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ORIGINAL PAPER

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Ecdysis of *Oesophagostomum*: possible involvement of eicosanoids and development of a bioassay

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Abstract Bioassays were developed and applied to test the role of eicosanoids and pH changes in ecdysis of *Oesophagostomum dentatum*. Exsheathment (80–100%) was achieved by subjecting third-stage larvae (L3) either to chlorine (hypochlorite assay) for 5 min or by incubating them in HCl followed by addition of $NaHCO_3$ (pH-change assay) with subsequent cultivation at 38.5°C/10% CO_2 for 1 week. Addition of the lipoxygenase (LOX) inhibitor diethylcarbamacine (DEC) to the larvae resulted in a reduction of the exsheathment rates which could be restored by the addition of leukotrienes (LT)B_4, LTC_4, LTD_4 and LTE_4. Addition of the cyclooxygenase (COX) inhibitor acetylsalicylic acid (ASA) also resulted in decreased exsheathment rates both in the hypochlorite and in the pH-change assays in a dose-dependent manner. However, the primary COX products (prostaglandins) were not able to reverse this effect, in contrast to LTC_4. It was concluded that: (1) both tests are suitable for bioassaying the effect of substances on exstheathment, and (2) eicosanoids involved in the control of exhseathment of L3 of *O. dentatum* are primarily LT.

Introduction

For strongylid nematodes, ecdysis (synonymous with exsheathment) of the infectious third-stage larva (L3) marks the transition from the free-living to the parasitic stage. Through exogenous stimuli, the L3 sheds its protective sheath (consisting of the cuticle of the previous stage) and subsequently penetrates the host tissue to undergo further development. Depending on the localisation of the parasite, gastrointestinal strongyles that dwell in the small intestines exsheath in the stomach, while those that are localised in the large intestines lose their sheath in the small intestines (Hertzberg et al. 2002). Although this biological phenomenon can be considered a target for intervention in the nematode's parasitic development, the mechanisms of exsheathment have not been studied in great detail. Rogers (1970, 1982) proposed that both exogenous (host-derived, i.e. changes in pH) and endogenous (parasite-derived, i.e. enzymatic activity of the worms excretory-secretory substances) factors play a role in the process. Metalloproteinases and other enzymes are implicated (Rogers 1982) and their activity can be specifically inhibited to disrupt ecdysis in *Haemonchus contortus* (Rogers and Brooks 1976). Characterisation of such proteases in recent studies gives supportive evidence for their role in ecdysis (Gamble et al. 1989; Zhan et al. 2002); however, final proof is still missing. Exsheathment is also one of the most obvious biological parameters of larval viability. It is quantifiable and can readily be applied to many strongylid species in vitro. It is therefore a practical tool for studying biochemical processes and also gene function in the early parasitic stage of nematode development. In vitro exsheathment can be achieved by the application of sodium hypochlorite to suspensions of L3 (Patel and Campbell 1997). However, this might not follow the physiological process of exsheathment (Conder and Johnson 1996). An artificial rumen was developed to mimic gastrointestinal physiology for the exsheathment of ovine nematode parasites (Hertzberg et al. 2002), but this system is not applicable to stringent high throughput, time-dependent measurements in functional studies.

The present study was undertaken to evaluate physical and biochemical parameters that may influence the

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ecdysis process and to apply exsheathment to viability assays for *Oesophagostomum dentatum*, the nodular worm of pigs inhabiting the large intestines.

Materials and methods

Parasite material

O. dentatum (strain Hannover) was maintained in pigs and faeces were collected for standard larval culture (Talvik et al. 1997). Faecal suspensions containing L3 were set up for small-scale agar migration as described by Talvik et al. (1997), and cleaned worms were kept in distilled water at 10°C for a maximum of 2 months. To account for batch-to-batch variation, larvae were used from different batches for the replication of the tests.

