IJP: Parasites and Wildlife 10 (2019) 170–183

Contents lists available at ScienceDirect

IJP: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

First elucidation of a blood fluke (*Electrovermis zappum* n. gen., n. sp.) life cycle including a chondrichthyan or bivalve

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ARTICLE INFO

Keywords:
taxonomy
Phylogenetics
Histology
Bivalvia
Elasmobranchii
Life cycle

ABSTRACT

We describe a new fish blood fluke (Digenea: Aporocotylidae: *Electrovermis zappum* n. gen., n. sp.) and its life cycle in the intertidal zone adjacent to Mobile Bay (north-central Gulf of Mexico). This is the first elucidated aporocotylid life cycle that includes a chondrichthyan definitive host or a bivalve intermediate host. The new species undergoes asexual reproduction within the gonad of the variable coquina clam before maturing in the heart of the lesser electric ray. These adults and cercariae had identical 28S, 18S, and ITS2 nucleotide sequences. The new genus is similar to *Ogawaia* Cutmor et al., 2018 by having an inverse U-shaped intestine, a looping testis, and a uterus having distinct ascending and descending segments. It differs by having a body that is ≥ 30 × longer than wide, a testis with >30 curves, an obvious cirrus sac enveloping an extremely elongate cirrus, an ovary anterior to the seminal vesicle, and a post-gonadal uterus. The new species further differs from the type species of *Ogawaia* (*Ogawaia glaucostegi* Cutmore et al., 2018) by having a massive seminal vesicle (>10% of body length), a cirrus sac enveloping an extremely elongate cirrus, and a slightly sinuous uterus. Histology confirmed gametogenesis in an infected coquina clam but no discernible cellular response to infection was observed. We also i) characterize a second morphologically and genetically distinct cercaria (perhaps representing an innominate chondrichthyan aporocotylid) infecting the green jackknife clam in Mississippi Sound (north-central Gulf of Mexico), ii) compare all known aporocotylid cercariae infecting estuarine and marine mollusks and polychaetes and iii) provide a key to identify those cercariae. A phylogenetic analysis including nucleotide sequences from adult and cercarial specimens of the newly collected fish blood flukes further supports the notion that chondrichthyan aporocotylids are monophyletic and use bivalves as the first intermediate host; perhaps unlike any other blood fluke lineage.

1. Introduction

Of the 1188 nominal extant chondrichthyan species, 123 (74 Squalomorphii and Galeomorphii; 49 Batoidea) range in the Gulf of Mexico (Felder et al., 2009; Weigmann, 2016). The fish blood flukes (Digenea: Aporocotylidae Odhner, 1912; see Bullard et al., 2009) comprise 165 species assigned to 39 genera. Of those, only nine infect chondrichthyans (Table 1). Another innominate species infects the heart of smalltooth sawfish, *Pristis pectinata* Latham, 1794 in the Gulf of Mexico (Bakenhaster et al., 2018), and that species is currently being described by us (MBW and SAB, in prep). Only *Chimaerohemecus trondheimensis* Van der Land, 1967, *Gymnurahemecus bulbosus* Warren et al., 2019, and *Ogawaia glaucostegi* Cutmore et al., 2018 are represented by nucleotide sequence data (Table 2).

The life cycles of aporocotylids include a mollusk or polychaete intermediate host (wherein the parasite undergoes clonal asexual reproduction) and a fish definitive host (wherein the parasite matures). A total of 18 aporocotylid life cycles have been elucidated. Eleven include freshwater hosts: *Sanguinicola armata* Plehn, 1905 (Sendersky and Dobrovolsky, 2004); *S. alseae* Meade and Pratt, 1965 (Meade and Pratt, 1965); *S. davisi* Davis, 1953 (Wales, 1958); *S. fontinalis* (Hoffman et al., 1985); *S. idahoensis* (Schell, 1974); *S. inermis* Plehn, 1905 (Kirk and Lewis, 1993); *S. klamathensis* Wales, 1958 (Wales, 1958); *S. lophophora* (Erickson and Wallace, 1959); *S. megalobramae* (Li, 1980); *S. occidentalis* (Bacha, 1966); *S. rutili* (Simón-Martin et al., 1987). Six include marine or estuarine hosts: *Aporocotyle simplex* Odhner, 1900 (Køie, 1982); *Cardicola forsteri* Cribb, Daintith, and Munday, 2000 (Cribb et al., 2011); *C. laruei* Short, 1953 (Siegel et al., 2018); *C. opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami, and Grabner, 2011 (Sugihara et al., 2014); *C. orientalis* Ogawa, Tanaka, Sugihara, and Takami, 2010 (Shirakashi et al., 2016); *C. parvus* Bullard, Baker, and de Buron, 2012 (Siegel et al., 2018). One includes an anadromous definitive host and a freshwater gastropod: *Paracardicoloides yamagutii* Martin, 1974 (Nolan and Cribb, 2004). Collectively, these life cycles are representative of

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https://doi.org/10.1016/j.ijppaw.2019.06.008
Received 15 May 2019; Received in revised form 17 June 2019; Accepted 18 June 2019
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Table 1
The blood flukes (Digenea: Aporocotylidae) infecting cartilaginous fishes (Chondrichthyes).

Parasite	Host	Site of infection	Locality	Reference
Chimaerohemecus trondheimensis Van der Land, 1967	rabbit fish, *Chimaera monstrosa* Linnaeus, 1758	dorsal aorta	NE Atlantic, off Bergen, Norway	Van der Land, 1967; Lockyer et al., 2003b
	spook fish, *Hydrolagus mitsukurii* (Jordan and Snyder, 1904) Nakaya, 1984	dorsal aorta and postcardinal vein around kidney	Saruga Bay, Japan	Kamegai et al., 2002
Electrovermis zappum Warren and Bullard n. gen., n. sp.	lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001	heart	Gulf of Mexico, off Fort Morgan, AL, USA	present study
Gymnurahemecus bulbosus Warren et al., 2019	smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801) Uyeno, 1983	heart	Gulf of Mexico, Mobile, AL, USA	Warren et al., 2019
Hyperandrotrema cetorhini Maillard and Ktari, 1978	basking shark, *Cetorhinus maximus* (Gunnerus, 1765) Springer, 1973	circulatory system; heart	Mediterranean Sea off Tunisia; Oslofjorden, Norway; North Sea off Montrose, Scotland	Maillard and Ktari, 1978; Smith, 1972
Hyperandrotrema walterboegeri Orélis-Ribeiro and Bullard ,2013	shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810	luminal surface (endocardium) of heart atrium and ventricle	Viosca Knoll, northern Gulf of Mexico, 123 km south/southwest of Dauphin Island, Alabama.	Orélis-Ribeiro et al., 2013
Myliobaticola richardheardi Bullard and Jensen, 2008	Atlantic stingray, *Hypanus sabinus* (Lesueur, 1824) Last et al., 2016	intertrabecular spaces of heart	Deer Island, Mississippi Sound, Northern Gulf of Mexico off Biloxi, Mississippi.	Bullard and Jensen 2008
Ogawaia glaucostegi Cutmore et al., 2018	giant shovelnose ray, *Glaucostegus typus* (Anonymous [Bennett], 1830) Compagno, Last, Stevens, and Alava, 2005	valves of conus arteriosus; ventricle	Moreton Bay, Queensland, Australia	Cutmore et al., 2018
Orchispirium heterovitellatum Madhavi and Hanumantha Rao, 1970	Bengal whipray, *Brevitrygon imbricata* (Bloch and Schneider, 1801) Last et al., 2016	mesenteric blood vessels	Western Bay of Bengal, waters off Waltair, India.	Madhavi and Hanumantha Rao, 1970
Selachohemecus benzi Bullard et al., 2006	blacktip shark, *Carcharhinus limbatus* (Valenciennes, 1839) Compagno, 1973	heart	Apalachicola Bay, Florida, USA Northern Gulf of Mexico, off Mississippi, USA	Bullard et al. 2006
Selachohemecus olsoni Short 1954	Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Richardson, 1837) Springer, 1964	heart	Alligator Harbor, Florida, USA Apalachicola Bay, Florida, USA Mississippi Sound, Mississippi, USA	Short 1954; Bullard et al., 2006

Table 2
DNA sequences used in the present study.

Parasite	Host	Locality	GenBank 28S Accession #	Reference
Aporocotylidae				
Aporocotylidae sp. cercaria NSW1	*Plebidonax deltoides* (Lamarck, 1818)	Stockton beach, new South Wales, Australia	MF503307	Cribb et al., 2017
Chimaerohemecus trondheimensis Van der Land, 1927	*Chimaera monstrosa* Linnaeus, 1758	NE Atlantic, off Bergen, Norway	AY157239	Lockyer et al., 2003b
Electrovermis zappum Warren and Bullard n. gen., n. sp.	*Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001	Gulf of Mexico, off Fort Morgan, AL, USA	MN244242	present study
Electrovermis zappum Warren and Bullard n. gen., n. sp. (cercarial sequence)	*Donax variabilis* Say, 1822	Gulf of Mexico, off Fort Morgan, AL, USA	MN244314	present study
Gymnurahemecus bulbosus Warren and Bullard, 2019	*Gymnura micrura* (Bloch and Schneider, 1801) Uyeno, 1983	Gulf of Mexico, Mobile, AL, USA	MH555432	Warren et al., 2019
Ogawaia glaucostegi Cutmore et al., 2018	*Glaucostegus typus* (Anonymous [Bennett], 1830) Compagno, Last, Stevens, and Alava, 2005	Moreton Bay, Queensland, Australia	MF503308	Cribb et al., 2017
Aporocotylidae sp. cercaria "Type 2"	*Solen viridis* Say, 1821	Northern Gulf of Mexico, Mississippi Sound, Mississippi, USA	MN244240	present study
Turtle blood flukes				
Hapalorhynchus gracilis Stunkard, 1922	*Chelydra serpentina* (Linnaeus, 1758)	Reelfoot Lake, TN, USA	AY604710	Snyder 2004
Spirorchis artericola (Ward, 1921)	*Chrysemys picta* (Schneider, 1783)	Reelfoot Lake, TN, USA	AY604704	Snyder 2004
Vasotrema robustum Stunkard, 1928	*Apalone spinfera* (LeSueur, 1827)	Nishnabotna River, IA, USA	AY604706	Snyder 2004

Table 3
Aporocotylid cercariae infecting marine and estuarine gastropods, bivalves, and polychaetes.

