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A surface lipid may control the permeability slump associated with entry into anhydrobiosis in the plant parasitic nematode *Ditylenchus dipsaci*

D. A. Wharton[1,*], L. Petrone[2], A. Duncan[3] and A. J. McQuillan[2]

Departments of Zoology[1], Chemistry[2] and Human Nutrition[3], University of Otago, PO Box 56, Dunedin, New Zealand

*Author for correspondence (e-mail: david.wharton@stonebow.otago.ac.nz)

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SUMMARY

The anhydrobiotic plant-parasitic nematode *Ditylenchus dipsaci* undergoes a decrease in permeability (the permeability slump) during the early stages of desiccation and this produces the slow rate of water loss necessary for its survival. There were no changes in annulation spacing, followed in individual nematodes by confocal microscopy, that would account for the permeability slump. Nile Red staining reveals that the surface of the nematode is coated with an extracuticular layer of lipid. This material can be seen in unstained desiccated nematodes where it forms an oil that adheres to the coverslip and to adjacent nematodes. The oily material leaves impressions on the coverslip (cuticle prints) after the nematode has detached upon rehydration. The presence of the surface lipid was confirmed using attenuated total reflection infrared spectroscopy. This material was shown to be a triglyceride and the proportion of fatty acids determined, using thin layer and gas chromatography. The production of the surface lipid material may be responsible for the permeability slump observed during the early phases of desiccation and its removal upon immersion in water may explain the paradox that cuticular permeability decreases during the permeability slump and yet desiccated nematodes are more permeable than are fully hydrated nematodes.

Key words: cuticle, desiccation, annulations, lipid, confocal microscopy, permeability, accessory layer, ATR–IR spectroscopy.

INTRODUCTION

Some nematodes can lose all their water and enter a state of anhydrobiosis, in which their metabolism comes reversibly to a standstill (Perry, 1999; Wharton, 2002a; Wharton, 2002b). A slow rate of water loss is essential for anhydrobiotic survival (Evans and Perry, 1976). Anhydrobiotic nematodes have been divided into two broad groups: slow-dehydration strategists, which rely on a slow rate of water loss from their environment, and fast-dehydration strategists, which control their own rate of water loss (Wharton, 2002b; Womersley, 1987). *Ditylenchus dipsaci* (Kühn) Filipjev is a plant-parasitic nematode that can survive direct exposure to 0% relative humidity (RH) and is thus a fast-rate strategist (Perry, 1977a; Wharton and Aalders, 1999).

There is a marked decrease in the rate of water loss from *D. dipsaci* during the early stages of desiccation: 'the permeability slump' (Wharton, 1996). This must involve a change in the permeability of the cuticle. Two mechanisms have been suggested for the permeability slump. If the cuticular annulations are more permeable areas of the cuticle, the permeability slump could be produced by a decrease in annulation spacing and a deepening of the annulation grooves (Rössner and Perry, 1975; Wharton, 1996; Wharton and Marshall, 2002). Alternatively there could be a physical change in the properties of the cuticle as it dries (Ellenby, 1968; Perry, 1977b).

Wharton and Marshall (Wharton and Marshall, 2002), using cold-stage field-emission scanning electron microscopy, were unable to demonstrate changes in annulation spacing that correlated with the permeability slump. However, these measurements were averages from different nematode samples frozen after differing periods of desiccation and this technique could not follow changes in individual specimens. Since both annulation spacing and the timing of the permeability slump may vary between individuals, any changes could have been obscured. Length changes during desiccation are not as clear if measurements from averages over several nematodes are compared with changes in individual nematodes (Wharton, 1996). We have used confocal microscopy to follow changes in the annulation spacing of individual nematodes during the early phases of desiccation. We have also used lipid and carbohydrate probes, confocal microscopy and other microscopical and analytical techniques to examine changes in the surface of the cuticle of *D. dipsaci* during desiccation that may be related to changes in cuticular permeability.

MATERIALS AND METHODS

Ditylenchus dipsaci Kühn (Filipjev) (oat race) was grown on carrot callus tissue at 20°C in the dark using the method described by Moody et al. (Moody et al., 1973). When nematodes appeared in numbers on the sides of the culture vessel they were harvested by macerating the carrot tissue in a blender, separated using a modified Baermann funnel technique (Hooper, 1986), washed into an artificial tap water (ATW) (Greenaway, 1970) and stored at 4°C. When sufficient nematodes had been accumulated they were transferred to a clay medium (Minsorb, OMYA NZ Ltd., Auckland, NZ), which was then dried at room temperature and stored at 4°C. When required for experiments nematodes were rehydrated in tapwater, separated from the Minsorb by allowing them to migrate through tissue paper, washed in ATW and stored at 20°C for at least a week before use. These samples comprise predominantly fourth-stage larvae (L4s) (Wharton, 1996).

Nematodes were exposed to 50% RH, 20°C for 30 min in a controlled humidity chamber, as described by Wharton and Aalders (Wharton and Aalders, 1999). This desiccation exposure is known to induce a lag phase, indicating entry into anhydrobiosis (Wharton and Aalders, 1999), and to produce a water content of about

0.23 g g^{-1} dry mass (Wharton, 1996). The samples were then immersed in ATW, the recovery counted at intervals and the time taken for 50% to recover calculated by probit analysis (Norusis, 1999). Survival was measured as the percentage recovery after 24 h in ATW.

Changes in annulation spacing observed by confocal microscopy and reflected laser light

Changes in cuticular annulation spacing were observed using a Zeiss LSM510 Axiovert 200 confocal laser scanning microscope operated in reflection mode using a 543 nm helium–neon laser. Nematodes were washed briefly in distilled water and a single nematode transferred to a humidity-controlled observation chamber, the relative humidity of which was controlled by an 81.77% (w/w) solution of glycerol in distilled water (Grover and Nicol, 1940), giving 50% RH. The temperature of the microscope room was controlled at 20°C. Most of the surface water was removed from the nematode and the observation chamber sealed with Vaseline. The nematode was observed until the remaining surface water evaporated and then images were taken at intervals during desiccation. The annulation spacing was determined by measuring the distance across ten annulations.

