# Changes in the lipophilicity of the surfaces of *Meloidogyne incognita* and *Haemonchus contortus* during exposure to host signals

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SUMMARY

The surfaces of plant and animal parasitic nematodes share certain lipids, which seem to be important in the infection process. The surfaces of 2 parasitic nematodes, *Meloidogyne incognita* and *Haemonchus contortus*, were activated by different pH buffers to allow the insertion of different fluorescent probes. The lipid analogue PKH26 and the surface charge indicator, cationized ferritin, were used as probes with these nematodes but labelled only the retaining 2nd-stage moulted cuticle of *H. contortus* 3rd-stage larvae ($L_3$). Shedding of the second moult of *H. contortus* $L_3$ was also visualized with PKH26 and cationized ferritin. The fluorescent anionic lipid probe 5-*N*-(octadecanoyl)-aminofluorescein (AF18) was inserted into the epicuticle layer of *M. incognita* 2nd-stage juveniles ($J_2$) and *H. contortus* $L_3$, and also of the second moult of *H. contortus* $L_3$. Incubation with tomato root diffusate caused modifications of the *M. incognita* surface allowing the insertion of AF18. Fluorescence with AF18 was significantly decreased after treating *M. incognita* $J_2$ with amiloride, a potent blocker of hydrogen and sodium ($H^+/Na^+$) antiporter. No surface fluidity was observed in *M. incognita* $J_2$ and *H. contortus* $L_3$ pre-treated with alkaline buffer when the lipid analogue AF18 was used in fluorescence recovery after photobleaching experiments. The significance of these findings to host infection processes is discussed.

Key words: *Meloidogyne incognita*, *Haemonchus contortus*, fluorescent probes, pH, root diffusate, surface fluidity.

## INTRODUCTION

The parasitic nematode *Haemonchus contortus*, which infects domestic and wild ruminants (Soulsby, 1982), and *Meloidogyne incognita*, which infects over 2000 plant species (Whitehead, 1997), are important because of the damage they cause and their ability to invade a diversity of hosts. Details of the mechanisms used by these parasites to invade their hosts are unknown. However, it seems that environmental cues from the host, such as temperature, pH and homologous host-tissue extracts, induce biochemical and physical surface changes which are important to the infection process of animal-parasitic nematodes (Philipp, Parkhouse & Ogilvie, 1980; Petronijevic, Rogers & Sommerville, 1986; Proudfoot *et al.* 1993*a*). Once the nematode enters in contact with their host tissues, rapid changes in the nematode surface lipid have been found (Proudfoot *et al.* 1993*a*) this might establish the initiation of the infection process. The nematode surface is rich in protein, carbohydrate and lipid molecules and it comprises 3 main layers: (1) epidermis, (2) epicuticle, and (3) surface coat. The latter consists of an additional envelope loosely attached to the epicuticle retained by some infective stages, such as *H. contortus* $L_3$ from the previous developmental larva ($L_2$), denominated also second moult or sheath (Maizels, Blaxter & Selkirk, 1993; Spiegel & McClure, 1997).

In the last decade, biophysical studies using lipid analogue probes have shown that surface coat and epicuticle of parasitic nematodes are selective to the insertion of these probes. Also, this lipid layer might be activated only when the nematode chages from pre-parasitic to post-parasitic stages *in vivo* (Kennedy *et al.* 1987; Proudfoot *et al.* 1993*b*; Roberts & Modha, 1997). Modha *et al.* (1997) suggested that a second signal such as cAMP plays an important role in the initiation of surface modifications in the mammalian nematode *Trichinella spiralis*. This paper uses fluorescent probes to assess and compare surface changes of the pre-parasitic stages of *M. incognita* and *H. contortus* caused by changes in pH and by exposure to root diffusate.

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## MATERIALS AND METHODS

### *Nematodes*

Two populations of parasitic nematodes were used: (a) *M. incognita* 2nd-stage juveniles ($J_2$) were obtained from infected roots of glasshouse grown aubergine (*Solanum melongena* L. cv. Blackbell). Plants were grown at 23 °C in a 16:8 h light:dark regime. Egg masses were picked from infected roots and juveniles were allowed to hatch in water at room temperature (RT); (b) infective larvae of the animal-parasitic nematode *H. contortus* ($L_3$) were obtained from the Moredun Institute (Edinburgh, UK). The infective larvae of *H. contortus* were kept in phosphate-buffered saline (PBS), pH 7, for 15 days at 4 °C and *M. incognita* were kept in distilled water (DW) at RT for 3 days, both nematodes were kept alive.