Host	Cercaria	Locality	Reference
GASTROPODA			
Cochliopidae (Tryon, 1866)			
Heleobia australis (Orbigny, 1835)	"Cercaria Aporocotylidae gen. sp. 1"	Arroyo Cangrejo, Buenos Aires province, Argentina	Merlo et al., 2014
BIVALVIA			
Donacidae Fleming, 1828			
Donax variabilis Say, 1822	*Cercaria asymmetrica* Holliman, 1961	Gulf Beach, Alligator Point, Franklin Co., Florida, USA	Holliman, 1961
	Electrovermis zappum Warren and Bullard n. gen., n. sp.	Northern Gulf of Mexico, Fort Morgan, Alabama, USA	present study
Pectinidae Rafinesque, 1815			
Argopecten irradians (Lamarck, 1819)	*Cercaria martini* Stunkard, 1983	Northwestern Atlantic Ocean, Woods Hole, Massachusetts, USA	Linton, 1915b; Stunkard, 1983
Pharidae H. Adams and A. Adams, 1856			
Ensis macha (Molina, 1872)	Aporocotylidae sp.	La Tapera, Argentina	Vázquez et al., 2013
Psammobiidae Fleming, 1828			
Plebidonax deltoides (Lamarck, 1818) (as *Donax*)	Aporocotylidae sp. NSW1	Stockton Beach, New South Wales, Australia	Cribb et al., 2017
Solecurtidae Orbigny, 1846			
Tagelus divisus (Spengler, 1794)	Aporocotylidae sp.	Northwestern Atlantic Ocean, Biscayne Bay, Florida, USA	Fraser, 1967
Solemyidae Gray, 1840			
Solemya velum Say, 1822	*Cercaria solemyae* Martin, 1944	Northwestern Atlantic Ocean, Woods Hole, Massachusetts, USA	Martin, 1944
Solenidae Lamarck, 1809			
Solen viridis (Say, 1821)	Aporocotylidae cercaria "type 2"	Northern Gulf of Mexico, Mississippi Sound, Mississippi, USA	present study
Veneridae Rafinesque, 1815			
Amiantis purpurata (Lamarck, 1818)	Aporocotylidae sp.	El Molino beach, San Matías Gulf, Argentina	Gilardoni et al., 2011
Chione cancellata (Linnaeus, 1767)	*Cercaria cristulata* Holliman, 1961	Northern Gulf of Mexico, Alligator Point, Florida, USA	Holliman,1961
Mercenaria capechiensis (Gmelin, 1791)	*Cercaria mercenariae* Wardle, 1979	San Luis Pass and Terramar Beach, Galveston Island, Texas, USA	Wardle, 1979
ANNELIDA			
Ampharetidae Malmgren, 1866			
Amphicteis gunneri (Sars, 1835)	*Cercaria amphicteis*, Oglesby, 1961	Estuary of Apalachicola River at Apalachicola, Franklin Co., Florida, USA	Oglesby, 1961
Serpulidae Rafinesque, 1815			
Hydroides dianthus Verrill, 1873 (Sabellida: Serpulidae)	*Cercaria loossi*, Stunkard, 1929	Northwestern Atlantic Ocean, Woods Hole, Massachusetts	Linton, 1915a; Stunkard, 1983
Terebellidae Johnston, 1846			
Reteterebella aloba Hutchings and Glasby, 1988	"aporocotylid type A"	off Port Lincoln, South Australia	Cribb et al., 2011
Amphitrite ornata (Leidy, 1855)	*Cardicola parvus* Bullard and de Buron, 2012	Oyster landing, North Inlet; Charleston, South Carolina, USA	Siegel et al., 2018
Amphitrite sp. Müller, 1771	*Cardicola fosteri* Cribb, Daintith, and Munday, 2000	Kushimoto, Wakayama Prefecture, Japan	Shirakashi et al., 2016
Artacama proboscidea Malmgren, 1866	*Aporocotyle simplex* Odhner, 1900	Øresund, north of the island Veen, Denmark	Køie, 1982
Enoplobranchus sanguineus (Verrill, 1873)	*Cardicola parvus* Bullard and de Buron, 2012	Oyster landing, North Inlet; Charleston, South Carolina, USA	Siegel et al., 2018
Lanassa nordenskioldi Malmgren, 1866	*Aporocotyle* sp. "morphologically indistinguishable" from *Aporocotyle simplex* Odhner, 1900	Seyðisfjörður, Eastern Iceland	Køie and Peterson, 1988
Lanicides vayssierei (Gravier, 1911)	*Cercaria hartmanae* Martin, 1952	Cape Boyds, Ross Island, Antarctica	Martin, 1952
Longicarpus modestus (Quatrefages, 1866)	*Cardicola fosteri* Cribb, Daintith, and Munday, 2000	off Port Lincoln, South Australia	Cribb et al., 2011
Nicolea gracilibranchis (Grube, 1878)	*Cardicola orientalis* Ogawa, Tanaka, Sugihara, and Takami, 2010	Kushimoto, Wakayama Prefecture, Japan	Shirakashi et al., 2016
Terebella lapidaria Linnaeus, 1767	*Cardicola laruei* Short, 1953	Oyster landing in North Inlet, South Carolina, USA	Siegel et al., 2018
Terebella sp. Linnaeus, 1767	*Cardicola opisthorchis* Ogawa, Ishimaru, Shirakashi, Takami, and Grabner, 2011	off Tsushima, Nagasaki Prefecture, Japan	Sugihara et al., 2014

approximately 10% of nominal aporocotylids.

No elucidated aporocotylid life cycle, i.e., one that confirms the identity of conspecific aporocotylid specimens from both hosts in the life cycle, includes a shark, skate, ray, or chimaera (Gnathostomata: Chondrichthyes) definitive host nor a bivalve intermediate host. Extant marine bivalves number approximately 8000 species assigned to 1100 genera in 99 families (Huber, 2010). Of the 23 known marine and estuarine aporocotylid intermediate hosts, only 9 comprise bivalves (Table 3). Prior to the present study, only a single aporocotylid cercaria from a bivalve host (*P. deltoides*) had been sequenced (Aporocotylidae sp. NSW1; Table 2; Cribb et al., 2017). Also, only one bivalve family (Veneridae Rafinesque, 1815; >650 spp.) (Huber, 2010) harbors more the one larval aporocotylid (Table 3).

KEY. Key to the known cercariae of Aporocotylidae Odhner, 1912 infecting marine gastropods, bivalves, and polychaetes*.

1a. Tail forked (furcocercous)	2
1b. Tail not forked (lacking furcae)	12
2a. Furcae symmetrical	3
2b. Furcae asymmetrical	9
3a. Lateral body spines present	4
3b. Lateral body spines absent	5
4a. Lateral body spines in ventrolateral rows	*Cercaria cristulata* (North-central Gulf of Mexico)
4b. Lateral body spines covering body surface	*Aporocotyle simplex* (North Sea), *Aporocotyle* sp. infecting *Lanassa nordenskioldi* (Norwegian Sea)
5a. Furcal fin fold present	6
5b. Furcal fin fold absent	8
6a. Body + tail < 500 μm long	7
6b. Body + tail ≥ 800 μm long	*Cercaria mercenariae* (Northwest Gulf of Mexico)
7a. Body fin fold present	*Cercaria loossi* (Northwest Atlantic Ocean)
7b. Body fin fold absent	cercaria infecting *Heleobia australis* (Southwest Atlantic Ocean)
8a. Developing in rediae	*Cercaria hartmanae* (Ross Sea, Southern Ocean)
8b. Developing in sporocysts only	*Cercaria martini* (Northwest Atlantic Ocean)
9a. Lateral body spines present	10
9b. Lateral body spines absent	11
10a. Four concentric oral spine rows	cercaria infecting *Solen viridis* (North-central Gulf of Mexico)
10b. Six concentric oral spine rows	*Cercaria asymmetrica, Electrovermis zappum* n. gen., n. sp. (North-central Gulf of Mexico)
11a. Furcal fin fold present	cercaria infecting *Amiantis purpurata* (Southwest Atlantic Ocean)
11b. Furcal fin fold absent	cercaria infecting *Plebidonax deltoides* (Tasman Sea, South Pacific Ocean)
12a. Tail spatulate; lateral body spines present	*Cercaria solyemae* (Northwest Atlantic Ocean)
12b. Tail cylindrical; lateral body spines absent	13
13a. Tail bearing fin fold	*Cercaria amphicteis* (North-central Gulf of Mexico)
13b. Tail lacking fin fold	cercariae of *Cardicola parvus* (Northwest Atlantic Ocean), *C. fosteri* (Northern Pacific Ocean; Great Australian Bight, South Pacific Ocean), *C. orientalis* (Northern Pacific Ocean), *C. laruei* (Northwest Atlantic Ocean), *C. opisthorchis* (Northern Pacific Ocean); and cercaria infecting *Reteterebella aloba* (Great Australian Bight, South Pacific Ocean)

*The cercariae infecting purplish tagelus, *Tagelus divisus* (Spengler, 1794) (Cardiida: Solecurtidae) (see Fraser, 1967) and razor clam, *Ensis macha* (Molina, 1782) (Adapedonta: Pharidae) (see Vázquez et al., 2013) are excluded because neither was morphologically described and no voucher specimen exists.

Herein, we describe a new aporocotylid and its life cycle in the intertidal zone of a high-energy beach adjacent to the mouth of Mobile Bay (north-central Gulf of Mexico). This is the first elucidated aporocotylid life cycle that includes a chondrichthyan definitive host (lesser electric ray, *Narcine bancroftii* [Griffith and Smith, 1834] Carvalho, 2001 [Torpediniformes: Narcinidae]) or a bivalve intermediate host (variable coquina clam, *Donax variabilis* Say, 1822 [Cardiida: Donacidae]). We also characterize another marine aporocotylid cercaria that infects the green jackknife clam, *Solen viridis* Say, 1821 (Adapedonta: Solenidae) and that shares a recent common ancestor with the monophyletic chondrichthyan blood flukes. A key to the known aporocotylid cercariae infecting estuarine and marine gastropods, bivalves, and polychaetes is provided.

2. Materials and methods

2.1. Specimen collection and preparation

Lesser electric rays were captured from the north-central Gulf of Mexico off Fort Morgan, Alabama (30°13′22. 61″N, 88°3′18. 57″W) during September 2012 and 2013, October 2014, May 2015, and July 2017 (n = 54) using hand nets (2012–2015) and a 10-m otter trawl (2017). This area comprises a high-energy beach habitat (Defeo et al., 2009). Each lesser electric ray was identified using the dichotomous key to McEachran and de Carvalho (2002) and by having dorsal disc coloration with few, scattered, incomplete ocelli and lacking dark brown spots covering the disc surface, base of tail, and dorsal and caudal fins. Live-captured lesser electric rays were placed in a flow-through seawater tank or aerated cooler prior to necropsy. At necropsy, each ray was killed by pithing before the heart and gill arches were excised intact and placed in separate columns (heart was bisected; gill arches separated). Two lesser electric rays captured in 2013 were pithed, iced, and examined for the purposes of extracting live flukes for DNA extraction: blood flukes from the heart of two lesser electric rays were wet-mounted on glass slides and examined with a compound light microscope equipped with differential interference contrast (DIC) optical components to confirm their identity, placed directly into 95% EtOH, and stored at −20 °C until DNA was extracted (see below). All tissues were examined with the aid of a stereo-dissecting microscope and fiber optic light source to isolate fluke specimens for morphology. The heart was teased apart with fine forceps to reveal adult blood flukes, and sediment from the gill and heart was examined for blood flukes with aid of a settling column.

