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Experimental Elucidation of the Life Cycle of *Rhinebothrium urobatidium* (Cestoda: Rhinebothriidea) from the Round Stingray (*Urobatis halleri*: Myliobatiformes) to First and Second Intermediate Hosts

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Abstract.—The life cycle of the cestode *Rhinebothrium urobatidium*, whose final host is the round stingray, *Urobatis halleri*, includes a copepod as the first intermediate host and small benthic fishes as second intermediate hosts. Hexacanth embryos within nonoperculate, untanned eggs collected from round stingray developed in the tidepool copepod *Tigriopus californicus* into caudate procercoids with an apical organ/sucker and cercomer, and rarely, bothridia. The procercoids, which developed in the body cavity within a membrane, adhered to the copepod intestine, were infective to arrow gobies, *Clevelandia ios*, within 15 d of exposure at 21°C. When infected copepods were fed to arrow gobies, procercoids developed into nonlacunate plerocercoids each bearing individually retractable bothridia and an apical sucker. Within 10 d of exposure to the second intermediate host, the larvae had migrated up the bile/cystic duct into the gall bladder, where they developed bothridia similar to those of adult worms. Between 30 to 51 d post-infection in the goby, plerocercoids approached the size of larvae found in natural infections, and the scolex became morphologically similar to that of adult worms from round stingrays. Only presumptive filiform microtriches (filitriches) were present on procercoids while both filitrichs and spiniform microtriches (spinitrichs) were present on plerocercoids. Identification of plerocercoids from experimental infections as those of *R. urobatidium* was confirmed through morphology of the scolex and using cytochrome c oxidase I sequences. The experimental transmission of *R. urobatidium* to first and second intermediate hosts provides improved understanding of the transmission and ontogeny of shark tapeworms. The biological characteristics of *U. halleri,* with its diverse parasite fauna, provide significant opportunities to examine the biology of an array of elasmobranch tapeworm taxa.

The Rhinebothriidea comprises approximately 24 genera and 143 species of eucestode tapeworms found as adults in the spiral intestine of batoid elasmobranchs (Caira and Jensen 2014; Ruhnke et al. 2017; Coleman et al. 2019a; Coleman et al. 2019b). While there are numerous reports of larval stages (metacestodes) of members of the Rhinebothriidea from various invertebrate and fish intermediate hosts (Chambers et al. 2000; Caira and Reyda 2005; Jensen and Bullard 2010), there are no publications that describe the experimental transmission of these parasites from egg to adult worm (Caira and Jensen 2014).

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This lack of information on the Rhinebothriidea and marine cestode life cycles in general is surprising considering the abundance and diversity of these worms. The sparsity of information on marine cestode life cycles is an impediment to a more complete understanding of tapeworm ontogeny and evolution (Beveridge 2001) and to a better understanding of the important role parasites play in the food chain of many ecosystems (e.g., see Lafferty et al. 2006).

During studies on fish and macroinvertebrate parasites of Anaheim Bay in Southern California, plerocercoids with a scolex resembling that of *R. urobatidium* (Young, 1955) (see Appy and Dailey 1977) were found in the gall bladder and bile duct of small benthic fish. Larvae were particularly abundant in arrow gobies, *Clevelandia ios*, (Jordon and Gilbert, 1882) and shadow gobies, Quietula y-cauda (Jenkins and Evermann, 1889) collected in four man-made tidal ponds. *Rhinebothrium urobatidium* is also one of the most abundant parasites of the round stingray, *Urobatis halleri* (Cooper, 1863), which is seasonally present in large numbers in the same tidal ponds (Jirik and Lowe 2010) where we observed plerocercoids in small benthic fishes. The purpose of this study is to describe the morphogenesis of the tapeworm in its intermediate hosts, and to confirm the identity of these plerocercoids as *R. urobatidium* with morphological features and DNA sequence data.

Materials and Methods

Round stingrays were collected at a number of collecting events from 2016 to 2018 in Anaheim Bay (hook and line, beach seine), Seal Beach (hook and line), San Diego Bay (hook and line), Long Beach Harbor (otter trawl), and Two Harbors, Catalina Island (by spear), California. A survey of fish parasites was conducted in Anaheim Bay in 2013 using an otter trawl, beach seine and minnow traps. In addition, small benthic fish examined for infection with *R. urobatidium* were collected in Anaheim Bay tidal ponds by beach seine in the fall of 2018. Arrow and cheekspot gobies, *Ilypnus gilberti* (Eigenmann and Eigenmann, 1889) (Gobiidae) used for experimental infections were collected in Cabrillo Salt Marsh, Los Angeles Harbor. For morphological and sequence data comparison with larvae obtained experimentally, adult *R. urobatidium* were collected from round stingrays at multiple localities in Southern California (see above) and plerocercoids were from gobies collected in Anaheim Bay.

Gravid proglottids of *R. urobatidium* taken from round stingrays were placed in seawater in small (25 ml) plastic cups, and, following expulsion of eggs, were removed from the cups. Eggs were rinsed with several changes of seawater. Eggs were examined with a compound microscope to confirm their morphology as that of *R. urobatidium*. Eggs were maintained in seawater at 21°C with daily seawater changes until use in experimental infections, which usually occurred within 3 d.

Experimental infections with *R. urobatidium* were carried out using the tidepool copepod *Tigriopus californicus* (Baker, 1912) (Harpacticoidea) obtained from a colony maintained at the Cabrillo Marine Aquarium as a first intermediate host, and wild caught arrow and cheekspot gobies as second intermediate hosts. None of 23 gobies examined at the time of collection in Cabrillo Salt Marsh were infected with *R. urobatidium*. At a subsequent collection, 1 of 31 gobies was infected with a single *R. urobatidium*. In addition, infections were attempted using ostracods [tentatively identified as *Cyprides beaconensis* (Leroy, 1943)] collected in tidal pools in San Diego Bay as a first intermediate host and commercially available mosquitofish [*Gambusia affinis* (Baird and Girard, 1854)] as a second intermediate host.

Eggs of *R. urobatidium* were pipetted into 25 ml plastic cups containing copepods or ostracods. After 8 hours, copepods and ostracods were placed in a loosely covered 125 ml plastic cup in an incubator at 21°C. Copepods were fed Nanno 3600 (Instant Algae) daily and ostracods were fed finely ground marine fish pellets once a week. Evaporation from the cups was compensated by adding distilled water. Five to ten copepods were examined for larvae of *R. urobatidium* at each sampling event over a 20 d period. Ostracods were examined 15 d post exposure (dpe) to eggs of *R. urobatidium*.

At 15 dpe two to five copepods experimentally infected with *R. urobatidium* were fed to both gobies and mosquitofish, that had been placed individually in 125 ml plastic cups. After eight hours, all copepods had been consumed and gobies and mosquitofish were removed from the individual cups and each species placed in a liter glass bowl at 21°C. Gobies and mosquitofish were fed ground dry marine aquarium fish pellets and frozen copepods. Gobies were examined at irregular intervals or at times when a fish was found moribund such as occurred at 51 dpe. Both arrow and cheekspot gobies were used in experimental infections although measurement data presented herein came only from plerocercoids removed from arrow gobies. All fish examined in the lab were euthanized using ~250 mg/l of Tricaine (MS222).

