In vitro cultivation of *Anisakis simplex*: pepsin increases survival and moulting from fourth larval to adult stage

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SUMMARY

This paper describes the *in vitro* cultivation of the 3rd-larval stage (L3) of *Anisakis simplex* to adulthood in a much simpler and easier to prepare medium than those described to date. The adult males obtained are between 3·8 and 6·5 cm long and the females between 4·5 and 8·0 cm. Some individually cultivated females laid eggs which had an average size of $44·4 \times 50·5$ μm. The culture conditions were as follows: medium RPMI-1640 supplemented with 20 % heat-inactivated fetal bovine serum and 1 % commercial pepsin, at pH 4·0 and a temperature of 37 °C, and in air atmosphere with 5 % CO_2. The pepsin was found to be the key to the success of the culture. The average survival of the worms in the culture increased from 50 to 88 days, due to the fact that the survival of the adults practically doubled (increasing by 1·9 times). Furthermore, the number of worms that completed the 4th moulting (M4) increased by 4·2 times, from 22·9 to 95·6 %. This culture medium may facilitate, due to its simplicity, the study of anisakids, or at least of *A. simplex*, constituting another step towards achieving a complete *in vitro* life-cycle for these parasites.

Key words: Anisakiosis, *Anisakis simplex*, *in vitro* cultivation, pepsin, moulting, survival.

INTRODUCTION

Anisakis simplex is a parasitic nematode of marine mammals, which infects a large variety of fish, among other hosts, during its larval stage. These parasitized fish may be ingested by humans, thus releasing the parasite larvae into the stomach, possibly resulting in anisakiosis if the fish were not adequately cooked.

In vitro cultivation of anisakids has had some success, but is difficult to carry out due to the fact that the culture media used to date are complex as regards both their composition and their preparation (Townsley *et al*. 1963; Van Banning, 1971; Grabda, 1976; Carvajal *et al*. 1981; Likely & Burt, 1989, 1992),

Our aim was to develop a culture medium that was more defined in its composition and simpler to prepare, to make it easier to obtain adult parasites. In this paper, the defined medium used, supplemented with fetal bovine serum and pepsin, allowed *in vitro* development of the 3rd larval stage (L3) of *Anisakis simplex* s.l. Rudolphi, 1809 to mature adults.

MATERIALS AND METHODS

The worms selected for our study were 3rd-stage larvae of *Anisakis simplex* s.l. Rudolphi, 1809 isolated from the host *Micromesistius poutassou* Risso, 1826

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(blue whiting), family Gadidae, purchased from the fish market of Granada (Southern Spain). The blue whiting, on the Atlantic and Mediterranean Spanish coasts, is frequently parasitized by *A. simplex* s.l. (Ruiz-Valero *et al*. 1992; Valero *et al*. 2000). The worms, found free in the host body cavity, were 20 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 (Hartwich, 1974; Petter & Maillard, 1988). Over 800 L3 were used in this study. Forty L3 (ca. 0·5 % of total) of *A. simplex* s.l. from the same hosts wee preserved at -70 °C for electrophoretic identification purposes. These larvae were analysed individually by isoenzyme electrophoresis using thick starch gel in a continuous buffer system (Nascetti *et al*. 1986; Martín-Sánchez, Paniagua & Valero, 1998; Iglesias, Martín-Sánchez, Adroher & Valero, unpublished observations).

Prior to cultivation, each larva was individually placed in an antibiotic-antifungal solution and axenized as described elsewhere (Iglesias, Valero & Adroher, 1997). Worms were cultured 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 37 °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). To the medium, where indicated, IFBS (40% v/v), horse blood (1% v/v), pepsin (1% p/v), L-cysteine (4 mM) or glutathione (4 mM) were added and the worms cultivated at several pHs.

The terms 'maximum survival' (S_{max}), 'survival 50' (S_{50}), and 'average survival' (S_{av}) in culture have previously been defined by us (Iglesias *et al.* 1997) 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.).

