Early development and life cycle of *Contracaecum multipapillatum* s.l. from a brown pelican *Pelecanus occidentalis* in the Gulf of California, Mexico

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ABSTRACT: The initial developmental stages of *Contracaecum multipapillatum* (von Drasche, 1882) Lucker, 1941 sensu lato were studied using eggs obtained from the uteri of female nematodes (genetically identified) found in a brown pelican *Pelecanus occidentalis* from Bahía de La Paz (Gulf of California, Mexico). Optical microscopy revealed a smooth or slightly rough surface to the eggs. Egg dimensions were approximately 53 × 43 µm, although after the larvae had developed inside, egg size increased to 66 × 55 µm. Hatching and survival of the larvae were greater at 15°C than at 24°C, and increased salinity resulted in a slight increase in hatching but seemed to reduce survival at 24°C, but not at 15°C. The recently hatched larvae measured 261 × 16 µm within their sheath. When placed in culture medium, the larvae grew within their sheath, and a small percentage (~2%) exsheathed completely (314 × 19 µm). The larvae continued to grow and develop once they had exsheathed, attaining mean dimensions of 333 × 22 µm. Although they did not moult during culture, optical microscopy revealed a morphology typical of third-stage larvae. Finally, the genetic identity between the larval parasites collected from mullet *Mugil curema* and adult female parasites collected from the brown pelican suggests a life cycle of *C. multipapillatum* in which the mullet are involved as intermediate/paratenic hosts and the brown pelicans as final hosts in the geographical area of Bahía de La Paz.

KEY WORDS: *Contracaecum multipapillatum* · Nematoda · Anisakidae · Fish parasite · Genetic identification · Development · Aquatic life cycle · Mullet · Brown pelican · Gulf of California

INTRODUCTION

Nematodes of the genus *Contracaecum* (Ascaridoidea: Anisakidae) parasitize fish-eating birds and marine mammals as their definitive hosts, while the larvae use planktonic crustaceans and fish as intermediate/paratenic hosts (Anderson 2000). If accidentally ingested, the third larval stage (L3) found in fish may infect humans, causing anisakidosis (contra-

caecosis) and possibly allergic reactions. In Germany, Schaum & Müller (1967) described the first human case of contracaecosis in the medical literature. Im et al. (1995) studied 107 cases of gastric anisakiasis in Cheju-do (South Korea), and after examining the collected larvae of the patients, found 1 larva of *Contracaecum* type A (= *C. osculatum* s.l.). Ishikura et al. (1996) later described 2 new cases in Hokkaido (Japan). All cases described to date have

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been caused by *C. osculatum* s.l. Recently, Shamsi & Butcher (2011) reported the first anisakidosis case in Australia caused by a larva of *Contracaecum* sp. Although no allergic reactions related to the ingestion of larvae of the genus *Contracaecum* in food have been described, these may well occur as they have been reported in other genera of anisakids, such as *Anisakis* (e.g. Daschner et al. 2012) and *Hysterothylacium* (Valero et al. 2003).

C. multipapillatum (von Drasche, 1882) Lucker, 1941 (sensu lato) consists of a complex of sibling species which parasitize fish-eating birds, principally of the family Pelicanidae (Mattiucci et al. 2010). The life cycle of these parasites is complex and has yet to be clarified. According to Huizinga (1967), the second larval stage (L2, with the L1 sheath) hatches from the egg into the water and is ingested by a copepod. It then frees itself from the sheath in the intestine of the copepod before passing to the coelom, where it grows without moulting. When the infected copepod is ingested by the first fish host (small planktivorous fish), L2 reaches the abdominal cavity, grows and moults to L3. When this fish is ingested by a larger, piscivorous fish, L3 grows in the abdominal cavity but does not moult. When this fish is ingested by a suitable fish-eating bird host, L3 lodges in the proventriculus where it grows, moulting twice to the adult stage. After fertilization of the female by the male, the eggs are passed in faeces from the final host to the water. The L2 larvae then develop inside the eggs, finally hatching, after which the larvae are free in the water until they are ingested by a new intermediate host.

Deardorff & Overstreet (1980) attempted experimental oral infection of chickens, ducks and rats with L3 of *C. multipapillatum* obtained from fish, without success, although a few larvae placed in the abdominal cavity of rats developed to the adult stage. At a later date, Vidal-Martínez et al. (1994) again tried to infect animals, this time including cats in the experiment. While the chickens, ducks and rats were not infected orally when using L4 collected from the Mayan cichlid *Cichlasoma urophthalmus*, the cats were not only infected, but the larvae also developed to the adult stage, at least 3 of these being found firmly attached to the intestine where they caused haemorrhaging similar to that associated with small ulcers. In addition, Barros et al. (2004) infected rabbits experimentally with *C. multipapillatum* L3 from fish, which caused gastric lesions. All of these authors

warned of the potential risks to human health resulting from the accidental ingestion of this parasite.

