Taxonomy and Systematics of Early Lineage Fish Blood Flukes (Digenea: Aporocotylidae)

by

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A thesis submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Auburn, Alabama
August 03, 2019

Keywords: *Acipensericola*, *Gymnurahemecus*, *Electrovermis*, life cycle

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ABSTRACT

The monophyletic fish blood flukes (Platyhelminthes: Digenea: Aporocotylidae Odhner, 1912) comprise ~166 species assigned to 40 genera infecting freshwater, marine, and estuarine fishes. They are occasional pathogens of cultured fishes, and are ancestral to the paraphyletic turtle blood flukes ("Spirorchiidae") and the human-pathogenic blood flukes (Schistosomatidae Stiles and Hassal, 1898). 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). Cartilaginous fishes are rarely examined for aporocotylids, and prior to my work, only seven species were described. Blood flukes described in this thesis were collected from 329 fish assigned to four genera in four families as well as from 1,185 bivalves from two families. I use alpha taxonomy, scanning electron microscopy (SEM), genetic sequence techniques (PCR; large subunit ribosomal DNA [28S], small subunit ribosomal DNA [18S], and internal transcribed spacer 2 [ITS2]), and molecular phylogenetic analysis (Baysian inference) to characterize four new species assigned to three new genera. I describe *Acipensericola glacialis* n. sp. from the heart of the lake sturgeon, *Acipenser fulvescens*, *Gymnurahemecus bulbosus* n. gen., n. sp. from the heart of the smooth butterfly ray, *Gymnura micrura*, *Electrovermis zappum* n. gen., n. sp. from the heart of the lesser electric ray, *Narcine bancroftii*, and a new genus and species from the heart of the smalltooth sawfish, *Pristis pectinata*). Further, I elucidate the life cycle of *E. zappum*, characterize a new cercaria infecting the green jackknife clam, *Solen viridis*, and compare all aporocotylid cercariae that infect marine and estuarine gastropods, bivalves, and polychaetes. This work culminates in the first descriptions of blood flukes infecting the order Torpediniformes, the families Acipenseridae, Gymnuridae, and Pristidae, as well as the first elucidated blood fluke life cycle that includes a chondrichthyan definitive host and a bivalve intermediate host. Further, I provide the 2nd genetic sequence from a blood fluke infecting an acipenseriform, and the 3rd and 4th genetic sequences from blood flukes infecting chondrichthyans, as well as the 2nd and 3rd sequences from cercariae

infecting bivalves. This work has resulted in publications in Systematic Parasitology, Parasitology Research, and International Journal for Parasitology: Parasites and Wildlife.

ACKNOWLEDGMENTS

I thank my advisor, mentor, and friend Stephen Ash Bullard (Aquatic Parasitology Laboratory, School of Fisheries, Aquaculture, and Aquatic Sciences [SFAAS], College of Agriculture [COA], Auburn University [AU], Auburn, AL) for sharing is expertise and experiences with me. I thank Ash for his curiosity in all things and his constant encouragement to better myself both in life and academia.

I am thankful to my committee members Cova Arias (Aquatic Microbiology Laboratory, SFAAS, COA, AU, Auburn, AL) and Nathan Whelan (United States Fish and Wildlife, SFAAS, COA, AU, Auburn, AL) for providing comments on this thesis; specifically, Cova's mentorship in molecular biology and Nathan's help with phylogenetic analyses. I also thank Delane C. Kritsky (Health Education Program, School of Health Professions, Idaho State University, Pocatello, ID) for his mentorship and his collaboration on several publications.

I thank my laboratory mates Raphael Orélis-Ribeiro and Jackson Roberts for their guidance in the laboratory and the field; Steven Ksepka and Haley Dutton for their supporters and friendship. I thank all members of the Aquatic Microbiology Laboratory, especially Stacey LaFrentz, Francisca Valverde, Wenlong (Colin) Cai, Alison Aceves, and Triet Truong for their technical assistance.

I thank Scott Snyder (University of Nebraska at Omaha, Omaha, Nebraska, USA), Matthew Womble (National Oceanic and Atmospheric Administration, Washington D. C., USA), and personnel of the Wisconsin Department of Natural Resources for helping collect lake sturgeon blood flukes; Marcus Drymon (Mississippi State University) and personnel of the Dauphin Island Sea Lab for facilitating the collection of smooth butterfly rays in Mobile Bay, AL; personnel with Florida Fish and Wildlife Commission for their collection of smalltooth sawfish in Florida.

I thank William Moser, Anna Phillips, Katie Ahlfield, and Karen Reed (all curatorial staff of the Smithsonian National Museum of Natural History: Department of Invertebrate Zoology,

Washington D.C.) for accepting and loaning type aporocotylids and Mal Bryant and Robert Adlard (Queensland Museum) for loaning type aporocotylids.

I thank my parents Robert and Patricia Warren for their love and support; specifically, my father for the late nights discussing life's journey and my mother for her high-energy spirit and "I can do anything" attitude. Thank you to my grandmothers Anne Tiller and Ingrid Warren for always being optimistic and joyful. Thank you to my late grandfathers G.C. Tiller and R.C. Warren for whom I would've collected fish with. Thank you to my brothers in arms James Watkins, Tyler Kellogg, Samarre Perez, Juan Garcia, and Eric Sprankle for their unwavering support and for always keeping me grounded.

Finally, I thank my wife Barrett Warren, who is my foundation in all things.

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***Published in Systematic Parasitology (Available online 08 September 2017)**

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ABSTRACT

Acipensericola glacialis n. sp. infects the heart of lake sturgeon, *Acipenser fulvescens* (Rafin- esque), in the Lake Winnebago System and differs from its only congener, *Acipensericola petersoni* Bullard, Snyder, Jensen & Overstreet, 2008, by having a dendritic intestine, deeply-lobed testes, a post-ovarian oötype, and a common genital pore that is medial to the dextral caecum. *Acipensericola petersoni* has a non-dendritic intestine, testes that are not deeply lobed, an oötype that is at level of the ovary (ventral to the ovary), and a common genital pore that is dorsal to the dextral caecum. Comparison of the large (28S) and small (18S) sub-unit ribosomal DNA and internal transcribed spacer 2 (ITS2) regions between specimens of *A. glacialis* n. sp. and *A. petersoni* revealed 13 (of 1,621 nt; 99.2% similarity in the 28S), 8 (of 1,841 nt; 99.9% similarity in the 18S), and 11 (of 442 nt; 97.5% similarity in the ITS2) nucleotide differences. Collectively, these results comprise an unexpectedly high degree of morphological and molecular similarity given the geographical (Mississippi River Basin vs Great Lakes Basin) and phylogenetic (Polyodontidae vs Acipenseridae) separation of these hosts but seemingly did not reject a previous hypothesis concerning lake sturgeon dispersal from the Mississippi Refugium following the Wisconsin glaciation *18,000 years ago. The new species is the first nominal blood fluke described from a sturgeon.

INTRODUCTION

The monophyletic fish blood flukes (Aporocotylidae Odhner, 1912) (Bullard et al., 2009; Orélis-Ribeiro et al., 2014) comprise approximately 150 accepted species of 35 genera infecting freshwater, marine, and estuarine fishes (Bullard 2014; Nolan et al., 2014, 2016; Ogawa et al., 2015; Orélis-Ribeiro & Bullard, 2015, 2016; Santoro et al., 2015; Yong et al., 2016a, b; Orélis-Ribeiro et al., 2017; Palacios-Abella et al., 2017). Freshwater fish blood flukes are little studied compared to marine species: only six of 35 (17%) accepted aporocotylid genera comprise blood flukes that mature in primary division freshwater fishes (Shimazu, 2007, 2013; Bullard et al., 2008; Truong & Bullard, 2013; Orélis-Ribeiro & Bullard, 2015, 2016). Those infecting North America's inland fishes are especially underexplored, totaling only eight species assigned to ''Sanguinicola'' Plehn, 1905 (*sensu lato*) (Van Cleave & Mueller, 1932; Fischthal, 1949; Davis, 1953; Wales, 1958; Erickson & Wallace, 1959; Meade & Pratt, 1965; Hoffman & Putz, 1966; Dechtiar, 1972a, b; Esch & Huffines, 1973; Schell, 1974; Hoffman et al., 1985; Muzzal, 2000) and monotypic *Acipensericola* Bullard, Jensen, Snyder & Overstreet, 2008 (see Bullard et al., 2008) (Table 1).

Sturgeons (Acipenseriformes: Acipenseridae) are among the most iconic and historically commercially valued freshwater fishes, including 25 extant species (19 of Acipenserinae; 6 of Scaphirhynchinae) (Birstein & DeSalle, 1998). Of those, eight range in North America (Page & Burr, 2011): the shortnose sturgeon *Acipenser brevirostrum* Lesueur; the lake sturgeon *Acipenser fulvescens* (Rafinesque); the green sturgeon *Acipenser medirostris* Ayres; the Atlantic sturgeon *Acipenser oxyrinchus* Mitchill (and the Gulf of Mexico sub-species *A. o. desotoi* Vladykov); the white sturgeon *Acipenser transmontanus* Richardson; the pallid sturgeon *Scaphirhynchus albus* Forbes & Richardson; the shovelnose sturgeon *Scaphirhynchus platorynchus* Rafinesque; and the

Alabama sturgeon, *Scaphirhynchus suttkusi* Williams & Clemmer. Perhaps because the blood and heart are seldom examined during routine parasitological examinations of fishes, only two sturgeons were previously recognized as hosts for blood flukes. Appy & Dadswell (1978; see Bullard et al., 2008) reported an innominate species of *Acipensericola* (as *Spirorchis* sp.) from the mesenteric blood vessels of a shortnose sturgeon, and Robin M. Overstreet (Gulf Coast Research Laboratory, Ocean Springs, Mississippi, USA) gave SAB two specimens of a species of *Acipensericola* that infected the heart of a Gulf sturgeon captured from Tampa Bay in 1977. Unfortunately, all of these specimens were lost, presumably destroyed, during Hurricane Katrina in 2005. As a result, aside from the materials described herein, we know of no extant specimen of a blood fluke that infects a sturgeon. Because several sturgeon species in North America are infected (personal observations, SAB), we predict that sturgeons in Europe and Asia also are infected by innominate fish blood flukes, likely of *Acipensericola* or another (other) closely related genus (genera).

Currently, nearly all sturgeon species have some degree of conservation status and are regulated (Birstein et al., 1997; Warren et al., 1997). Because of this, the logistics of lethally sampling them for blood fluke infections are challenging (cumbersome regarding scientific collection permits) or impossible (illegal). This is unfortunate from a parasitological perspective because the lineage of fishes comprising Acipenseriformes (including also the paddlefishes, Polyodontidae) represents ‘‘living fossils’’ with which to explore the evolutionary origins of particular parasite groups, parasite-host cophyly, and biogeography. Perhaps the most accessible and abundant sturgeon in North America, the lake sturgeon is relatively unique among North American sturgeons as it is the only wholly freshwater species of *Acipenser* there (Choudhury & Dick, 1993, 2001; Dick et al., 2006; Page & Burr, 2011) and that includes limited recreational

and commercial fisheries. Myriad parasitological surveys of lake sturgeon exist (Linton, 1889, 1901; MacCallum, 1895; Stafford, 1904; Wilson, 1916; Pearse, 1924; Hopkins, 1934; Bangham & Hunter, 1939; Schneberger & Woodbury, 1944; Bangham, 1955; Choudhury & Dick, 1991, 1992, 1993, 1994, 1996a, b, 1998a, b, c; Choudhury et al., 1990, 1996; Hoffman, 1970; Dechtiar, 1972a, b); however, none has detected a blood fluke infection. Hoffman and Dechtiar both worked on and reported blood fluke infections in other fishes (Hoffman & Putz, 1966; Hoffman et al., 1985; Dechtiar, 1972a, b; Dechtiar & Nepszy, 1988). Hence, we doubt that these infections were being missed but rather attribute the lack of reporting to the rarity of blood fluke infections in lake sturgeon populations.

Rarely are parasitologists granted legal permissions to sample sturgeons for endoparasites. Since 1941, the Wisconsin Department of Natural Resources (WDNR) has managed a limited take spearfishery for lake sturgeon in the Lake Winnebago System. The fishery is sustainable, i.e. comprising the largest and most intensively managed stock of lake sturgeon (Bruch, 1998, 1999), culturally integral to local communities, and offers opportunities for biological data to be collected from harvested lake sturgeons. During February 2001, 2008, 2014, and 2017, we sampled hearts of lake sturgeon harvested during this event in collaboration with WDNR personnel. Herein, we describe a new species of *Acipensericola* and compare it with the only known congener, *Acipensericola petersoni* Bullard, Snyder, Jensen & Overstreet, 2008, which infects the American paddlefish, *Polyodon spathula* (Walbaum) (Acipenseriformes: Polyodontidae) in the Mississippi and Tennessee rivers (Mississippi River Basin). The new species is the first nominal species of the Aporocotylidae reported from any sturgeon and only the second named species of *Acipensericola*.

Materials and Methods

Lake sturgeon from both Lake Winnebago and the ‘‘Upriver Lakes’’ (Lakes Butte des Morts, Win- neconne and Poygan) were harvested during the annual sturgeon spear fishery that occurs each February. Lake sturgeon hearts were collected at registration stations during the 2001, 2008, 2014, and 2017 seasons. The heart of each dead sturgeon was excised intact, placed in sample bag, bisected to expose its lumen, stored in a portable shanty to prevent freezing, and examined with the aid of a stereo-dissecting microscope and fiber optic light source immediately or after several hours. The fluid and sediment from each bag was settled, rinsed, and decanted several times before finally examining it with the same microscope and light. Flukes intended as whole-mounts and for molecular biology were placed in vials of 5% neutral buffered formalin and stored at room temperature or placed in 95% ethanol and stored at -20°C until DNA was extracted, respectively. Specimens for morphology were rinsed 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 a Leica DM 2500 microscope with differential interference contrast (DIC) optical components and a drawing tube, and measured using an ocular micrometer. Measurements are reported in micrometres (μm) as the range followed by the mean ± standard deviation and number of specimens measured (n) in parentheses. Data for ventrolateral transverse spine rows, ventral sensory papillae, and anterior sucker spines (large and small) in the large adult specimens are presented as repeated measures, i.e. the n value is the total number of structures measured rather than the total number of specimens measured. Scientific names including taxonomic authorities and dates for fishes follow Eschmeyer et al. (2016). Morphological terms and nomenclature for flukes follow Bullard et al. (2008, 2009). Type- and voucher- materials are deposited in the United States National

Museum (USNM, Washington, D.C., USA). We regard an adult blood fluke as a specimen that has discernable gonads, terminal genitalia, or an egg; whereas, a specimen having none of those features is a schistosomulum or juvenile (e.g. Bullard & Overstreet, 2004, 2008; Bullard, 2014; Orélis-Ribeiro & Bullard, 2015).

Four specimens of the new species and one specimen of *A. petersoni* were processed for molecular biology [large sub-unit ribosomal DNA (28S), small sub-unit ribosomal DNA (18S), and internal transcribed spacer 2 (ITS2)]. Total genomic DNA (gDNA) was extracted using DNeasyTM Blood and Tissue Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol except that the incubation period with proteinase-K was extended to overnight and that the final elution step was performed using only 100 µl of elution buffer to increase the final DNA concentration. Amplification and sequencing used the set of primers described in Orélis-Ribeiro et al. (2017). PCR amplifications used a total volume of 50 µl with 2 µl of DNA template, 0.4 µM of each primer along with 19 buffer, 2.5 mM MgCl2, 1 mM dNTP mixture and 0.3 µl Taq polymerase (5 U/µl) (Promega, Madison, Wisconsin, USA). The thermocycling profile was an initial 5 min at 95°C for denaturation, followed by 40 repeating cycles of 94°C for 30 s for denaturation, 50°C for 30 s for annealing, and 72°C for 2 min for extension, followed by a final extension for 5 min at 72°C. All PCR reactions were carried out in a MJ Research PTC-200 (BioRad, Hercules, California, USA). PCR products (10 µl) were verified on a 1% agarose gel and stained with ethidium bromide. PCR products were purified by microcentrifuge with the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA) according to the manufacturer's protocol, except that the last elution step was performed with autoclaved nanopure H2O rather than the provided buffer. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Reactions were sequenced using BigDye

terminator version 3.1, cleaned-up with magnetic beads (CleanSeq dye terminator removal kit), and analyzed using ABI 3730 XL or 3730 Genetic Analyzer. Sequence assembly and analysis of chromatograms were performed with BioNumerics version 7.0 (Applied Maths, Saint- Martens-Latem, Belgium). All nucleotide sequence data were deposited in GenBank: *A. glacialis* n. sp. (28S: MF186849–MF186852; 18S: MF186853; ITS2: MF186854–MF186857); *A. petersoni* (28S: KY2438 79; 18S: KY243874; ITS2: KY243884).

Family Aporocotylidae Odhner, 1912

Genus *Acipensericola* Bullard, Snyder, Jensen & Overstreet, 2008

Acipensericola glacialis Warren & Bullard n. sp.

Type-host: *Acipenser fulvescens* (Rafinesque), (Acipenseriformes: Acipenseridae), lake sturgeon.

Type-locality: Lake Winnebago (43°530 35.1900 N, 88°28003.8100W), Wisconsin, USA.

Other locality: Lake Butte des Morts (44°060 41.9300 N, 88°43016.8400W), Wisconsin, USA.

Type-material: Holotype (USNM 1422361), paratypes (USNM 1422362–1422367).

Site in host: Heart lumen.

Prevalence and intensity of infection: Estimated based on adult parasites. Approximately 50 lake sturgeon were sampled annually during 2001, 2008, 2014, and 2017. In the 2001 sample, 2 of 50 (estimated prevalence of 4%) lake sturgeon had 1 specimen of *A. glacialis* n. sp. each (estimated mean intensity of 1). In the 2008 sample, 4 of 50 lake sturgeon (8%) had a total of 30 specimens (mean intensity of 7.5). In the 2014 sample, none of 50 lake sturgeon was infected. In the 2017 sample, 5 of 50 lake sturgeon (10%) had 1, 1, 1, 1 and 2 specimens each (mean intensity of 1.2).

Representative DNA sequences: GenBank Nos. MF18 6849–MF186852 (28S); MF186853 (18S); MF1868 54–MF186857 (ITS2).

Comparative material examined: Paratypes of *Acipensericola petersoni* (Bullard's collection).

ZooBank registration: To comply with the regulations set out in article 8.5 of the amended 2012 version of the International Code of Zoological Nomenclature (ICZN, 2012), details of the new species have been submitted to ZooBank. The Life Science Identifier (LSID) for *Acipensericola glacialis* n. sp. is urn: lsid: zoobank.org:act: 4719F6C5-5478-483E- 84B2-E971761BEBBB.

Etymology: The Latin specific epithet *glacialis* (glacier) refers to the hypothesized re-colonization of the Great Lakes Basin by lake sturgeon from the Mississippi Refugium approximately 18,000 years ago, subsequent to the Wisconsin glaciation.

Description (Figs. 1–3)

Large adults [Based on 2 whole-mounted specimens; USNM coll. nos. 1422361 and 1422362.] Body flat, ventrally concave, anterior and posterior ends tapering equally, 2,109 and 4,062 long, 545 and 1,145 wide, 3.59 and 3.99 longer than wide (Fig. 1C); dorsal body surface with honeycomb-like features; ventral body surface smooth. Body spines spike-like, arranged in ventrolateral transverse rows, 7–14 (9 ± 2, n = 20) long; proximal end broadly rounded; distal end with sharp tip protruding slightly from tegument (Fig. 3G). Spine rows 5–16 (10 ± 4; n = 20) in breadth (measured as a perpendicular to long axis of body), numbering 158 and 182 per side of body, indistinct in posterior region of some specimens, not contiguous posteriorly (Figs. 1C, 3G). Rosethorn spines absent (Fig. 1C). Ventrolateral nerve cord becoming confluent with paired cord 55 and 120 or 3% of body length from posterior body end; ventrolateral nerve commissure 250 and 310 or 8% and 12% of body length from anterior body end, 55 and 100 in breadth, 30 (2) in diameter, perpendicular to midline of body (Fig. 1C). Ventral sensory papillae 2–11 (6.5 ± 2.8; n = 20) in diameter, appearing conical under light microscopy, between ventrolateral nerve cord and lateral body margin, less dense medially. Anterior sucker bowl-shaped, centered on

mouth, demarcated from anterior body end by peduncle, spinous on inner anteroventral surface only, 103 and 155 in diameter or 14% and 19% of body width (Figs. 1C, 3C). Anterior sucker spines conical, directed posteriorly, clustered, not occurring in clearly delineated rows, comprising large and small spines; large spines 28 and 30 in number, approximately 5–10 (7.6 ± 1.4; n = 20) long, 1–3 (1.5 ± 0.6; n = 20) wide; small spines 16 (1) in number, 1 (4) long, seemingly at the limits of light microscopy, < 1 wide (Fig. 3C). Pharynx occupying space between anterior sucker and nerve commissure, highly muscular, 115 and 185 long or 41% and 45% of oesophagus length, 80 and 133 wide or 39 and 89 oesophagus width, with muscular wall 35 and 45 thick (Figs. 1C, 3C). Oesophagus medial, straight, ventral to anterior

nerve commissure, extending posteriad approximately 280 and 415 long or 11% and 16% of body length, 10 and 45 wide, with wall 5 and 25 thick immediately posterior to pharynx; posterior oesophageal swelling immediately posterior to pharynx, 25 and 145 wide or 59 and 69 width of anterior oesophagus, 85 and 200 long or 30% and 48% of oesophagus length, with wall 10 and 20 thick. Intestine inverse U-shaped (Fig. 1C), with long posterior caeca only and no anterior caeca, dendritic, with lateral diverticula, extending to near posterior end of body, bifurcating immediately posterior to oesophageal swelling, 500 and 730 long or 18% and 24% of body length from anterior body end, extending posteriad in parallel 1,351–3,020 (2,153) or 67% and 74% of body length, ending 215–640 (393) or 10% and 11% of body length from posterior body end, 40–105 (73) wide, not extending laterally beyond ventrolateral nerve cord, containing granular material within lumen in some individuals; granular material blackish, probably comprising hematin (perhaps from digested erythrocytes).

