Acta Zoologica (Stockholm) **90**: 75–86 (January 2009) doi: 10.1111/j.1463-6395.2008.0343.x

The Florida amphioxus (Cephalochordata) hosts larvae of the tapeworm *Acanthobothrium brevissime*: natural history, anatomy and taxonomic identification of the parasite

Nicholas D. Holland,[1] Terry G. Campbell,[2] James R. Garey,[2] Linda Z. Holland[1] and Nerida G. Wilson[1]

[1]Marine Biology Research Division, Scripps Institution of Oceanography, La Jolla, CA 92093-0202, USA; [2]Department of Biology, University of South Florida, Tampa, FL 33620, USA

Keywords:
lancelet, stingray, cestode, tetraphyllidean, *Acanthobothrium brevissime*

Accepted for publication: 28 May 2008

Abstract

Holland, N. D., Campbell, T. G., Garey, J. R., Holland, L. Z. and Wilson, N. G. 2009. The Florida amphioxus (Cephalochordata) hosts larvae of the tapeworm *Acanthobothrium brevissime*: natural history, anatomy and taxonomic identification of the parasite. — *Acta Zoologica* (Stockholm) **90**: 75–86.

Plerocercoid larvae of a tapeworm are frequently found in the hindgut lumen of the Florida amphioxus (*Branchiostoma floridae*) in central west Florida. About three-quarters of the adult amphioxus are parasitized. On average, each adult amphioxus hosts about five tapeworm larvae. The residence time of the parasites in the amphioxus gut appears to be in the order of several months, which is considerably shorter than the potential lifetime of the host. The living larvae range in length (when fully extended) from 300 to 850 m and are approximately cone-shaped, tapering to a point posteriorly and bearing a single large sucker anteriorly. Toward the anterior end of the body are four hookless bothridia, each indented by three loculi plus an inconspicuous accessory sucker. The larvae initiate the early stages of hook formation when they are cultured for a few days in urea-saline (mimicking the gut fluid of the definitive host, which is an elasmobranch). The tapeworm larvae are identifiable to genus and species on the basis of correspondences between their nuclear ribosomal DNA genes and those of adult specimens of *Acanthobothrium brevissime* recovered from the spiral valve of a stingray from the same environment.

Nicholas Holland, E-mail: nholland@ucsd.edu

Introduction

Amphioxus is an invertebrate chordate that has long been studied intensively for the insights it provides into the evolutionary origin of the vertebrates (Stokes and Holland 1998). In spite of this scrutiny, however, amphioxus has only rarely been reported to harbour commensals or parasites, either protozoan or metazoan (Table 1). We were thus surprised to discover that the Florida amphioxus (*Branchiostoma floridae*) – which we were dissecting to make subtractive complementary DNA libraries – was parasitized by larval tapeworms (phylum Platyhelminthes, class Cestoda). All the larvae were of a single morphological type and were found in the gut lumen. Although the terminology for naming larval tapeworms (also called metacestodes) is somewhat unsettled, the ones parasitizing amphioxus were clearly plerocercoids (as defined by Chervy 2002). We followed up our discovery with a five-year study of the parasitism and found that these cestode larvae were commonly present in amphioxus collected throughout the region of central west Florida.

Here we (1) describe the morphology, behaviour and length–frequency distribution of the larvae, (2) trigger the larva-to-adult transition *in vitro*, (3) determine the prevalence and intensity of the infection from a sample of several hundred amphioxus containing more than a thousand tapeworm larvae, and (4) identify the larvae to genus and species by comparing their nuclear ribosomal DNA (rDNA) sequences with those of taxonomically described adult tapeworms. Molecular phylogenetic approaches are increasingly being used to match life history stages of cestodes (Brickle *et al.* 2001; Dezfuli *et al.* 2002; Agustí *et al.* 2005; Anzar *et al.*

Table 1 Previous reports of parasites and commensals of amphioxus (*Branchiostoma* spp.)

Parasite or commensal	Host species	Tissue location	Reference(s)
Protozoans			
Coccidian apicomplexan	*B. lanceolatum*	In intestinal wall	Pollard (1893)[1]
Hymenostome ciliate	*B. lanceolatum*	In atrial cavity	Codreanu (1928)[2]
Ciliate[3]	*B. floridae*[4]	On oral cirri	Boschung and Gunter (1962)
Metazoans			
Trematode	*B. lanceolatum*	In coelom	Burchardt (1900)[5]
Nematode	*B. belcheri*	In atrium?	Nishikawa 1981[6]

[1] Questioned by Labbé (1896), who thought it was really a gregarine, but evidently seen again by Burchardt (1900), who supported its coccidian affinities and named it *Branchiocystis amphioxi*.
[2] Named *Frontonia branchiostomae*, and seen previously by Pollard (1893) and Dragesco (1953).
[3] Not identified to any lower taxonomic category.
[4] As *B. caribaeum* in original publication.
[5] Single specimen, possibly a metacercaria.
[6] Single specimen (second specimen seen near original locality by G. W. Rouse in 1998, unpublished).

2007). In the present instance, we sought the adult tapeworms in local stingrays, which are the definitive hosts of many marine tapeworms and which are known to be major predators of amphioxus in west Florida (Stokes and Holland 1992). Studies like the present one are important in the light of what Caira *et al.* (2006) have called 'the remarkable lack of detailed data available on the life-cycles of most marine cestode groups.'

Materials and Methods

Collection of amphioxus; demonstration of the infection site; length distribution of the tapeworm larvae; prevalence and intensity of the infection

Several hundred specimens of the Florida amphioxus (*B. floridae* Hubbs 1922) were collected by shovel and sieve at a depth of about 1 m in central west Florida. Twenty collections were made over 5 years (Table 2) at seven different locations (Fig. 1). The length of each living amphioxus was measured to the nearest millimetre. To establish where the tapeworm larvae were located in the body of the host, we fixed five adult amphioxus (collected on 17 July 2002) in Bouin's fluid, embedded them in paraffin, and prepared them as complete series of 10- m thick serial sections stained with 0.5% aqueous toluidine blue. For all subsequent collections, we dissected each amphioxus in a dish of seawater to remove the larvae from the gut lumen. After removal from the host, the living larvae were easy to detect and count because of their sustained cycle of extension and retraction (in one instance, quantified from recordings made through a dissecting microscope fitted with a video camera).

We established the length distribution for a sample of 58 larvae from amphioxus collected at Apollo Beach on 9 August 2006. For each larva, the living length at maximum extension was measured with an ocular micrometer. To facilitate the comparison of these living lengths with literature values for other larval cestodes (which are often measured from preserved material), we also determined the effects of fixation and critical point drying on this parameter. The same larvae that had been measured alive were fixed overnight in 95% ethanol and measured a second time. Finally, following

Table 2 Numbers of amphioxus collected with dates and locations

Collection date	Location (see Fig. 1)	Number of juveniles[1]	Number of adults[2] M	F	U[3]
17 July 2002[4]	CC	0	3	2	0
12 July 2003	SA	0	6	6	0
16 July 2003	AB	0	4	5	1
23 July 2003	SP	0	5	5	0
23 July 2003[5]	PI	0	4	5	1
31 July 2003	MK	1	5	5	1
7 July 2004	PI	4	9	4	11
8 July 2004	AB	0	3	7	0
9 July 2004	SP	0	6	4	0
9 July 2004	SA	0	5	5	0
2 August 2005	PI	0	5	5	0
2 August 2005	HI	15	4	4	2
3 August 2005	AB	0	2	8	0
4 August 2005	SP	0	5	5	0
9 August 2006[6]	AB	0	8	8	0
9 August 2006	HI	0	8	8	0
14 August 2006	PI	0	8	8	0
26 August 2006	SP	0	8	8	0
18 March 2007	AB	0	1	1	18
19 March 2007	SP	0	10	10	0

[1] Juvenile lengths: 0.7 cm (size at settlement) to 2.3 cm (Stokes 1996).
[2] Adult lengths: ≥ 2.3 cm (Stokes and Holland 1996).
[3] Unsexable amphioxus (all juveniles and some adults) = gonads not visible.
[4] Fixed for histology and serially sectioned.
[5] All tapeworm larvae collected were fixed in 95% ethanol for DNA extraction.
[6] Some of the larvae were cultured in urea-saline to stimulate further development.