C. multipapillatum s.l. has been found both in commercially important fish of the mullet family Mugilidae (as L3) and in brown pelicans *Pelecanus occidentalis* (as L3, L4 and adults) from Mexico and other regions of America (Deardorff & Overstreet 1980, Iglesias et al. 1998, 2011, Valles-Ríos et al. 2000, Valles-Vega 2011, 2014). Furthermore, in these areas, where mullets form part of the diet of these birds, infections have been found in brown pelicans as young as 2 wk (Humphrey et al. 1978).

The present study describes the early developmental stages of *C. multipapillatum* s.l. and the first attempt at their *in vitro* culture in nutritive media in order to further our knowledge of the biology of this parasite, to determine its life cycle and to describe eggs and larvae using optical microscopy.

MATERIALS AND METHODS

Host and parasite collection

A total of 353 adult nematodes were obtained from the digestive tract of male adult brown pelican from Bahía de La Paz, Baja California Sur, Mexico (Fig. 1). Six females of *Contracaecum multipapillatum* s.l., identified at CICIMAR de La Paz (Mexico) using the morphological characteristics of males that accompanied them (Lucker 1941, Yamaguti 1961), were sent

Fig. 1. Geographical zone where mullet *Mugil curema* were collected and a brown pelican *Pelecanus occidentalis* was captured (■) for this study

to the Department of Parasitology at the University of Granada (Spain) where they were dissected to extract the eggs. These females were then analysed for molecular identification (see below). The third larval stage (L3) of *C. multipapillatum* s.l. from mullet *Mugil curema* captured in the same geographical area were collected for genetic identification.

Molecular identification

For genetic identification of the L3 larvae of *C. multipapillatum* from mullet and of the adult female *C. multipapillatum* collected from the brown pelican, PCR of the ribosomal fragment ITS1–5.8S–ITS2 of the nuclear genome was carried out. In brief, the procedure described by Ausubel et al. (2002) was employed to extract the genomic DNA of every parasite. The PCR amplification primers NC5 (forward) and NC2 (reverse) described by Zhu et al. (2000) were employed. The expected size of the amplified fragment was ~1000 bp. PCR products were run through electrophoresis in 1% agarose gel and visualized with SYBR Gold dye and a transilluminator. The DNA was purified and sequenced by MacroGen (South Korea).

Analysis of sequences

In brief, the sequences were edited and aligned with Clustal X (version 2.0). A phenetic analysis was carried out. Trees were constructed using neighbour-joining (NJ) analysis, based on Kimura-2-parameter (K2P) distance (Kimura 1980) values, using MEGA 5.05 (Tamura et al. 2011). The reliabilities of the measure of stability of the branches were evaluated using nonparametric bootstrap analysis (Felsenstein 1985) for the NJ tree, with 1000 bootstrap replicates.

Maintenance and cultivation

The eggs were taken from the most external part of the uteri, placed in tubes of tap water, centrifuged at $600 \times g$ for 10 min and the supernatant decanted. Next, 9‰ NaCl solution was added, and the tubes were centrifuged at $600 \times g$ for 10 min, after which the eggs were placed in an antibiotic-antimycotic solution for 30 min for axenization (Iglesias et al. 1997). The eggs were then washed with sterile 9‰ NaCl solution and centrifuged at $600 \times g$. After resuspension in this saline solution, the solution was

divided into 3 aliquots which were then centrifuged, decanted and the eggs resuspended in a volume of sterile NaCl solution at 9, 28 and 35‰, respectively. Aliquots of 1 ml with ~200 eggs were placed in the wells of sterile polystyrene culture plates, in triplicate, which were incubated separately at 15 and 24°C. The development of the eggs was monitored to distinguish the different stages, and we recorded hatching and survival of the larvae in the saline solutions, which were renewed weekly. After 1 mo under these conditions, the saline solution in the wells was changed to a solution of Grace's insect medium supplemented with 2% v/v basal medium Eagle's vitamins (100×) solution, 1 mM L-cysteine, 1 g l⁻¹ glucose, 20% v/v heat-inactivated foetal bovine serum and 1% v/v RPMI-1640 amino acid solution (50×), adjusting pH to 7.2, without changing the other experimental conditions and the observation of larval development. The culture medium was renewed weekly. The experiments were deemed to be completed when dead larvae outnumbered live larvae.

RESULTS

The genetic analyses of the ITS1–5.8S–ITS2 sequences of both adult female *Contracaecum* collected from the brown pelican and L3 larvae collected from mullet in Bahía de La Paz showed 100% identity (Table 1; K2P = 0; Fig. 2).

During the first developmental stages (up to the morula), the eggs from the uteri of female *C. multipapillatum* s.l. placed in different saline solutions measured (mean ± SD) $53.4 ± 3.4 × 43.1 ± 4.1$ μm (n = 13). When the larvae developed inside the eggs, these increased in size up to $65.6 ± 5.4 × 54.8 ± 4.5$ μm (n = 12). Optical microscopy observations revealed that the eggs had either 2 layers with a smooth covering or 3 layers with a slightly rough covering (Fig. 3).