Similarly, other terms are defined as follows: 'L5-survival' (S_{L5}) is the arithmetic mean of the days that worms survive after the 4th to 5th stage moult (M4). 'L4-survival' (S_{L4}) is the arithmetic mean of the days from M3 to 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 media, sera and reagents were purchased from Sigma Chemical Co. (St Louis, MO, USA) and Bio-Whittaker (Walkersville, MD, USA). Pepsin was obtained from Probus (Barcelona, Spain). The commercial pepsin used in our experiments was analysed by SDS–PAGE (Laemmli, 1970). Protein concentration was determined according to the method of Lowry *et al.* (1951).

RESULTS

Forty L3 of *A. simplex* s.l. collected from *M. poutassou* were electrophoretically identified as *A. simplex* s.s. (39) or *A. pegreffii* (1) (Nascetti *et al.* 1986).

The greatest moulting percentages to L4 were obtained in the presence of 5% CO_2, no matter which medium was used. The best parameters both for survival and moulting occurred in the nutritive medium RPMI (Table 1). Consequently, the following experiments were carried out in the RPMI medium in air atmosphere +5% CO_2.

When the culture was carried out at different pHs, it was observed that at any pH used, all or almost all the L3 reach L4, but L5 were only produced at pH 4·0 (1♂, 3♀; 28·6% of the initial larvae). In this medium, the moult to L4 occurred between days 3 and 4; the moult to L5 occurred between days 30 and 42, and S_{L5} was 26·7 days (Fig. 1).

Moulting to L4 under natural conditions involves a change from the pH in fish tissues to that in the stomach of the final host. Therefore, we set up an experiment in which the larvae were subjected to a change in pH on the 5th day (from pH 7·2 to pH 2·0, 3·0 and 4·0), when all the parasites had moulted to L4. Moulting to L5 only occurred between days 43 and 71 in the medium in which the pH was changed to 4·0, 5 L5 were produced (20% of the initial larvae; 3♂, 2♀) with a S_{L5} of 39·2 days. No statistically significant differences were observed with the control at pH 4·0 from the beginning of the culture (4 L5, 19% of the initial larvae; 2♂ and 2♀; M4 between days 54 and 64 of the culture; S_{L5} 31·0 days). Other manipulations of pH produced suboptimal development. The high survival rates in these media at pH 4·0 should be noted.

Pepsin is a component of the gastric juice in the stomachs of mammals. For this reason, we added pepsin to the culture media at various times during cultivation. In the first assay, at pH 7·2, moulting to L5 did not occur with or without pepsin. However, when the assay was carried out at pH 4·0, there was a highly significant difference between the medium with or without pepsin added from the beginning (S_{av}, $P < 1 \times 10^{-6}$; S_{L5}, $P < 0.003$). Some moulting to L5 occurred in all media at pH 4·0 and reached 100% in the medium where pepsin was added at the beginning. This M4 occurred between 20 and 60 days of cultivation. The later the pepsin was added to the culture, the lower the S_{av} and percentage moulting to L5 (Table 2). Opposite results occurred when the pepsin was eliminated from (i.e. not added to) the culture medium at different culture times (Fig. 2). On the other hand, 1 female, grown in the medium with pepsin added on the 14th day, began ovoposition on the 97th day of cultivation, 51 days after moulting to L5. This worm laid 15442 eggs in 16 days (with a maximum of 5672 eggs laid on 1 day).

Other changes in the culture medium resulted in reduced moulting and survival times compared with the pepsin-added media. The addition of either glutathione or L-cysteine alone, to the culture medium inhibited the moult to L5. Even with pepsin, L-cysteine prevented the moult to L5 and glutathione significantly reduced the numbers moulting. Similarly, increasing the percentage of IFBS or adding horse blood alone, inhibited the moult to L5. With pepsin and increased IFBS, the numbers moulting were reduced. In this experiment, in the control medium RPMI with pepsin, at pH 4·0, 22 worms moulted to L5 (91·6%) between days 22 and 46, of which 10 were male and 12 female, with a S_{L5} of 56·1 days. One of the latter laid eggs on day 101 (55 days after M4). The total number of eggs counted was 190793, laid during the period ranging from days 101 to 143 (42 days). The maximum number of eggs counted in 1 day was 16790 on day 113.