Experiment A: induction of ecdysis by mimicking the gastrointestinal environment (pH-change assay)

During their transport through the gastrointestinal tract, strongylid larvae face two pH changes, one on entry into the stomach where the pH decreases and one on exiting this acidic environment. Since passage times are so far unknown, larvae were exposed to an acidic environment (hydrochloric acid; HCl) for different time periods followed by neutralisation with sodium hydrogen carbonate ($NaHCO_3$). In detail, 250 µl HCl (0.1, 0.25, or 0.5 M) was added to 50 µl larvae in distilled water and larvae were incubated for 1, 2, 6, or 8 h. For neutralisation 1 ml $NaHCO_3$ (0.1 or 0.25 M) was then added and the final pH was determined. Exsheathment rates (%) were determined on days 1, 2, 3, 4, 5, and 6 afterwards. During treatment and observation, larvae were incubated at 38.5°C with 10% CO_2. Controls consisted of larvae in distilled water incubated in parallel. Viability of the larvae was estimated by sodium hypochlorite-induced exsheathment at the end of each trial.

Experiment B: development of an ecdysis bioassay for L3 of *O. dentatum* (hypochlorite assay)

Addition of 20 µl of sodium hypochlorite (10–14% free chlorine) to 1 ml of a larval suspension results in exsheathment within 5 min at room temperature. The sheath is not lysed but at the cranial part the "cap" detaches and the larva actively exits the intact sheath (Fig. 1).

Inhibition of exsheathment after addition of test substances was calculated as follows:

$$Ex_i = \frac{sL}{sL + eL}$$

(sL = number of ensheathed larvae,
eL = number of exsheathed larvae;
Exi = inhibition of exsheathment)

Viable larvae kept at 10°C for less than 2 months all left their sheath ($Ex_i = 0$) within 5 min. A value of $Ex_i = 1$ corresponds to 100% inhibition. The exsheathment stimulus (hypochlorite) was titrated to an Ex_i value of 0.5 corresponding to 1.1–3.5% free chlorine depending on the batch of chemicals and larvae. In the following, the value for Ex_i is always given as a median of all samples in the same group.

The choice of test substances that were evaluated was based on earlier observations that eicosanoids are produced by *O. dentatum* in vitro and that their inhibition

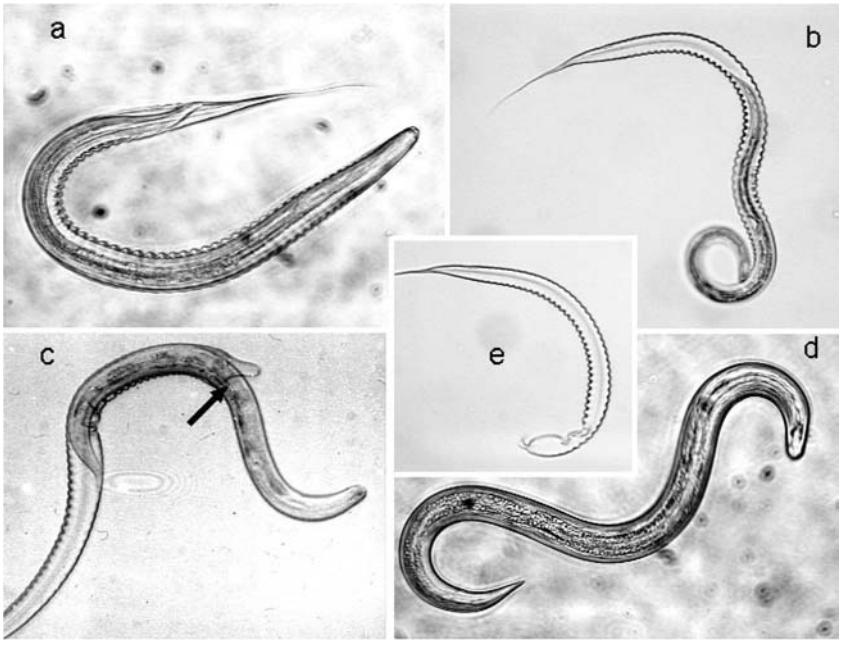

Fig. 1 Exsheathment of *Oesophagostomum dentatum* L3 induced by addition of sodium hypochlorite. **a** Ensheathed larva; **b**, **c** course of exsheathment (*arrow* marks apical opening in the sheath); **d** exsheathed larva; **e** empty, intact sheath

Table 1 Inhibitors of eicosanoid production.*COX* Cyclooxygenase (prostaglandin pathway), *LOX* lipoxygenase (leukotriene-thromboxane pathway), *PG* prostaglandin, *LT* leukotriene