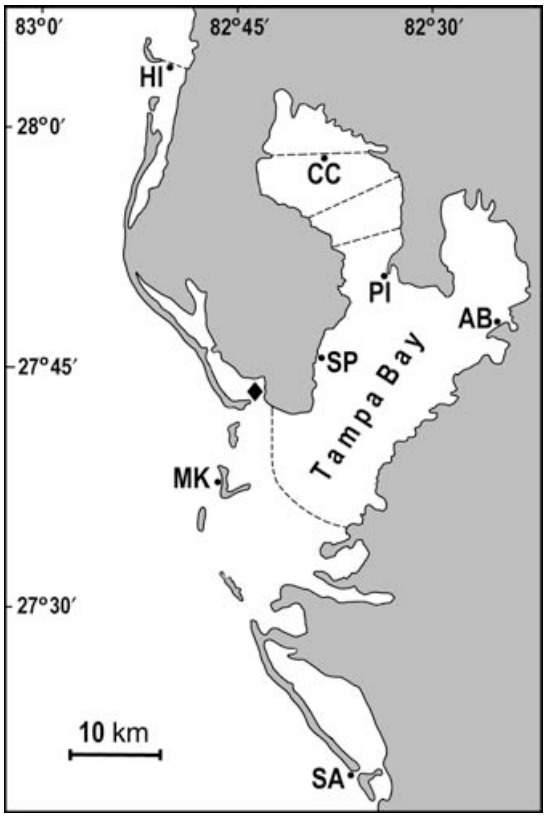

Fig. 1—Map of Tampa Bay, Florida and vicinity showing where amphioxus collections were made from 2002 though 2007. The dashed lines indicate major bridges. Collection sites, from top to bottom, are: HI, Honeymoon Island; CC, Courtney Campbell Causeway; PI, Picnic Island; AB, Apollo Beach; SP, Saint Petersburg; MK, Mullet Key; SA, Sarasota (New Pass). The diamond marks a stingray collection site (explained in text).

critical point drying, the sample was prepared for scanning electron microscopy (SEM) by methods detailed in the next section; only 26 of these larvae could be measured because the others were either lost during processing for SEM or not appropriately orientated for length measurement. In addition, from counts of tapeworm larvae, we determined the *prevalence* of infection (percentage of amphioxus containing one or more larval tapeworms) and the *intensity* of infection (number of larval tapeworms per amphioxus).

Preparing larvae for SEM observation; triggering larval-to-adult transition in vitro

For morphological study, several dozen larvae were fixed in freshly prepared 4% paraformaldehyde in 0.1 M MOPS buffer (pH 7.5) containing 1 mM EGTA, 2 mM $MgSO_4$ and 0.5 M NaCl (referred to below simply as PFA). After fixation overnight in the refrigerator, the specimens were rinsed briefly in distilled water, dehydrated through an ethanol series, critical-point-dried from CO_2, attached to stubs with double-sided adhesive tape, sputter-coated with a mixture of gold and palladium, and viewed in a Cambridge Instruments 360 scanning electron microscope. We also fixed some additional larvae immediately in 95% ethanol, and the preservation, as judged from SEM preparations, was almost as good as for the PFA-fixed specimens.

On morphological grounds, the larvae were clearly tetraphyllideans, formerly considered a well-defined order, but now known to be a paraphyletic grouping (Waeschenbach *et al.* 2007). Even more exactly, the larvae belonged to one of three genera – *Acanthobothrium*, *Calliobothrium* or *Platybothrium* – all of which have adult stages inhabiting the gut lumen of elasmobranchs. Adults of these genera can be distinguished from their hook morphology so we stimulated our larvae to begin hook growth in an attempt to identify them generically. Sixty living tapeworm larvae were isolated from amphioxus and washed in several changes of seawater. Half were cultured in 10 mL seawater as a control, and half were cultured in 10 mL of a medium mimicking elasmobranch gut fluid (the approach of Hamilton and Byram 1973) with the following composition: 250 mM NaCl, 4.4 mM KCl, 5.1 mM $MgCl_2$ and 10 mM Tris–(hydroxymethyl) amino methane-maleate buffer at pH 7.2 with 300 mM urea. The seawater and the ersatz gut fluid were maintained at 25 C and renewed twice daily. At each of three time-points (30 h, 60 h and 100 h), 10 control larvae and 10 experimental larvae were fixed in PFA and processed for SEM as described in the preceding paragraph.

Collection and taxonomic identification of adult tapeworms; matching rDNA sequences between larval and adult tapeworms

We assumed that one or more species of elasmobranchs in Tampa Bay would be parasitized by an adult tapeworm conspecific with the larval stage infecting amphioxus. Because we had already determined that stingrays are major predators of the Florida amphioxus (Stokes and Holland 1992), we collected adults of three species of stingray in Tampa Bay (*Rhinoptera bonasus*, *Dasyatis sabina* and *Dasyatis say*). The spiral valves were removed, cut into half a dozen pieces, fixed in a large volume of 95% ethanol, and shaken to dislodge adult tapeworms living there. We found that each stingray was parasitized by adult tapeworms, often representing several species, as determined using SEM. However, only one stingray yielded adult tetraphyllidean tapeworms that could conceivably be conspecific with the larval tapeworms from amphioxus. This stingray was a 53-cm long female specimen of *D. say* (the bluntnose stingray) netted on 3 January 2007 at a depth of 2 m at the location indicated by the diamond in Fig. 1. The spiral valve yielded several dozen adults of the tapeworm of interest. Some of these ethanol-fixed worms were prepared for SEM, and others were stained as whole mounts in Mayer's haematoxylin for optical microscopic study.

For molecular analysis, we extracted DNA from ethanol-fixed tapeworm tissue from larvae taken from *B. floridae* and

from adults taken from *D. say*. To correlate the life history stages of cestodes, recent studies have used variable regions of either 18S rDNA (Dezfuli *et al.* 2002) or 28S rDNA (Brickle *et al.* 2001; Agustí *et al.* 2005; Anzar *et al.* 2007). We used both. For the 18S rDNA extraction, we started with three pooled larvae and two pooled adults, from which we amplified 2067 base pairs (from stem 4 to stem 50′) with the 18S4C and 18S2C primers of Winnepenninckx *et al.* (1994). Polymerase chain reaction (PCR) products were purified by agarose gel electrophoresis and cloned for sequencing with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). For sequencing we used vector-specific T7 and T3 primers, the original PCR primers, and 10 forward (f) and reverse (r) primers based on Winnepenninckx *et al.* (1994): 18S1 (f) 5′-GTTTTCATTGGCCATGA-3′; 18S3 (f) 5′-TGGGTGGTGGTGCATGGC-3′; 18S3+ (f) 5′-GCAATAACAGGTCTGTG-3′; 18S5 (r) 5′-ACCATACTCCCCCCGGAACC-3′; 18S5+ (r) 5′-TGAACCCATGCCGACAT-3′; 18S6 (f) 5′-ACGGGTAACGGGGAATCAGGGTTCG-3′; 18S7 (r) 5′-CGAACCCTGATTCCCCGTTACCCGT-3′; 18S8 (f) 5′-TGCAGTTAAAAAGCTCGTAGTTG-3′; 18S9 (r) CAACTACGAGCTTTTTAACTGCA-3′; 18S (r) 5′-CTAAGGGCATCACAGACC-3′. Contiguous sequences for each gene were assembled and edited using SEGMEN II (DNAStar Inc., Madison, WI).

For the 28S rDNA, a single larva and a single adult cestode underwent extraction with a DNeasy blood and tissue kit (Quiagen, Valencia, CA). We amplified approximately 750 base pairs (encompassing the D2 loop) with the 300F and ECD2 primers of Littlewood *et al.* (2000). The amplified products were gel-purified, and bi-directionally sequenced on an ABI automated sequencer using a Big Dye Readymix reaction kit (ABI-Perkin Elmer, Foster City, CA.). The sequences were edited, and the forward and reverse sequences were reconciled using SEQUENCHER v.4.7 (Gene Codes Corp., Ann Arbor, MI). Voucher specimens of the tapeworm larvae (fixed either in 4% PFA or 95% ethanol) have been deposited in the Benthic Invertebrates Collection at Scripps Institution of Oceanography (Voucher numbers: SIO-BIC-PT50-52).

