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Chapter 21

Hysterothylacium aduncum

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1. Background

1.1. Introduction

Hysterothylacium aduncum (Rudolphi, 1802) Deardorff and Overstreet, 1981 is a parasitic nematode that infects a wide variety of aquatic species worldwide. It was first described over two centuries ago, under the name *Ascaris adunca*. Some controversy still surrounds its taxonomy, due to its close relation to the anisakids with which it shares many morphological and structural characteristics (Hartwich, 1974). Although it was included in the family Anisakidae for many years, most authors currently place it in the family Raphidascaridae; both of these families belong to the superfamily Ascaridoidea.

There are more than 70 known species of the genus *Hysterothylacium* Ward & Magath, 1917. *H. aduncum* is the most abundant and widely distributed, particularly in marine environments, although it is also found in freshwater.

Since first being described, this nematode has been reclassified several times, being known previously as (WoRMS, 2019): *Ascaris adunca* (Rudolphi, 1802), *Ascaris clavata* (Rudolphi, 1809), *Contracaecum aduncum* (Rudolphi, 1802), *Contracaecum clavatum* (Rudolphi, 1802) Baylis, 1920 and *Thynnascaris adunca* Rudolphi. Furthermore, many synonyms have also been recognized (see WoRMS, 2019).

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1.2. Distribution of Hysterothylacium aduncum

Hysterothylacium aduncum is a parasitic nematode belonging to a ubiquitous genus whose natural development takes place in a great number of aquatic host species, among which are many cold and temperate marine teleost fish and even some freshwater species (Moravec et al., 1985; Yoshinaga et al., 1987a,b). It is one of the most widespread helminths in teleost fish in the North Atlantic Ocean (Balbuena et al., 1998) and is the most abundant nematode parasite of fish in the North Sea (Klimpel and Rückert, 2005). It is also widely distributed throughout the Southern Hemisphere (Carvajal et al., 1995) as far as Antarctic waters, where adults have been found (Rocka, 2006).

Many authors have reported the presence of these nematodes in commonly consumed fish in orders of great economic importance including Clupeiformes, Gadiformes, Perciformes and Pleuronectiformes. These are often heavily parasitized by larval and/or adult stages, with high prevalence (up to 100%) as a function of catch area and/or age of the fish. Among commercial species that may show high prevalence and/or intensity are *Clupea harengus* (herring); *Engraulis encrasicolus* (anchovy); *Gadus morhua* (cod); *Merluccius merluccius* (European hake); *Merluccius australis* (southern hake); *Scomber japonicus* (chub mackerel); *Scophthalmus maximus* (turbot); *Lepidorhombus whiffiagonis* (m egrim); *Sardina pilchardus* (sardine); *Trachurus trachurus* (horse mackerel); *Micromesistius poutassou* (blue whiting); *Phycis blennoides* (greater forkbeard); and *Trisopterus luscus* (pouting) (Adroher et al., 1996; Álvarez et al., 2002; Andersen, 1993; Carvajal et al., 1995; Costa et al., 2018; Huang, 1988; Moravec and Nagasawa, 2000; Navone et al., 1998; Petter and Maillard, 1988; Rello et al., 2008a,b, 2009; Ruiz-Valero et al., 1992; Sanmartín et al., 1989; Valero et al., 2000, 2006).

Parasitization appears to depend on diverse factors, such as host species, latitude or zone of capture, season, size or age of fish (Adroher et al., 1996; Andersen, 1993; Chenoweth et al., 1986).

1.3. Life cycle of Hysterothylacium aduncum

The adults of *H. aduncum* occupy the intestinal lumen of the definitive fish host. Once fertilized by the male, the female releases eggs which pass into the aquatic medium (usually marine) with the faeces of the fish. The first larval stage (L1), followed by the second (L2) and third (L3), develop inside the egg. It is unclear whether the larva hatches as L3 in the sea, covered by a sheath (the cuticle of L2), to then live free until ingestion by its first invertebrate intermediate host, usually a crustacean (particularly copepods). According to Køie's experimental data (Køie, 1993), it seems more likely that the eggs containing the L3 are ingested by the crustaceans, first intermediate hosts, the sheathed L3 freeing itself in the host's intestine. Regardless of its mode of entry into the intestine, it is here that the L3 loses its sheath and penetrates the haemocoel, where it grows. In these hosts, or in other invertebrates which prey on them (intermediate/paratenic hosts, see Fig. 21.1), the L3 must attain a minimum size (~1.5 mm) to be able to infect the second intermediate/paratenic host, small fish. The L3 passes from the intestinal lumen to the abdominal cavity and mesenteries, where it continues to grow. In order to infect the definitive host, a larger fish preying on smaller fish, the L3 must attain a minimum size (>3.0 mm). It then occupies the intestinal lumen where it continues to develop, moulting to L4 and then to a male or female adult nematode (Hurst, 1984; Køie, 1993). It has also been suggested that the growth of a small L3 infecting the abdominal cavity of a fish, which can also act as a definitive host, may result in the nematode reaching the optimum size to infect this host and being able to return to the intestinal lumen, establish itself and reinitiate its development to sexually mature adult, thus completing the parasitic life cycle (Andersen, 1993; Iglesias et al., 2002).

