In vitro cultivation of *Hysterothylacium aduncum* (Nematoda: Anisakidae) from 3rd-stage larvae to egg-laying adults

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SUMMARY

This is the first demonstration of the *in vitro* development of the 3rd-stage larvae (L3) of *Hysterothylacium aduncum* to the adult. This was achieved in a semi-defined medium that is easy to prepare and to reproduce. The L3, collected from the peritoneal cavity of horse mackerel (*Trachurus trachurus*), were individually inoculated into RPMI-1640 medium +20% heat-inactivated fetal bovine serum (IFBS). It has been demonstrated that the optimum temperature for development is around 13 °C and is stimulated by the presence of 5% CO_2 in the growth atmosphere, increasing the percentage moulting to the 4th larval stage (L4) by 1·9-fold (from 44 to 82%) and the average survival of the nematodes by 1·6 times (from 60 to 96 days). When the larvae were grown at different pHs, optimum development occurred at pH 4·0. Under these conditions, all the larvae moulted to the L4 and more than two-thirds transformed to the adult stage – in which 25–30% of the females laid eggs – and reached an average survival of over 4 months. When this medium was supplemented with 1% (w/v) of commercial pepsin, all the larvae reached the adult stage, at least 45% of the females oviposited, laying around 12-fold more eggs per female than in the medium without pepsin. The mean size of the eggs (non-fertilized) obtained was $56{\cdot}8 \times 47{\cdot}6$ μm. The mean length of the adult males obtained was between 3·2 and 5·2 cm and the females were between 3·0 and 6·5 cm. The adult specimens were morphologically identified as *Hysterothylacium aduncum aduncum*. This culture medium (RPMI-1640+20% (v/v) IFBS+1% commercial pepsin, at pH 4·0, 13 °C and 5% CO_2 in air) could facilitate the identification of at least some of the larvae of the genus *Hysterothylacium* – and perhaps other anisakids – for which the specific identification and the biological study of these parasites is often difficult.

Key words: fish parasite, Anisakidae, *Hysterothylacium*, *in vitro* cultivation, survival, moulting, pepsin.

INTRODUCTION

Hysterothylacium aduncum is a widely distributed species, which parasitizes teleost fishes worldwide including some freshwater species (Moravec, Nagasawa & Urawa, 1985; Yoshinaga, Ogawa & Wakabayashi, 1987). Several authors have demonstrated the presence of these nematodes in fish frequently consumed by humans (Huang, 1988; Petter & Maillard, 1988; Sanmartín, Quinteiro & Ubeira, 1989; Ruiz-Valero *et al*. 1992; Andersen, 1993; González & Carvajal, 1995; Adroher *et al*. 1996; Navone, Sardella & Timi, 1998; Moravec & Nagasawa, 2000; Valero *et al*. 2000).

To date, 3 different types of anisakidosis have been recognized in relation to the causal agents involved: anisakiosis (caused by *Anisakis*), pseudoterranovosis (caused by *Pseudoterranova*) and contracaecosis (caused by *Contracaecum*). The implication of *Hysterothylacium* spp. as a potential causal agent of anisakidosis has been widely discussed (Petter, 1969; Vermeil *et al*. 1975; Norris & Overstreet, 1976; Overstreet & Meyer, 1981; Huang, 1988). Yagi *et al*. (1996) have described for the first time a case of non-invasive anisakidosis, by consumption of raw fish, caused by an immature adult of *H. aduncum*. Moreover, it seems that this parasite is also responsible for allergic reactions when ingested with the fish (Fernández-Caldas *et al*. 1998).

González & Carvajal (1995) and González (1998) have revealed that this nematode is a common parasite, with a growing incidence in salmon cultured on the Chilean coast. Although, these authors do not consider this nematode to generate severe clinical conditions in the fish they parasitize, they do believe that the stress induced in parasitized fish can predispose them to colonization by other more pathogenic organisms, reducing the yield of these marine cultures. Moreover, a pathogenic effect of the larvae of *H. aduncum* on herring larvae (*Clupea harengus*) has been reported and also on cod (*Gadus morhua*) resulting in high mortality in the former (Balbuena *et al*. 2000; Karlsbakk *et al*. 2001). From these data, one can imagine the huge economic impact that this parasite can have. Therefore, *in vitro* maintenance and development of *H. aduncum* is a