To construct phylogenetic trees, we included the following species (with GenBank accession numbers for their 18S and 28S rDNA, respectively): one distant outgroup (*Caryophyllaeus laticeps*, AJ287488, AF286911), four relatively close relatives of *Acanthobothrium* (*Pachybothrium hutsoni*, EF095246, EF095260; *Phoreiobothrium* sp., AF286994, AF286954; *Prosobothrium armigerum*, AF286995, AF286956; and *Platybothrium auriculatum*, AF124470, EF095264), and previously published sequences for *Acanthobothrium* (*Acanthobothrium parviuncinatum*, EF095250, EF095264; *Acanthobothrium* sp. 1, AF286993, AF286953; *Acanthobothrium* sp. 2, AF126067, 28S sequence not available), and two sequences determined during the present study (adult *Acanthobothrium brevissime*, EU660530, EU170363; and the tapeworm larva isolated from amphioxus, EU660529, EU170364). The sequences were aligned with CLUSTALX v.1.83.1 (Chenna *et al.* 2003) – the alignment is available on TreeBASE (www. treebase.org/). Parsimony analyses were conducted using a heuristic search in PAUP* v4.0b10 (Swofford 2002) with tree bisection–reconnection branch swapping. Branch support was estimated using 1000 bootstrap replicates.

Results

Tapeworm larvae: site of infection, behaviour and length distribution

As determined by histological reconstructions, the tapeworm larvae in the body of amphioxus are limited to the lumen of the posterior third of the digestive tract, which comprises the iliocolon and the intestine (as defined in Ruppert 1997). When the parasites are present, the gut wall of amphioxus shows no obvious signs of damage (Fig. 2A). Sectioning also shows that the larvae are plerocercoids, in that they include no conspicuous fluid spaces (Fig. 2B). The tapeworm larvae, when dissected from their host and observed alive in a dish of seawater, repeatedly alternate between full extension and contraction to a little over half their maximum length (Fig. 2C). They crawl anteriad along the bottom of the dish by alternately applying and detaching their bothridia. Videotape analysis (Fig. 3) demonstrates that the cycle of elongation and contraction has a periodicity of about 3 s at 25 C. When kept in a dish of seawater, the larvae maintain this activity incessantly until they die after about 3.5 days. Although we could not directly observe larvae living undisturbed in the amphioxus hindgut lumen, we think it unlikely that they engage in such sustained activity there.

Lengths of living larvae, when measured in the extended state (Fig. 4), ranged from about 300 to 850 m (mean ± SD; 564 ± 128 m, $n = 58$). After overnight fixation in 95% ethanol, the same larvae ranged from about 250 to 600 m long (mean 378 ± 91 m, $n = 58$). In the SEM preparations, the larvae that were measurable ranged from about 200 to 400 m long (mean 287 ± 54 m, $n = 26$). Larval morphology (alive, ethanol-fixed, or in SEM) did not differ qualitatively over the entire length spectrum.

Detailed morphology of the tapeworm larvae described from SEM

Each larva is approximately cone-shaped, with a tapered posterior end and a broad anterior end bearing a single anterior sucker (Fig. 2D,E, AntS). Four bothridia (= sucking organs) are located toward the anterior end. Each bothridium is sessile (i.e. not stalked), and its sucking face is divided by two transverse septa (= costae) into three concavities (= loculi = areolae) (labelled 1–3 in Fig. 2D). Anterior to the loculi, each bothridium is shallowly indented by an accessory sucker (Fig. 2D, AcS). The entire surface of the larva is covered with crowded filiform microtriches (Fig. 2F).

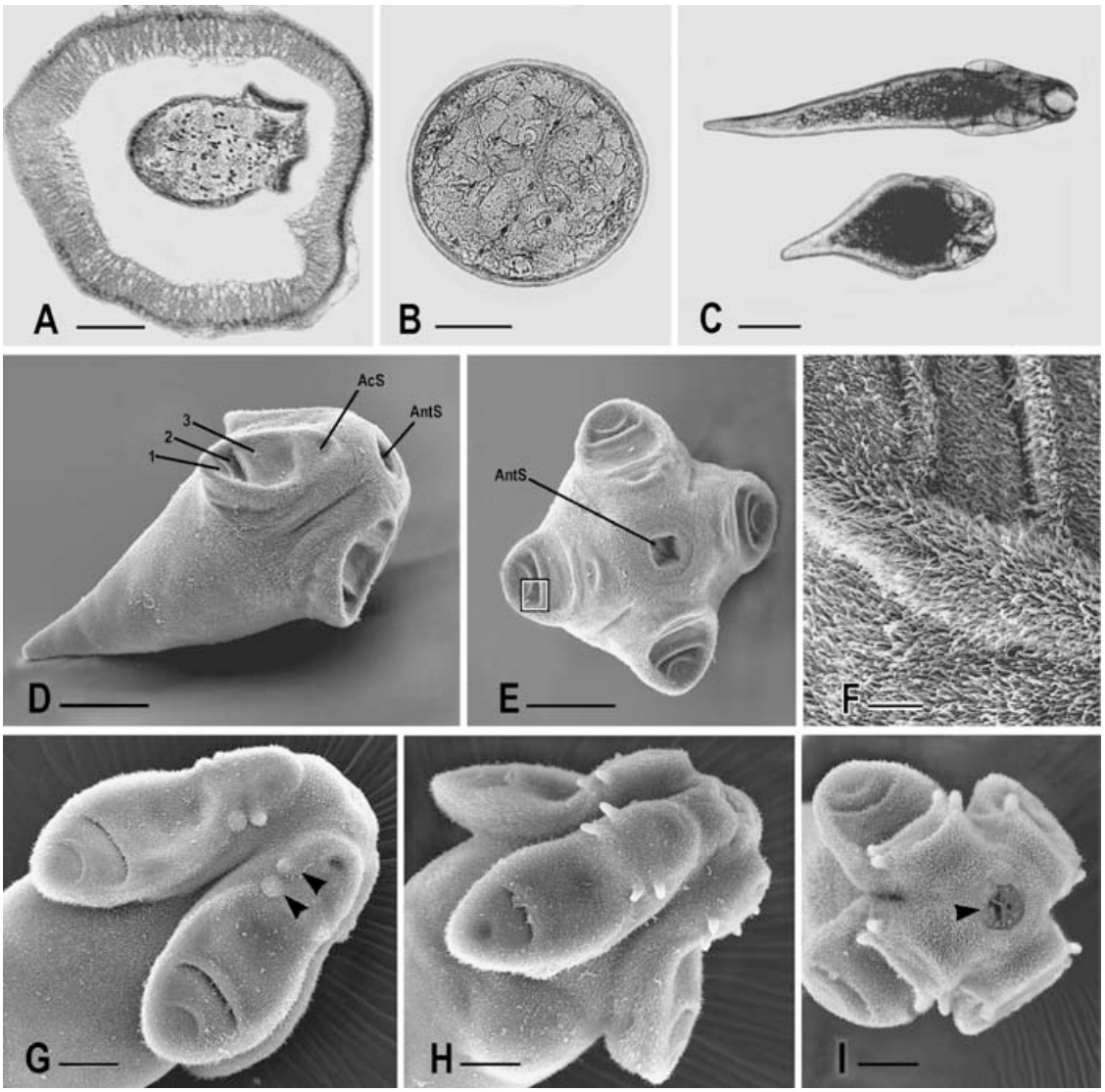

Fig. 2—Plerocercoid larvae of a tapeworm from the intestinal lumen of the Florida amphioxus (*Branchiostoma floridae*). —**A**. Light micrograph of a cross-section of the amphioxus intestine with a larva within the lumen. Scale bar: 100 m. —**B**. Light micrograph of a cross-section of a larva. Scale bar: 30 m. —**C**. Living larvae, upper one fully extended; lower one fully contracted. Scale bar: 100 m. —**D**. Scanning electron micrograph (SEM) of larva in side view; 1, 2, 3, and AcS indicate, respectively, the three loculi and the accessory sucker on the sucking face of one of the four bothridia; AntS indicates the anterior sucker at the anterior end of the larva. Scale bar: 50 m. —**E**. SEM of larva in anterior view; AntS indicates the single anterior sucker; the area in the rectangle is enlarged in the next panel. Scale bar: 50 m. —**F**. SEM of surface detail in the rectangle in previous panel. Scale bar: 5 m. —**G**. SEM of anterior end of larva cultured for 30 h in urea-saline; rudiments of the bothridial hooks (two of which are indicated by arrowheads) have appeared. Scale bar: 20 m. —**H**. SEM of anterior end of larva cultured for 60 h in urea-saline; the bothridial hooks are larger than in the preceding panel. Scale bar: 20 m. —**I**. SEM of anterior end of larva in H, showing that the anterior sucker (arrowhead) is still present. Scale bar: 20 m.