Staining with wheat germ agglutinin

Nematodes were stained with wheat germ (*Tritium vulgaris*) agglutinin (WGA: Sigma-Aldrich, Uckland, NZ), conjugated to fluorescein isothiocyanate (FITC), using 1 mg ml^{-1} in phosphate-buffered saline (PBS, pH 7.4) for 15 min and then washed three times in PBS. They were then observed using epifluorescence on a Zeiss Axiophot Photomicroscope with a 450–490 nm exciter filter and a 520 nm barrier filter or on the confocal microscope with argon ion laser excitation at a wavelength of 488 nm. Unstained controls were examined for autofluorescence under the Zeiss Axiophot Photomicroscope. WGA/FITC-stained specimens were also used, after washing six times in distilled water, to follow changes in annulation spacing during desiccation in a humidity-controlled observation chamber on the confocal microscope as described above.

Staining with Nile Red

Nematodes were stained with Nile Red [9-diethylamino-5H-benzo(α)phenoxazine-5-one; Sigma]. Nile Red is a benzophenoxazone dye that is intensely fluorescent in organic solvents, with its fluorescence maxima depending upon the relative hydrophobicity of its surrounding environment, but which is poorly soluble and is fully quenched in water. It can thus be used as a selective and sensitive stain for lipids (Fowler and Greenspan, 1985; Greenspan et al., 1985). 10 µl of a stock solution of Nile Red (10 mg ml^{-1} in ethanol) was added to 1 ml of PBS in an Eppendorf tube and vortexed to mix. This was added to nematodes on a coverslip. The nematodes were stained for 15 min at room temperature and then washed three times in PBS. The coverslip was inverted onto a microscope slide for observation. Samples were observed using epifluorescence on a Zeiss Axiophot Photomicroscope with a 546–558 nm exciter filter and a 590 nm barrier filter or with a 450–490 nm exciter filter and a 520 nm barrier filter. Samples were also observed using the confocal microscope with laser excitation at a wavelength of 543 nm (helium–neon laser). The staining of material left behind after the removal of nematodes by gentle ATW washing was also examined.

Other observations of surface material

Nematodes were allowed to dry from a small droplet of ATW on a coverslip at 50% RH, 20°C for 3 days. The coverslip was then inverted onto a microscope slide and the edges of the coverslip sealed with nail varnish. The contact areas between the nematodes and the coverslip were observed using a Zeiss Photomicroscope and differential interference contrast (DIC) optics. Similar observations were made on nematodes dissected from fresh carrot tissue, washed thoroughly in distilled water to ensure that they were free of any contaminating material (migrated through tissue paper twice and then washed three times in distilled water), allowed to dry at 0% RH (over silica gel), 50% RH or 98% RH (saturated solution of K_2SO_4) (Winston and Bates, 1960), at 20°C for 24 h on a clean coverslip and then mounted and observed as above.

Nematodes were also observed for the presence of surface material using confocal microscopy and reflected laser light at 543 nm (helium–neon laser) after desiccation in a controlled humidity chamber (50% RH, 20°C).

Attenuated total reflection infrared (ATR–IR) spectroscopy

Internal reflection spectroscopy is based on the existence of an evanescent wave in a medium of lower index of refraction (n) in contact with an optically denser medium in which a light is introduced (Harrick, 1967). The evanescent electric field decays exponentially in the rarer medium and a penetration depth (d_p) is defined as the decay of such an electric field by 63%.

In this work, a three-reflection diamond-coated ZnSe prism (ASI SensIR Technologies, Norwalk, CT, USA) of 3 mm diameter sampling area was used to collect the infrared spectra. For a diamond prism [index of refraction (n)=2.35] in contact with water (n=1.33) d_p is 1.35 µm at 1650 cm^{-1} (Vigano et al., 2005). Thus the sampling depth is of the order of a few micrometers. This configuration provides three contacts across the surface between the radiation and the sample (Fig. 1). The prism surface was cleaned prior to each experiment by polishing with 0.015 µm Al_2O_3 powder (BDH, polishing grade) on a wet polishing microcloth (Buehler, Lake Bluff, IL, USA) and then rinsed with deionised water (Millipore, Milli-Q, Billerica, MA, USA). Infrared spectra were obtained using a Digilab FTS 4000 infrared spectrometer equipped with a KBr beamsplitter and a Peltier-cooled DTGS (Deuterated Tri-Glycine Sulfate) detector. Dried air was used to purge the optical bench and contained a variable amount of CO_2 which is evident as a variable intensity band in the spectra at about 2350 cm^{-1}. All the infrared spectra show interferences in the region 1800–2300 cm^{-1} due to the diamond prism absorptions. Win-IR Pro version 3.4 software was used to analyze the spectra recorded from 64 co-added scans at 4 cm^{-1} resolution. The background spectrum was from the bare diamond prism. An air conditioning unit in the room in which the spectrometer was located produced a controlled temperature of 20°C and a measured relative humidity of 27–28% (±1%).

About 20 nematodes in distilled water were transferred to the prism surface and most of the water removed using a fine pipette. The sample was then allowed to desiccate in air and an infrared spectrum collected every 1 min for 15 min. The spectrum was initially dominated by the water signal but this declined as desiccation proceeded until the spectrum was stable (with-worm spectrum: after 15 min). Distilled water was added to the sample, the nematodes gently removed, the surface of the prism allowed to dry and a further spectrum collected (without-worm spectrum). The surface of the prism was then washed with diethyl ether and another spectrum collected (ether-extraction spectrum).