Owing to the ubiquity of the parasite and the variety of conditions in the different ecosystems, in each ecosystem the life cycle will follow mainly one or other route to the definitive host.

The number of invertebrates that can serve as host to *H. aduncum* is very large, comprising more than 100 species, mainly arthropods (copepods, mysids, isopods, amphipods, euphausiids, decapods),

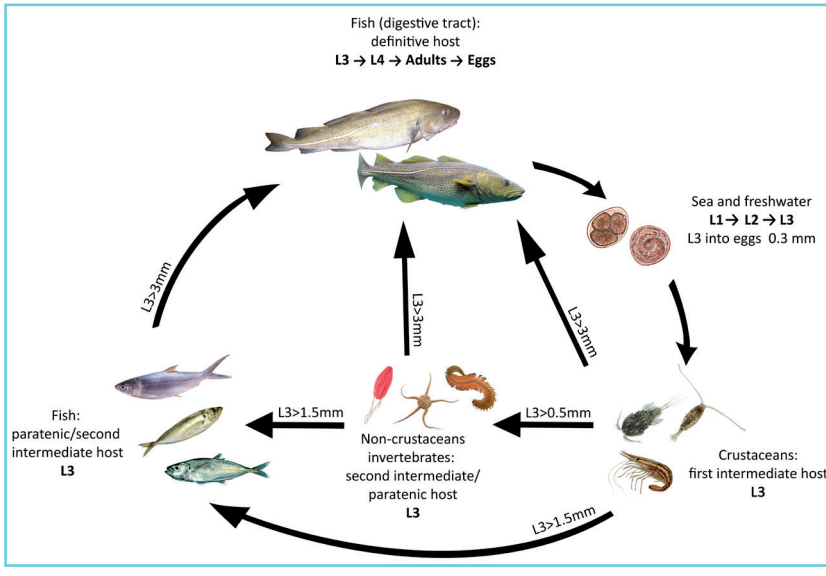

Fig. 21.1 Life cycle of *Hysterothylacium aduncum*, according to Køie (1993). L1, L2 and L3 are the first, second and third larval stages of this parasitic nematode in the eggs. L3 are also found in the body cavity of all intermediate and paratenic hosts throughout the life cycle of the worm. The fourth larval stage (L4) and adults develop in the digestive tract of the definitive hosts. Note that L3 is the infective stage for all hosts, and a minimum size of this L3 is necessary to infect subsequent hosts in the life cycle. Credit: Drawing by Lola Molina-Fernández.

with some chaetognaths, ctenophores, cnidarians and echinoderms (Køie, 1993; Marcogliese, 1996; Navone et al., 1998).

As mentioned previously (Section 1.2), many marine tele-ost fish can serve as intermediate/paratenic hosts of this parasite. Furthermore, *H. aduncum* appears to be able to complete its life cycle in freshwater because larvae and adults have been found in freshwater invertebrates and fish. It has been shown experimentally that fertilized eggs can hatch in freshwater and that the larvae can develop in freshwater crustaceans, such as mysids, infecting fish of continental waters such as *Oncorhynchus mykiss* (rainbow trout) (Rokicki, 2005; Yoshinaga et al., 1987a).