As already mentioned, these morphological features are consistent with larvae belonging to one of three genera (*Acanthobothrium*, *Calliobothrium* or *Platybothrium*), all of which live in the spiral valve lumen of elasmobranchs. Adults of these tetraphyllidean genera can be distinguished morphologically because their bothridia bear distinctively shaped hooks. Therefore, we isolated tapeworm larvae from the amphioxus gut and induced them to initiate hook formation by culturing them in a medium mimicking elasmobranch gut fluid (the approach of Hamilton and Byram 1973). After a treatment of 30 h, the larvae developed nascent bothridial hooks (Fig. 2G, arrowheads). On each bothridium, these hooks appeared as four small protrusions (an anterior and a posterior one on either side) in a region between the loculi

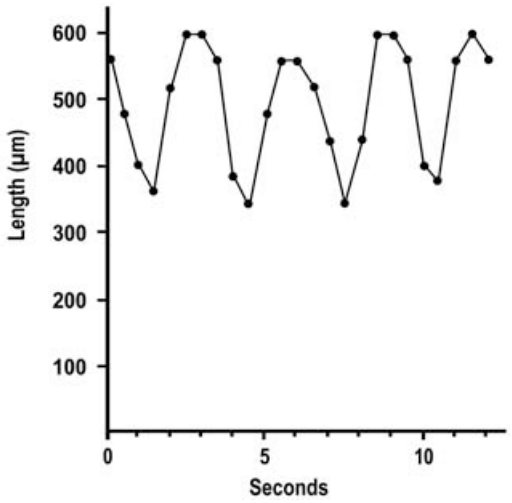

Fig. 3—The elongation/contraction cycle of a tapeworm larva removed from the gut lumen of amphioxus and observed in sea water (25 C).

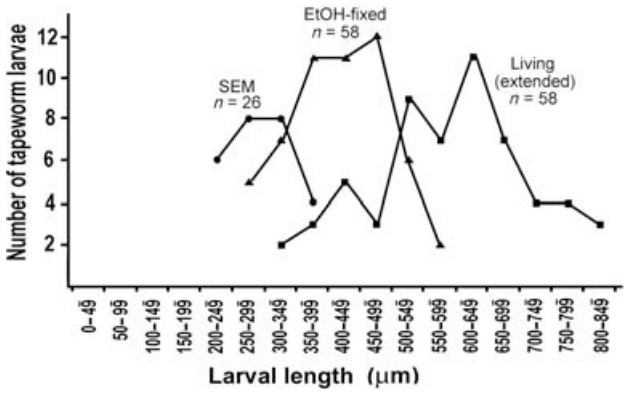

Fig. 4—Length–frequency distributions of tapeworm larvae from the gut of amphioxus. The curve at the right (square data points) shows the extended lengths of a sample of 58 living larvae; the curve in the middle (triangular data points) shows the lengths of the same 58 larvae after ethanol fixation; and the curve at the left (circular data points) shows the lengths of 26 of the foregoing larvae after preparation for Scanning electron microscopy.

and the accessory sucker. After 60 h of culture, the protrusions were longer and, on either side of each bothridium, the more anterior protrusion was now located slightly lateral to the posterior one (Fig. 2H,I). These data indicate that the larvae do not belong to the genus *Platybothrium*, but cannot distinguish between the other two genera, because we could not tell whether the neighbouring hooks on either side of each bothridium were separate as in *Calliobothrium* or united basally as in *Acanthobothrium*. In contrast to the larvae cultured in urea-saline, the larvae in seawater underwent no morphological changes. Both the experimental and the control larvae maintained their incessant elongation/contraction cycle for 3.5 days, but had died or were moribund by 100 h.

Prevalence and intensity of infection of amphioxus by tapeworm larvae

Out of the total sample of amphioxus (Table 2), 109 were males, 113 were females and 54 lacked visible gonads and were unsexable. The sex ratio of the amphioxus sampled did not differ significantly from 1 : 1 ($\chi^2_{[1]} = 0.07, P > 0.05$). The unsexable amphioxus were either juveniles under the minimum size for sexual maturity (20 in all) or adults that had evidently spawned out and not yet started to ripen again (34 in all). For the total sample of amphioxus, we determined the prevalence of infection, which is the percentage of hosts harbouring one or more tapeworm larvae.

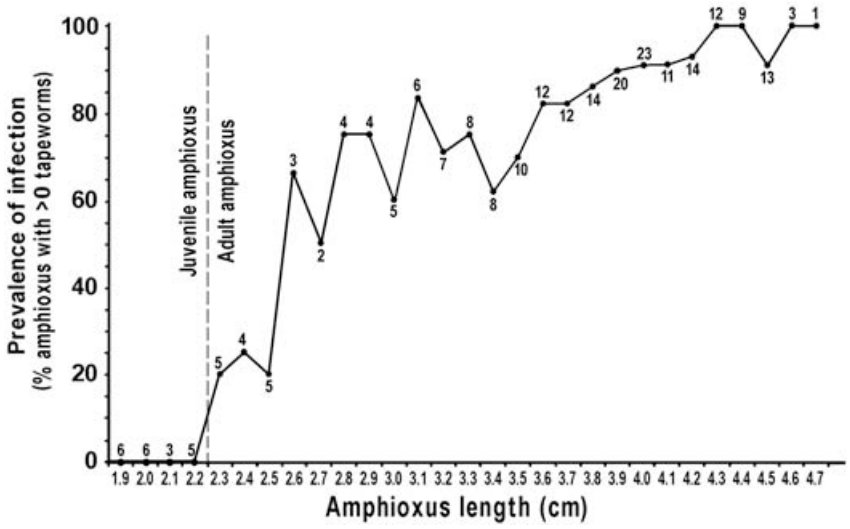

Fig. 5—Prevalence of infection (percentage of amphioxus infected with one or more larvae) in all the juvenile and adult amphioxus from all 18 summer collections. The percentage of infected animals (i.e. those harbouring one or more tapeworm larvae) is plotted for each 1-mm length class, and the number of individual amphioxus used to calculate each data point is indicated.

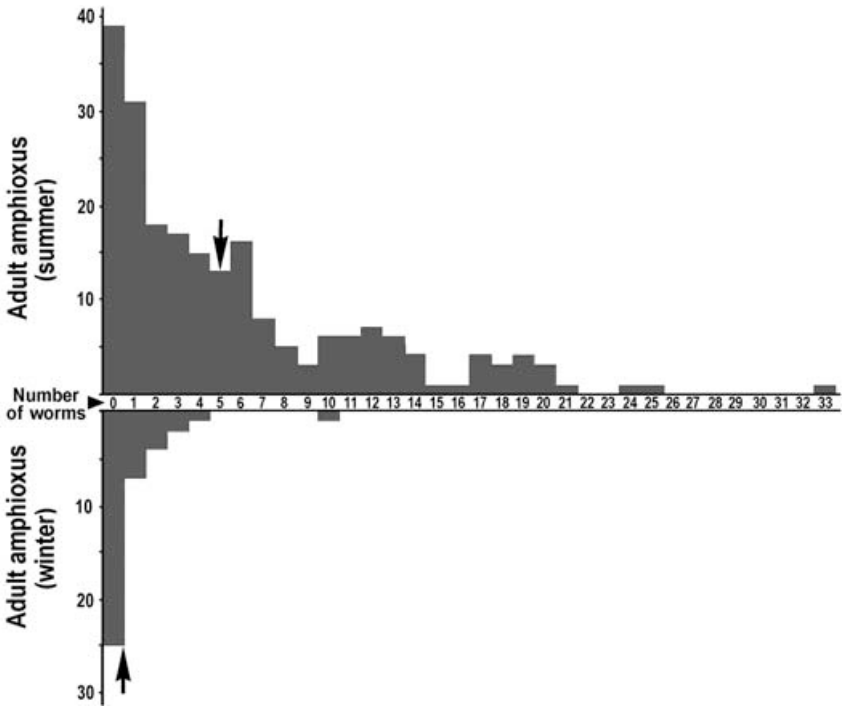

Fig. 6—Intensity of infection for all adult animals collected in the summer (above horizontal axis) and winter (below horizontal axis). The downward and upward arrows indicate, respectively, the mean intensity of infection in summer and winter.

The mean prevalence of infection (84.6%) did not differ significantly between males and females ($\chi^2_{[1]} = 0.1$, $P > 0.05$). Note that the foregoing calculation excluded any amphioxus that could not be identified as either a male or a female. In contrast, Fig. 5 plots the prevalence of infection for *all* the amphioxus (sexable or not) collected during the summer. The juvenile amphioxus – defined as shorter than 2.3 cm, the minimum length at sexual maturity (Stokes and Holland 1996) – were never infected. For amphioxus longer than 2.3 cm, the prevalence of infection progressively increased with body length. For all the amphioxus, the prevalence of infection differed significantly between summer (76% for 254 amphioxus) and winter (38% for 40 amphioxus) ($\chi^2_{[1]} = 6.9$, $P > 0.05$).

