WATER LOSS AND MORPHOLOGICAL CHANGES DURING DESICCATION OF THE ANHYDROBIOTIC NEMATODE *DITYLENCHUS DIPSACI*

DAVID A. WHARTON

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

Summary

Ditylenchus dipsaci **is an anhydrobiotic nematode which can withstand direct exposure to extreme desiccation. Water loss has been determined gravimetrically and morphological changes quantified by light microscopy. Water loss occurs in two distinct phases, with a permeability slump 2 min after the onset of desiccation. The permeability slump remains after treatment with sodium azide or carbon dioxide but disappears after heat treatment. There is a marked decrease in body length during the first 2 min of desiccation but diameter decreases throughout the desiccation period, mainly as a result of a** decrease in the thickness of the hyaline layer. These observations suggest that one mechanism by which the nematode controls water loss during desiccation is by narrowing the groove between annulations. They also provide measurements on living nematodes which can be compared with more detailed observations using electron microscope techniques.

Key words: *Ditylenchus dipsaci*, nematode, anhydrobiosis, desiccation, morphology.

Introduction

Some nematodes can survive the loss of all their body water and enter a state of anhydrobiosis in which their metabolism comes reversibly to a standstill (Barrett, 1991; Crowe *et al.* 1992). The study of anhydrobiosis has a long history (Keilin, 1959), but it is only recently that we have begun to understand some of the mechanisms involved. A slow rate of water loss is thought to be particularly important for anhydrobiotic survival (Evans and Perry, 1976; Higa and Womersley, 1993). Sugars, particularly trehalose and sucrose, are synthesised by some organisms in response to desiccation stress and these protect membranes by preventing membrane fusion and phase transitions during desiccation and rehydration (Crowe *et al.* 1992). However, some nematodes are unable to survive severe desiccation stress even if trehalose synthesis has been triggered by mild desiccation (Higa and Womersley, 1993; Womersley, 1990). This suggests that the adaptations required for anhydrobiotic survival are more complex than the synthesis of a single protective compound.

The desiccation stress and the rate of water loss that nematodes can tolerate are related to the conditions to which they are exposed in their natural habitat (Womersley, 1987). They can be divided into slow-dehydration and fast-dehydration strategists, although some nematodes seem to fall into an intermediate group (Allan and Wharton, 1990). *Ditylenchus dipsaci* is a fast-dehydration strategist and can tolerate direct exposure to 0 % relative humidity (Perry, 1977a). This nematode may itself possess adaptations that

ensure a slow rate of water loss in a highly desiccating environment, whereas a slow-dehydration strategist relies on the surrounding soil or other habitat losing water slowly. Coiling and clump formation are also adaptations that will reduce the rate of water loss but individual nematodes could decrease water loss by reducing their cuticular permeability (Evans and Perry, 1976).

Water loss during desiccation of *D. dipsaci* has been determined using interference microscopy (Perry, 1977*b*). However, this technique cannot monitor water content continuously during desiccation and measurements were only taken at 5 min intervals, which may be too infrequent to indicate changes in the rate of water loss. Studies on other nematode species have also used interference microscopy to determine water contents or rates of water loss (e.g. Ibrahim and Perry, 1993; Pickup and Rothery, 1991; Ellenby, 1968*a*). Hendriksen (1982) determined water loss in a *Plectus* sp. by measuring the loss of tritiated water after various periods of desiccation. Although this technique was used to determine water contents at intervals of a few seconds, water loss from samples was not monitored continuously and the amount of variability in the data was too great to indicate any changes in the rate of water loss during desiccation. Gravimetric determinations would allow the water content to be monitored continuously during desiccation. The only such measurements of water loss from nematodes isolated from their substratum are for *Aphelenchus avenae* (Crowe and Madin, 1975; Higa and Womersley, 1993). This

nematode is a slow-dehydration strategist and will only survive if dried in large clumps at high relative humidity, the rate of water loss being dependent upon the size of the clump and the relative humidity. Measurements were made too infrequently in these studies to indicate any changes in the rate of water loss during the initial stages of desiccation. The drying characteristics of clumps will be different from those of a sample where all the nematodes are exposed directly to desiccation.

A slow rate of water loss may allow the synthesis of protective compounds, such as trehalose, and the orderly packing of body components, thus preventing structural disruption (Evans and Perry, 1976). *D. dipsaci* (Wharton and Barrett, 1985), *Anguina tritici* (Bird and Buttrose, 1974) and *A. avenae* (Crowe *et al.* 1978) have been shown to maintain their structural integrity following desiccation. Structural changes have been examined using scanning or transmission electron microscopy. These techniques involve chemical and physical processing which may have caused changes in dimensions. Crowe *et al.* (1978) used scanning electron microscopy to determine changes in body length and diameter and the frequency of cuticular folds during desiccation. Preparation of specimens for scanning electron microscopy using freeze drying or critical-point drying can cause changes in dimensions (Beckett *et al.* 1984), and different fixation and preparative techniques are known to affect the dimensions of nematodes (Stynes and Bird, 1980). Light microscope observations on living specimens are free of artefact and can be used to confirm ultrastructural studies. There have been no previous studies to quantify changes in anhydrobiotic nematodes at the light microscope level during desiccation, although changes during rehydration have been described (Wharton *et al.* 1985).

In this paper, gravimetric measurements are used to determine the pattern of water loss from *D. dipsaci* during the initial stages of desiccation and morphological changes at the light microscope level during desiccation are quantified.