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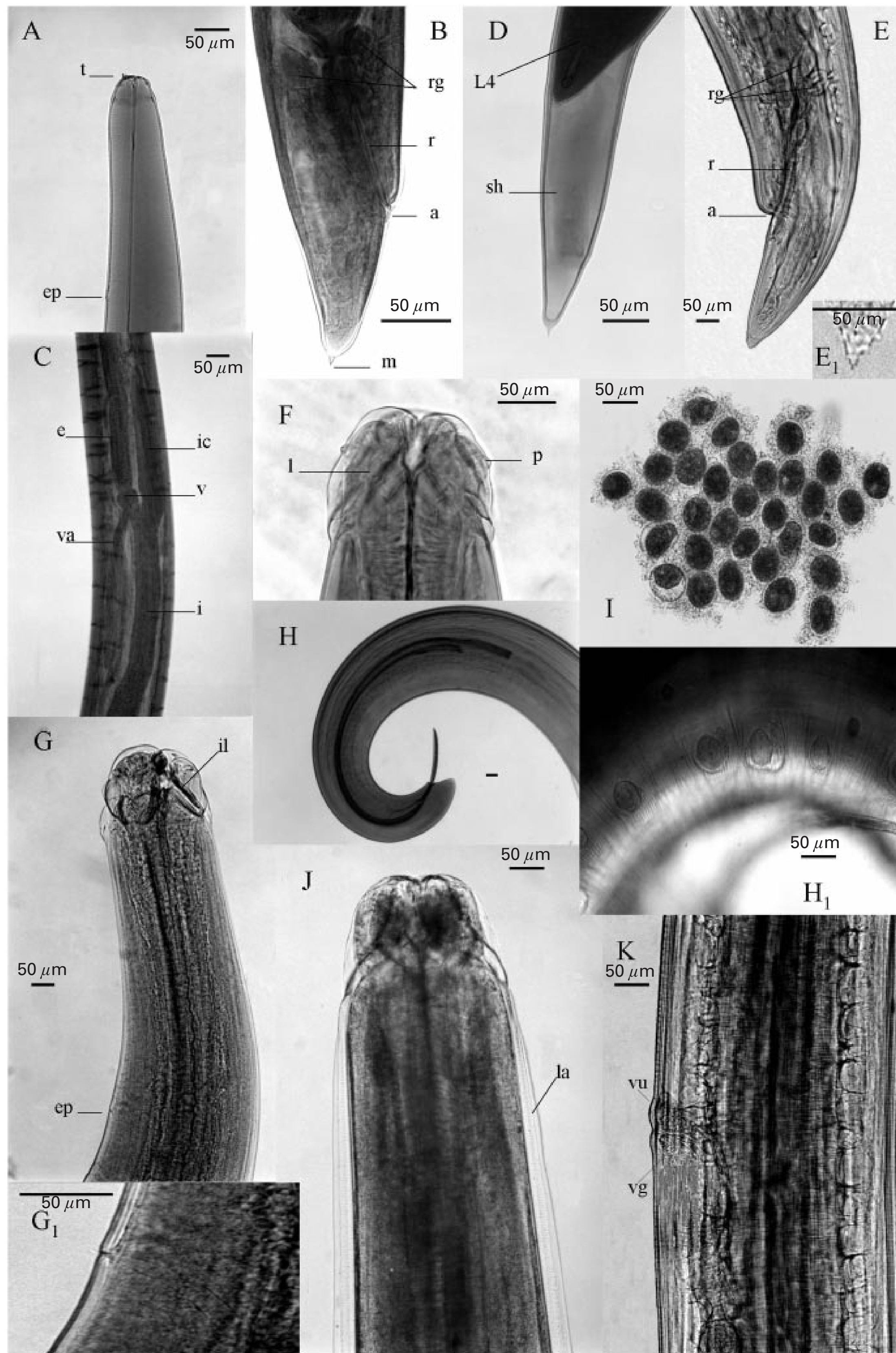

Fig. 1. *Hysterothylacium aduncum* from fish (L3) and culture (L4 and adults). (A) Anterior and (B) posterior ends of L3. (C) Intestinal caecum and ventricular appendage of L3. (D) Posterior end of larvae during M3. (E) Posterior (detail in E1) and (F) anterior extremities of L4. (G) Anterior end of adult showing the interlabia and excretory pore (detail in G1). (H) Posterior end of adult male showing the spicules. (H1) Detail showing pre-anal papillae. (I) Cluster of non-fertilized eggs laid by an adult female. (J) Anterior part of adult showing the lips and lateral alae. (K) Detail of the vulva and vagina of an adult female. a, anus; e, oesophagus; ep, excretory pore; i, intestine; ic, intestinal caecum; il, interlabium; l, lip; la, lateral alae; m, mucron; p, papilla; r, rectum; rg, rectal glands; sh, sheath; t, tooth; v, ventricule; va, ventricular appendage; vg, vagina; vu, vulva.

useful tool to study the different larval stages and the adult of this species. This will facilitate morphological identifications where required and help to understand the different aspects of their biology and to develop effective control measures in cultured fish populations. To date, *H. aduncum* has only been maintained *in vivo* (Yoshinaga *et al*. 1987; Køie, 1993; Balbuena *et al*. 1998). This technique is complex, requiring maintenance of the hosts in fish tanks in the laboratory and their correct handling and infection. We have established *in vitro* culture conditions in a simple growth medium that is easy to prepare with commercial products, which allow development of egg-laying adults from 3rd-stage larvae collected from fish.