1.4. *Problematic issues concerning* Hysterothylacium aduncum

1.4.1. Aquaculture

In the coastal waters of Chile, *H. aduncum* is a common parasite of growing importance in marine cage aquaculture, particularly in salmonids (Carvajal et al., 1995). In addition to the implications for the health of farmed fish, and possible economic losses as a result, infection by *H. aduncum* also leads to an increase in energy expenditure by the parasitized fish (González, 1998) and a subsequent increase in food consumption. Although these authors do not consider that the parasite seriously harms the fish, they believe that the stress it causes may facilitate colonization by other pathogenic organisms, thus reducing yield. In contrast, a study on the pathogenic effect of *H. aduncum* on herring (*C. harengus*) larvae in experimental cultures revealed high mortality (Balbuena et al., 2000; Karlsbakk et al., 2003). They were also highly damaging for the cultivated larvae of cod (*G. morhua*) fed on plankton obtained from their natural habitat (Karlsbakk et al., 2001). Feeding with natural plankton, or with a wet fish-based diet, may be the principal source of infection by this nematode for farmed fish (Bricknell et al., 2006). Berland (1987) attributed mortality in farmed rainbow trout (*O. mykiss*) to the presence of *H. aduncum*, which was possibly introduced via the undried fish on which they were fed. Furthermore, in the viscera of farmed Atlantic salmon, Sepúlveda et al. (2004) found *H. aduncum*, and Marty (2008) reported anisakid larvae from histological sections of the viscera of the same farmed fish species. Mo et al. (2014) demonstrated the presence of this parasite in runts of Atlantic salmon (*Salmo salar*) farmed at sea off southwest Norway, but not in quality salmon for human consumption. Runts are individual fish with clear signs of poor performance over time and abnormal appearance and are thus not processed for human consumption. These studies show that the presence of these parasites affects the yield and quality of the product produced in marine farms.

1.4.2. Human consumption

Numerous studies have also shown the role of *H. aduncum* as a potential aetiological agent of anisakidosis in humans (Huang, 1988; Norris and Overstreet, 1976; Overstreet and Meyer, 1981; Petter, 1969; Vermeil et al., 1975). Yagi et al. (1996) described the first case of non-invasive anisakidosis, through consumption of raw fish (cod and salmon), caused by an immature adult female *H. aduncum,* which was eventually expelled in the faeces. The symptoms were reported as chronic abdominal pain and diarrhoea. Norris and Overstreet (1976) and Overstreet and Meyer (1981) attributed cases of human anisakidosis to two larvae of the genus *Hysterothylacium*. Furthermore, this parasite may also be responsible for allergic reactions in humans, following its ingestion with fish (Fernández-Caldas et al., 1998; Valero et al., 2003). Despite these studies, many authors feel that there is still insufficient clinical and/or experimental evidence demonstrating that *H. aduncum* can cause digestive or allergic disorders in humans.

2. Facilities

To minimize the risk of fungal and/or bacterial contamination of cultures, a specialized laboratory is preferable. However, a clean, draught-free room equipped as described in section 3 will suffice.

3. Equipment and reagents

Basic:

- Laminar flow cabinet
- CO_2 incubator with humidity and temperature control below room temperature (see section 4.4)
- Inverted microscope
- Optical/stereoscopic microscope
- Autoclave

- pH meter
- Precision balance
- Laboratory centrifuge
- Sterilizing filtration equipment (depending on the equipment chosen, a vacuum/pressure pump may be required)
- Membrane filters, 0.22-µm pore size.
- Scissors
- Dissecting needles or probe with hooked curve
- Petri dishes
- Plates of 24/12/6/4 wells and/or wide-mouth culture flasks.
- Glass staining blocks or small Petri dishes
- Syringes/needles/pipettes
- 70° ethanol
- NaCl solutions
- Antibiotic/antimycotic solution
- Culture media
  - RPMI-1640
  - Commercial pepsin
  - Fetal bovine serum.

4. Procedure

4.1. Source and isolation of parasites

As reported in section 1.2, many fish species are parasitized by this nematode. Horse mackerel (*T. trachurus*) from the northeast Atlantic were used in the present study, due to their relatively high parasite load (Adroher et al., 1996) and low price. Fish of between 25 and 35 cm in length are usually the most heavily parasitized. If horse mackerel are absent from the study zone, or have a low level of parasitization, other host fish can be employed (see 'Parasite isolation' Summary template).

After acquisition, the fish were kept cold while being transported to the laboratory. *H. aduncum* L3 were extracted by dissecting the fish along the *linea alba* and searching for the larvae, either embedded in the intestinal mesenteries and free or encapsulated in the viscera.