### *Fluorescent probes*

Nematodes incubated in different pH buffers and in root diffusate were observed under light microscopy to confirm their movement. The lipid insertion account and the FRAP-technique were made using only nematodes in movement.

### *AF18*

The fluorescent anionic lipid probe 5-*N*-(octadecanoyl)-aminofluorescein, AF18 (Molecular Probes) was used as described by Kennedy *et al.* (1987) and Modha *et al.* (1997) to label the surface coat and epicuticle of parasitic nematodes. The effect of pH on insertion of AF18 in *H. contortus* $L_3$ and *M. incognita* $J_2$ was determined using phosphate-buffered saline (PBS) at pH 4, 7 and 10. Nematodes were incubated in 0·5 ml of each buffer at RT for 10 min. A stock solution of lipid probe AF18 was prepared at 2 mg/ml in ethanol. Then, 10 $\mu$l of lipid fluorescent probe AF18 (stock) was added to each buffer for 15 min, after which it was removed by 2 washes with 0·5 ml of appropriate buffer of 3 min each. A third wash used buffer at pH 7 to equilibrate all parasites to the same pH before measuring fluorescence.

### *PKH26*

Stock solutions of the lipid probe PKH26 (Sigma) were prepared following the manufacturer's instructions (1 $\mu$l of probe per 100 $\mu$l of diluent 'C') and used as described by Modha, Kusel & Kennedy (1995) to label the surface coat of *T. spiralis*. Infective larvae of *H. contortus* and 2nd-stage juveniles of *M. incognita* were incubated in 0·5 ml of PBS, pH 10, with PKH26 for 2–3 min at RT. Two washes were made with 0·5 ml of PBS at pH 10 followed by a third wash with PBS, pH 7, before measuring the fluorescence.

### *Cationized ferritin*

Fluorescein isothiocyanate (FITC)-conjugated cationized ferritin (Molecular Probes) is generally used to label anionic sites (Himmelhoch, Kisiel & Zuckerman, 1977). A stock solution of 2 mg/ml diluted in distilled water was used as follows: *H. contortus* and *M. incognita* infective stages were incubated in 0·5 ml of PBS at pH 7 to which 4 $\mu$l of FITC-cationized ferritin were added for 15 min at RT, as described by Proudfoot *et al.* (1993*b*). Three washes were made with 0·5 ml of PBS at pH 7 before examination of the nematodes by fluorescence microscopy.

### *Quantification of fluorescence*

The fluorescence of infective stages of *H. contortus* $L_3$ and *M. incognita* $J_2$ labelled with AF18 lipid probe was measured using a Leitz MPV quantitative photometric system. The area of parasite surface to be measured was set at 400 $\mu m^2$ (Proudfoot *et al.* 1993*a*). Ten live nematodes were measured and 3 readings were made at 3 different positions on each specimen. From these experimental reading and an average background fluorescence reading was subtracted for each specimen. Measurements of fluorescence were made in arbitrary units and Student's *t*-test was applied after subtracting the background fluorescence readings. Data were considered significant at $P < 0{\cdot}0001$.

### *Effect of tomato root diffusate on* M. incognita

Tomato plants were grown in a 10 cm diameter pot in sterilized peat loam soil for 4 weeks and root diffusate was collected by leaching pots with 10 ml of distilled water, pH 6·5. One hundred *M. incognita* $J_2$ were incubated in 2 ml of tomato root diffusate for 30 min at RT and were then washed 3 times in PBS at pH 7 to maintain ionic balance. Nematodes were then incubated in 0·5 ml of PBS at pH 7 containing 10 $\mu$l of AF18 lipid probe for 15 min at RT. They were washed 3 more times in PBS, pH 7, at RT before measuring flourescence as described above. Infective 2nd-stage juveniles were incubated in PBS at pH 4 as negative controls because previous assays showed a low percentage of lipid insertion at this pH.