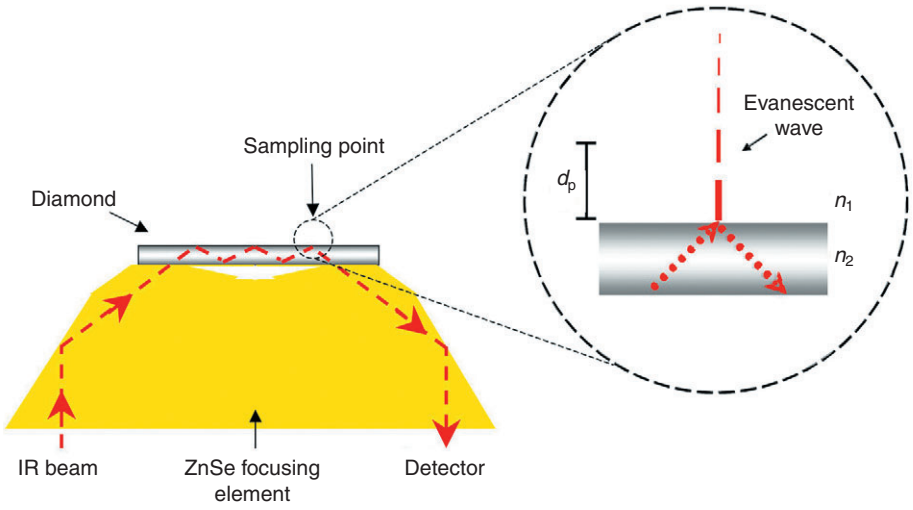

Fig. 1. Schematic diagram of the three-internal reflection diamond-coated ZnSe attenuated total reflection infrared (ATR–IR) prism (left). Penetration depth (d_p) and exponential decay of the evanescent wave in the medium with a lower refractive index (n_1<n_2) are shown in detail for one of the points of reflection (right).

To isolate the nematode surface lipid material, nematodes were washed thoroughly with distilled water and transferred to a watchglass. The surface water was removed and the nematodes desiccated at 0%, 50% or 98% RH at 20°C for 24 h. Distilled water was then added, the nematodes allowed to rehydrate for 10 min and then carefully removed using a fine pipette. The remaining water was then gently removed and the watchglasses dried at 0% RH, 20°C for 1 h. Diethyl ether (100 µl) or chloroform:methanol (2:1 v/v; 100 µl) was added and the samples transferred to gas chromatography (GC) vials and sealed. Samples (5 µl) were transferred to the surface of the ATR prism, the solvent allowed to evaporate, and the spectrum collected.

Thin layer chromatography and gas chromatography

Thin layer chromatography (TLC) separation was carried out in order to purify and to separate the nematode surface lipid material from any phospholipids coming from the worms' cuticle outer layer. Nematode surface lipid material was prepared as described above and taken up in 100 µl methanol. The sample was applied to a silica-gel-coated aluminium plate.

The developing solvent system and staining reagent for neutral lipids were *n*-hexane:diethyl ether:acetic acid (85:15:1, v/v/v) and ANS 0.1% (8,1-anilinonaphthalene sulphonic acid). Phosphatidylcholine (PC) and glycerol triacetate (TAG) were used as standards. The lipid to be examined (50 µl) and each standard (50 µl) were applied to the TLC plate, left for 40 min and dried at room temperature. The triglyceride region was scraped off and poured into a test tube for the subsequent methylation reaction.

Three millilitres of a 6% (v/v) sulphuric acid in methanol solution were added to the isolated lipid fraction as a methylating reagent. Fatty acids were methylated by acid-catalysed transesterification at 80°C for 2 h. After cooling to room temperature, 2 ml of hexane followed by 1 ml of water were added to the sample with 2 min of vortexing separating each addition. The upper hexane layer containing the fatty acid methyl esters (FAMEs) was removed, dried under N_2 flow, reconstituted in 70 µl of hexane and stored at –20°C until gas chromatographic analysis. Sulphuric acid was AnalaR grade (BDH Chemicals, Poole, England).

The hexane in which the FAMEs were dissolved was evaporated under a stream of nitrogen and the FAMEs reconstituted in 70 µl of hexane for analysis of fatty acid composition by GC. FAMEs were separated using a HP-225 capillary column, 30 m×0.25 mm i.d., 0.25 µm film (Agilent Technologies, Santa Clara, CA, USA) equipped with a flame ionization detector. The gas chromatographic system consisted of a 5890 GC equipped with an autosampler (HP7673) and Chem Station integration (all Hewlett Packard, Avondale, PA, USA). The column oven was held at a temperature of 180°C for 5 min, then programmed to increase at 1°C min^{-1} to 210°C and held for 5 min. The total runtime was 45 min.

Fatty acid peaks were identified by retention time matching with authentic standards. A composite standard was used, made from commercially available methyl esters (NuCheck Prep, Elysian, MN, USA and Sigma, St Louis, MO, USA).

RESULTS

Survival and recovery after exposure to 50% RH indicates that our samples enter anhydrobiosis and survive these conditions. The 50% recovery time after nematode exposure to desiccation at 50% RH, 20°C for 30 min was 3.48 h (95% c.i.: 3.30–3.64). Recovery after 24 h in ATW was 99.2±0.1% (mean ± 1 s.e.m., *N*=4).

Changes in annulation spacing

Cuticular annulations could be observed using reflected laser light but changes in annulation spacing were difficult to follow with this technique, since nematodes often moved during the desiccation series. There was clear labelling of the surface of the nematode after staining with WGA (Fig. 2). The amphids and the opening of the excretory duct were also stained. There was no autofluorescence associated with the cuticle in unstained controls, although droplets in the intestine did show autofluorescence. The WGA labelling observed by epifluorescence or confocal microscopy appeared to be concentrated at, or confined to, the annulations and enabled the major annulations (which extend to the lateral alae) and the minor annulations (which do not) to be distinguished (Fig. 2).