Testes intercaecal, non-contiguous, an anterior testicular column plus 1 testis posteriorly, highly dendritic, 6 in number, comprising 5 testes (t1–t5) orienting in a single testicular column

plus 1 separate testis (t6) posteriorly, each approximately equal in diameter; anterior testicular column (t1–t5) 560 and 1,040 long or 26% and 27% of body length; anterior- most testis in column 455 and 1,070 long or 22% and 26% of body length from caecal bifurcation, 98 and 280 wide or 18% and 24% of body width at widest level; posterior-most testis at level of distal ends of caeca, 225 and 460 long or 11% of body length, 135 and 300 wide or 25% and 26% of body width, 255 and 546 or 12% and 13% of body length from posterior margin of anterior testicular column; post-testicular space 138 and 210 or 5% and 7% of body length (Fig. 1C). Vasa efferentia comprising myriad fine ducts entwining throughout testes, with anterior and posterior trunks linking t1–t5 and posterior-most testis (Fig. 2C); anterior trunk of vasa efferentia extending posteriad 88 and 190 or 4% and 5% of body length from posterior margin of t5 before uniting with posterior trunk of vasa efferentia, 13 (2) wide (Fig. 2C); posterior trunk of vasa efferentia extending anteriad 155 and 355 or 7% and 9% of body length from anterior margin of t6, uniting with anterior trunk of vasa efferentia and forming vas deferens, 10 and 13 wide; vas deferens 83 and 250 long, 8 and 15 wide, extending dorso-laterally, ventral to ovary, joining with cirrus-sac and seminal vesicle in dextral half of body (Figs. 2C, 3D). Cirrus-sac thin-walled, enclosing seminal vesicle; seminal vesicle ovoid, occupying space between t5 and t6, 53 and 108 long, 33 and 98 wide or 1.19 and 1.69 longer than wide, orienting toward dextral body margin and posterior body end; extruded cirrus 60 and 70 long, 35 and 50 wide, directing posteriad (Fig. 2C), appendix-like, lacking spines; post-cirrus-sac space 455 and 850 or 21% and 22% of body length. Common genital pore medial to dextral intestinal cecum.

Ovary medial, transverse, inter-caecal, immediately posterior to t5, 115 and 140 long or 3% and 5% of body length, 138 and 460 wide or 25% and 40% of body width, 1.29 and 3.39 wider than long, dorsal to vas deferens and seminal vesicle; post-ovarian space 440 and 1,020

long or 21% and 25% of body length (Figs. 1C, 2C). Oviduct sinistral, functioning as oviducal seminal receptacle, extending posteriad from posterior margin of ovary approximately at level of, or immediately posterior to, junction of anterior and posterior branches of vasa efferentia, looping once or twice (one loop is posterior to oötype) before joining with oötype at midline, 13 and 60 in maximum width (Figs. 2C, 3D). Laurer's canal not visible in stained, whole-mounted specimens. Primary vitelline collecting duct sinistral, ventral to gonads, extending posteriad and coursing between or slightly ventral to testicular column and sinistral cecum, extending 93 and 350 posteriad from level of the posterior margin of t5, uniting with oviduct dorsally immediately before joining oötype (Figs. 2C, 3D). Oötype spherical, inter-caecal, inter-testicular, post-ovarian, medial to and at level of cirrus-sac, 20 and 63 in diameter; Mehlis' gland distinct, appearing extensively lobed surrounding oötype (Figs. 2C, 3D). Uterus near level of ovary, inter-caecal, extending directly anteriad from oötype along midline, passing ventral to ovary, extensively convoluted and extending sinuously dextrad between ovary and t5, extending posteriad ventral to dextral side of ovary and dextral to cirrus-sac before curving mediad to connecting with common genital pore, 13 and 38 in maximum width (Figs. 2C, 3D). Uterine eggs not present in mounted specimens. Excretory vesicle oblong, 78 and 145 long, 3 and 18 wide; excretory pore subterminal, dorsal, 43 and 55 or 1% and 2% of body length from posterior body end (Fig. 1C).

Small adults [Based on 20 whole-mounted specimens; USNM coll. nos. 1422363–1422367.] With features of large adult specimens (see above) except the following. Body 920–2,073 (1,706 ± 280; n = 18) long, 273–570 (430 ± 74; n = 18) wide, 2.1–5.3 (4 ± 0.7; n = 18) 9 longer than wide (Fig. 1A, B); body spines 10–13 (11) long (Fig. 3E, F); spine rows 8–13 (10) in breadth, numbering 132–153 (143) per side of body. Ventrolateral nerve cord confluence 25–135

(59±25; n=18) or 2–7% (3±1%; n=18) of body length from posterior body end (Figs. 1, 2); ventrolateral nerve commissure 110–245 (183 ± 44; n = 18)or8–13%(11 ± 2%;n = 18) of body length from anterior body end, 40–78 (57 ± 11; n = 18) in breadth, 10–30 (16 ± 5; n = 18) in diameter (Fig. 1A, B). Ventral sensory papillae 3–9 (5) in diameter. Anterior sucker 45–103 (73 ± 19; n = 18) in diameter or 10–23% (17 ± 3%; n = 18) of body width (Fig. 3A, B); large spines of anterior sucker 16–37 (24) in number, approximately 5–12 (8.3) long, 1–3 (2.5) wide; small spines of anterior sucker 4–28 (15) in number, approximately \ 1 long and \ 1 wide among 10 spines measured. Pharynx 60–125 (102 ± 18; n=18) long or 32–54% (43±6%; n=18) of oesophagus length, 55–95 (74 ± 11; n = 18) wide or 2.8–16 (7 ± 3; n = 18) 9 oesophagus width, with muscular wall 10–33 (26 ± 6; n = 18) thick (Figs. 1A, B, 3A, B). Oesophagus 125–336 (241±46; n=18) long or 12–19% (14±2%; n = 18) of body length,5–23(12 ± 5;n = 18)wide, with wall 3–20 (9 ± 5; n = 18) thick at level immediately posterior to pharynx; posterior oesophageal swelling 25–73 (46 ± 14; n = 18) wide or 2.2–18 (7 ± 5; n = 18)9 width of anterior oesophagus, 53–105 (88 ± 14; n = 18) long or 26–42% (37 ± 4%; n = 18) of oesophagus length, with wall 5–18 (12 ± 3; n = 18) thick. Intestine 193–500 (383±73;n=18) or 19–25%(22±2%;n=18) of body length from anterior body end, extending posteriad in parallel 680–1,420 (1,154 ± 183; n = 18) or 64–74% (68 ± 3%; n = 18) of body length, ending 108–250 (183 ± 44; n = 18) or 6–16% (11 ± 2%; n = 18) of body length from posterior body end, 20–48 (31 ± 7;n = 18)wide(Fig. 1A,B).

Anterior testicular column 270–640 (516 ± 84; n = 18) long or 19–38% (30 ± 3%, n = 18) of body length; anterior-most testis in column 193–418 (276±60; n=18) long or 12–24% (16±3%; n = 18) of body length from caecal bifurcation, 25–135 (63 ± 28; n = 18) wide or 6–26% (14 ± 5%; n = 18) of body width at widest level; posterior-most testis 118–225 (160 ± 26; n = 18) long or 8–13% (9 ± 1%; n = 18) of body length, 48–125 (73±19; n=18) wide or 11–24%

(17±3%; n = 18) of body width, 300–980 (610) or 30–56 (37)% of body length from posterior margin of anterior testicular column; post-testicular space 75–450(160±80;n=18)or6–34% (10±6%; n = 18) of body length (Fig. 1A, B). Anterior trunk of vasa efferentia 53–108 (83 ± 18; n = 13) or 3–6 (5)% of body length from posterior margin of t5 before uniting with posterior trunk of vasa efferentia, 4–16 (10 ± 3; n = 18) wide (Fig. 2A, B); posterior trunk of vasa efferentia 78–195 (132 ± 35; n = 18) or 4–12% (8 ± 2%; n = 18) of body length from anterior margin of t6, 5–14 (9 ± 2; n = 18) wide; vas deferens 25–85 (55 ± 16; n = 18) long, 5–14 (9 ± 3; n = 18) wide (Fig. 2A, B). Seminal vesicle 25–105 (47 ± 18; n = 18) long, 18–50 (28 ± 8; n = 18) wide or 1–2.8 (1.7 ± 0.5; n = 18) 9 longer than wide; extruded cirrus 15–40 (26 ± 8; n = 18) long, 8–33 (17 ± 7; n = 18) wide; post-cirrus-sac space 158–500 (362 ± 94; n = 18) or 11–26% (21 ± 4%; n = 18) of body length.

Ovary 53–165 (86 ± 28; n = 18) long or 3–10% (5 ± 2%; n = 18) of body length, 40–175 (80 ± 35; n=18) wide or 12–40% (18±7%;n=18) of body width, 0.5–2.2 (1 ± 0.4; n = 18) 9 wider than long; post-ovarian space 170–515 (377 ± 94; n = 18) long or 12–28% (22 ± 4%; n = 18) of body length (Fig. 1A, B). Oviduct 8–25 (16 ± 4; n = 18) in maximum width (Fig. 2A, B). Primary vitelline collecting duct extending posteriad 148–225 (184) (Fig. 2A, B). Oötype 15–35 (24 ± 5; n = 18) in diameter. Uterus considerably less convoluted than in large adult specimens, 8–23 (14 ± 4; n = 18) in maximum width (Fig. 2A, B). Uterine eggs not observed. Excretory vesicle 45–100 (77) long, 3–10 (5) wide; excretory pore 3–35 (21) or approximately 1% of body length from posterior body end (Fig. 1A, B).

Molecular results

Four specimens (1 large adult and 3 small adults) of *A. glacialis* n. sp. were processed for molecular biology. PCR and sequencing of the 28S, 18S, and ITS2 (4, 1, and 4 resulting

sequences, respectively) resulted in an alignment of 1,621, 1,841 and 442 nucleotides, respectively. We detected no intraspecific variability among these sequences, i.e. the four 28S and four ITS2 sequences of the new species were identical, respectively. A comparison of these sequences to those derived from a specimen of *A. petersoni* (ex heart of American paddlefish from the Tennessee River) revealed 13 (99.2% similarity in the 28S), 8 (99.9%, 18S), and 11 (97.5%, ITS2) nucleotide differences. Two of the 11 differences in the ITS2 sequences comprised base pair insertions at alignment positions 107–108 in *A. petersoni*.

Discussion

Specimens of the new species were assigned to *Acipensericola* based on the presence of spike-like tegumental body spines arranged in ventrolateral transverse rows, a large bowl-shaped anterior sucker, inverse U-shaped caeca, a column of inter-caecal testes, and an inter-testicular ovary. Based on the results of the present study, we herein emend the generic diagnosis of *Acipensericola* (see Bullard et al., 2008): anterior sucker having large spines on inner ventrolateral surface plus small spines anterior to mouth; intestine smooth or dendritic (with or without lateral diverticula); testes smooth or dendritic. The only nominal congener, *A. petersoni*, infects American paddlefish. The new species differs from *A. petersoni* by having a dendritic intestine, deeply-lobed testes, a post-ovarian oötype, and a common genital pore that is medial to the dextral caecum. *Acipensericola petersoni* has a non-dendritic intestine, testes that are not deeply lobed, an oötype that is at level of the ovary (ventral to the ovary), and a common genital pore that is dorsal to the dextral caecum. Further, large adult specimens of the new species differed from comparable-sized paratypes and vouchers of *A. petersoni* by having [150 (vs < 140 in *A. petersoni*) tegumental spine rows per lateral body margin, a short testicular column that is 1/4 (vs 1/3–1/2) of the body length and that terminates anteriad approximately 1/4 (vs 1/5) of the

body length from the caecal bifurcation, a short ovary that is 1/20 (vs 1/10) of the body length, and a distal portion of the uterus that is ventral (vs lateral) to the ovary.

The life-cycle of *A. glacialis* n. sp. intrigues us given the size range of the flukes we observed herein (see also Bullard & Overstreet, 2004, 2008; Bullard, 2014; Orélis-Ribeiro & Bullard, 2015; Palacios-Abella et al., 2017), the low prevalence of infection in heart (< 6% mean prevalence over four years of sampling), the small number of large adult specimens observed in heart, and the strong spawning site fidelity and spawning seasonality of lake sturgeon (lake sturgeon move into shallow, riverine habitats to spawn during spring; see Bruch & Binkowski, 2002). Perhaps cercarial shedding is geographically widespread in the lake system and occurs throughout the year; which would result in a wide range of different-aged (= different-sized) fluke specimens infecting lake sturgeon. Alternatively, if cercarial shedding occurs during spring and within lake sturgeon spawning sites, then the presence of an apparent continuum of small to large fluke specimens infecting lake sturgeon may suggest that conspecific flukes experience different growth rates. The notion that the heart is not the preferred site of infection for *A. glacialis* n. sp. is supported by the fact that most specimens of *A. glacialis* n. sp. in heart were small. Perhaps larger specimens of *A. glacialis* n. sp. more frequently infect other tissue sites, e.g. mesenteric blood vessels (Appy & Dadswell, 1978). Noteworthy also is that the intensity and prevalence of infection by *A. glacialis* n. sp. may differ between male and female lake sturgeon because only male lake sturgeon make successive spawning runs (unpublished data RPK; Bruch, 1999; Bruch & Binkowski, 2002; Bruch et al., 2001) and because males and females typically having a 2 year and 3–5 years spawning periodicity, respectively. Also possible is that adults of *A. glacialis* n. sp. are long-lived (surviving in the fish host for 12 months) such that an infected lake sturgeon can harbor different year classes of *A. glacialis* n. sp. Screening snails in Lake

Winnebago for aporocotylid infections, examinations of extra-cardiac sites in lake sturgeon, and histopathology of gill, mesenteric blood vessels, and intestine of lake sturgeon would be helpful in exploring these various aspects of the life cycle of *A. glacialis* n. sp.

The blood flukes infecting basal actinopterygians, i.e. *Acipensericola* spp. infecting sturgeons and paddlefish, and those infecting some turtles are morphologically similar, which may indicate that they share a recent, common ancestor. Species of *Acipensericola* resemble those of *Spirorchis* MacCallum, 1918 by having a spinous anterior sucker, a pharynx, inverse U-shaped caeca nearly reaching the posterior body end, a pre-ovarian and inter-caecal testicular column, a Laurer's canal, and a common genital pore as well as by lacking a ventral sucker (Bullard et al., 2008; Roberts et al., 2016). Yet, considerable morphological differences separate these genera: species of *Acipensericola* differ from those of *Spirorchis* by the combination of having an anterior sucker that is demarcated from the anterior body end by a peduncle, ventrolateral tegumental body spines, a smooth oesophagus (lacking diverticula), a post-ovarian testis, a sinistral vitelline collection duct, a massive and convoluted uterus, multiple uterine eggs, and a dorsal common genital pore that is dextral as well as by lacking a median oesophageal diverticula and metraterm. Species of *Spirorchis* have an anterior sucker that is not demarcated from the anterior body end by a peduncle, an aspinous tegument (lacking ventrolateral tegumental body spines), an oesophagus with myriad characteristic and highly distinctive diverticula, a median oesophageal diverticulum (absent in *Spirorchis elegans* Stunkard, 1923; see Platt, 1993; Roberts et al., 2016), a transverse vitelline collecting duct that is ventral to the caeca and genitalia, a single uterine egg, an obvious metraterm, and a dorsal common genital pore that is sinistral as well as lacks a post-ovarian testis and uterine convolutions. The turtle blood flukes of *Spirhapalum* Ejsmont, 1927 and *Plasmiorchis* Mehra, 1939 also have U-shaped

caeca that extend to near the posterior body end and a column of testes that are pre-ovarian and inter-caecal. Species of *Acipensericola* further resemble those of *Spirhapalum* by having a post-ovarian testis (Ejsmont, 1927; Tkach et al., 2009). *Spirhapalum* and *Plasmiorchis*, however, are easily differentiated from *Acipensericola* by having a ventral sucker (Mehra, 1934, 1940; Sinha, 1934; Mehrotra, 1973; Gupta & Mehrotra, 1975). Forthcoming molecular phylogenetic analyses will help further test relationships between *Acipensericola* spp. and the morphologically-similar lineages of turtle blood flukes; perhaps recasting the notion of aporocotylid monophyly as well as the concept that the nominal blood fluke families comprise blood fluke species that, collectively and rather conveniently, are regarded as infecting fishes or turtles but not both.

Based on molecular and biogeographic evidence, Birstein & DeSalle (1998) suggested that the lake sturgeon lineage originated approximately 90–80 million years ago (Upper Cretaceous Period) but that the lake sturgeon came to occupy its present geo- graphic range in the Great Lakes Basin relatively recently, *18,000 years ago, following the Wisconsin glaciation. These authors and others (Guénette et al., 1993; Ferguson & Duckworth, 1997) speculated that the Mississippi Refugium (Mississippi River Basin) was the origin of lake sturgeon. If so, this is interesting in light of the morphological and molecular similarity between the new species and its only nominal congener (*A. petersoni*), which infects an acipenseriform (paddlefish) that has a present-day distribution limited to the Mississippi River Basin (Bullard et al., 2008). Perhaps the high degree of morphological and molecular similarity between *A. petersoni* and *A. glacialis* n. sp. can be explained by this relatively recent separation of their definitive hosts. Of interest also will be to ultimately test the phylogenetic interrelationships of sturgeon and paddlefish aporocotylids in light of recent biogeographic and phylogenetic hypotheses for acipenseriforms. For example, do phylogenetically-related *Acipensericola* spp. infect phylogenetically-related

sturgeon lineages/genera or do phylogenetically-unrelated but geographically-overlapping sturgeons share blood flukes?

Acknowledgements

We thank Scott Snyder (University of Nebraska at Omaha, Omaha, Nebraska, USA), Matthew Womble (National Oceanic and Atmospheric Administration, Washington D. C., USA), and personnel of the Wisconsin Department of Natural Resources for helping collect lake sturgeon blood flukes. Funding This study was supported financially by and is a contribution of the Southeastern Cooperative Fish Parasite and Disease Laboratory. Initial funding was provided by several grants from the National Science Foundation's Division of Environmental Biology (NSF-DEB 1112729, 1051106, 1048523) to SAB.