Materials and methods

Ditylenchus dipsaci (Kuhn) Filipjev was harvested from a natural infection in garlic, which was stored dry at room temperature. The nematodes were separated from the garlic using the Baermann funnel technique (Hooper, 1986*a*), washed three times in tap water and washed and stored in an artificial tap water (ATW: Greenaway, 1970) at 4 °C for at least 48 h. They were allowed to warm to room temperature and the viability was checked before use (by counting the number moving: 100 % active).

Humidity chambers consisted of airtight, 700 ml plastic boxes with specimens supported on a wire mesh platform. An 81.77 % (w/w) solution of glycerol in distilled water was used to maintain a relative humidity (RH) of 50 % at 20 °C (Grover and Nicol, 1940).

Water loss determinations

The water loss from samples was measured using a Sartorius M3P microbalance, which had been allowed to equilibrate at room temperature and relative humidity (measured using a digital hygrometer/thermometer, Radio Spares Components Ltd, stock no. 212-124) for 2 h before use. Nematodes were transferred to preweighed aluminium foil discs and the surface water was removed using a fine pipette and filter paper spills. The nematodes were spread out during this process to prevent clumping. The sample was immediately transferred to the weighing pan of the balance and the mass recorded at 30 s intervals for 10 or 30 min. It was then dried at 60 °C for 24 h and reweighed to determine the dry mass. Mass loss was also monitored from nematodes that had been heat-treated at 65 °C for 1 h and allowed to cool to room temperature before the removal of surface water and in nematodes which had been anaesthetised by bubbling carbon dioxide through a suspension for 10 min or treated by immersion in 0.01 mol l^{-1} sodium azide for 15 min (Hooper, 1986*b*).

Samples were also transferred to a humidity chamber (50 % RH, 20 °C) and allowed to desiccate for various periods. They were then weighed, dried at 80 °C for 1 h, reweighed and the water content after desiccation calculated. To determine the water content of fully hydrated nematodes, samples were weighed immediately after the removal of surface water. A logarithmic curve was fitted to the data for 5–30 min of desiccation and used to calculate water contents for subsequent experiments.

Changes in dimensions during desiccation

To determine length changes during desiccation, nematodes were transferred to glass slides in ATW. A hydrated control sample was photographed using a microflash under a Zeiss Axiophot Photomicroscope. A stage micrometer was also photographed at the same magnification for calibration. The nematode images were projected onto paper using a photographic enlarger, and the length of each nematode was drawn by tracing along its centre with a pencil. The image of the calibration specimen was traced at the same enlargement. Nematode lengths were determined using Sigma-Scan (Jandel Scientific) with a digitising tablet and a personal computer which enables the measurement of curvilinear lengths by tracing over the pencil image of the nematode with the graphics tablet cursor after calibrating the system.

The lengths of nematodes desiccated for 2, 5, 10, 20 or 30 min were determined as above after covering the specimens with liquid paraffin to prevent further desiccation before photographing.

Nematode larval stages may be distinguished by looking for discontinuities in length/frequency curves (Wilson, 1976). The range of lengths of fourth-stage larvae (L4s) was determined using this method and the mean L4 length for each desiccation period calculated.

To determine length and diameter changes in individual nematodes, a single L4 was transferred to a microscope slide in ATW. The specimen was photographed as before, the surface water removed and the specimen transferred to a humidity chamber. The specimen was photographed after

desiccation for 2, 5, 10, 20 and 30 min, being rapidly returned to the humidity chamber after each photograph. Length was measured as above, diameter was measured directly from the negative using an X7 Peak Scale Lupe (Agar Aids). The volume was calculated using the formula for a cylinder. The results were expressed as the percentage change from the dimensions of the hydrated nematode, to allow for the different initial lengths of individual specimens.

Changes in the hyaline layer and intestine during desiccation

L4s were transferred to a microscope slide in ATW or were desiccated for 2, 5, 10, 20 and 30 min at 50 % RH, 20 °C, and covered with liquid paraffin and a coverslip. The nematodes were photographed under a ×100 oil-immersion objective using a microflash and differential interference contrast (DIC) optics on a Zeiss Axiophot photomicroscope. Measurements were made using an X7 Peak Scale Lupe of the total diameter, the diameter of the intestine and the thickness of the hyaline layer (the layer between the cuticle and the intestine on both sides of the nematode). Twenty individual L4s were measured for each sample.

Changes in annulation spacing during desiccation

The surface water was removed from L4s as before and they were either transferred immediately to liquid paraffin or desiccated for 2, 5, 10, 20 or 30 min at 50 % RH, 20 °C, and covered with liquid paraffin and a coverslip. The cuticular annulations were photographed using a microflash, a ×100 objective lens and DIC optics. The annulation spacing was determined by measuring the distance between 10 annulations on a print using dividers.