Measurements of changes in annulation spacing of individual nematodes during the early stages of desiccation were made using reflected laser light, and directly, on WGA-labelled specimens (Fig. 3). Only specimens where the same area of the cuticle could be clearly observed from the beginning to the end of the desiccation period were measured. Specimens measured by reflected laser light showed a decrease in annulation spacing of 9%, 3% and 14.7%. In WGA-labelled nematodes three specimens showed no change in annulation spacing during desiccation whilst two showed only small changes (1.7% and 4.5% decreases). In a time series taken by

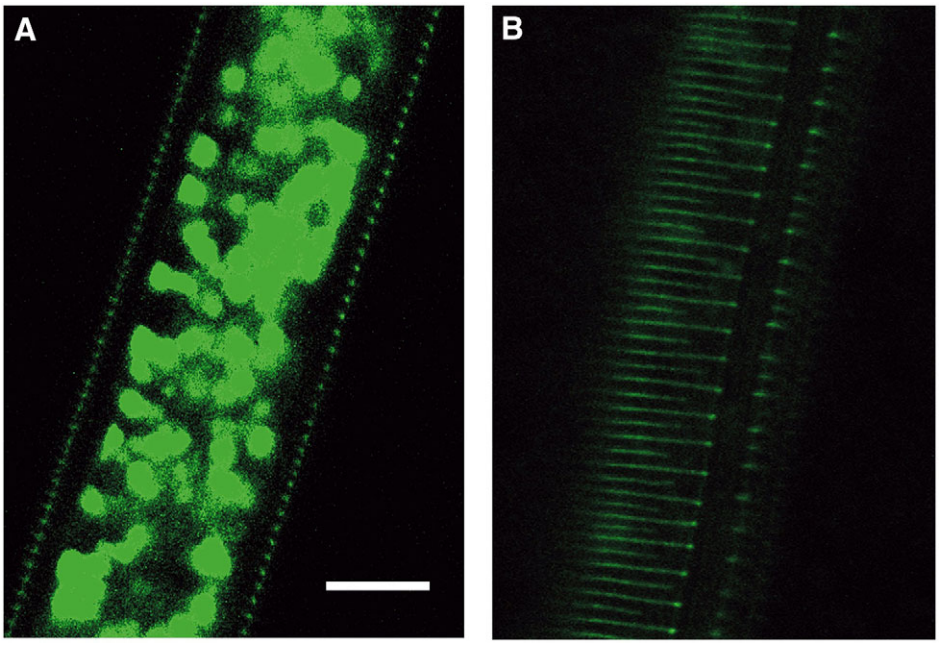

Fig. 2. Wheat germ agglutinin–fluorescein isothiocyanate (WGA–FITC)-stained *D. dipsaci* fourth-stage larvae (L4s) observed by confocal microscopy, focused on the centre (A) or surface (B) of the nematodes. Surface labelling of the cuticle can be observed and appears to be confined to the annulations. Scale bar, 10 μm.

confocal microscopy little change in the spacing or appearance of the annulations could be seen whereas the droplets in the intestine were observed to move during desiccation. To test whether annulation spacing changed with time, the percentage decrease in annulation spacing was calculated. There was no significant effect of time on the decrease in annulation spacing for either the WGA specimens (linear regression after arcsin transformation: r^2=0.01, $F_{1,70}$=0.681, P>0.05) or for those measured by reflected laser light (r^2=0.051, $F_{1,40}$=2.139, P>0.05).

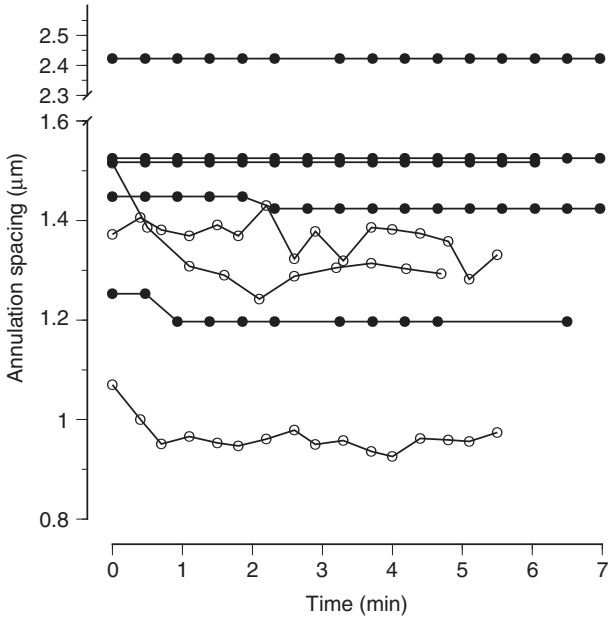

Fig. 3. Changes in annulation spacing of individual nematodes observed by confocal microscopy during desiccation of *D. dipsaci* fourth-stage larvae (L4s) at 50% RH, 20°C using reflected laser light (open symbols) or on Wheat germ agglutinin–fluorescein isothiocyanate (WGA–FITC)-stained specimens (closed symbols).

Lipid material on the nematode surface

There was no staining with Nile Red in PBS of fully hydrated nematodes and no autofluorescence observed in unstained specimens observed by epifluorescence microscopy. Desiccated nematodes, however, became permeable to the dye and showed extensive staining of internal structures. The staining of surface material was noted, with the nematodes appearing to be covered with droplets (Fig. 4). The staining of this material was variable, appearing in 0–69% of nematodes in a sample. No consistent relationship was found between the time of exposure to desiccation and the appearance of this material. However, the greatest proportion exhibiting these droplets was in samples desiccated at 50% RH, 20°C for 5 min on a coverslip, stained in Nile Red for 15 min and washed twice in PBS; with care being taken to ensure that changes of liquid were gentle. The droplets appeared to be associated with the cuticular annulations and the grooves of the lateral alae (Fig. 4).