The eggs exhibited negative buoyancy in all tested saline solutions, depositing on the bottom of the culture wells. Most of the eggs (>85%) left to incubate in the different saline solutions developed as far as forming larvae (Fig. 4A,B) which later hatched (Fig. 4A,C) in large numbers (61–98%). However, the speed of development and the survival of the larvae differed according to incubation conditions. At 15°C (Table 2), salinity did not seem to significantly affect the mean hatch percentage (average range 80–97%) or the survival of the hatched larvae (75–82%, mean percentage of larvae alive at the end of the experiment). Furthermore, a notable increase was observed in the slope of the eclosion curve from Day 11 onwards (not

Table 1. Kimura-2-parameter standard genetic distance in the ITS1 sequence between *Contracaecum multipapillatum* of this study and other *Contracaecum* spp. of birds. *Ascaris suum* was used as an outgroup. GenBank accession numbers are in brackets

Contracaecum	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1. *C. multipapillatum* (*Mugil curema*)	–																
2. *C. multipapillatum* (*Pelecanus occidentalis*)	0.00	–															
3. *C. multipapillatum* (AM940057.1)	0.06	0.06	–														
4. *C. multipapillatum* B & C (KC437337.1)	0.06	0.06	0.00	–													
5. *C. multipapillatum* (AM940059.1)	0.06	0.06	0.00	0.00	–												
6. *C. multipapillatum* D (KC4373338.1)	0.06	0.06	0.00	0.00	0.00	–											
7. *C. rudolphii* (FM210251.1)	0.32	0.32	0.30	0.30	0.30	0.30	–										
8. *C. rudolphii* F (JF424597.1)	0.33	0.33	0.32	0.32	0.32	0.32	0.01	–									
9. *C. variegatum* (FM177532.1)	0.33	0.33	0.32	0.32	0.32	0.32	0.01	0.01	–								
10. *C. rudolphii* B (AJ634783.1)	0.33	0.33	0.32	0.32	0.32	0.32	0.01	0.01	0.01	–							
11. *C. rudolphii* A (AJ634782.1)	0.31	0.31	0.30	0.30	0.30	0.30	0.03	0.02	0.02	0.02	–						
12. *C. eudyptulae* (FM177561.1)	0.32	0.32	0.30	0.30	0.30	0.30	0.02	0.02	0.02	0.01	0.02	–					
13. *C. bioccai* (JF424598.1)	0.32	0.32	0.30	0.30	0.30	0.30	0.05	0.04	0.04	0.04	0.05	0.04	–				
14. *Contracaecum* sp. (JF424599.1)	0.34	0.34	0.32	0.32	0.32	0.32	0.06	0.06	0.05	0.05	0.07	0.05	0.03	–			
15. *C. septentrionale* (AJ634784.1)	0.34	0.34	0.33	0.33	0.33	0.33	0.05	0.05	0.04	0.04	0.05	0.04	0.04	0.06	–		
16. *C. microcephalum* (FM177525.1)	0.34	0.34	0.32	0.32	0.32	0.32	0.09	0.05	0.08	0.09	0.10	0.06	0.09	0.09	0.09	–	
17. *C. bancrofti* (EU839572.1)	0.31	0.31	0.28	0.28	0.28	0.28	0.14	0.14	0.14	0.13	0.15	0.13	0.13	0.16	0.13	0.13	–
18. *Ascaris suum* (AB571302.1)	0.49	0.49	0.47	0.47	0.47	0.47	0.38	0.37	0.36	0.36	0.37	0.36	0.38	0.39	0.38	0.34	0.34

shown). When the same experiment was carried out at 24°C, the notable increase in the slope of the hatching curve occurred towards Day 13 and did not seem to be affected by the salinity of the medium (not shown). However, the hatch percentage was slightly lower than at 15°C (Table 2), while there was a slight increase in mean hatch percentage (71–80%) with increasing salinity. On the other hand, the mean percentage of larvae alive at the end of the experiment was lower at 24°C (30–55%) than at 15°C (75–82%), apparently influenced by the salinity, such that survival decreased as salinity increased (Table 2). In summary, increasing the temperature from 15 to 24°C reduced the percentage of hatching and survival of the larvae; the increase in salinity reduced larval survival at 24°C, with a slight increase in hatching at either temperature.

Recently hatched larvae showed a boring tooth, oesophagus and a developing intestine, which frequently presented 1 or 2 large clear areas in both anterior and posterior zones (Fig. 5A), as well as a striated sheath corresponding to the cuticle of the previous larval stage (Fig. 5). As the larvae developed, the nerve ring, the excretory duct, the ventriculus, the ventricular appendix — with differing degrees of development — and the rectum could also be observed (Fig. 5B). The intestinal caecum could not be observed at this stage, at least not during the period of maintenance.