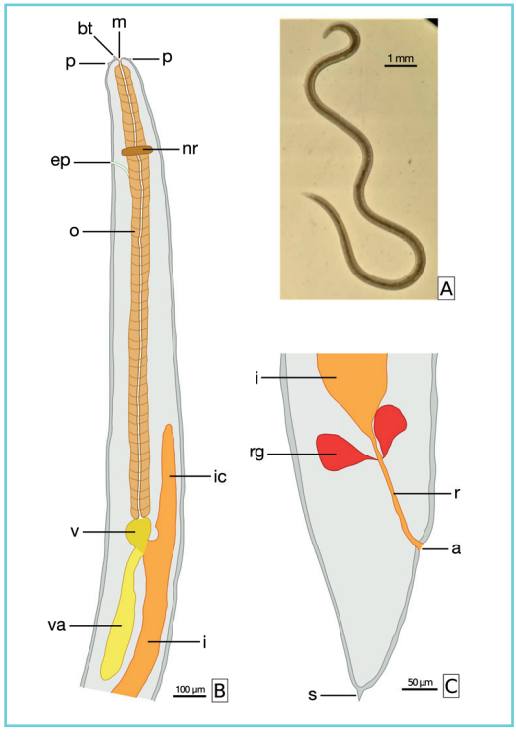

Fig. 21.2
Hysterothylacium aduncum L3. A: A freshly collected L3 from the mesenteries of a blue whiting, a paratenic host fish. B and C: Drawings of the anterior (B) and posterior (C) end of the worm, showing its external morphology and digestive system. Abbreviations: a, anus; bt, boring tooth; ep, excretory pore; i, intestine; ic, intestinal caecum; m, mouth; nr, nerve ring; o, oesophagus; p, papilla; r, rectum; rg; rectal gland; s, spine; v, ventricle; va, ventricular appendage. Credit: Drawing by Irene Adroher-Benítez.

4.2. Parasite identification

The visceral cavity may also contain larvae of other parasitic nematodes, the most common in marine fish being L3 of *Anisakis simplex* s.l., although L3 of other species of the genus *Hysterothylacium* or other anisakids may also be present, depending on factors such as geographical provenance of the fish.

The L3 of *H. aduncum* (Fig. 21.2) extracted from the fish are whitish and elongated, generally between 5 and 20 mm in length and 0.10–0.15 mm in width. Microscopic examination of the anterior extremity revealed a small protuberance, or tooth, and three poorly differentiated incipient labia. A small spine is observed at the posterior extremity. Using external morphology, these are difficult to differentiate from the larvae of other ascaridoid nematodes, particularly anisakids, and it is thus necessary to locate the excretory pore. In the anisakids this is found at the anterior extremity

between the subventral labia, whereas in the genus *Hysterothylacium* it is found in a lateral position close to the nerve ring. In *H. aduncum*, it is located 0.12–0.38 mm from the anterior extremity (mean, 0.24 mm) and is clearly visible under optical microscopy. The digestive system can also be observed. The mouth leads into a cylindrical muscular oesophagus (length, 0.96–2.32 mm; mean, 1.37 mm) finishing in a more or less spherical ventricle (glandular oesophagus) connected to the intestine. Both the ventricle and intestine show characteristic prolongations of slightly unequal length, one towards the anterior extremity, known as the intestinal cecum (length, 0.31–0.96 mm; mean, 0.54 mm) and another towards the posterior extremity, known as the ventricular or oesophageal appendage (length, 0.34–0.82 mm; mean, 0.50 mm). The distance from the anus to the posterior extremity of the larvae ranges from 0.10 to 0.29 mm (mean, 0.20 mm). These values were determined in our laboratory (Ruiz-Valero, 1991), although there may be some variation due to the source of the parasite or its level of development. Other data for morphological identification can be found in Petter and Maillard (1988), Petter et al. (1995) and Yoshinaga et al. (1987a). The low genetic variability of this parasite obviates the need for molecular identification (Klimpel et al., 2007).

4.3. Preparation of the culture medium

The medium developed by Iglesias et al. (2001, 2002) for the culture of *A. simplex* s.l. and *H. aduncum* contains:

- RPMI-1640 medium
- Commercial pepsin 1% w/v
- Fetal bovine serum, heat-inactivated, 20% v/v.

After mixing and dissolving all the components, pH is adjusted to 4.0 with HCl 0.1 N.

The medium is then sterilized by filtration through 0.22-µm pore membrane filters and stored in 100–500-ml flasks. A sample is taken from each flask under sterile conditions and incubated for a minimum of 48–72 h at 37°C, in order to determine whether bacterial

and/or fungal contamination has taken place. The remainder of the medium is frozen at –20°C, until required. If samples show contamination, the medium is filtered again until sterile. After thawing the frozen medium for use, it should not be refrozen. It is thus useful to estimate the volume of the storage flasks, depending on consumption of the medium during cultivation.