We also calculated the mean intensity of infection (Fig. 6), which is the average number of tapeworm larvae per amphioxus (also known as the worm load). Mean intensity of infection was calculated separately for summer and winter collections for all amphioxus longer than the minimum size at sexual maturity. The mean intensity in summer was about five tapeworm larvae per amphioxus, significantly higher than the mean intensity in winter, which was only about one tapeworm larva per amphioxus ($\chi^2_{[1]} = 157.6$, $P > 0.05$). The maximum intensity was also higher in summer (33 larvae per amphioxus) than in winter (10 larvae per amphioxus).

It is unlikely that the prevalence and intensity of the tapeworm infection influences amphioxus behaviour, thereby changing the probability that a given amphioxus will be eaten by a stingray. The stingray vacuums the sand containing the shallowly buried amphioxus, which it catches on the gill rakers (Stokes and Holland 1992). The predator does not use visual cues to capture the amphioxus, so there is little chance that parasite-induced changes in prey behaviour would increase the likelihood of capture.

Identification of adult tapeworms to species on the basis of morphology

Of several kinds of adult tapeworms we collected from the spiral valves of Tampa Bay stingrays, only one (from the bluntnose stingray, *D. say*) had a morphology close to that of the larval tapeworms from amphioxus – in that each bothridium was dented by three loculi and an accessory sucker. The adult tapeworms (Fig. 7A) were a few mm long and comprised a scolex and half a dozen relatively elongated proglottids that were acraspedote (nonoverlapping). The largest (posterior) proglottids easily became detached from the rest of the body of the tapeworm, so, in life, the bodies of some of the worms probably included several more proglottids than we saw in our fixed material.

There are relatively few testes per proglottid – Fig. 7(B) shows 22 testes: seven prevaginal, two postvaginal and 13 antiporal (terminology from Campbell 1969). Moreover, the two lobes of the ovary in the larger proglottids are asymmetrical (Fig. 7C, Ov). The scolex is characterized by four bothridia, each divided into three loculi (labelled 1 to 3 in Fig. 7D) by two transverse septa. Anterior to the loculi, each bothridium sprouts a pair of bifurcated bothridial hooks (Fig. 7D, BH), and anterior to the hooks is a muscular pad bearing the accessory sucker (Fig. 7D, MP and AcS). As measured from

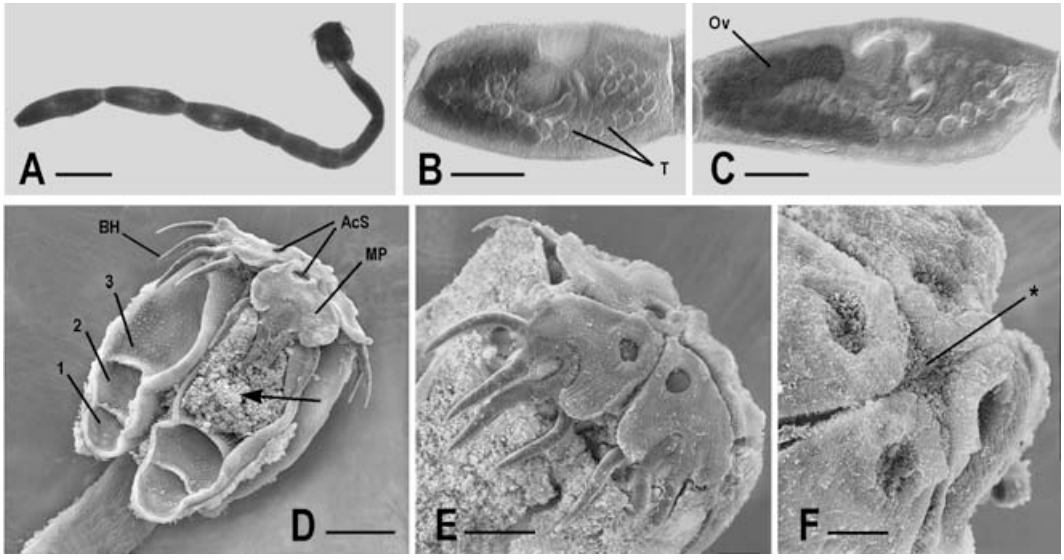

Fig. 7—Adults of a tapeworm (*Acanthobothrium brevissime*) from the spiral valve of bluntnose stingray (*Dasyatis say*). —**A**. Light micrograph of entire worm. Scale bar: 500 m. —**B**. Light micrograph of a relatively young proglottid in which the testes (T) are conspicuous. Scale bar: 100 m. —**C**. Light micrograph of an older proglottid containing an asymmetrical ovary (OV), in that the antiporal branch extends farther anterior than the other branch. Scale bar: 100 m. —**D**. Scanning electron micrograph (SEM) of adult scolex; each bothridium is divided into three loculi (labelled 1–3) and is associated anteriorly with a pair of bifurcated bothridial hooks (BH) and a muscular pad (MP) bearing an accessory sucker (AcS); the arrow indicates debris derived from the gut wall of the stingray host. Scale bar: 100 m. —**E**. SEM enlargement of the bothridial hooks. Scale bar: 50 m. —**F**. SEM demonstrating the absence of an anterior sucker (AntS) at the anterior end of the scolex (marked with an asterisk). Scale bar: 50 m.

SEM specimens (Fig. 7E), each bifurcated bothridial hook consists of an anterior handle (about 30 m long), an inner prong (about 85 m long), and an outer prong (about 75 m long). There is no anterior sucker at the anterior end of the scolex (Fig. 7F). In sum, these morphological characters define the adult stage of *A. brevissime* Linton (1908) (especially as re-described in more detail by Campbell in 1969). Of the numerous species of *Acanthobothrium* found in the western Atlantic Ocean (Ghoshroy and Caira 2001), *A. brevissime* is morphologically closest to *A. lineatum* from Virginia (Campbell 1969), *A. zapterycum* from Argentina (Ostrowski de Núñez 1971) and *A. tasajerasi* from Colombia (Brooks 1977); however, the testes of *A. lineatum* and *A. zapterycum* are much more numerous than those of *A. brevissime*, and the bothridial hooks of *A. tasajerasi* are 50% longer than those in *A. brevissime*.

Identification of the larval tapeworms to species on the basis of rDNA sequences

Our PCR primers amplified 2067 base pairs of 18S rDNA and about 725 base pairs of the 28S rDNA, fragments that were variable enough to distinguish among species in a genus. These fragments were sequenced for adults of *A. brevissime* from the stingray and for tapeworm larvae from amphioxus. Phylogenetic analyses using parsimony were carried out separately for the 18S rDNA sequences and the 28S rDNA sequences (Fig. 8A,B, respectively). For the 18S rDNA sequences, pairwise comparison shows that the adult *A. brevissime* diverged by about 3% from its congeners, but only 0.1% from the larval tapeworm. Similarly, for the 28S rDNA sequences, the adult *A. brevissime* diverged by about 6% from its congeners, but was identical to the larval tapeworm. Thus the tapeworm larvae from amphioxus and the tapeworm adults from the stingray belong to the same species – namely *A. brevissime*.

Discussion

Exact taxonomic correlations between different life cycle stages of marine tapeworms

Several approaches have been used to correlate life cycle stages within tapeworm species. In two favourable cases, different life cycle stages of marine tapeworms were matched at the species level on the basis of shared morphological peculiarities (Carvajal 1977; Caira and Ruhnke 1991). More commonly, however, life cycle stages have been correlated by isolating larvae from an intermediate host and feeding them to a suspected definitive host. Such experiments are usually designed so that the definitive host, usually an elasmobranch, is free from tapeworms at the start of the experiment – as the result of eating artificial food from birth, or starving for a long time, or being treated with a vermifuge (Monticelli 1888; Curtis 1911; Young 1954; Sakanari and Moser 1989).