Finally, the mean length of the adults obtained was 4·4 ± 0·3 cm for males (range 3·8–6·5 cm) and 4·9

Table 1. *Influence of* 5 % CO_2 *on M3 and survival of* Anisakis simplex *larvae incubated in different media*

Medium†	nL3‡	Atmosphere	$S_{av} \pm$ s.e.	S_{50}	S_{max}	nL4(%)§
SS	18	Air	$10{\cdot}5 \pm 0{\cdot}5$	10	16	1 (5·5%)
SS	24	Air $+5$% CO_2	$10{\cdot}0 \pm 0{\cdot}4^{ns}$	10	15	24 (100%)
PBS	21	Air	$7{\cdot}3 \pm 0{\cdot}5$	7	13	0
PBS	26	Air $+5$% CO_2	$18{\cdot}9 \pm 1{\cdot}7*$	19	35	22 (84%)
RPMI	25	Air	$6{\cdot}4 \pm 0{\cdot}2$	7	10	0
RPMI	22	Air $+5$% CO_2	$24{\cdot}9 \pm 0{\cdot}8*$	22	37	22 (100%)

†SS, 0·9% NaCl solution; PBS, phosphate-buffered saline, pH 7·2; RPMI, RPMI-1640+20% IFBS, pH 7·2.
‡Initial number of parasites.
§Percentage of L3 that reached L4 stage. nL3 and nL4, Number of larvae in 3rd and 4th stages. Survival rates (maximum survival S_{max}, survival 50 S_{50}, and average survival S_{av} as defined in the Materials and Methods section. s.e., Standard error.
*Statistical comparison with the same medium without/with CO_2 using Student's t distribution: $P < 1 \times 10^{-5}$; ns, not significant.
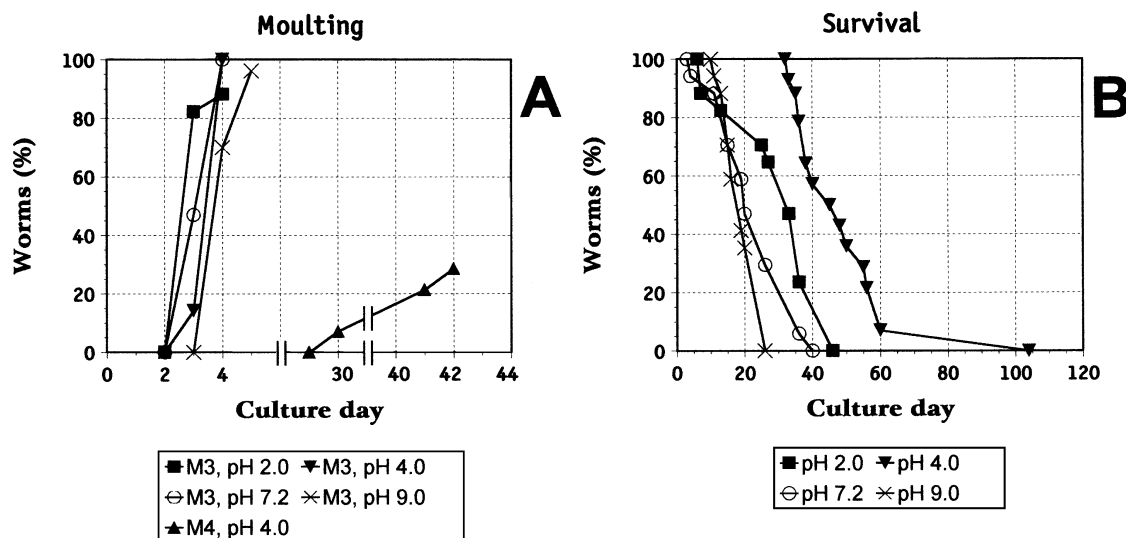

Fig. 1. Effect of pH on the *in vitro* moulting (A) and survival (B) of *Anisakis simplex* larvae cultured on RPMI-1640 plus 20% IFBS. (A) Percentage of worms that completed M3 and M4 throughout the culture time at different pHs. (B) Percentage of worms alive throughout the culture time at different pHs.