4.4. Axenization of parasites

All biotic contamination of the parasite must be eliminated to obtain an axenic culture. This requires preparation of an antibiotic/antimycotic solution (80 mg gentamicin sulfate, 0.625 mg amphotericin B, 10,000 IU sodium penicillin G, 10 mg streptomycin sulfate and 4.5 ml Hanks' balanced salt solution (HBSS), for a final solution volume of 10 ml; Iglesias et al., 1997) in which the larvae are placed (in glass staining blocks, culture wells or small Petri dishes, sterile in all cases) for 20–30 min in a sterile environment at room temperature. Alternatively, antibiotic/antimycotic solutions for axenization of parasites are also available commercially.

4.5. Individual cultivation of parasites

Individual cultivation is most suitable for developmental studies, as identification of the stages of the parasite is easier. After axenization of the larvae, each is placed in a well of a 12–24-well culture plate with 1 ml of sterile culture medium. The plate is then placed in an incubator at 15°C, in a humid atmosphere of air +5% CO_2. Although cultivation without added CO_2 (using an incubator without CO_2) – and even without pepsin – is possible, the culture becomes desynchronized and yield is lower (Iglesias et al., 2002). The frequency of observation of the cultures depends on the type of study. However, since it is necessary to check for bacterial and/or fungal contamination, daily observation is recommended, at least for the first 72 h after addition of the medium.

As the parasites grow and moult they can be transferred to plates with six or fewer wells so as not to limit growth, although development does not appear to be affected. The larvae will undergo two

moults, M3 and M4, attaining adulthood, but will grow more in larger than in smaller wells with limited space. The moulting to L4 generally takes place between days 3 and 5 of cultivation, while moulting to the adult stage usually occurs between 3 and 4 weeks after the start of cultivation (mean, 23 days; Iglesias et al., 2002). It may be necessary to add a larger volume of medium (2–3 ml) to wells of greater diameter to cover the parasite. The medium should also be renewed weekly due to the accumulation of waste products released by the parasite. Moulting can be detected through the appearance of a sheath, this being the old cuticle that has been shed. These should be removed from the well on changing the medium, to facilitate detection of the next sheath to be shed.

4.6. Changing the medium

The used culture medium should be removed and replaced with fresh medium once per week, always under sterile conditions, in the laminar flow cabinet.

4.7. Group culture

The procedure for group culture is similar to that of individual culture except that the parasites are placed together in the medium. This method is necessary for fertile eggs to be obtained.

The authors generally use 6–10 larvae for group culture, in a wide-mouthed culture flask (for easier handling) with 30–50-ml capacity (alternatively Petri dishes of 6–10-cm diameter can be used, depending on number and size of larvae) and containing approximately 1 ml of culture medium per parasite, under the same conditions as for individual culture and with the medium replaced weekly. Larger flasks with a greater volume of medium should be employed when large numbers of worms are required. In this case it is desirable to renew the medium more often to reduce the concentration of potentially toxic metabolites, which may increase the mortality rate.

Alternatively, if the parasites are to be paired according to sex, they can be cultivated individually to adulthood and their sex determined

by observation of the vulva in the female and the pre- and post-anal papillae in the male, using an inverted microscope. They can then be placed together in flasks, Petri dishes of diameter 3–4 cm or culture plates with 4–6 wells.

4.8. Egg production and development

Group culture results in the release of eggs by the females (Fig. 21.3), generally within 1–2 months of starting cultivation (Adroher et al., 2004; Iglesias et al., 2002). The eggs can be isolated from the culture medium by centrifugation at low speed (~500 × *g*, 5 min), washed with sterile saline solution at 0.9% w/v NaCl, resuspended in sterile solution of NaCl at 2.8% w/v, placed in wells in culture plates and incubated under the same conditions as for their development (Fig. 21.3). Development of the egg is complete in 2–3 weeks. This process can be accelerated by raising the temperature, such that, at 20–24°C, it takes approximately one week (González, 1998; Køie, 1993). At this point the egg contains the L3, which is infective for the first intermediate host, although only a small proportion hatch (2–10%), at a size of approximately 150–300 µm (Adroher *et al.*, 2004). However, other authors have obtained high levels of success