MATERIALS AND METHODS

The worms selected for our study were 3rd-stage larvae (L3) of *H. aduncum* (Rudolphi, 1802) Deardorff & Overstreet, 1981 isolated from the host *Trachurus trachurus* L. 1758 (horse mackerel), family Carangidae, purchased from the fish market of Granada (Southern Spain). The horse mackerel, on the Atlantic and Mediterranean Spanish coasts, is frequently parasitized by *H. aduncum* (Adroher *et al*. 1996). The worms, found free in the host body cavity, were 8 mm or more in length, and were collected with the help of a needle with a blunt tip, placed on a Petri dish with 0·9 % NaCl solution and washed in it several times. The worms were observed individually under an inverted microscope and those which showed any kind of internal or external damage were discarded. They were then identified according to morphological features (Yoshinaga *et al*. 1987; Petter & Maillard, 1988; Petter & Cabaret, 1995). Over 500 L3 were used in this study.

Prior to cultivation, each larva was placed in an antibiotic–antifungal solution and axenized as described elsewhere (Iglesias, Valero & Adroher, 1997; Iglesias *et al*. 2001). Worms were cultured individually on a sterile polystyrene 24-well tissue-culture plate. The culture medium (1 ml) was placed into each well with 1 parasite. The culture plates were then placed in an incubator at 13 °C and 5 % CO_2 in humid air (except when indicated), and the culture medium was renewed twice a week. The worms were observed daily for mobility, moulting and survival.

The culture medium (RPMI) was RPMI-1640 plus 20 % (v/v) heat-inactivated fetal bovine serum (IFBS). The worms were cultivated at several pHs. To the medium, where indicated, pepsin, trypsin, L-cysteine (4 mM) or glutathione (4 mM) were added.

The terms 'maximum survival' (S_{max}), 'survival 50' (S_{50}), and 'average survival' (S_{av}) in culture have been defined by us (Iglesias *et al*. 1997, 2001) as follows: S_{max} is the day the last living nematode in the experiment dies. S_{50} is the day on which 50 % of nematodes in the experiment are dead and S_{av} is the arithmetic mean day of death of each nematode in the experiment. The data are expressed as the mean ± standard error (S.E.). 'L5-survival' (S_{L5}) is the arithmetic mean of the days that worms survive after the 4th–5th-stage moult (M4). $M4_{av}$ is the arithmetic mean of the culture day on which the M4 of each nematode in the experiment is completed. The data are expressed as the mean ± S.E. *L4* indicates the larvae that completed M3 (i.e. the 3rd to 4th-stage moult), and *L5* the larvae that completed M4 to adulthood. A statistical comparison of the culture data was made using the Student's *t*-distribution. The survival and moulting curves were analysed using the Cox regression.

The media, sera and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Bio-Whittaker (Walkersville, MD, USA). Commercial pepsin was obtained from Probus (Barcelona, Spain). This commercial pepsin was analysed by SDS–PAGE (Laemmli, 1970). Protein concentration was determined according to Lowry *et al*. (1951).

RESULTS

The nematodes collected from the abdominal cavity of *T. trachurus* were identified as 3rd-stage larvae of *H. aduncum* (Fig. 1) (Yoshinaga *et al*. 1987; Petter & Maillard, 1988; Petter & Cabaret, 1995).

The first phase of testing of culture conditions on larval survival was a comparison of different culture media at different temperatures. The best survival rates and percentage moulting to L4 occurred in RPMI medium at 13 °C. At higher and lower temperatures, survival and development were reduced and at 37 °C larvae only survived for a few hours (results not shown).

In an attempt to determine the effect of CO_2 on nematode development, larvae were kept in different media in an atmosphere enriched with 5 % CO_2. The percentages of M3 were greater in the presence of CO_2 (Table 1). In the RPMI medium, under the same conditions, there was more development and the M3 occurred in 14 of the L3 (more than 80 %) between days 4 and 21 and the M4 of 3 L4 (17·6 % of initial larvae; 1 male and 2 females) between days 34 and 39. Survival and moulting were notably higher in RPMI + CO_2 than in the other conditions and media tested (Table 1). As a consequence, all the following tests were performed in RPMI medium and in an atmosphere enriched with 5 % CO_2.