### *Amiloride analogue and pH-mediated by AF18 insertion*

Amiloride is a potent blocker of the $Na^+/H^+$ antiport of mammalian cells which acts to prevent intracellular alkalization (Proudfoot *et al.* 1993*a*). Amiloride analogue I3110 (Molecular Probes) was prepared at 15 mM in methanol as a stock solution. Amiloride was incubated before treatment of *M. incognita* $J_2$ as follows: a hundred *M. incognita* $J_2$

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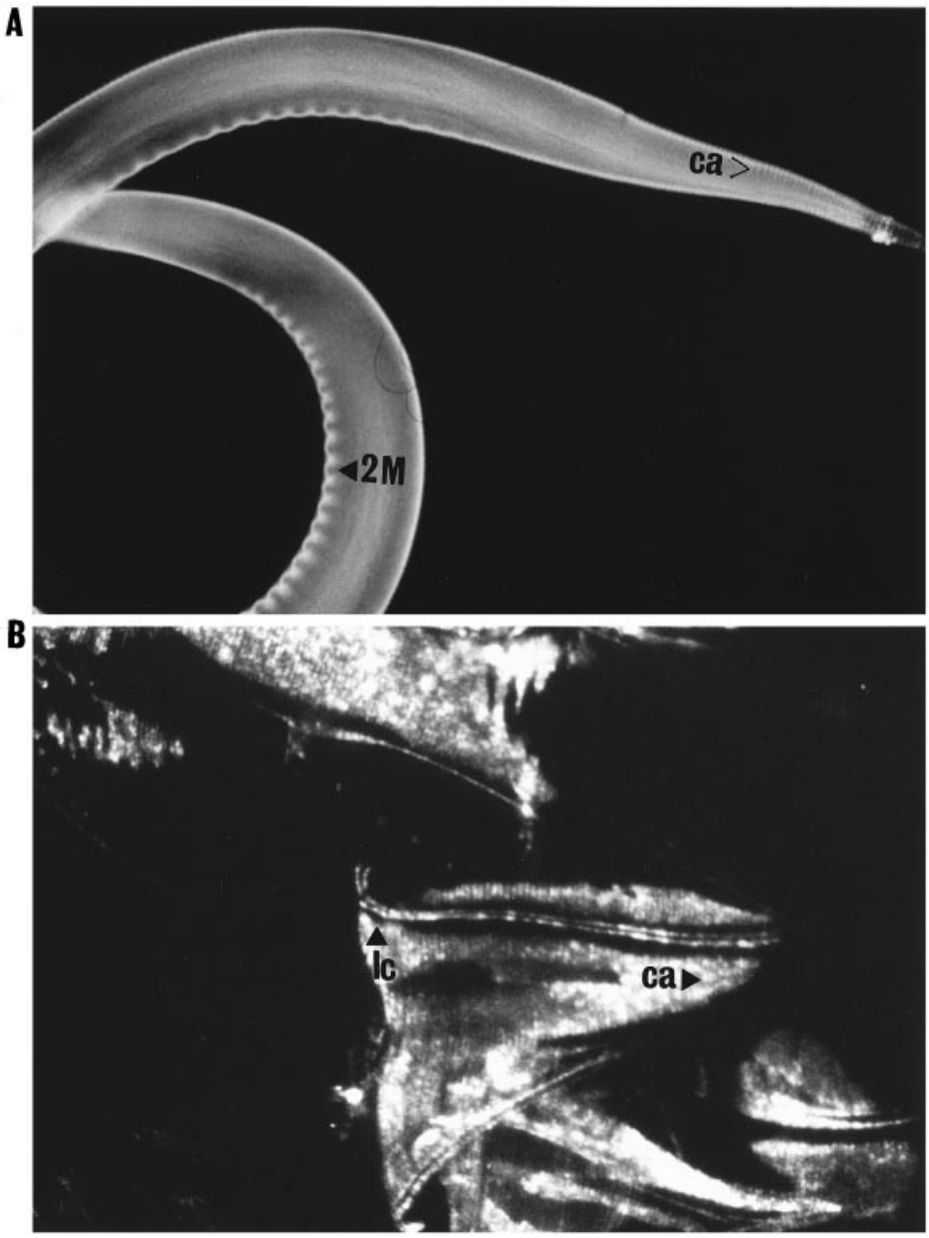