Test substance	Concentrations	Inhibition of	Reversion assay
ASA: acetylsalicylic acid	0.1–0.25–0.5–1.0 mg/ml (0.6–5.6 mmol/l)	COX	1 mg/ml ASA + PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2; (5×10^{-8}–5×10^{-6} mmol/l each); LTC_4 (8×10^{-7} mmol/l)
DEC: diethylcarbamacine	0.5–1.3–2.5–5.1 mg/ml (2.6–25.5 mmol/l)	LOX	1.3 mg/ml DEC + LTD_4 (9×10^{-9}–9×10^{-7}), LTE_4 (12×10^{-9}–12×10^{-7}), LTB_4 (15×10^{-8}–15×10^{-6}), LTC_4 (8×10^{-9}–8×10^{-6})

leads to retarded growth and viability (Daugschies 1995, 1996). We therefore used inhibitors of prostaglandin H-synthase (cyclooxygenase; COX) and lipoxygenase (LOX) as described in Table 1. To exclude non-specific toxic effects on larval viability, acetylsalicylic acid (ASA)-inhibited larvae were treated with prostaglandins (PG) or leukotrienes (LT), and diethylcarbamacine (DEC)-inhibited larvae were treated with LT to reverse the effect of inhibition (Table 1). A minimum of ten samples (approximately 100 larvae each) was tested and each trial was repeated at least twice on different days. Controls were incubated in the respective solvents.

In order to determine the effect of ASA on ecdysis under the conditions investigated in experiment A, larvae were treated with 0.1 M HCl for 6 h, neutralised by 0.25 M $NaHCO_3$ and cultured as above. Additionally, ASA was added in concentrations of 0.6, 1.4 and 5.6 mmol/l together with $NaHCO_3$; in these cases solvent controls were used instead of distilled water.

Statistical analysis

The average counts for exsheathed/unexsheathed larvae are given as median values. Statistical calculations were performed using SPSS for Windows. To test for significant differences, the Mann-Whitney U-test was applied. For evaluation of correlations, the Spearman rank cor-