Variable coquina clams were collected from Fort Morgan (sympatric with the infected lesser electric rays) during November 2017 (n = 532), June 2018 (n = 363), and September 2018 (n = 279) using a fine mesh (4 millimeter [mm]) circular sieve to gather sand before elutriation in 20 liter (L) buckets. Two (one was infected) green jackknife clams were collected from the north-central Gulf of Mexico approximately 6 km north/northwest of the west end of Horn Island (N30°14′35. 6″; W88°46′52. 9″) by SAB during July 2005 using an A-frame dredge with a fine mesh cod-end bag. All variable coquina clams were maintained alive in an aerated 144 L cooler prior to isolation and necropsy. Live variable coquina clams (60) were isolated into 20 medium (55 mm in diameter) stender dishes (3 clams per stender dish) and observed for cercarial shedding but most were crushed outright and examined for cercarial infections. Live cercariae and sporocysts were illustrated using a Leica DM 2500 and a Leica DMR microscopes (Leica, Wetzler, Germany) equipped with DIC, measured using an ocular micrometer, and illustrated using a drawing tube.

Adult flukes for morphology were routinely heat-killed on glass slides using a butane hand lighter under little or no coverslip pressure as per Roberts and Bullard (2017). Cercariae and sporocysts for morphology were isolated in stender dishes filled with fresh seawater. One stender dish was left for 12 h or until dead specimens were observed. Those specimens were then vialed in 10% neutral buffered formalin (n.b.f.). Cercariae and sporocysts in a second stender dish were fixed by adding one drop of 5% n.b.f. to the dish approximately every 60 s while swirling the cercariae and sporocysts. Adult flukes, cercariae, and sporocysts were stained by rinsing with distilled water, cleaned with fine brushes to remove any debris, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, dehydrated using an ethanol series, cleared in clove oil, permanently mounted in Canada balsam, illustrated using Leica DM 2500 and Leica DMR (Leica, Wetzler, Germany) microscopes each equipped with DIC, measured using an ocular micrometer, and illustrated using a drawing tube. Specimens for scanning electron microscopy (SEM) were washed with de-ionized water, dehydrated through a graded EtOH series, pipetted onto a 45 micrometer (μm) mesh cut to fit within a

30 µm microporous specimen capsule, critical point dried in liquid CO_2, mounted on SEM aluminum stubs with double-sided carbon tape, sputter-coated with gold palladium (19.32 g/cm3; 25 mA), and viewed with a Zeiss EVO 50VP SEM. Measurements are reported in µm as the range followed by the mean, +/- standard deviation, and sample size in parentheses. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer et al. (2016). Morphological terms and nomenclature for blood flukes follows Bullard et al. (2006, 2009), Bullard and Jensen (2008), and Warren et al. (2019). Type and voucher materials are deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

2.2. Histology

Variable coquina clams intended for histopathology were maintained alive for 72 h in the laboratory (allowing time for clams to purge sand from gut), fixed whole in 10% n.b.f. (a wooden dowel was inserted between the valves of each clam to allow immediate penetration of n.b.f. to soft tissues), and dissected such that visceral mass was separated from each valves, and rinsed in de-ionized water for 2 h. The visceral mass was then bisected, dehydrated through an EtOH series, embedded under vacuum pressure in paraffin, sectioned at 4 µm, mounted on glass slides, de-paraffinized, routinely stained with hematoxylin and eosin, and photographed with aid of a compound light microscope. Nomenclature for bivalve histology follows DeVilliers (1975) and McElwain and Bullard (2014).

2.3. DNA extraction and PCR amplification

Total genomic DNA (gDNA) was extracted (1 adult specimen of the new species; 1 pooled sample of cercariae from variable coquina clam; 1 pooled sample of cercariae from green jackknife clam) using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) as per the manufacturer's protocol with one exception: the proteinase-K incubation period was extended overnight and the final elution step used 100 microliter (µl) of elution buffer to increase the final DNA concentration. Amplification and sequencing of the large subunit ribosomal DNA (28S), small subunit ribosomal DNA (18S), and internal transcribed spacer (ITS2) used the set of primers described in Orélis-Ribeiro et al. (2017). PCR amplifications were performed with a cycling profile identified in Warren et al. (2017). All PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California, USA). PCR products (12 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifugation with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) according to manufacturer's protocols except that the last elution step was performed with autoclaved nanopure H_2O rather than with the provided buffer. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Reactions were sequenced using BigDye terminator version 3.1, cleaned with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using an ABI 3730 XL or 3730 Genetic Analyzer. Sequence assembly and analysis of chromatograms were performed with Geneious version 11.1.5 (http://www.geneious.com). All nucleotide sequence data were deposited in GenBank (Table 2).

2.4. Phylogenetic analysis

The phylogenetic analysis included two sequences of the new species (one adult; one cercarial) and one cercarial sequence from the green jackknife clam plus all sequences taken from nominal aporocotylids infecting chondrichthyans and the cercarial sequence of Cribb et al. (2017) (Table 2). The out-group was represented by sequences from adults of the turtle blood flukes *Hapalorhynchus gracilis* Stunkard, 1922, *Spirorchis artericola* (Ward, 1921), and *Vasotrema robustum* Stunkard, 1928. Out-group sequences were chosen based on previous publications and their proven reliability in recovering trees that match morphological assessments of the group (Cribb et al., 2017; Warren et al., 2019). Sequences were aligned using MAFFT (Katoh and Standley, 2013). JModelTest 2 version 2.1.10 was implemented to perform statistical selection of the best-fit models of nucleotide substitution based on Bayesian information Criteria (BIC) (Darriba et al., 2012). Aligned sequences were reformatted (from.fasta to.nexus) using the web application ALTER (Glez-Peña et al., 2010) to run Bayesian inference (BI). BI was performed in MrBayes version 3.2.5 (Ronquist and Huelsenbeck, 2003) using substitution model averaging ("nst-mixed") and a gamma distribution to model rate-heterogeneity. Defaults were used in all other parameters. Three independent runs with four Metropolis-coupled chains were run for 5,000,000 generations, sampling the posterior distribution every 1000 generations. Convergence was checked using Tracer v1.6.1 (Rambaut et al., 2014) and the "sump" command in MrBayes: all runs appeared to reach convergence after discarding the first 25% of generation as burn-in. A majority rule consensus tree of the post burn-in posterior distribution was generated with the "sumt" command in MrBayes. The inferred phylogenetic tree was visualized using FigTree v1.4.3 (Rambaut et al., 2014) and further edited for visualization purposes with Adobe Illustrator (Adobe Systems).

3. Results

3.1. *Electrovermis* Warren and Bullard n. gen (Fig. 1 − 4)

3.1.1. Generic diagnosis

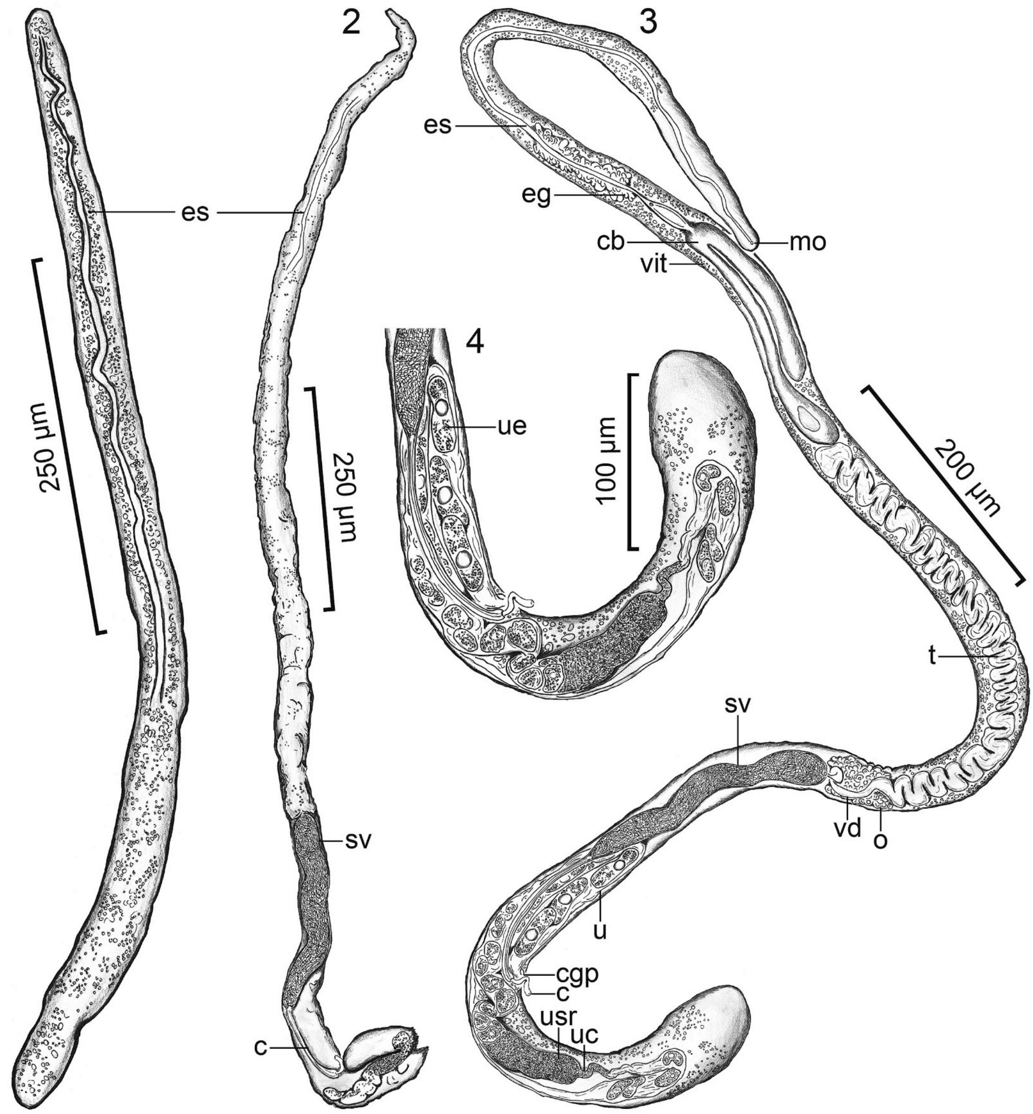
Body extremely elongate, dorsoventrally flattened, having anterior end tapering and posterior end bluntly rounded, aspinous, lacking lateral tubercles. Rosethorn-shaped spines lacking. Nervous system indistinct. Anterior sucker aspinous, lacking peduncle, diminutive. Mouth on mid-ventral surface of anterior sucker. Pharynx absent. Oesophagus extending sinuously posteriad along mid-line for 1/4 of body length; posterior oesophageal swelling present. Intestinal caeca inverse U-shaped, asymmetrical; posterior caeca shorter than oesophagus, connecting to oesophagus ventrally, lacking diverticula, terminating in anterior half of body. Testis single, medial, looped, lacking lobed margins, wholly posterior to intestine. Vas deferens short, extending posteriad from testis. Internal seminal vesicle distinct, longer than vas deferens, enveloped by cirrus sac. Cirrus long, 65% of seminal vesicle length, curving sinistrally before everting. Auxiliary external seminal vesicle lacking. Ovary medial, post-caecal, post-testicular; post-ovarian space comprising 1/3 of body length. Vitellarium follicular, diffuse, distributed throughout body. Oviduct and oötype indistinct. Laurer' s canal absent. Uterus post-gonadal, dorsal to posterior-most end of seminal vesicle, not extensively convoluted, extending anteriad before crossing midline and extending posteriad; uterine eggs large, occupying 67% of uterus, oblong, vacuous. Common genital pore dorsal, post-gonadal. Excretory vesicle indistinct.