$\pm 0{\cdot}2$ cm for females (range 4·5–8·0 cm). The mean size of the eggs was $44{\cdot}4 \pm 0{\cdot}5 \times 50{\cdot}5 \pm 0{\cdot}7$ μm ($n = 50$).

To determine the purity of the commercial pepsin used in our experiments, it was analysed by SDS–PAGE (Laemmli, 1970). A single band of approximately 35 kDa was revealed. This band matched the band of purified and crystallized pepsin from Sigma Chemical Co. No protein impurities were revealed in the lane of pepsin from Probus. Other experiments performed with 0·01% pepsin (EC 3.4.23.1) from Sigma Chemical Co. showed similar results to ones with 1% pepsin from Probus (results not shown).

DISCUSSION

Of the forty L3 analysed by electrophoresis, 39 were identified as *A. simplex* s.s.; 1 was identified as *A. pegreffii*. We thus assumed that all (or nearly all) larvae used in the experiments of the *in vitro* cultivation were *A. simplex* s.s., since all hosts were caught off the Atlantic coast of Spain where this parasite is predominant over *A. pegreffii* (Nascetti *et al.* 1986).

The maximum development and survival of the larvae of *A. simplex* was obtained, as expected, in the nutritive medium in the presence of 5% CO_2 (Table 1; Sommerville & Davey 1976; Mercer *et al.* 1986; Iglesias *et al.* 1997). Although the pH of the gastric juices in mammals is close to 2, we obtained a maximum survival and development in the culture at pH 4·0. However, the gastric conditions in cetaceans are different to those of land mammals. Cetaceans, final hosts of *A. simplex*, present a multi-chambered stomach, generally with 4 chambers. The worms are generally, but not exclusively, located in the non-glandular stomach or forestomach. This is probably because it is the chamber in which the larvae usually are released from the food, when bacterial digestion begins and due to the peristaltic movements of this chamber. The larvae are fixed intimately to the gastric mucous where M3 occurs,

Table 2. *Influence of pepsin on the* in vitro *cultivation of* Anisakis simplex *larvae at pH 4·0*

(nL3 and nL5 are the number of worms in 3rd larval or adult stage, respectively. Survival and moulting rates (average survival S_{av}, maximum survival S_{max}, survival 50 S_{50} and L5-survival SL5, average M4 $M4_{av}$) as defined in the Materials and Methods section. S.E., Standard error).

Medium†	nL3‡	$S_{av} \pm$ S.E.	S_{50}	S_{max}	nL5 (%)§	$M4_{av} \pm$ S.E. (range)	♂:♀	SL5 $\pm$ S.E.
Pepsin added at start	13	$91·3 + 4·1$***	97	111	13 (100%)	$39·1 + 2·8^{ns}$ (20–52)	5:8	$51·8 + 5·3$**
Pepsin, added on 5th day	15	$87·0 + 6·5$***	85	134	13 (86·6%)	$41·9 + 2·5$* (27–61)	6:7	$49·9 + 7·0$*
Pepsin, added on 14th day	11	$63·8 + 11·2^{ns}$	48	158	6 (54·5%)¶	$38·0 + 4·7^{ns}$ (25–59)	2:4	$48·6 + 16·7^{ns}$
Pepsin, added on 21st day	12	$60·5 + 11·2^{ns}$	41	154	5 (41·6%)	$31·8 + 2·9^{ns}$ (25–38)	3:2	$56·2 + 17·4^{ns}$
Without pepsin	15	$50·0 + 3·7$	50	85	3 (20·0%)	$34·3 + 0·3$ (34–35)	2:1	$31·5 + 2·4$

