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Osmoregulation in the Antarctic nematode *Panagrolaimus davidi*

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SUMMARY

A technique for determining the internal osmotic concentration of a small nematode using a modified nanolitre osmometer is described and used to investigate osmoregulation in the Antarctic nematode *Panagrolaimus davidi*. This technique enables the osmotic concentration to the measured with an accuracy of ±12 mmol kg^{-1}. The pattern of melting in the nematode's different body compartments suggests that it is the osmolality of its pseudocoelomic fluid that is being measured. *Panagrolaimus davidi* maintains its internal osmotic concentration above that of the external medium and is thus an hyperosmotic regulator. The nematode achieves regulation under hyposmotic stress more rapidly than under hyperosmotic stress.

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Key words: nanolitre osmometer, internal osmotic concentration, nematode, osmoregulation, melting point.

INTRODUCTION

Many free-living nematodes live in soil, the osmotic concentration of which varies according to its degree of hydration. *Panagrolaimus davidi* Timm 1971 is an Antarctic nematode associated with ornithogenic soils (Porazinska et al., 2002) in coastal areas that are ice-free during spring and summer but which receive sufficient meltwater from adjacent snowbanks to support the growth of algae and/or moss. The water content of these sites varies from saturated to completely dry (Wharton, 1998) and the nematode would thus be expected to face changes in external osmotic concentration (Wharton, 2003), as do other free-living nematodes (Wright, 1998).

Most free-living nematodes are small (<1 mm long) and extracting fluid for analysis is difficult. Their body cavity (the pseudocoel) may account for only a small proportion of their total volume, perhaps 2% (Wright and Newall, 1976). Measurements of internal osmotic concentration have thus been restricted to large parasitic species (Davey, 1995) and there have been no such measurements on a free-living nematode. Studies on osmoregulation in small nematodes have relied on indirect methods, such as measuring changes in volume or length and water flux (Clarke et al., 1978; Forster, 1998; Wright and Newall, 1976).

The accurate measurement of length and diameter (d) is difficult in a nematode, which may be moving rapidly in some test solutions. Any errors in diameter are particularly serious (because volume $\propto d^2$), and the nematode may have to be restricted in a small volume of water to ensure that it is in focus under a microscope (Wright and Newall, 1980). Water flux could result in a change in turgor pressure, rather than volume, due to the limitations imposed by the cuticle (Harris and Crofton, 1957). Uneven collapse under hyperosmotic stress in different parts of the body may render the calculation of volume from length and d inaccurate (Wharton, 1986).

Water flux can be measured by comparing wet and dry masses or by using tritiated water. However, this requires large numbers of nematodes. Interference microscopy allows the water content of individual nematodes to be determined and is perhaps the best current technique for investigating the osmoregulation of small nematodes (Forster, 1998; Perry et al., 1980; Wright and Newall, 1980).

A nanolitre osmometer allows the melting point, and hence osmotic concentration, of small amounts of fluids to be measured. It has been used to measure fluid from animal parasitic nematodes as small as 3.75 mm long (Fusé et al., 1993; Kirk et al., 2002). Nematodes are transparent and, if frozen on a microscope cold stage and their melting point determined with sufficient accuracy, their internal osmotic concentration could be measured. In this paper I describe the modification of the sample holder of a nanolitre osmometer to act as a microscope cold stage and its use in measuring the internal osmotic concentration of *P. davidi* after exposure to osmotic stress.

MATERIALS AND METHODS

A balanced salt solution (BSS) was prepared to reflect the osmotic environment experienced by the nematode in its natural soil environment. This was based on that used by Piggott et al. (Piggott et al., 2000) and consisted of the following salt concentrations: NaCl, 7.14 mmol l^{-1}; KCl, 0.86 mmol l^{-1}; $MgCl_2$, 1.61 mmol l^{-1}; $Ca(NO_3)_2$, 3.47 mmol l^{-1}; $CaSO_4$, 5.15 mmol l^{-1}; $MgSO_4$, 1.73 mmol l^{-1}. To culture nematodes a balanced salt solution agar (BSSA) was prepared, consisting of 1% agar, 0.1% nutrient broth, made up in BSS. After autoclaving and pouring agar into standard Petri dishes, plates were sealed in plastic bags (to prevent evaporation), stored upside down at 4°C and used within one month of preparation. Nematodes for experiments were cultured by washing a stock plate with BSS, transferring 100 µl to a fresh BSSA plate, spreading the liquid with a sterile spreader and incubating at 20°C. These experimental plates were used within one week of preparation. These techniques are similar to those used by Lamitina et al. (Lamitina et al., 2004).