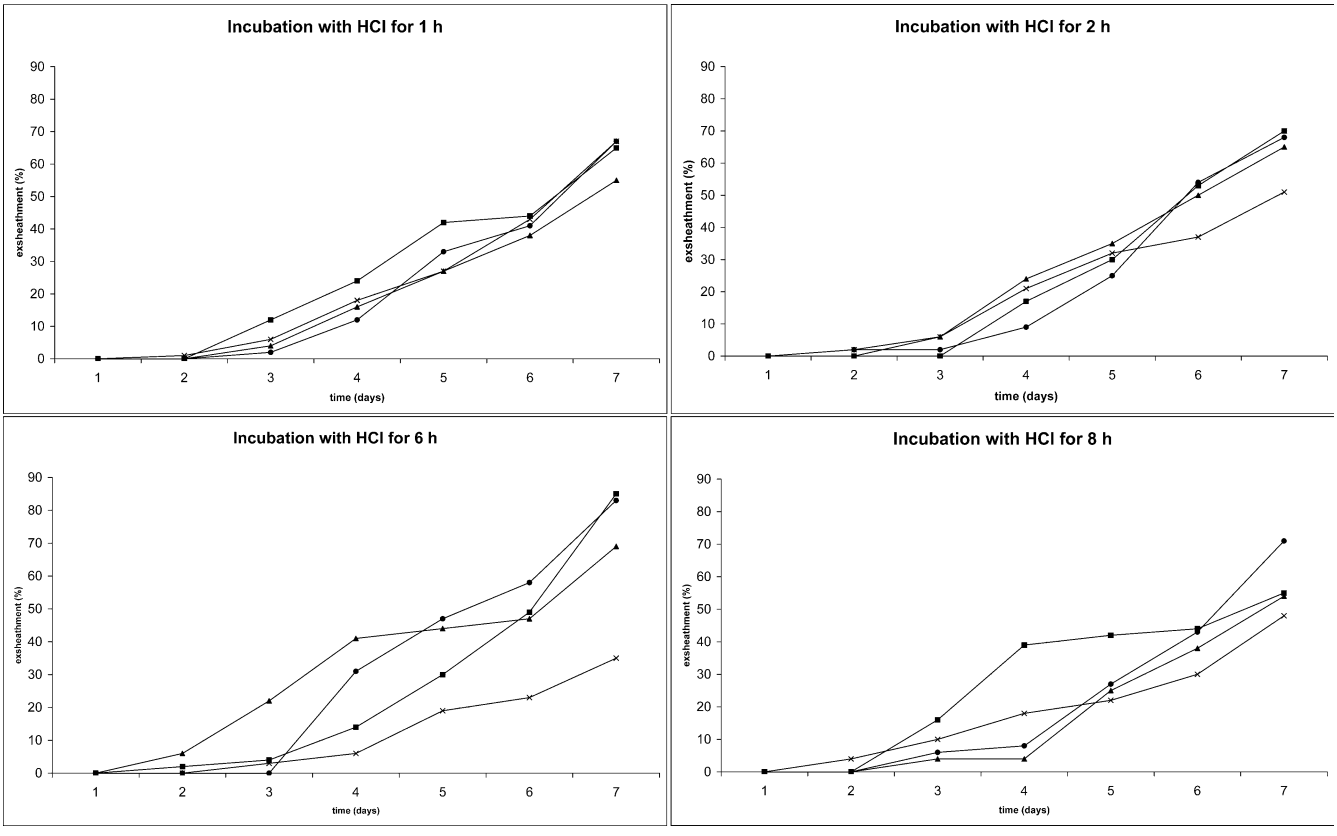

Fig. 2 Exsheathment rates (Ex_i) of larvae incubated with hydrochloric acid (HCl) for 1–8 h in different concentrations followed by incubation with sodium hydrogen carbonate ($NaHCO_3$). *Filled squares* 0.25 M HCl - 0.25 M $NaHCO_3$, *filled triangles*: 0.25 M HCl - 0.1 M $NaHCO_3$, *filled circles*: 0.1 M HCl - 0.25 M $NaHCO_3$, *X*: 0.5 M HCl - 0.25 M $NaHCO_3$

relation coefficient (r_S) was calculated. $P \leq 0.05$ was considered significant, $P \leq 0.001$ highly significant.

Results

Exsheathment rates by changes in pH

The pH after neutralisation varied from slightly acidic to slightly basic (5.5–8.5). Incubation in water or HCl without neutralisation or after the addition of 0.1 M $NaHCO_3$ did not result in exsheathment. Independently of the incubation conditions, the exsheathment rates increased over the 7 days of observation in all other samples (Fig. 2). The highest exsheathment rates (85 and 83%, respectively) were seen at a final pH of 8.0 (0.1 or 0.25 M HCl followed by 0.25 M $NaHCO_3$) after 6 h of incubation in HCl.

Development of an exsheathment bioassay for third-stage larvae

Incubation with LOX-inhibitors

DEC displayed a dose-dependent inhibitory effect on the exsheathment of the larvae when hypochlorite was added immediately after the addition of DEC to the medium. While 2.6 mmol/l had no significant effect ($Ex_i = 0$) compared to the negative controls, Ex_i was 0.39 with 6.4 and 0.89 and 0.93 with 12.8 and 25 mmol/l, respectively, which was highly significantly different ($P < 0.001$) compared to the low concentrations and the controls (Fig. 3) with an r_S of 0.92 ($P < 0.01$). Incubation of larvae in 25.5 mmol/l DEC decreased the sensitivity of the larvae to hypochlorite stimulation. This effect was obvious after an incubation period of 1 h, and persisted, although on a somewhat lower level, over the whole incubation period of 24 h. Incubation with a lower dose of 6.4 mmol/l DEC also clearly inhibited exsheathment, although less distinctly and with maximum values after 3 h. Inhibition of exsheathment was completely reversible as the larvae were fully susceptible to NaOCl stimulation after removal of DEC by repeated washing. The effect of DEC was always significant compared to the controls (not shown).

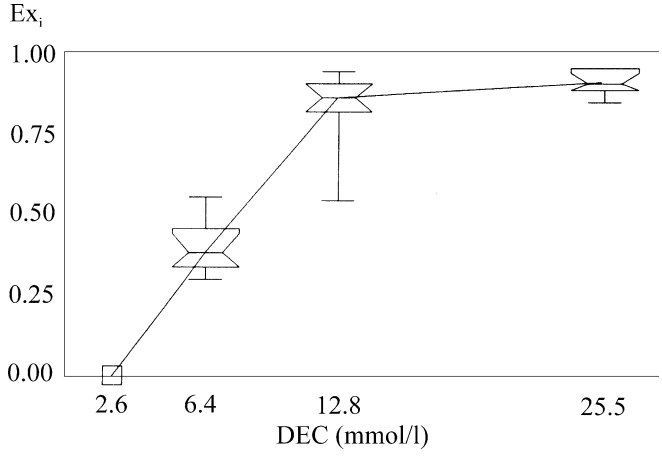

Fig. 3 Exsheathment rates (Ex_i) of *O. dentatum* L3 after incubation with LOX-inhibitor DEC in different concentrations