Larvae isolated from copepods and dissected from gobies were observed live under a petroleum jelly-rimmed coverslip and then heat killed using the flame of a match prior to measuring. Measurements of plerocercoids were made on worms heat-killed prior to placing the coverslip to avoid effects of compression by the coverslip and attachment/flattening of bothridia adhering to the slide/coverslip. Measurements, drawings, and photographs were made using a Wild M12 compound microscope fitted with an optical graticule, drawing tube, and a Canon EOS 60e digital camera. Hand drawings were scanned and inked using Adobe Illustrator. The contrast and brightness of photographs and micrographs were sometimes enhanced using Adobe Photoshop. Unless stated otherwise, measurements are given in micrometers (μm).

Selected specimens used for scanning electron microscopy (SEM) included: adult worms from round stingray, plerocercoids from naturally and experimentally infected arrow goby, and procercoids from experimentally infected *T. californicus*. Specimens were placed directly into fixative or were heat killed with steaming hot water, fixed in 1.25% glutaraldehyde, post-fixed in 1% osmium tetroxide, dehydrated in an ethanol series, critical point dried using $C0_2$, and mounted on stubs. Specimens were coated with gold/palladium using an Emitech K550x sputter coater (Quorum Technologies, Ltd, Kent, UK) and imaged using a Hitachi S3000N variable pressure SEM (Hitachi, Troy, MI). For histology, approximately 15 infected copepods were fixed in Alcohol-Formalin-Acetic Acid (AFA), embedded in mass in paraffin, sectioned at 6–8 μm, and stained with hematoxylin and eosin. The morphology of adult worms was examined using a compound microscope by placing worms on a microscope slide and compressing slightly with a coverslip. This allowed accurate counting of loculi and testes.

Specimens obtained from experimental trials and from wild caught round stingrays were fixed in 95% ethanol for molecular analyses. Total genomic DNA was extracted from these samples using the Qiagen DNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. A 572 base pair region of the cytochrome c oxidase I (COI) gene was amplified via the polymerase chain reaction (PCR) using Cestoda-specific primers nLCO (5′-TTT ACT YTR GAY CAT AAG CGT-3′), and BEN-5 (5′-AAG CAG AAC CAA TTT ACG ATC-3′), according to Reyda and Marques (2011). Successful PCR reactions, determined via electrophoresis, were cleaned using MultiScreen HTS plates (Millipore

Corporation, Bedford, MA) and sequenced via Laragen, Inc. (Los Angeles, CA). Alignments to close relatives were performed in Sequencher v4.10.1 and distance matrices (Jukes-Cantor model) were created using Geneious v8.0.2. Sequences of *R. urobatidium* obtained from experimental infections were compared to *R. urobatidium*, *Ruptobothrium ditesticulum* (Appy and Dailey, 1977) Coleman et al., 2019 and *R. gravidum* Friggens and Duszynski, 2005 from wild caught *U. halleri*, collected during the current study. COI sequences generated during this study are available via GenBank (Acc. #MN416032-MN416047).

Tapeworm ontogenetic and morphological terminology generally follows that of Chervy (2002) for larval stages (but see Discussion), (Caira et al. 1999) for scolex attachment organs, Conn and Swiderski (2008) for embryonic envelopes, Chervy (2009) for microtriches; Jensen and Bullard (2010) for larval types, and Hilliard (1960) and Bylund (1975) for hexacanth hooks. Miller and Lea (1972) was used to identify fishes.

Results

In the 2013 survey of fish parasites in Anaheim Bay, 75.6% (N = 41) of round stingray were found to be infected with 1 to 53 adult *R. urobatidium*. The only other rhinebothriidean species present included *R. gravidum* (85.4%) and *R. ditesticulum* (26.8%). Presumptive *R. urobatidium* plerocercoids were found in the gall bladder and bile ducts of several species of small benthic fish including 22.7% (N = 22) of longjaw mudsuckers (*Gillichthys mirabilis* Cooper, 1864) 23.1% (N = 26) of arrow and shadow gobies and 6.3% (N = 32) of staghorn sculpins (*Leptocottus armatus* Girard, 1854). These larvae were absent in topsmelt [*Atherinops affinis* (Ayres, 1860)] (N = 30), California killifish (*Fundulus parvipinnis* Girard, 1854) (N = 37), shiner surfperch (*Cymatogaster aggregata* Gibbons, 1854) (N = 16), yellowfin goby [*Acanthogobius flavimanus* (Temminck and Schlegel, 1845)] (N = 9), and barred sand bass (*Paralabrax nebulifer* (Girard, 1854)) (N = 60). *Rhinebothrium urobatidium* was particularly common in the tidal ponds where in 2013, 100% of arrow goby were infected with 2 to 54 worms. In the fall of 2018, 93% of 43 arrow and cheekspot gobies collected in Anaheim Bay tidal ponds were found to be infected with 1 to 57 plerocercoids per fish.

Apolytic gravid proglottids of *R. urobatidium* (Fig. 1A) in the spiral intestine of round stingrays were initially distinguished/segregated from other gravid proglottids by a combination of moderate size relative to the gravid proglottids of other tapeworm species, uniform white appearance, a smooth surface and an indistinct midbody genital pore. Identification as *R. urobatidium* was confirmed by examining egg morphology using a compound microscope. When placed in seawater, eggs extruded from an opening midway along the proglottid (Fig. 1A). Eggs of *R. urobatidium* taken directly from gravid proglottids were nonoperculate and enclosed by a transparent outer coat with a robust gradually tapering terminally rounded filament extending from one pole (Figs. 1B, 2A). Within this clear membrane the hexacanth embryo was surrounded by inner and outer envelopes. Expelled eggs held in seawater did not attach to one another or become tanned. Embryos were active within the envelopes. Hexacanth hooks were not uniform in size or shape (Table 1, Fig. 2B). The medial (third) pair were longer than the lateral (first) or medio-lateral (second) pairs and had a more recurved blade tip, and the mediolateral pair were shorter than the medial pair and had a wider handle and blade. The lateral pair was the most delicate with a gently curved blade tip and thin handle.