Results
Water loss determinations

The water loss from a nematode sample is shown in Fig. 1. A similar pattern was observed in two other samples (data not shown). In each case, the drying curve was divided into two phases: an initial rapid water loss, followed by slower constant rate of loss. The change in the rate of water loss occurred at
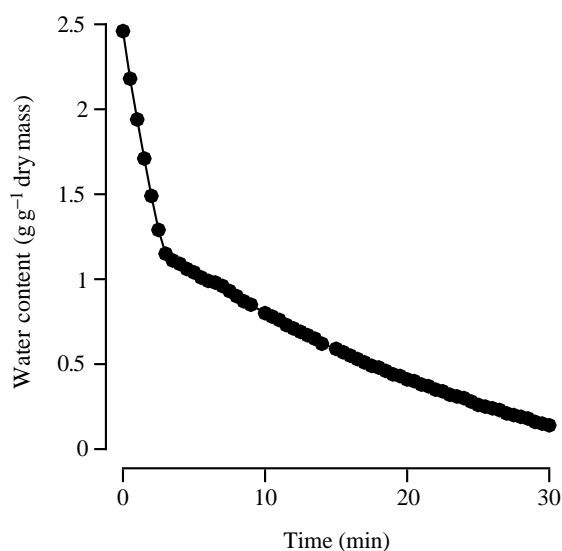

Fig. 1. Water loss from a sample of fourth-stage larvae (L4s) of *Ditylenchus dipsaci* monitored continuously on a microbalance.

the following times and water contents: sample 1, 3 min, 53.5 %; sample 2, 2 min, 56.7 %; sample 3, 2.5 min, 48.0 %. The water losses from a heat-treated sample, from an untreated control sample and from samples of nematodes anaesthetised with carbon dioxide or $0.01 \, \text{mol} \, l^{-1}$ sodium azide are compared in Fig. 2. Similar patterns of water loss were observed in two other samples for each treatment (not shown). Heat-treated samples lost water rapidly and no discontinuity in the drying curve was observed. The drying curves of untreated controls and of nematodes treated with carbon dioxide or $0.01 \, \text{mol} \, l^{-1}$ sodium azide were similar. Discontinuities were observed with all three treatments but were less evident in the sodium-azide-treated samples. These treatments had an effect on the appearance of the nematodes. After exposure to heat or carbon dioxide, the nematodes were straight and inactive and after treatment with sodium azide they were tightly coiled. Unanaesthetised nematodes have a variety of postures, often retaining the waves of their sinusoidal locomotion. The water

Table 1. *Water contents of* Ditylenchus dipsaci *after desiccation at 50 % relative humidity, 20 °C*

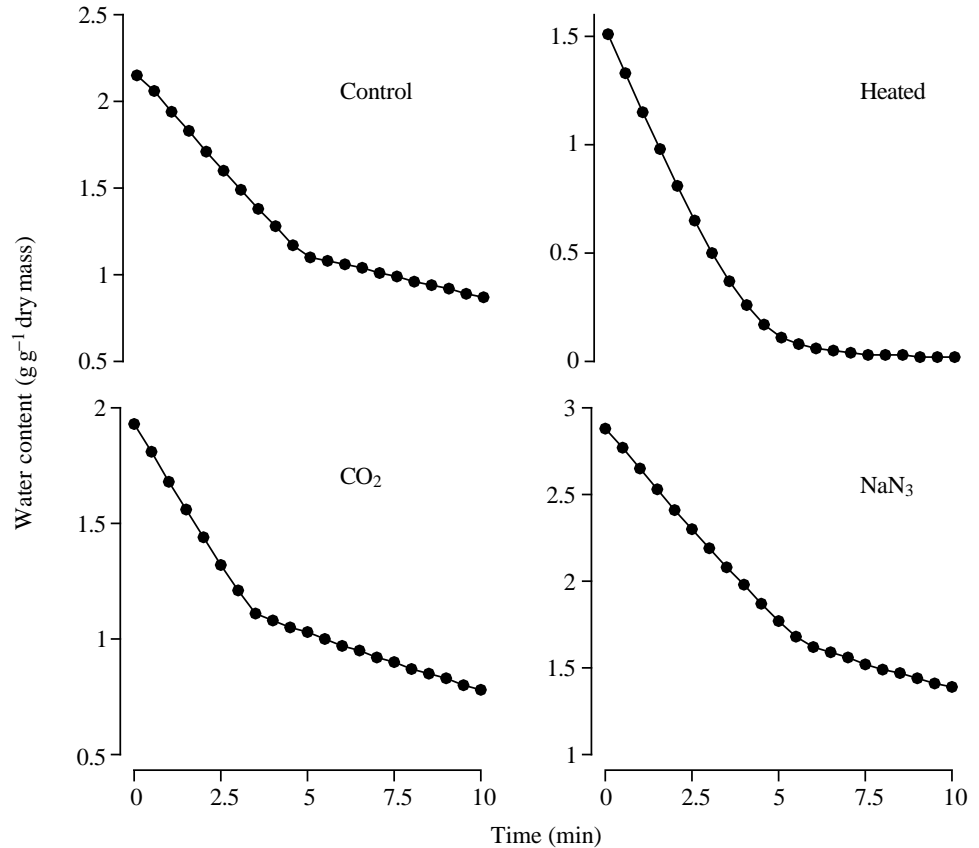| Time (min)	Water content (g water g^{-1} dry mass)	Observed water content (%)	Calculated water content, 5–30 min (%)	Calculated water content, all data (%)	Water content value used (%)
0.001	4.8	78.3	207.5	158.6	78.3
2	0.88	45.4	60.9	50.3	45.4
5	0.79	43.4	43.3	37.3	43.3
10	0.42	28.8	29.9	27.4	29.9
20	0.28	18.6	16.5	17.5	16.5
30	0.23	7.7	8.7	11.8	8.7
r^2			0.805	0.755	
χ^2 (all data)			84.8	43.7	0.423
χ^2 (5–30) min			0.423	2.563	0.423