†RPMI-1640 + 20% IFBS adjusted at pH 4·0. Pepsin is added at 1%.
‡Initial number of parasites. All worms reached L4 stage.
§Percentage of L3 that reached L5 stage.
¶In this medium, 1 female laid 15442 eggs over 16 days.
Statistical comparison with medium without pepsin using Student's t distribution: *$P < 0·02$; **$P < 0·003$; ***$P < 5 \times 10^{-5}$; ns, not significant.
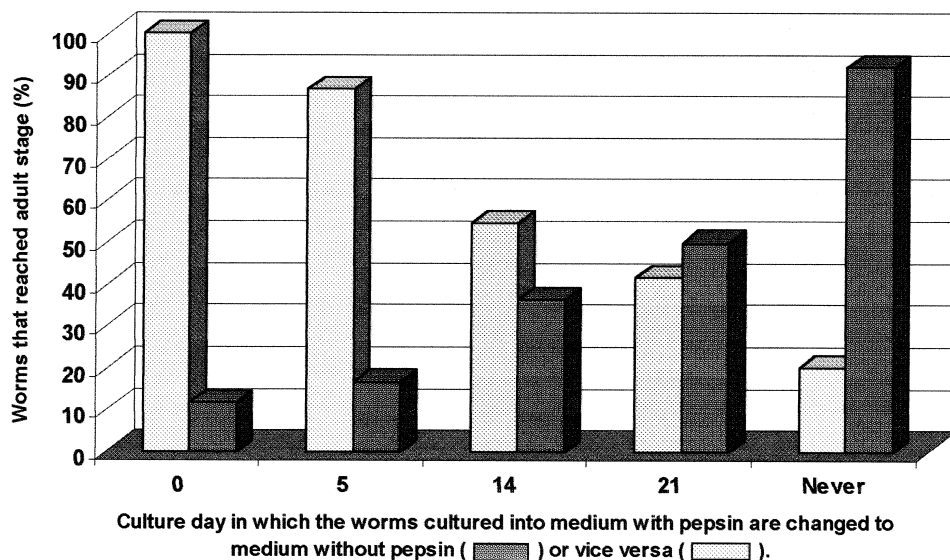

Fig. 2. Effect of pepsin (at 1% p/v) into the RPMI-1640 plus 20% IFBS medium on the *in vitro* development of *Anisakis simplex* larvae at pH 4·0.

but the subsequent moult takes place in the stomach lumen and, therefore, the adults are normally free or only superficially attached to the stomach wall (Kikuchi *et al.* 1967 (cited by Podolska, Piusiński & Rokicki, 1997); Young & Lowe, 1969; Smith & Wootten, 1978; Smith, 1989; Olsen, Aagnes & Mathiesen, 1994*a*). However, Højgaard (1999) has recently reported a cluster of adults in the fore-stomach wall of a long-finned pilot whale. The pH of the forestomach ranges between 5·4 and 7·3 depending on the diet of the cetaceans, at least in minke whales (Olsen *et al.* 1994*a*; Olsen *et al.* 2000). In the second stomach or fundic chamber, where HCl and pepsin are produced, the pH is 5·0–5·3, whereas in the other chambers the pH is 3·2–3·6 (Olsen *et al.* 1994*b*).

The change in pH, after M3 *in vitro*, did not lead to any improvement in the results. Nor did the results improve when reducing substances were added, such as L-cysteine and glutathione, the former having been cited as improving the yield of the *in vitro* cultivation of another anisakid, *Contracaecum osculatum*, which matures in the stomach of seals (Likely & Burt, 1992). It might be worth noting that *A. simplex* is found in seal stomachs but does not reach maturity there whereas *C. osculatum* does (Young & Lowe, 1969; Smith & Wootten, 1978). This could also be related to the fact that the pH of the monogastric stomach of seals during digestion remains within the range of 1·5–3·5 (Mikkelsen, 1998), being lower than that recorded in the stomach content of the whales.

In addition to the pH, the pepsin may also be a stimulus necessary in the development of anisakids, at least of *A. simplex*, in the stomach of marine mammals. On the one hand, L3 of *A. simplex* prove more resistant to digestion by pepsin than by other proteases of animal and vegetal origin *in vitro*

Table 3. Summary of the data on the *in vitro* cultivation of *Anisakis simplex* in RPMI-1640 ($+20\%$ IFBS) medium at pH 4·0 added with 1 % pepsin

(Results are expressed as mean $\pm$ standard error of the number "n" of experiments. For all these experiments 137 worms were used. Survival rates (average survival S_{av}, L4-survival SL4, and L5-survival SL5) as defined in the Materials and Methods section. The survival curves were significantly different by the Kaplan-Meier test (confidence 95 %). %L5: Percentage of L3 that reached L5 stage.)

