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DIFFERENTIAL SCANNING CALORIMETRY STUDIES ON THE CYSTS OF THE POTATO-CYST NEMATODE *GLOBODERA ROSTOCHIENSIS* DURING FREEZING AND MELTING

D. A. WHARTON[1,*] AND H. RAMLØV[2]

[1]*Department of Zoology, University of Otago, PO Box 56, Dunedin, New Zealand* and [2]*Chemical Institute, University of Copenhagen, Universitetsparken 5, DK-2100 Copenhagen 0, Denmark*

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Summary

Differential scanning calorimetry (DSC) was used to determine the thermal events associated with freezing and melting of the cysts of the potato-cyst nematode *Globodera rostochiensis*. There were no thermal events during the cooling of dry cysts from 5 to −60 °C and warming back to 5 °C, indicating the absence of water freezing in the dry cysts. During heating of dry cysts from 5 to 80 °C, two overlapping endothermic events were observed at 55 °C, indicating the irreversible destruction of the permeability barrier of the eggshell by the melting of the lipids which constitute the lipid layer.

The first exothermic event (T=−9 °C) during the cooling of hydrated cysts indicates the presence of an ice-nucleating agent. A broad exotherm at −38 °C is due to the freezing of eggs. DSC thus confirms that the eggshell prevents exogenous ice nucleation and allows the eggs to supercool in the presence of external ice. The enthalpy of the egg exotherm increased during hydration of the cysts. The temperature of the egg exotherm was elevated after heating of the sample to 70 °C. This is thought to be due to the loss of trehalose from the eggs following the destruction of the permeability barrier of the eggshell.

During melting, three endothermic events were observed. These became merged after the destruction of the permeability barrier of the eggshell by heating, and only two peaks were observed in isolated eggs. The sample is thus considered to consist of three freezable compartments: (1) the water surrounding the cyst, (2) the solution between the cyst wall and the eggs and (3) the egg contents.

Key words: *Globodera rostochiensis*, nematode, differential scanning calorimetry, cold tolerance, cyst, freezing, melting.

Introduction

Nematodes can endure subzero temperatures in contact with water either by surviving the freezing which results from exogenous ice nucleation from the surrounding water (inoculative freezing) or by preventing ice nucleation by the presence of a structure such as an eggshell or a sheath (Wharton, 1995).

The female of the potato-cyst nematode *Globodera rostochiensis* becomes gravid and then dies; the body wall of the nematode is tanned to form a protective cyst. The cyst wall forms a spherical container holding approximately 300 eggs. The eggs develop to second-stage larvae which do not hatch until they receive a stimulus from the potato host. The eggshell can prevent inoculative freezing when the surrounding water freezes, allowing the enclosed larva to supercool in the presence of external ice, with a mean supercooling point observed by cryomicroscopy of −38.2±0.1 °C and hatching occurring after exposure to −35 °C (Perry and Wharton, 1985; Wharton *et al.* 1993).

Differential scanning calorimetry (DSC) can be used to measure heat flux during freezing and melting of samples and is increasingly being used in studies of invertebrate cold tolerance. It has been used in the study of antifreezes, ice-nucleating activity, subzero glass transitions and to measure ice contents in insects (Block, 1994). DSC has not been previously applied to the study of nematode cold tolerance. The cold tolerance mechanism of the cyst of *G. rostochiensis* is fairly well understood, which allows us to interpret the results obtained by DSC and to assess the usefulness of this technique for studies of nematode cold tolerance.

Materials and methods

Cysts of *Globodera rostochiensis* Ro1 (Woll.), grown on potato cultivar Désirée in pots, were taken from a single generation harvested in 1986 and stored dry at 5 °C after extraction from the soil. Artificial tap water (ATW) was used for rehydration of the cysts (Greenaway, 1970).

*Author for correspondence.

The differential scanning calorimeter (DSC) used was a Perkin-Elmer DSC-7, equipped with an intercooler II cooling system. The sample was sealed in an aluminium pan and an empty pan was used as a reference. The calorimeter was calibrated with gallium (T_m=30 °C, ΔH_m=80 J g^{-1}), water (T_m=0 °C) and *n*-decane (T_m=−30 °C). Samples were weighed before and after the DSC run, the top of the aluminium pan was punctured and heated at 70 °C for 24 h, reweighed and the dry mass and water content calculated.