In regions of the coverslip where nematodes had adhered during desiccation and then detached during the staining regime, clear impressions of the cuticle ('cuticle prints') (Bird, 1988) were observed. These also appeared to be made up of droplets but left clear impressions of the cuticular annulations and lateral alae (Fig. 5).

In unstained desiccated nematodes, surface oily material could be seen adhering to the coverslip and to adjacent nematodes using DIC optics and seen smeared from the surface using confocal microscopy (Fig. 6). Similar material was observed in nematodes freshly dissected from carrots before exposure to desiccation, and exposed to 0%, 50% or 98% RH.

Confirmation of the presence of surface lipid by attenuated total reflection infrared spectroscopy and its characterisation by thin layer chromatography and gas chromatography

For ATR–IR spectroscopy, nematodes were placed on the diamond prism and infrared spectra recorded at 1 min intervals until the water dried out and no further changes were observed [with-worm spectrum (Fig. 7A)]. The distinctive aliphatic absorptions found at 2956 cm^{-1} (CH_3 symmetric stretch), 2923 and 2852 cm^{-1} (asymmetric and symmetric CH_2 stretch), 1452 cm^{-1} (CH_2 bending), 1392 cm^{-1} (CH_2 scissoring) (Cieślik-Boczula et al.,

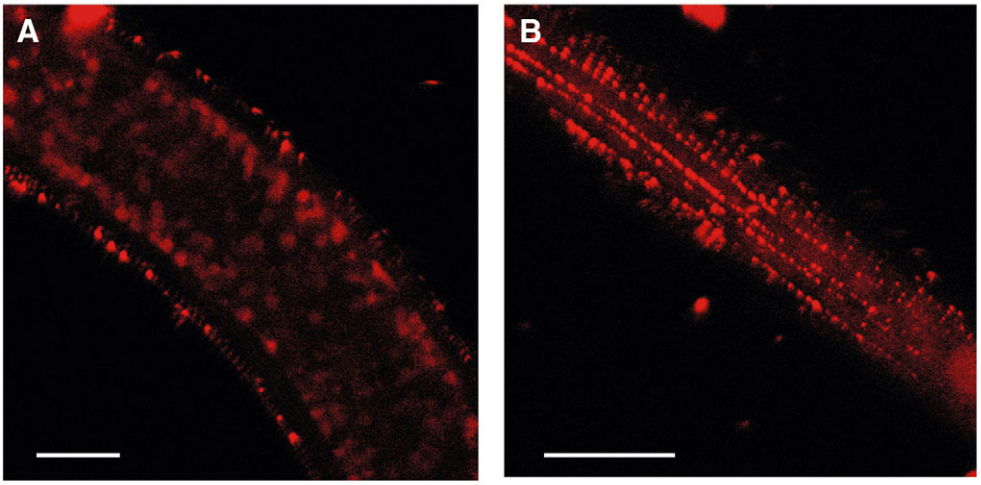

Fig. 4. *D. dipsaci* L4s desiccated at 50% RH, 20°C for 5 min, stained with Nile Red and observed by confocal microscopy focused on the centre (A) or surface (B) of the nematodes. Lipid droplets are associated with the surface of the cuticle, particularly with the cuticular annulations and the grooves of the lateral alae. Scale bars, 10 μm.

2008), along with the ester group peak at 1744 cm^{-1} (Nara et al., 2002), clearly show the presence of lipids. This lipid signal first appeared after 7 min desiccation. The with-worms spectrum (Fig. 7A) shows also the characteristic protein vibrational modes peaking at 3289, 1633 and 1546 cm^{-1}, which are assigned respectively to amide A (N–H stretch), amide I (C=O stretch) and amide II (N–H bend, C–N stretch) (Barth, 2007). The band centered at 1237 cm^{-1} arises from phosphodiester (PO_2^-) asymmetric stretch observed in phospholipids, and the corresponding PO_2^- symmetric stretch is found at 1079 cm^{-1} (Wang et al., 2006). In addition, the broad band in the 1200–1000 cm^{-1} region, peaking at 1035 cm^{-1}, is related to C–H, C–OH, C=O and C–O–C stretching/bending modes in polysaccharides (Kacurakova and Mathlouthi, 1996). Peaks in the regions 1900–2150 cm^{-1} and 2200–2400 cm^{-1} are variable and are due to absorptions from the diamond prism and the CO_2 gas, respectively.

Another infrared spectrum was recorded after removal of the nematodes from the diamond prism [without-worms spectrum (Fig. 7B)]. The characteristic peaks of lipids were still evident at 2955, 2923, 2853, 1460 and 1744 cm^{-1}, whereas the signals resulting from proteins (1636 and 1546 cm^{-1}) and polysaccharides (broad band peaking at 1022 cm^{-1}) appeared only as weak peaks.

The diamond surface was subsequently washed with diethyl ether and the spectrum collected (ether-extraction spectrum; Fig. 7C). The bands arising from proteins and polysaccharides were still visible at 1638, 1546 and 1021 cm^{-1}. Therefore, after the ether wash the lipid peaks disappeared but most of the protein and the polysaccharide peaks remained on the prism.

The infrared spectrum of the isolated lipid from the nematode surface exposed to 50% RH (Fig. 8A) had peaks at 2958, 2922, 2852, 1721, 1462 and 1377 cm^{-1}, all associated with lipid characteristic absorptions and there were no peaks associated with protein, polysaccharide or phospholipid. The overlapped infrared spectra of the lipid material from the nematode surface produced at different relative humidities showed that more lipid is produced at 0% and 50% RH than at 98% RH (Fig. 8B).