Medium (n)	S_{av}	SL4	SL5	%L5	♂:♀ ratio
W/o pepsin (4)	50·3 ± 0·4	38·2 ± 5·2	30·2 ± 1·2	22·9 ± 2·2	1·02 ± 0·35
With pepsin (3)	87·8 ± 2·1	32·0 ± 2·0	56·8 ± 3·1	95·6 ± 2·4	0·89 ± 0·17
Student t distribution	$P < 0.003$	N.S.	$P < 0.0003$	$P < 0.00006$	N.S.

N.S. Not significant

Table 4. *Anisakis simplex in vitro* culture in the literature

Reference	Medium	Conditions	M3 (%L4)	M4 (%L5)	Egg size (μm)	Adult length (cm)
Van Banning (1971)	Pepsin digested beef liver + beef blood	34–37 °C pH 2·0 Air	4 or more days p.i.† (−)	26–98 days post-M3 (−)	40 × 50	♂ 3·5–7·0 ♂ 4·5–15·0
Grabda (1976)	Pepsin digested bovine liver + fresh cattle blood*	36–37 °C pH 2·0 Air	4–7 days p.i. (−)	12–14 days post-M3 (50 %)	39–42 × 41–43	♂ 5·7–7·2 ♀ 5·6–10·4
Sommerville & Davey (1976)	Medium 199	37·5 °C pH 7·0 Air + 5 % CO_2	3–5 days p.i. (96·7 %)	No	No	No
Carvajal et al. (1981)	Homogenated fresh bovine liver*	34 °C pH 2·0 Air	Yes (−)	Yes (42 %)	–	–
Iglesias et al. (1997)	RPMI-1640 + fetal bovine serum	37 °C pH 7·2 Air + 5 % CO_2	4–5 days p.i. (100 %)	No	No	No
This work	RPMI-1640 + fetal bovine serum + pepsin	37 °C pH 4·0 Air + 5 % CO_2	3–4 days p.i. (100 %)	16–57 days post-M3 (95·6 %)	44·4 × 50·5	♂ 3·8–6·5 ♀ 4·5–8·0

*These media were based on the medium of Van Banning (1971).
†p.i., Post-inoculum.
−, Not reported. %L4: Percentage of L3 that reached L4 stage. %L5: Percentage of L3 that reached L5 stage.

(Dziekońska-Rynko, Rokicki, & Jabłonowski, 1997). On the other hand, although the secretion of various substances with proteolytic activity have been described in anisakids (Sakanari, 1990), to our knowledge none similar to pepsin has yet been cited, which could obviate the need for an exogenous source of this enzyme.

Furthermore, the majority of the media used for the cultivation of *A. simplex* and other gastric nematodes are complex media which at some point in their elaboration require a pepsic digestion of one of their components (Van Banning, 1971; Grabda, 1976). In no case is there any suggestion of the total elimination of the pepsin. Indeed, pepsin has been used in the *in vitro* cultivation of other gastric nematodes (Douvres & Malakatis, 1977; Douvres, 1979).

Additionally, it can be affirmed that the addition of pepsin favours the development of the parasites the earlier it is added to the maintenance medium, although it does not appear to be necessary in the first days of *in vitro* cultivation of L3. According to these results, it seems that the pepsin is required, if not by L3, then by L4 from when this larva is formed and possibly by adults, as these have a greater mean survival rate S_{L5} in media with pepsin (48–56 days) than in the control without it (31 days). It has also been demonstrated that the proteases play an important role in the ecdysis process 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).

In summary, CO_2, pH and pepsin are factors which influence the development of *A. simplex*, at least *in vitro*. The results obtained by us using this medium, which is simpler than that used up to now by other authors, show it to be useful for cultivation, as the yield, measured as a percentage of adults obtained, is much higher than that obtained by other

authors, when reported, and the size of the adults is similar, although the maximum length of the females obtained by other authors is greater than that obtained in our cultures (Tables 3 and 4).

Further studies are necessary to clarify the effect of pepsin and other factors on these parasites and to achieve an *in vitro* complete life-cycle of the anisakids in the laboratory.

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