3.1.2. Differential diagnosis

Body ≥30 × longer than wide; aspinous, lacking lateral tubercles. Anterior sucker aspinous, lacking peduncle, diminutive. Pharynx absent. Posterior oesophageal swelling present. Intestinal caeca inverse U-shaped, asymmetrical, terminating in anterior half of body, lacking diverticula. Testis single, looped, lacking lobed margins, curving >30 times. Internal seminal vesicle distinct, longer than vas deferens, 1/2 of body width, enveloped by cirrus sac. Cirrus long, >60% of seminal vesicle length. Ovary medial, post-caecal, post-testicular, wholly anterior to seminal vesicle and uterus. Uterus post-gonadal, dorsal to posterior-most end of seminal vesicle, not extensively convoluted; uterine eggs large, occupying 67% of uterus. Laurer's canal absent. Common genital pore dorsal, sinistral, post-caecal, post-gonadal.

3.1.3. Taxonomic summary

Type and only nominal species: Electrovermis zappum n. sp.



Figs. 1–4. *Electrovermis zappum* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) infecting the heart of the lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001 (Torpediniformes: Narcinidae) **(1)** Body of shistosomulum (voucher, USNM No. 1578577), ventral view. **(2)** Body of shistosomulum (larger) Voucher (USNM No. 1578576), ventral view. **(3)** Body of adult (holotype, USNM No**.** 1578574), ventral view. **(4)** Genitalia of holotype, ventral view. Oesophagus (es), oesophageal gland (eg), caecal bifurcation (cb), mouth (mo), vitellarium (vit), testis (t), ovary (o), vas deferens (vd), seminal vesicle (sv), common genital pore (cgp), uterus (u), and cirrus (c), uterine seminal receptacle (usr), and uterine constriction (uc).

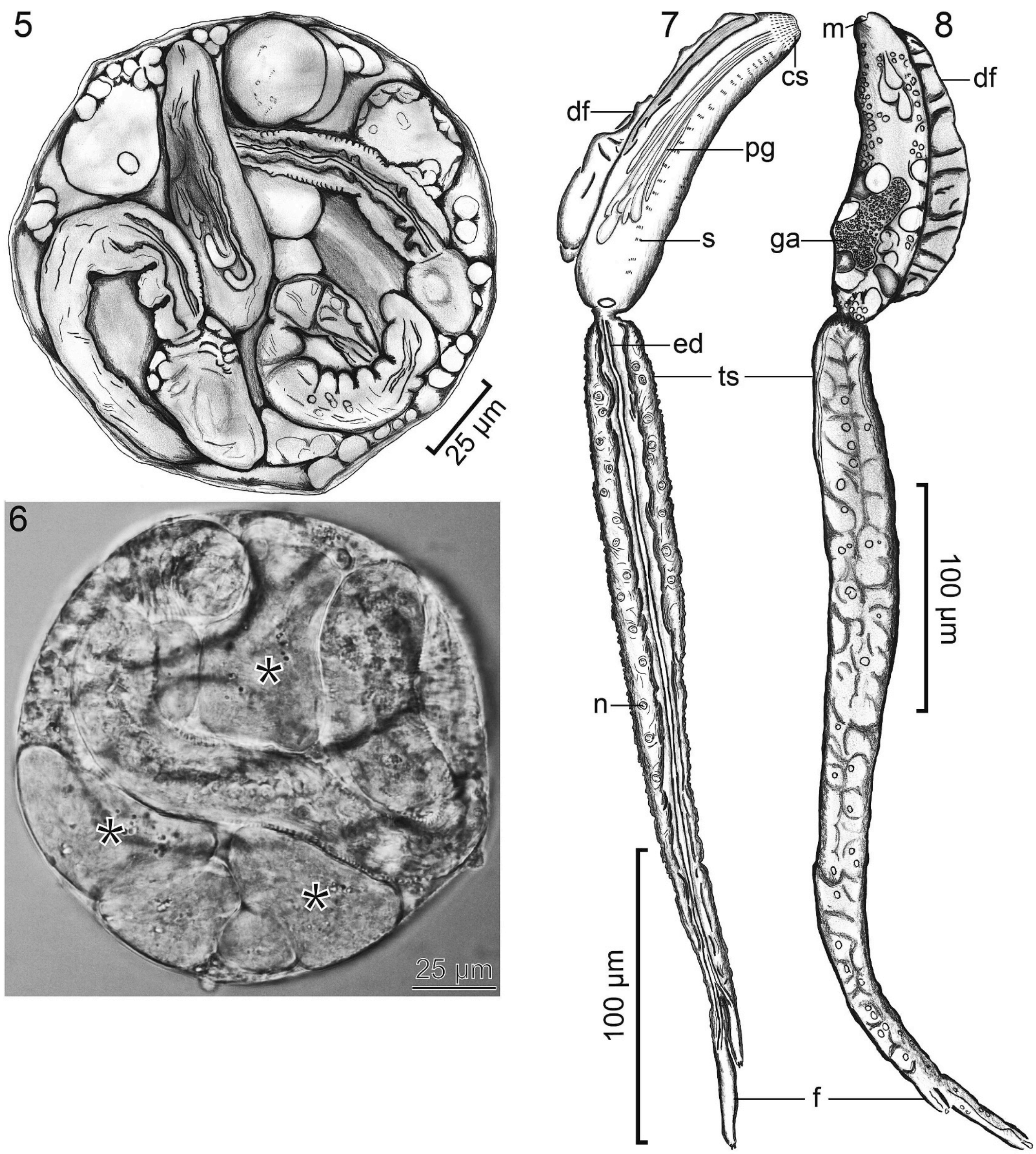
Etymology: "*Electro*" refers to the type host of the type species of the new genus and "*vermis*" is for worm.

3.2. Electrovermis zappum Warren and Bullard n. gen. (Fig. 1 – 12)

3.2.1. Diagnosis of adult (based on two stained, whole-mounted specimens and observations of two live adults collected from the heart of the lesser electric ray, N. bancroftii)

Body 1590 and 1780 long, 53 and 55 wide at greatest width, 30 and 32 × longer than wide (Fig. 3), aspinous. Ventrolateral nerve-cord, primary, and secondary commissure not evident in whole-mount. Mouth 3 in diameter, 9 from terminal end (Fig. 3). Oesophagus 425 and 500 in total length or 27% and 28% of body length, 11 and 13 in maximum width (at level of pre-caecal dilation); pre-caecal dilation 38 long, 11 wide. Caecal bifurcation 440 and 515 or 28% and 29% of body length from anterior body end; caeca extending posteriad in parallel, asymmetrical, dextral caecum 205 long or 12% of body length, 25 wide or 45% of body width, sinistral caecum 150 long or 8% of body length, 18 wide or 33% of body width, containing granular material within lumen of one individual (Fig. 3); post-caecal space 975 from posterior margin of the body.

Testis 250 and 325 long or 16% and 18% of body length, 25 and 30 wide or 47% and 55% of body width, 10 and 11 × longer than wide, post-caecal, curving 33 and 34 times (Fig. 3) widening posterior until narrowing and becoming confluent with vas deferens. (Fig. 3); post-testicular space 651 long or 37% of body length. Vas deferens 28 and 38 long, 8 and 10 wide, emanating from postero-ventral portion of testis, extending posteriad, looping just before becoming confluent with cirrus-sac. Cirrus-sac 335 long or comprising 19% of body length, max width equaling the width of seminal vesicle, having extremely thin wall approximately <1 thick, including seminal vesicle and cirrus; seminal vesicle 160 and 215 long or 10% and 12% of body length, 23 and 28 wide or 43% and 51% of body width at level of vesicle (Fig. 3), filling breadth of cirrus sac, extending sinuously posteriad before narrowing and ending 120 from common genital pore. Cirrus extremely long, 140 long or 65% of seminal vesicle length, 4 wide or 14% of seminal vesicle width, extending posteriad, gradually curving before reaching sinistral margin and common genital pore (Figs. 3 and 4); everted cirrus 15 long or 11% of total cirrus length. Common genital pore 288 or 16% of body length from posterior end of body,



Figs. 5–8. Sporocyst and cercaria of *Electrovermis zappum* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) infecting variable coquina clam, *Donax variabilis* Say, 1822 (Bivalvia: Cardiida: Donacidae). **(5)** Sporocyst showing four cercarial bodies among several germ bodies, ventral view. **(6)** Photo of live sporocyst showing three germ bodies (*). **(7)** Body of live cercaria, ventral view. **(8)** Body of mounted cercaria (USNM No. 1578578–1578583), ventral view. Mouth (mo), concentric spines (cs), dorsal fin fold (df), penetration gland (pg), lateral body spines (s), gonadal anlage (ga), excretory duct (ed), tail stem (ts), nuclei (n), and furca (f).