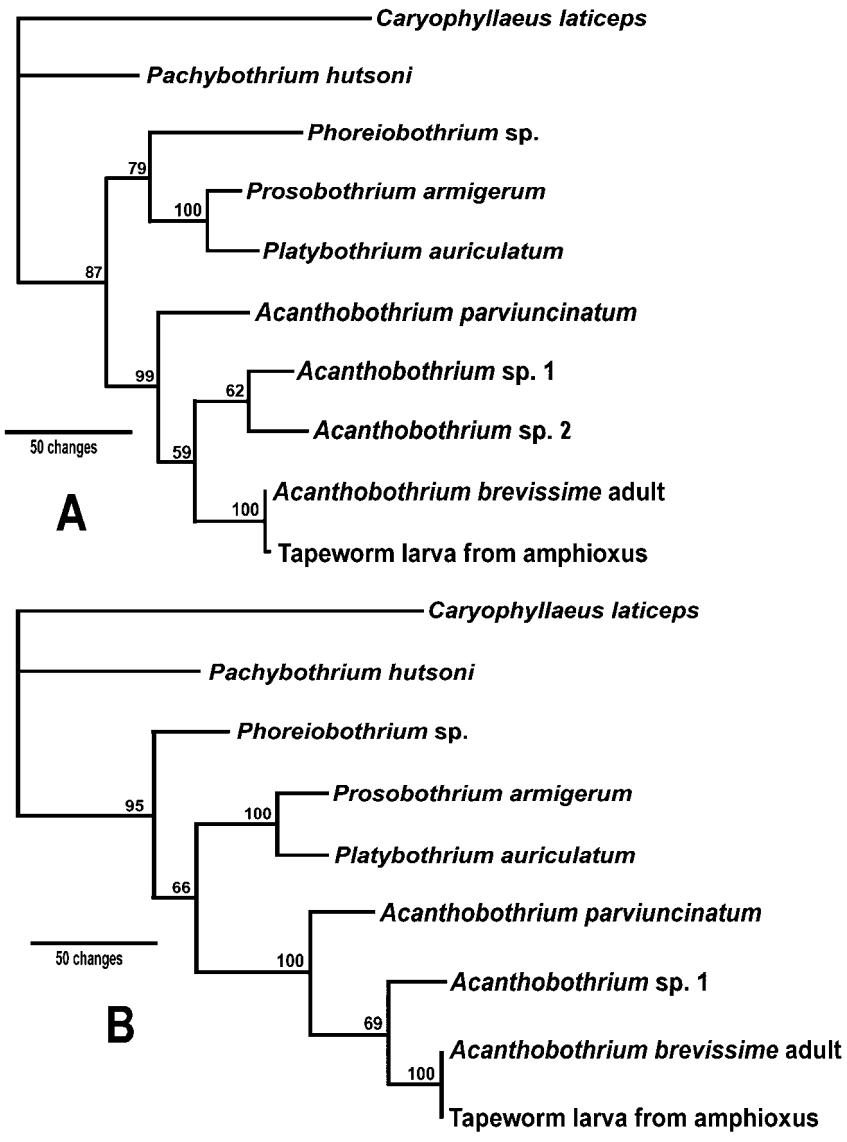

Fig. 8—Most parsimonious trees (constructed with PAUP* v4.0b10) based on recombinant DNA (rDNA) from a basal cestode (*Caryophyllaeus*), four relatively close relatives of *Acanthobothrium* (*Pachybothrium*, *Phoreiobothrium*, *Prosobothrium* and *Platybothrium*); and three species of *Acanthobothrium*. The numbers at the nodes are bootstrap values. —**A**. 18S rDNA differing by only 0.1% between tapeworm larvae from amphioxus and the adult of *Acanthobothrium brevissime* from the stingray. —**B**. 28S rDNA identical between tapeworm larvae from amphioxus and the adult of *A. brevissime* from the stingray.

Alternatively, feeding the definitive host with a great abundance of larvae can produce so many adult tapeworms that they drown out any signal from tapeworms already present (Reichenbach-Klinke 1957). To date, most feeding experiments have demonstrated transfer from the intermediate to the definitive host, and only a few (Riser 1949; Sakanari and Moser 1989) have demonstrated the reciprocal link from a definitive host back to an intermediate host. In comparison to such transmission experiments, it is less labour intensive and just as convincing to correlate life-cycle stages within tapeworm species by comparing their 18S rDNA (Dezfuli *et al.* 2002; present study) or 28S rDNA (Brickle *et al.* 2001; Agustí *et al.* 2005; Anzar *et al.* 2007; present study). This approach is becoming applicable to an ever-wider spectrum of species as more molecular sequences accumulate in databases.

Reliability of prevalence and intensity data for larval stages of tapeworms

Larval stages of tapeworms (including all of the medically important species) are most commonly encysted in solid tissues and so are difficult to recover quantitatively from the host. Therefore, accurate measurements of the prevalence and intensity of infection by larval tapeworms are exceptional (for example, the meticulous studies of Adjei *et al.* 1986; Tolonen *et al.* 2000; Gurney *et al.* 2006; Hammerschmidt and Kurtz 2007). More commonly, the numerical data for encysted tapeworm larvae have been, at best, semiquantitative underestimations (e.g. Saini *et al.* 1997; Kim and Powell 2004). In the present study, two factors contributed to the accuracy of our measurements of prevalence and intensity of infection for the larval stages of *A. brevissime* living in

amphioxus. First, the larvae could readily be recovered from the host, because they live free in the gut lumen instead of being encysted in solid tissues, and, second, the living larvae, once freed from the amphioxus, call attention to themselves by their incessant cycle of extension and retraction.

Dynamics of the larval stage of A. brevissime *in amphioxus*

Although the data in the present paper are essentially static, three dynamic aspects of the larval stage in the life cycle of the tapeworm can be inferred from the length–frequency distribution of the larvae and from the summer vs. winter comparisons of prevalence and intensity. First, it is likely that tapeworm embryos (whatever they might be – see below) enter the amphioxus gut via the mouth and then transform into larvae very quickly, because, although we isolated 1234 larvae in the present study, we never detected anything that could possibly have been interpreted as a tapeworm embryo. Second, after the larvae are produced in the amphioxus gut, they increase in length by nearly 300% over a period of several months. And, third, the residence time of the larvae in the gut is relatively short relative to the potential lifetime of host. Amphioxus lives for several years (Stokes 1996), yet we found that both the prevalence and intensity of the larval infection fall markedly in the 6 months between late summer and late winter. Taken together, these data suggest that tapeworm embryos enter the amphioxus gut and very rapidly differentiate into larvae that grow for a few months and then tend to leave the host – either because they die or because they are expelled alive with the faeces. The declines in prevalence and intensity during winter could be related to one or more of the following: a decrease in the supply of tapeworm embryos from the environment, low feeding rates by amphioxus, or an elevated larval death rate or exit rate.

Toward elucidating the complete life cycle for A. brevissime

In tapeworms generally, the embryos are released from the ripe proglottids. This release may occur while the proglottids are still in the gut lumen of the definitive host or after they have been expelled with the host's faeces. Depending on the tapeworm species, there are two main kinds of embryos. The first are non-ciliated and may be deposited in either terrestrial or aquatic environments, and the second are ciliated coracidia that hatch in water and then swim. For marine tapeworms, Cheng (1966) and Katkansky *et al.* (1969) claimed to have observed the production of coracidia, whereas Reichenbach-Klinke (1955) and Avdeev and Avdeeva (1986) only assumed that such swimming embryos were produced but did not demonstrate them directly. In contrast, Freeman (1982) and Ubelaker (1983) emphatically stated that the studies of Cheng (1966) and Katkansky *et al.* (1969) were based on faulty observations. In the light of this controversy, it would be especially interesting to study the embryonic stage of *A. brevissime*, from emission from the ripe proglottids, through entry via the mouth of amphioxus (which Ruppert *et al.* 2000 determined can take in particles up to about 100 m in diameter), to differentiation into the larval stage within the gut of amphioxus. It should be relatively straightforward to isolate stingray faeces in dishes of seawater to see if tapeworm embryos hatch out. Any embryos obtained in this way could be fed to a sample of larva-free amphioxus (it is possible to raise the hosts in the laboratory on artificial diets), which could then be sampled at frequent intervals to elucidate the transition between the embryonic and larval stages of the tapeworm.

The demonstration of the embryo-to-larva transition would reveal all the stages required for a complete life cycle of *A. brevissime*. However, a simple cycling from the larval stage in the Florida amphioxus (*B. floridae*) to the adult stage in the bluntnose stingray (*D. say*) and back does not exclude other possible scenarios. Indeed, the life cycle is already known to be potentially more complex, because adults of *A. brevissime* have been found not only in *D. say* (Linton 1908; Goldstein 1964; present study), but also in *D. sabina* (Goldstein 1964; Henson 1975), *Dasyatis americana* (Campbell 1969), *Raja eglanteria* (Campbell 1969), and possibly *Myliobatis peruvianus* (Manuel 1991) (although the last report, being the only record of *A. brevissime* in the Pacific Ocean, needs additional verification).