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FIGURE LEGENDS

Fig. 1 *Acipensericola glacialis* n. sp. ex heart of *Acipenser fulvescens*. Ventral view. A, Small adult specimen (paratype, USNM 1422364). B, Small adult specimen (paratype, USNM 1422365). C, Large adult specimen (holotype, USNM 1422361). *Abbreviations*: as, anterior sucker; ph, pharynx; nc, nerve commissure; es, oesophagus; cb, caecal bifurcation; t1–t6, testes 1–6; o, ovary; ep, excretory pore

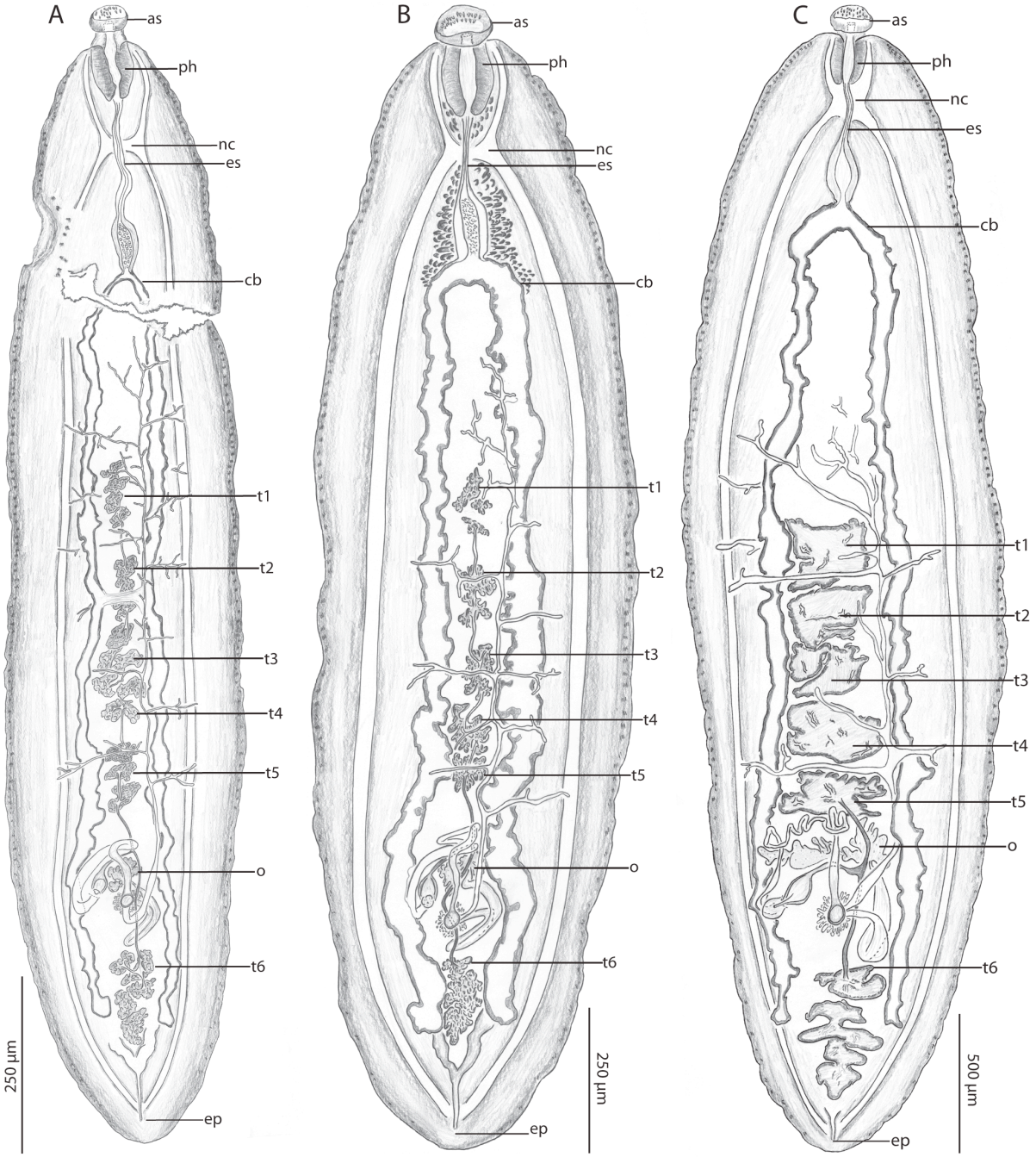
Fig. 2 *Acipensericola glacialis* n. sp. ex heart of *Acipenser fulvescens*. Ventral view. A, Small adult specimen (paratype, USNM 1422364). B, Small adult specimen (paratype, USNM 1422365). C, Large adult specimen (holotype, USNM 1422361). *Abbreviations*: t5, posterior-most testis of testicular column; ave, anterior trunk of vasa efferentia; u, uterus; vd, vas deferens; o, ovary; vs, seminal vesicle; ic, inverted cirrus; vt, primary vitelline collecting duct; oo, ootype; pve, posterior trunk of vasa efferentia; ov, oviduct with sperm and serving as oviducal seminal receptacle; t6, posterior-most testis

Fig. 3 *Acipensericola glacialis* n. sp. ex heart of *Acipenser fulvescens*. Ventral view. A–C, Anterior sucker. A, Small adult specimen (paratype, USNM 1422364). B, Small adult specimen (paratype USNM 1422365). C, Large adult specimen (holotype, USNM 1422361). D, Genitalia about ootype of large adult specimen (holotype, USNM 1422361). E–G, Ventrolateral transverse spine rows. E, Large adult specimen (holotype, USNM 1422361). F, Small adult specimen (paratype, USNM 1422364). G, Small adult specimen (paratype, USNM 1422365). *Abbreviations*: as, anterior sucker; ls, large spines; mo, mouth; ph, pharynx; vd, vas deferens; u, proximal portion of uterus; sv, seminal vesicle; vt, vitelline duct; ov, oviduct; oo, ootype; mg, Mehlis' gland

Table 1. Nominal species of fish blood flukes (Digenea: Aporocotylidae) infecting primary division North American freshwater fishes.

Parasite	Type host	Site	Type locality	Reference
Acipensericola glacialis n. sp.	lake sturgeon, *Acipenser fulvescens* (Rafinesque, 1817) (Acipenseriformes: Acipenseridae)	heart lumen	Lake Winnebago and Lake Butte des Morte, Wisconsin	present study
Acipensericola petersoni Bullard, Snyder, Jensen, and Overstreet, 2008	American paddlefish, *Polyodon spathula* (Walbaum, 1792) (Acipenseriformes: Polyodontidae)	heart lumen	Mississippi River and Tennessee River (Mississippi River Basin)	Bullard et al. 2008
Sanguinicola lophophora Erickson and Wallace, 1958	spottail shiner, *Notropis hudsonius* (Clinton, 1824) (Cypriniformes: Cyprinidae)	blood vessels, fins	Lake Francis Isanti Co., and Lake Johanna, Ramsey Co., Minnesota	Erickson and Wallace, 1959
Saguinicola davisi Wales, 1958	rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae)	branchial blood vessels	Mount Shasta Hatchery, Oregon	Wales, 1958
Sanguinicola klamathensis Wales, 1958	cutthroat trout, *Oncorhynchus clarkii clarkii* (Richardson, 1836) (Salmoniformes: Salmonidae)	hepatic blood vessels	Klamath Hatchery, Oregon	Wales, 1958
Sanguinicola alseae Meade and Pratt, 1965 (as *Cardicola*)	cutthroat trout, *Oncorhynchus clarkii* clarkii (Richardson, 1836) (Salmoniformes: Salmonidae)	branchial and hepatic blood vessels	Alsea River, Benton Co., Oregon	Meade and Pratt, 1965
	rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae)	branchial and hepatic blood vessels	Alsea River, Benton Co., Oregon	Meade and Pratt, 1965
Sanguinicola idahoensis Schell, 1974	rainbow trout, *Oncorhynchus mykiss* (Walbaum, 1792) (Salmoniformes: Salmonidae)	branchial blood vessels	Clearwater River, Nez Perce and Lewis Cos., Idaho	Schell, 1974
Sanguinicola fontinalis Hoffman, Fried, and Harvey,	brook trout, *Salvelinus fontinalis* (Mitchill, 1814)	branchial, renal, and cardiac blood vessels	Susquehanna River, Pennsylvania	Hoffman et al., 1985

1985	(Salmoniformes: Salmonidae)			
	longnose dace, *Rhinichthys cataractae* (Valenciennes, 1842) (Cypriniformes:Cyprinidae)	branchial, renal, and cardiac blood vessels	Susquehanna River, Pennsylvania	Hoffman et al., 1985
Sanguinicola occidentalis Van Cleave and Mueller, 1932	walleye, *Stizostedion vitreum* (Mitchill, 1818) (Perciformes: Percidae)	heart	Oneida Lake, New York	Van Cleave and Mueller, 1932
Sanguinicola huronis Fischthal, 1949	largemouth bass, *Micropterus salmoides* (Lacepede, 1802) (type host) (as Huro) (Perciformes: Centrarchidae)	mesenteric blood vessels	Lost Land Lake, Sawyer Co., Wisconsin	Fischthal, 1949
	smallmouth bass, *Micropterus dolomieu* Lacepede, 1802 (Perciformes: Centrarchidae)	mesenteric blood vessels	Lost Land Lake, Sawyer Co., Wisconsin	Fischthal, 1949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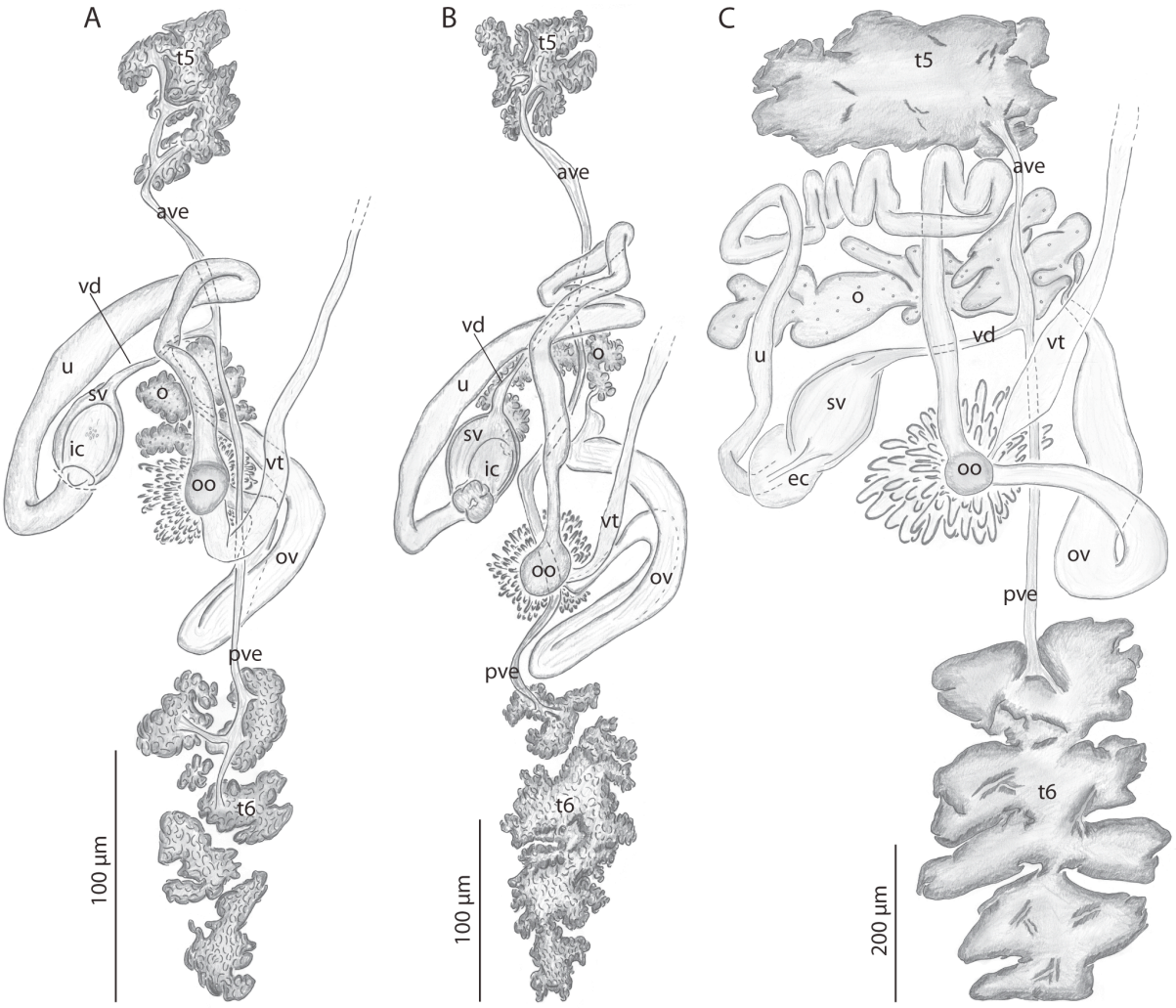
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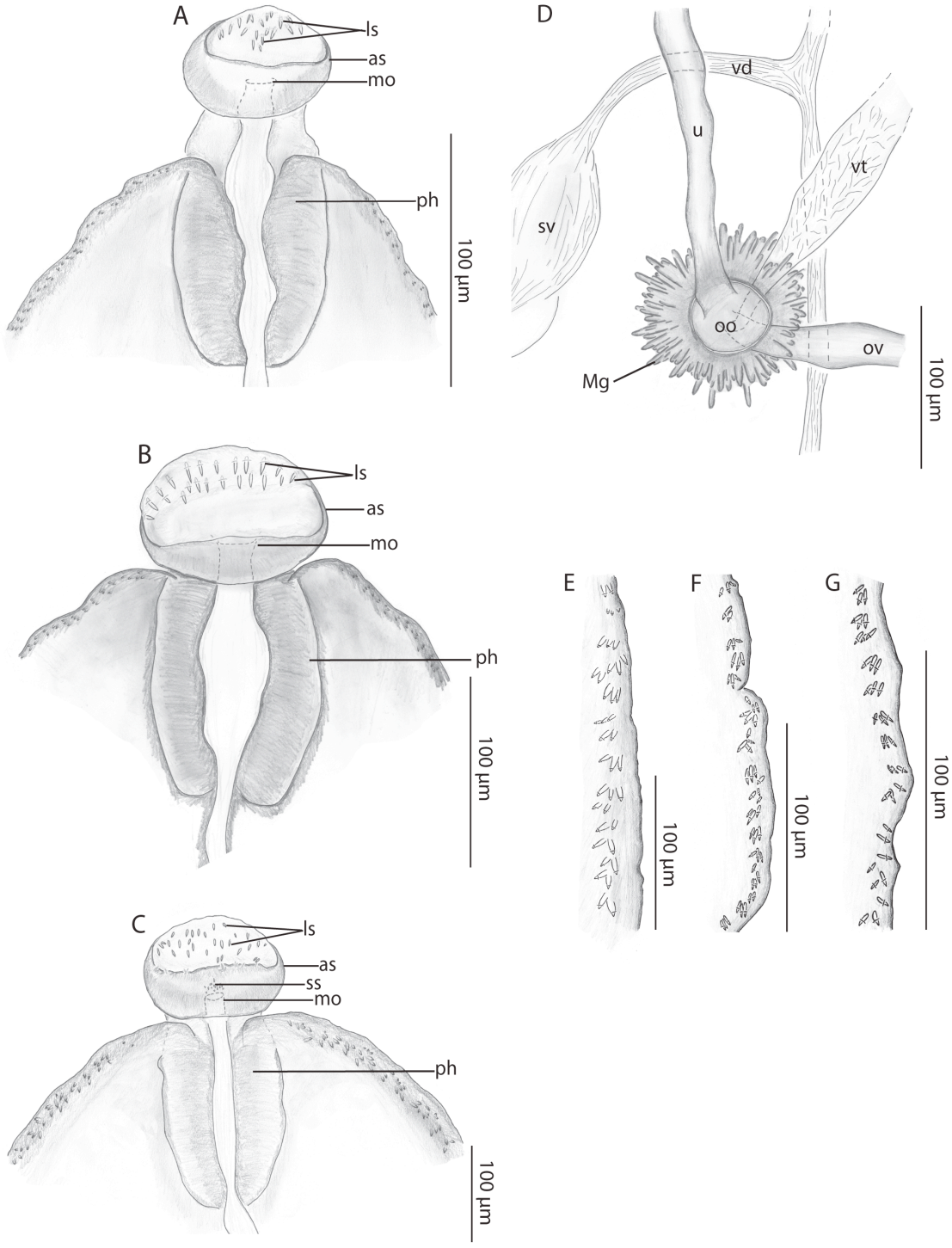
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CHAPTER 2: *GYMNURAHEMECUS BULBOSUS* GEN. ET SP. NOV. (DIGENEA: APOROCOTYLIDAE) INFECTING SMOOTH BUTTERFLY RAYS, *GYMNURA MICRURA* (MYLIOBATIFORMES: GYMNURIDAE) IN THE NORTHERN GULF OF MEXICO, WITH A TAXONOMIC KEY AND FURTHER EVIDENCE FOR MONOPHYLY OF CHONDRICHTHYAN BLOOD FLUKES

***Published in Parasitology Research (Available online 14 January 2019)**

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ABSTRACT

Gymnurahemecus bulbosus gen. et sp. nov. infects the heart of smooth butterfly rays, *Gymnura micrura* in the Gulf of Mexico. *Gymnurahemecus* differs from all other accepted aporocotylid genera by having one column of C-shaped lateral tegumental spines, a medial oesophageal bulb anterior to a diverticulate region of the oesophagus, inverse U-shaped intestinal caeca, a non-looped testis, an oviducal ampulla, a Laurer's canal, and a post-caecal common genital pore. The new species, the shark blood flukes (*Selachohemecus* spp. and *Hyperandrotrema* spp.), and the chimaera blood fluke *Chimaerohemecus trondheimensis* are unique by having C-shaped lateral tegumental spines. *Selachohemecus* spp. and the new species have a single column of lateral tegumental spines, whereas *Hyperandrotrema* spp. and *C. trondheimensis* have 2–7 columns of lateral tegumental spines. The new species differs from *Selachohemecus* spp. most notably by having an inverse U-shaped intestine. The other ray blood flukes (*Orchispirium heterovitellatum*, *Myliobaticola richardheardi*, and *Ogawaia glaucostegi*) differ from the new species by lacking lateral tegumental spines, a medial oesophageal bulb, and a Laurer's canal and by having a looped testis. Phylogenetic analysis using large subunit ribosomal DNA (28S) indicated that the new species is sister to the clade that includes the other

sequenced adult blood fluke (*O. glaucostegi*), which infects a ray in Australia. These results agree with and extend previous morphology- and nucleotide-based phylogenetic assertions that the blood flukes of early-branching jawed craniates (Chondrichthyes) are monophyletic and phylogenetically separated from the blood flukes of later-branching ray-finned fishes (Actinopterygii: Euteleostei).

INTRODUCTION

The fish blood flukes (Digenea: Aporocotylidae Odhner, 1912; see Bullard et al. [2009]) that infect sharks, skates, and rays (Chondrichthyes: Elasmobranchii) plus chimaeras (Chondrichthyes: Holocephali) presently comprise eight nominal species assigned to six genera: *Selachohemecus olsoni* Short, 1954 from the Atlantic sharpnose shark, *Rhizoprionodon terraenovae* (Richardson) (Short 1954; Bullard et al. 2006); *Selachohemecus benzi* Bullard et al., 2006 from the blacktip shark, *Carcharhinus limbatus* (Valenciennes) (Bullard et al. 2006); *Chimaerohemecus trondheimensis* van der Land, 1967 from rabbit fish, *Chimaera monstrosa* Linnaeus and spookfish, *Hydrolagus mitsukurii* Jordan and Snyder (van der Land 1967; Kamegai et al. 2002); *Orchispirium heterovitellatum* Madhavi and Rao, 1970 from the Bengal whipray, *Brevitrygon imbricata* (Bloch and Schneider) (Madhavi and Rao 1970; Bullard and Jensen 2008); *Myliobaticola richardheardi* Bullard and Jensen, 2008 from the Atlantic stingray, *Hypanus sabinus* (Lesueur) (Bullard and Jensen 2008); *Hyperandrotrema cetorhini* Malliard and Ktari, 1978 from the basking shark, *Cetorhinus maximus* (Gunnerus) (Maillard and Ktari 1978; Orélis-Ribeiro et al. 2013); *Hyperandrotrema walterboegeri* Orélis-Ribeiro et al., 2013 from shortfin mako shark, *Isurus oxyrinchus* Rafinesque (Orélis-Ribeiro et al. 2013); and *Ogawaia glaucostegi* Cutmore et al., 2018 from the giant shovelnose ray, *Glaucostegus typus* (Anonymous [Bennett]) (Cutmore et al. 2018). They collectively infect the heart, kidney, mesenteric blood

vessels, and gill epithelium of four sharks and three rays in the northwestern Atlantic Ocean (Gulf of Mexico) (Short 1954; Bullard et al. 2006; Bullard and Jensen 2008; Orélis-Ribeiro et al. 2013) and Indian Ocean (Madhavi and Rao 1970; Maillard and Ktari 1978). The single blood fluke named from chimaeras has been reported from off Norway (van der Land 1967) and Japan (Kamegai et al. 2002), but another congener may exist off Greenland (Karlsbakk et al. 2002). A new species of blood fluke infecting the heart of smalltooth sawfish, *Pristis pectinata* Latham in the eastern Gulf of Mexico off Florida, USA, is being described by us (MBW and SAB) and will comprise the ninth named blood fluke infecting a chondrichthyan. Compared to the taxonomic diversity of blood flukes reported from ray-finned fishes (Actinopterygii), those infecting early branching fish lineages, especially chondrichthyans, remain vastly underexplored.

Herein, we propose a new genus and describe a new species of blood fluke from the heart of smooth butterfly rays, *Gymnura micrura* (Bloch and Schneider), (Myliobatiformes: Gymnuridae) captured in the northern Gulf of Mexico. This is the fourth species described and fourth genus proposed for a blood fluke infecting a ray.

MATERIALS AND METHODS

A total of 74 smooth butterfly rays were captured using a 10-m otter trawl during summer 2016 (n = 54) and 2017 (n = 20) from the mouth of Mobile Bay (30°13'22.61"N, 88°3'18.57"W) (northern Gulf of Mexico). Each smooth butterfly ray was identified using the dichotomous key of McEeachran and de Carvalho (2002) and by having a tail without serrated spines and no tentacle on the posterior margin of the spiracle. Live smooth butterfly rays were removed from the trawl and placed in a flow-through seawater tank prior to necropsy. At necropsy, each smooth butterfly ray was killed by pithing before the heart and all gill arches were excised intact, placed in separate sample bags (heart was bisected; gill arches separated), exposed to 60ºC

freshwater, shaken vigorously, and preserved in 5-10% neutral buffered formalin. Gill arches, gill filaments, and corresponding sediment from the sample bags were examined using a stereo-dissecting microscope for the presence of adults and eggs of the new species. Several gill filaments from each set of gill arches were excised, wet-mounted on a glass slide, and examined with the aforementioned compound microscopes to detect blood fluke eggs in the branchial arterioles and gill epithelium (observations of eggs will be reported elsewhere). Twenty live smooth butterfly rays captured in 2017 were pithed, placed on ice, and examined for the purposes of extracting blood flukes for DNA extraction: one adult blood fluke was isolated from the heart of two smooth butterfly rays, wet-mounted on a glass slide to confirm their identity, placed directly into 95% EtOH, and stored at −20°C until DNA was extracted (see below). All bagged, formalin-fixed tissues were examined with the aid of a stereo-dissecting microscope and fiber optic light source to isolate blood flukes for morphology. The heart was teased apart with fine forceps to reveal adult blood flukes, and sediment from each sample bag was examined for blood flukes with aid of a settling column.