At the 4 different pHs tested, it was observed that more than 66 % of L3 moulted to L4. The moult of the L4 to L5 (M4) usually occurs (> 67 % of larvae) at pH 4·0 and only occasionally (< 18 % of larvae) at pH 7·2 and did not occur at either pH 2·0

Table 1. Influence of CO_2 on M3 and survival of *Hysterothylacium aduncum* larvae incubated in different media

(nL3, nL4 and nL5: number of larvae in 3rd, 4th and adult stage, respectively. Survival rates (maximum survival S_{max}, survival 50 S_{50}, average survival S_{av}, and L5-survival S_{L5}) as defined in Materials and Methods section. Results of survival rates are expressed as mean of culture days ± standard error (S.E.).)

Medium†	nL3‡	Atmosphere	S_{av} ± S.E.§	S_{50}	S_{max}	nL4 (%)‖	nL5 (%)‖	S_{L5} ± S.E.
SS	25	Air	6·6 ± 0·3	7	10	0	–	–
SS	24	Air + 5% CO_2	10·4 ± 0·7*	9	17	1 (4·1%)	0	–
PBS	28	Air	10·9 ± 0·8	10	21	3 (10·7%)	0	–
PBS	26	Air + 5% CO_2	10·9 ± 0·8$^{N.S.}$	10	23	9 (34·6%)	0	–
RPMI	16	Air	59·6 ± 8·4	48	122	7 (43·7%)	0	–
RPMI	17	Air + 5% CO_2	95·5 ± 12·2*	105	182	14 (82·3%)	3 (17·6%)	105·0 ± 5·3

† SS, 0·9% NaCl solution; PBS, phosphate-buffered saline, pH 7·2; RPMI, RPMI-1640 + 20% IFBS, pH 7·2.
‡ Initial number of parasites.
§ Statistical comparison with the same medium without/with CO_2 using Student's *t*-distribution: * $P < 0.02$; (N.S.) not significant.
‖ Percentage of L3 that reached L4 or L5 stage.

Table 2. Effect of pH on *in vitro* moulting and survival of *Hysterothylacium aduncum* larvae

(nL3, nL4 and nL5: number of worms in 3rd larval, 4th larval or adult stage, respectively. Survival and moulting rates (average survival S_{av}, maximum survival S_{max}, survival 50 S_{50}, and L5-survival S_{L5}) as defined in Materials and Methods section. Results of survival rates are expressed as mean of culture days ± standard error (S.E.).)

Medium†	nL3‡	S_{av} ± S.E.§	S_{50}	S_{max}	nL4 (%)‖	nL5 (%)‖	S_{L5} ± S.E.§
RPMI, pH 2·0	12	32·5 ± 4·9**	33	58	8 (66·6%)	0	–
RPMI, pH 4·0	25	123·9 ± 9·9	116	220	25 (100%)	17 (68%)	111·8 ± 11·8
RPMI, pH 7·2	24	92·5 ± 9·3*	90	190	18 (75%)	3 (12·5%)	93·0 ± 9·5$^{N.S.}$
RPMI, pH 9·0	12	26·5 ± 3·5**	29	40	9 (75%)	0	–

† RPMI-1640 + 20% (v/v) IFBS adjusted at pH indicated.
‡ Initial number of parasites.
§ Statistical comparison with the medium at pH 4·0, using Student's *t*-distribution: * $P < 0.05$; ** $P < 3 \times 10^{-6}$; (N.S.) not significant.
‖ Percentage of L3 that reached L4 or L5 stage.

or pH 9·0. Approximately 25% of females oviposited. Moreover, all the survival parameters determined were higher at pH 4·0 (see Table 2).

In its development from L3 to adult, *H. aduncum* is to be found in the stomach, pyloric caeca and intestine of the definitive host (Deardorff & Overstreet, 1981; Moravec *et al.* 1985; González & Carvajal, 1994; Navone *et al.* 1998; Moravec & Nagasawa, 2000). Since the L3 encounters different pHs in the different regions of the digestive tract of the fish, we designed an experiment in which we cultured larvae in medium at pH 4·0 until their moult to L4, after which they were transferred to a medium with pH 7·2 and vice versa, i.e. L3 at pH 7·2 and L4 at pH 4·0. Larvae L3 cultured at pH 4·0 and pH 7·2 moulted to L4. Those L4 transferred from pH 4·0 to pH 7·2 did not develop further. Of these L4 transferred from pH 7·2 to pH 4·0, 70·6% moulted to the L5 with an S_{L5} of 82·5 days, and with approximately 30% of females ovipositing (results not shown).