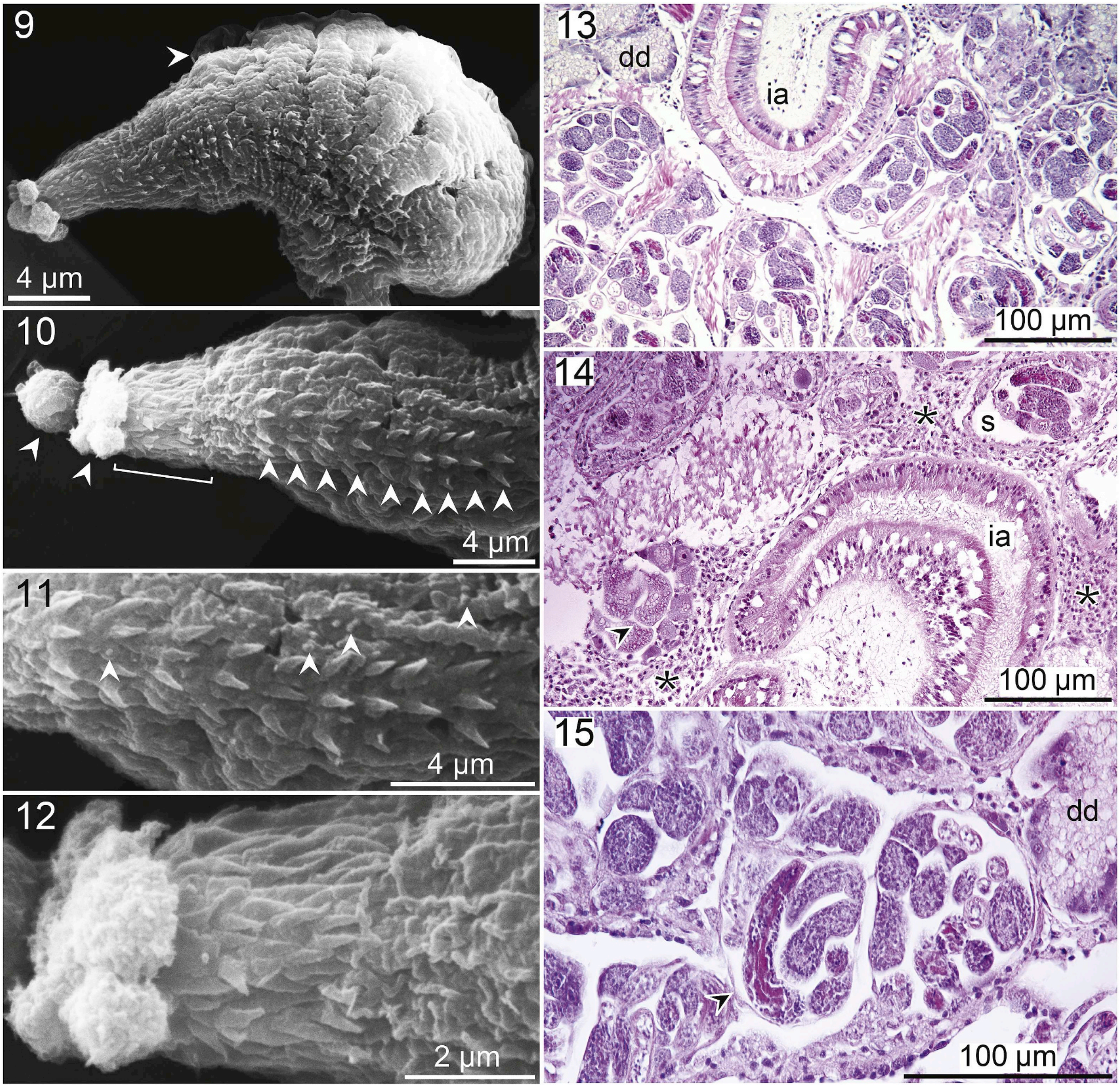
bordering sinistral body margin, 45 from dextral body margin (Fig. 3).

Ovary medial, lobed, 43 and 58 long or 3% of body length, 23 and 30 wide or 43 and 55% of body width at level of ovary, 1.9 × longer than wide, immediately post-testicular. Oviduct and oötype indistinct. Vitellarium having follicles compacted in dense lobules, distributed throughout entire body; common collecting duct indistinct. Uterus extending directly posterior 75 and 122 long before looping back anteriad, displaying marked constriction 140 from posterior end, expanding for 100 as uterine seminal receptacle (resembling seminal vesicle), before sharply turning sinistral creating constriction just posterior to common genital pore before returning anterior, eggs observed in adult specimens (Figs. 3 and 4); total ascending portion 281 and 358 long or 18% and 20% of body length, 16 and 23 wide, with wall <1 thick, extending anteriad and dorsal to cirrus and posterior region of seminal vesicle before coursing sharply back posterior to form descending portion; descending portion short, 94 and 140 long or 30% and 42% of ascending uterus length, 15 and 21 wide, (Figs. 3 and 4). Uterine eggs 43 in length or 31% of descending uterus length, 10 in width or 67% of descending uterus width, containing a large oval shell with a spheroid body 7 in diameter, surrounded by several smaller, dense lipid-like bodies (Figs. 3 and 4). Excretory bladder indistinct.

3.2.2. Description of sporocyst and cercaria (based on seven fixed, whole-mounted cercariae, 10 specimens prepared for SEM, and live cercariae collected and photographed from Donax variabilis)

Sporocyst spheroid, thin-walled, enveloping 5–7 (5 ± 0.8, 21) cercariae, germ sacs present, 88–140 (114 ± 12, 21) in diameter (Figs. 5 and 6). Rediae not observed.

Body of cercaria non-acetabulate, apharyngeate, non-ocellate, 73–110 (84 ± 11, 15) long, 20–30 (26 ± 3.4, 15) wide or 2.5–4.8 × longer than wide, with dorsal fin fold (Figs. 7–9), having spines distributed along lateral body margin (Figs. 7, 9–11). Spines of lateral body margin protruding from tegument approximately 0.7–1 (8), having pointed tips, distributed in transverse rows along lateral body margin of body; transverse spine rows numbering approximately 21–25 (3) per side of body (Figs. 7, 9–11), each comprising 3–4 spines (Figs. 9–11), approximately



Figs. 9–15. Scanning electron microscopy and histopathology of cercaria of *Electrovermis zappum* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) infecting variable coquina clam, *Donax variabilis* Say, 1822 (Bivalvia: Cardiida: Donacidae). **(9)** Whole body, arrow = dorsal fin fold. **(10)** Body, white arrows = possible secretion masses from penetration glands; white bar = anterior-most end including concentric spines; white arrows = lateral body spine rows, lateral view **(11)** Higher magnification of lateral body margin, arrows = tegumental papillae. **(12)** Higher magnification of anterior body end, showing space between spines of anterior sucker and those of the lateral body margin. **(13)** Histological section of infected gonad adjacent to intestinal arms (ia) and digestive diverticulum (dd). **(14)** Histological section showing infiltration of hemocytes (*) surrounding intestinal arm (ia), spororcysts (sp), and ooctyes (arrow). **(15)** Higher magnification of sporocyst containing developed cercaria (arrow) adjacent to digestive diverticulum (dd).

2.5–3 (9) in breadth. Fin fold extensively membranous vulnerable to fixation artifact, observed intact in live, SEM, and one whole-mounted specimen (Figs. 7–9), dorsomedial, 4–13 (11 ± 4.3, 4) in maximum height (in posterior half of body), extending from posterior most concentric spine row of anterior sucker and terminating 8 or 9% of body length from body terminus. Sensory papillae on body circular in shape, approximately <1 in diameter, distributed about body surface (Figs. 9–11). Anterior sucker 3.5–4 (3) long, 4–4.5 (3) wide, spinous (Figs. 7, 9–12); anterior sucker spines minute, visible with SEM at 8000× magnification only (indistinct in whole-mounted specimens), distributed in concentric rows (*cf.* spinous anterior sucker of teleost blood flukes), protruding from tegument approximately 0.75–1 (10), forming 6 concentric pre-oral rows (Fig. 12); mouth <1 in diameter (see Fig. 13).

Tail brevifurcate (Figs. 7 and 8), comprising a tail stem and a pair of furcae; tail stem 153–265 (227 ± 35, 15) long or 1.9–3.5 × body width or 7.6–13.3 × longer than wide, 18–30 (22 ± 3.2, 15) wide or 0.6–0.8 × body width; base 8–15 (10 ± 3, 15) wide at connection to body; tegument appearing rigid, jagged in live specimens (Fig. 7), filled with cellular masses and associated nuclei (Fig. 7); excretory duct running medial along length of tail, bifurcating and extending to tips of furcae, lacking obvious fin fold (Figs. 7 and 8); furcae asymmetrical, appearing boot-shaped in lateral view (Fig. 8), lacking fin fold; longest furca 23–45 (35 ± 8, 15) long or 3 × longer than shortest furca, 5–8 (5.8 ± 1.2, 15) wide or 4.1–8.6 × longer than wide; shortest furca 10–15 (16 ± 3.8, 15) long or 30–65% of longest furca length (Figs. 7 and 8).

3.2.3. *Diagnosis of schistosomulum (based on six stained, whole-mounted specimens from the heart of the lesser electric ray, N. bancroftii)*

Body 730–1420 (1088 ± 255, 5) long, 38–55 (42 ± 7, 5) at greatest width, 19–33 × longer than wide (Fig. 1 – 2), aspinous. Oesophagus 75

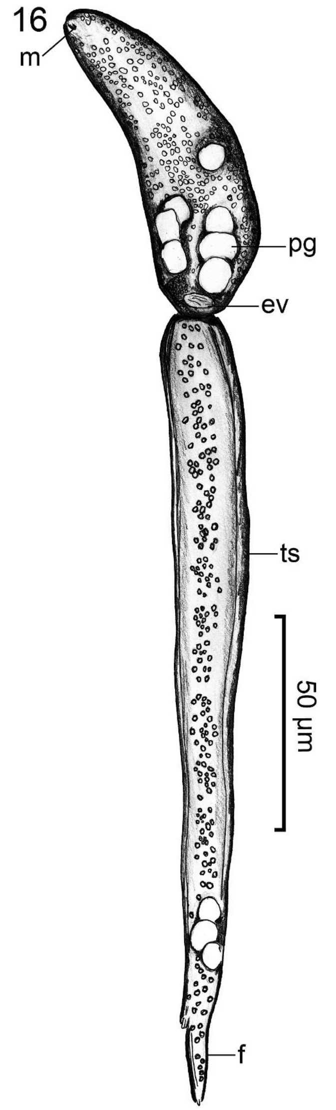


Fig. 16. Cercaria infecting green jackknife clam, *Solen viridis* Say, 1821 (Bivalvia: Adapedonta: Solenidae). **(16)** Body of mounted cercaria (USNM No. 1578587–1578589), ventral view. Mouth (mo), penetration gland (pg), excretory vesicle (ev), tail stem (ts), and furca (f).

and 450 long, 2–7 at greatest width (Fig. 1 – 2). Cirrus sac 83 long, 3 wide; seminal vesicle 203 long or 14% of body length, 28 wide or 65% of body width at level of vesicle (Fig. 2), everted cirrus 13 long, 4 wide.

3.2.4. Taxonomic summary

Type and only reported hosts: Lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001 (Torpediniformes: Narcinidae) and variable coquina clam, *Donax variabilis* Say, 1822 (Bivalvia: Cardiida: Donacidae).

Site in host: Heart lumen (*N. bancroftii); gonad* (*D. variabilis*).

Type locality: Fort Morgan, Alabama (30°13′30.21″N, 88° 0′34.18″W), north-central Gulf of Mexico, USA.

Prevalence and intensity of infection: 14 of 54 (prevalence = 26%) lesser electric rays sampled in September 2012, 2013, October 2014, May 2015, and July 2017 were infected by 1 specimen of *E. zappum* each (mean intensity = 1.0). Six of 1174 (prevalence = 0.5%) of variable coquina clams had several hundred sporocysts and cercariae.