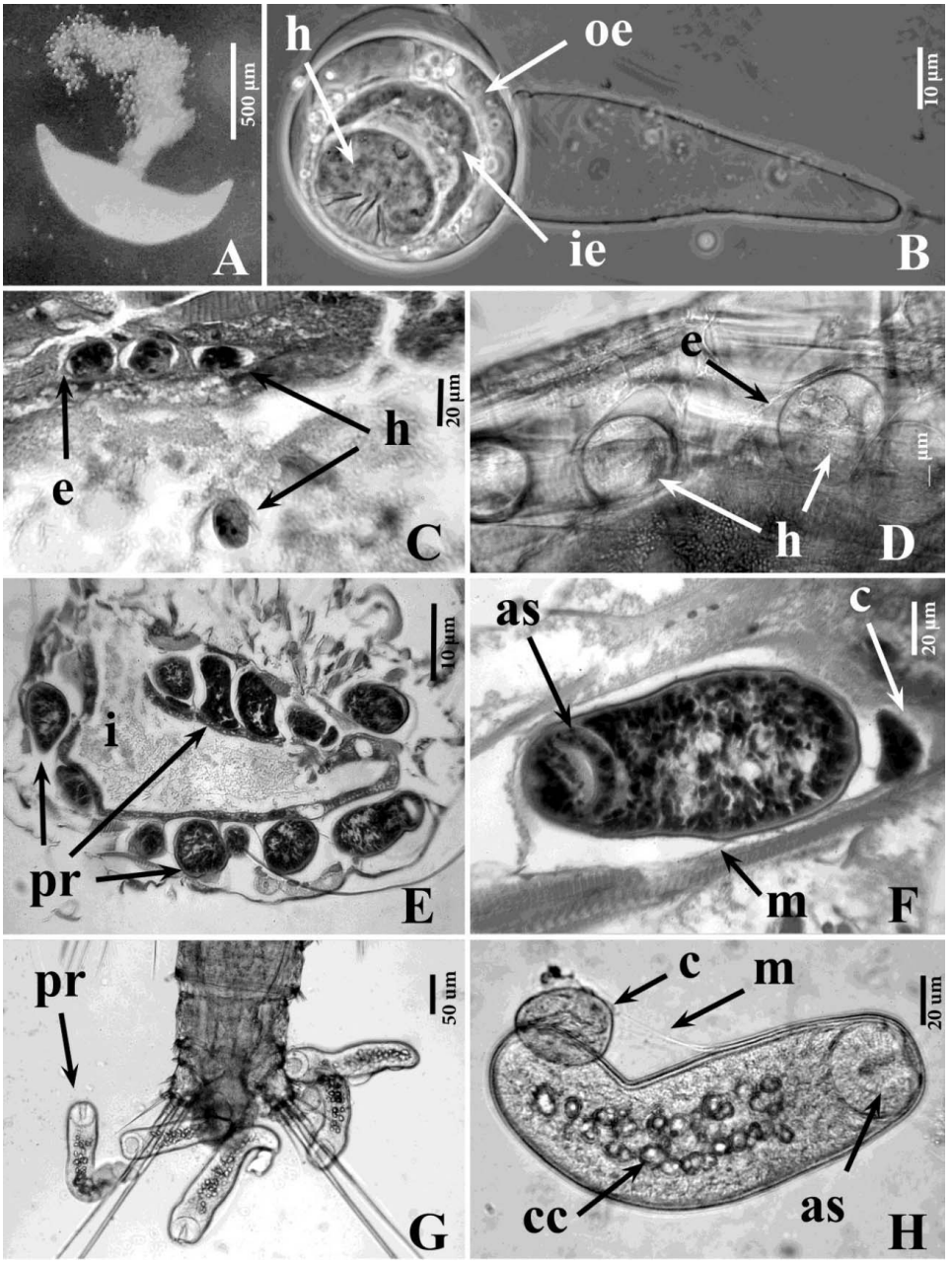

Fig. 1. Photographs of the development of *R. urobatidium* in *T. californicus,* held at 21°C. (A) Gravid proglottid expelling eggs. (B) Egg containing hexacanth embryo under coverslip pressure. (C) Hexacanth embryo in copepod intestinal lumen and in hemocoel at 8 hours post-exposure. (D) Hexacanth embryo within an envelope in copepod hemocoel. (E) Procercoids within a membrane in the copepod hemocoel at 10 d post-exposure (dpe). (F) Proceroid within a membrane at 10 dpe. (G) Procercoids isolated from a copepod at 14 dpe. (H) Mature procercoid within a membrate at 14 dpe. Abbreviations: as – apical sucker, c – cercomer, cc – calcareous corpuscles, h – hexacanth embryo, ie – inner envelope, m–membrane, oe – outer envelope, pr – procercoid.

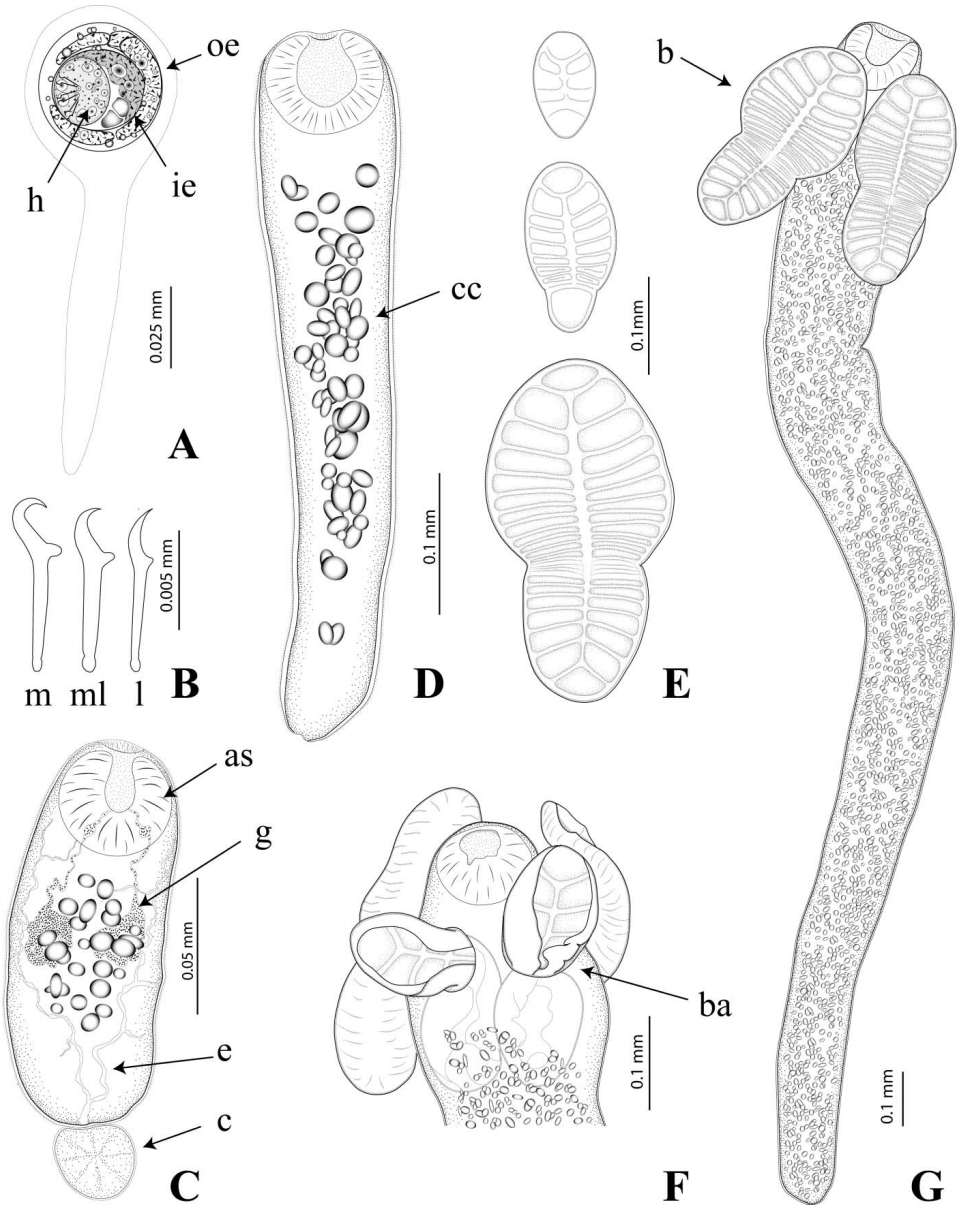

Fig. 2. Line drawings of *R. urobatidium* egg, and heat-killed procercoids from *T. californicus* and procercoids and plerocercoids from the arrow goby at 21°C. (A) Egg. (B) Hexacanth hooks. (C) Procercoid with cercomer at 10 d post-exposure (dpe). (D) Procercoid from arrow goby at 8 dpe. (E) Stages in bothridial development of the plerocercoid. (F) Plerocercoid with bothridia partially retracted. (G) Habitus of plerocercoid at 60 dpe. Abbreviations: as - apical sucker, b - bothridia, ba - bothridial aperture, c - cercomer, cc - calcareous corpuscles, g - gland cells and ducts, e - excretory system, ie - inner envelope, l - lateral hook, m - medial hook, ml - mediolateral hook, oe - outer envelope.