Specimens for morphology were rinsed 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 microscopes each equipped with differential interference contrast (DIC) optical components, measured using an ocular micrometer, and illustrated using a drawing tube. Blood fluke measurements are reported in micrometers (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. (2018). Morphological terms and nomenclature for blood flukes follows Bullard

et al. (2006; 2009), Bullard and Jensen (2008), and Orélis-Ribeiro et al. (2013). Type and voucher materials are deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.) and the Auburn University Museum of Natural History (AUMNH, Auburn, Alabama).

Using the two EtOH-preserved and microscopically-identified blood flukes, total genomic DNA (gDNA) was extracted 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 µL of elution buffer to increase the final DNA concentration. Amplification and sequencing of the large subunit ribosomal DNA (28S) used the set of primers described in Orélis-Ribeiro et al. (2017). PCR amplifications were performed according to Warren et al. (2017) with one exception: the annealing temperature was 61°C for 30 s. DNA sequencing was performed by ACGT, Incorporated (Wheeling, Illinois, USA). Sequence assembly and analysis of chromatograms were performed with Geneious version 11.0.5 (http://www.geneious.com; Kearse et al. [2012]). All nucleotide sequence data were deposited in GenBank (Table 1).

The phylogenetic analysis included two sequences of the new species plus the taxa included in Cribb et al. (2017) (Table 1). The outgroup comprised the three turtle blood fluke taxa *Hapalorhynchus gracilis* Stunkard, 1922, *Spirorchis artericola* (Ward, 1921), and *Vasotrema robustum* Stunkard, 1928. 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

verision 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).

RESULTS

Gymnurahemecus gen. nov. (Figs. 1–10)

Diagnosis: Body 6–10× longer than wide, dorsoventrally flattened, ventrally concave, having anterior and posterior ends tapering equally, spinous; lateral tegumental spines C-shaped, directed ventrally, each on a muscular peduncle, distributing in a single ventrolateral column, not continuous anteriorly nor posteriorly. Rosethorn-shaped spines lacking. Nervous system comprising paired lateral nerve cords. Anterior sucker aspinous, lacking peduncle, diminutive, occupying space between anterior-most lateral tegumental spines. Mouth on mid-ventral surface of anterior sucker. Pharynx not evident. Oesophagus extending sinuously posteriad along mid-line for 1/4–1/3 of body length, with middle portion having an oesophageal bulb; oesophageal bulb delimited anteriorly and posteriorly by marked constrictions. Intestinal caeca inverse U-shaped, connecting to oesophagus ventrally, lacking diverticulae, terminating in anterior half of body. Testis single, medial, occupying middle 1/3 of body. Auxiliary external seminal vesicle

lacking. Cirrus-sac present, enveloping internal seminal vesicle and cirrus. Ovary sinistral, post-caecal, post-testicular; post-ovarian space comprising 1/3 of body length. Oviducal ampulla present. Laurer's canal present, opening on dorsal surface. Oötype medial, posterior to genitalia, comprising an inconspicuous ovoid chamber. Uterus post-gonadal, not extensively convoluted, extending anteriad from oötype before crossing midline and extending posteriad; uterine eggs spheroid, thin-shelled. Vitellarium follicular, asymmetrical posteriorly, filling space between nerve commissure to ovary; common vitelline collecting duct extending from dextral branch of vitellarium. Common genital pore dorsal, post-gonadal, anterior to level of oötype. Excretory vesicle small, medial, visible in posterior most region of specimen body.

Differential diagnosis: Body 6–10× longer than wide; lateral tegumental spines C-shaped, distributing in 1 column. Oesophageal bulb present. Intestinal caeca inverse U-shaped, terminating in anterior half of body. Testis single. Ovary sinistral, post-testicular. Oviducal ampulla and Laurer's canal present. Oötype posterior to genitalia. Common vitelline collecting duct extending from dextral branch of vitellarium. Common genital pore post-gonadal, anterior to level of oötype.

Taxonomic summary

Type and only nominal species: Gymnurahemecus bulbosus sp. nov.

Etymology: *Gymnurahemecus* refers to the type species infecting the blood of a gymnurid.

***Gymnurahemecus bulbosus* sp. nov.**

(Figs. 1−10)

Diagnosis of adult specimens (based on 10 whole-mounted specimens and 2 SEM prepared specimens): Body 830–1130 (986 ± 101, 9) long, 88–155 (135 ± 22, 9) at greatest width, 6–10 × longer than wide (Figs. 1−3). Lateral tegumental spines 83–97 (91 ± 4, 8) per side of body or a

total of 168–193 (183 ± 7.4, 8), ending 13–25 (19 ± 4.2, 8) or 1–3% (2 ± 0.05, 8) of body length from posterior end of body, base slightly bifurcate at posterior margin, tissue not associated with base on anterior-most lateral tegumental spines (Fig. 4), approximately equal in size throughout length of body; lateral tegumental spines in anterior region 5–8 (6.2 ± 1.2, 9) long, 1–2 (1.6 ± 0.5, 9) wide; lateral tegumental spines in mid-body and posterior region 4–8 (5 ± 1.3, 9) long, 1–2 (1.2 ± 0.4, 9) wide (Fig. 5); peduncles supporting lateral tegumental spines approximately equal in size throughout length of body; peduncles in anterior region of body 5–8 (7.1 ± 1.1, 9) long, 3–5 (3.9 ± 0.8, 9) wide; peduncles in mid-body and posterior region of body 5–7 (6 ± 0.5, 9) long, 2–6 (3.4 ± 0.8, 9) wide. Ventrolateral nerve-cord 760–970 (851 ± 107, 3) long, 8–10 (9 ± 1, 3) wide near mid-body at widest level, 13–18 (15 ± 2.5, 3) from body margin. Primary commissure perpendicular to mid-line of body, connecting ventrolateral nerve-cords, 78–125 (109 ± 19, 5) or 6%–14% (11% ± 0.2, 5) of body length from anterior end of body, 20–30 (25 ± 5, 5) across width of worm, 8–10 (9.2 ± 1.1, 5) in breadth; (Figs. 1–3, 6, 7); secondary commissure and nerve cords not evident in wholemounts.

Mouth 2–3 (2.4 ± 0.5, 7) in diameter, 6–9 (7 ± 1, 7) from terminal end of anterior sucker (Figs. 1–3). Oesophagus 238–325 (288 ± 31, 7) in total length or 25%–35% (29% ± 0.03, 7) of body length, 13–30 (24 ± 4, 7) in maximum width (at level of oesophageal bulb), ventral to primary nerve-commissure, comprising several distinct segments (anterior portion, pre-oesophogeal bulb dilation, medial oesophageal bulb, diverticulate portion, and pre-caecal dilation); anterior portion a narrow duct extending directly or slightly sinuously posteriad, 105–145 (128 ± 13, 7) long or 41%–48% (44% ± 0.02, 7) of oesophagus length, 1–3 (2.5 ± 0.75, 8) wide or 1%–2% (1.8% ± 0.003, 8) of body width; medial oesophageal bulb a markedly laterally-expanded chamber occupying approximate middle portion of oesophagus, 70–98 (90 ± 11, 6)

long or 23%–33% (30% ± 4, 6) of oesophagus length, 18–30 (24 ± 4, 6) wide or 15%–20% (18% ± 2, 6) of body width, immediately following a slightly expanded portion of oesophagus, having wall 2–3 (2 .3± 0.5, 6) thick; diverticulate portion having a wall comprising diverticulae-like structures, 32–53 (39 ± 8, 6) long or 10%–18% (13% ± 0.03, 6) of oesophagus length, 8–20 (12 ± 4, 6) wide or 6%–14% (8% ± 0.02, 6) of body width, immediately following medial oesophageal bulb, having wall 2–3 (2.5 ± 0.5, 6) thick. Oesophageal gland enveloping oesophagus pre-caecal dilation, diverticulate portion, and posterior portion of oesophageal bulb, 63–135 (103 ± 23, 8) long or 21%–52% (36% ± 8, 8) of oesophagus length, 28–48 (39 ± 7, 8) wide or 26%–33% (29% ± 2, 8) of body width. Caecal bifurcation 175−360 (280 ± 69, 8) or 17%−38% (29% ± 7, 8) of body length from anterior body end; caeca extending posteriad in parallel, 127−192 (162 ± 23, 8) long or 15%−19% (16% ± 1, 8) of body length, 14–31 (25 ± 5.5, 8) wide, ventral to lateral nerve cord, containing granular material within lumen of some individuals (Fig. 1–3).

Testis 203–345 (286 ± 45, 8) long or 24%–32% (29% ± 2, 8) of body length, 18–55 (36 ± 13, 8) wide or 15%–36% (27% ± 7, 8) of body width, 6–15 (9 ± 3.4, 8) × longer than wide, intercaecal (Figs. 1–3), processes extending dorsoventrally. Post-testicular space 210–455 (349 ± 73, 8) long or 20%–41% (36% ± 7, 8) of body length. Vasa efferentia comprising interconnecting meshwork of fine ducts entwined throughout testicular tissue, 10–13 (2) in diameter, extending primarily dorsoventrally and along ventral surface of testis, coalescing in posterior region of testis; vas deferens 30–75 (46 ± 16, 6) long, 3–8 (4 ± 2, 6) wide, emanating from postero-ventral portion of testis, extending dextrad lateral to ovary before curving mediad and posteriad and extending posteriad aside ascending uterus for a short distance before becoming confluent with cirrus-sac. Cirrus-sac 103–168 (151 ± 22, 7) long or comprising 71%–

81% (77% ± 0.04, 7) length of male terminal genitalia, 15–33 (25 ± 5, 7) wide or 16%–22% (18% ± 0.02, 7) of body width, having extremely thin wall approximately 1–2 (2 ± 0.5, 8) thick, including seminal vesicle and cirrus; seminal vesicle 98–180 (150 ± 19, 7) long, 18–30 (26 ± 4.3, 7) wide, anterior half filling breadth of cirrus sac, posterior half filling 39%–60% (51% ± 0.06, 7) breadth of cirrus sac, containing sperm in 6 of 10 specimens, extending sinuously posteriad before narrowing and curving anterodorsally. Common genital pore 155–220 (184 ± 20, 8) or 17%–20% (18% ± 1, 8) of body length from posterior end of body, 28–50 (38 ± 7, 8) from sinistral body margin, 50–85 (65 ± 12, 8) from dextral body margin (Figs. 1–3, 9, 10).

Ovary sinistral to testis, slightly lobed, 25–53 (41 ± 10, 8) long or 3%–5% (4% ± 0.08, 8) of body length, 18–63 (40 ± 15, 8) wide or 20%–47% (28% ± 9, 8) of body width, 0.7–1.8 (1 ± 0.4, 8) × wider than long, immediately post-testicular, ventral to lateral nerve-cords; post-ovarian space 275–405 (344 ± 43, 7) long or 33%–36% (34% ± 4, 7) of body length (Figs. 9, 10). Oviduct (including oviducal ampulla) 153–288 (243 ± 43, 8) long, 6–9 (7 ± 1.1, 6) wide; oviducal ampulla 13–30 (21 ± 6, 6) long or 6%–12% (8% ± 2, 6) of oviduct length, 10–28 (17 ± 6.3, 6) wide. Laurer's canal 10–33 (22 ± 9, 6) long, 3–5 (5 ± 0.7, 6) wide, sinistral to oviducal seminal ampulla (Figs. 9, 10). Oötype 13–28 (18 ± 5.2, 7) long, 8–15 (13 ± 2.3, 7) wide, posterior to all other genitalia (Figs. 9, 10). Vitellarium having follicles compacted in dense lobules, occupying space dorsal and lateral to testis and caeca; common collecting duct 178–385 (281 ± 68, 7) long, 8–13 (10 ± 1.5, 7) wide. Uterus extending directly anteriad from oötype, 155–298 (251 ± 45, 8) long or 18–32 (25 ± 0.04, 8) of body length, 13–30 (22 ± 5, 8) wide, with wall 2 (8) thick; ascending portion extending sinuously anteriad and dorsal to seminal vesicle before coursing diagonally across mid-line, containing eggs in 5 of 10 specimens (Figs. 1, 9), containing sperm in 3 of 10 specimens (superficially resembling seminal vesicle), curves dextrally before

immediately curving anteriorly and sinistral to posterior margin of ovary before connecting with descending portion; descending portion 28–65 (52 ± 12, 8) long or 17%–28% (20 ± 4, 8) of ascending uterus length, 15–28 (21 ± 4, 8) wide, with wall 1 (8) thick, containing eggs in 5 of 10 specimens (Figs. 1, 9), extending posteriad before connecting with metraterm; metraterm 73–110 (92 ± 10, 8) long or 1.4–2.6 (1.8 ± 0.4, 8) × longer than the descending uterus, 15–28 (21 ± 4, 8) wide, comprising distal-most portion of female reproductive tract, demarcated from descending uterus by obvious constriction (Figs. 9, 10), with eggs in 3 of 10 specimens, with wall 2 (8) thick. Uterine eggs 18–25 (21 ± 2.8, 7) in diameter or 51%–82% (71% ± 14, 6) of uterus width, containing a large spheroid body plus several smaller, dense lipid-like bodies, with thin shell (Fig. 9). Excretory bladder small, 4–10 (7.5 ± 2.3, 7) long, 2–7 (4.2 ± 1.5, 7) wide, medial (Figs. 1–3).

Taxonomic summary

Type and only reported host: smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae).

Site in host: Heart lumen.

Type locality: Mobile Bay (30°13'22.61"N, 88° 3'18.57"W), North central Gulf of Mexico.

Prevalence and intensity of infection: Seven of 54 (prevalence = 13%) smooth butterfly rays sampled in 2016 were infected by 1, 1, 1, 1, 2, 2, and 4 specimens of *G. bulbosus* (mean intensity = 1.7). Three of 20 (15%) smooth butterfly rays sampled in 2017 were infected by 1 specimen of *G. bulbosus* each (mean intensity = 1.0).

Specimens deposited: Holotype (USNM 1530997), paratypes (USNM 1530998−1531000), GenBank Nos. (28S: MH555432 and MH555433).

Etymology: The specific epithet "*bulbosus"* refers to the distinct oesophageal bulb of the new species.

Sequence comparison and phylogenetic results

The amplified 28S fragment from the two specimens of the new species comprised 1,622 nucleotides (MH55432 and MH555433-GenBank). These were identical (100% similar) to each other; differed from the 28S sequence of *C. trondheimensis* (AY157239) by 256 nucleotides (15%); and differed from those of *O. glaucostegi* and Aporocotylidae sp. NSW1 by 371 (32%) and 349 (30%) nucleotides, respectively. The recovered 28S phylogeny (Fig. 11) placed the new species within a clade that included sequences from two adult blood flukes (*C. trondheimensis* and *O. glaucostegi*) infecting chondrichthyans as well as that of a cercaria extracted from a bivalve. Expectedly, the BI tree recovered four clades comprising the blood flukes of marine actinopterygians, euryhaline elopomorphs, freshwater cercariae, and marine chondrichthyans.

Key for identification of fish blood flukes (Aporocotylidae) infecting chondrichthyans

1. Body spinous, lateral tegumental spines (LTSs) C-shaped, each on a muscular peduncle……...…….....…….....2

   Body aspinous…….....3

2. Intestinal caeca X-shaped, oviducal ampulla absent……5

   Intestinal caeca inverse U-shaped, oviducal ampulla present…….....6

3. Adults infecting mesenteric vessels; body margin having lateral tubercles…….....*Orchispirium heterovitellatum*

   Adults infecting heart; body lacking lateral tubercles……..4

4. Body minute (< 1 mm long), testis curving < 15 times…....*Myliobaticola richardheardi*

Body extremely elongated (≥ 4 mm long), testis curving > 15 times…….…….…….…….....*Ogawaia glaucostegi*

5. Body minute (< 1.4 mm long), LTSs numbering >170 per side of body…….……..…….....*Selachohemecus olsoni*

Body large (≥1.4 mm long), LTSs <100 per side of body….....*Selachohemecus benzi*

6. LTSs distributed in a single column…..…….....*Gymnurahemecus bulbosus*

LTSs distributed in multiple columns or lateral field……..7

7. Caeca short, terminating at level of genital pores……..…….....*Chimaerohemecus trondheimensis*

Caeca elongate, extending to near posterior body end, terminating posterior to genitalia……...…….....8

8. Body 2 × longer than wide, mid-body LTSs <20 μm long……...……...*Hyperandrotrema cetorhini*

Body 7–8 × longer than wide, mid-body LTSs ≥25 μm long……...……..*Hyperandrotrema walterboegeri*

Taxonomic remarks

The new genus is most similar to *Hyperandrotrema* (two species, both infecting lamniform sharks) and *Chimaerohemecus* (monotypic, infecting chimaeras) by the combination of having a minute, diminutive, aspinous anterior sucker that is not demarcated from the body, C-shaped lateral tegumental spines each associated with a muscular peduncle, intestinal caeca that are inverse U-shaped (lacking anterior caeca) and that lack diverticulae, an internal seminal vesicle and cirrus sac, an oviducal ampulla, a Laurer's canal, an oötype that is posterior to the remainder of the genitalia, a dextral vitelline duct (posteriorly asymmetrical vitellarium), and a common

genital pore (van der Land 1967; Maillard and Ktari 1978; Orélis-Ribeiro et al. 2014). The new genus can be easily differentiated from *Chimaerohemecus* and *Hyperandrotrema* by the combination of having a single column of lateral tegumental spines, a medial oesophageal bulb anterior to a diverticulate region of the oesophagus, intestinal caeca that terminate in the middle 1/3 of the body, a sinistral, post-caecal, post-testicular ovary, and a post-caecal common genital pore. This combination of features differentiates the new genus, *Chimaerohemecus*, and *Hyperandrotrema* from the remaining chondrichthyan blood fluke genera, i.e., *Selachohemecus* (comprised of two species, both infecting carcharhiniform sharks) (Short 1954; Bullard et al. 2006) plus *Orchispirium*, *Myliobaticola*, and *Ogawaia* (each monotypic, infecting rays) (Madhavi and Rao 1970; Bullard and Jensen 2008; Cutmore et al. 2018). Further, these features distinguish the new genus and its relatives infecting chondrichthyans from all genera of blood flukes that infect ray-finned fishes (Actinopterygii). *Selachohemecus* is unique among chondrichthyan blood fluke genera by having X-shaped intestinal caeca and an oötype at level of the seminal vesicle as well as by lacking an oviducal ampulla (the oviducal seminal receptacle in *S. olsoni* and *S. benzi* comprises a swelling of the proximal portion of the oviduct) and Laurer's canal. *Orchispirium*, *Myliobaticola*, and *Ogawaia* are distinct by having a looped testis and a post-caecal common genital pore as well as lacking lateral tegumental spines, an oviducal ampulla (*Orchispirium* and *Myliobaticola* have an oviducal seminal receptacle comprising a swelling of the proximal portion of the oviduct), and a Laurer's canal.

DISCUSSION

Because chondrichthyans are the earliest branching, extant lineage of jawed vertebrate (Craniata: Gnathstomata), their blood flukes comprise an obvious and requisite group with which to test hypotheses concerning fish blood fluke monophyly and blood fluke-craniate cophyly. This

area of research has progressed considerably since the late 1990's, when it was accepted that the blood flukes of fishes exhibited no detectable level of phylogenetic host specificity (Smith 1997a). The present study, comprising only the 2nd and 3rd published elasmobranch and chondrichthyan blood fluke 28S sequences (Cribb et al. 2017), respectively, provides further evidence of a monophyletic chondrichthyan blood fluke lineage. This result agrees with and extends previous morphology- and/or nucleotide-based phylogenetic assertions that the blood flukes of early-branching jawed craniates are monophyletic and phylogenetically distant from the blood flukes of ray-finned fishes (Actinopterygii: Euteleostei) (Bullard et al. 2006; 2008; Bullard and Jensen 2008; Cribb et al. 2017; Orélis-Ribeiro et al. 2013; 2014; 2017). Clearly, the chondrichthyan blood flukes share morphological features that set them apart as unique and differentiate them from other blood flukes infecting ray-finned fishes, turtles, crocodiles, birds, and mammals (present study; Bullard et al. 2006; Orélis-Ribeiro et al. 2013). However, relatively low taxon sampling for chondrichthyan blood flukes used in molecular phylogenetic studies limits our ability to thoroughly test these patterns. Further, no life cycle for a chondrichthyan blood fluke is known. Aporocotylidae sp. NSW1 is a cercarial sequence, and the definitive host for that taxon is indeterminate; however, Cribb et al. (2017) assumed that it matured in an elasmobranch because it claded with *O. glaucostegi,* and that clade is sister to *C. trondheimensis* (Cutmore et al. 2018).