A more interesting question is whether the larvae of *A. brevissime* occur in intermediate hosts other than the Florida amphioxus. In Chesapeake Bay, adults of *A. brevissime* parasitize elasmobranchs (Campbell 1969), even though the Florida amphioxus does not live there or anywhere else along the east coast of the USA. However, another amphioxus species – *Branchiostoma virginiae* – does live along the east coast of the USA (Poss and Boshung 1996) and could conceivably serve as the alternative host for *A. brevissime* in that region.

There are also indications that, as alternatives to amphioxus, molluscs might sometimes serve as intermediate hosts for *A. brevissime*. Cake (1976), in a study of molluscs in the Gulf of Mexico, previously found larval tapeworms morphologically very close to the one described in the present study living in one gastropod (*Polinices duplicatus*) and several bivalves (*Agropecten irradians*, *Macoma constricta*, *Ensis minor*, *Pseudomiltha floridana*, *Raeta plicatella* and *Tagelus divisus*). At least in the bivalves, the tapeworm larvae were not free in the gut lumen but were enclosed in cysts in the molluscan connective tissue. In another study of molluscs from the Gulf of Mexico, Wardle (1974) also discovered tapeworm larvae infecting two bivalves (*Tagelus plebeius* and *Macoma constricta*). Intriguingly, these larvae were living free in the molluscan gut lumen, were characterized by rudimentary bothridial hooks, and appeared virtually identical to the *A. brevissime* larvae described here after a 60-h treatment with urea-saline. Wardle (1974) guessed that the adult stage of the tapeworm larva from these bivalves was *Acanthobothrium dujardinii* [which is actually *A. brevissime* according to Campbell (1969)], which had been found in the gut of a stingray from the same environment.

An additional potential complexity in the life cycle is that *A. brevissime* larvae might sometimes be found in second intermediate hosts (even if not absolutely required for completion of the life cycle). It has been experimentally demonstrated that some marine tapeworms (not tetraphyllideans) have life cycles that include a second intermediate host – either a carnivorous mollusc (Cake 1977) or a teleost (Sakanari and Moser 1989). There is some evidence that at least some tetraphyllideans also pass through larval stages in second intermediate hosts. Possible examples are the relatively large (several mm long) plerocercoid larvae of tetraphyllideans that have been found in carnivorous molluscs in the Gulf of Mexico (Regan 1963; Friedl and Simon 1970). Stingrays are opportunistic feeders (Gilliam and Sullivan 1993) and would be expected to prey on such molluscs. In conclusion, sequencing studies with rDNA should demonstrate whether the life cycle of *A. brevissime* can potentially include (1) intermediate hosts other than amphioxus and/or (2) second intermediate hosts.

Acknowledgements

Laboratory space and facilities in the Department of Biology at the University of South Florida were generously supplied by John M. Lawrence. We are deeply indebted to Evelyn York and Mathilde Paris for help with our SEM and cinematography, respectively. We are also extremely grateful to Brent L. Winner and Bret Pittinger of the Florida Fish and Wildlife Conservation Commission's Independent Monitoring Program for capturing stingrays for us.

References

Adjei, E. L., Barnes, A. and Lester, R. J. G. 1986. A method for estimating possible parasite-related host mortality, illustrated using data from *Callitetrarhynchus gracilis* (Cestoda: Trematoda) in lizardfish (*Saurida* spp.). – *Parasitology* **92**: 277–243.

Agustí, C., Anzar, F. J., Olson, P. D., Littlewood, D. T. J., Kostadinova, A. and Raga, J. A. 2005. Morphological and molecular characterization of tetreaphyllidean merocercoids (Platyhelminthes: Cestoda) of striped dolphins (*Sternella coeruleoalba*) from the Western Mediterranean. – *Parasitology* **130**: 461–474.

Anzar, F. J., Agustí, C., Littlewood, D. T. J., Raga, J. A. and Olson, P. D. 2007. Insight into the role of cetaceans in the life cycle of the tetraphyllideans (Platyhelminthes: Cestoda). – *International Journal of Parasitology* **37**: 243–255.

Avdeev, V. V. and Avdeeva, N. V. 1986. Occurrence of cestodes of the order Tetraphyllidea in marine invertebrates and fishes and probable schemes of their development [in Russian]. – *Parazitologiya* **20**: 448–454.

Boshung, H. V. and Gunter, G. 1962. Distribution and variation of *Branchiostoma carabaeum* in Mississippi Sound. – *Tulane Studies in Zoology* **9**: 245–257.

Brickle, P., Olson, P. D., Littlewood, D. T. J., Bishop, A. and Arkhipin, A. I. 2001. Parasites of *Loligo gahi* from waters off the Falkland Islands, with a phylogenetically based identification of their cestode larvae. – *Canadian Journal of Zoology* **79**: 2289–2296.

Brooks, D. R. 1977. Six new species of tetraphyllidean cestodes, including a new genus, from a marine stingray *Himanthura schmardae* (Werner, 1904) from Colombia. – *Proceedings of the Helminthological Society of Washington* **44**: 51–59.

Burchardt, E. 1900. Beiträge zur Kenntnis des *Amphioxus lanceolatus*, nebst einem ausführlichen Verzeichnis der bisher über *Amphioxus* veröffentlichen Arbeiten. – *Jenaische Zeitschrift für Naturwissenschaft* **34**: 719–832 + pl. XVIII–XXVI.

Caira, J. N. and Ruhnke, T. R. 1991. A comparison of scolex morphology between the plerocercoid and the adult of *Calliobothrium verticillatum* (Tetraphyllidea: Onchobothriidae). – *Canadian Journal of Zoology* **69**: 1484–1488.

Caira, J. N., Scholz, T. and Gregoriev, B. B. 2006. Cestode systematics and phylogeny move forward. – *Systematic Parasitology* **65**: 171–174.

Cake, E. W. 1976. A key to larval cestodes of shallow-water benthic molluscs in the Northern Gulf of Mexico. – *Proceedings of the Helminthological Society of Washington* **43**: 160–171.

Cake, E. W. 1977. Experimental infection studies with bothridio-plerocercoids of *Rhinebothrium* sp. (Cestoda: Tetraphyllidea) and two intermediate molluscan hosts. – *Northeast Gulf Science* **1**: 55–59.

Campbell, R. A. 1969. New species of *Acanthobothrium* (Cestoda: Tetraphyllidea) from Chesapeake Bay, Virginia. – *Journal of Parasitology* **55**: 559–570.

Carvajal, J. 1977. Description of the adult and larva of *Caulobothrium myliobatidis* sp. n. (Cestoda: Tetraphyllidea) from Chile. – *Journal of Parasitology* **63**: 99–103.

Cheng, T. C. 1966. The coracidium of the cestode *Tylocephalum* and the migration and fate of this parasite in the American oyster, *Crassostrea virginica*. – *Transactions of the American Microscopical Society* **85**: 246–255.

Chenna, R., Sugawara, H., Koike, T., Lopez, R., Gibson, T. J., Higgins, D. G. and Thompson, J. D. 2003. Multiple sequence alignment with the CLUSTAL series of programs. – *Nucleic Acids Research* **31**: 3497–3500.

Chervy, L. 2002. The terminology of larval cestodes or metacestodes. – *Systematic Parasitology* **52**: 1–33.

Codreanu, R. 1928. Un infusoire nouveau (*Frontonia branchiostomae* n. sp.) commensal de l'amphioxus (*Branchiostoma lanceolatum* Pall.). – *Société Roumaine de Biologie* **98**: 1078–1080.

Curtis, W. C. 1911. The life history of the *Scolex polymorphus* of the Woods Hole region. – *Journal of Morphology* **22**: 819–853.

Dezfuli, B. D., Capuano, S. and Congiu, L. 2002. Identification of life cycle stages of *Cyathocephalus truncatus* (Cestoda: Spathebothriidea) using molecular techniques. – *Journal of Parasitology* **88**: 632–634.

Dragesco, J. 1953. Contribution a la connaissance d'un infusoire commensal de l'amphioxus: *Frontonia branchiostomae* (Codreanu). – *Vie et Milieu* **4**: 605–607.

Freeman, R. S. 1982. Do any *Anonchotaenia*, *Cyathocephalus*, *Echineibothrium* or *Tetragonocephalum* (= *Tylocephalum*) (Eucestoda) have hookless oncospheres or coracidia? *Journal of Parasitology* **68**: 737–743.