Specimens deposited: Holotype (USNM 1578574), vouchers (USNM 1578575–1578586), GenBank Nos. (28S: MN244242 and MN244314; 18S: MN244243 and MN244244; ITS2: MN244245 and MN244246).

Etymology: The specific epithet "*zappum"* refers to the electric charge delivered by the lesser electric ray.

3.2.5. Taxonomic remarks

Adults of the new species are most similar to those of aporocotylids that infect batoids (*O. heterovitellatum*, *M. richardheardi*, and *O. glaucostegi* (Madhavi and Hanumantha Rao, 1970; Bullard and Jensen, 2008; Cutmore et al., 2018; Table 1) (excluding *G. bulbosus*) by having a diminutive anterior sucker that lacks spines, an asymmetrical and inverse U-shaped intestine, a looped testis that is post-caecal, an internal seminal vesicle and cirrus sac, and a post-caecal common genital pore as well as by lacking lateral tegumental spines. *Electrovermis zappum* differs from *O. heterovitellatum* by the combination of having a vermiform (vs. fusiform) body, an inverse U-shaped caeca that is smooth (vs. dendritic), and a testis that is post-caecal (vs. intercaecal) and by lacking lateral tubercles and testicular lobes. The new species can be differentiated from *M. richardheardi* by the combination of having a body that is 32 × longer than wide (vs. 3 × longer than wide), a straight to sinuous oesophagus (vs. sharply curved), a testis that is 10 × longer than wide (vs. 2.7) and consisting of 34 curves (vs. 10), a seminal vesicle occupying 1/2 the body width (vs. <1/4), a uterus that is located dorsal to the posterior-most extremity of the seminal vesicle (vs. flanking the seminal vesicle), and a seminal vesicle and uterus that are sinuous to straight (vs. extensively convoluted). *Electrovermis zappum* differs from *O. glaucostegi*, by the combination of having a body that is smaller, by 1/2 that of *O. glaucostegi* and 32 × longer than wide (vs. 17–28), a testis that is 1/6 **of** the body length (vs. >1/3) and that has 34 curves (vs. 52), positioned >1/3 from the terminal body margin (vs. >1/6), a seminal vesicle that is >10% of the total body length (vs. 4%) and occupies 1/2 of the body width (vs. >1/4), an obvious cirrus sac enveloping an extremely elongate cirrus that is > 1/2 of the seminal vesicle length, an ovary that is anterior to the seminal vesicle (vs. lateral), a uterus that is posterior to the testis and ovary (vs. overlapping and lateral to both), and a sinuous or straight (vs. convoluted) uterus. The remaining chondrichthyan blood fluke genera (*Hyperandrotrema* Maillard and Ktari, 1978, *Chimaerohemecus, Gymnurahemecus,* and *Selachohemecus* Short, 1954 (Short, 1954; Van der Land, 1967; Maillard and Ktari, 1978; Bullard et al., 2006; Orélis-Ribeiro et al., 2013; Warren et al., 2019)) all include species having large, C-shaped lateral tegumental spines. The new species lacks lateral tegumental spines altogether.

The sporocyst of the new species and that of other aporocotylids infecting bivalves is spheroid (Figs. 5 and 6), whereas the sporocyst of aporocotylids infecting polychaetes is elongate with tapered (spindle-shaped) or rounded ends (Cribb et al., 2011; Sugihara et al., 2014; Shirakashi et al., 2016; Siegel et al., 2018). We could not discern a morphological difference between the sporocyst of *E. zappum* and Holliman's (1961) description of the sporocyst of *Cercaria asymmetrica* Holliman, 1961. The diameter of the sporocyst of the new species is < 1/2 of the maximum size of those infecting *Plebidonax deltoides* (Lamarck, 1818). The number of developing cercariae within a sporocyst is likely taxonomically important. The sporocyst of the new species has 5–7 cercariae and that of *C. asymmetrica* has 4–8.

The cercaria of *E. zappum* is most similar to *C. asymmetrica* and the cercaria that infects *P. deltoides* (Cribb et al., 2017) in that it is apharyngeate, non-ocellate, brevifurcate, and spinous as well as by infecting a marine bivalve. The new species differs from the cercaria infecting *P. deltoides* by having lateral body spines (Figs. 9 and 10). Cribb et al. (2017) did not mention the presence or absence of lateral body spines but they are clearly absent in their SEM images. We did not observe a morphological difference between *E. zappum* and the description of *C. asymmetrica* (Holliman, 1961; to our knowledge no voucher specimen of *C. asymmetrica* was deposited in a museum). We observed spheroid masses about the distal end of the anterior sucker of the cercaria of *E. zappum* (Figs. 9 and 10; 12). At least superficially, these masses resemble those described for *A. simplex* (see Køie, 1982) and likely comprise secretions from the penetration glands.

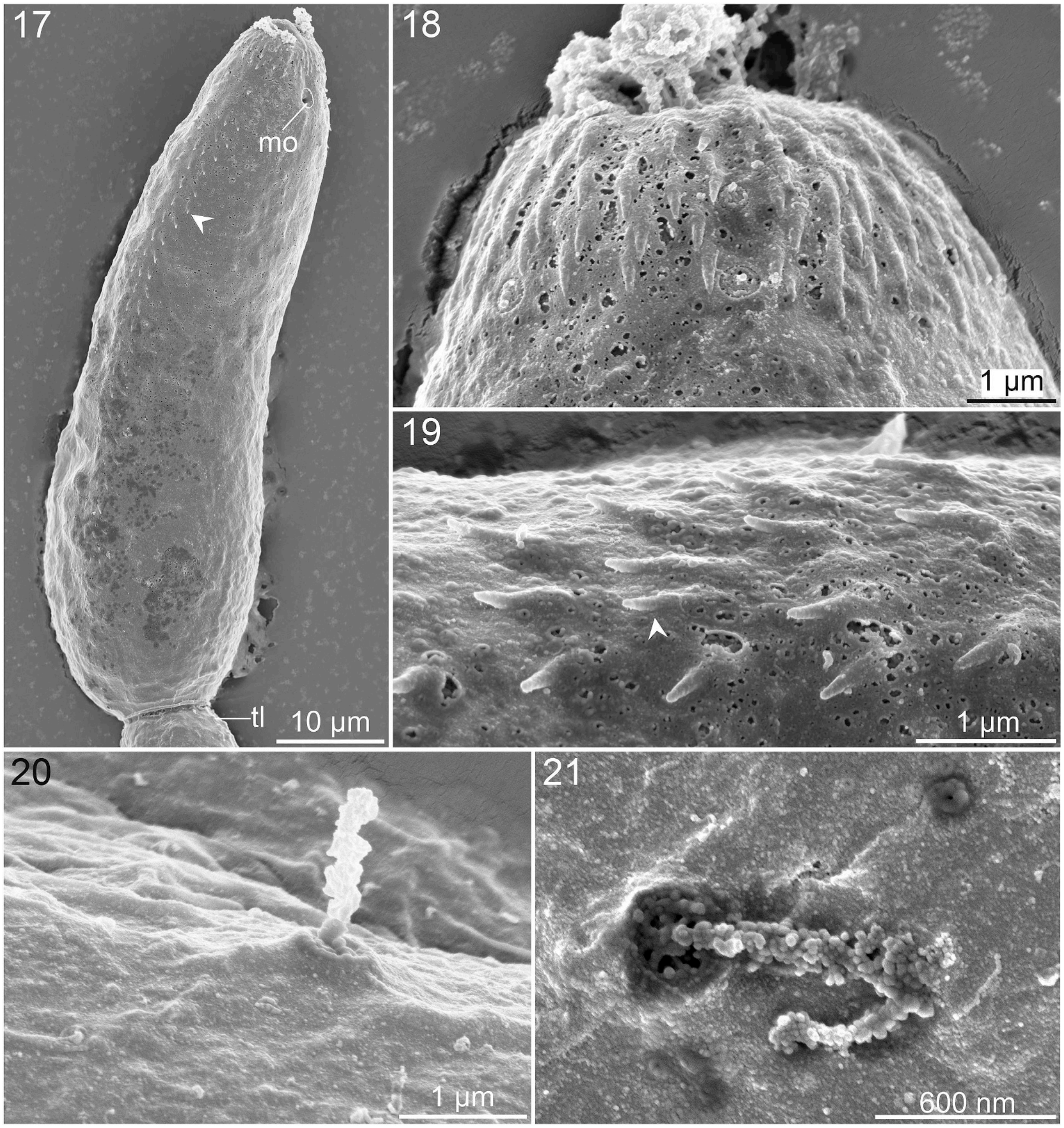


Fig. 17–21. Cercaria infecting green jackknife clam, *Solen viridis* Say, 1821 (Bivalvia: Adapedonta: Solenidae). **(17)** Cercarial body showing mouth (m), anterior-most row of spines (arrow), and connection with tail (tl). **(18)** Anterior end showing concentric rows of minute spines about anterior body end, lateral view. **(19)** High magnification view of spine (arrow) and spine rows in anterior region of cercarial body near mouth, lateral view. **(20 & 21)** Granular material near tegumental pore.

3.3. Aporocotylidae sp. ex. Solen viridis (Fig. 16 − 21)

3.3.1. Description of sporocyst and cercaria (based on 5 fixed, whole-mounted cercariae, specimens prepared for SEM, and live cercariae collected and photographed from a single crushed bivalve, S. viridis)

Sporocyst spheroid, thin-walled, enveloping fewer than 12 cercariae, 91–133 (103 ± 15, 8) in diameter. Rediae not observed.

Body of cercaria non-acetabulate, apharyngeate, non-ocellate, 79–89 (83 ± 4, 6) long, 27–30 (28 ± 1.1, 6) wide or 2.6–3.3 × longer than wide, recurved ventrally, kidney bean-shaped (Figs. 16 and 17), with dorsal fin fold (Fig. 16), having spines distributing along lateral body margin. Spines of lateral body margin protruding from tegument approximately 0.25 in anterior body region (Fig. 19), having pointed tips, spine tips in posterior region of body margin embedded in tegument, distributed in transverse rows along lateral body margin of body; transverse spine rows numbering approximately 24–27 per side of body of a total of 48–54 total rows (Figs. 17 and 19), each comprising 3–4 spines (Fig. 19), approximately 1.3–2 (4) in breadth. Fin fold extensively membranous, vulnerable to fixation artifact, observed in live specimens only, dorsomedial, 15 in maximum height. Body pores probably secretory in nature (or possibly representing "penetration glands") and probable sensory pores; secretory pores having secretion appearing as a loose conglomeration of small spheroid droplets (Figs. 20 and 21); sensory pores with central nipple-like structure surrounded by several laterally-directed extension, appearing as a spoked wheel in SEM, distributed about body surface as well as terminally on anterior body end (Figs. 20 and 21). Anterior sucker 7 long, 5 wide, spinous (Fig. 18); anterior sucker spines minute, visible with SEM at 1700× magnification only (indistinct in live and whole-mounted specimens), distributed in concentric rows (*cf.* spinous anterior sucker of teleost blood flukes), protruding from tegument approximately <1, forming four concentric pre-oral rows (Fig. 18).