Table 1. Morphometrics in micrometers (μm)* of eggs and hexacanth larvae of *R. urobatidium*.

Parameter	
Number	10
Total length	144 ± 7.0
	129–157
Maximum width	48 ± 2.3
	45–51
Filament length	92 ± 6.3
	79–103
Filament width	14 ± 1.0
	12–15
Outer envelope length	40 ± 1.8
	37–42
Outer envelope width	35 ± 1.7
	32–37
Inner envelope length	29 ± 1.4
	27–32
Inner envelope width	23 ± 2.3
	20–25
Hexacanth length	22 ± 1.2
	21–24
Hexacanth width	16 ± 1.2
	14–18
Embryonic Hook Length	
Lateral Pair	8.2 ± 0.14
	7.9–8.4
Medio-lateral Pair	8.3 ± 0.19
	8.1–8.7
Medial Pair	9.0 ± 0.27
	8.6–9.5

* Mean ± standard deviation over the range.

Experimental transmission of *R. urobatidium* to copepods was conducted in excess of 20 times, and in all cases 100 percent of copepods became infected with up to 48 larvae per copepod. Mortality of copepods was common in the first days of infection. Two attempts to infect ostracods were not successful. At eight hours post-exposure, hexacanth embryos were present in copepod tissues lining the digestive tract, where they were surrounded by a clear space and thin membrane (Figs. 1C, D). Larvae penetrated the gut along its entire length but were found less frequently in the urosome (hindgut). As larvae grew they elongated, and by 5 dpe they had started to develop a cercomer and an apical organ/sucker. By 8 dpe calcareous corpuscles were present, the apical sucker appeared fully developed, and the cercomer was oval and connected to the body proper (Figs. 1F, G, H; 3A, B) by a narrow isthmus (Fig. 3C). Procercoids were clustered around the copepod digestive tract and surrounded by a thin membrane (Figs. 1E, F, H; 3B, C). Hexacanth hooks were not visible on the cercomer, but were occasionally observed in the posterior body of mature procercoids. While growth of some larvae continued through at least 20 dpe (Fig. 4A), elongation slowed following 8 dpe and by 11 dpe some larvae were near the maximum size of larvae examined at 20 dpe. Glandular cells were present in the anterior midbody of the procercoid, with ducts extending into the area of the apical sucker (Fig. 2C). The excretory

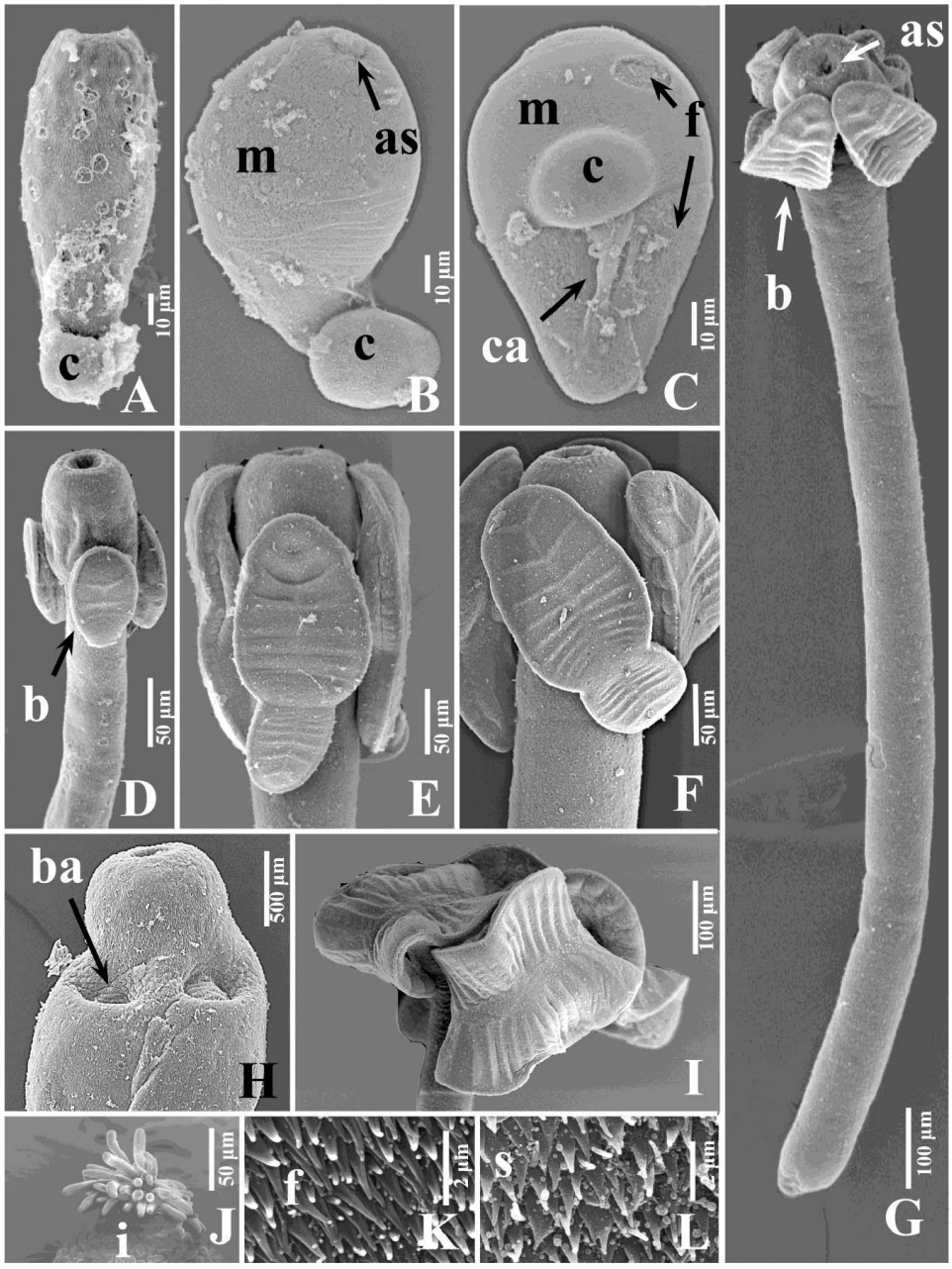

Fig. 3. Scanning electron micrographs of *R. urobatidium* in experimental (A–E, G–H, J–K) and natural infections (F, I). A. Procercoid from *T. californicus* at 15 d post-exposure (dpe). B, C. Procercoid from *T. californicus* with enclosing membrane at 15 dpe. D. Plerocercoid from arrow goby at 30 dpe. E, G. Plerocercoid from arrow goby at 51 dpe. F, H. Plerocercoid from natural infection of arrow goby. I. Adult scolex of specimens from round stingray. J. Bodies of plerocercoids extending from bile duct into the intestinal lumen of arrow goby. K. Procercoid microtriches at 15 dpe. L. Plerocercoid microtriches at 51 dpe. Abbreviations: as - apical sucker, b - bothridium, ba - bothridial aperture, c - cercomer, ca - cercomer attachment, f - filithriches, i - intestine, m - membrane, s - spinitriches.