The nematode surface lipid material was analysed by GC after TLC separation. Little material was detected from the phospholipid region (polar lipids) of the TLC plate (<0.05% of the total), and most lipid was associated with the triglyceride (non-polar lipid) region of the plate. The relative composition in mass percentage of the chain lengths of the free fatty acid methylated esters that constitute the triglycerides of the nematode surface lipid are shown in Table 1. The profile is dominated by saturated fatty acids with the only unsaturated fatty acid present in any quantity (1.3% of the total) being C18:3*n*-3 (α-linolenic). The main chain lengths of saturated fatty acids detected, in order of abundance, were: C8 (caprylic), C16 (palmitic), C6 (caproic), C12 (lauric), C18 (stearic), C10 (capric) and C14 (myristic).

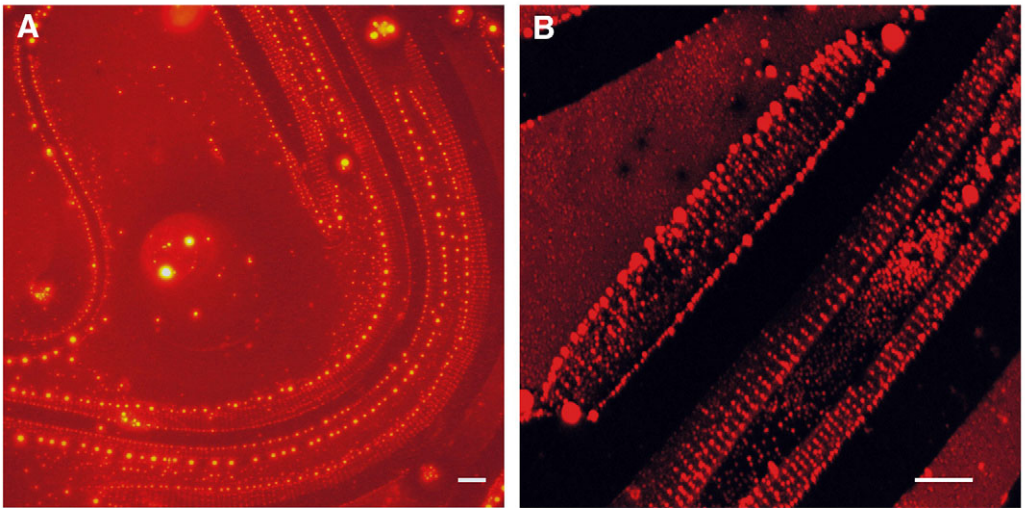

Fig. 5. Cuticle prints. Samples were prepared as for Fig. 4 and the material adhering to the coverslip, after nematode removal, was then observed by epifluorescence (A) or confocal (B) microscopy. Cuticle prints stained with Nile Red can be observed, leaving clear impressions of the cuticular annulations and lateral alae. Scale bars, 10 μm.

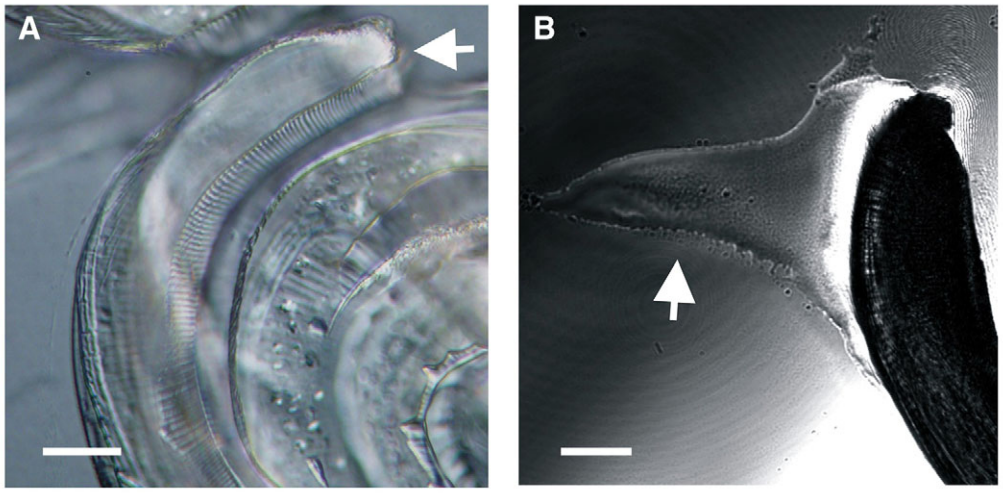

Fig. 6. *D. dipsaci* fourth-stage larvae (L4s) allowed to dry on a coverslip and then imaged through the coverslip using DIC optics on a Zeiss photomicroscope using a ×100 objective lens (A) or using confocal microscopy and reflected laser light (B). Oily material is observed between the nematodes and the coverslip (arrow in A) or smeared from the surface of the nematodes, as a result of their movement (arrow in B). Scale bars, 10 μm.

DISCUSSION

The presence of a lag phase and subsequent recovery after exposure to 50% RH, 20°C for 30 min indicates successful entry into anhydrobiosis (Wharton, 2002b; Wharton and Aalders, 1999). The water content of *D. dipsaci* L4s after desiccation at 20°C for 24 h, determined gravimetrically (Wharton and Aalders, 1999), was 0.45±0.06 g g^{-1} dry mass (mean ± s.e.m., *N*=4: 30.8% w/w water content) at 98% RH, 0.02±0.01 g g^{-1} dry mass (2.3%) at 50% RH but was not measurable at 0% or 33% RH. Some water is retained by this nematode at 98%, 76% and 50% RH but exposure to 0 or 33% RH results in the loss of all measurable water (Wharton and Aalders, 1999).

D. dipsaci L4s undergo a permeability slump during the early stages of desiccation (2–6 min), which slows their rate of water loss and thus facilitates survival (Wharton, 1996; Wharton and Marshall, 2002). There was no consistent change in the annulation spacing of individual nematodes, observed by confocal microscopy in unstained specimens, by reflected laser light or in specimens labelled with WGA, during this period that correlates with the permeability slump. There is a 4% change in length during the first 2 min of desiccation (Wharton, 1996). Any corresponding change in annulation spacing may be too small to measure.