Although it has been extensively demonstrated that morphologically-similar fish blood flukes mature in phylogenetically-related craniates (Bullard et al. 2006; 2008; Bullard and Jensen 2008; Orélis-Ribeiro et al. 2013; 2014; 2017), no molecular phylogenetic evidence definitively supports a strict cophyly hypothesis for the blood flukes of craniates (Orélis-Ribeiro et al. 2017). For example, existing blood fluke phylogenies, including ours herein, fail to recover a topology

reflective of the current deep classification system for the subphylum Craniata (see Nelson et al. 2016), i.e., the blood flukes of cartilaginous fishes are monophyletic and sister to all remaining craniate blood flukes. Instead, the recovered phylogenies generally show a monophyletic Aporocotylidae, which is sister to the paraphyletic turtle blood flukes ("Spirorchiidae") and monophyletic Schistosomatidae (Fig. 11) (Orélis-Ribeiro et al. 2014; Cribb et al. 2017). Again, low taxon sampling among the blood flukes of early-branching craniates is a contributing factor to this conundrum, and this lack of information underscores the importance of robust taxonomic descriptions coupled with molecular sequence data from chondrichthyan blood flukes. Sequences from blood flukes infecting chondrichthyans (especially sharks), early-branching actinopterygians (Acipenseriformes [sturgeons and paddlefishes], Polypteriformes [bichirs], Amiiformes [bowfin], Lepisosteiformes [gars]), and early-branching members of Teleosteomorpha (see Nelson et al. 2016) could result in a topology that mirrors that of craniates (Chondrichthyes sister to Actinoptergyii + Sarcopterygii). Obviously, obtaining a blood fluke from a hagfish (Myxiniformes) or a lamprey (Petromyzontiformes) would be a breakthrough in this regard.

The recovered 28S phylogeny (Fig. 11) indicate four fish blood fluke lineages corresponding to the phylogenetic and ecological affiliation of their definitive hosts: marine actinopterygians, euryhaline elopomorphs, freshwater cercariae, and marine chondrichthyans. Cribb et al. (2017) recovered the same clades and discussed them; however, they recovered the blood flukes infecting rays and chimaeras as sister to those infecting freshwater actinopterygians. Based on only a few life cycles from marine and freshwater fish blood flukes, Orélis-Ribeiro et al. (2014) suggested a dichotomy between marine and freshwater fish blood flukes that infect polychaetes and bivalves vs. those that infect snails, respectively. Doubtless, previous speculation on the

evolutionary history of the blood flukes is wanting for information about their life cycles. Although several workers have described cercariae morphologically ascribed to the fish blood flukes (Smith 1997b), Aporocotylidae sp. NSW1 (MF503307) is the only published cercarial sequence that clades with adult blood flukes infecting chondrichthyans (Cribb et al. 2017). Although no life cycle for a chondrichthyan aporocotylid is known and despite not knowing the identity of the definitive host for the aforementioned cercaria, Cribb et al. (2017) speculated that all elasmobranch blood flukes utilize a bivalve intermediate host rather than a gastropod or polychaete (this assumes also that all blood flukes of marine ray-finned fishes utilize polychaetes as exemplified so far by *Aporocotyle simplex* Odhner, 1900 [see Køie 1982]; *Cardicola forsteri* Cribb et al., 2000 [see Cribb et al. 2011]; *Cardicola parvus* Bullard et al., 2012 [see Bullard et al. 2012; Siegel et al. 2018]; and *Cardicola laruei* Short, 1953 [see McVay et al. 2011; Siegel et al. 2018] only). They predicted that this could explain the phylogenetic separation between the blood flukes that mature in chondrichthyans and those of actinopterygians.

Regarding hosts and blood fluke ancestry, within the marine actinopterygian clade (Fig. 11), it is noteworthy that *Elopicola* spp. are sister to all of the blood flukes infecting the remaining marine actinopterygians. The hosts for *Elopicola* spp. comprise euryhaline fishes, ladyfishes (*Elops* spp.: Elopidae), and tarpons (*Megalops* spp.: Megalopidae) (Orélis-Ribeiro et al. 2017) that spend considerable periods of their life in freshwater or low salinity estuarine waters (Zale and Merrifield 1989; Adams and Cooke 2015). Bullard (2014) documented eggs and schistosomula larvae of *Elopicola nolancribbi* Bullard, 2014 in the gill epithelium and blood of juvenile ladyfish (*Elops saurus* Linnaeus) in a northern Gulf of Mexico estuary; suggesting that the life cycle was being completed in that estuary or a nearby river(s). Species of *Elopicola* Bullard, 2014 and *Paracardicoloides* Martin, 1974 infect fishes with a leptocephalus larva

(Euteleostei: Elopomorpha), are morphologically similar, and are sister taxa based on ITS2 sequence data (Bullard 2014; Orélis-Ribeiro et al. 2017); all of which strongly suggest cophyly among elopomorphs and their blood flukes. Nolan and Cribb (2004) demonstrated the life cycle of *P. yamagutii*, which infects a riverine freshwater snail (*Posticobia brazieri* [Smith] [Littorinimorpha: Tateidae]) and matures in the Australian long-finned eel, *Anguilla reinhardtii* Steindachner. The assertion that related blood flukes infect related intermediate hosts predicts that *Elopicola* spp. (like the related *P. yamagutii*) use gastropods rather than bivalves or polychaetes. Cercariae ascribed to the fish blood flukes by molecular barcoding and phylogenetic inference (i.e., Aporocotylidae sp. W5003, *Sanguincola* cf. *inermis*, and Aporocotylidae sp. W5004; see Cribb et al. [2017]) also infect gastropods but are distantly related to *Elopicola* spp. Hence, Cribb et al.'s (2017) tree and ours herein (if *Elopicola* spp. have a gastropod intermediate host), suggest that gastropod blood flukes are paraphyletic.

We do not know the intermediate host for *G. bulbosus*, but based upon Cribb et al.'s (2017) prediction, it should be an estuarine or littoral bivalve. Scant information is available on the life histories of the smooth butterfly rays throughout their range; however, this fish is considered to be a relatively non-vagile resident of estuaries, with females producing offspring year-round (Yokota et al. 2006; 2012). Given the residency of this elasmobranch, the host is likely littoral or estuarine. Examinations of estuarine invertebrates in the northern Gulf of Mexico continue (MBW) in an attempt to isolate infected intermediate hosts, especially bivalves.

Phylogenetic analyses involving the chimaera blood fluke *C. trondheimensis* and the ray blood flukes consistently produce long branches (Bullard et al. 2008; Bray et al. 2012; Orélis-Ribeiro et al. 2013; 2014; 2017; Santoro et al. 2015; Yong et al. 2016a; 2016b; 2018; Cribb et al. 2017; Siegal et al. 2018). We speculate that the genetic differences that produce these long

branches are indicative of a long phylogenetic separation from other blood flukes and that morphological distinctness is reflected by genetic distinctness. The inferred long branches could also be a result of limitations to taxon sampling associated with extinction in chimaera and ray blood flukes (and their hosts). Furthermore, unresolved, deep relationships among chondrichthyan and ray-finned fish blood flukes (Fig. 11) are likely a result of long branch artifacts. Greater taxon and character sampling will ultimately be needed to resolve relationships among these major lineages.

Acknowledgements

We thank Marcus Drymon (Mississippi State University) for facilitating the collection of smooth butterfly rays in Mobile Bay, AL.

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FIGURE LEGENDS

Figs. 1–5 *Gymnurahemecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). (**1**) Body of holotype (USNM No. **XXXXX**), ventral view. (**2**) Body of paratype (USNM No. **XXXXX**), dorsal view. (**3**) Body of paratype (USNM No**. XXXXX**), ventral view. Mouth (mo), oesophagus (os), nerve commissure (nc), oesophageal bulb (ob), vitellarium (vit), caecal bifurcation (cb), testis (t), vasa efferentia (ve), ovary (o), common genital pore (cgp), oötype (oo), and excretory vesicle (ev). Bars = 150 μm. (**4**) Anterior-most ventrolateral tegumental spines and supporting peduncles. Dorsal view. (**5**) Mid-body ventrolateral tegumental spines and supporting peduncles. Black shading indicates exposed portion of lateral tegumental spine. Bar = 4 μm.

Figs. 6–8 *Gymnurahemecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). Oesophagus of adults demonstrating morphological consistency. All specimens show mouth (mo), oesophagus (os), nerve commissure (nc), pre-oesophageal bulb dilation (pb), oesophageal bulb (ob), oesophageal gland (og), diverticulated portion (dp), and pre-cecal dilation (pd). Bar = 100 μm. (Oesophageal gland surrounds oesophagus but its enveloping portion is not illustrated so as not to obscure the oesophagus.)

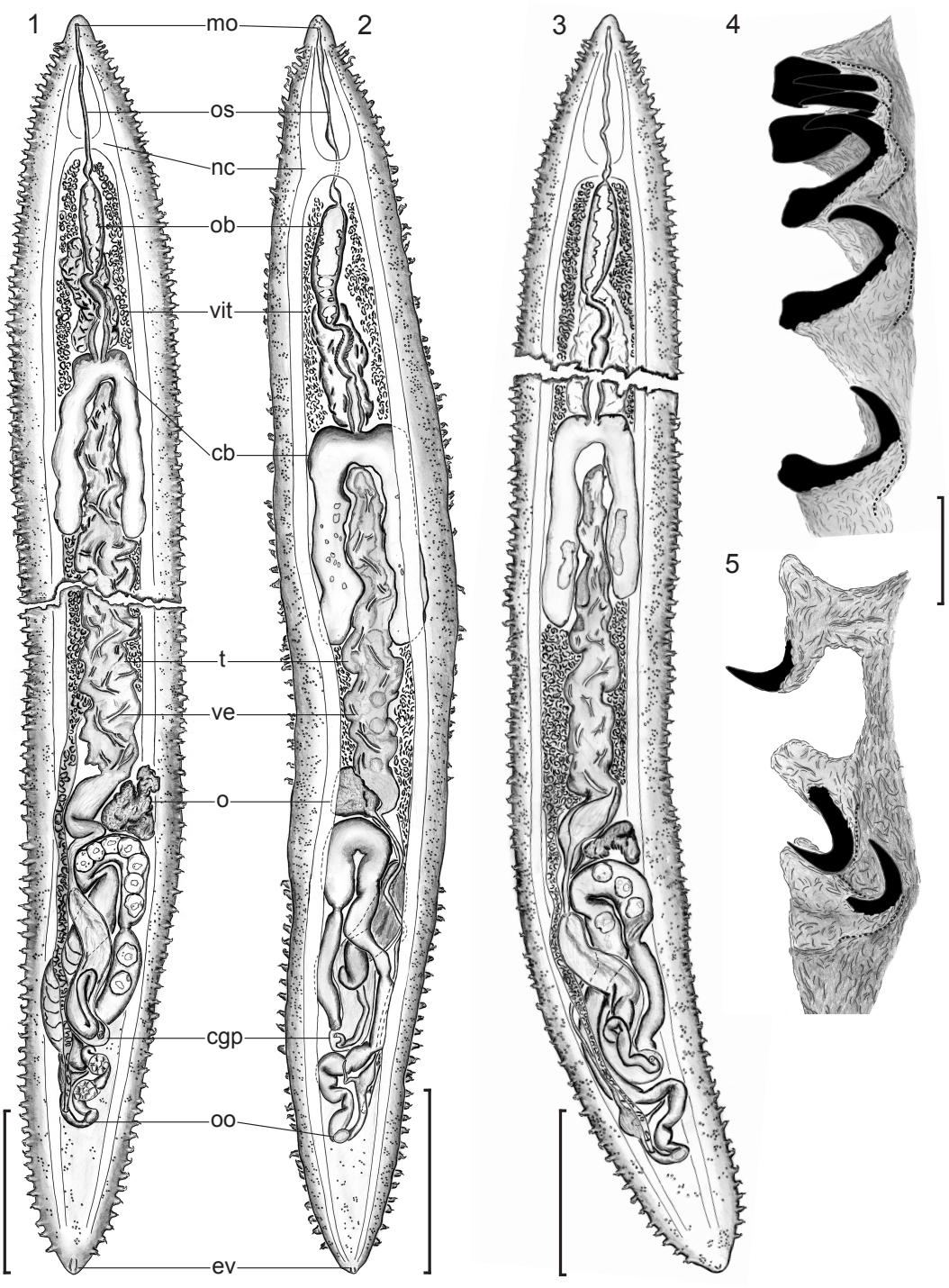
Figs. 9–10 *Gymnurahemecus bulbosus* Warren and Bullard gen. et sp. nov. (Digenea: Aporocotylidae) from the heart of the smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider) (Myliobatiformes: Gymnuridae). (**9**) Genitalia of holotype (USNM No. **XXXXX**), ventral view. (**10**) Genitalia of paratype (USNM No. **XXXXX**), dorsal view. Testis (t), ovary (o), vitelline duct (v), vas deferens (vd), oviduct (ov), ascending and descending portions of the uterus (u), common genital pore (cgp), seminal vesicle (sv), metraterm (met), Laurer's canal (lc), oviducal ampullae (oa), and oötype (oo). Bars = 100 μm. Note that nerve cords are drawn dorsal so as not to obscure the genitalia.

Fig. 11 Phylogenetic relationships of blood flukes reconstructed using Bayesian inference with the large subunit ribosomal DNA (28S) gene. Clades are indicated by colors: blue = marine actinopterygians, green = euryhaline elopomorphs, yellow = freshwater cercariae, red = marine chondrichthyans, and black = turtle blood fluke outgroup. Numbers aside tree nodes indicate posterior probability. Scale bar is in substitutions per site.

Table 1 DNA sequences used in the present study.

Blood fluke	Host	Locality	GenBank 28S Accession #	Reference
Aporocotylid cercaria W5003 Brant et al., 2006	*Plotiopsis balonnensis* (Conrad)	Victoria River, Northern Territory, Australia	AY858878	Brant et al. 2006
Aporocotylid cercaria W5004 Brant et al., 2006	*Glyptophysa gibbosa* (Gould)	Mary River, Northern Territory, Australia	AY858879	Brant et al. 2006
Aporocotylid cercaria NSW1 Cribb et al., 2017	*Plebidonax deltoides* (Lamarck)	Stockton beach, new South Wales, Australia	MF503307	Cribb et al. 2017
Aporocotyle argentinensis Smith, 1969	*Merluccius hubbsi* Marini	off north Patagonia, Argentina	JX094803	Hernández-Orts et al. 2012
Aporocotyle mariachristinae Hernández-Orts et al., 2012	*Genypterus blacodes* (Forster)	off north and central Patagonia, Argentina	JX094802	Hernández-Orts et al. 2012
Aporocotyle spinosicanalis Williams, 1958	*Merluccius merluccius* (Linnaeus)	off Orkney islands, NE Atlantic Ocean	AY222177	Olson et al. 2003
Cardicola beveridgei Yong et al., 2016b	*Lutjanus argentimaculatus* (Forsskål)	off Lizard Island, Australia	KX523188	Yong et al. 2016b
Cardicola bullardi Yong et al., 2016b	*Scomberomorus munroi* Collette and Russo	Moreton Bay, Queensland, Australia	KX523190	Yong et al. 2016b
Cardicola forsteri Shirakashi et al., 2016	*Thunnus orientalis* (Temminck and Schlegel)	off Wakayama Prefecture, Japan	KT119353	Shirakashi et al. 2016
Cardicola opisthorchis Ogawa et al., 2011	*Thunnus orientalis* (Temminck and Schlegel)	off Wakayama Prefecture, Japan	HQ324227	Ogawa et al. 2011
Cardicola suni Yong et al., 2016b	*Chanos chanos* (Forsskål)	Moreton Bay, Queensland, Australia	KX463511	Yong et al. 2016b
Chimaerohemecus trondheimensis van der Land, 1927	*Chimaera monstrosa* Linnaeus	NE Atlantic, off Bergen, Norway	AY157239	Lockyer et al. 2003b
Elopicola nolancribbi Bullard, 2014	*Elops saurus* Linnaeus	Gulf of Mexico, off Ship Island, MS	KY243880	Orélis-Ribeiro et al. 2017
Elopicola bristowi Orélis-Ribeiro et al., 2017	*Elops hawaiensis* Reagan	Eastern Sea, off Nha Trang, Vietnam	KY243881	Orélis-Ribeiro et al. 2017
Elopicola franksi Orélis-Ribeiro et al., 2017	*Megalops atlanticus* Valenciennes	Gulf of Mexico, off FL	KY243882	Orélis-Ribeiro et al. 2017
Gymnurahemecus bulbosus gen. et sp. nov.	*Gymnura micrura* (Bloch and Schneider)	Gulf of Mexico, mouth of Mobile Bay, AL	MH555432	Present study
Gymnurahemecus bulbosus gen. et sp. nov.	*Gymnura micrura* (Bloch and Schneider)	Gulf of Mexico, mouth of Mobile Bay, AL	MH555433	Present study
Neoparacardicola nasonis Yamaguti, 1970	*Naso unicornis* (Forsskål)	off Lizard Island, Australia	AY222179	Olson et al. 2003