Fig. 1. Labelling of the 2M of *Haemonchus contortus* $L_3$ with FITC-PKH26 lipid probe. (A) Strong fluorescence after activation of the nematode with PBS, pH 10. (B) Shedding of the lipid layer was observed a few min after insertion. 2M, second moult; lc, lateral chord; ca, cuticle annulations.

were incubated in PBS, pH 4 and 10, and PBS, pH 10, containing 100 $\mu$M amiloride for 30 min at RT, after which 10 $\mu$l of AF18 lipid stock solution was added directly to samples for incubation for 15 min at RT. Several washes were made in appropriate buffer (pH 4 or 10) followed by 2 further washes in buffer at pH 7 to equilibrate the nematodes to the same buffer before taking readings.

*Fluorescence recovery after photobleaching*

The fluorescence recovery after photobleaching (FRAP) technique has been used to study the lateral diffusion of plasma membrane components (Peters, 1981; Wolf, 1988). The application of the FRAP technique on nematode epicuticle using fluorescent probes shows either mobility or immobility on a small nematode area after bleaching with an attenuated laser beam which is irreversible. When the lipid properties are mobile, fluorescent lipids will diffuse into the bleached area after bleaching. However, if the lipid layer is immobile the fluorescent lipids in the nematode membrane will stay in the same position after bleaching, with no lateral diffusion (DL) or fluorescence recovery (%R) (Proudfoot *et al.* 1993*b*; Modha *et al.* 1995).

*Haemonchus contortus* $L_3$ and *M. incognita* $J_2$ were incubated in PBS, pH 10, for 10 min and 2 washes were made in PBS, pH 10, with a last wash in PBS, pH 7, to equilibrate nematode surfaces before

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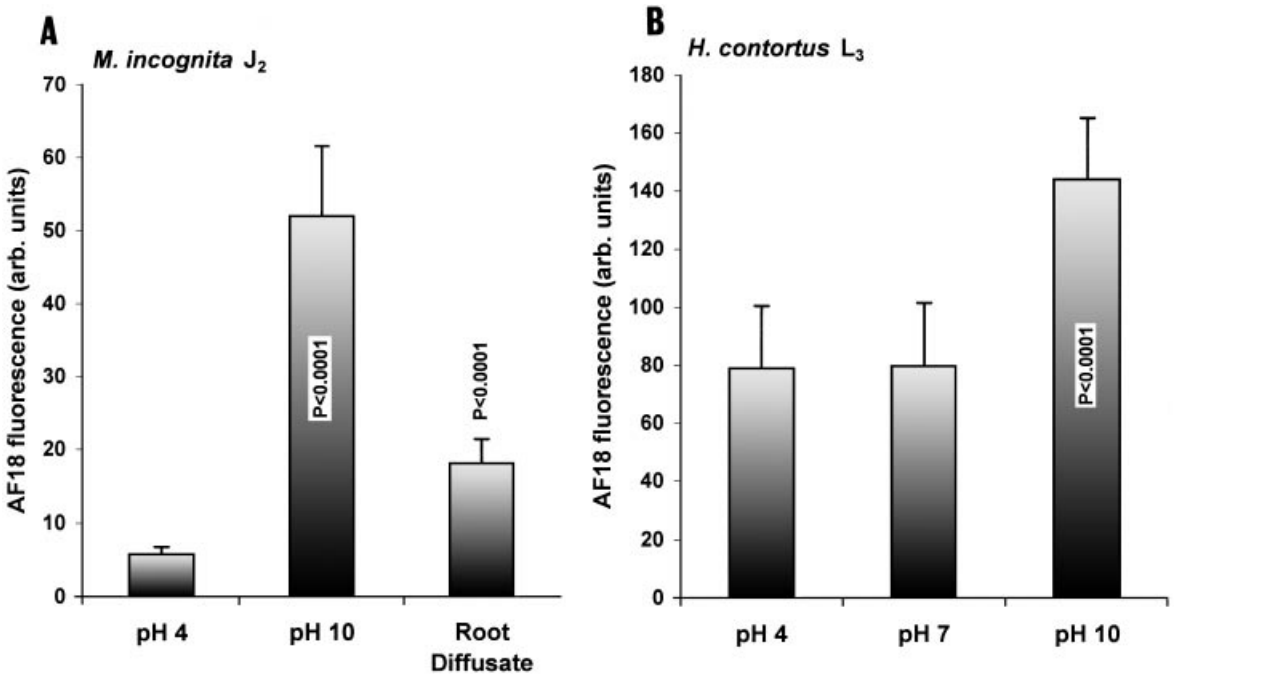