Live larvae collected from the previous experiment at 24°C were placed in wells with 1 ml of Grace's modified medium at the same temperature. These larvae were ensheathed (Fig. 5) and measured 261.0 ± 38.1 × 15.8 ± 2.9 µm (n = 23), or 297.8 ± 16.3 µm (n = 8) including the sheath. The larvae tended to be highly mobile during this stage, exhibiting a flicking motion, but with periods at rest. Some larvae adhered to each other or to fibrous substrates in the culture medium, using the posterior end of the sheath, which finishes in a point (Fig. 5C and see Video S1 in the Supplement at www.int-res.com/articles/suppl/d125p167_supp/). After 14 d under these conditions, the larvae had grown sufficiently to fill the sheath while some started to free themselves upon reaching a length of around 315 µm (314.4 ± 51.1 × 18.5 ± 2.0 µm, n = 7). The larvae freed themselves from the sheath at different times using rapid movements. However, some failed to achieve this under our experimental conditions (Fig. 6A). Over the 25 d of experiments, ~2% of the larvae exsheathed, growing more than 70 µm (333.4 ± 39.2 × 21.9 ± 5.8 µm, n = 3) with no new moulting observed. The freed larvae exhibited a more serpentine and

Fig. 2. Neighbour-joining reconstruction between sequences of *Contracaecum multipapillatum* obtained in this study (in **bold**, also showing the host) and sequences of *Contracaecum* species collected from birds, obtained from the NCBI database, with the tree inferred from the ITS1 data set. The numbers on the tree branches represent the percentage of bootstrap resampling (with 1000 replicates). *Ascaris suum* was used as an outgroup. GenBank accession numbers are in front of species names
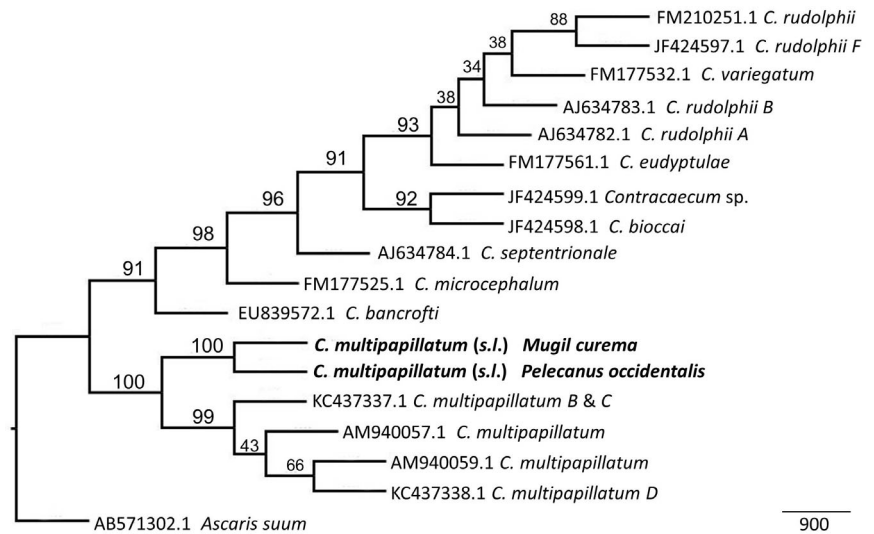
less vigorous type of movement than that of the ensheathed larvae and tended not to adhere as much to the substrates of the medium using the posterior end. At the end of the experiment, more than 50% of the larvae were dead. When culture was carried out at 15°C, a similar pattern was observed, although exsheathment did not occur within the timespan of the experiment but was observed later. Exsheathed larvae observed by optical microscopy also revealed an oesophagus, ventriculus, ventricular appendix, incipient intestinal cecum, intestine, rectum and the excretory cell, excretory duct and excretory pore (Fig. 6). From when the larvae began to exsheathe the intestinal contents were seen to actively move,

Fig. 3. Eggs of *Contracaecum multipapillatum* s.l. under an optical microscope. Egg 'a' has 3 layers, with the outer layer slightly rough. Egg 'b' has 2 smooth layers and contains larvae, and egg 'c' is empty after hatching of the larvae, with the exit hole visible (*)

alternating between the ventriculus and the rectum (see Video S2).

DISCUSSION

The nematodes collected from the brown pelican and mullet were identified as belonging to the complex of species of *Contracaecum multipapillatum* s.l. (K2P < 0.07), clearly separated from other species and complexes of *Contracaecum* that infect birds (K2P > 0.30; Table 1, Fig. 2), as shown by phenetic analysis.