In the following tests, we attempted to simulate the reducing conditions of the gastrointestinal habitat by adding L-cysteine or glutathione. Both substances, added individually, reduce the S_{av} of the nematodes to half or less and also the S_{L5} (differences were significant in all cases; see Table 3). In relation to the moults, M3 does not seem to be affected but M4 is inhibited by almost 90% in the presence of glutathione. However, this inhibition is reverted largely by addition to the culture medium of 1% (w/v) of commercial pepsin (Table 3).

The presence of proteolytic activity in the gastrointestinal tract of the fish was simulated by adding trypsin or pepsin, digestive proteases active at neutral and acid pHs, respectively. The addition of trypsin (Sigma Chemical Co.) to the culture medium at pH 7·2 (Table 4) led to a reduction in M3 corresponding to a 63·6% inhibition of moulting at a concentration of 0·01% of trypsin accompanied by a significant reduction of the survival parameters (S_{av} from 91·5 to 56·9 days; $P < 0.01$).

Table 3. Influence of glutathione (G) and L-cysteine (C) on *in vitro* culture of *Hysterothylacium aduncum* larvae at pH 4·0

(nL3, nL4 and nL5: number of worms in 3rd larval, 4th larval or adult stage, respectively. Survival and moulting rates (average survival S_{av}, maximum survival S_{max}, survival 50 S_{50}, L5-survival S_{L5}, and average M4 $M4_{av}$) as defined in Materials and Methods section. Results of survival rates and $M4_{av}$ are expressed as mean of culture days ± standard error (S.E.). N.D., Not determined.)

Medium†	nL3‡	S_{av} ± S.E.§	S_{50}	S_{max}	nL4 (%)‖	$M4_{av}$ ± S.E.§	nL5 (%)‖	S_{L5} ± S.E.§
RPMI	10	137·0 ± 16·1	113	249	10 (100 %)	40·2 ± 5·9	8 (80·0 %)	103·0 ± 16·6
RPMI + G	12	74·3 ± 14·6**	47	164	11 (91·6 %)	N.D.	1 (8·3 %)	16·0
RPMI + C	11	70·1 ± 8·7**	61	114	11 (100 %)	N.D.	7 (63·6 %)	40·5 ± 11·8*
RPMIP	23	160·5 ± 12·7	150	279	23 (100 %)	23·1 ± 0·6****	23 (100 %)	124·5 ± 11·9
RPMIP + G	21	64·5 ± 6·3***	63	143	18 (85·7 %)	N.D.	14 (66·6 %)	37·5 ± 7·5***
RPMIP + C	21	75·8 ± 6·9***	74	141	21 (100 %)	N.D.	18 (85·7 %)	52·6 ± 6·6***

† RPMI-1640 + 20 % IFBS (RPMI) adjusted at pH 4·0 added with glutathione (G) or L-cysteine (C). RPMIP (pH 4·0) contains 1 % (w/v) commercial pepsin.
‡ Initial number of parasites.
§ Statistical comparison using Student's *t*-distribution: * $P < 0·02$; ** $P < 1 \times 10^{-5}$ to compare with RPMI medium; *** $P < 2 \times 10^{-5}$ to compare with RPMIP medium. **** $P < 0·01$ to compare RPMI/RPMIP media.
‖ Percentage of L3 that reached L4 or L5 stage.

Table 4. Effect of trypsin (T) and pepsin (P) on the *in vitro* culture of *Hysterothylacium aduncum* larvae

(nL3, nL4 and nL5: number of worms in third larval, fourth larval or adult stage, respectively. Survival and moulting rates (average survival S_{av}, maximum survival S_{max}, survival 50 S_{50}, L5-survival S_{L5}, and average M4 $M4_{av}$) as defined in Materials and Methods section. Results of survival rates and $M4_{av}$are expressed as mean of culture days ± standard error (S.E.). N.D., Not determined.)

Medium†	nL3‡	S_{av} ± S.E.§	S_{50}	S_{max}	nL4 (%)‖	$M4_{av}$ ± S.E.§	nL5 (%)‖	S_{L5} ± S.E.§
RPMI, pH 7·2	12	91·5 ± 10·6	77	190	10 (83·3 %)	–	0	–
RPMI + 0·01 %T	11	56·9 ± 5·8*	59	93	4 (36·3 %)	–	0	–
RPMI + 0·0025 %T	11	67·9 ± 5·6$^{N.S.}$	74	101	10 (90·9 %)	N.D.	1 (9·1 %)	15·0
RPMI, pH 4·0	12	134·3 ± 10·1	119	195	12 (100 %)	27·8 ± 1·6	12 (100 %)	105·6 ± 10·0
RPMI + 0·01 %P	8	114·8 ± 10·2	101	183	8 (100 %)	N.D.	8 (100 %)	78·7 ± 15·1
RPMI + 0·0025 %P	12	111·0 ± 9·7	101	180	12 (100 %)	N.D.	12 (100 %)	85·2 ± 9·0
RPMIP	11	130·2 ± 9·8	112	200	11 (100 %)	22·6 ± 1·8**	11 (100 %)	107·6 ± 9·2