Fig. 2. Water loss from a sample of *D. dipsaci* L4s monitored continuously on a microbalance for untreated controls, heat-treated samples and nematodes treated with carbon dioxide or sodium azide.

contents of samples immediately after transfer to the microbalance and after drying for a further 10 min are compared in Fig. 3. Heat-treated samples lost water fastest whilst samples treated with sodium azide lost water slowest. The effect of treatment on water content was significant, both immediately after transfer to the microbalance (analysis of
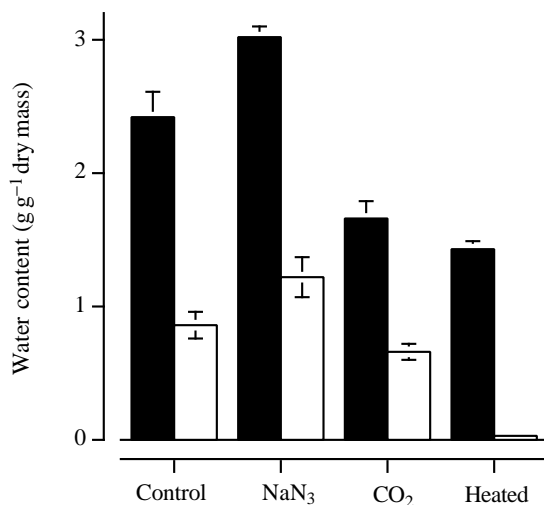

Fig. 3. Mean water contents of controls, heat-treated samples and nematodes treated with carbon dioxide or sodium azide immediately after transfer to the microbalance (filled columns) and after 10 min of further desiccation (open columns). $N=7$ (controls) or $N=3$ (other treatments); vertical lines represent the standard error of the mean.

variance: $F=12.1$, $P=0.0006$) and after 10 min of desiccation ($F=14.9$ $P=0.0002$). After 10 min of desiccation, the water content of heat-treated samples was significantly lower than those of the other treatments, whilst sodium-azide-treated samples had a significantly higher water content than CO_2-treated samples or controls (least-significant difference, $P<0.05$). Room temperatures and humidities during these experiments were 17–20 °C and 55–63 % RH.

Water loss at 50 % RH, 20 °C, is shown in Fig. 4. The drying curve was again divided into two phases. The initial rate of water loss was rapid, followed by a slower rate of water loss after 2 min of desiccation. Overall, the data could be modelled by a logarithmic curve. Using the data for all time intervals gave the better fit to the data for 0 and 2 min but overestimated water contents for the longer time intervals (Table 1). Restricting the curve fit to the data for 5–30 min gave a better fit for these time intervals. The water content has therefore been taken to be the observed values for 0 and 2 min and for 5–30 min the calculated values from the log curve fitted to these data.

Changes in dimensions during desiccation

Length/frequency distributions indicate that the majority of nematodes in the sample are L4s (for the control sample: 76.7 % L4s, 13.8 % L3s, 9.5 % adults). There is a marked shift in the length/frequency distribution between hydrated control nematodes and those after 2 min of desiccation (Fig. 5). The decrease in length is significant ($t=8.107$, $P<0.0001$). Less
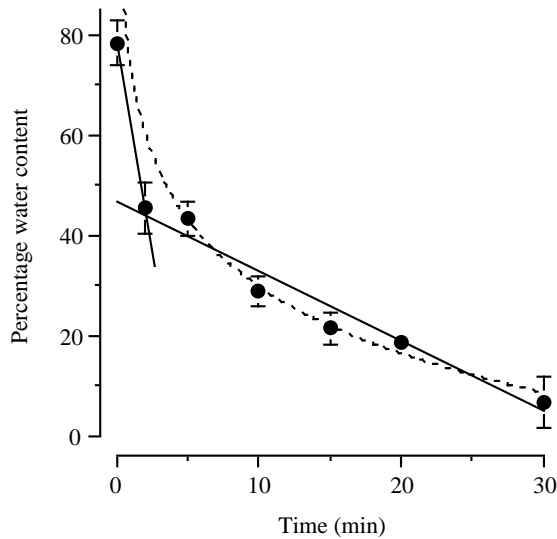
Fig. 4. The rate of water loss from *D. dipsaci* L4s upon exposure to 50 % RH, 20 °C. The straight lines are the linear regression lines for the data from 0 to 2 min (r^2=0.79, P<0.02) and from 2 to 30 min (r^2=0.78, P<0.001). The dashed curve is the least-squares fit to the equation $y=-44.4\log x+74.3$ for data from 5 to 30 min (r^2=0.805, P<0.001). The vertical lines represent the standard error of the mean (N=4).

and is responsible for most of the decrease in volume. Diameter continues to decrease throughout the desiccation period. The decreases in dimensions during desiccation are significant (factorial ANOVA after arcsin transformation: length, F=21.38; diameter, F=14.71; volume, F=23.79; P<0.0001). The decrease in volume during water loss is not as great as would be predicted if water loss were to produce a corresponding decrease in volume (Fig. 8; χ^2>100, P<0.0001).