Samples consisted of (1) dry cysts, (2) batches of five cysts rehydrated for 1, 2 or 3 days and (3) eggs dissected from 20 cysts after 4 days of rehydration. The dry cysts were cooled from 5 to −60 °C at a cooling rate of −1 °C min^{-1}. They were then heated from −60 to 5 °C at 1 °C min^{-1} and then from 5 to 80 °C at 10 °C min^{-1}. They were allowed to cool rapidly to 5 °C and then the heating from 5 to 80 °C at 10 °C min^{-1} was repeated (Fig. 1).

Five cysts rehydrated for 1, 2 or 3 days in ATW and eggs dissected from 20 cysts after 4 days of rehydration were cooled from 5 to −60 °C at a rate of −1 °C min^{-1}. They were then heated rapidly to −5 °C, allowed to stabilise and then warmed from −5 to 5 °C at 0.5 °C min^{-1}. The sample was then heated rapidly to 70 °C and held at this temperature for 5 min to destroy the permeability barrier of the eggshell. They were then cooled rapidly to 5 °C and the cooling/warming sequence described above was repeated (Fig. 1). An ATW control was subjected to a freezing/warming cycle as described above.

Results

Dry cysts

No exothermic events were detected during cooling of dry cysts from 5 to −60 °C and no endothermic events were detected during warming from −60 to 5 °C. During heating from 5 to 80 °C, however, two overlapping endotherms were detected with peaks at 57 and 59 °C (Fig. 2). There was also a shift in the baseline. When heating from 5 to 80 °C was repeated, however, the endotherms were substantially reduced and the baseline shift did not occur. The water content of dry cysts was estimated to be 0.09 g H_2O g^{-1} dry mass.

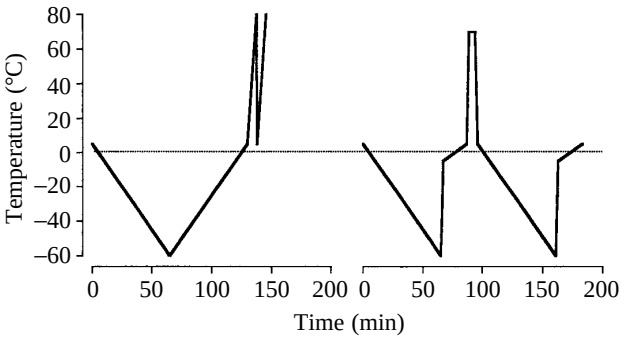

Fig. 1. Cooling and heating regimes in the differential scanning calorimeter applied to dry cysts (left) and hydrated cysts and eggs (right).

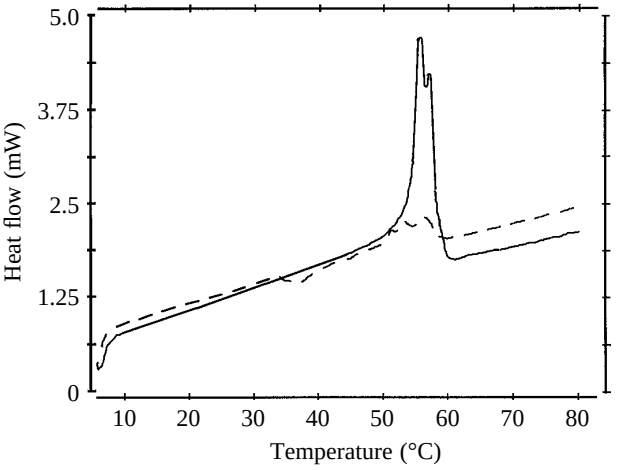

Fig. 2. DSC trace showing heat flow during heating of dry cysts from 5 to 80 °C (solid line). The broken line shows the repeat run after cooling the sample to 5 °C.

