fig. 3). The freezing of the nematodes was initiated by the freezing of the medium, with at most a further 6 °C of supercooling after the formation of external ice.

There was a marked increase in the staining of eggs with 1 % acid fuchsin following exposure to 60 °C (Fig. 4), suggesting that the permeability barrier of the eggshell is destroyed by heat between 50 °C and 60 °C. There was, however, no increase in the freezing of heat-treated eggs after cooling to – 10 °C (Fig. 4). The effect of temperature on staining is significant (factorial ANO-

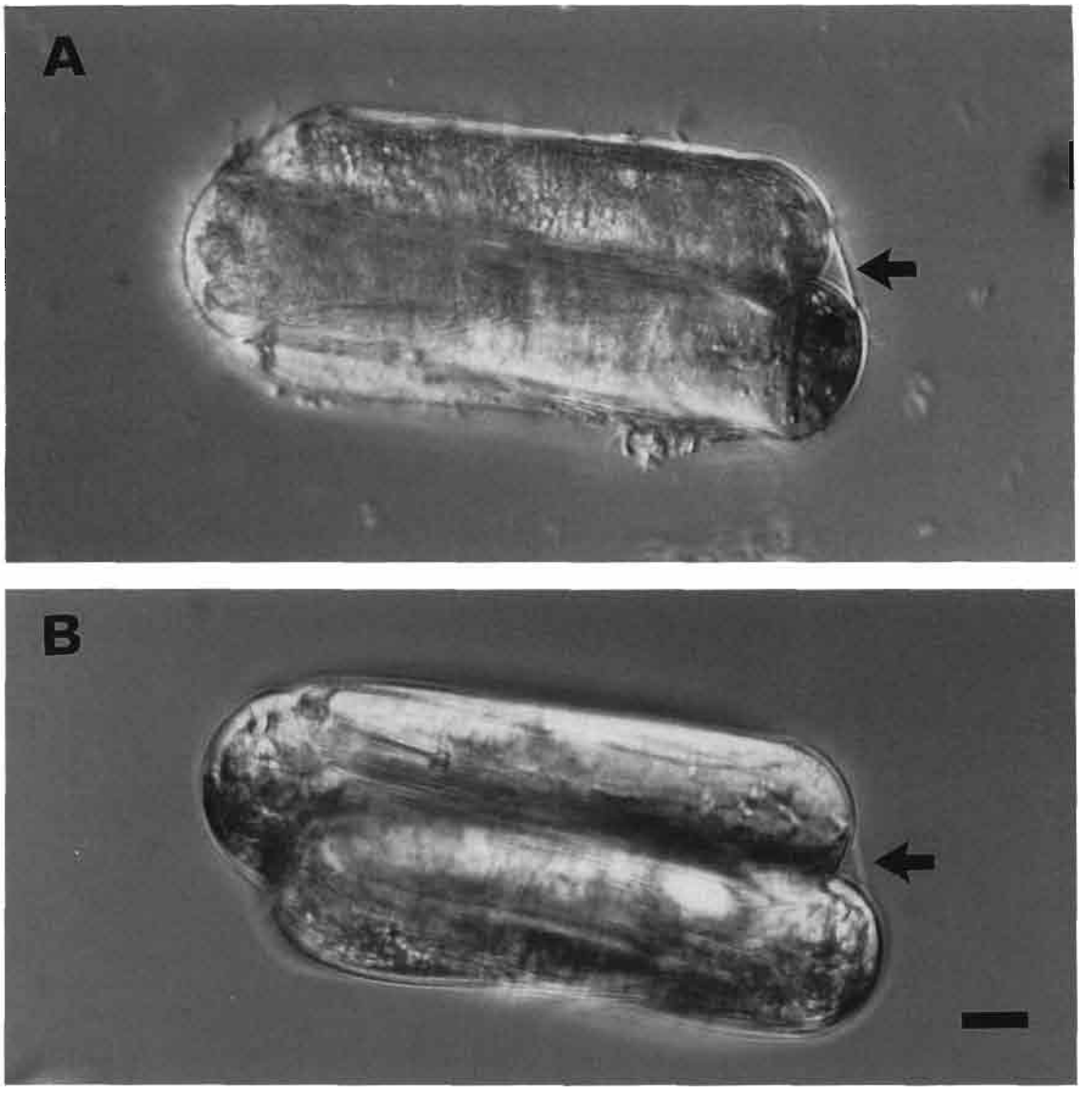

Fig. 6. Photomicrographs of eggs of *Globodera rostochiensis* taken using differential interference contrast optics. A : Untreated egg; B : Egg exposed to 10 % NaOCl for 30 min at room temperature. Note the decrease in the thickness and rigidity of the eggshell after NaOCl treatment (arrows). *(Scale bar = 10 µm.)*

VA after arcsin transformation : $F = 164.8$, $p < 0.001$) but there is no significant effect on egg freezing ($F = 2.4$, $p > 0.1$).