Plates containing test media of different osmotic concentrations were prepared to expose nematodes to osmotic stress. These consisted of 1% agar (no nutrient broth) made up in the following solutions: hyposmotic, distilled water; isosmotic, BSS; hyperosmotic, BSS containing 0.2 mol l^{-1} NaCl; and BSS containing

0.1 mol l^{-1}, 0.3 mol l^{-1} or 0.4 mol l^{-1} NaCl. Media were autoclaved and poured into small (50 mm diameter) Peri dishes and stored as before. The osmotic pressure of the liquid on the surface of these agar plates was measured by gently pressing a sample disc onto the surface of the plate and waiting until it was saturated with liquid. The sample disc was then transferred to the sample chamber of a previously calibrated vapour pressure osmometer (Wescor Vapro 5520, Logan, UT, USA) and its osmolality measured. The osmolalities of media and solutions used in this study are given in Table 1.

A mounted eyelash was used to transfer individual 4th-stage larvae (L4s) of *P. davidi* from BSSA to the surface of the test media plate, which was then incubated at 20°C for various periods of time before the internal osmotic concentration of the nematode was measured, as described below. To test the effect of the test media on the activity and survival of *P. davidi*, 20 L4s or adults were transferred to the surface of the agar plates and incubated at 20°C for 24 h. The proportion of nematodes showing spontaneous activity was then counted. The nematodes were then washed off the surface of the plates with BSS, transferred to a watchglass, incubated for a further 1 h at 20°C and the proportion showing spontaneous activity counted.

The use of a nanolitre osmometer to measure internal osmotic concentrations of nematodes

The sample holder of a nanolitre osmometer (Otago Osmometers: www.otago-osmometers.com) was modified to hold 6 mm-diameter glass coverslips. Short lengths of capillary tube were made by drawing out the end of a Pasteur pipette in a bunsen flame and breaking into the required lengths. These were filled with either Milli-Q water (Millipore, Milli-Q Water Purifying System, Billerica, MA, USA) (0 mmol kg^{-1}) or a 1000 mmol kg^{-1} standard (Wescor, Logan, UT, USA) and one of each transferred to a glass coverslip in a drop of mineral oil (Cargille's A, Cedar Grove, NJ, USA). Nematodes were then transferred quickly using a mounted eyelash from the surface of the test medium to the oil on the coverslip, care being taken not to transfer too much liquid adhering to the surface of the nematode. The coverslip was transferred to the sample holder of the osmometer head, a second coverslip was placed on top of the sample and more oil added to fill the space between the two coverslips. The glass cover of the osmometer head was replaced, the head mounted on the stage of a Zeiss Axiophot photomicroscope (Carl Zeiss Inc., Thornwood, NY, USA) and observed using a ×10 objective lens. Usually several nematodes were visible in the field of view. The osmometer head was cooled by water at 5°C from a refrigerated circulator (Colora Messtechnik, GmbH, Lorch, Germany) and provided with a dry air supply to prevent condensation.

The nanolitre osmometer was set to cool rapidly, until the nematodes and standards froze. The temperature was then increased rapidly to within 1°C of the melting point of the 1000 mmol kg^{-1} standard and then slowly until the last ice crystal in the standard melted. The melting point of the 1000 mmol kg^{-1} standard was noted. The temperature was then increased further until signs of melting appeared in the nematodes. Photographs were taken at 1 min intervals using a Canon Powershot A640 digital camera (Canon USA, Lake Success, NY, USA), controlled by AxioVision v. 4.6 software (Zeiss) run on an Insite PC (Auckland, New Zealand). The magnitude of the temperature increase between photographs was decreased as the melting point of the nematode was approached, with the aim of measuring the melting point with an accuracy of ±0.01°C. When all the nematodes had melted the melting point of the 0 mmol kg^{-1} standard (Milli-Q water) was determined.