Hydrated cysts

Thermal events during heating and cooling of batches of five cysts rehydrated for 1, 2 and 3 days are summarised in Table 1. The pattern observed during cooling of the samples was similar in all three samples with a large first exotherm at about −9 °C (I, Fig. 3A). Small second exotherms were observed in the 2 day and 3 day samples (II, Fig. 3A), with peaks at −12 and −18 °C respectively. A much broader exotherm with a peak at −39 °C represents the freezing of the eggs (III, Fig. 3A,B). The onset and peak of the egg exotherm were similar in all three samples, but the size of the egg exotherm, measured as enthalpy per gram of sample, increased with increasing period of rehydration (Table 1). The detail in the egg exotherm (Fig. 3B) suggests that the freezing of individual eggs can be detected. After the sample had been heated to 70 °C, there was a shift in the peak of the egg exotherm to approximately −21 °C but the first exotherm was not affected. A consistent trend in the percentage water content of the samples with rehydration was not found as the cyst samples included varying amounts of excess water (Table 1). The ATW control showed a single exotherm at −20.2 °C. The exotherm of ATW controls was significantly lower than that of the first exotherm of cyst/egg samples (means ± 1 S.D., ATW, −16.73±3.38, N=3; cyst/eggs, −8.0±0.82, N=4; t-test, t=−4.378, d.f.=2, P<0.05).

Three endotherms were observed during the melting of the samples (Fig. 4A); a similar pattern was observed in the 1, 2 and 3 day hydration samples. After heating the samples to 70 °C, the three endotherms appeared to merge into a single endotherm (Fig. 4B).

Isolated eggs

Eggs dissected from 20 cysts produced two exotherms on cooling from 5 to −60 °C, with a first exotherm at −7 °C and an egg exotherm peak at −38.5 °C. There was no exotherm at around −16 °C. After heating, there was a shift in the egg exotherm to −21.7 °C.

Table 1. *Freezing events in the cysts of* Globodera rostochiensis *measured by differential scanning calorimetry*

Parameter	1 day of hydration	2 days of hydration	3 days of hydration	Eggs	ATW control
First exotherm (°C)	–9	–8	–8	–7	–20.2
Egg exotherm peak (°C)	–38.7	–38.8	–38.8	–38.5	–
Egg exotherm onset (°C)	–37.6	–37.9	–37.1	–36.4	–
Egg exotherm enthalpy ($J\,g^{-1}$)	–34.3	–43.6	–48.8	–43.6	–
First exotherm, after heating (°C)	–9	–9	–9	–10	–
Egg exotherm peak, after heating (°C)	–20.8	–20.1	–21.3	–21.7	–
Egg exotherm onset, after heating (°C)	–20.7	–13.4	–21.3	–21.7	–
Egg exotherm enthalpy, after heating ($J\,g^{-1}$)	–13.1	–44.6	–40.5	–38.0	–
Wet mass of sample (mg)*	0.86	0.77	0.98	0.95	0.07
Water content of sample ($g\,g^{-1}$ dry mass)*	1.77	1.20	1.45	4.00	–

ATW, artificial tap water.
*Including water surrounding the cysts/eggs.

The pattern during melting was simpler than that observed for whole cysts, with only two endotherms being observed (IV and VI, Fig. 5A). After heating, the first endotherm (IV) was more prominent and became shifted to a higher temperature (Fig. 5B).

Discussion

The absence of exotherms during cooling, and of endotherms during warming from −60 to 5 °C, indicates the absence of water freezing in the dry cysts of *G. rostochiensis*. DSC studies on the cysts of the brine shrimp *Artemia salina* indicate that freezing of water does not occur at hydration levels below $0.3\,g\,H_2O\,g^{-1}$ dry mass (Ramløv and Hvidt, 1992). The water content of dry cysts of *G. rostochiensis* was estimated as $0.09\,g\,H_2O\,g^{-1}$ dry mass.