Changes in the hyaline layer and intestine during desiccation

The diameter of the intestine and the nematode and the thickness of the hyaline layer decrease during desiccation (Fig. 9). During the initial stages of desiccation, changes in the intestine result in a decrease in the diameter of the nematodes, with the hyaline layer not decreasing in thickness. After 2 min of desiccation, however, changes in the hyaline layer are responsible for most of the decrease in diameter and there is little change in the diameter of the intestine. Body diameter, intestinal diameter and hyaline layer thickness all show significant decreases during desiccation (ANOVA: F=22.2, 9.84 and 67.9, respectively; P<0.0001).

Changes in annulation spacing during desiccation

Annulation spacing decreases during desiccation (Fig. 10). The decrease in annulation spacing with time is significant (F=8.68, P<0.0001).

Discussion

Water loss from *D. dipsaci* occurs in two phases, with a marked decrease in the rate of water loss after 2–5 min of desiccation. This does not appear to be due to the loss of any free water remaining around the nematodes since the water content determined gravimetrically at the start of the desiccation period agrees well with that determined by other

pronounced changes in length/frequency distributions were observed at longer desiccation periods. The mean length of L4s decreases as they lose water, as does the mean length of the total nematode sample (Fig. 6). Most of the decrease in length occurs in the initial stages of desiccation.

The length, diameter and volume of individual L4s decrease as they lose water (Fig. 7). Most of the decrease in length occurs during the first 2 min of desiccation. Diameter decreases at a faster rate than length, after the first 2 min of desiccation,
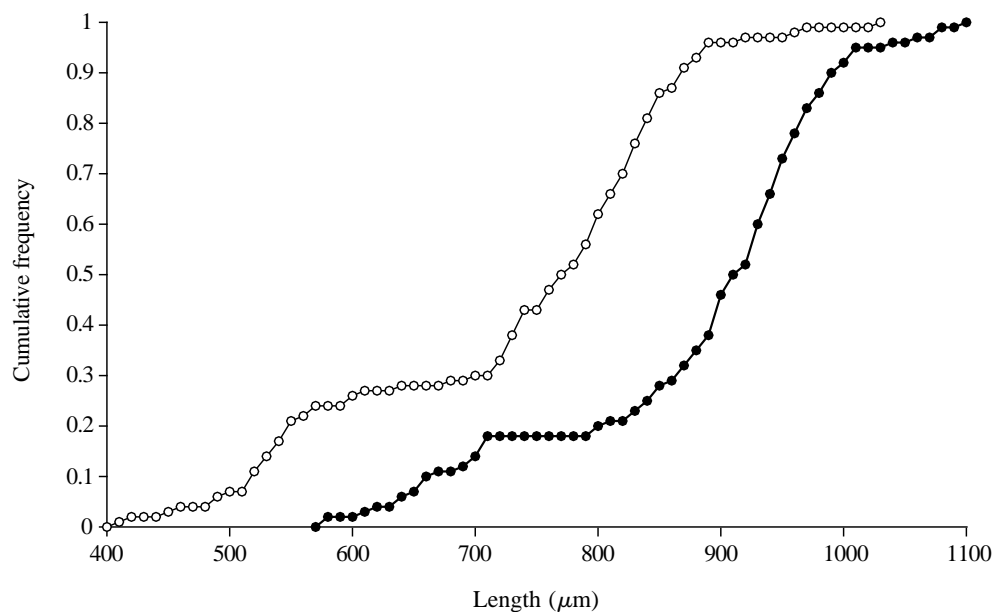

Fig. 5. Length/frequency plots of *D. dipsaci* for hydrated controls (●) and after desiccation for 2 min (○). Discontinuities are taken to represent the size limits of different instars. *N*=114 (control) and *N*=90 (2 min).
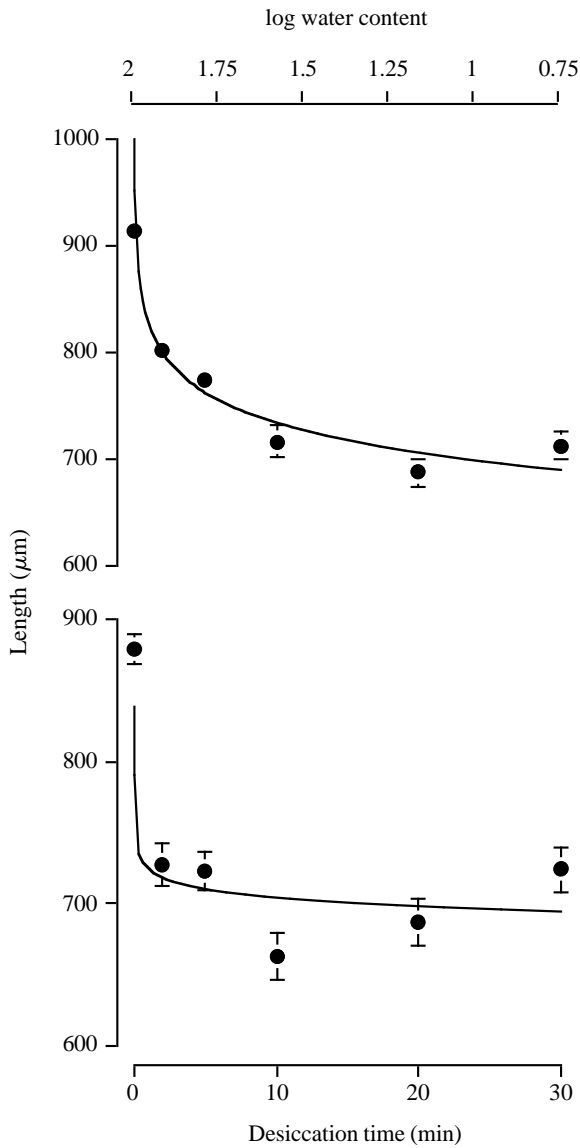
Fig. 6. Changes in mean length during desiccation of L4s (top) and all stages in the sample (bottom). The vertical lines represent the standard error of the mean. N=62–114 (all stages), N=48–84 (L4s).