Most of the eggs collected from the uteri of *C. multipapillatum* s.l. females obtained from the brown pelican were already embryonated, probably as a result of the time elapsed between the collection of the nematodes and the removal of the eggs. Huizinga (1967) reported that the eggs collected from the uteri of females were either not embryonated or were in a 2- or 4-cell state. Various authors have described a certain degree of embryonation (up to the morula stage) in both anisakid eggs taken from the uterus and those passed in faeces of the host (Thomas 1937, Grabda 1976, Brattey 1990). However, Adroher et al. (2004) reported that the eggs spontaneously liberated in culture by females of *Hysterothylacium aduncum*, a gastrointestinal anisakid of fish, were not embryonated. The eggs collected in the present study measured approximately 53 × 43 µm. Vidal-Martínez et al. (1994) reported eggs measuring 53 × 38 µm, Huizinga (1967) reported 65 × 58 µm, and Lucker (1941) reported ca. 60 × 50 µm. Although the last 2 measurements are greater than those found in the present study, they are similar to those for the
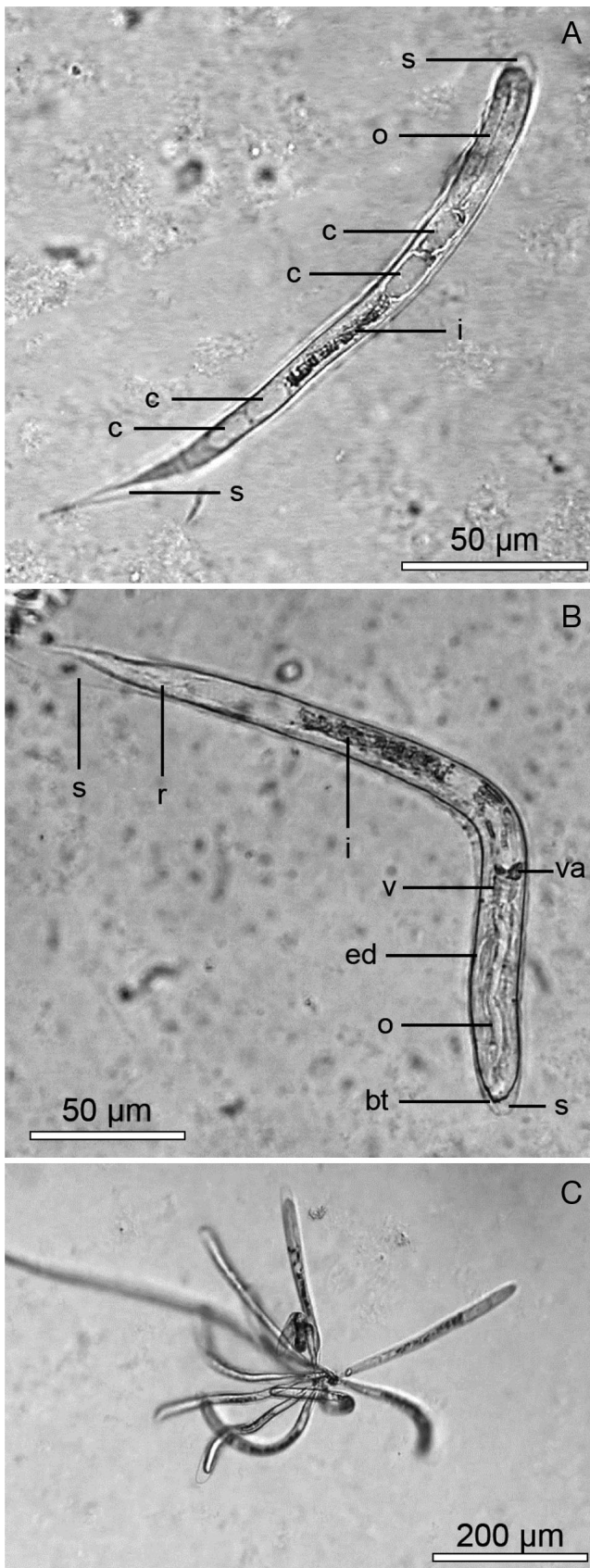
Fig. 4. Eggs and larvae of *Contracaecum multipapillatum* s.l. during development. (A) Larvae within eggs, with larvae pushing the eggshell to hatch (∗), larvae hatching (¤), empty eggs, larvae with sheath and some exsheathed larvae (§); note that these are larger than ensheathed larvae. (B) Larva in an egg, with the boring tooth (bt), oesophagus (o), intestine (i) and sheath (s) visible. (C) Larva during hatching

Table 2. Effect of salinity and temperature on development and hatching of eggs and survival of hatched larvae of *Contracaecum multipapillatum* s.l. Data are mean (range) for eggs from 2 different females, except where marked. Experiments were performed in triplicate

Temp (°C)	NaCl (‰)	Unhatched eggs (%)	Hatched eggs (%)	Larvae alive (%)
15	9[a]	12	88	75
	28	19 (12–27)	80 (73–88)	82 (74–90)
	35	3 (2–5)	97 (95–98)	81 (79–82)
24	9	29 (19–39)	71 (61–81)	55 (48–62)
	28	25 (22–28)	75 (72–78)	40 (36–44)
	35	20 (18–21)	80 (79–82)	30 (12–47)
[a]Eggs from a single female				

eggs with the developed larvae inside, which measured up to 66 × 55 µm, probably due to the lack of rigidity and elasticity of the egg shell associated with the movement of the larva inside the egg, as suggested by Adroher et al. (2004). The eggs exhibited negative buoyancy in all solutions, as in other anisakids (Huizinga 1966, Adroher et al. 2004). This could explain why mullet, which are catadromous fish, are usually infected with *Contracaecum* in endemic zones, since they feed on detritus, micro-algae and benthic organisms in sandy- or muddy-bottomed coastal areas where the eggs and larvae of these parasites can be found, either free or within copepod hosts (Salgado-Maldonado & Barquín-Álvarez 1978, Deardorff & Overstreet 1980, Chávez López &

Fig. 5. Ensheathed larvae of *Contracaecum multipapillatum* s.l. (A) Newly hatched larva with developing intestine containing large clear areas at both ends. (B) Hatched larva that is older than the larva in (A), showing more advanced development. (C) Larvae in a cluster attached to the substrate and to each other by the posterior end of their sheaths. bt: boring tooth; c: clear areas; ed: excretory duct; i: intestine; o: oesophagus; r: rectum; s: sheath; v: ventriculus; va: incipient ventricular appendix

Montoya Mendoza 1988, Juárez-Arroyo & Salgado-Maldonado 1989).