No change corresponding to the permeability slump was measured (the average of several specimens) by cold-stage field-emission scanning electron microscopy on unfixed, frozen samples observed at 0, 2 and 5 min of desiccation (Wharton and Marshall, 2002). There were, however, changes in the appearance of the annulations after longer periods of desiccation (10–30 min), with the annulation grooves becoming more prominent and the minor annulations extending closer to the margins of the lateral alae (Wharton and Marshall, 2002). Given the lack of change in annulation spacing, however, it seems unlikely that the permeability slump involves the narrowing or deepening of the annulation grooves (Rössner and Perry, 1975; Wharton, 1996; Wharton and Marshall, 2002).

The labelling of the annulation grooves by WGA suggests that the material in the grooves is different from that covering the general surface of the cuticle or that it is concentrated at the grooves. A similar pattern of labelling of *D. dipsaci* J4s has been observed using a monoclonal antibody raised against surface antigens (Palmer et al., 1992) and using concanavalin A and limulin, which bind to

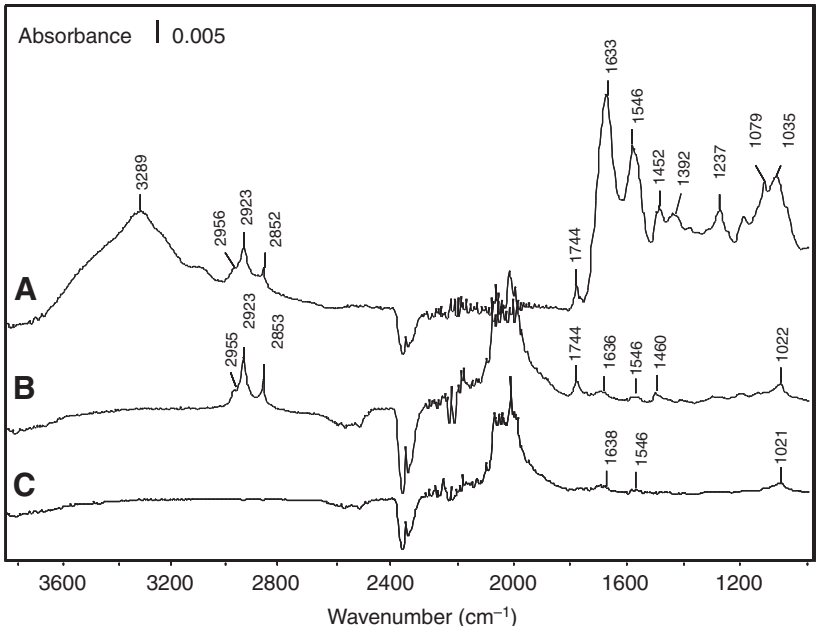

Fig. 7. Attenuated total reflection infrared (ATR–IR) spectra from the dry nematodes (with-worms; A), the oily secretion left after the nematodes removal (without-worms; B), and the spectrum after ether-extraction (C). Absorbance scale is the same for all spectra. Background was the bare diamond prism.

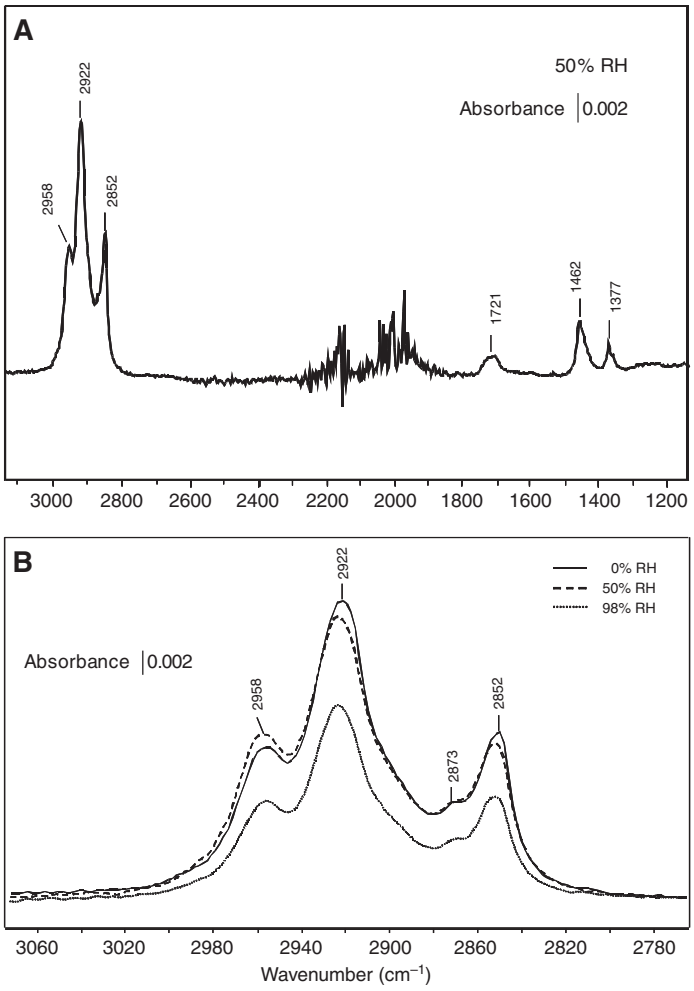

Fig. 8. (A) Attenuated total reflection infrared (ATR–IR) spectrum of purified nematode surface lipid from nematodes exposed to 50% RH and (B) comparison of part of the spectra from those exposed to 0%, 50% or 98% RH. Background was the bare diamond prism.

surface carbohydrates (Durschnerpelz and Atkinson, 1988). The presence of surface carbohydrates is also indicated by WGA, which binds to *N*-acetylglucosamine and/or *N*-acetylneurominic acid (sialic acid) residues (Jansson et al., 1986; Wright, 1984).