The effect of exposure to PRD for 24 h on egg freezing compared to controls in ATW is shown in Table 1. There is no significant effect of PRD on freezing compared with the ATW control (t test : $t = 0.48$, $p > 0.1$).

Treatment with 10 % NaOCl had a marked effect on the freezing of eggs (Fig. 5). The proportion frozen at

Table 1. The effect of exposure to potato root diffusate (PRD) for 24 hrs on freezing of *Globodera rostochiensis* eggs at – 10 °C.

Medium	Replicates	% Frozen	S.E.
PRD	6	1.0	0.4
ATW	6	1.6	1.1

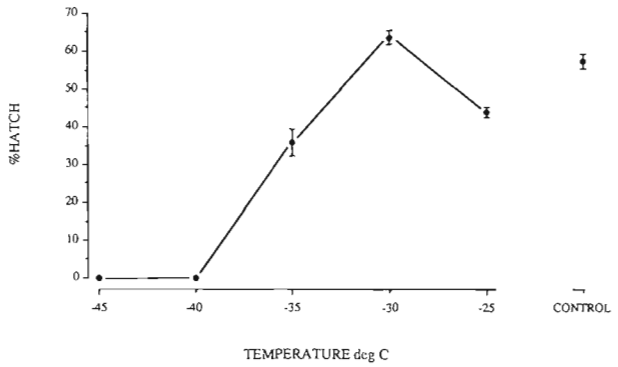

Fig. 7. Effect of exposure to subzero temperatures on the hatching of eggs of *Globodera rostochiensis* during exposure to PRD in standard hatching tests. The control was maintained at 20 °C. (Vertical lines are standard errors; three replicates, 20 cysts/replicate.)

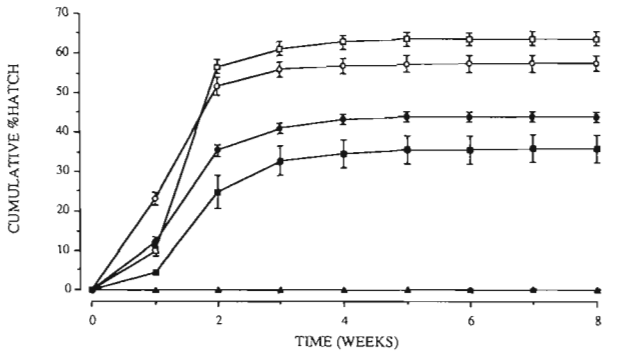

Fig. 8. Effect of exposure to subzero temperatures on the cumulative hatch of eggs of *Globodera rostochiensis* in standard hatching tests. (Vertical lines are standard errors; three replicates, twenty cysts/replicate; ●: – 25 °C; □: – 30 °C; ■: – 35 °C; ▲: – 40 °C; ○: control.)

– 15 °C increased following treatment with NaOCl for 20 min or more. Most of these eggs froze when the surrounding water froze. The effect of treatment time on freezing was significant (factorial ANOVA: $F = 31.9$, $p < 0.001$). Treatment with NaOCl caused a marked decrease in the thickness of the eggshell, which was reduced to a thin layer which more closely followed the contours of the unhatched J2 (Fig. 6).

In hatching tests juveniles emerged from cysts exposed to subzero temperatures in the range – 25 °C to – 35 °C (Fig. 7). There was little hatching (< 0.5 %), however, after exposure to – 40 °C and – 45 °C. The effect of temperature on hatching was significant (repeated measures ANOVA, after arcsin transformation: $F = 193$, $p < .01$), even if the data for – 40 °C and – 45 °C was excluded ($F = 31.6$, $p < .01$). Exposure to subzero temperatures did not appear to delay hatching with most juveniles hatching during the second week in both experimental and control groups (Fig. 8).