The photographs were assembled in PowerPoint (Microsoft Corporation, Redmond, WA, USA) as a decreasing temperature sequence and the temperature at which each nematode melted determined. The observed melting points of the 1000 mmol kg^{-1} and 0 mmol kg^{-1} standards were plotted against their actual melting points (1000 mmol kg^{-1} standard=–1.86°C, 0 mmol kg^{-1} standard=0°C) and a regression line was calculated, the equation for which was then used to correct the observed nematode melting points to their actual melting points. Osmolality was calculated using the relationship that 1 mmol kg^{-1} produces a freezing point depression of 0.00186°C.

To test the accuracy of this technique, capillary tubes containing 290 mmol kg^{-1} standard (Wescor) were added to a sample with no nematodes. The melting point was measured as for the nematode samples and compared with the actual melting point and osmolality of the standard (–0.54°C, 290 mmol kg^{-1}).

Nematode osmoregulation experiments

Nematodes (L4s) were transferred individually, using a mounted eyelash, from BSSA to the surface of hyposmotic, isosmotic or hyperosmotic plates and incubated at 20°C for 10 min, 30 min, 2 h, 4 h or 24 h. They were then transferred to the sample holder of the nanolitre osmometer and their internal osmotic concentration determined, as described. Nematodes were also transferred to the surface of agar plates containing BSS and 0.1 mol l^{-1}, 0.3 mol l^{-1} or 0.4 mol l^{-1} NaCl, incubated for 24 h at 20°C and their internal osmotic concentration determined using the nanolitre osmometer.

Whether internal osmotic concentrations were significantly different at various times after exposure to hyposmotic, isosmotic or hyperosmotic stress was tested using a one-way analysis of variance (ANOVA) with either the Scheffe *post-hoc* test for the comparison of means or Tamhane's T2 (if Levene's test indicated that variances were not equal) on the statistics package SPSS (SPSS Inc., Chicago, IL, USA). The relationship between internal and

Table 1. Osmolality of media and solutions used in this study, and the effect of test media on the activity of *Panagrolaimus davidi*

Material	Osmolality (mmol kg^{-1})	Spontaneous activity (%) after 24 h	Activity (%) after transfer to BSS
BSS	42±1*		
BSSA	100±4		
1% agar, dH_2O (hyposmotic)	55±4	100±0*	100±0*
1% agar, BSS (isosmotic)	93±2	100±0	100±0
1% agar, BSS, 0.1 mol l^{-1} NaCl	275±2	100±0	100±0
1% agar, BSS, 0.2 mol l^{-1} NaCl (hyperosmotic)	478±2	100±0	100±0
1% agar, BSS, 0.3 mol l^{-1} NaCl	658±7	100±0	100±0
1% agar, BSS, 0.4 mol l^{-1}NaCl	845±4	6.7±4.4	98.3±1.7

*Means ± standard error (*N*=3). BSS – balanced salt solution; BSSA – balanced salt solution agar.

Table 2. Tests of the accuracy of melting point and osmolality measurement of a 290 mmol kg^{-1} standard

Run	Measured melting point (°C)	Actual melting point (°C)	Calculated melting point (°C)*	Error (°C)	Osmolality error (mmol kg^{-1})
1	–2.14	–0.54	–0.56	0.02	11
2	–1.29	–0.54	–0.54	0	0
3	–1.36	–0.54	–0.59	0.05	27
4	–1.22	–0.54	–0.52	0.02	11
Mean				0.02	12

*From the melting points of the 0 mmol kg^{-1} and 1000 mmol kg^{-1} standards.

external osmotic concentration was examined by linear regression using SPSS.

Patterns in ice crystal location and melting

To see whether there were any patterns in the location of ice crystals and the sequence of their melting, the series of photographs was examined to determine the part of the nematode where the melting of the last ice crystal occurred. This may indicate structures associated with the elimination of excess water, because the excretory system empties in the anterior region of the nematode (Wharton and Ferns, 1995) and the intestine in the posterior.

The body was divided longitudinally into three approximately equal regions: head, middle, and tail. To acquire sufficient data for analysis the different time intervals of exposure to hyperosmotic, hyposmotic or isosmotic conditions were aggregated. A Chi-squared test was used to determine whether there was a significant difference between the observed distribution and that expected if melting occurred at random in different body regions, using the statistics programme R (R Development Core Team; http://www.R-project.org).