techniques (Perry, 1977b; Wharton et al. 1988). Interference microscopy measurements of water contents during desiccation also indicate that the rate of water loss is much greater between 0 and 5 min than between 5 and 10 min (Perry, 1977b). A similar decrease in permeability during desiccation has been described in anhydrobiotic tardigrades (Crowe, 1972; Wright, 1989a) and is called the 'permeability slump' (Wright, 1989a). This appears to be a property of the lipid-rich intracuticle which may undergo hydration-dependent changes in permeability (Wright, 1989b).

The water loss from heat-treated nematodes is much more rapid and shows no discontinuities in the water loss curve. The cuticular permeability barrier of *D. dipsaci* is heat-labile and the cuticle undergoes an irreversible increase in permeability after heating (Wharton et al. 1988). In the absence of this
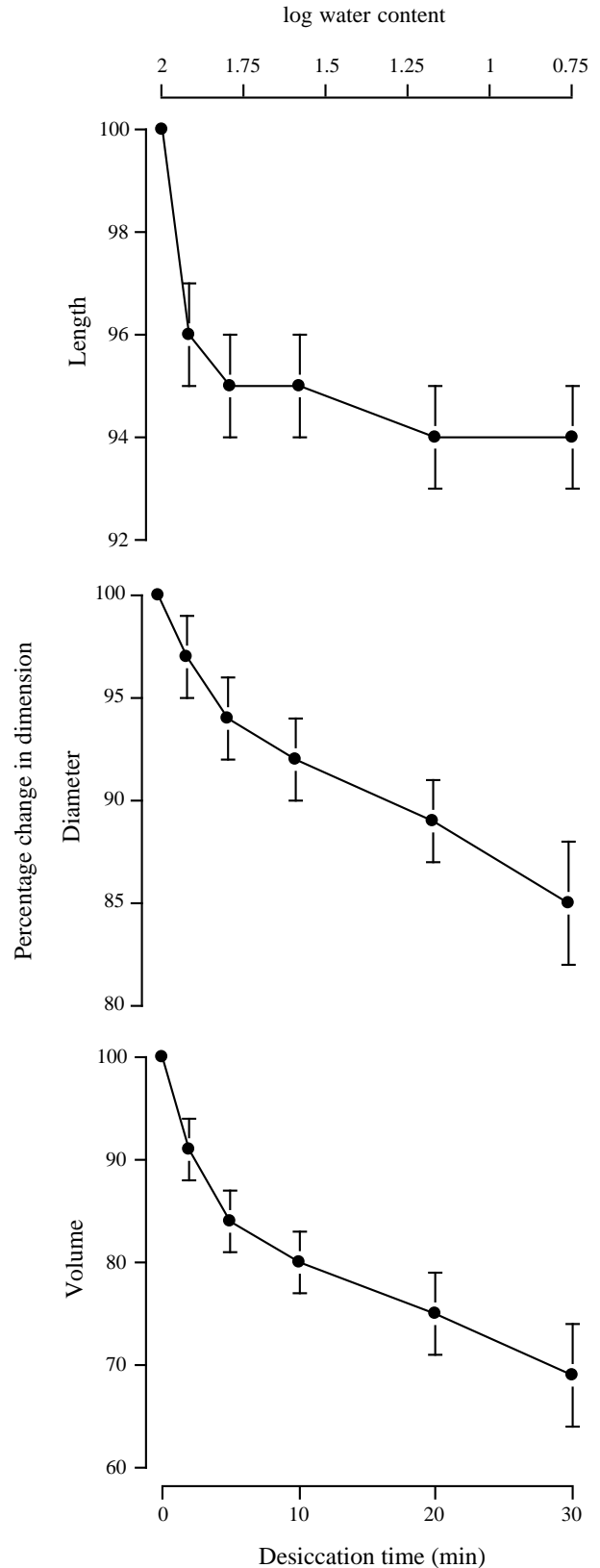

Fig. 7. Changes in length (top), diameter (middle) and volume (bottom) of individual *D. dipsaci* L4s during desiccation, expressed as percentage changes from the value for the hydrated nematode in ATW. The vertical lines represent the standard error of the mean (N=9).
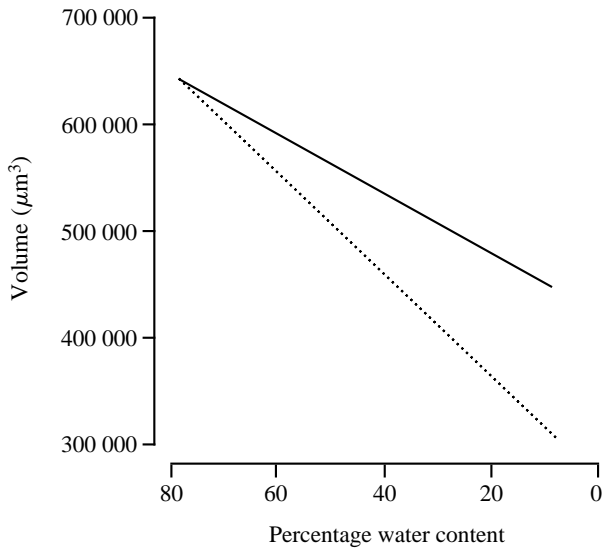
Fig. 8. The decrease in volume during water loss from individual *D. dipsaci* L4s (solid line) compared with that predicted if water loss were to produce a corresponding decrease in volume (dotted line).