Tail brevifurcate (Fig. 16), comprising a tail stem and a pair of furcae; tail stem 156–234 (202 ± 28, 8) long or 1.8–2.5 × body length, 17–28 (22 ± 3.7, 7) wide or 7.0–11.7 × longer than wide or 0.6–0.8 × body width; base 7–10 (8.6 ± 1.1, 5) wide at connection to body, lacking obvious fin fold (Fig. 16) but perhaps having extremely thin, delicate fold; furcae asymmetrical, appearing boot-shaped in lateral view, lacking fin fold; longest furca 17–22 (18 ± 2.1, 5) long or 6 × longer than shortest furca, 5 (5) wide or 3.4–4.0 × longer than wide; shortest furca 3–5 (4.2 ± 0.8, 5) long or 14–29% of longest furca length (Fig. 16).

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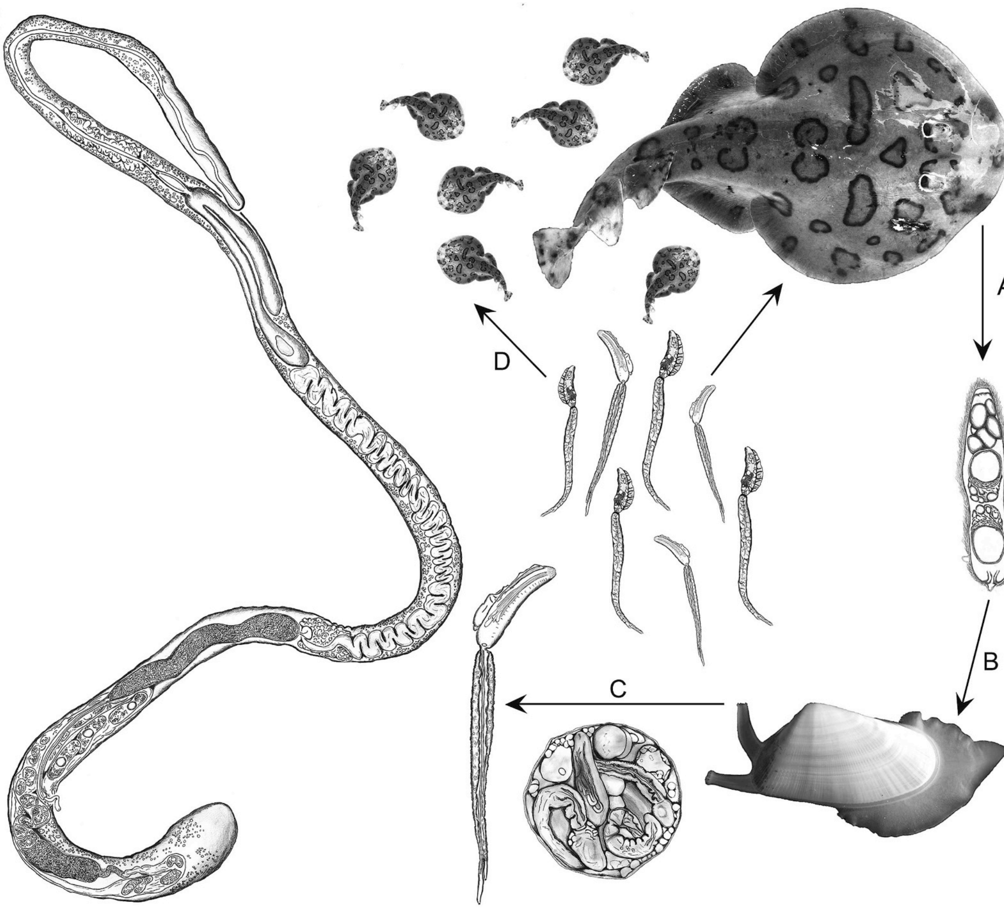

Fig. 22. Life cycle of *Electrovermis zappum* Warren and Bullard n. gen., n. sp. (Digenea: Aporocotylidae) infecting the heart of the lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834) Carvalho, 2001 (Torpediniformes: Narcinidae), and the variable coquina clam, *Donax variabilis* Say, 1822 (Bivalvia: Cardiida: Donacidae). **(22)** Letters indicate the life history: **A)** egg or miracidium emerges from definitive host, *N. bancroftii*; **B)** miracidium infects the intermediate host, *D. variabilis*; **C)** clonal asexual reproduction occurs in sporocyst and cercariae emerge; **D)** cercariae infect neonates, juveniles, or adults of *N. bancroftii*.

3.3.2. Taxonomic summary

Type and only reported host: Green jackknife clam, *Solen viridis* Say, 1821 (Bivalvia: Adapedonta: Solenidae).

Type locality: Mississippi Sound, ~6 km north/northwest of the west end of Horn Island, Mississippi (30°14′35.6″N, 88° 46′52.9″W), northern Gulf of Mexico, USA.

Prevalence and intensity of infection: One of 10s of green jackknife clams had hundreds of cercariae.

Specimens deposited: Vouchers (USNM 1578587–1578589), GenBank Nos. (28S: MN244240).

3.3.3. Taxonomic remarks

The sporocyst is similar in size to those of *C. asymmetrica* and *E. zappum*; however, it is < 1/2 of the maximum diameter of the sporocyst infecting *P. deltoides*. The sporocyst from *S. viridis* differs from these other sporocysts by having as many as 12 cercariae, whereas that of *E. zappum* and *C. asymmetrica* had 5–7 and 4–8, respectively. The new cercaria is most similar to *C. asymmetrica*, *E. zappum*, and the cercaria that infects *P. deltoides* in that it is apharyngeate, non-ocellate, brevifurcate, spinous, and by the presence of a dorsal fin fold and lacking furcal fin folds. It differs from *C. asymmetrica* and *E. zappum* by the

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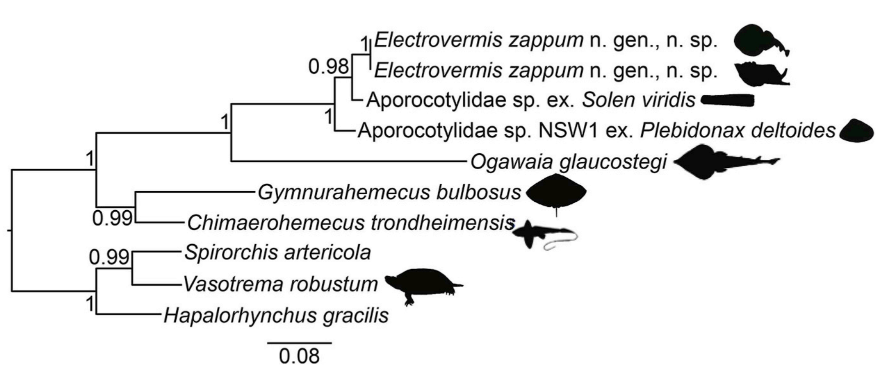

Fig. 23. Phylogenetic relationships of chondrichthyan blood flukes and innominate cercariae reconstructed using Bayesian inference with the large subunit ribosomal DNA (28S) gene. Numbers aside tree nodes indicate posterior probability. Scale bar is in substitutions per site.

combination of having 4 rows of concentric anterior spines (vs. 6) (Fig. 12; 18), lateral body spines that protrude 0.25 from the tegumental surface (vs. 0.7–1), and having furcae that are 2 × shorter in length. The new cercaria differs from the cercaria infecting *P. deltoides* by having lateral body spines.

3.4. Phylogenetic results

PCR and sequencing of the 28S resulted in 1559 (adult of *E. zappum*), 1571 (cercariae *of E. zappum*), and 2360 (cercariae from *S. viridis*) nucleotides. That for the 18S resulted in 1867 (adult of *E. zappum*) and 1928 (cercariae *of E. zappum*) nucleotides. That for the ITS2 resulted in 427 (adult of *E. zappum*), 470 (cercariae *of E. zappum*), as well as 441 and 438 (cercariae from *S. viridis*) nucleotides. The aligned 28S, 18S, and ITS2 fragments representing the adult and cercaria of the new species were identical (100% similarity). The 28S of *O. glaucostegi* (MF503308), Aporocotylidae sp. NSW1 (MF503307), and Aporocotylidae sp. ex. *S. viridis* (MN244240) differed from that of the new species by 309 (28%), 68 (7%), and 48 (5%) nucleotides, respectively. The ITS2 differences among these taxa were 169 (39%), 54 (13%), and 49 (7%), respectively. The new species differed from *G. bulbosus* (MH555432) by 357 (31%) (28S), 264 (14%) (18S), and 193 (40%) (ITS2) nucleotides.

The 28S phylogeny recovered the new species sister to Aporocotylidae sp. ex. *S. viridis*, with those species sharing a recent common ancestor with Aporocotylidae sp. NSW1 (Fig. 23). All of those species are sister to *O. glaucostegi*. *Gymnurahemecus bulbosus* and *C. trondheimensis* share a recent common ancestor (indicating paraphyly of the batoid blood flukes (Warren et al., 2019)) and are sister to the remaining chondrichthyan blood flukes plus the cercarial sequences from *S. viridis* and *P. deltoides*; both of which have indeterminate fish hosts.

3.5. Histopathology of infected Donax variabilis

We sectioned four tissue blocks that generated a total of 580 paraffin sections on 116 slides. Sporocysts and cercariae of the new species infected gonad but also occupied the spaces between the digestive diverticulum, intestinal arms, stomach, and crystalline sac (Figs. 13–15). Developing cercariae were within sporocysts (Fig. 15). Fewer than 10 oocytes that bordered sporocysts were observed in all of the examined sections; some were surrounded by an aggregate of basophilic, granular cells (possibly infiltrating immune response hemocytes or nutritive cells [Cheng, 1996]) (Fig. 14). Uninfected variable coquina clams (control tissue) had normal gonad and no demonstrable pathological change.

4. Discussion

4.1. Hosts, habitat, and the host-parasite relationship

The lesser electric ray is a small, relatively lethargic batoid that is capable of discharging an electric shock and that seasonally inhabits shallow, littoral marine (high salinity) waters in the northern Gulf of Mexico (Rudloe, 1989; Carlson et al., 2016; Carlson et al., 2017). During August–October, lesser electric rays (mature females, mature males, and neonates) are abundant within the shallow subtidal zone (< 1–2 m). The literature holds few accounts of the general biology and habits of this ray; perhaps because these rays are typically buried beneath the sand and are difficult to observe without a search image for their spiracles (the only part of the ray that is visible if the ray is buried in sand). Lay persons and some biologists who walk in the subtidal sand flats that harbor lesser electric rays might assume that they have stepped on a flatfish (Pleuronectiformes spp.) rather than a buried lesser electric ray. Moreover, the spiracles of the lesser electric ray superficially resemble the openings of various invertebrate burrows that pepper these subtidal sand flats (Adkison and Heard, 1995). Adult lesser electric rays are usually in waters > 1 m in depth but neonates can be observed swimming along the ~10–30 cm sand ledge that marks the lower portion of the swash zone (a sub-surface feature sculpted by breaking wave action on the beach; lower intertidal zone/upper subtidal zone). SAB has on numerous occasions observed neonate lesser electric rays (~15 cm total length) aside juvenile Florida pompano (*Trachinotus carolinus* [Linnaeus 1766] Robins and Ray, 1986) evidently feeding on macrobenthic crustaceans displaced by wave action along the base of this ledge.