Table 2 Concentrations of leukotrienes (*LT*) and their effect on DEC (6.4 mmol/l) inhibited larvae. *** $P \leq 0.001$, *n.s.* not significant (compared to controls without LT)

Sample (mmol/l)	Median Ex_i	Significance compared to the control
control w/o LTB_4	0.54	
LTB_4 (1.5×10^{-7})	0.56	n.s.
LTB_4 (1.5×10^{-6})	0.62	n.s.
LTB_4 (1.5×10^{-5})	0.46	***
control w/o LTC_4	0.54	
LTC_4 (8×10^{-9})	0.66	n.s.
LTC_4 (8×10^{-8})	0.52	n.s.
LTC_4 (8.0×10^{-7})	0.32	***
LTC_4 (8.0×10^{-6})	0.54	n.s.
control w/o LTD_4	0.64	
LTD_4 (9.0×10^{-9})	0.67	n.s.
LTD_4 (9.0×10^{-8})	0.41	***
LTD_4 (9.0×10^{-7})	0.47	***
control w/o LTE_4	0.56	
LTE_4 (1.2×10^{-8})	0.50	n.s.
LTE_4 (1.2×10^{-7})	0.30	***
LTE_4 (1.2×10^{-6})	0.54	n.s.

To test for the specificity and reversibility of DEC inhibitory effects, LT were added to DEC inhibited larvae (6.4 mmol/l; $Ex_i = 0.5$) (Table 2).

LTB_4 was added in concentrations of 1.5×10^{-7}–1.5×10^{-5} mmol/l. Only the highest concentrations of LTB_4 were able to significantly ($P < 0.05$–0.001) reverse the effect of DEC compared to the control and the lower concentrations.

LTC_4 was used in concentrations of 8.0×10^{-9}–8.0×10^{-6} mmol/l. Concentrations of 8.0×10^{-7} reversed the inhibitory effect significantly ($P < 0.001$) while lower doses were ineffective and the highest concentration was obviously toxic since inhibition was not further reduced but increased to a level similar to the controls (Table 2). When the highest concentration was excluded r_S was -0.33 ($P < 0.05$).

While LTD_4 in a concentration of 9.0×10^{-9} did not reverse inhibition of exsheathment, the higher doses of 9.0×10^{-7} and 9.0×10^{-8} were able to reduce inhibition by DEC in a dose-dependent manner ($r_s = -0.43$; $P < 0.05$).

LTE_4 was ineffective at concentrations of 12×10^{-9} and 12×10^{-7} but decreased inhibition significantly at 12×10^{-8} mmol/l.

Incubation with COX inhibitors

ASA at concentrations of 0.6–5.6 mmol/l was able to inhibit exsheathment after 5 min of hypochlorite stimulation at all concentrations in a dose-dependent manner ($r_S = 0.76$; $P < 0.01$) with $Ex_i = 0.07$ for 0.6 mmol/l

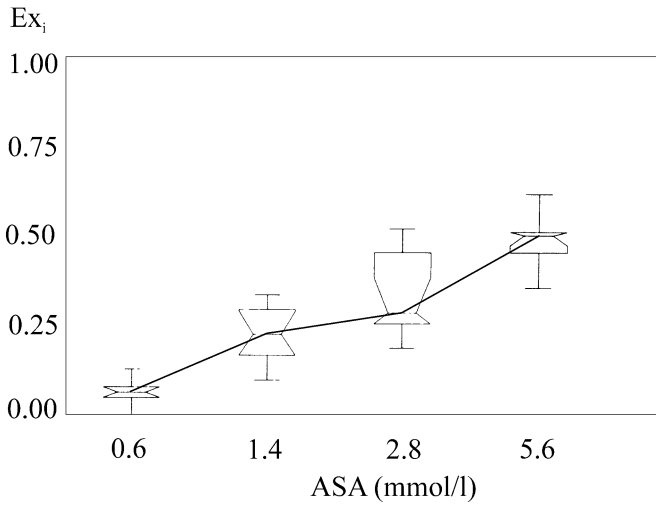

Fig. 4 Exsheathment rates (Ex_i) of *O. dentatum* L3 after incubation with COX-inhibitor ASA in different concentrations

ASA, $Ex_i = 0.23$ for 1.4 mmol/l, $Ex_i = 0.29$ for 2.8 mmol/l and $Ex_i = 0.51$ for 5.6 mmol/l (Fig. 4). The differences were significant for concentrations of 1.4 mmol/l or more ($P < 0.05$–0.001). Stimulation with hypochlorite for 10 min resulted in an even more pronounced effect ($r_S = 0.96$; details not shown).