† RPMI-1640 + 20 % IFBS adjusted at pH 4·0 for media with purified pepsin (%P) or 7·2 for media with purified trypsin (%T). RPMIP (pH 4·0) contains 1 % (w/v) commercial pepsin.
‡ Initial number of parasites.
§ Statistical comparison using Student's *t*-distribution: (*) Media at pH 7·2: RPMI is only statistically different with the medium with 0·01 % trypsin, * $P < 0·01$. (**) Media at pH 4·0: there are not significant differences statistically in the survival parameters, but $M4_{av}$ shows significant differences, ** $P < 0·05$.
‖ Percentage of L3 that reached L4 or L5 stage.

The addition of 1 % commercial pepsin (Probus) to the RPMI growth medium at pH 4·0 (RPMIP) improved the survival parameters, 100 % of M3 and M4 were obtained, and the $M4_{av}$ was reduced from 28 to 23 days ($P < 0·05$) (see Table 4 and Fig. 2). Moreover, the percentage of egg-laying females increased and also the mean number of eggs laid by the females rose from 2238 in medium without pepsin to 27015 in medium with commercial pepsin. When these experiments were repeated at different concentrations of purified pepsin (Sigma Chemical Co.) the results were more similar to those obtained with commercial pepsin as the concentration of purified pepsin used increased. Electrophoretic analysis of commercial pepsin produced a single band of protein that coincided with that of purified pepsin.

In the experiments carried out with RPMIP medium, all the individuals moulted to L5 between days 12 and 35 of culture. A total of 16 individuals were females and 18 males. A total of 7 females oviposited (45 % of the females), 6 of these started the oviposition between days 46 and 98 of culture and 1 at 220 days. The most prolific female started ovipositing on day 63, 41 days after the moult to L5 and a total of 70980 eggs were counted in an interval of 117 days. The mean size ± S.E. of the eggs obtained is $56·8 \pm 0·6 \times 47·6 \pm 0·5$ μm ($n = 50$). The mean length ± S.E. of the adults obtained in the RPMIP medium was 4·0 ± 0·3 (3·2–5·2) cm for the males and 4·1 ± 0·6 (3·0–6·5) cm for the females (Fig. 1). The adult specimens were identified as *Hysterothylacium aduncum aduncum*, in accordance with Petter &