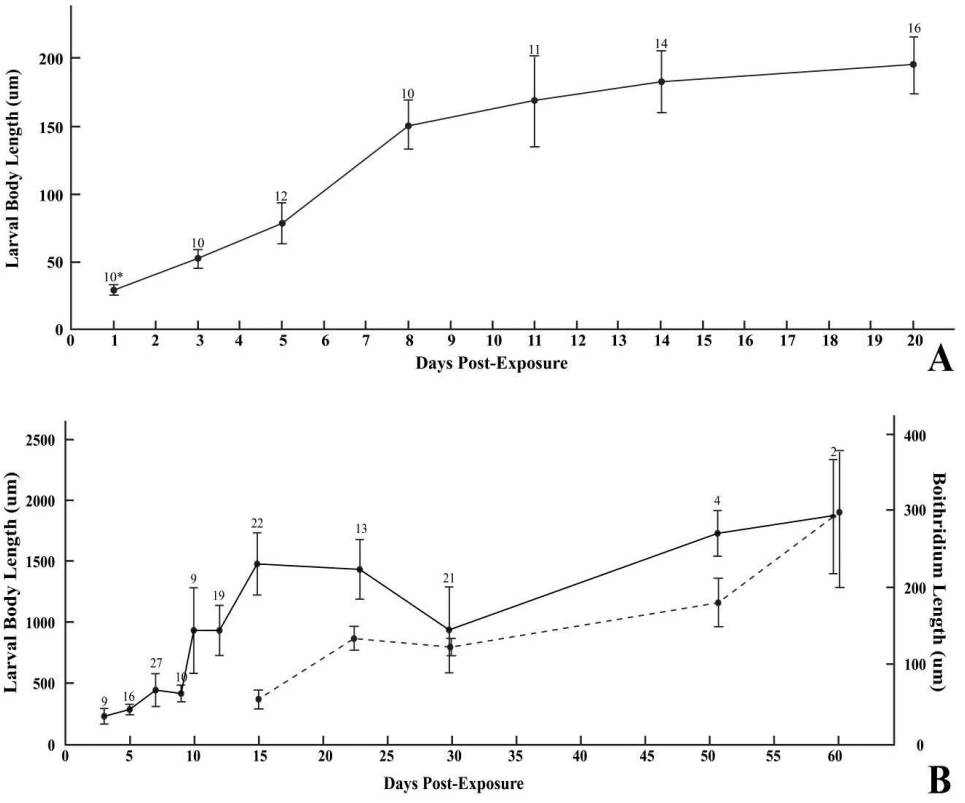

Fig. 4. Growth of *R. urobatidium* in intermediate hosts held at 21°C: A. Procercoids in *T. californicus*. B. Procercoids/plerocercoids in arrow goby. Solid line is total length of larvae while dashed line is change in length of bothridia.

system consisted of a posterior common duct, which bifurcated in two lateral branches extending into the anterior end of the larvae. During development in the copepod, larvae usually did not move freely within the hemocoel, but adhered to the external surface of the digestive tract (Fig. 1E). No obvious bothridial development occurred within 20 dpe. However, one of two larvae present in a copepod at 30 dpe had developed rudimentary bothridia and was moving in the hemocoel.

Gobies experimentally infected with *R. urobatidium* procercoids from copepods harbored 11 to 44 larvae. At 5 dpe, procercoids with the apical sucker, but without a cercomer (Fig. 2D), were present between villi in the anterior intestine of experimentally infected gobies (Fig. 5A). By 7 dpe some larvae, which had increased in length (Fig. 4B), were present in the bile duct and gall bladder (Fig. 5B); those in the gall bladder had attached to its inner wall by the apical sucker. Bothridia began forming in some larvae by 10 dpe as indistinct anterior swellings (Fig. 5C), and subsequently became septate oval bothridia, which developed anterior and posterior lobes as they increased in size (Figs. 2E; 3D, E, G; 5D). Septa at the anterior end formed first, with septa increasing in number and definition as the infection progressed (Fig. 2E). By 51 to 60 dpe, 36 to 38 loculi were present in the longer plerocercoids (Figs. 2E, G; 3G; 5E). During development, the anterior bothridial lobe was larger than the posterior lobe, and more loculi were present in the anterior lobe than in