Friedl, F. E. and Simon, J. L. 1970. A tetraphyllidean tapeworm larva from the marine snail *Fasciolaria tulipa* in Florida. – *Journal of Parasitology* **56**: 400–401.

Ghoshroy, S. and Caira, J. N. 2001. Four new species of *Acanthobothrium* (Cestoda: Tetraphyllidea) from the whiptail stingray *Dasyatis brevis* in the Gulf of California, Mexico. – *Journal of Parasitology* **87**: 354–372.

Gilliam, D. and Sullivan, K. M. 1993. Diet and feeding habits of the southern stingray *Dasyatis americana* in the central Bahamas. – *Bulletin of Marine Science* **52**: 1007–1013.

Goldstein, R. J. 1964. Species of *Acanthobothrium* (Cestoda:

Tetraphyllidea) from the Gulf of Mexico. – *Journal of Parasitology* **50**: 656–661.

Gurney, R. H., Johnston, D. J. and Nowak, B. F. 2006. The effect of parasitism by trypanorhynch plerocercoids (Cestoda, Trypanorhyncha) on the digestive enzyme activity of *Carcinus maenas* (Linneaus, 1758) (Decapoda, Portunidae). – *Crustaceana* **79**: 663–675.

Hamilton, K. A. and Byram, J. E. 1973. Tapeworm development: the effects of urea on a larval tapeworm. – *Journal of Parasitology* **60**: 20–29.

Hammerschmidt, K. and Kurtz, J. 2007. *Schistocephalus solidus*: establishment of tapeworms in sticklebacks – fast food or fast lane. – *Experimental Parasitology* **116**: 141–149.

Henson, R. N. 1975. Cestodes of elasmobranch fishes of Texas. – *Texas Journal of Science* **26**: 401–406.

Hubbs, C. L. 1922. A list of lancelets of the world with diagnoses of five new species of *Branchiostoma*. – *Occasional Papers of the Museum of Zoology of the University of Michigan* (number 105): 1–16.

Katkansky, S. C., Warner, R. W. and Poole, R. L. 1969. On the occurrence of larval cestodes in the Washington clam, *Saxidomus nuttalli*, and the gaper clam, *Tresus nuttalli*, from Drakes Estero, California. – *California Fish and Game* **55**: 317–322.

Kim, Y. K. and Powell, E. N. 2004. Surfclam histopathology survey along the Delmarva mortality line. – *Journal of Shellfish Research* **23**: 429–441.

Labbé, A. 1896. Recherches zoologiques, cytologiques et biologiques sur les coccides. – *Archives de Zoologie Expérimentale et Générale (Sér. 3)* **4**: 517–624 + pl. I–VIII.

Linton, E. 1908. Helminth fauna of the Dry Tortugas. – *Papers from the Tortugas Laboratory of the Carnegie Institution of Washington* **1**: 159–190 + pl. I–XI.

Littlewood, D. T. J., Curini-Galletti, M. and Herniou, E. A. 2000. The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. – *Molecular Phylogenetics and Evolution* **16**: 449–466.

Manuel, T. V. 1991. Nuevos helmintos parasitos en peces elasmobranquios de la costa Peruana. – *Boletin de Lima* (Number 73): 25–28.

Monticelli, S. 1888. Contribuzioni allo studio della fauna elmintologica del golfo di Napoli. – *Mitteilungen aus der zoologischen Station zu Neapel* **8**: 85–152 + pl. VI–VII.

Nishikawa, T. 1981. Considerations on the taxonomic status of the lancelets of the genus *Branchiostoma* from the Japanese waters. – *Publications of the Seto Marine Biology Station* **26**: 135–156.

Ostrowski de Núñez, M. 1971. Estudios preliminaries sobre la fauna parasitaria de algunos elamobranquios del litoral Bonaerense, Mar Del Plata, Argentina. I. Cestodes y trematodes de *Psammobatis microps* (Günther) y *Zapteryx breirostris* (Müller y Henle). – *Physis* **30**: 425–446.

Pollard, E. C. 1893. A new sporozoon in amphioxus. – *Quarterly Journal of Microscopical Science* **34**: 311–316 + pl. XXIX.

Poss, S. G. and Boshung, H. T. 1996. Lancelets (Cephalochordata: Branchiostomatidae): how many species are valid? *Israel Journal of Zoology* **42** (Suppl.): 13–66.

Regan, J. D. 1963. A cestode plerocercoid from the crowned conch. – *Quarterly Journal of the Florida Academy of Sciences* **26**: 184–187.

Reichenbach-Klinke, H. H. 1955. Die Entwicklung der Larven bei der Brandwurmordnung Tetraphyllidea Braun 1900. – *Abhandlungen der Braunschewigischen Wissenschaftlichen Gesellschaft* **8**: 61–72.

Reichenbach-Klinke, H. H. 1957. Artzugehörigkeit und Entwicklung der als *Scolex pleuronectis* Müller bekanten Cestodenlarven (Cestoidea, Tetraphyllidea). – *Zoologischer Anzeiger* **20** (Suppl.): 317–324.

Riser, N. W. 1949. *Studies on Tetraphyllidea*. PhD Dissertation, Stanford University, Palo Alto, CA.

Ruppert, E. E. 1997. Cephalochordata. In Harrison, F. W. and Ruppert, E. E. (Eds): *Microscopic Anatomy of Invertebrates: Hemichordata, Chaetognatha, and the Invertebrate Chordates*, Vol. 15, pp. 349–504. Wiley-Liss, New York.

Ruppert, E. E., Nash, T. R. and Smith, A. J. 2000. The size range of suspended particles trapped and ingested by the filter-feeding lancelet *Branchiostoma floridae* (Cephalochordata: Acrania). – *Journal of the Marine Biology Association of the United Kingdom* **80**: 329–332.

Saini, P. K., Webert, D. W. and McCaskey, P. C. 1997. Food safety and regulatory aspects of cattle and swine cysticercosis. – *Journal of Food Production* **60**: 447–453.

Sakanari, J. and Moser, M. 1989. Complete life cycle of the elasmobranch cestode, *Lacistorhynchus dollfusi* Beveridge and Sakanari, 1987 (Trypanorhyncha). – *Journal of Parasitology* **75**: 806–808.

Stokes, M. D. 1996. Larval settlement, post-settlement growth and secondary production of the Florida lancelet (=amphioxus), *Branchiostoma floridae*. – *Marine Ecology Progress Series* **130**: 71–84.

Stokes, M. D. and Holland, N. D. 1992. Southern stingray (*Dasyatis americana*) feeding on lancelets (*Branchiostoma floridae*). – *Journal of Fish Biology* **41**: 1043–1044.

Stokes, M. D. and Holland, N. D. 1996. Reproduction of the Florida lancelet (*Branchiostoma floridae*): spawning patterns and fluctuations in gonad indexes and nutritional reserves. – *Invertebrate Biology* **115**: 349–359.

Stokes, M. D. and Holland, N. D. 1998. The lancelet. – *American Scientist* **86**: 552–560.

Swofford, D. L. 2002. *PAUP**. Sinauer, Sunderland, MA.

Tolonen, A., Rita, H. and Peltonen, H. 2000. Abundance and distribution of *Diphillobothrium ditremum* Creplin (Cestoda: Pseudophyllidea) plerocercoids in benthic whitefish in northern Finnish Lapland. – *Journal of Fish Biology* **57**: 15–28.

Ubelaker, J. E. 1983. The morphology, development and evolution of tapeworm larvae. In Arme, C. and Pappas, P. W. (Eds): *Biology of the Eucestoda*, Vol. 1, pp. 235–296. Academic Press, London.

Waeschenbach, A., Webster, B. L., Bray, R. L. and Littlewood, D. T. J. 2007. Added resolution among ordinal level relationships of tapeworms (Platyhelminthes: Cestoda) with complete small and large subunit nuclear ribosomal RNA genes. – *Molecular Phylogenetics and Evolution* **45**: 311–325.

Wardle, W. J. 1974. *A survey of the occurrence, distribution and incidence of infection of helminth parasites of marine and estuarine Mollusca from Galveston, Texas*. PhD Dissertation, Texas A & M University, College Station, Texas.

Winnepenninckx, B., Bakeljau, T. and De Wachter, R. 1994. Small ribosomal subunit RNA and the phylogeny of Mollusca. – *Nautilussupplement* **2**: 98–100.

Young, R. T. 1954. A note on the life cycle of *Lascistorhynchus tenuis* (Van Beneden, 1858), a cestode of the leopard shark. – *Proceedings of the Helminthological Society of Washington* **21**: 112 Only.