Fig. 2. Effect of pH on (A) *Meloidogyne incognita* $J_2$ epicuticle and (B) *Haemonchus contortus* $L_3$ 2M and epicuticle insertion of the fluorescent probe AF18. Infective stages from both nematodes were incubated with PBS at different pH values or root diffusate, at RT. Relative fluorescence values were expressed in arbitrary units, error bars are standard deviation ($n = 30$).

readings. Then PBS, pH 10, and AF18 lipid probe were added for 15 min at RT, as described above. FITC–BSA (Sigma) in 90 % glycerol was used as a standard for calibration of the spot size and as a control for detection of maximal fluorescence recovery after photobleaching (%R); the intensity of this fluorescence is arbitrarily referred to as 100 %. A Lexel model 85 argon laser, adjusted for use with a Leitz microscope was used to make measurements: the radius of the spot of the attenuated laser beam was 1 $\mu$m and the bleach time 400 msec at a pulse frequency of 96 Hz. Using these settings FITC–BSA repeatedly showed 100 % recovery of fluorescence after photobleaching (%R).

## RESULTS

### *Selective insertion of fluorescent probes and subsequent surface shedding*

The fluorescent anionic lipid probe PKH26 failed to label the *M. incognita* $J_2$ surface coat at pH 10. In contrast, exposure to PKH26 at pH 10 for only 2–3 min modified *H. contortus* $L_3$ second moult allowing the insertion of PKH26. The cuticle surface of *H. contortus* $L_3$ second moult was shed a few minutes after activation and labelling with PKH26. Fig. 1A–B displays the shedding in sheets with the imprint of the cuticle.

Fluorescein isothiocyanate (FITC)-conjugated cationized ferritin failed to label the surface of *M. incognita* $J_2$. In contrast, it labelled the second moult of *H. contortus* $L_3$, which was subsequently shed from the nematode (not shown).

### *Insertion of the AF18 lipid probe on* H. contortus *and* M. incognita *surfaces*

*Haemonchus contortus* $L_3$ achieved 70 (arb. units) of FITC-lipid probe AF18 insertion after exposure to PBS at pH 4 and 7. In contrast, *M. incognita* $J_2$ resisted the insertion of AF18; at pH 4 it reached only 8 (arb. units), which was considered negative. *H. contortus* $L_3$ and *M. incognita* $J_2$ exposed to PBS, pH 10, reached 144 and 52 (arb. units) of insertion, respectively. Both values were significantly greater than the degree of insertion at pH 4 and pH 7, $P < 0{\cdot}0001$ (Fig. 2A–B). The labelling of *H. contortus* $L_3$ epicuticle and the second moult with AF18 is shown in Fig. 3A–B.

Incubation of *M. incognita* $J_2$ with tomato root diffusate pH 6·5, for 30 min allowed insertion of the lipid probe AF18 into the nematode epicuticle. The lipid insertion (18 arb. units) was significantly greater ($P < 0{\cdot}0001$) than in the control group (Fig. 2A).

The insertion of AF18 at pH 10 into *M. incognita* $J_2$ epicuticle was partially blocked (8 arb. units) by pre-incubation of the nematodes with the amiloride analogue I3110 (Fig. 4).

### *AF18 fluorescence recovery after photobleaching (FRAP)*

The insertion of the lipid probe on the nematode surface did not show mobility after incubation with alkaline buffer (pH 10) or tomato root diffusate used in *H. contortus* $L_3$ and *M. incognita* $J_2$ epicuticle, respectively.