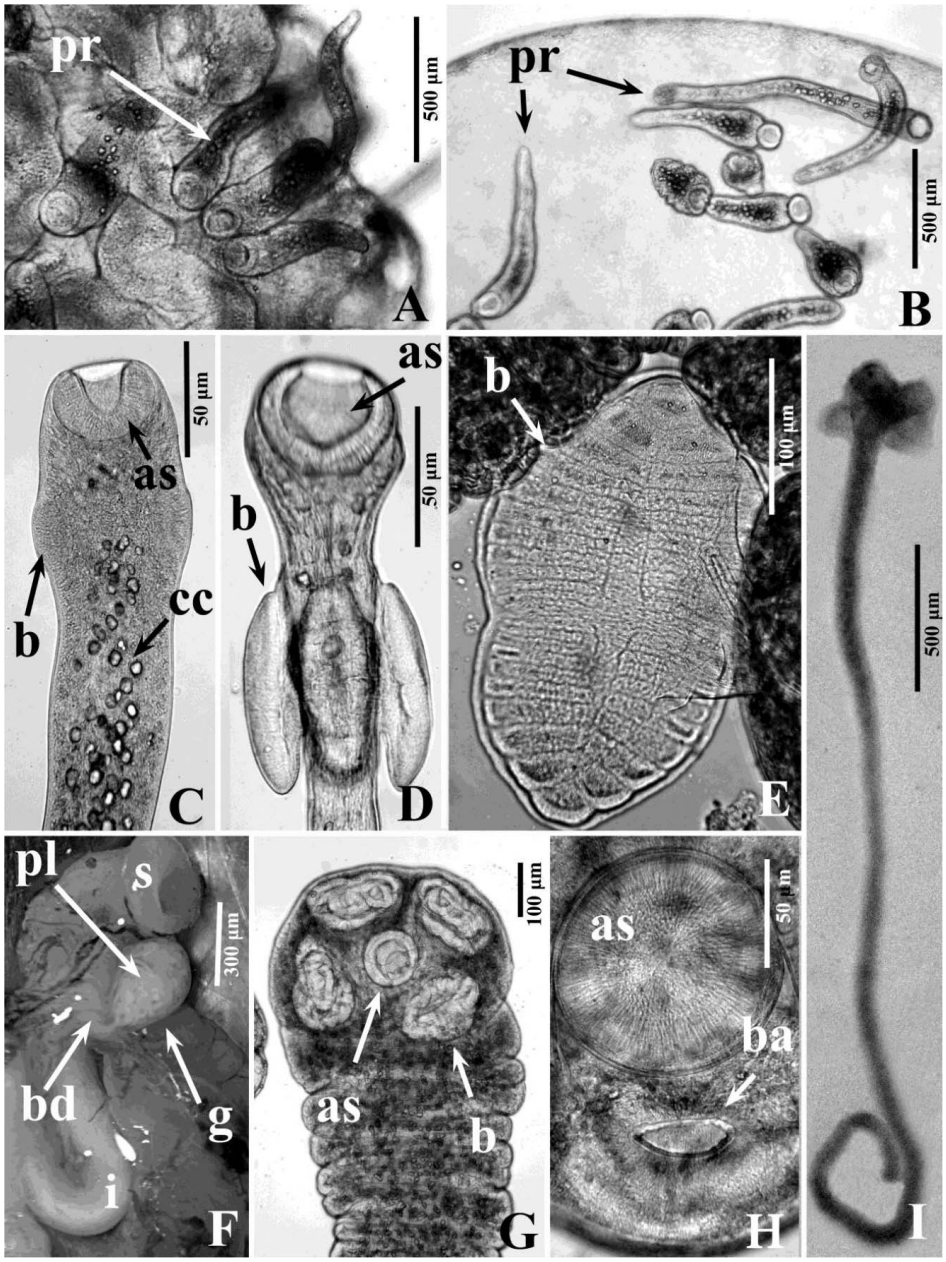

Fig. 5. Photographs of *R. urobatidium* larvae in arrow goby held at 21°C: (A) Procercoids among the goby intestinal villi. (B) Procercoids attached to the inner wall of the gall bladder at 10 d post-exposure (dpe). (C) Procercoid beginning to differentiate into a plerocercoid at 15 dpe. (D) Plerocercoid with developing bothridia at 30 dpe. (E) Developed bothrium attached to coverslip at 60 dpe. (F) Digestive tract of goby with plerocercoids in the gall bladder and bile duct. (G) Plerocercoid with bothridia and apical sucker retracted at 60 dpi. (H) Aperture of a retracted bothridium. I. Extended plerocercoid from a naturally infected arrow goby. Abbreviations: as - apical sucker, b - bothridia, ba - bothridial aperture, bd - bile duct, cc - calcareous corpuscles, g - gall bladder, i - intestine, im - inner envelope, oe - outer envelope, m - microtriches, pr - procercoid, pl - plerocercoid, s - stomach.

Table 2. Morphometrics in micrometers (μm)* of larval stages of *R. urobatidium* in copepods (*T. californicus)* and arrow gobies.

Parameter	Procercoid (20 dpe)†	Plerocercoid (51–60 dpe)†	Plerocercoid Natural Infection
Number	14	6	7
Body length	196 ± 16.3 167–223	1778 ± 377.9 1338–2414	2808 ± 922.5 1237–3906
Body width	68 ± 7.1 59–85	151 ± 38.9 89–203	704 ± 308.9 238–1125
Apical sucker length	40 ± 2.6 35–44	92 ± 16.8 75–116	75 ± 14.9 46–94
Apical sucker width	41 ± 2.5 36–44	98 ± 11.9 83–116	105 ± 27.3 47–128
Cercomer length	34 ± 6.6 21–46	-	-
Cercomer width	36 ± 4.4 30–43	-	-
Bothridium length		229 ± 81.1 170–387	327 ± 60.6 213–382
Bothridium width		130 ± 45.3 98–220	101 ± 75.4 85–190
Number of loculi		32–38	34–40

* Mean ± standard deviation over the range.
† dpe = days post-exposure.

the posterior lobe (Figs. 2E; 3E). In heat-killed specimens, the shape and reflexion of the scolex/bothridia was similar to that in heat-killed plerocercoids and adult worms from natural infections (Figs. 3G, I). By 51 to 60 dpe, some plerocercoids approached the length of larvae present in naturally infected gobies (Table 2). Plerocercoids were densely packed with calcareous corpuscles and had excretory canals similar to those seen in the procercoid. In heavy experimental and natural infections, the gall bladder and the bile duct were congested with plerocercoids (Fig. 5F) and in one case, the posterior portion of the larvae extended out of the bile duct into the intestine (Fig. 3J). In some cases, procercoids (larvae without bothridial development) were still present in the bile duct and gall bladder as late as 30 dpe. The bodies of plerocercoids were highly elastic (Fig. 5I), and the bothridia and apical sucker could individually be retracted into the scolex (Figs. 2F; 3H; 5G, H). The pedicel retracted into the scolex followed by the posterior portion of bothridium and then the anterior portion. Plerocercoids viewed in the gall bladder of fish invariably were attached to the inner bladder wall with the apical sucker and with bothridia retracted. In both experimental and natural infections, developed plerocercoids were present in the biliary system but not in the intestine. No plerocercoids that could be attributed to a different morphotype were found in the bile/cystic duct or gall bladder of gobies although plerocercoids of other tapeworms (believed to be *Acanthobothrium* spp. and *Parachristianella* sp.) were present in the lumen of the posterior intestine and rectum. Mosquitofish did not become infected in two trial exposures.

Presumptive filitriches were present on procercoids (Fig. 3K) but absent on the cercomer. In plerocercoids, filitriches were present adjacent to the apical sucker, and spinitriches were present on the corpus sometimes mixed with filitriches (Fig. 3L). Microtriches became sparse toward the posterior of the plerocercoid.

Table 3. Genetic relationship of *R. urobatidium* (*R.u.*) from experimental infections with natural infections of *R. urobatidium*, at different localities, *R. gravidum* (*R.g.*) and *R. ditesticulum* (*Ru.d.*) based on mitochondrial cytochrome oxidase 1. Numbers represent the difference in base pairs in a 465 base pair region.

						R. urobatidium					
Species	Stage	N*	Locality†	***Ru. d.*** adult AB	***R.g.*** adult LAH	adult LBH	adult SB	adult SDB	plero. AB	plero. 30 dpe	plero. 60 dpe
Ru. d.	adult	1	AB	–	123	117–118	117–118	117–118	117	117	117
R. g.	adult	1	LAH		–	117	117–119	117–118	118	117	117
R. u.	adult	4	LBH			**0–2**	1–3	0–1	1–2	0–1	0-1
	adult	2	SB				**3**	1–3	1–2	1–2	1-2
	adult	4	SDB					**0–2**	1–2	0–1	0-1
	plero.	1	AB						–	1	1
	plero.	2	30							**0**	0
	plero.	1	60								–

* N = number of specimens sequenced.

† AB = Anaheim Bay, LAH = Los Angeles Harbor, LBH = Long Beach Harbor, SB = Seal Beach, SDB = San Diego Bay; plero. = plerocercoid. dpe = days post exposure.

Cytochrome c oxidase I sequences of experimental plerocercoids were nearly identical to those from plerocercoids and adult *R. urobatidium* from natural infections, with only 1–3 basepair differences out of a total 465-bp region, compared to 117–119 bp mismatch with other rhinebothriidean species (Table 3).