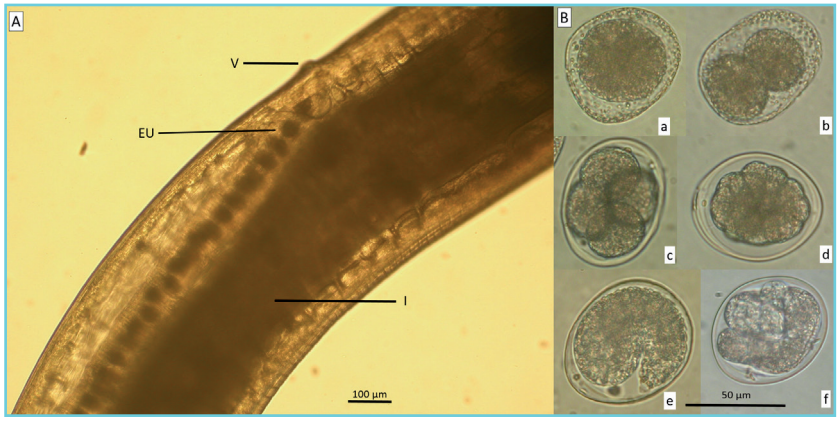

Fig. 21.3 *Hysterothylacium aduncum*. A: Detail of the vulva (V) and uterus with eggs (EU) of an adult female worm (I, intestine). B: Fertilized eggs at various stages of development: (a) Newly laid; (b) 2-cell state; (c) 4-cell state; (d) morula; (e) embryo; (f) larva. Credit: Authors' original.

with hatching in seawater, at temperatures up to 24°C (González, 1998), or at 5°C and 2% salinity (Balbuena et al., 1998). The L3 which hatch are found within the L2 sheath, from which they are rarely able to free themselves, at least under culture conditions.

4.9. Note

It is possible to continue the life cycle of the parasite in the laboratory, using suitable hosts. To achieve this, copepods (or other invertebrates that act as first hosts) must be infected with the developed eggs of the parasite and then fed to fish (second hosts), to infect them. See Balbuena et al. (1998, 2000), González (1998), Køie (1993) and Yoshinaga et al. (1987a).

A similar procedure, at 36°C, has been used for the culture of *A. simplex* s.l., but without the development of fertile eggs. *Anisakis* seems to be more demanding with regard to the conditions necessary for attaining sexual maturity (see Iglesias et al., 2001).

5. Summary templates

Hysterothylacium aduncum **(Rudolphi, 1802) Deardorff and Overstreet, 1981**	
Parasite isolation	
Fish or paratenic/intermediate host species required for parasite collection	Many fish species can be used as a source of this parasite. We work with horse mackerel (*T. trachurus*) from the NE Atlantic Ocean
Susceptible host characteristics	Horse mackerel of length 25–35 cm
Environment of infected hosts	Greater prevalence in larger fish and, especially, in winter (in our geographic area), although parasites occur throughout the year
Identification of infected individuals	Exploration of the body cavity and intestinal mesenteries of fish
Parasite stage(s) collected	L3
Length (mm) of parasite stages	5–20
Site of infection, including target tissues if relevant	Visceral cavity, free and on the viscera, and in the intestinal mesenteries
Isolation technique(s)	Manual removal

Additional isolation requirements	Optic or stereoscopic microscope for observation and identification of the parasites
Collection media	Collection in cold tap water or saline solution (0.9% w/v NaCl)
Post-processing	Washing in tap water or saline solution (0.9% w/v NaCl)
Transport/maintenance conditions	N.A.
Monitoring requirements	N.A.
Optimal fixative(s) if using	N.A.
Other relevant information	In geographic areas where the horse mackerel is not common or is poorly parasitized, another host fish can be used. To determine which fish species is most suitable, it is necessary to review the epidemiological studies of the area or, in their absence, carry out a survey

***In vitro* culture**	
Axenic	Yes
With co-culture	No
Source of inoculum	L3 from host fish
Quantification of infective stages	Macroscopic larvae. Easy quantification
Dose of inoculum	Individual or group cultivation
Culture conditions	15°C, in humid air +5% CO_2
Monitoring of culture	Observation by inverted microscope
Renewal of culture medium, timing and harvesting	Renewal of culture medium once per week. Timing up to L4, 3–5 days. Timing up to adults, 3–4 weeks. Harvesting of fertilized eggs:,1–2 months
Parasite stages cultured	L3 from fish, L4, adults, eggs: L1, L2 and L3 in eggs. Hatched L3
Storage	Cryopreservation not achieved
Attenuation of virulence	Not investigated
Other relevant information	It is possible to continue the life cycle of the parasite in the laboratory using suitable hosts. Suitable crustaceans (first hosts) must be infected with the developed eggs of the parasite and then fed to fish (second hosts) to infect them. See main text

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