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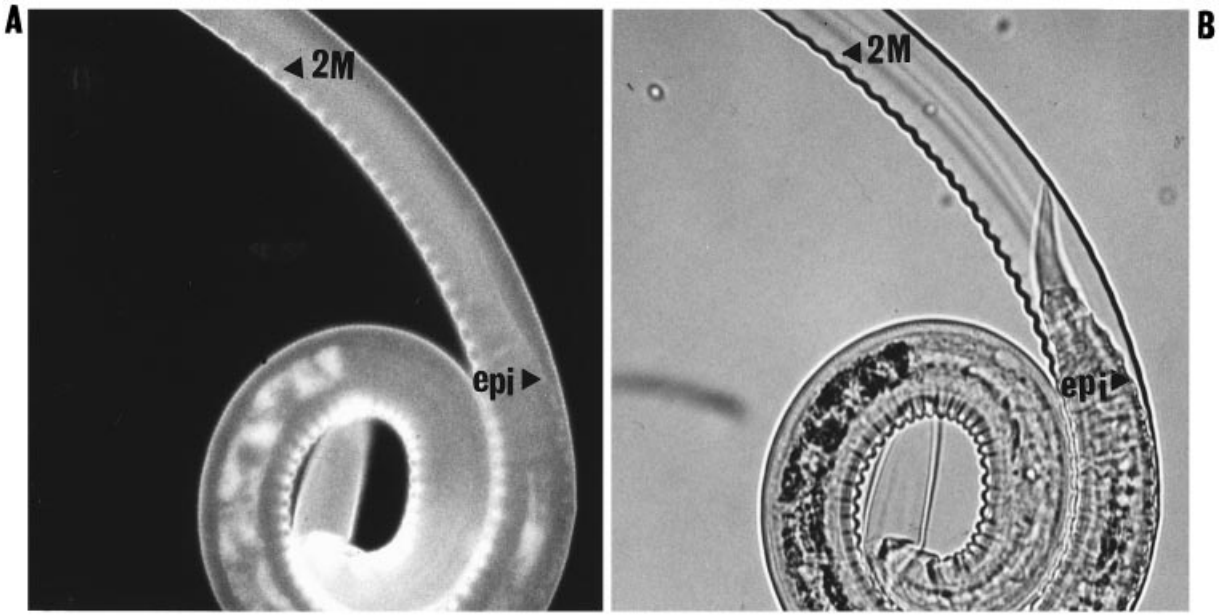


Fig. 3. *Haemonchus contortus* $L_3$ treated with FITC-AF18 fluorescent lipid analogue and PBS, pH 10. The strong reaction of FITC-AF18 on the nematode surface allowed observation of 2 positions of insertions: on the nematode second moult and on the epicuticle. (A) Fluorescence micrograph. (B) Bright field. 2M, second moult; epi, epicuticle.

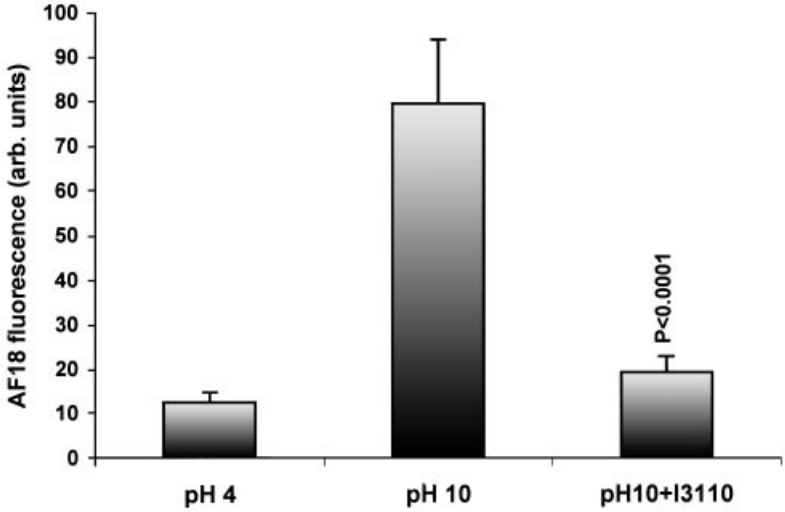


Fig 4. Effect of amiloride analogue I3110 on the insertion of the fluorescent lipid probe AF18 into the surface of *Meloidogyne incognita* $J_2$. Two control groups were used: PBS, pH 4 (negative) and PBS, pH 10 (positive). Fluorescent values are given in arbitrary units and error bars show standard deviation. Differences were significant ($P < 0{\cdot}0001$). A total of 30 readings was taken for each experimental group.