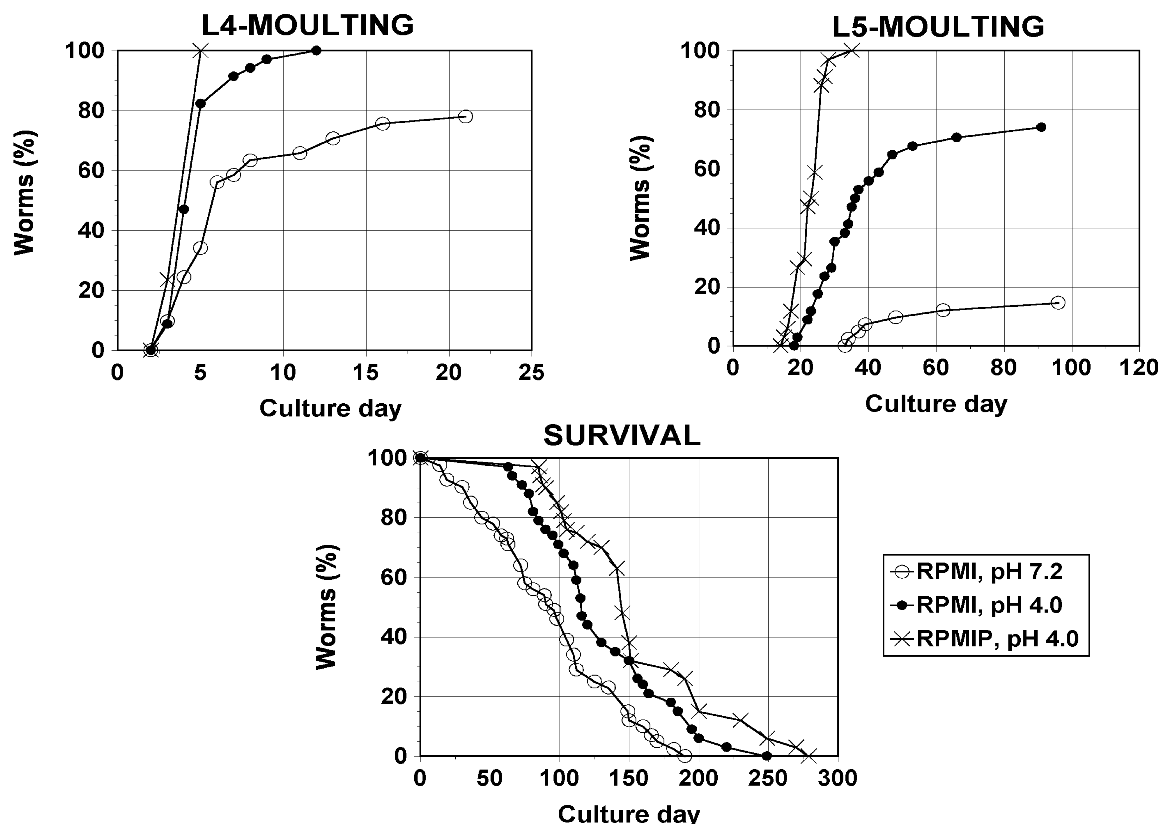

Fig. 2. Comparative development of larvae of *Hysterothylacium aduncum* cultured on RPMI-1640 plus 20% IFBS (RPMI) at pH 7·2, and at pH 4·0 without or with 1% commercial pepsin (RPMIP). For all these experiments 110 worms were used. The survival and moulting curves were analysed by the Cox regression (at confidence 95%) showing the significant influence of pH on L4-moulting ($P = 0·0008$), L5-moulting ($P < 0·0001$) and survival ($P = 0·0031$) curves and the pepsin on the L5-moulting ($P < 0·0001$) curve.

Cabaret (1995). Finally, the statistical Cox regression analysis of the *H. aduncum* survival and moulting curves demonstrated the significant influence of the pH 4 on the 3 curves and of the pepsin on the L5-moulting curve (see Fig. 2).

DISCUSSION

For the survival and development of *H. aduncum in vitro* the correct culture temperature was essential, around 13 °C, a temperature common for many seas where a mixture of the hosts of these cosmopolitan parasites are found. In the *in vitro* culture of *H. aduncum*, atmospheric CO_2 stimulates ecdysis to L4, since this occurs with greater synchrony and in a larger percentage than in media without CO_2. As expected, the best survival and moulting results are achieved in the nutritive medium RPMI, in which occasionally some L5 are produced, always in the presence of CO_2. Although *H. aduncum* can also develop in the absence of CO_2, its presence stimulates development of these parasites as also occurs in other anisakids such as *Anisakis simplex* (Sommerville & Davey, 1976; Mercer *et al*. 1986; Iglesias *et al*. 1997 and 2001). This could be related to the high levels of HCO_3^- in the intestinal fluids of many fish (Grosell *et al*. 2001).

Although the precise localization of the L4 and the adults of *H. aduncum* inside the final fish hosts has not yet been clearly established, it seems that these can be found in any region of the host's digestive tract (stomach, pyloric caeca and intestine) (Deardorff & Overstreet, 1981; Moravec *et al*. 1985; González & Carvajal, 1994; Navone *et al*. 1998; Moravec & Nagasawa, 2000). The pH of the different regions differs in the fish, as also occurs in other vertebrates, from an acid pH in the stomach (pH ~ 3·0), to a neutral pH (~ 7) in the pyloric caeca, to an increasingly alkaline pH (pH ~ 7·8–8·5) progressing along the intestine towards the rectum. It therefore appears that this nematode has a great tolerance to pH since it developed to L4 and survived for longer than 1 month at extreme pH values. Culture at pH 4·0 and 7·2 notably favoured parasite development resulting in formation of the adults and higher S_{av} than at 3 months. However, at pH 4·0 the worms moult earlier, higher values are obtained for all moulting and survival parameters and more than 25% of the females can lay eggs (egg-laying females were not obtained at any other pH), demonstrating that pH 4·0 is the most favourable for development

of these parasites. On the other hand, moulting of the nematodes to L4 occurs in a largely unsynchronized manner. This phenomenon could be associated with the different degree of maturity that larvae can present when collected from the host, although an attempt was made to collect specimens of a similar length always 8 mm or more. The direct association between length of L3 and their subsequent development has already been demonstrated for another anisakid, *A. simplex* (Iglesias *et al*. 1997).