According to Huizinga (1967), the shell of the eggs of *C. multipapillatum* is thin with 2 layers: a transparent, adhesive and lightly mamillated outer layer and a thin inner vitelline layer. Although some eggs in the present study had 2 apparently smooth layers, many seemed to have 3 layers, the outermost layer being slightly rough (Fig. 3). Among anisakid eggs, a double covering, with a smooth or slightly undulating outer layer is frequently present (see Anderson 2000 for references); however, a triple covering with a rough outer layer has been reported in another anisakid (Adroher et al. 2004).

Many authors, working with different anisakids, have shown that the development of the eggs is directly related to the maintenance temperature, such that they develop more rapidly with increasing temperature, with a maximum around 13–20°C. Temperature has also been shown to affect the infectivity of the larvae for the first host, at least in the anisakid *Pseudoterranova decipiens* (see Palm 1999 for references). The salinity of the medium has also been shown to have an effect in *Anisakis simplex*, with increasing numbers of eggs hatched when salinity was 10‰ or greater (Højgaard 1998). In the present study, hatching of *C. multipapillatum* s.l. was greatest at 15°C, tending to increase slightly with salinity, both at 15°C and at 24°C (Table 2). The fact that we found only slight differences with temperature could be due to working within a range of high temperatures (15–24°C). The hatching percentages reported for different species of anisakids vary greatly, not only due to experimental conditions but also to the species and strains employed (see Bier 1976 for references). Hatching was high in the present study (61–98%).

The decrease in the percentage of live larvae at the end of the experiment at 24°C compared to at 15°C and its relationship with salinity at 24°C may be due to a more rapid development/metabolism of the larvae as a result of the higher incubation temperature and the efforts of the larvae to maintain osmotic equilibrium. Both would require greater
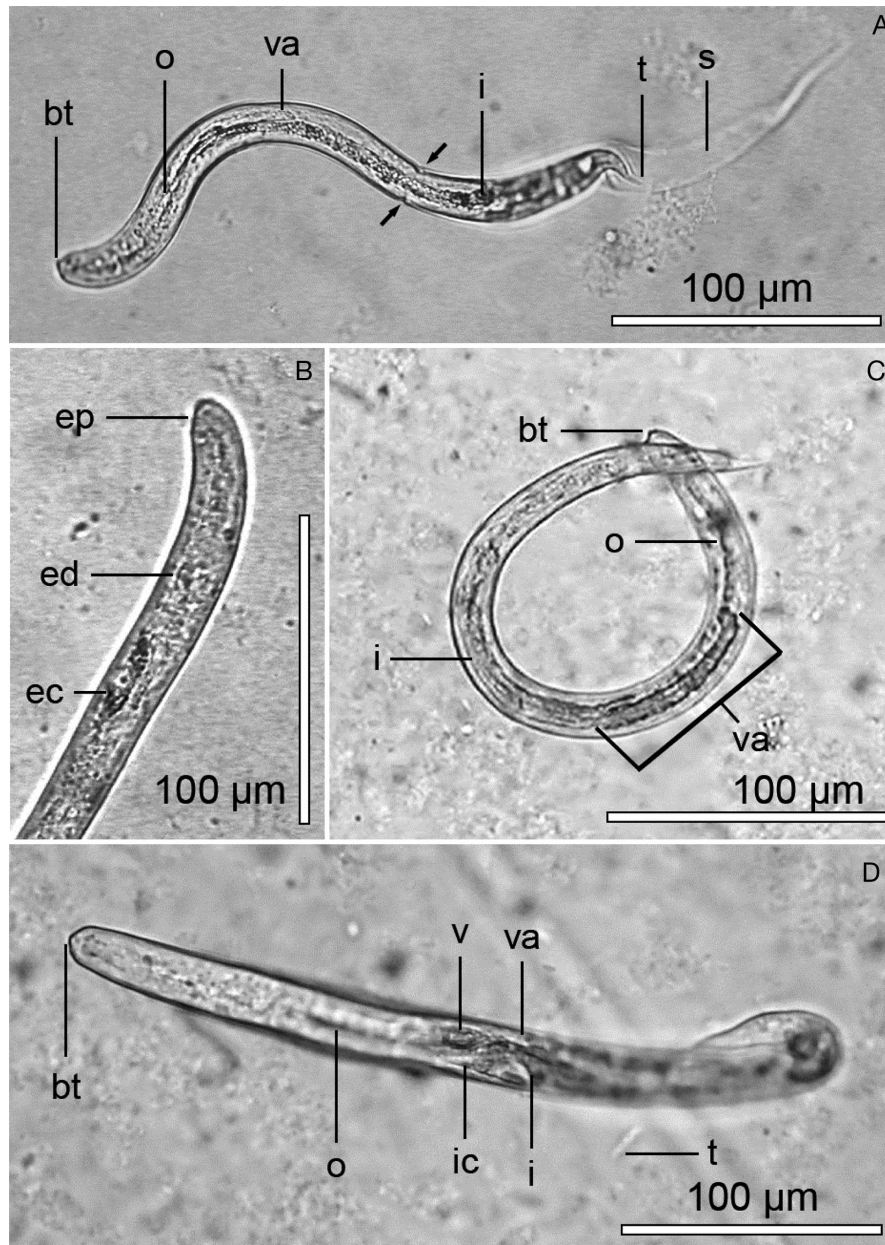
Fig. 6. (A) Exsheathing larva and (B–D) exsheathed larvae of *Contracaecum multipapillatum* s.l. bt: boring tooth; ec: excretory cell; ed: excretory duct; ep: excretory pore; i: intestine; ic: incipient intestinal caecum; o: oesophagus; s: sheath; t: tail; v: ventriculus; va: ventricular appendix. The arrows in panel (A) indicate the strangulation caused by the sheath during larval exsheathment