We interpret the endotherms observed during heating of the dry cysts as representing melting or phase transitions of the lipids of the lipid layer of the eggshell and the destruction of its permeability barrier. DSC indicates that there are at least two lipids responsible. The change appears to be irreversible, as the peaks were much reduced during a second heating of the sample after cooling. The permeability barrier of the eggshell of *G. rostochiensis* to 1% Acid Fuchsin is irreversibly destroyed between 50 and 60 °C (Wharton *et al.* 1993). An irreversible change in the permeability of the lipid layer during heating occurs in a variety of nematode eggs (Wharton, 1980). The picture is, however, complex. The temperature at which an apparent change in permeability takes place varies according to the substance used to identify it (dyes, fixatives, water) and the way in which the eggs are exposed (continuously or after cooling). This is, therefore, thought not to be a simple critical temperature transition, but to represent a series of phase changes or melting events in the complex mixture of lipids that make up the lipid layer (Barrett, 1976; Wharton, 1980). The eggshell of *G. rostochiensis* consists of 9% lipid (Clarke *et al.* 1967). Lipid is located in the vitelline layer and the lipid layer, which consists mainly of lipoprotein membranes (Perry *et al.* 1982). The lipid layer is, however, thought to be the main permeability barrier of the eggshell (Wharton, 1980).

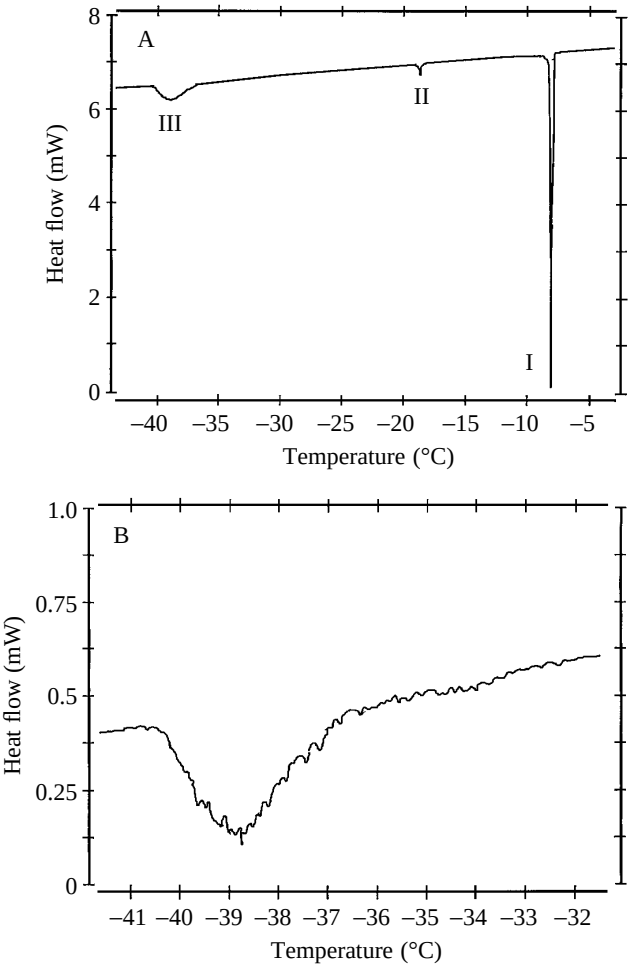

Fig. 3. (A) Thermal events (I–III) during the cooling from −5 to −60 °C of five cysts rehydrated for 3 days. (B) Detail of egg exotherm III shown in A.