Fluorescence recovery after photobleaching (FRAP) analysis of *M. incognita* $J_2$ and *H. contortus* $L_3$ showed that AF18 was unable to diffuse on the nematode surface after photobleaching, achieving a low %R (< 20%). Although the surface of both nematodes was activated *in vitro* with PBS, pH 10, exhibiting an increase in lipophilicity, there was no change in AF18 %R after photobleaching (Fig. 5A–B).

## DISCUSSION

The indication that the components of nematode surfaces are metabolically active and respond to environmental changes has been supported by previous studies (Kennedy *et al.* 1987; Proudfoot *et al.* 1993*a*) and this work shows similar results with the plant-parasitic nematode, *M. incognita* and with the animal parasite, *H. contortus*. The surfaces of these nematodes were activated by alkaline buffer and/or tomato root diffusate, allowing insertion of the anionic fluorescent probe cationized ferritin, AF18 and/or PKH26, demonstrating that changes in the nematode cuticle surface lipophilicity were induced by alkaline buffer and root diffusate.

Particularly notable was the AF18 labelling of the epicuticle of *M. incognita* $J_2$ and *H. contortus* $L_3$ and even of the second moult of *H. contortus*. Greatest

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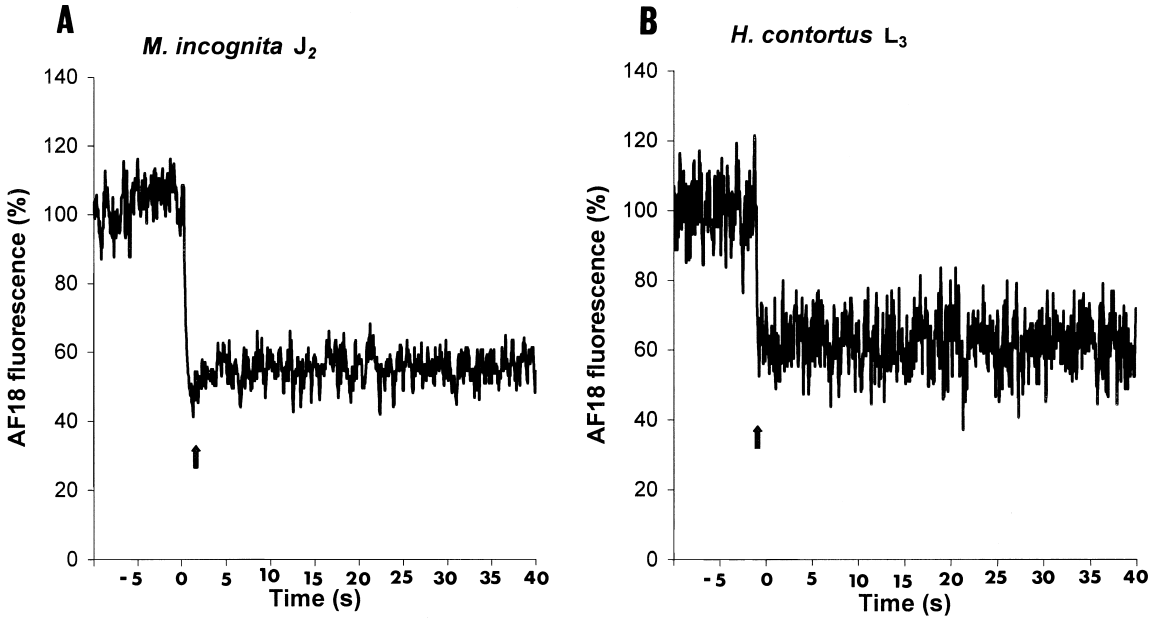


Fig. 5. Lateral lipid fluidity of *Meloidogyne incognita* $J_2$ and *Haemonchus contortus* $L_3$ surfaces after treatment with PBS, pH 10, and FITC-AF18. Surface fluidity was measured by fluorescence recovery after photobleaching (FRAP). The bleaching flash is indicated by the arrow. Percentage recovery ( %R) of fluorescence in both *M. incognita* (A) and *H. contortus* (B) was $< 20\,\%$.

insertion with AF18 was observed at pH 10. However, insertion of this probe at pH 4 and/or pH 7 was also observed for both nematodes.