energy expenditure by the larvae, still ensheathed, resulting in their exhausting their energy reserves within the saline medium, which lacks exogenous energy substrates. Similar results were obtained for *C. rudolphii* s.l. collected from cormorants (Dzie-końska-Rynko & Rokicki 2010). The high rate of hatching and survival of the larvae in all of the saline solutions used in our study (Table 2), particularly at

15°C, suggests that the life cycle of this parasite can take place both in the sea and in fresh or brackish water. This has also been suggested for other anisakids such as *C. osculatum baicalensis*, *H. adun-cum* and *P. decipiens* (Sudarikov & Ryzhikov 1951, Yoshinaga et al. 1987, Burt et al. 1990). The presence or absence of light during culture at 24°C had no significant effect on the results (not shown).

Much debate surrounds the larval stage which hatches from anisakid eggs. Køie (1993), Køie & Fagerholm (1993) and Køie et al. (1995) found that a larva is enclosed by 2 cuticles before hatching and is consequently third-stage, at least in *H. aduncum*, *C. osculatum*, *A. simplex* and *P. decipiens.* Smith et al. (1990) successfully infected trout intraperitoneally with larvae of *C. osculatum* which had recently been hatched (~0.4 mm in length), and they studied the development of the larvae up to 13 mm and with L3 characteristics, without observing any moulting. Thomas (1937) had already shown that it was the L3 that emerged from the egg of *C. spiculigerum* (=*C. rudolphii*) after 2 moults inside it. Similar results were reported by Moravec (2009). In this respect, in the other anisakids mentioned it has not been possible so far to demonstrate the existence of new moults after hatching and before reaching the final host, suggesting that, at least in most anisakids, the L3 hatches from the egg. However, other authors believe that it is the L2 that hatches from the egg (Huizinga 1966, 1967, Measures & Hong 1995, Bartlett 1996).

To our knowledge, our study is the first to attempt the laboratory culture of hatched *C. multipapillatum* larvae in a nutritive medium. If the proposal of Huizinga (1967) is correct, then the larvae obtained from hatching the eggs of *C. multipapillatum* s.l. must be L2, despite the belief of many authors that the larvae hatching from the eggs of the anisakids studied are L3 (see above). The size of the recently hatched larvae (261 × 16 μm) is smaller than the 362 × 13 μm reported by Huizinga (1967). These larvae exhibited a flicking motion, which has also been described in other species of anisakids (Davey 1969, McClelland & Ronald 1974a, Adroher et al. 2004), and they were often attached to each other by the posterior end of their sheaths, forming clusters, or to eggshells or to substrates in the medium (Video S1), in a similar way to that described for hatched larvae of *C. spiculigerum* (= *C. rudolphii*), *P. decipiens* or *A. simplex* (Thomas 1937, Huizinga 1966, McClelland & Ronald 1974b, Højgaard 1998). The culture medium, at 24°C, permitted some growth in the larvae, leading to exsheathment and a subsequent increase in diameter up to 22 μm as well as a change to a slower, less vigorous serpentine swimming movement. This change has been observed in other anisakids such as *H. aduncum* (R. Benítez & F. J. Adroher unpublished) and *C. osculatum* (Davey 1969). As other authors have also shown, an increase in temperature results in more rapid development of the eggs and larvae (McClelland 1990, Measures 1996). This would ex-

plain the exsheathment in the medium at 24°C, but not at 15°C, in the 25 d of the experiment, although, at the latter temperature, this was observed some days after completing data collection. However, in no case were additional moults observed.