For closer observations of melting patterns and to determine which compartment of the body melted last (and hence the osmolality of which compartment was measured in the preceding experiments), a ×40 objective lens was used with a plastic sleeve for the lens and a cover for the osmometer head, which allowed the lens to focus on the specimen without condensation occurring. Differential Interference Contrast (DIC) optics were used. The thickness of the osmometer head and the lack of a long-working-distance condensor did not permit the correct alignment of the microscope but nevertheless the use of DIC optics enhanced the observation of ice in the nematodes.

Nematodes were transferred from BSSA to a drop of BSS, Milli-Q water or BSS containing 0.2 mol l^{-1} NaCl on a 6 mm coverslip. They were exposed to the solution for 10 min at 20°C, transferred to the osmometer head and a second coverslip added. The osmometer head was mounted on the stage of the Axiophot photomicroscope and the sample rapidly frozen. The temperature was increased to about –10°C and then more slowly at 1 min intervals, reaching a rate of 0.1°C min^{-1}, within 2°C of the melting temperature of the nematode. Photographs of the intestinal region of a nematode were taken at 1 min intervals, assembled in PowerPoint as before and the order in which different body compartments melted determined (intracellular in the muscle and intestinal cells, extracellular in the intestinal lumen and the pseudocoel).

RESULTS

The test of the accuracy of the nanolitre osmometer method for determining the internal osmotic concentration of nematodes indicated that melting points could be determined to an accuracy of ±0.02°C and hence osmolality to an accuracy of ±12 mmol kg^{-1} (Table 2).

The melting of ice crystals inside the body of nematodes could be clearly seen (see supplementary material Movie 1) and the melting point determined using the modified nanolitre osmometer. Changes in internal osmotic concentration of *P. davidi* measured using this technique after exposure to isosmotic, hyposmotic or hyperosmotic conditions for various periods of time are shown in Fig. 1.

There was a small decrease in internal osmotic concentration during the first 10 min of exposure to hyposmotic conditions; followed, after 30 min, by a restoration of osmotic concentration to that measured before exposure (Fig. 1). The effect of time on osmolality under hyposmotic conditions was significant (F=12.96, P<0.05) with the osmolality after 10 min being significantly lower than that at the other time intervals (Tamhane's T2: P<0.05), apart from at 4 h (P>0.05).

Under hyperosmotic conditions internal osmotic concentration increased to a peak after 2 h exposure, followed by a decline (Fig. 1). After 24 h exposure to hyperosmotic conditions internal osmotic concentration was still elevated, compared with that measured before exposure. The effect of time on osmolality under hyperosmotic conditions was significant (F=104.5, P<0.05); with the osmolalities at different time intervals being significantly different (Scheffe: P<0.05) apart from at 10 min, 30 min and 24 h (P>0.05). Under isosmotic conditions internal osmotic concentration varied within a narrow range (Fig. 1: 215–324 mmol kg^{-1}), the effect of time on osmolality was significant (F=2.747, P<0.05) but the only significant

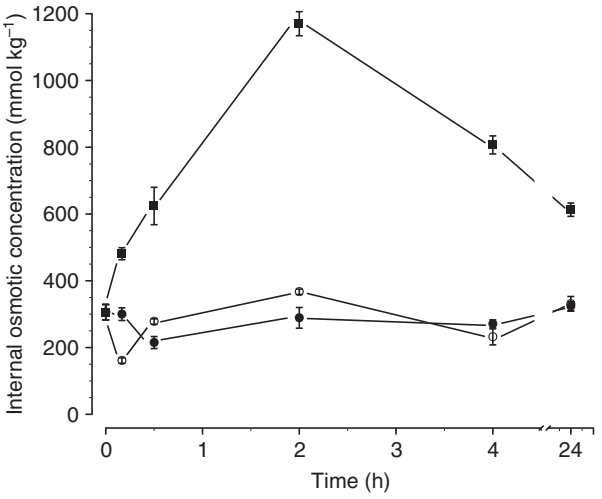

Fig. 1. Changes in internal osmotic concentration with time in *Panagrolaimus davidi* during exposure to isosmotic (closed circles), hyposmotic (open circles) and hyperosmotic (closed squares) conditions on the surface of agar plates. Vertical lines are standard errors (N=5–13).