Ogawaia glaucostegi Cutmore et al., 2018	*Glaucostegus typus* (Anonymous [Bennett])	Moreton Bay, Queensland, Australia	MF503308	Cribb et al. 2017
Paradeontacylix buri Ogawa et al., 2015	*Seriola quinqueradiata* Temminck and Schlegel	off Miyazaki Prefecture, Japan	AB904154	Ogawa et al. 2015
Paradeontacylix grandispinus Repullés-Albelda et al., 2008	*Seriola dumerili* (Risso)	off Kagoshima Prefecture, Japan	AM489596	Repullés-Albelda et al. 2008
Paradeontacylix ibericus Repullés-Albelda et al., 2008	*Seriola dumerili* (Risso)	off Murcia, Spain	AM489593	Repullés-Albelda et al. 2008
Plethorchis acanthus Martin, 1975	*Mugil cephalus* Linnaeus	Brisbane River, Queensland, Australia	AY222178	Olson et al. 2003
Psettarium jimbaranense Yong et al., 2016a	*Arothron reticularis* (Bloch and Schneider)	off Bali, Indonesia	KX284693	Yong et al. 2016a
Psettarium nolani (Bray et al. 2012) Yong et al., 2016	*Arothron meleagris* (Anonymous)	off Moorea, French Polynesia	AY157174	Lockyer et al. 2003a
Psettarium sinense (Liu, 1977) Orélis-Ribeiro et al., 2014	*Takifugu rubripes* (Temminck and Schlegel)	Fuzhou, China	EU368853	Chen et al. 2008
Sanguinicola cf. *inermis* Plehn, 1905	*Lymnaea stagnalis* (Linnaeus)	Warminia-Mazury Region, Poland	AY222180	Olson et al. 2003
Skoulekia meningialis Alama-Bermejo et al., 2011	*Diplodus vulgaris* (Geoffroy St. Hilaire)	off Valencia, Spain	FN652293	Alama-Bermejo et al. 2011
Turtle blood flukes				
Hapalorhynchus gracilis Stunkard, 1922	*Chelydra serpentina* (Linnaeus)	Reelfoot Lake, TN	AY604710	Snyder 2004
Spirorchis artericola (Ward, 1921)	*Chrysemys picta* (Schneider)	Reelfoot Lake, TN	AY604704	Snyder 2004
Vasotrema robustum Stunkard, 1928	*Apalone spinifera* (LeSueur)	Nishnabotna River, IA	AY604706	Snyder 2004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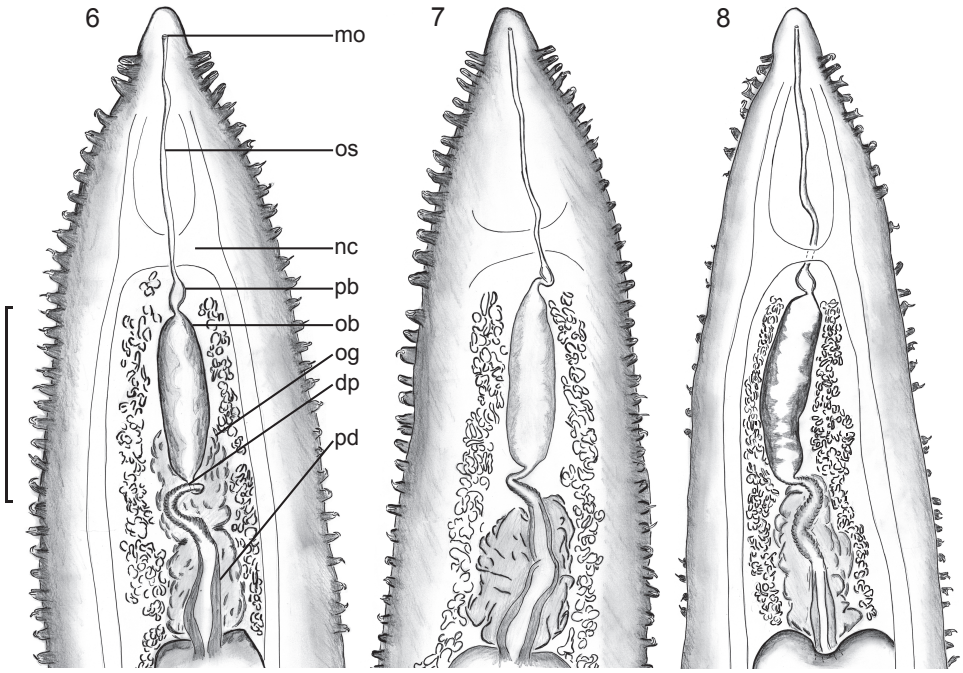
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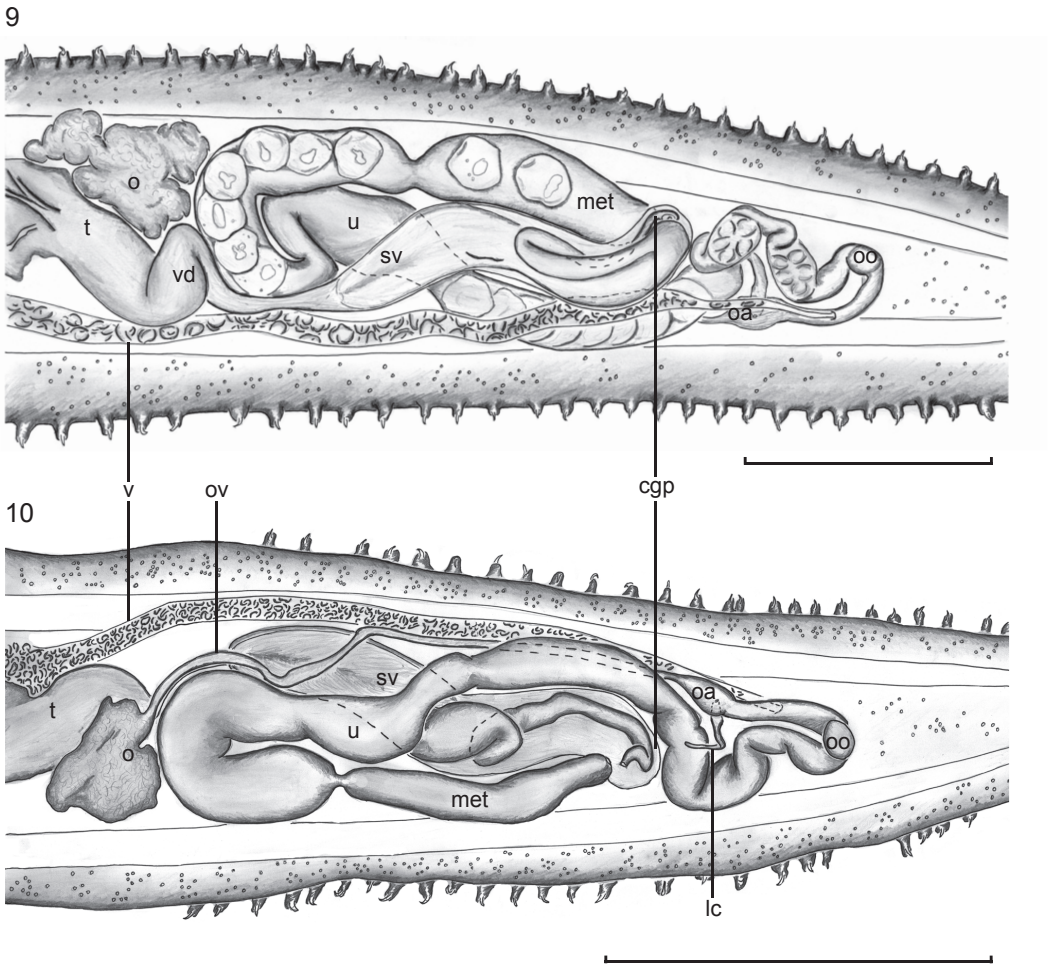
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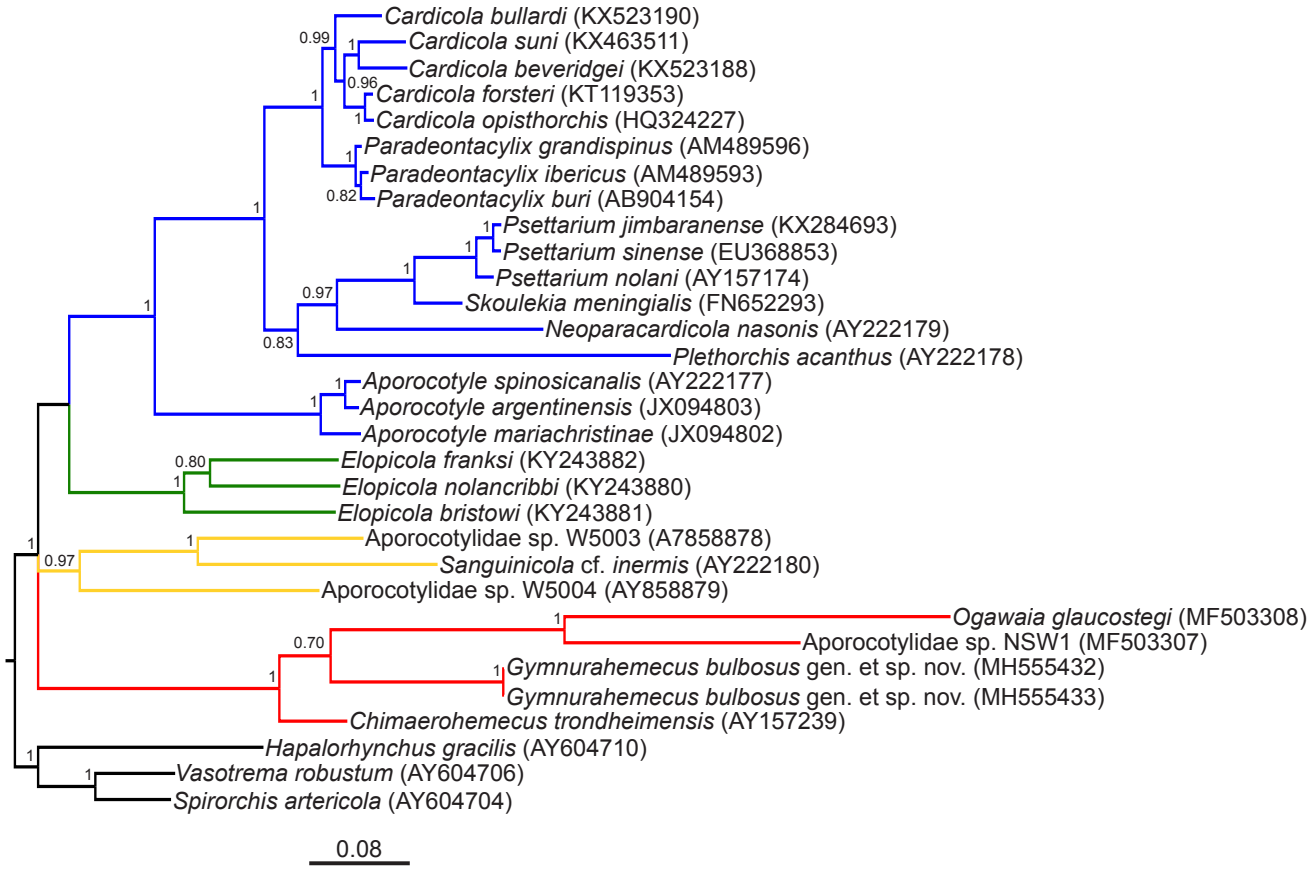
Cardicola bullardi (KX523190)
Cardicola suni (KX463511)
Cardicola beveridgei (KX523188)
Cardicola forsteri (KT119353)
Cardicola opisthorchis (HQ324227)
Paradeontacylix grandispinus (AM489596)
Paradeontacylix ibericus (AM489593)
Paradeontacylix buri (AB904154)
Psettarium jimbaranense (KX284693)
Psettarium sinense (EU368853)
Psettarium nolani (AY157174)
Skoulekia meningialis (FN652293)
Neoparacardicola nasonis (AY222179)
Plethorchis acanthus (AY222178)
Aporocotyle spinosicanalis (AY222177)
Aporocotyle argentinensis (JX094803)
Aporocotyle mariachristinae (JX094802)
Elopicola franksi (KY243882)
Elopicola nolancribbi (KY243880)
Elopicola bristowi (KY243881)
Aporocotylidae sp. W5003 (A7858878)
Sanguinicola cf. inermis (AY222180)
Aporocotylidae sp. W5004 (AY858879)
Ogawaia glaucostegi (MF503308)
Aporocotylidae sp. NSW1 (MF503307)
Gymnurahemecus bulbosus gen. et sp. nov. (MH555432)
Gymnurahemecus bulbosus gen. et sp. nov. (MH555433)
Chimaerohemecus trondheimensis (AY157239)
Hapalorhynchus gracilis (AY604710)
Vasotrema robustum (AY604706)
Spirorchis artericola (AY604704)
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CHAPTER 3: FIRST ELUCIDATION OF A LIFE CYCLE FOR A BLOOD FLUKE (*ELECTROVERMIS ZAPPUM* N. GEN., N. SP.) THAT INCLUDES A CHONDRICHTHYAN OR BIVALVE

***Aceepted in International Journal for Parasitology: Parasites and Wildlife (Accepted XX XXX 2019)**

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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* 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, Cribb, and Yong, 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 discernable 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.

Keywords: taxonomy, phylogenetics, histology, Bivalvia, Elasmobranchii, life cycle

1. Introduction

Of the 1,188 nominal extant chondrichthyan species, 123 (74 Sqaulomorphii 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 and Bullard, 2019*,* and *Ogawaia glaucostegi* Cutmore, Cribb, and Yong, 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, Fried, and Harvey, 1985 (Hoffman et al., 1985); *S. idahoensis* Schell, 1974 (Schell, 1974); *S. inermis* Plehn, 1905 (Kirk and Lewis, 1993); *S. klamathensis* Wales, 1958

(Wales, 1958); *S. lophophora* Erickson and Wallace, 1959 (Erickson and Wallace, 1959); *S. megalobramae* Li, 1980 (Li, 1980); *S. occidentalis* Bacha, 1966 (Bacha, 1966); *S. rutili* Simón-Martín, Rolo-Vásquez and Simón-Vicente, 1987 (Simón-Martín 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* Nolan and Cribb, 2004 (Nolan and Cribb, 2004). Collectively, these life cycles are representative of 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 8,000 species assigned to 1,100 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. deltoids*) 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).

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 of 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 et al. (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 microlitre (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. (Figs. 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.

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. sp. (Figs. 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. 3–4); everted cirrus 15 long or 11% of total cirrus length. Common genital pore 288 or 16% of body length from posterior end of body, 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. 3–4); 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. 3–4). 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. 3–4). 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. 5–6). 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 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 8,000 × 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.

Tail brevifurcate (Figs. 7–8), 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. 7–8); 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. 7–8).

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 (Figs. 1−2), aspinous. Oesophagus 75 and 450 long, 2–7 at greatest width (Figs. 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 1,174 (prevalence = 0.5%) of variable coquina clams had several hundred sporocysts and cercariae.

Specimens deposited: Holotype (USNM XXXXXXX), paratypes (USNM XXXXXXX), GenBank Nos. (28S: XXXXXXXX and XXXXXXXXX; 18S: XXXXXXXX and XXXXXXXXX; ITS2: XXXXXXXX and XXXXXXXXX).

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, 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. 5–6), 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; Seigel 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. 9–10). 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. 9–10; 12). At least superficially, these masses resemble those described for *A. simplex* (see Køie, 1982) and likely comprise secretions from the penetration glands.

3.3 Aporocotylidae sp. ex. *Solen viridis* (Figs. 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. 16–17), 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, 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. 20–21); 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. 20–21). Anterior sucker 7 long, 5 wide, spinous (Fig. 18); anterior sucker spines minute, visible with SEM at 1,700 × 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).

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: Holotype (USNM XXXXXXX), paratypes (USNM XXXXXXX), GenBank Nos. (28S: XXXXXXXX, ITS2: XXXXXXXX).

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 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 1,559 (adult of *E. zappum*), 1,571 (cercariae *of E. zappum*), and 2,360 (cercariae from *S. viridis*) nucleotides. That for the 18S resulted in 1,867 (adult of *E. zappum*) and 1,928 (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* (XXXXXXXX) 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's (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; Seigel 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; Olgesby, 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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FIGURE LEGENDS

Figures 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. XXXXX), ventral view. **(2)** Body of shistosomulum (larger) Voucher (USNM No. XXXXX), ventral view. **(3)** Body of adult (holotype, USNM No**.** XXXXX), 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).

Figures 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 (USNM No. XXXXX), ventral view. **(8)** Body of mounted cercaria (USNM No. XXXXX), 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).

Figures 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), sporocysts (sp), and ooctyes (arrow). **(15)** Higher magnification of sporocyst containing developed cercaria (arrow) adjacent to digestive diverticulum (dd).

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

Figures 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.

Figure 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*.

Figure 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.

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 and Bullard, 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 Malliard 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	Malliard 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	Atlantic stingray, *Hypanus*	intertrabecular	Deer Island,	Bullard and

Jensen, 2008	*sabinus* (Lesueur, 1824) Last, Manjaji-Matsumoto, Naylor, and White, 2016	spaces of heart	Mississippi Sound, Northern Gulf of Mexico off Biloxi, Mississippi.	Jensen, 2008
Ogawaia glaucostegi Cutmore, Cribb, and Yong, 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 Rao, 1970	Bengal whipray, *Brevitrygon imbricata* (Bloch and Schneider, 1801) Last, Manjaji-Matsumoto, Naylor, and White, 2016	mesenteric blood vessels	Western Bay of Bengal, waters off Waltair, India.	Madhavi and Rao, 1970
Selachohemecus benzi Bullard, Overstreet, and Carlson, 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	XXXXXXX	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	XXXXXXX	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, Cribb, and Yong, 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	XXXXXXX	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*, Olgesby, 1961	Estuary of Apalachicola River at Apalachicola, Franklin Co., Florida, USA	Olgesby, 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	Seigel 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	Seyðisfjörður, Eastern Iceland	Køie and Petersen, 1988

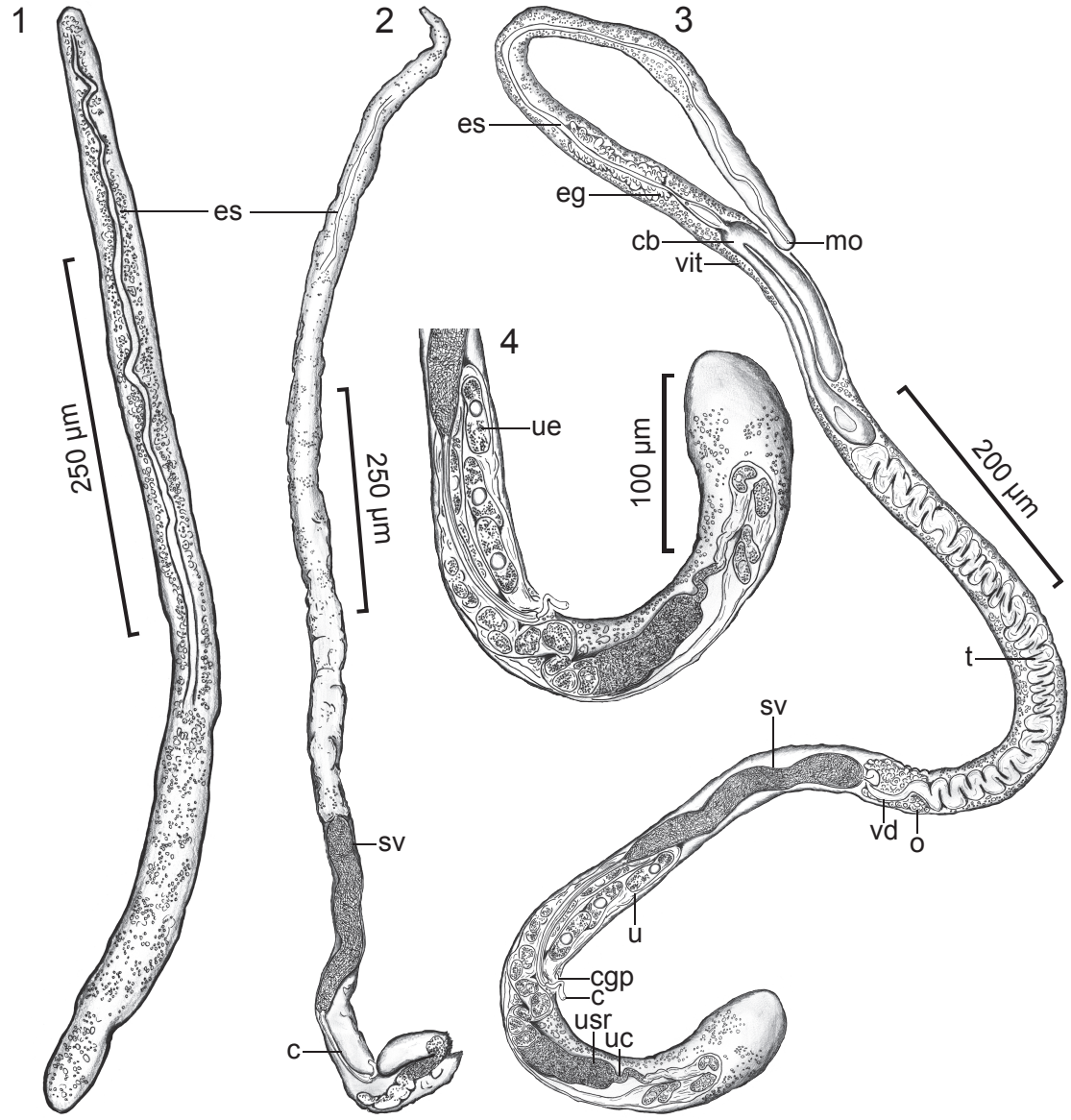
	Aporocotyle simplex Odhner, 1900		
Lanicides vayssierei (Gravier, 1911)	*Cercaria hartmanae* Martin, 1952	Cape Boyds, Ross Island, Antarctica	Martin, 1952
Longicarpus modestus (Quatrefages, 1866)	*Cardicola fosteri* Cribb, Daintith, and Munday, 2000	off Port Lincoln, South Australia	Cribb et al., 2011
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