After incubation of *M. incognita* $J_2$ with root diffusate the level of insertion of AF18 was relatively high. This suggests that alkaline buffer and root diffusate provide signals that induce similar effects on the surface of *T. spiralis* (Proudfoot *et al.* 1993*a*; Modha *et al.* 1997). Partial inhibition of the uptake of AF18 by amiloride in *M. incognita* $J_2$ suggests the involvement of this antiporter system in the regulation of intracellular pH during the infection process (Proudfoot *et al.* 1993*a*).

Unlike the lipid probe AF18, PKH26 labelled the second moult of *H. contortus* $L_3$ and PKH26 did not label the SC of *M. incognita* $J_2$. A few minutes after labelling with PKH26 the cuticle surface was shed.

The charge indicator, cationized ferritin did not label the *M. incognita* $J_2$ and the same result was obtained with the $J_2$ of the plant-parasitic nematode *Globodera pallida* (not shown), indicating that these 2nd-stage juveniles might have a few negative charges on their cuticle surfaces. It is also possible that anionic sites were not accessible to the cationic ferritin; perhaps cationic detergents might help to uncover these sites and help to confirm the net charge of these plant-parasitic nematodes. Factors such as age and surface integrity were considered important in this study, and freshly hatched $J_2$ were always used for the experiments.

Himmelhoch & Zuckerman (1983) suggested that cationized ferritin insertion into the surface coat of the free-living nematode *Caenorhabditis elegans* depends on the sulphate group, as demonstrated by blockage of this group by Alcian blue. Most likely, a similar organization of the sulphate groups on *H. contortus* $L_3$ second moult might have allowed the insertion of cationized ferritin at physiological pH.

*Haemonchus contortus* surface dynamics allowed this nematode to shed bound substances such as cationized ferritin and PKH26, as demonstrated in *Strongyloides ratti* (parasite of mouse) and *T. spiralis* with the same labelling (Proudfoot *et al.* 1993*b*; Modha *et al.* 1995). Shedding of the nematode cuticle surface might be an important aspect of the survival mechanisms operating in these nematodes (Philipp *et al.* 1980; Blaxter *et al.* 1992; Ashman *et al.* 1995).

Fluorescence recovery after photobleaching (FRAP) analysis showed that fluidity on the nematode surface was restricted after photobleaching. This result is in agreement with studies of animal-parasitic nematodes, which also failed to show fluidity after photobleaching *in vitro* (Proudfoot *et al.* 1993*a*). Modha *et al.* (1997) observed that lipid layer fluidity (100 %) occurred in *T. spiralis* larvae during penetration of the intestinal epithelial cells. This effect could be mimicked by generating high concentrations of cAMP in the parasite using membrane-permeant caged cAMP. These authors suggest that not only cAMP but also calcium [$Ca^{+}$] are participating in the activation of the nematode surface in the living parasite.

The infection of the abomasum (the last stomach compartment of ruminants) with *H. contortus* causes the stomach pH to be raised above normal levels (more than pH 3). As well as pH, calcium levels have been shown to be important in the exsheathing

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process of the infective larvae of *H. contortus* (Petronijevic *et al*. 1986; Nichols *et al*. 1988). These two host signals might combine to activate the pre-parasitic nematode surface during the infection process *in vivo*.

The importance of an increase in pH during surface changes in *M. incognita* $J_2$ is suggested by the inhibition of uptake of AF18 by the amiloride analogue I31100. The activity might be different in *M. incognita* as measured by the increase in AF18 insertion, which would allow the signalling mechanisms triggered during infection to be analysed, as in *T. spiralis*. Fractionation of root diffusate may permit identification of molecules involved in triggering these surface changes.

In summary, the lipid surface of plant and animal-parasitic nematodes showed similar properties in allowing the insertion of the lipid probe AF18. In contrast, *M. incognita* $J_2$ and *H. contortus* $L_3$ showed selective insertion of the lipid probe PKH26. The insertion of FITC-cationized ferritin on *H. contortus* $L_3$ suggests a negative charge on the nematode surface. However, the restricted labelling by this fluorescent probe of the *M. incognita* $J_2$ surface suggests a few negative charges. The cuticle surface is very important for the host–parasite interaction in plant and animal-parasitic nematodes. We have shown that they have some similarities in terms of lipophilicity and that they are able to perceive and respond to specific host cues. The lipid activity of nematode surfaces shown in this study might be associated with their behaviour *in vivo*.

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