barrier, water loss follows a smooth curve, as would be expected during the unimpeded physical loss of water from the sample. The permeability slump remains after the nematodes have been treated with sodium azide or carbon dioxide. This suggests that the permeability slump is due to a change in the physical properties of the cuticle as it dries, rather than to a mechanism dependent upon muscular contraction. However, the treatments used may have had effects other than simply inhibiting muscular contraction. Sodium azide treatment resulted in the nematodes becoming tightly coiled, whereas they straightened after treatment with carbon dioxide. These different postures affect the rate of water loss and sodium-azide-treated samples lost water more slowly than controls. The specimens may also have recovered rapidly after the removal of the carbon-dioxide-saturated water surrounding them and subsequent exposure to air. The permeability slump observed during the desiccation of tardigrades is also observed in dead animals, suggesting that it is not dependent upon the changes in body shape which result in tun formation (Wright, 1989a).

The destruction of the permeability barrier of the cuticle by heat treatment and by brief exposure to diethyl ether suggests that the epicuticle is the main permeability barrier as it is superficial and contains lipid (Wharton *et al.* 1988). The cuticular permeability barrier is damaged by desiccation and is repaired during rehydration. This repair occurs over several hours and has a metabolic basis (Wharton *et al.* 1988). In contrast, the decrease in the rate of water loss during desiccation, which reflects a decrease in permeability, occurs rapidly and does not have a metabolic basis. This suggests that the permeability slump occurs by a mechanism other than a change in the composition of the epicuticle or muscular contraction.

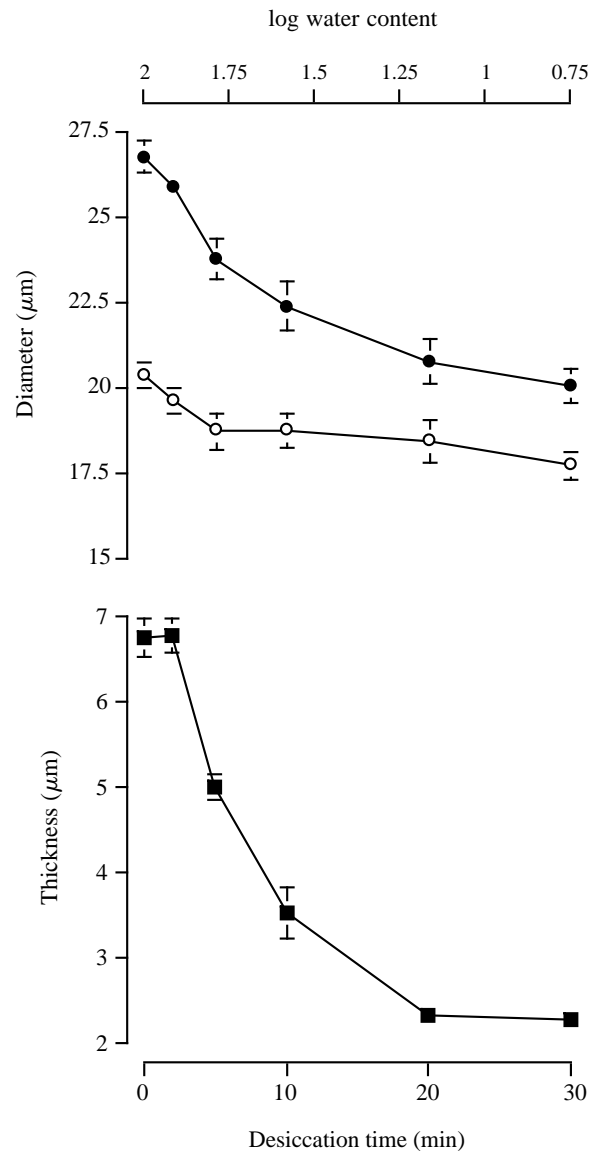Ellenby (1968b) and Perry (1977b) have suggested that the

Fig. 9. Changes in body diameter (●), diameter of the intestine (○) and the thickness of the hyaline layers (■) during desiccation. The vertical lines represent the standard error of the mean (*N*=20).

cuticle of *D. dipsaci* dries first, resulting in a decrease in permeability which slows the rate of drying of deeper tissues. It is difficult to visualise a mechanism for this if the epicuticle is the only permeability barrier. Water lost from the cuticle will be replaced by water from deeper layers and the cuticle will dry at the same rate as the rest of the body. The cuticle could only dry at a faster rate than the underlying tissues if there is a further permeability barrier between them. This could be achieved by decreasing the permeability of cuticular layers deeper than the epicuticle. There are significant changes in the thickness of both the cortical and basal zones of the cuticle during desiccation and rehydration (Wharton and Barrett, 1985).