Variable coquina clams are dense within the intertidal zone, exclusively within the swash zone. Dense populations of perhaps thousands of variable coquina clams per m^2 (Simone and Dougherty, 2004; Cobb et al., 2011) are typical within Gulf of Mexico high-energy beach habitat during summer and early Fall (Rudloe, 1989; personal observations MBW and SAB). We collected the infected variable coquina clams during the only time of year, in our experience, that one can observe large numbers of lesser electric rays in that region of the Gulf of Mexico. Because we have observed large numbers of adult and neonate lesser electric rays simultaneously with variable coquina clams shedding cercariae, we plan to test the hypothesis that *E. zappum* cercarial shedding is synchronized with the pupping of lesser electric rays. Although we anecdotally observed lesser electric rays during other activities, we conducted targeted parasite sampling during one month within each of 5 years (September 2012; 2013; October 2014; May 2015; July 2017). Hence, the existing data do not permit us to determine the frame of time wherein this life cycle takes place.

At least for the period of time we sampled, cercariae of *E. zappum* and adults and neonates of the lesser electric ray co-mingle in the shallow subtidal waters of the northern Gulf of Mexico. We speculate that during Fall (August–October) the miracidium hatches from the egg (egg is either ejected from gill epithelium or the egg hatches while embedded in gill epithelium); perhaps as these rays enter the subtidal zone (sand flats) to pup (Fig. 22A). There, the miracidium infects variable coquina clams that densely populate the swash zone (Fig. 22B). These variable coquina clams harbor the sporocyst, which liberates multitudes of cercariae (Fig. 22C) that, once shed, are washed down the slope of the swash zone by receding wave action and into the subtidal waters that evidently comprise a haven for adult and neonate lesser electric rays (Fig. 22D). To decrease the amount of speculation regarding the timing of this life cycle, we plan to enumerate the density and prevalence of eggs of *E. zappum* in lesser electric ray gill and experimentally expose neonate lesser electric rays to shedding cercariae to fill in these gaps in knowledge.

4.2. Comparing morphological attributes of aporocotylid cercariae infecting marine and estuarine invertebrates

The literature holds accounts of 21 marine fish blood fluke cercariae infecting 23 intermediate hosts of Gastropoda, Bivalvia, and Annelida (Table 3). No adult fish blood fluke has a ventral sucker, we herein diagnose all marine fish blood fluke cercariae as such: body lacking ventral sucker (non-acetabulate), apharyngeate, non-ocellate, ventrally curved, tubular; anterior sucker with concentric spine rows; dorsal fin fold present or absent; lateral body spines present or absent; tail stem bristles present or absent; furcae present or absent, symmetrical or asymmetrical; furcal fin fold present or absent; furcal bristles present or absent (KEY). One cercaria, *Cercaria hartmanae* Martin, 1952, needs to be reassessed because its family affiliation is uncertain. Køie (1982) re-described it based on Martin's type material and determined that Martin (1952) misinterpreted the intestinal anlage as a ventral sucker. Based on Martin's (1952) description and without examining museum materials nor citing Køie (1982), de Buron et al. (2018) concluded that *C. hartmanae* was a turtle blood fluke because Martin's (1952) description included ventral sucker present. Based on Køie (1982) re-description, *C. hartmanae* is likely not a turtle blood fluke. New collections and genetic sequencing of *C. hartmanae* should be made to confirm and resolve this issue.

Synapomorphies for aporocotylid cercariae are lacking in the literature but our morphological comparisons herein (KEY) suggest that cercarial morphology could inform aporocotylid ancestry and host affiliation (e.g., differentiating cercariae of batoid and shark blood flukes). This seems especially promising regarding the morphological features of the furcae, dorsal fin folds, and penetration glands (KEY). Regarding furcae, asymmetrical furcae diagnose a group of bivalve-infecting aporocotylid cercariae, including the new species. Five cercariae (of which three have been sequenced; Table 2; Fig. 23) have asymmetrical furcae, infect bivalves, and are monophyletic (Holliman, 1961; Gilardoni et al., 2011; Cribb et al., 2017; KEY). Two cercariae ranging in the Gulf of Mexico have symmetrical furcae with a body and tail fin fold (Holliman, 1961; Wardle, 1979). A 3rd bivalve cercaria has a non-furcate, short, paddle-shaped tail (*Cercaria solemyae* Martin, 1944). Because there are no other cercariae with this tail morphology, we suspect that *C. soleymae* represents a new species, perhaps representing a new genus. Further, all species infecting marine ray-finned fishes (Actinopterygii) of those that have been elucidated (except one; *A. simplex*), have a tube-like tail stem (vs. paddle-shaped tail stem; *C. solemyae*), lack furcae, and infect polychaetes. Regarding the dorsal fin fold, all bivalve-infecting aporocotylids, which likely all mature in chondrichthyans, have a dorsal fin fold except *C. solemyae*; further separating *C. solemyae* from all other cercariae that infect bivalves, polychaetes, or gastropods. The known blood fluke cercariae from all marine ray-finned fishes (Actinopterygii) lack a dorsal fin fold (Køie, 1982; Cribb et al., 2011; Sugihara et al., 2014; Shirakashi et al., 2016; Siegel et al., 2018; Table 3; KEY). Regarding the penetration glands, *C. asymmetrica* has seven penetration glands, and *Cercaria cristulata* Holliman, 1961 has eight (Holliman, 1961); whereas *Cercaria mercenaria* Wardle, 1979 and *C. soleymae* have 10 (Martin, 1944; Wardle, 1979). Only four of the 12 polychaete-infecting fish blood flukes have been characterized regarding their penetration glands: three of four have 10 penetration glands and *C. orientalis* has 14 (Stunkard, 1929; Martin, 1952; Oglesby, 1961; Køie, 1982; Shirakashi et al., 2016) (Table 3). Collectively, these features are difficult to image and visualize because they are delicate and vulnerable to fixation artifact; reinforcing the importance of observing and illustrating live cercariae whenever possible.

4.3. Phylogenetic relationships

Our results herein support the hypotheses that chondrichthyan blood flukes are monophyletic, albeit there has evidently been some host-switching among chondrichthyan lineages, and that they may exclusively infect bivalves as intermediate hosts (Cribb et al., 2017). Despite the fact that chondrichthyan blood flukes remain underrepresented in phylogenetic studies of the Schistosomatoidea (see Orélis-Ribeiro et al., 2014; Cribb et al., 2017; Hernández-Mena et al., 2017; Pérez-Ponce de León and Hernández-Mena, 2019; Warren et al., 2019), the addition of a few new taxa helps explore their systematics and host relationships. The present study brings the total number of nominal chondrichthyan blood fluke nucleotide sequences to four (Table 2; Fig. 23). We also included two cercarial sequences (Aporocotylidae sp. "type 2" described herein; Aporocotylidae sp. NSW1) that have been presumed (Cribb et al., 2017) to mature in chondrichthyans (Footnote: Previous to the present study, the only existing bivalve aporocotylid sequence was Aporocotylidae sp. NSW1) (Table 2; Cribb et al., 2017). Cribb et al.'s (2017) phylogenetic analysis recovered this cercarial sequence within a clade of adult aporocotylids infecting chondrichthyans (Bullard et al., 2006; Bullard and Jensen, 2008; Orélis-Ribeiro et al., 2013; Warren et al., 2019) and presumed that the cercaria ultimately infected a chondrichthyan. This sequence was reported as *O. glaucostegi* (as cf. *Myliobaticola* sp.) in de Buron et al. (2018), who mistook Cribb et al. (2017) as having elucidated a life cycle for a chondrichthyan blood fluke. If those cercariae are eventually revealed to mature in chondrichthyans, then all chondrichthyan blood fluke sequences are monophyletic (Cribb et al., 2017; Warren et al., 2019). Those working with larval trematodes, which have complex life cycles, should use caution in assuming that phylogenetic affiliation predicts the identity of the definitive host; since this would ignore the possibility that any fluke has "switched" definitive hosts (see example below). That said, no evidence so far suggests that chondrichthyan blood flukes are paraphyletic.

Considering their host affiliations, the phylogeny recovered herein is neither concordant with the branching order nor the tree topology of the latest batoid phylogeny (Last et al., 2016). This suggests that, based on available evidence, there is no support for cophyly (phylogenetically-related parasites infecting phylogenetically-related hosts) between these parasites and their definitive hosts. Most obvious in this regard is that the batoid blood flukes are paraphyletic (Fig. 23) because *G. bulbosus* shares a recent common ancestor with *C. trondheimensis*, a chimaera blood fluke. Further, *E. zappum* (infecting a narcinid) clades with *O. glaucostegi* (infecting a glaucostegid) and is sister to the clade comprising *G. bulbosus* (infecting a gymnurid) and *C. trondheimensis* (infecting a chimaerid). Last et al.'s (2016) phylogeny recovered the gymnurids sister to the glaucostegids, which share a recent common ancestor with the narcinids. The two bivalve cercarial sequences clade with *O. glaucostegi* and *E. zappum*, and no congruence is evident between the aporocotylid phylogeny herein and Combosch et al.'s (2017) bivalve phylogeny. Combosch et al. (2017) recovered *Donax* sp. (Donacidae) sister to *Plebidonax* sp. (Psammobiidae), which are sister to the Solenidae. The recovered phylogeny herein suggests that the cercaria of *S. viridis* (Solenidae) shares a recent common ancestor with the new species (*D. variabilis*; Donacidae) and that they together are sister to the cercaria infecting *P. deltoides* (Psammobiidae). Hence, a cophyly hypothesis is rejected at the intermediate host level as well because *E. zappum* does not share a recent common ancestor with the cercaria infecting *P. deltoides* (a species within the same family [Donacidae]), and together are sister to the cercaria infecting *S. viridis.*

Acknowledgements

We thank Cova R. Arias (Aquatic Microbiology Laboratory [AML], Auburn University) for supporting DNA extraction and PCR and Steven P. Ksepka (Auburn University) for their assistance in collecting bivalves. This work was funded by Federal Aid in Sport Fish Restoration, Project #AL-F-F19AF00146 (Alabama Department of Conservation and Natural Resources, Marine Resources Division), Auburn University's Intramural Grants Program, the Southeastern Cooperative Fish Parasite and Disease Project, Alabama Agriculture Experiment Station, and U.S. Department of Agriculture.

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