Discussion

The three-host life cycle of *R. urobatidium* includes a first intermediate host copepod where a caudate procercoid with an apical organ/sucker and cercomer develops, a second intermediate host small benthic fish in which a nonlacunate plerocerocoid bearing individually retractable bothridia and apical sucker develops in the biliary system, and the final host, the round stingray. Eggs of *R. urobatidium* include inner and outer envelopes, which are likely homologous with embryonic envelopes recognized by Conn and Swiderski (2008). The outermost clear membrane with a single rounded process may be the outer coat formed in some cestodes (Conn and Swiderski 2008). Other rhinebothriidean species including *R. abaiensis* Healy, 2006 (Healy 2006) and *R. gravidum* and *R. ditesticulum* (pers. obs.) have a transparent outer coat, although the eggs of these species all possess a process extending from each end of the egg. Other tapeworm species have similar outer coats (e.g. *Proteocephalus* spp. of the order Onchoproteodephalidea (Scholz 1999). The size and shape of these eggs is certainly a microecological adaptation that enhances transmission (Jarecka 1961) either by providing buoyancy, being attractive/refractive, and/or by mimicking a particular food (e.g. phytoplankton) of the first intermediate host.

The development of a procercoid with a cercomer and apical organ/sucker is a common ontogenetic feature among the Cestoda (Freeman 1973, Jarecka 1975, Chervy 2002), but within the Rhinebothriidea this stage has only been described for *Pseudanthobothrium* Baer, 1956 (Jarecka and Burt 1984). Unlike in *R. urobatidium*, the cercomer of *Pseudanthobothrium* spp. contains large refractile bodies. In addition, the body (protoscolex) of *Pseudathobothrium* is enclosed with a clear membrane within which the larva moves freely and separately from the cercomer (Jarecka and Burt 1984). In contrast, a clear membrane

observed in *R. urobatidum* surrounds the entire procercoid, including the cercomer, and the body of the procercoid is directly attached to the cercomer. In *R. urobatidum* the movement of the procercoid is constrained within the membrane, perhaps by virtue of its attachment to the cercomer.

Among acetabulate fish tapeworms, development of a procercoid with an apical sucker and a cercomer, as in *R. urobatidium*, is also found in *Acanthobothrium olseni* (Mudry and Dailey 1971), *A. hispidum* (Riser 1956), and similar to initial development in species of *Proteocephalus* Wineland, 1858 (Scholz 1999). In most *Proteocephalus* spp. the formation of cercomer and apical sucker is quickly followed by the development of four suckers similar to what is found in the adult worms (Scholz 1999). In contrast, *R. urobatidium* did not develop bothridia in the first 20 d in the copepod, and larvae without bothridia fed to gobies at 15 dpe, developed bothridia similar to the adult worm. Thus in *R. urobatidium* reared in *T. californicus*, the development of bothridia is not required in order to infect the second intermediate host as it apparently is in *Proteocephalus*. The pattern in *R. urobatidum* is more similar to that in the bothriate cestodes such as some bothriocephalideans or diphyllobothriideans, in which the procercoid does not develop an adult-like scolex until it enters the second intermediate fish host (Freeman 1973).

The presence of microtriches on procercoids has been observed in other cestode orders (Davydov et al. 1995). While not studied in detail herein, a transition of microthrix type from procercoid to plerocercoid of *R. urobatidium* has been reported for other tapeworms (Hopkins and Charles 1969). Such a transition is not surprising in light of general ontogenetic tegumental changes that occur in other tapeworm species (Davydov et al. 1995).

The membrane surrounding the procercoid of *R. urobatidium* appears to be of parasite rather than host origin and similar membranes have been observed in other cestode species. Invertebrate immune responses typically involve a cellular response and encapsulation (van der Veen and Kurtz 2002; Rowley and Powell 2007). Such responses were not evident in *T. californicus* infected with *R. urobatidium*. In contrast, ultrastructural and *in vitro* studies have variably described a surface coat in the spathebothriidean *Cyathocephalus truncates* (Pallas 1781) (Okada 1990), an electron-translucent fibrillar secretion around the procercoid of the bothriocephalid *Triaenophorus nodulosus* (Pallas, 1781) (Davydov et al. 1995), a surface filamentous coat in the caryophyllidea *Archigetes sieboldi* Leuckart, 1878 (Poddubnaya et al. 2003), and an outer surface or outer layer in the diphyllobothriidean Schistocephalus solidus (Müller 1776) (Jakobsen et al. 2012; Marwaha et al. 2013). The membrane/layer sometimes described as carbohydrate (Jacobson and Doyle 1996) or shed outer surface (Jakobsen et al. 2012) has been purported to help the parasite evade the immune responses of the invertebrate intermediate host (Hammerschmidt and Kurtz 2005) or to provide protection from enzymatic action when the procercoid is passing through the vertebrate host digestive tract (Kurtz and Franz 2003; Marwaha et al. 2013). An ultrastructural/cytochemical study would be needed to better compare the membrane found in *R. urobatidium* with those found surrounding the procercoids of these other tapeworms. Absence of bothridial development in *R. urobatidium* in the copepod may also be related to the presence of this membrane. This is supported by our observation of a single larva of *R. urobatidium* with bothridia moving freely in the copepod hemocoel at 30 dpe. In addition, at least rudimentary bothridia are present in larvae of *R. gravidum* and *A. parvinuncinatum,* which move freely in the hemocoel of *T. californicus* (pers. obs.). This may suggest that conditions in the copepod hemocoel trigger or allow development of bothridia.

The presence or absence of bothridia in the larvae in the first intermediate host may also be related to the trophic demands of transmission. In *Proteocephalus* spp., development

of acetabula/scolex in the first intermediate host precedes subsequent development to an adult in the digestive tract of the final host in a usual two-host life cycle (Scholz 1999). In acetabulate fish tapeworms in a three-host cycle, such as *R. urobatidium*, the absence of bothridial development may be related to subsequent location of the larva in the second intermediate host; e.g., bothridia may be an impediment to larval parasites that migrate into ducts (e.g. *R. urobatidium*) or the body cavity [e.g., Type 5 larva of Chambers et al. (2000) and *R. ditesticulum* (pers. obs.)] of the second intermediate host. In any case, while the ontogenetic stages (procercoid and plerocercoid/merocercoid) of acetabulate tapeworms appear similar across taxa, the timing of larval morphogenesis from one to the other in the first intermediate host appears to be variable and likely a species-specific adaptation to trophic requirements of transmission.

The plerocercoid of *R. urobatidium* conforms to Jensen and Bullard's (2010) Type VII, which has an apical sucker and four stalked bothridia that are individually and totally retractable into the scolex. This general type of larva has been reported from the digestive system of a number of fish species from the Great Barrier Reef (Chambers 2000) and in the Gulf of Mexico (Jensen and Bullard 2010). The larval worms described by Brooks and Brothers (1997) from arrow goby and the shadow goby, *Quietula y-cauda* (Jenkins and Evermann, 1889) (Gobiidae) in San Diego Bay, California are most likely *R. urobatidium*. Small benthic fish in Anaheim Bay are commonly infected with *R. urobatidium,* especially in the man-made tidal ponds. The heavy use of these tidal ponds by gestating round stingray in the late summer and fall (Jirik and Lowe 2012) evidently provides a focus of infection of small benthic fish with *R. urobatidium*. The occurrence of rhinebothriidean larvae in fish is not always consistent with the feeding habits of final hosts, which consume few fish (Jensen and Bullard 2010). This is also true for *R. urobatidium*, in which the round stingray's diet consists of only 7.5% fish (Babel 1967).