During desiccation, oily material that stains with Nile Red appears on the surface of the cuticle. The fluorescence of Nile Red is greatly enhanced in organic solvents and hydrophobic lipids but is fully quenched in water. It can thus be used as a selective and sensitive stain for lipids (Fowler and Greenspan, 1985), suggesting that the droplets observed contain lipid. Nile Red also stains hydrophobic proteins, although the fluorescent enhancement is weaker than that produced by lipids (Sackett and Wolff, 1987). The lipid nature of this material in *D. dipsaci* is also suggested by its oily appearance on the surface of desiccated specimens and the formation of droplets by this material upon the addition of water. The material is easily lost after immersion of the nematodes in water (and hence during staining procedures) but is closely applied to the surface of desiccated nematodes. Remnants of this material adhere to a coverslip from which desiccated nematodes have detached after the addition of water. This leaves impressions of the cuticular annulations and lateral alae. A similar phenomenon was observed by Bird (Bird, 1988) in *Anguina agrostis* L2 after labelling with WGA–FITC and termed ‘cuticle prints’. Cuticle prints were observed in some WGA–FITC-labelled samples of *D. dipsaci* but these were not as clear as in samples labelled with Nile Red.

Table 1. Chain length and double bond content from the gas chromatography analysis of the free fatty acid methylated esters obtained by hydrolysis of the triglyceride fraction of the nematode surface lipid

Chain length:double bond	Mass percentage (%)
C6:0	12.69
C8:0	27.64
C10:0	5.93
C12:0	11.93
C12:1	3.75
C14:0	5.45
C16:0	24.31
C18:0	6.99
C18:3n-3	1.31

The lipid nature of this material was confirmed by ATR–IR spectroscopy and GC analysis. In contrast to staining with Nile Red, which could not be reliably obtained, detection by ATR–IR spectroscopy was reliable and reproducible, and was capable of detecting very small quantities of material (using less than 20 nematodes). The lipid material was easily purified by dissolving the material retained on a glass surface in an organic solvent, following the desiccation of nematodes and their subsequent removal by gentle washing with water. The lipid was identified by GC analysis as being a triglyceride and its main chain lengths were found to be saturated fatty acids, mainly caprylic, palmitic and caproic acid. In fact, the infrared spectra (Fig. 8) show the dominant presence of CH_2 characteristic absorptions (stretching and bending modes), indicating an elevated content of saturated lipids. In contrast to the surface lipid material, a total lipid analysis of *D. dipsaci* (by TLC–GC) is dominated by unsaturated fatty acids; particularly oleic acid, which constitutes 66.6% of the total lipid content (Krusberg, 1967).

The nematode epicuticle consists of lipid and protein and the surface coat of the nematode cuticle contains carbohydrates, as is shown in the with-worms spectrum (Fig. 7A). The surface lipid described here contains neither proteins nor carbohydrates. Only alkyl and ester moieties are observed by ATR–IR spectroscopy (Fig. 8) and the presence of esterified fatty acids was confirmed by GC analysis (Table 1). It thus does not appear to be part of the surface coat or the epicuticle but to form a material that overlies the cuticle but is not part of it. This is also suggested by the appearance of the oily material seen coating the surface of the desiccated nematodes when observed by DIC and confocal microscopy. In nematodes dried on the surface of the diamond prism (Fig. 7) the surface lipid signal first appeared after 7 min of desiccation. However, it was probably secreted earlier than this since the signal would have at first been obscured by the signal from water inside and surrounding the nematodes.

There are a few reports in the literature that suggest the presence of extracuticular lipid material on the surface of some species of nematodes. Water loss from infective larvae of *Nippostrongylus brasiliensis* may be reduced by a thin layer of lipid, derived from the skin and hairs of the host (Lee, 1972). The accessory layer covering the cuticle of some of the larval stages of *Trichinella spiralis* and *T. pseudospiralis* contains lipid (Lee, 2002). A variety of plant-parasitic cyst nematodes, of the genus *Heterodera*, produce cuticular exudates that contain lipids and which appear to originate

from secretions of the epidermis (Endo and Wyss, 1992). Additional material on the surface of the cuticle [a 'lipophilic coating' (Womersley et al., 1998)] is produced by desiccated larvae of the plant parasites *Anguina amsinckiae* (Womersley et al., 1998) and *Ditylenchus myceliophagus* (Perry, 1999). The nature of this material, and its origin, is uncertain but it could be similar to the lipid coating described here in *D. dipsaci*. Although such evidence is lacking, lipid coatings could be widespread in nematodes and play important roles in desiccation survival and other aspects of nematode biology.

The oily material formed on the surface of *D. dipsaci* during desiccation is perhaps best referred to as an accessory layer. This term is used to refer to layers associated with the nematode cuticle but which are not part of the cuticle itself (Lee, 2002). The production of this surface lipid material occurs rapidly upon exposure to desiccation and may account for the permeability slump observed during desiccation. Since this material is washed away by immersion in water it does not impede the uptake of water during the rehydration of desiccated nematodes. This may explain the paradox that cuticular permeability decreases during the permeability slump (Wharton, 1996) and yet desiccated nematodes are more permeable, upon immersion in water, than are fully hydrated nematodes (Wharton et al., 1988).

LIST OF ABBREVIATIONS

ATR–IR	Attenuated total reflection infrared
ATW	artificial tap water
DIC	differential interference contrast
d_p	penetration depth
FAMEs	fatty acid methyl esters
FITC	fluorescein isothiocyanate
GC	gas chromatography
L4s	fourth-stage larvae
n	index of refraction
PC	phosphatidylcholine
RH	relative humidity
TAG	glycerol triacetate
TLC	thin layer chromatography
WGA	wheat germ agglutinin

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