According to Huizinga (1967), exsheathment takes place in the intestine of the copepod, the first host of the parasite, as has also been observed in *C. osculatum* (Davey 1969). The larvae grow within the sheath, often filling it completely, before freeing themselves of it. We observed larvae in the process of exsheathing in which the pressure exerted by the sheath on the body of the larva at the point of exit can be seen to be strangling it (Fig. 6A). In fact, in the cultures some larvae were seen to be unable to exsheathe completely. Although it is not known whether digestive enzymes of the copepod are needed to facilitate or stimulate exsheathment, proteases have been shown to be involved in hatching, exsheathment and moulting in nematodes (reviewed by Malagón et al. 2013). Finally, the description of recently exsheathed larvae (Fig. 6) is very similar to that of L3 found in the fish hosts of *C. multipapillatum* s.l., although less developed and smaller. This suggests that it could be the L3 larval stage that hatches from the egg in this nematode, as occurs in other anisakids (see above). These larvae appear to have a functional intestine, since, on exsheathing, intense movement of the intestinal content was observed (Video S2). This was probably a result of entering into direct contact with the medium on escaping from the sheath, although it is not known whether the larvae at this stage were able to ingest food.

The high prevalence of infection by *Contracaecum* spp. in mullet (95% in *Mugil curema* and 100% in *M. cephalus*) and pelicans (83% in *Pelecanus occidentalis*) in the area of La Paz, Mexico (Iglesias et al. 1998, Valles-Vega 2011, 2014), which probably maintains the life cycle of *C. multipapillatum* s.l. in this geographical zone (Fig. 7), could be explained by the following: the density of the eggs of *C. multipapillatum* s.l., which is greater than that of seawater, their ability to develop and the survival of the larvae at both temperate (15°C) and high temperatures (24°C) and at different salinities (9, 28 and 35‰), and, finally, on the one hand, the catadramous behaviour and benthic feeding habits of the mullet, and, on the other hand, the frequent consumption of these fish by pelicans (Humphrey et al. 1978). Cannon (1977) noted that *Contracaecum* and *Thynnascaris* (=*Hysterothylacium*) occur principally in bottom feeders.

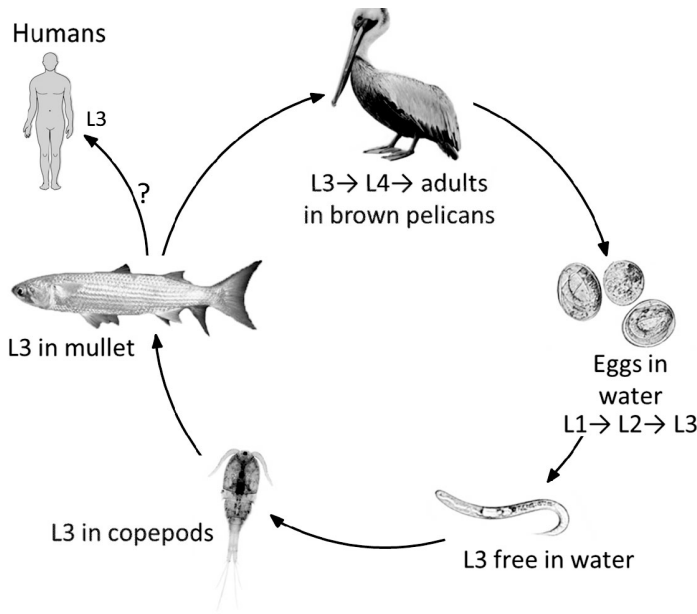Regarding the possible first crustacean host of the parasite in this zone, Valles-Vega (2014) made sev-

Fig. 7. Tentative life cycle of *Contracaecum multipapillatum* s.l. in the geographical area of Bahía de La Paz, Gulf of California, Mexico

eral unsuccessful attempts to experimentally infect omnivorous marine copepods such as *Acartia clausi*, *A. lilljeborgi* and *Centropages furcatus* from Bahía de La Paz or *Calanus helgolandicus*, *Centropages typicus* and *Acartia* sp. from Motril waters (northern Alborán Sea in the western Mediterranean Sea), with recently hatched larvae. However, Huizinga (1967) was able to experimentally infect the freshwater copepod *Cyclops vernalis*.

Previous studies in Bahía de La Paz showed that surface water temperature varies during the annual cycle from a minimum average of 20.5°C in winter to an average maximum of 31°C in summer, not significantly varying until 100 m depth (Obeso-Nieblas et al. 2008, Guevara-Guillén et al. 2015). Salinity shows minimal annual changes depending on river inputs and evaporation, ranging between averages of 34.5‰ in winter and 39‰ in summer (Chávez-Sánchez 2012). Finally, in the study area, Palomares-García (1996) showed that the copepods, intermediate hosts of *Contracaecum*, occur throughout the year, and their abundance is related to the seasonal hydrographic changes, with up to 129 species of copepods documented (Aceves-Medina et al. 2007 for references), the most abundant being *A. clausi*, *A. lilljeborgi* and *C. furcatus* (S. Hernández-Trujillo unpublished data). There are also mullet (intermediate/paratenic hosts) and pelicans (final hosts) throughout the year (I. Valles-Vega unpublished data). These data show that the life cycle of *C. multipapillatum* s.l.

is likely to occur throughout the year in this area of the Gulf of California, since, as we have shown, *C. multipapilatum* L3 from the mullet and *C. multipapilatum* adults from the brown pelican correspond to the same species (K2P = 0).

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