While the presence of larval shark tapeworms in the gall bladder and bile/cystic duct of a fish host does not appear to be a common occurrence (e.g. see Jensen and Bullard 2010) larvae that might be attributed to *Rhinebothrium* or related taxa have been reported from the biliary system of fish (Linton 1897, 1905; Chandler 1935; Overstreet 1968; Jensen 2009). In tapeworms of groups other than elasmobranchs, acystic larvae of *Valipora* spp. (Cyclophyllidea: Gryporhynchidae), parasites of birds, are also found in the gall bladder of bony fishes (Jarecka 1970; Scholz and Salgado-Maldonado 2001). Larval helminths exploit intermediate host tissues to avoid the gut, maximize their growth, and minimize mortality (Chubb et al. 2009; Parker et al. 2009a, b). The specialized location of *R. urobatidium* in the biliary system, which might seem to be an inhospitable environment, would provide the benefits of being in host tissues or body cavities while avoiding the need to penetrate host tissues. In particular, *R. urobatidium* plerocercoids in the biliary system avoid energy expenditure associated with withstanding gut peristalsis, possibly avoid host immune response due to minimal host/parasite contact, and evidently find adequate nutrition to significantly increase their size. The protruding of the posterior ends of larvae into the intestinal lumen could be a means to obtain nutrition (Fig. 3J) although this was only observed in heavy experimental infections of the arrow goby.

The larvae of *R. urobatidium*, like those of at least some other *Rhinebothrium* species (see Jensen and Bullard 2010), possess neither evaginated bothridia (plerocercoid) nor an invaginated scolex (merocercoid). Instead, bothridia of *R. urobatidium* are individually retractable; they are withdrawn, without invagination of the bothridia, into the scolex. In discussing larval cestode terminology Chervy (2002) recognized the difficulty in the use of scolex retraction and invagination as a feature in defining larval types but felt it

may be a useful feature. Other authors have also noted difficulty in assigning a designation to the bothridia of plerocercoids (Rocka 2003; Guagliardo et al. 2009). While larval *R. urobatidium* from gobies do not appear to fit neatly into the definition of a plerocercoid or merocercoid it seems premature to create new or resurrect terminology in light of the absence of transmission and ultrastructural studies on the thousands of shark tapeworm metacestodes. Therefore, we have utilized the term plerocercoid, which is consistent with the terminology used by Jensen and Bullard (2010) for similar larvae.

The number of bothridial loculi present in the plerocercoid of *R. urobatidium* approaches or reaches the number of loculi in adult worms (Table 2; Appy and Dailey 1977), suggesting that in regions where the local elasmobranch tapeworms are known, plerocercoids of *Rhinebothrium* may be attributed to an adult form and thus a species based on the bothridial morphology of the plerocercoid. In addition to morphological similarity between experimental plerocercoids and adult worms, and plerocercoids from natural infections, mitochondrial DNA sequence data confirmed the finding that among rhinebothriidean tapeworms commonly found as adults in round stingray (i.e., *R. gravidum* and *R. ditesticulum*) in Anaheim Bay and Southern California, larvae from experimental infections are those of *R. urobatidium* (Table 3). Similarly Laskowski and Rocka (2014) found fewer than five base pair differences in COI sequence between plerocercoids from marbled rockcod, *Notothenia rossii* Richardson, 1844, and the onchoproteodephalidean *Oncobothrium antarticum* Wojciechowska, 1990 from the skate *Bathyraja eatonii* (Gunther 1876), a finding also confirmed with lsrDNA, which not surprisingly showed no molecular differences. In contrast, Jensen and Bullard (2010) found that congeners of some shark tapeworms may have identical COI sequences and recommended caution in identification of larvae or adults to species using COI. However, even absent genetic data, *R. urobatidium* can be distinguished from other rhinebothriidean tapeworms occurring in round stingray. *Ruptobothrium ditesticulum*, which is relatively scarce in round stingray in Anaheim Bay, has bothridia comprising two separate lobes, very large gravid proglottids and eggs with filaments at both poles. The bothridia of *R. gravidum* are not indented/lobed, their gravid proglottids have crenulated surface, prominent genital atrium and eggs have filaments at both poles. *Rhinebothrium urobatidium* exhibits substantial growth in both intermediate hosts. Procercoid growth was asymptotic and reached growth arrest at larval maturity (GALM) (Ball et al. 2008; Parker et al. 2009a) between 8 and 11 dpe at 21°C (Fig. 4). In contrast, while the morphological development and bothridial growth of the plerocercoids proceeded sequentially, growth in length of the larvae was quite variable. This is likely a fixation artifact related to highly contractile plerocercoid, and a less-than-optimum experimental design including the examination of only a single goby at each sampling point, and possible over-infection of small (~2 cm) goby hosts. In particular, extremely high intensity of infection has been shown to cause delayed development in the intermediate host (Benesh 2010). The goby examined at 30 dpe in the present study (Fig. 4) was infected with 44 larvae, which completely filled the gall bladder and greatly expanded the width of the bile duct. It should also be noted that experimental plerocercoids at 51 to 60 dpe did not reach the maximum size of larvae from natural infections (Table 2). Additional experimental studies will be necessary to better determine when growth arrest occurs in the plerocercoid and at what point in development plerocercoids become infective to the final host.

While recent years have seen a rise in morphological/taxonomic and phylogenetic information on elasmobranch tapeworms (e.g. Caira and Jensen 2014; Caira et al. 2014; Caira and Jensen 2017), there are few experimental/ultrastructural studies on any of the

nine orders of elasmobranch tapeworms. This is in contrast to the significant advances in experimental and ultrastructural studies of some mammalian and avian tapeworms (e.g. Michaud et al. 2006; Hammerschmidt and Kurtz 2009) including the sequential in vitro development of the procercoid and pleroceroid of *Schistocephalus solidus* (Jakobson et al. 2012). The present study experimentally confirms the identity of elasmobranch tapeworm plerocercoids found in small benthic fish as *R. urobatidium,* and for the first time describes morphogenesis of larval stages from first to second intermediate hosts. However, it still falls short of documenting the exact transmission that occurs in nature. It seems clear that *R. urobatidium* has a three-host life cycle, but the species of the natural first invertebrate (copepod) intermediate host(s) of *R. urobatidium* is still unknown, and worms have yet to be experimentally transmitted to the final host to determine time to maturity and longevity. Finding copepods infected with *R. urobatidium* in the marine environment is a difficult undertaking (although not impossible – see Marcogliese 1995), and use of wild-caught round stingrays for experimental studies is not feasible since regardless of size or locality of capture, they are invariably infected with a menagerie of tapeworm species. We are currently studying the use of oral anthelminthics to remove digestive tract parasites from wild-caught stingrays and husbandry of stingrays born live in captivity to allow more complete transmission studies. Round stingray is a particularly good host for experimental studies since it is well-studied locally (Babel 1967; Hoisington and Lowe 2005; Vaudo and Lowe 2006; Mull et al. 2008; Plank et al. 2010; Jirik and Lowe 2012; Lyons et al. 2017), is abundant and easily caught in shallow waters where large congregations of rays result in foci of parasite transmission, is small and relatively easy to maintain/feed in captivity, and above all, it has a diverse tapeworm fauna including representatives from a number of tapeworm orders (Rhinebothriidea, Trypanorhyncha, Diphyllidea, and Onchoproteocephalidea).

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