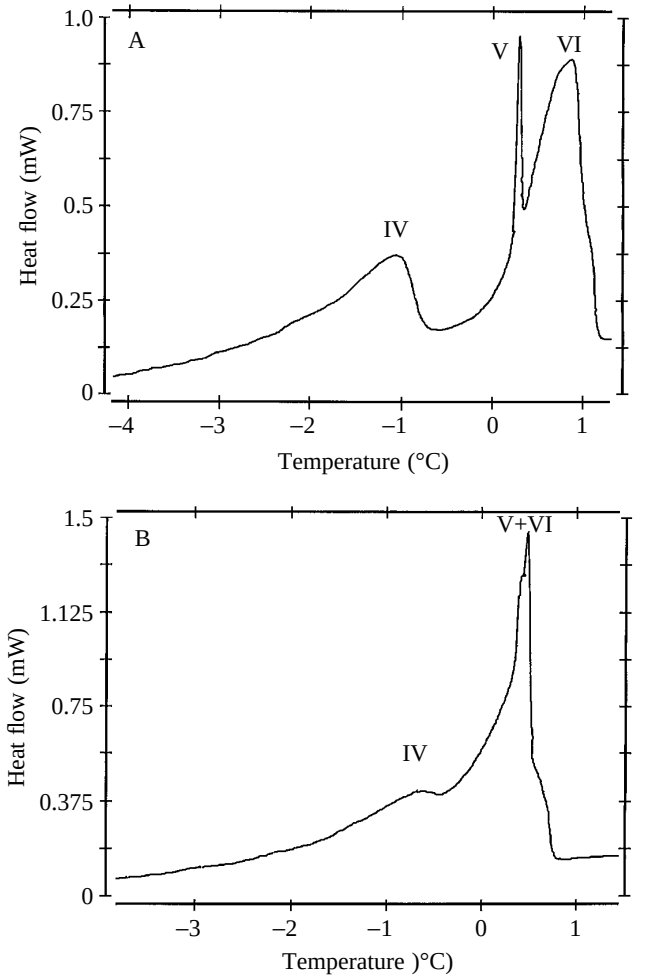

Fig. 4. (A) Melting endotherms of cysts rehydrated for 3 days and heated from −5 to 5 ˚C. (B) Melting endotherms for the same sample following heating to 70 ˚C.

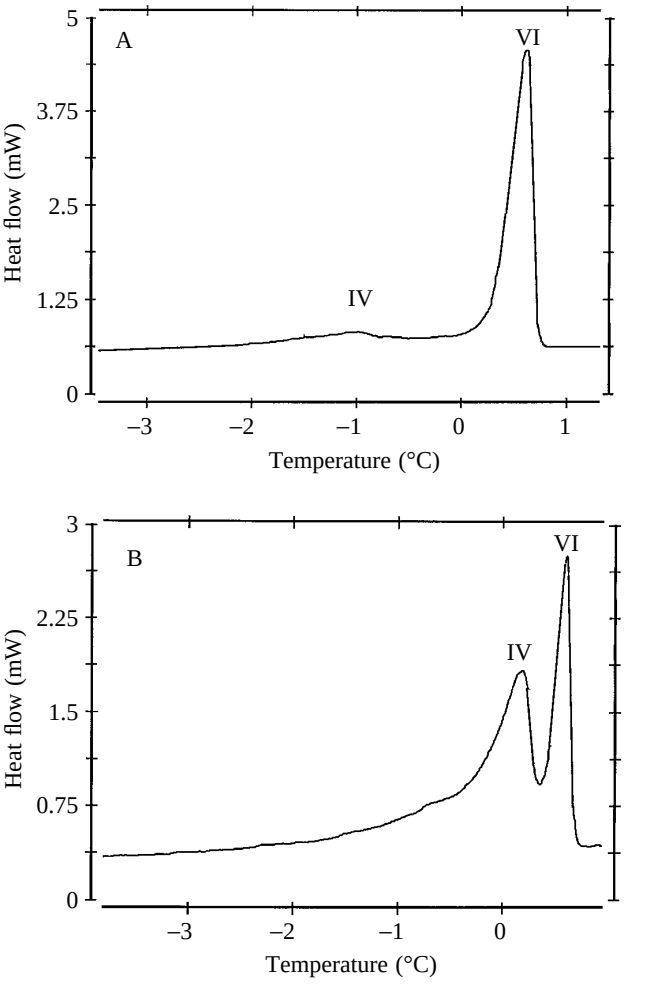

Fig. 5. (A) Melting endotherms of isolated eggs heated from −5 to 5 ˚C. (B) Melting endotherms for the same sample following heating to 70 ˚C.

The exotherms observed during the cooling of hydrated cysts from 5 to −60 ˚C result from the freezing of three compartments at different temperatures (Fig. 6). The first exotherm (I, Fig. 3A) is presumably due to the freezing of the water surrounding the cyst. This occurred at a higher temperature than the ATW control (Table 1), indicating the presence of an ice-nucleating agent. This is probably a property of the cyst wall. The first exotherm was also observed in eggs dissected from cysts, but small amounts of cyst wall material may well have remained after the dissection and acted as ice-nucleating sites. The ice-nucleating activity was not destroyed by heating. The second exotherm (II) is interpreted as being due to the freezing of the solution between the cyst wall and the eggs. The third exotherm (III) is due to the freezing of the egg contents, which have a high trehalose content.