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.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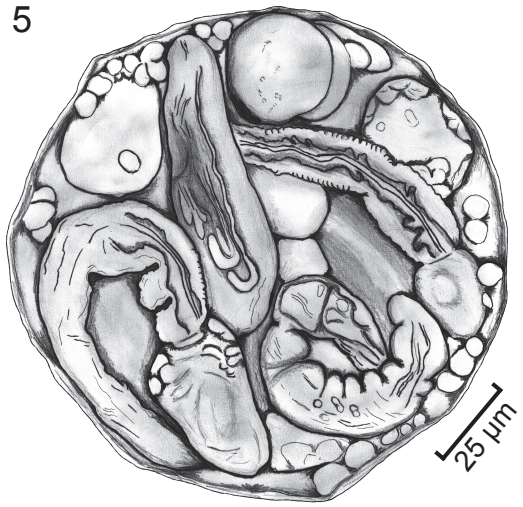
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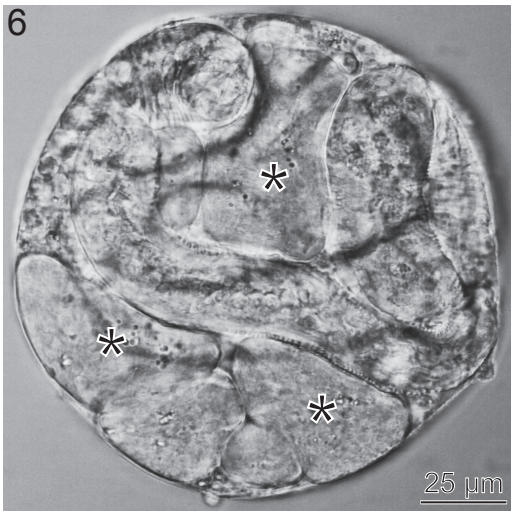
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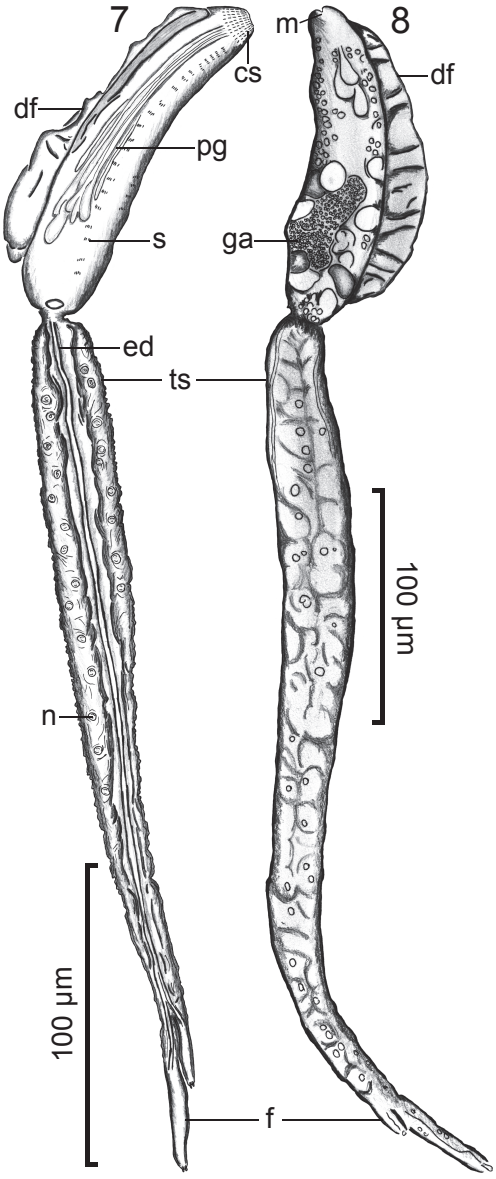
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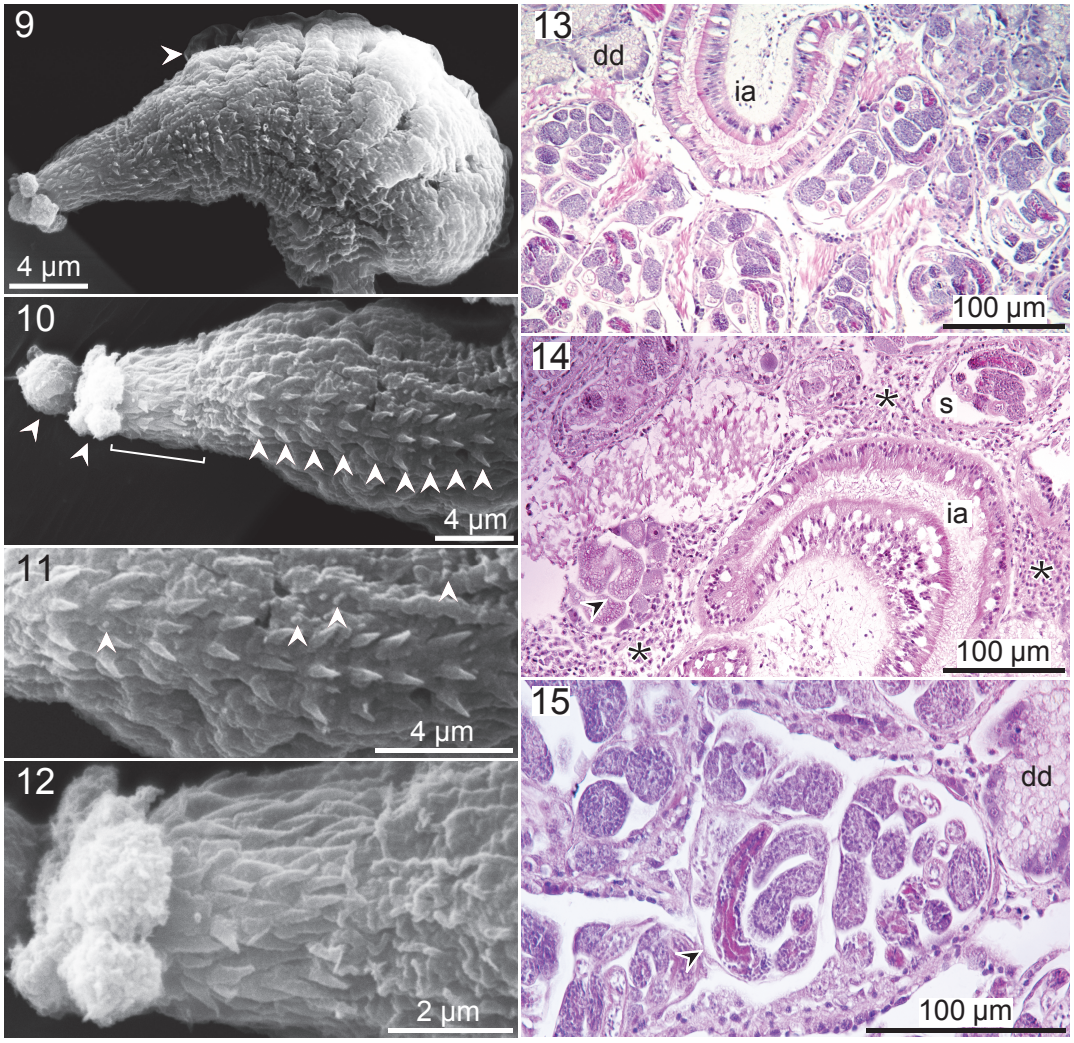
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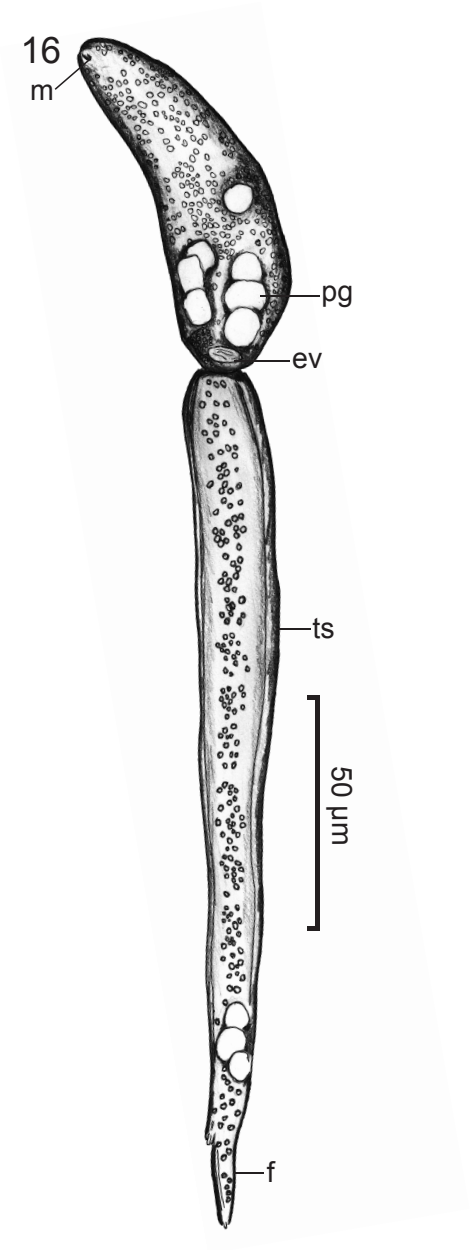
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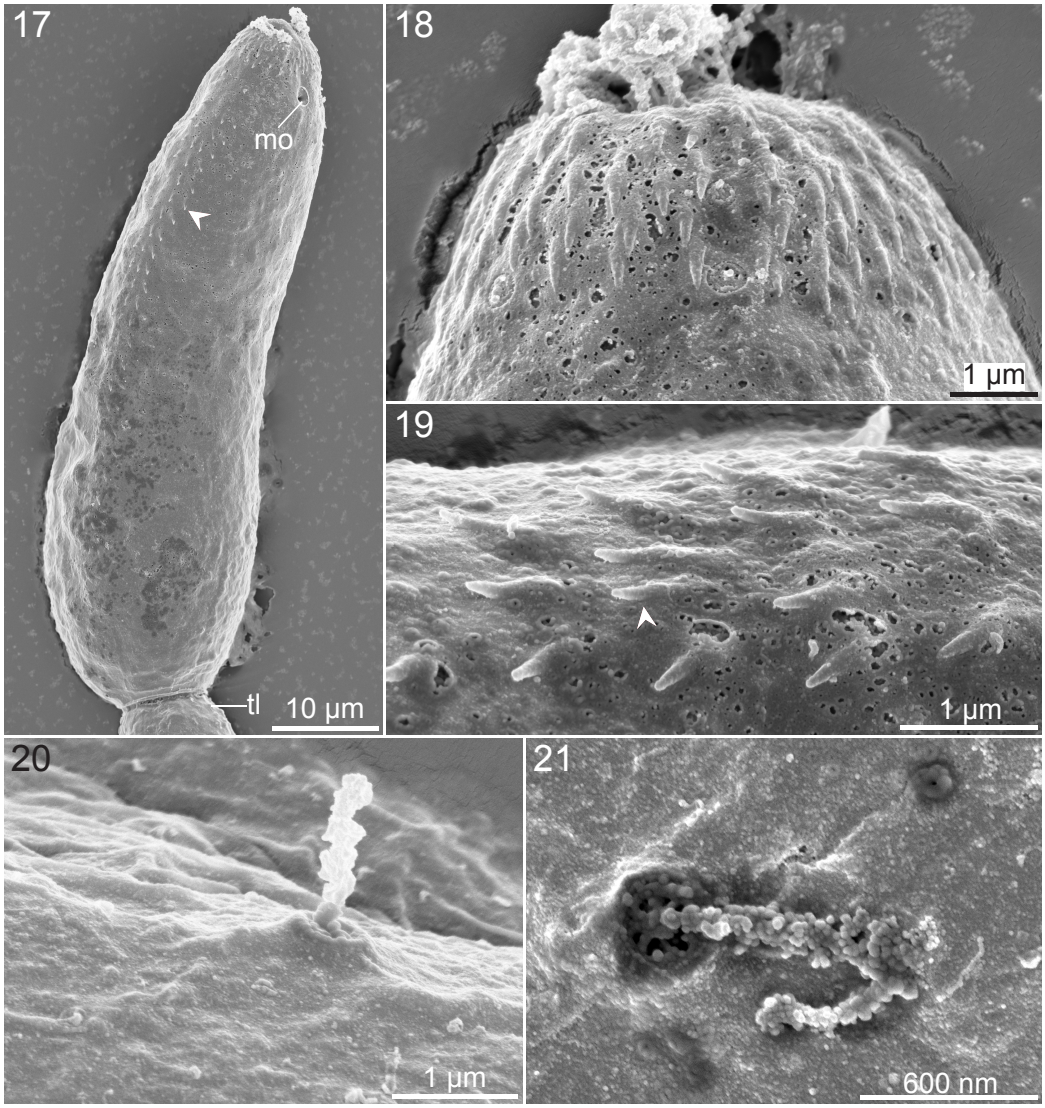
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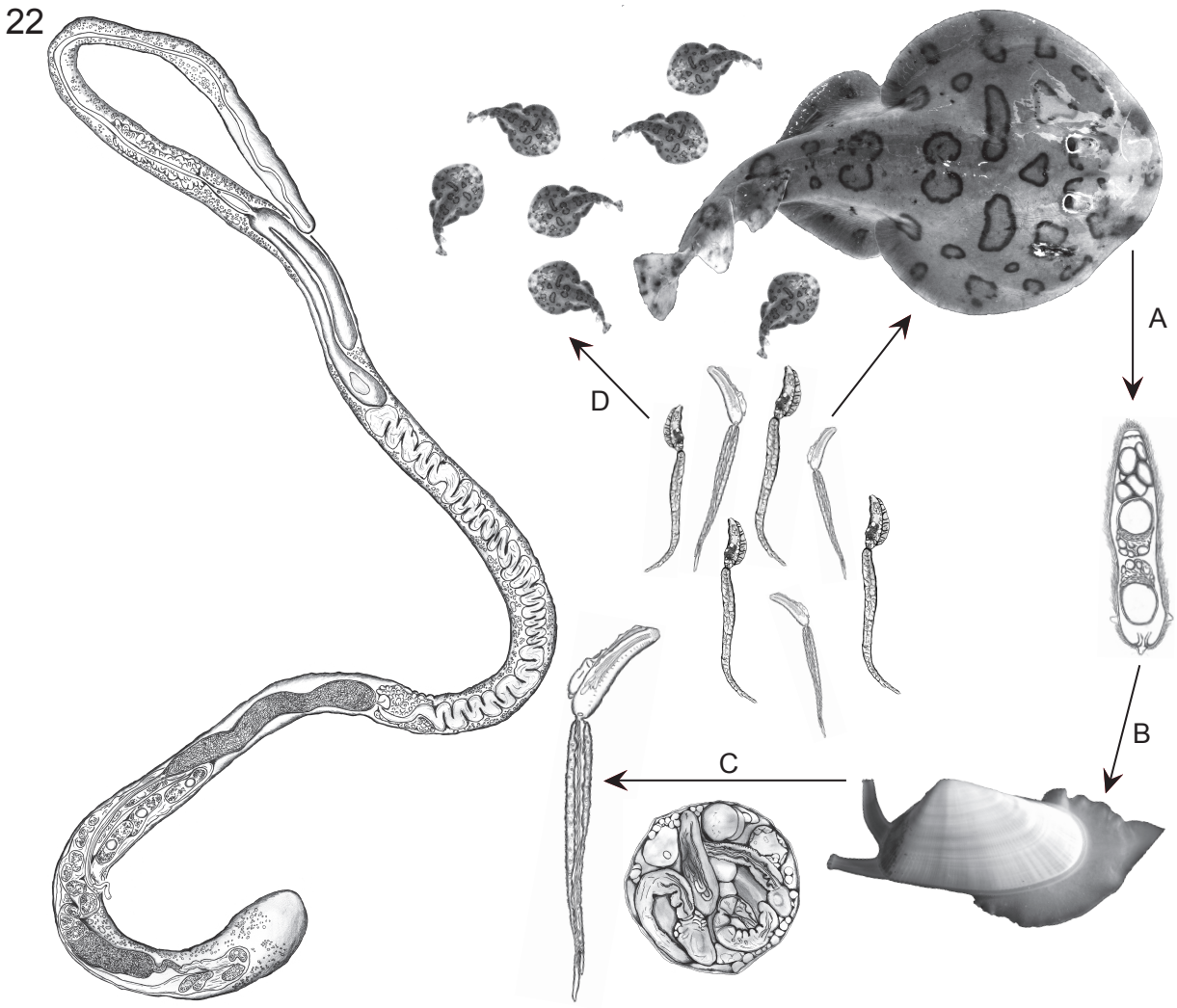

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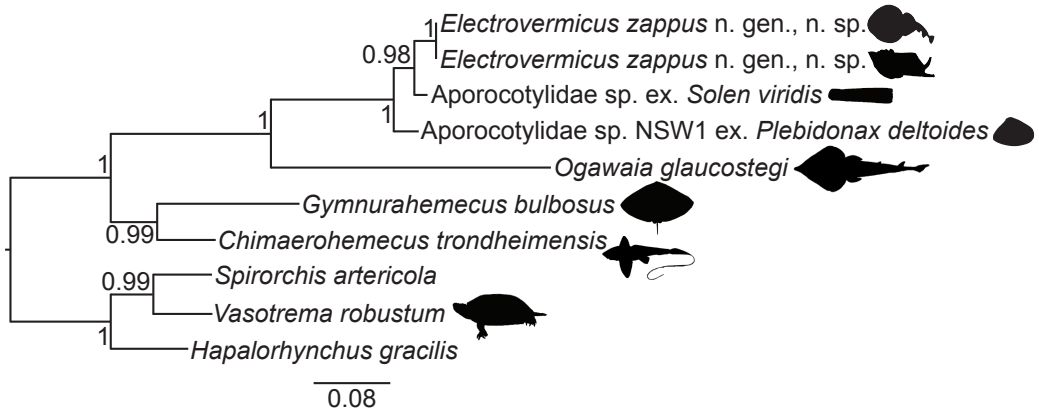
1
Electrovermicus zappus n. gen., n. sp.
0.98
Electrovermicus zappus n. gen., n. sp.
Aporocotylidae sp. ex. Solen viridis
1
1
Aporocotylidae sp. NSW1 ex. Plebidonax deltoides
Ogawaia glaucostegi
1
Gymnurahemecus bulbosus
0.99
Chimaerohemecus trondheimensis
0.99
Spirorchis artericola
Vasotrema robustum
1
Hapalorhynchus gracilis
0.08

CHAPTER 4: A NEW GENUS AND SPECIES (DIGENEA: APOROCOTYLIDAE), INFECTING THE CRITICALLY ENDANGERED SMALLTOOTH SAWFISH, *PRISTIS PECTINATA* (RHINOPRISTIFORMES: PRISTIDAE)

***Submitted to Folia Parasitologica**

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ABSTRACT

The new genus and species (Digenea: Aporocotylidae) infects the heart of the smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae) in the eastern Gulf of Mexico. The new genus, along with the other nominal blood fluke genera including species that infect batoids, are similar by having an inverse U-shaped intestine, a curving testis, and all lack tegumental spines. The new species differs from all of the other blood flukes infecting batoids by having an elongate body (>50 × longer than wide), a curving testis having >100 curves, and an ovary wholly anterior to the uterus. It differs from *Ogawaia glaucostegi* Cutmore, Cribb et Yong, 2018, the only other blood fluke infecting a rhinopristiform, by having a body that is >50 × longer than wide (vs. <30), a testis that is >75 × longer than wide (vs. <40), and >100 curves (vs. <70), an ovary positioned wholly anterior to (vs. lateral and dorsal to) the seminal vesicle, a uterus positioned wholly posterior to (vs. overlapping and lateral to both) the testis and ovary, and a sinuous (vs. convoluted) uterus. The new description adds to the small group of nominal chondrichthyan blood flukes that lack tegumental spines (*O. glaucostegi*, *Myliobaticola richardheardi* Bullard et Jensen, 2008, *Electrovermis zappum* Warren et Bullard, *in review*). Blood flukes infecting batoids not only differ just by lacking spines, but also by having a curving testis. These morphological differences agree with Shirai's (1996) assessment and proposal of

the two elasmobranch groups, in that blood flukes infecting species within Galea are morphological distinct from those infecting species within Squalea. Based on the morphological similarity we infer that the new species shares a recent common ancestor with *O. glaucostegi*. The discovery of the new species brings the total number of chondrichthyan blood flukes to 11 species assigned to nine genera.

Keywords: taxonomy, systematics, Endangered Species Act, Chondrichthyes, Elasmobranchii, fish blood fluke

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More than half (90 of 166 spp. assigned to 40 genera) of the nominal fish blood flukes (Digenea: Aporocotylidae Odhner, 1912; see Bullard et al. 2009) have been discovered in the last 20 years. Of these, 19 have been described from North America (Table 1), with five infecting chondrichthyans (Bullard et al. 2006, Bullard and Jensen 2008, Orélis-Ribeiro et al. 2013, Warren et al. 2019, Warren and Bullard *in review*). No blood fluke has been described from the sawfishes (Pristidae) and only one (*Ogawaia glaucostegi* Cutmore, Cirbb et Yong, 2018) has been described from the order (Rhinopristiformes).

The smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae), was the first elasmobranch to be listed as an endangered species under the United States Endangered Species Act (Simpfendorfer 2005). The International Union for Conservation of Nature (IUCN) classified it as critically endangered in 2006 (Adams et al. 2006). Gill netting, shrimp trawling, and the use of purse seines led to the decline of sawfish populations, thus increasing their conservation status (Simpfendorfer 2000). Currently, 55 parasites have been reported collectively from sawfishes (Pristidae: one species of *Anoxypristis* White et Moy-Thomas, 1941 and four

species of *Pristis* Linck, 1790) (23 cestodes [Southwell 1927, Watson and Thorson 1976, Campbell and Beveridge 1996, 2009, Beveridge and Campbell 2005, Schaeffner and Beveridge 2012, 2013, Cielocha et al. 2014, Bakenhaster et al. 2018, Caira et al. 2018]; nine monogenoids [Watson and Thorson 1976, Cheung and Nigrelli 1983, Ogawa 1991, Chisholm and Whittington 1997, 2000, Kearn et al. 2010, Kritsky et al. 2017]; seven copepods [Ogawa 1991, Morgan et al. 2010, Bakenhaster et al. 2018]; four digeneans [Bakenhaster et al. 2018]; four isopods [Moreira and Sadowsky 1978, Bakenhaster et al. 2018]; four leeches [Bakenhaster et al. 2018]; three nematodes [Bruce et al. 1994, Bakenhaster et al. 2018]; one branchiuran [Bakenhaster et al. 2018]). Of these 55, only one innominate aporocotylid (described herein) has been reported (Bakenhaster et al. 2018).

We herein diagnose a new species of blood fluke infecting the smalltooth sawfish from the eastern Gulf of Mexico. The new species is the first described blood fluke from a sawfish (Pristidae) and the second blood fluke described from a rhinopristiform (Cutmore et al. 2018).

MATERIALS AND METHODS

Smalltooth sawfish were reported stranded or dying to the Florida Fish and Wildlife Conservation Commission's Fish and Wildlife Research Institute (FWRI) Sawfish Hotline from the eastern Gulf of Mexico. When on-site monitoring of the sawfish by FWRI personnel began, the sawfish was alive but progressively weakened and died. Within 1.5 hr postmortem, the fish was packed on ice and shipped to the FWRI Charlotte Harbor Field Laboratory in Port Charlotte, Florida, where it was kept on ice until necropsy the following morning. All organs were excised and transported to FWRI headquarters in St. Petersburg, Florida, for parasitological evaluation. At necropsy, the heart and gill arches were excised intact and placed in separate columns (heart was bisected; gill arches separated). 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. Adult flukes for morphology were routinely heat-killed on glass slides using a butane hand lighter under little or no coverslip pressure.

Adult flukes 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. Measurements are reported in micrometers (μm) as the range followed by the mean 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), Warren et al. (2019), and Warren and Bullard *in review*). Specimens of a related aporocotylid (*O. glaucostegi*) were borrowed from the Queensland Museum (South Brisbane, Australia). Type and voucher materials of the new species were deposited in the National Museum of Natural History's Invertebrate Zoology Collection (USNM, Smithsonian Institution, Washington, D. C.).