Evidence for the cuticle drying at a faster rate than the deeper tissues comes from interference microscope
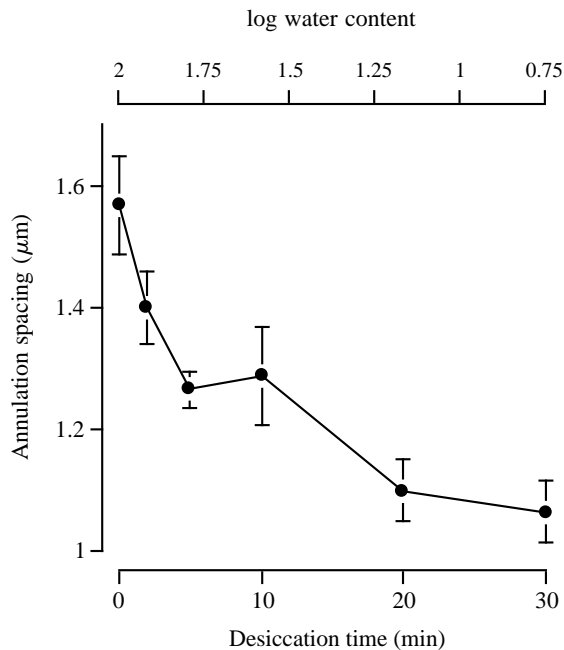
log water content

Fig. 10. Changes in cuticular annulation spacing during desiccation. The vertical lines represent the standard error of the mean (N=10).

observations which indicate that the periphery of the nematode has a higher refractive index, and hence lower water content, shown by a greater displacement of the interference fringes at the periphery (Ellenby, 1968*b*). Perry (1977*b*), however, could not observe these peripheral fringe displacements in specimens that had been desiccated at 0 % or 50 % RH for 5 and 10 min. Peripheral fringe displacements were only observed in specimens with lower water contents. The muscle cells and hypodermis form a distinct layer, the hyaline layer, which can be distinguished under the light microscope and which changes in thickness during desiccation and rehydration (Wharton *et al.* 1985; present results). Differences in the water contents of the hyaline layer and the intestinal cells may be responsible for the peripheral fringe displacements observed by interference microscopy. As the intestinal cells contain large amounts of lipid (Wharton and Barrett, 1985), it is possible that they lose water more slowly than the overlying tissues.

An alternative explanation for the decrease in the rate of water loss after 2 min of desiccation is that more permeable areas of the cuticle are removed from contact with the air. Anhydrobiotic tardigrades contract their bodies into a compact 'tun', reducing the surface area and removing more permeable areas of the cuticle from contact with air; this reduces their overall permeability (Crowe, 1972; Wright *et al.* 1992). The reduction in surface area appears to have the greater effect, however, and the invagination of more permeable areas of the cuticle may be of minor significance. Tun formation is also not responsible for the permeability slump observed in tardigrades (Wright, 1989*a*). Changes in both length and diameter are responsible for volume, and hence surface area, changes in *D. dipsaci*. The greatest length change, however, occurs during the first 2 min of desiccation and coincides with the decrease

in the rate of water loss. The reduction in surface area is about 7 %, whereas the decrease in the rate of water loss is about 74 %. The decrease in water loss is thus much greater than can be explained by a decrease in surface area.

The observed reduction in length is reflected in a decrease in the spacing of the cuticular annulations. Cuticular annulation spacing decreases throughout the desiccation period, however, and not just during the first 2 min. A decrease in annulation spacing could be achieved by an increase in the depth of the annulations or by a narrowing of the groove between the annulations. In *Rotylenchus robustus*, there is a narrowing of the grooves in desiccated specimens (Rössner and Porstendörfer, 1973). It has been suggested that the exchange of substances through the cuticle occurs *via* these grooves (Rössner and Perry, 1975). If the groove between the annulations is a more permeable area of the cuticle, narrowing of the groove could reduce the overall permeability of the cuticle. In *D. dipsaci*, there is a decrease in annulation spacing in anhydrobiotic nematodes (Wharton and Barrett, 1985), although no increase in the depth of the annulation grooves was observed. Some of the monoclonal antibodies raised against the oat race of *D. dipsaci* labelled the annulation grooves, suggesting that the grooves contain a material that is different from that on the surface of the rest of the cuticle (Palmer *et al.* 1992).

After 2 min of desiccation, decreases in volume result mainly from decreases in the diameter of the nematode. Decreases in diameter occur throughout the desiccation period, initially as a result of a decrease in the diameter of the intestine and later as a result of decreases in the thickness of the hyaline layer. The hyaline layer increases in thickness during rehydration (Wharton *et al.* 1985), reflecting changes in the muscle cells and epidermis (Wharton and Barrett, 1985). Length and diameter changes produce a smaller decrease in volume than would be predicted from the water lost. The high concentration of lipid droplets within the intestinal cells of the nematode (Wharton and Barrett, 1985) may mean that there is less water to be lost from the intestine than from other tissues. Changes in the diameter of the intestine are complete after 5 min of desiccation. Water loss from the intestinal cells will be from the spaces between the lipid droplets and would not be reflected in overall changes in the dimensions of the nematode. Changes in the appearance of nematodes after desiccation and rehydration are shown in Wharton *et al.* (1985).

The decrease in length observed during the first 2 min of desiccation coincides with the permeability slump. This suggests that one mechanism by which the nematode controls water loss during desiccation is by narrowing the groove between annulations. This permeability slump also occurs in anaesthetised nematodes, suggesting that it is the result of a change in the physical properties of the cuticle rather than the result of muscular contraction. The light microscope measurements on living nematodes should now be compared with more detailed observations using electron microscope techniques.

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