Prostaglandins (PGD_2, PGE_2, $PGF_{2\alpha}$, PGI_2) that could possibly reverse the inhibitory effect of ASA (5.6 mmol/l corresponding to an Ex_i of 0.43–0.54) were added in concentrations of 0.05, 0.1, 0.5, 1.0, or 5.0 $x10^{-6}$ mmol/l. Additionally, LTC_4 (8×10^{-7} mmol/l) which had a reversing effect on DEC-mediated inhibition, was also tested to evaluate a possible LOX-independent mechanism. None of the PGs had a significant effect on the exsheathment of ASA-treated larvae ($Ex_i = 0.46$–0.54). LTC_4, however, significantly enhanced the exsheathment ($Exi = 0.46$; $P < 0.05$) compared to the controls ($Ex_i = 0.54$).

Inhibition of exsheathment with ASA using the pH-change assay

Larvae were incubated with 0.1 M HCl for 6 h followed by neutralisation with 0.25 M $NaHCO_3$ and simultaneous treatment with ASA as above. The pH after 7 days was 9.5.

Compared to the controls ($Ex_i = 0.85$) ASA-treated larvae had Ex_i values of 0.74 (0.6 mmol/l), 0.67 (1.4 mmol/l) and 0.58 (5.6 mmol/l). Incubation over 7 days showed that ASA in concentrations of 1.4 mmol/l or more significantly reduced exsheathment in a dose-dependent manner (Fig. 5).

Discussion

Exsheathment after entry into the host's body marks the change from the free-living to the parasitic life style for strongylid nematodes (Sommerville 1957). It can be induced in vitro by proteolytic enzymes or changes in pH (e. g. by the addition of the highly basic sodium hypochlorite or carbon dioxide for acidification); however, interspecific differences can be quite substantial (Silverman and Podger 1964). We chose *O. dentatum* as a reference model since larvae can be produced in vitro in high numbers and exsheathment can regularly be induced by short-term incubation with sodium hypochlorite. In contrast to the more intensively studied trichostrongylids of ruminants (*Haemonchus*, *Ostertagia*, *Trichostrongylus*) which live in the more anterior part of the gut and exsheath in the stomach, *Oesophagostomum* inhabits the first half of the large intestines, and exsheathment in vivo occurs mainly in the small intestines (Hertzberg et al. 2002). Therefore, it must be assumed that the stimuli for ecdysis vary for these species and that extrapolation of a common mechanism should be undertaken with care. This is supported by the fact that the exsheathment technique used for *H. contortus* (Slocombe and Whitlock 1970) with

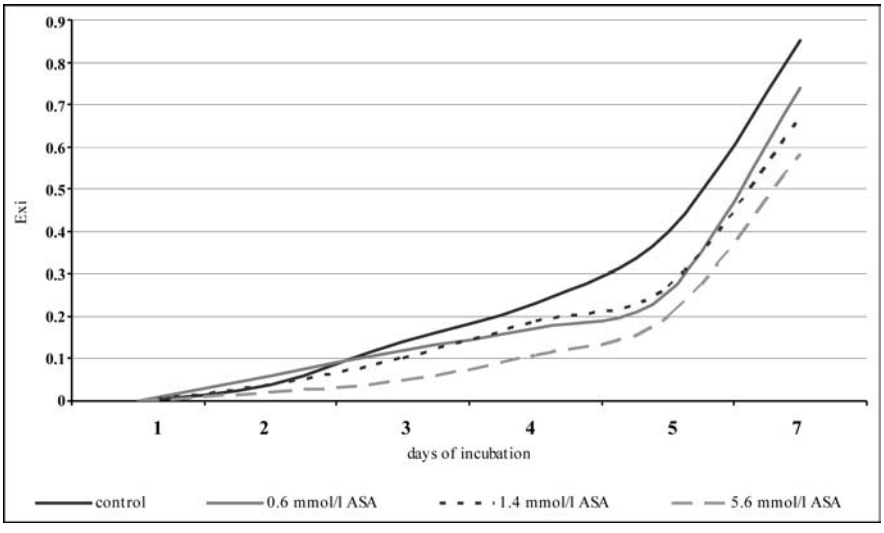

Fig. 5 Inhibition of exsheathment by ASA using the pH-change assay. Differences were significant ($P \leq 0.5$) for 1.4 and 5.6 mmol compared to the control and to 0.6 mmol/I ASA at the end of the trial