Endotherms observed during the melting of hydrated cysts (Fig. 4A) can also be interpreted as the melting of three distinct compartments (Fig. 6): the egg contents (IV), the water between the eggs and the cyst wall (V) and the water surrounding the cysts (VI). The presence of three peaks in the melting thermogram of hydrated cysts indicates the presence within the sample of three compartments of different composition and therefore with different melting points. Isolated eggs showed only two peaks in the melting endotherm, indicating that one of the compartments in the intact cyst

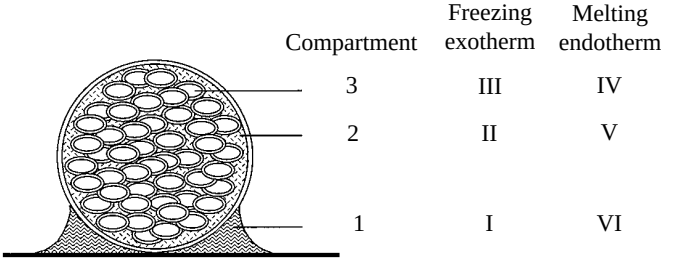

Fig. 6. Diagram showing the water compartments in samples of the cysts of *Globodera rostochiensis* and their corresponding freezing exotherms and melting endotherms: (1) the water surrounding the cyst, (2) the solution between the cyst wall and the eggs and (3) the egg contents.

consists of the space between the cyst wall and the eggs. After heating the sample, the three peaks appeared to merge into one (Fig. 4B). This can be interpreted as resulting from mixing following the destruction of permeability barriers.

The egg exotherm peak of hydrated cysts (III, Fig. 3A), and of eggs dissected from cysts, occurred at about $-38\,^{\circ}C$ (Table 1). This equates well with the supercooling points of eggs in ATW determined using a cryomicroscope stage, which had a mean supercooling point of $-38.2\pm0.1\,^{\circ}C$ (Wharton *et al.* 1993). DSC thus confirms that the eggshell of *G. rostochiensis* prevents exogenous ice nucleation and allows the eggs to supercool in the presence of external water. The enthalpy of the egg exotherm increased in magnitude with increasing time of rehydration, indicating that the eggs continued to take up water during a 3 day period.

The eggs of *G. rostochiensis* contain 6.4% trehalose (on a dry mass basis) at a concentration of $0.34\,mol\,l^{-1}$ (Clarke and Hennessy, 1976). Trehalose is located in the perivitelline space between the unhatched second-stage larva and the eggshell and is retained within the egg by the limited permeability of the lipid layer of the eggshell. Hatching is in response to some factor released by the potato host and involves an increase in the permeability of the eggshell, allowing trehalose to diffuse out of the egg, releasing osmotic stress and activating the larva (Clarke and Perry, 1977; Clarke *et al.* 1978). The destruction of the permeability barrier upon heating would also allow trehalose to diffuse out of the egg.

The eggs of *Nematodirus battus* accumulate trehalose during chilling, and there is a corresponding depression of their supercooling point to $-37.17\pm0.76\,^{\circ}C$ after chilling for 8 weeks (Ash and Atkinson, 1986). The low supercooling points observed in *G. rostochiensis* and *N. battus* are presumably mainly due to the small volume of water and the absence of ice nucleators within the egg. However, the high concentration of trehalose within the egg will also depress the supercooling point. Ash and Atkinson (1986) found that the supercooling point of $0.5\,\mu l$ droplets of water was depressed from -10.5 to $-22.0\,^{\circ}C$ by the addition of trehalose. We therefore interpret the elevation in the onset temperature of the egg exotherm after heating of the sample, observed in our study, as being due to the loss of trehalose from the egg contents after the destruction of the permeability barrier of the eggshell.

The use of DSC has produced a clearer understanding of the events occurring during the freezing and melting of the cysts of *G. rostochiensis* and should prove useful in studies of nematode cold tolerance.

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