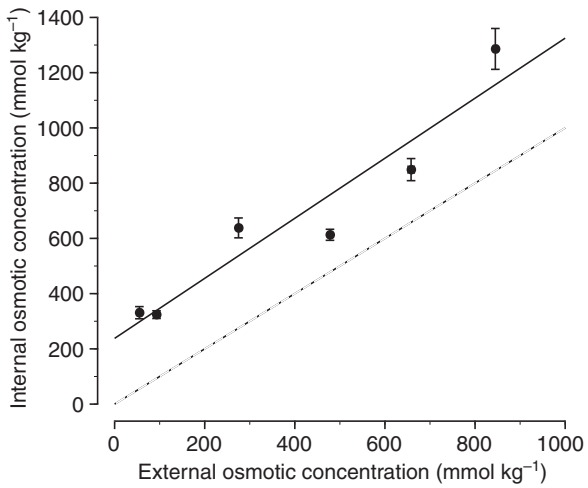

Fig. 2. Changes in internal osmotic concentration with external osmotic concentration in *Panagrolaimus davidi* exposed for 24 h on the surface of agar plates containing different concentrations of NaCl. The dotted line is if the internal osmotic concentration equalled that of the external medium. Vertical lines are standard errors (*N*=5–10). The solid line is the calculated regression line.

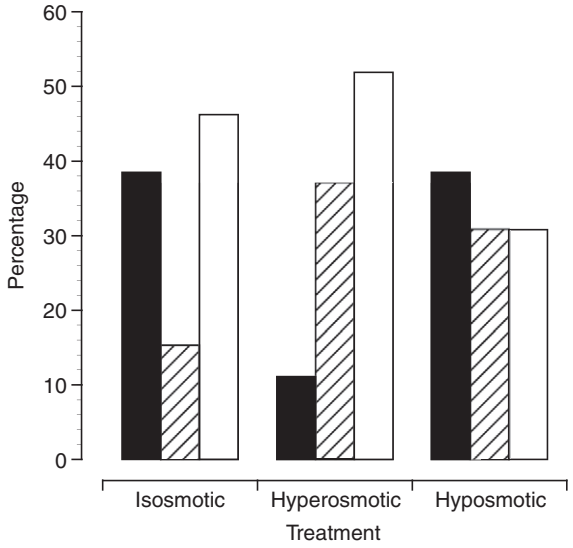

Fig. 3. The location of the melting of the last ice crystal, with respect to the head (closed bars), middle (hatched bars) or tail (open bars) of *Panagrolaimus davidi* under isosmotic, hyperosmotic or hyposmotic conditions. *N*=13–27.

difference in a *post-hoc* test was between 30 min and 24 h (Tamhane's T2: $P<0.05$).

The nematodes maintained their internal osmotic concentration above that of the external osmotic concentration across a range of osmolalities when exposed on the surface of agars containing 0–0.4 mol l^{-1} NaCl for 24 h (Fig. 2). Linear regression provides a good fit to the relationship between internal and external osmotic concentration (R^2=0.798, $F_{1,44}$=173.4). Nematodes were spontaneously active on the surface of agar plates, with the exception of those plates containing 0.4 mol l^{-1} NaCl where the nematodes were largely inactive and had a shrunken appearance. These nematodes recovered activity after washing with BSS (Table 1).

There were no significant differences between the location where the last ice crystal melted with respect to the head, tail or middle of the nematodes under isosmotic or hyposmotic conditions (Fig. 3, Chi-squared test, $P>0.05$). Under hyperosmotic conditions the melting of the last ice crystal occurred significantly more frequently in the tail of the nematode ($P<0.05$). Melting occurred in intracellular compartments first, with the pseudocoel melting last (Table 3, Fig. 4) (see also Movie 2 in supplementary material).

DISCUSSION

A modified nanolitre osmometer allowed the melting point, and hence the internal osmotic concentration of a small free-living nematode, *P. davidi*, to be measured. Since the pseudocoel is the last compartment to melt, it is likely to be the osmotic concentration of the pseudocoelomic fluid that is being measured.

The nematode maintains its internal osmotic concentration hyperosmotic to the external medium across a range of external osmotic concentrations. It may also be considered to be an osmoconformer, in the sense that its internal osmotic concentration increases as the external osmotic concentration increases (Willmer et al., 2005). It thus behaves in a similar fashion to other free-living nematodes, and the free-living stages of parasitic nematodes, which also appear to be hyperosmotic regulators (Clarke et al., 1978; Forster, 1998; Lamitina et al., 2004; Stephenson, 1942; Wharton et al., 1983; Wright and Newall, 1976; Wright and Newall, 1980).