$Na_2B_4O_7$ does not induce exuveation in *O. dentatum* (unpublished observations). The aim of our study was to further characterise physical and biochemical parameters in vitro that might influence ecdysis and consequently establishment and development of this parasite in vivo.

Physical parameters of exsheathment

Exuveation with sodium hypochlorite does not alter larval viability in comparison to that induced under physiological conditions (Eckert 1967). Our observations show that the uvea of *O. dentatum* is not simply lysed by the action of hypochlorite, but that the larva actively leaves it through a predetermined opening close to the apical cap, similar to exsheathed as a physiological process (Silverman and Podger 1964). While ecdysis induced by hypochlorite is accomplished within 5 min, the pH-change assay took several days to finish and was not as effective. The reasons for this are unknown; most likely other factors present in the gut add to the effect of the pH change that could not be mimicked in vitro. However, both tests are suitable for bioassaying.

Biochemical parameters of exsheathment

We were able to show that sodium hypochlorite-induced exsheathment can be reduced by inhibitors of eicosanoid synthesis. It is difficult to determine the possible modes of action, since the metabolism of eicosanoids and their precursors (arachidonic acid and other long-chained polyunsaturated fatty acids) appear to vary substantially between different organisms (Frandsen and Bone 1989). Consequently, the concentrations of inhibitors may differ greatly between mammalian and nematode systems. Parasite-derived eicosanoids that are released by nematodes at high concentrations (for references see Daugschies and Joachim 2000) are not available (and probably not necessary) for intrinsic functions, therefore, inhibition of their production can, at least initially, be compensated for by reduced release. Thus, high concentrations of inhibitors might be necessary to produce appreciable effects. Lasting harmful effects of the inhibitors were not seen in our system since the inhibition was reversible. However, LTC_4 and LTE_4 at higher doses appeared to be toxic for the larvae, since the level of exsheathment inhibition was similar to that of DEC itself. DEC was formerly used as an anthelmintic against filarial and strongylid infections and may act as inhibitor of endogenous parasitic LOXs, although the mode of action is not known and DEC has no filaricidal effect in vitro (reviewed by Daugschies and Joachim 2000). It is a LOX inhibitor that can also inhibit COX and other enzymes non-specifically. The inhibitory effect of DEC was abrogated by substitution of leukotrienes at high concentrations, indicating that the action of DEC was both specific (inhibition of LOX) and reversible.

Another enzyme possibly involved in this process is leucine aminopeptidase (LAP) which can acts as a dipeptidase in the synthesis of LT and was also implemented in exsheathment of *H. contortus* (Sommerville 1957), although it could not be demonstrated histochemically in *Oesophagostomum radiatum* (Douvres and Thompson 1973). The actual role of LAP in ecdysis remains controversial (Daugschies and Joachim 2000).

Similarly to DEC, ASA also had an inhibitory effect on exsheathment in high concentrations in both the hypochlorite and the pH-change assay, and the addition of leukotrienes could neutralise its inhibitory effects, whereas prostaglandins had no effect on ASA-induced inhibition. Since ASA is a specific COX inhibitor in mammalian cells, our results indicate that in *O. dentatum* other metabolic pathways involving leukotrienes may be susceptible to ASA.

In general, it can be assumed that leukotrienes, but not prostaglandins, play a role in the ecdysis of *O. dentatum*. This is in contrast to studies on the role of eicosanoids in the migration and growth of *O. dentatum*, biological functions which are both reduced by inhibition of prostaglandin production (Daugschies 1995, 1996; Daugschies and Ruttkowski 1998).

Further studies should shed more light on the role of eicosanoids and related compounds in the biology of nematodes and, ultimately, provide insight into possible mechanisms of developmental interruption of parasite development.

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