Discussion

The eggshell of *G. rostochiensis* prevents exogenous ice nucleation from the freezing of external water and allows the egg to supercool in contact with ice in the medium. The hatched J2, however, is seeded by exogenous ice nucleation. J2s will still hatch from cysts after cooling to temperatures down to – 35 °C at 0.5 °C/min but not after exposure to – 40 °C and – 45 °C. This correlates with the freezing of eggs, which occurs at – 38.2 ± 0.1 °C during cooling at 0.5 °C/min. The eggs thus employ a freeze avoiding strategy but die when freezing eventually occurs. Hatched J2s cannot survive freezing (Perry & Wharton, 1985) and are thus not cold tolerant. Exposure to subzero temperatures down to – 35 °C did not delay hatching, compared with controls; thus there is no evidence for a cold-induced diapause. Although the majority of eggs exhibited low supercooling points in contact with water, a few (2.1 %) froze by exogenous ice nucleation when the medium froze; in these eggs the eggshell may have been damaged. The effect of cooling and warming rates on cold tolerance may be critical (Baust & Rojas, 1985). It is probable that nematodes are similarly affected by rates and may exhibit different freezing responses when cooled at rates which more closely approximate those experienced in nature.

The eggshell of *G. rostochiensis* consists of three layers: the vitelline layer, the chitinous layer and the inner lipid layer (Perry *et al.*, 1982). Exposure to high temperatures causes an irreversible increase in the permeability of the eggshell to acid fuchsin. This is thought to be due to a phase change or melting of the lipids that make up the lipid layer (Barrett, 1976; Wharton, 1980). Exposure to PRD also causes an increase in eggshell permeability to water and trehalose (Perry & Clarke, 1981). However, neither exposure to high temperature nor exposure to PRD resulted in the loss of the ability to prevent exogenous ice nucleation. This does not, therefore, appear to be a property of the lipid layer of the eggshell. The lipid layer and the trehalose content of the perivitelline fluid surrounding the juvenile are central to the hatching mechanism (Perry, 1989) and help protect the unhatched juvenile from desiccation (Perry, 1983). The trehalose content of the perivitelline fluid of *Nematodirus battus* has a cryoprotective role (Ash & Atkinson, 1986) but in *G. rostochiensis* exposure to PRD, with concomitant permeability change of the lipid layer and loss of trehalose (Perry, 1989), does not alter the ability to prevent exogenous ice nucleation. Thus, in *G. rostochiensis* eggs the trehalose content of the perivitelline fluid is less important than the eggshell for the cold tolerance of the unhatched J2s, although it may affect the supercooling ability of the eggs and this merits further study.

Exposure to 10 % NaOCl destroyed the ability of the eggshell to prevent exogenous ice nucleation. This was accompanied by a noticeable thinning of the eggshell, which was reduced to a thin membrane surrounding the unhatched J2. This is presumably due to the dissolution of the outer layers of the eggshell. Clarke and Hennessy (1980) did not observe a gradual thinning of the eggshell in hypochlorite but an abrupt rupturing. However, this occurred in hypochlorite which had been diluted with NaOH which maintained a high pH. Hypochlorite diluted with distilled water shows a rapid fall in pH and is much less effective in stimulating emergence from cysts (Clarke & Hennessy, 1980). Smedley (1936) observed that the eggs of *Heterodera schachtii* retained a thin membrane after exposure to NaOCl. Dissolution in NaOCl is thought to indicate the presence of quinone-tanned proteins (Brown, 1950). The structure and chemistry of the chitinous layer is consistent with it comprising chitin microfibrils in association with quinone-tanned proteins (Perry *et al.*, 1982). The loss of the ability of the eggshell to prevent exogenous ice nucleation after treatment with NaOCl suggests that the chitinous layer is responsible but this requires confirmation, perhaps by electron microscopy, that it is the chitinous layer that is removed by NaOCl treatment.

Nematodes which freeze in contact with water are seeded by exogenous ice nucleation but may be freezing tolerant (Wharton & Brown, 1991). The sheath of the infective juveniles of *Trichostrongylus colubriformis* can prevent exogenous ice nucleation and allow the nematode to supercool (Wharton & Allan, 1989). In this paper we have shown that the eggshell of *G. rostochiensis* can also prevent exogenous ice nucleation and allow the enclosed J2 to adopt a freeze avoiding strategy when exposed to subzero temperatures in contact with water.

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