Panagrolaimus davidi fails to regulate its volume in single salt solutions, including 0.1–0.4 mol l^{-1} NaCl (Viglierchio, 1974). However, single salt solutions may give an inaccurate picture of a nematode's osmoregulatory abilities. *Enoplis brevis* and *Enoplis*

Table 3. Melting sequence of body compartments of *Panagrolaimus davidi*

Treatment	Run #	MC	IC	IL	PS
BSSA → BSS	1	1	2	3	4
	2	1	2	3	4
	3	1	2	2	3
BSSA → Milli-Q	1	1	2	3	4
	2	1	n.o.	2	3
	3	1	2	n.o.	3
BSSA → 0.2 mol l^{-1} NaCl in BSS	1	1	2	3	3
	2	1	1	3	4
	3	1	2	3	3

MC – muscle cells (intracellular), IC – intestinal cells (intracellular), IL – intestinal lumen, PS – pseudocoel, n.o. – not observed, BSS – balanced salt solution, BSSA – balanced salt solution agar.

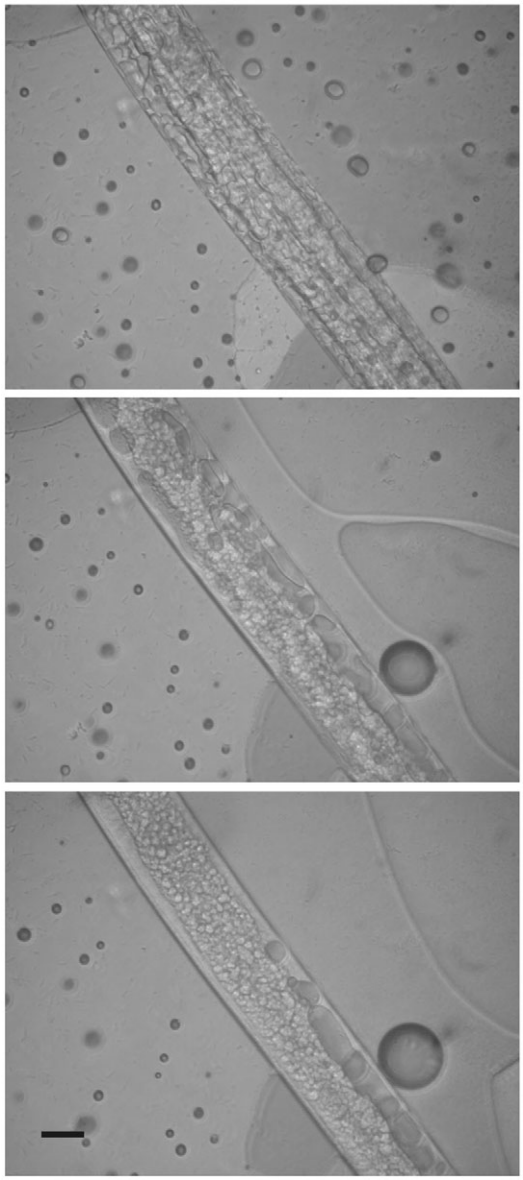

Fig. 4. Melting sequence (top to bottom) of *Panagrolaimus davidi* after transfer from BSSA (balanced salt solution agar) to BSS (balanced salt solution) for 10 min. Scale bar=20 μm.

communis cannot volume regulate in 0.55 mol l^{-1} NaCl but do regulate their volume when Ca^{++} and K^{+} are present (Wright and Newall, 1976; Wright and Newall, 1980). The entomopathogenic nematode, *Heterorhabditis* sp. regulates its length in balanced salt solutions but shows only partial regulation in single salt solutions (Piggott et al., 2002).

Hyperosmotic regulation in nematodes may be related to the need to maintain a positive internal pressure that, in conjunction with the limitations on expansion imposed by the cuticle, provides the antagonistic system for the longitudinal muscles; because nematodes lack circular muscles (Harris and Crofton, 1957). If the nematode was hyposmotic to its surroundings, water would be lost and the nematodes become inactive. Infective larvae of *Trichostrongylus colubriformis* lose water and become inactive in media of high osmotic concentration (Wharton et al., 1983), as do *P. davidi* (Wharton and To, 1996). However, inactivity could result from the increased ionic stress involved, rather than a loss of internal pressure. In *Caenorhabditis elegans* the mechanical properties of the cuticle appear to be more important in maintaining body stiffness than the internal hydrostatic pressure (Park et al., 2007).