Glutathione and L-cysteine are chemical agents capable of simulating the reducing conditions of vertebrates' gastrointestinal tracts. However, none of these supplements managed to improve the survival parameters or the rate of moulting to L5, as also occurred previously with *A. simplex* (Iglesias *et al*. 2001), cetaceans parasite. Although in a previous study, Likely & Burt (1992) achieved an improved rate of development in *Contracaecum osculatum*, pinnipeds parasite, by adding L-cysteine to the culture medium. However, the differences between the hosts and, therefore, the habitat of these 3 anisakids must be taken into consideration.

Localization of the L4 and adults of *H. aduncum* throughout the gastrointestinal tract of its hosts also implies a tolerance or resistance to the different enzymes present there. In this respect, maintenance of the parasite in the presence of trypsin (at pH 7·2) or pepsin (at pH 4·0) did not produce any deterioration of its cuticle visible by optical microscope nor did it notably affect its development in comparison to controls, except when trypsin is added at 0·01 % leading to a reduction in M3 and, as a result, also in survival of the nematodes.

Addition of commercial pepsin to the culture medium led to an improvement in moulting percentage to L5, to 100 %, improved synchrony of M3 (occurring between days 3 and 8 of culture instead of between days 3 and 12) and an increase in the percentage of egg-laying females and the number of eggs laid by them.

Since pepsin is a protease capable of digesting proteins in an acidic environment, it could act by breaking down serum proteins present in the medium permitting improved protein assimilation by the parasite and/or releasing certain active peptides that could act as stimuli for the parasite. On the other hand, it has also been demonstrated that the proteases play an important role in the ecdysis of some nematodes, as they are involved in the digestion processes of the old cuticle, enabling it to be expelled (Rogers, 1970; Matthews, 1982; Lustigman *et al*. 1996; Rhoads, Fetterer & Urban, 1997).

At pH 4·0, the oviposition occurs asynchronically ranging from 13 to 192 days after the moult to L5. In media containing pepsin, more eggs are usually detected, suggesting that pepsin stimulates the egg-laying potential of the females and/or their complete maturation. The eggs are a similar size as those described by other authors (Moravec *et al*. 1985; Moravec & Nagasawa, 2000).

Finally, the length of the adults obtained *in vitro* indicates that the *in vitro* maintenance conditions used are suitable for parasite development and even comparable to *in vivo* conditions. For example, the total length of the males ranges between 32 and 52 mm, and the females between 30 and 65 mm, always within the range of lengths reported by different authors for adults collected from the intestinal tracts of different host fish (e.g. Moravec *et al*. 1985; Andersen, 1993; Petter & Cabaret, 1995; Moravec & Nagasawa, 2000). Experimental infection of fish (*Salmo gairdneri*) has also led to the development – 2 months after infestation – of adults of similar sizes (♂ 24–43 mm and ♀ 36–51 mm) to those obtained in natural infections (Yoshinaga *et al*. 1987).

On the basis of the results obtained we can hypothesize that the L3 ingested by the fish with its prey, remain free inside the digestive tract after gastric digestion and, if they have a sufficient degree of development, will remain in the stomach to facilitate their development to sexually mature adults (acid pH and presence of pepsin). They, in principle, can wander around different regions of the digestive tract where the fertilized females can lay their eggs so that these are shed to the exterior with the host's faeces. If the L3 in the stomach have not reached a suitable degree of development, they can pass through to the intestine (where the neutral–alkaline pH and trypsin inhibit their moulting) and cross the intestinal mucosa to the mesentery and abdominal cavity, areas more frequently inhabited by the L3. The L3 in these regions can mature and, either in this host or in a new host if this has been preyed on, return to the stomach and complete their development to sexually mature adults (Andersen, 1993), as in other species of the genus *Hysterothylacium* (Yoshinaga, Ogawa & Wakabayashi, 1989; Rye & Baker, 1992). In any case, there are still many aspects of the developmental cycle of *H. aduncum* that remain to be clarified and require further research.

It is interesting that the medium used here is also suitable for the development of other anisakids such as *A. simplex* (Iglesias *et al*. 2001). This indicates a physiological similarity between both parasites in spite of obvious differences in their life-cycles, and the possible usefulness of this medium, at a suitable temperature, for other anisakids.

In summary, the effect of pH, CO_2 and pepsin on development of *H. aduncum* has been studied and a simple growth medium has been developed with commercial products that is easy to prepare and in which L3 of *H. aduncum* can develop to the sexually mature adult. This can enable a precise identification of the parasite based on morphological characteristics of the adult. The research we are carrying out in our laboratory is aimed at completing the life-cycle of this anisakid *in vitro*.

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