There have been rather few measurements of internal osmotic concentration in nematodes and such measurements in previous studies have been restricted to larger parasitic species that are of sufficient size for samples of pseudocoelomic fluid to be extracted (Table 4). The internal osmotic concentration of *P. davidi* is within the range of that measured in other species (237–471 mmol kg^{-1}). The range of measurements is rather narrow, considering the wide range of external osmotic concentrations used in these studies, suggesting that nematode internal osmotic concentrations are tightly controlled.

Osmoregulation by *P. davidi* under hyposmotic stress (Milli-Q water) is achieved much more rapidly than under hyperosmotic stress. Various nematode structures have been suggested to be involved in removing the excess water that enters during hyposmotic stress (Wright, 2004; Wright and Newall, 1976). The tubular type of secretory–excretory system found in secernentean nematodes seems to be involved in this function, since the rate of pulsation, or filling and emptying (Wharton and Sommerville, 1984), of the excretory ampulla is related to the degree of hyposmotic stress (Atkinson and Onwuliri, 1981; Croll et al., 1972; Nelson and Riddle, 1984; Waddell, 1968; Weinstein, 1952). In some species the filling and emptying of the intestine may be involved (Stephenson, 1942). There were, however, no differences in the melting patterns of different body compartments of *P. davidi* under hyposmotic, isosmotic and hyperosmotic conditions that might indicate which structures were involved in water removal during hyposmotic stress.

Table 4. Internal osmotic concentrations in nematodes

Species	External medium	External osmotic concentration (mmol kg^{-1})	Internal osmotic concentration (mmol kg^{-1})	Reference
*Ascaris suum**	Pig intestinal fluid	468	355	Hobson et al., 1952
Aspiculuris tetraptera	Mouse intestinal fluid	Not determined	371	Anya, 1966
Pseudoterranova decipiens	40% ASW†	400	471	Fusé et al., 1993
	1% IO†	296	237	Stormo et al., 2009
Anguillicola crassus	Seawater‡	1000	311	Kirk et al., 2002
Panagrolaimus davidi	BSSA	93	324	This study
Angusticaecum sp.	Tap water	1	389	Pannikar and Sproston, 1941

*The pig species of *Ascaris* is now known to be *A. suum* and not *A. lumbricoides* (Anderson, 2000).
†ASW, artificial seawater, nematodes exposed for various periods; IO – Instant Ocean, 3 days.
‡Two weeks in natural seawater, plasma from uninfected eels maintained in similar fashion had an osmolality of 330 mmol kg^{-1}.

Under hyperosmotic stress (478 mmol kg^{-1}) the nematode's internal osmotic concentration increased to over 1170 mmol kg^{-1} after 2 h, before declining to 613 mmol kg^{-1} after 24 h. These changes are consistent with the model of osmoregulation under hyperosmotic stress proposed for *C. elegans* and a general model of the response of animal cells (Strange, 2007). Water is rapidly lost under hyperosmotic stress and shrinkage occurs. $Na^+/K^+/2Cl^-$ cotransporters or Na^+/H^+ and Cl^-/HCO_3^- exchange mechanisms are activated, resulting in regulatory cell volume increase by salt uptake. The high ionic concentrations that result can damage cells so the inorganic ions are replaced by compatible organic osmolytes, such as glycerol. *Caenorhabditis elegans* shows an increase in glycerol concentration under hyperosmotic stress (Lamitina et al., 2004). Whether this also occurs in *P. davidi* is not known but this nematode does synthesise trehalose and glycerol at low temperatures (Wharton et al., 2000) and these could be acting as compatible osmolytes (Yancey, 2005). The infective larvae of *Steinernema carpocapsae* synthesise both glycerol and trehalose under hyperosmotic conditions (Qiu and Bedding, 2002).

Panagrolaimus davidi has the ability to osmoregulate under both hyposmotic and hyperosmotic stress, enabling its survival in a stressful and variable Antarctic terrestrial environment. It maintains its internal osmotic concentration hyperosmotic to its surroundings; thus, facilitating its locomotion in range of osmotic conditions.

LIST OF SYMBOLS AND ABBREVIATIONS

BSS	balanced salt solution
BSSA	balanced salt solution agar
d	diameter
DIC	Differential Interference Contrast
L4	4th-stage larva

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