RESULTS

New genus Warren et Bullard n. gen. (Figs. 1−8)

Generic diagnosis of adult (based on nine whole-mounted specimens; USNM coll. nos. XXXXX−XXXXX): Body extremely elongate, dorsoventrally flattened, having anterior and posterior ends tapering equally, aspinous. Rosethorn-shaped spines absent. Nervous system

indistinct. Anterior sucker aspinous, lacking peduncle, diminutive. Mouth subterminal. Pharynx absent. Oesophagus extending sinuously posteriad along midline for ≤1/4 of body length; posterior oesophageal swelling present. Intestine inverse U-shaped, asymmetrical; posterior caeca slightly shorter than oesophagus, connecting to oesophagus ventrally, lacking diverticula, terminating in anterior half of body. Testis single, medial, curving, lacking lobed margins, wholly posterior to intestine. Vas deferens short, extending posteriad from testis. Cirrus-sac present, enveloping internal seminal vesicle and cirrus. Internal seminal vesicle distinct, longer than vas deferens. Cirrus short, >8% of seminal vesicle length, curving sinistrally before everting. Auxiliary external seminal vesicle absent. Common genital pore dorsal, post-gonadal, far anterior and sinistral to oötype. Ovary medial, post-caecal; post-ovarian space comprising ≥1/6 of body length. Vitellarium follicular, diffuse, slightly asymmetrical, filling space between caecal bifurcation and testis. Laurer's canal absent. Oötype dextral, posterior to common genital pore, comprising an inconspicuous ovoid chamber. Uterus post-gonadal, not extensively convoluted, extending posteriad from oötype before curving anteriad before crossing midline and extending posteriad; uterine eggs oblong. Excretory vesicle small, medial, with arms, visible in posterior most region of body.

Differential diagnosis: Body approx. 50–70 × longer than wide; aspinous, lacking lateral tubercles. Anterior sucker aspinous, lacking peduncle, diminutive. Pharynx absent. Posterior oesophageal swelling present. Intestine inverse U-shaped, asymmetrical; posterior caeca terminating in anterior half of body, lacking diverticula. Testis single, curving, lacking lobed margins, curving >100 times. Internal seminal vesicle distinct, longer than vas deferens, enveloped by cirrus sac. Cirrus short, >8% of seminal vesicle length. Common genital pore post-caecal, post-gonadal, far anterior and sinistral to oötype. Ovary medial, post-caecal, dorsal to

posterior portion of testis, wholly anterior to uterus. Laurer's canal absent. Oötype posterior to common genital pore. Uterus post-gonadal, dorsal and flanking seminal vesicle, not extensively convoluted; uterine eggs small, occupying 1/3 of uterus.

Taxonomic summary

Type-species: New genus and new species Warren et Bullard n. sp. (Digenea: Aporocotylidae)

Type host: Smalltooth sawfish, *Pristis pectinata* Latham, 1796 (Rhinopristiformes: Pristidae).

New species Warren et Bullard n. sp. (Figs. 1−8)

Diagnosis of adult (based on nine whole-mounted specimens; USNM coll. nos. XXXXX−XXXXX):

Body 580–6680 (6178; 4) long, 100–160 (116; 4) at greatest width, 49–67 × longer than wide (Fig. 1). Nerve commissures and ventrolateral nerve-cords not evident in whole-mounts. Mouth 2–4 (3; 6) in diameter, 7–10 (8; 4) from terminal end of body (Fig. 2). Oesophagus 1030–1420 (1232; 5) in total length or 15–25% (21%; 5) of body length, 13–20 (16; 5) in maximum width (at level of pre-caecal dilation), with oesophageal wall gradually thickening and thinning throughout oesophagus, (Figs. 1–4); oesophageal gland enveloping oesophagus anterior to pre-caecal dilation, 33–73 (50; 6) long or 3–5% (4%; 5) of oesophageal length, 25–53 (31; 6) wide or 29–66% (44%; 5) of body width (Figs. 1, 3, 4). Caecal bifurcation 1030−1480 (1248; 5) or 15−27% (21%; 4) of body length from anterior body end; caeca extending posteriad in parallel, asymmetrical, dextral caecum 730−1300 (946; 4) long or 13–19% (16%; 4) of body length, sinistral caecum 520−1145 (767; 4) long or 9–17% (13%; 4) of body length, mean posterior caeca 13−41 (24; 4) wide or 24–51% (35%; 4) of body width (Figs. 1, 4), containing granular material within lumen of some individuals.

Testis 1950–3820 (2703; 6) long or 37–63% (46%; 4) of body length, 20−34 (26; 7) wide, occupying 50–60% (56%; 4) of body width, 78–191 (108; 6) × longer than wide, post-caecal, curving 109–129 (119; 6) times (Figs. 1, 5) until narrowing and becoming confluent with vas deferens. (Figs. 6–8); post-testicular space 1110–1460 (1268; 6) long or 19–22% (21%; 4) of body length. Vas deferens 20–50 (30; 6) long, 8–10 (8; 6) wide, emanating from postero-ventral portion of testis, extending posterior for a short distance before connecting to the cirrus sac (Figs. 6–8). Cirrus-sac having extremely thin wall 1–3 (2.4; 7) thick (Figs. 7, 8), including seminal vesicle and cirrus; seminal vesicle extending sinuously posteriad, 353–555 (444; 7) long or 6–8% (7%; 4) of body length, 13–20 (15; 7) wide or 14–26% (19%; 4) of body width, running between ascending and descending portions of the uterus, ultimately narrowing and curving sinistral towards body margin (Figs. 1, 6–8); cirrus 40–78 (55; 3) long or 9–14% (11%; 3) of seminal vesicle length, 5–10 (7.6; 5) wide or 33–71% (53%; 5) of seminal vesicle width (Figs. 7, 8); everted cirrus short, observed in two specimens, 10 and 18 (14; 2) long, 8 and 10 (9; 2) wide, cirrus pore 10–18 (13; 2) in diameter. Common genital pore 630–830 (735; 7) or 9–14% (12; 4) of body length from posterior end of the body, 8–13 (10; 7) from sinistral body margin, 60–88 (76; 7) from dextral body margin (Figs. 6–8).

Ovary medial, 123–140 (132; 6) long or 2–3% (2%; 4) of body length, 38–65 (55; 7) wide or 45–52% (49%; 4) of body width, 2–3 (2.3; 6) × longer than wide, appears as aggregation of ova anterior to male and female reproductive ducts; post-ovarian space 1010–1250 (1105; 7) long or 17–19% (18%; 4) of body length (Figs. 6–8). Oviduct originating from posterior margin of ovary, sharply curving twice before running dextrad and widening to form distal portion (Figs. 7, 8); distal potion of oviduct a narrow tube extending approximately in parallel with and between body margin and ascending uterus, 653–796 (704: 6) long or 11–12% (11.3; 3) of body length,

10–18 (14; 6) in maximum width, curving sinistral toward medial body line body, then curving dextral before connecting with primary vitelline duct (Figs. 6–8). Primary vitelline duct 702–1050 (806; 6) long, 5–10 (8; 6) wide, indistinct anterior of ovary. Laurer's canal not observed. Oötype 25–30 (27; 6) long, 23–30 (26; 6) wide (Figures 6–8). Uterus extending directly posterior from oötype 618–856 (709; 7) long, 18–28 (24; 7) in maximum width, before curving back anteriorly 133–238 (175; 7) from posterior end and connecting to ascending portion (Figs, 7, 8); total ascending portion 403–698 (548; 7) long or 13–17% (15%; 3) of body length, 30–53 (43; 7) wide (Figs. 7, 8), sharply curving sinistrally to posterior margin of ovary, dorsal to seminal vesicle, before connecting with descending portion; descending portion extends posteriorly 297–416 (360; 7) long or 5–6% (5.7; 3) of body length, 20–35 (28; 7) wide. Uterine eggs 8–13 (10; 7) in diameter or 27–50% (36%; 7) of uterus width, containing many small dense bodies, with thin shell (Figs. 7, 8). Excretory vesicle 10–43 (19; 5) long, 3–15 (7; 5) wide, with arms 93 (1).

Taxonomic summary

Type and only reported host: Smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae).

Site in host: Heart lumen.

Type locality: Eastern Gulf of Mexico

Prevalence and intensity of infection: one smalltooth sawfish sampled was infected with 13 specimens of the new species.

Specimens deposited: Holotype (USNM XXXXXXX), paratypes (USNM XXXXXXX − XXXXXXX).

DISCUSSION

The new species is most similar to *O. glaucostegi* and the other blood flukes that infect batoids (Madhavi and Rao 1970, Bullard and Jensen 2008, Warren and Bullard *in review* [excluding *Gymnurahemecus bulbosus* Warren et Bullard, 2019]) by the combination of having a diminutive anterior sucker that lacks spines, an asymmetrical, inverse U-shaped intestine, a curving testis, an internal seminal vesicle and cirrus sac, and a post-caecal common genital pore, as well as by lacking lateral tegumental spines. It differs from *O. glaucostegi* by the combination of having a body that is >50 (vs. <30) × longer than wide, a testis that is >75 (vs.<40) × longer than wide, and has >100 (vs. <70) curves, an ovary positioned wholly anterior to (vs. lateral and dorsal to) the seminal vesicle, a uterus positioned wholly posterior to (vs. overlapping and lateral to both) the testis and ovary, and a sinuous (vs. convoluted) uterus. The new species differs from *Orchispirium heterovitellatum* Madhavi et Rao, 1970 and *Myliobaticola richardheardi* Bullard et Jensen, 2008 by the combination of having a body that is that is >50 (vs. 3) × longer than wide, a testis with >100 (vs. 21 and 10, respectively) curves, and a uterus that is sinuous (vs. extensively convoluted). Further, *O. heterovitellatum* differs by having lateral tubercles along the body, an intestine bearing diverticula, and a testis that is inter-caecal and that has lobed margins. The new species does not have lateral tubercles along the body margin, diverticula, and lobed margins along the testis, as well as a testis that is wholly post-caecal. *Myliobaticola richardheardi* further differs by having a winding (vs. sinuous) oesophagus and seminal vesicle. The new species differs from *Electrovermis zappum* Warren et Bullard, *in review* by the combination of having a body that is >50 (vs. <40) × longer than wide, a testis that is >30% (vs. <20%) of the body length and has >100 (vs. <40) curves, a seminal vesicle that occupies <17% (vs. >40%) of the body, a cirrus that is <15% (vs. 65%) of the seminal vesicle length, and an ovary that is positioned <20% (vs. >30%) of the body length from the posterior body end.

The new species is the fifth chondrichthyan blood fluke that has been characterized as lacking spines, and all of these species infect batoids (Madhavi and Rao 1970, Bullard and Jensen, 2008, Cutmore et al. 2018, Warren and Bullard *in review*). Based on the morphological similarity we infer that the new species shares a recent common ancestor with *O. glaucostegi*. The remaining nominal chondrichthyan blood flukes have large C-shaped spines; four infecting sharks: *Hyperandrotrema cetorhini* Malliard et Ktari 1978, *H. walterboegeri* Orélis-Ribeiro et Bullard, 2013*, Selachohemecus olsoni* Short, 1954, *S. benzi* Bullard, Overstreet et Carlson, 2006; one infecting a holocephalan: *Chimaerahemecus trondheimensis* Van der Land, 1967; and one infecting a batoid: *G. bulbosus* (Short 1954, Van der Land 1967, Malliard and Ktari 1978, Bullard et al. 2006, Orélis-Ribeiro et al. 2013, Warren et al. 2019). Shirai (1996) formalized two distinct lineages of elasmobranchs: Galea (Orectolobiformes, Lamniformes, and Carcharhiniformes) and Squalea (Chlamydoselachiformes, Hexanchiformes, Echinorhiniformes, Dalatiiformes, Centrophoriformes, Squaliformes, and all batoids). The morphological comparisons of the nominal chondrichthyan blood flukes support this classification. For example, species of *Hyperandrotrema* and species of *Selachohemecus* infect lamniform and carcharhiniform sharks (Galea), respectively and species of both genera have C-shaped spines. *Ogawaia glaucostegi*, *M. richardheardi*, *O. heterovitellatum*, and *E. zappum* all infect batoids (Squalea) and all lack tegumental spines. However, *C. trondheimensis* and *G. bulbosus* contradict this hypothesis by having C-shaped spines and infecting a holocephalan and a batoid, respectively. Further, none of the nominal chondrichthyan blood flukes that have C-shaped spines have a curving testis, like those infecting batoids (excluding *G. bulbosus*). *Chimaerahemecus trondheimensis*, *H. cetorhini*, *H. walterboegeri*, and *G. bulbosus* also have an oötype type located posterior to all other genitalia, unlike the species of *Selachohemecus* that has

a oötype located anterior to the common genital pore (Van der Land 1967, Malliard and Ktari 1978, Bullard et al. 2006, Orélis-Ribeiro et al., 2013, Warren et al. 2019). Additionally, *S. olsoni* and *S. benzi* are unlike the nine other chondrichthyan blood flukes by having an X-shaped (vs. inverse U-shaped) intestine. This character state has only been observed in fish blood flukes infecting ray-finned fish (Actinopterygii) lineages (Bullard and Overstreet 2006, Bullard 2010). These morphological differences are vital to understanding the systematics of the underrepresented chondrichthyan blood flukes.

Acknowledgments. We thank Florida Fish and Wildlife Conservation Commission for facilitating the collection of the new species; and Mal Bryant and Robert Adlard (Queensland Museum) for loaning type aporocotylids (*O. glaucostegi*). This study was supported by research grants from the Alabama Marine Resources Division, Auburn University Vice President for Research and Economic Development, Southeastern Cooperative Fish Parasite and Disease Project (Alabama Department of Conservation and Natural Resources), Alabama Agriculture Experiment Station, and US Department of Agriculture. Earlier exploratory aspects of this project were supported by grants from the National Science Foundation.

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FIGURE LEGENDS

Figs. 1–2 New genus and species Warren et Bullard (Digenea: Aporocotylidae) from the heart of the smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae). (**1**) Body of holotype (USNM No. XXXXX), ventral view. Mouth (mo), oesophagus (os), intestine (in), vitellarium (vit), testis (t), ovary (ov), seminal vesicle (sv), uterus (u), common genital pore (cgp), oötype (oo). Bar = 1000 μm. (**2**) High magnification of anterior sucker, holotype (USNM No. XXXXX), ventral view showing mouth (m). Bar = 50 μm.

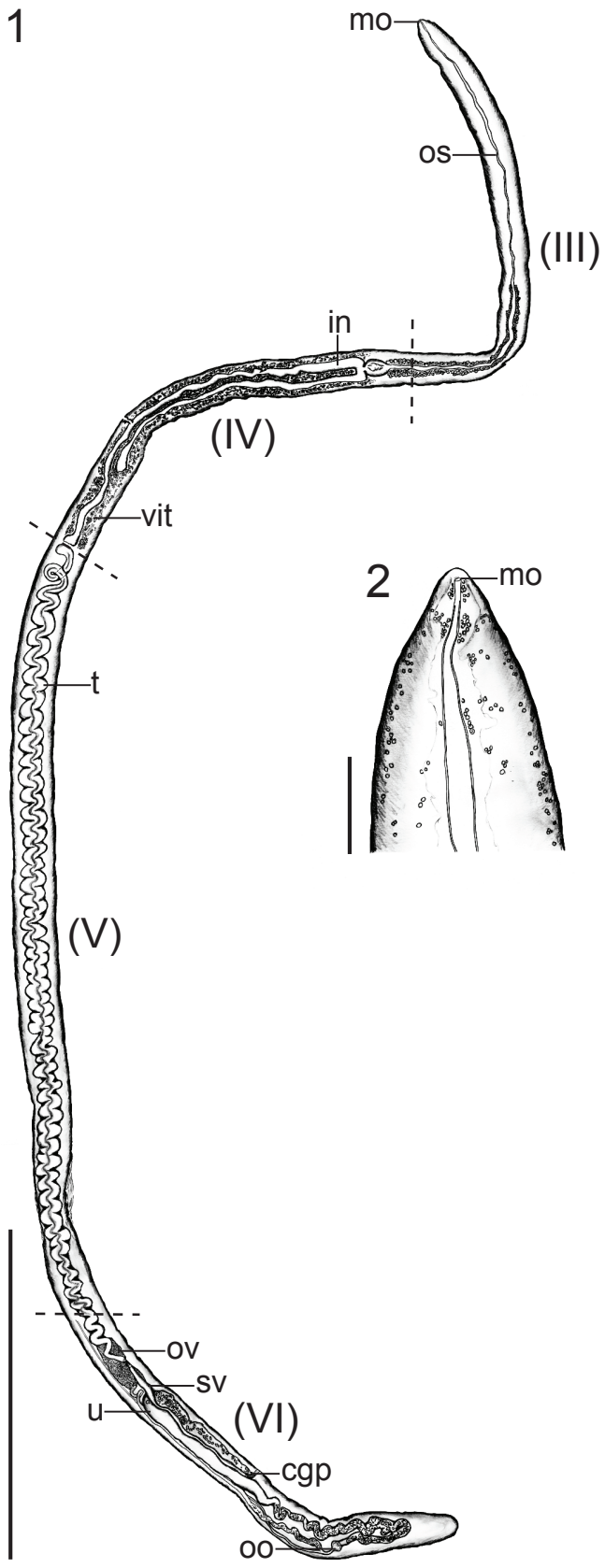
Figs. 3–6 New genus and species Warren et Bullard (Digenea: Aporocotylidae) from the heart of the smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae). (**3**) Anterior body segment (III) showing mouth (mo), oesophagus (os), oesophageal gland (og). (**4**) Body segment (IV) showing oesophageal gland (og), caecal bifurcation (cb), vitellarium (vit). (**5**) Body segment (V) showing testis (t), vasa efferentia (ve). (**6**) Posterior body segment (VI) showing ovary (ov), vas deferens (vd), seminal vesicle (sv), oviduct (o), ascending uterus (au), descending uterus (du), common genital pore (cgp), cirrus (c), vitelline duct (v), oötype (oo). Bar = 250 μm.

Fig. 7 New genus and species Warren et Bullard (Digenea: Aporocotylidae) from the heart of the smalltooth sawfish, *Pristis pectinata* Latham, 1794 (Rhinopristiformes: Pristidae). (**7**) Genitalia of paratype (USNM No. XXXXX), ventral view showing testis (t), vas deferens (vd), ovary (ov), seminal vesicle (sv), oviduct (o), ascending uterus (au), descending uterus (du), common genital pore (cgp), everted cirrus (ec) vitelline duct (v), oötype (oo). Bar = 250 μm.

Table 1. Fish blood flukes (Digenea: Aporocotylidae) described from North America since 2000.

Parasite	Host	Locality	Reference
Acipensericola glacialis Warren et Bullard, 2017	lake sturgeon, *Acipenser fulvescens* Rafinesque, 1817	Lake Winnebago, Wisconsin, USA	Warren et al. 2017
Acipensericola petersoni Bullard, Jensen et Overstreet, 2008	American paddlefish, *Polyodon spathula* (Walbaum, 1792)	Mississippi River and Tennessee River (Mississippi River Basin), USA	Bullard et al. 2008
Cardallagium anthicum (as *Psettarium*) Bullard et Overstreet, 2006	cobia, *Rachycentron canadum* (Linnaeus, 1766)	Northern Gulf of Mexico approx. 50 km south.southeast of Ocan Springs, Mississippi, USA	Bullard and Overstreet 2006
Cardicola currani Bullard et Overstreet, 2004	red drum, *Sciaenops occelatus* (Linnaeus, 1766)	Northern Gulf of Mexico, Davis Bayou, Mississippi Sound, Ocean Springs, Mississippi, USA	Bullard and Overstreet 2004
Cardicola langeli Bullard, 2013	sheepshead, *Archosargus probatocephalus* Walbaum, 1792	Northern Gulf of Mexico, off Horn island, Mississippi sound, Mississippi, USA	Bullard 2013
Cardicola nonamo Bullard, 2009	white seaperch, *Phanerodon furcatus* Girard, 1854	Eastern Pacific Ocean, Monterey Bay, California, USA	Bullard 2009
Cardicola palmeri Bullard et Overstreet, 2004	black drum, *Pogonias cromis* (Linnaeus, 1766)	Mississippi Sound off Point Cadet, Biloxi, Mississippi, USA	Bullard and Overstreet 2004
Cardicola parvus Bullard, 2012	Atlantic croaker, *Micropogonias undulatus* (Linnaeus, 1766)	South Atlantic Bight, off Cow Island, North Carolina, USA	Bullard et al 2011
Elaphrobates euzeti Bullard et Overstreet, 2003	red snapper, *Lutjanus campechanus* (Poey, 1860)	Northern Gulf of Mexico approx. 50 km south.southeast of Ocan Springs, Mississippi, USA	Bullard and Overstreet 2003
Electrovermis zappum Warren et Bullard, *in review*	lesser electric ray, *Narcine bancroftii* (Griffith and Smith, 1834)	Northern Gulf of Mexico, off Fort Morgan, AL, USA	Warren and Bullard *in review*
Elopicola franksi Orélis-Ribeiro et Bullard, 2017	Atlantic tarpon, *Megalops atlanticus* (Valenciennes, 1847)	North Captiva Island and Bayboro Harbor, off Florida, Gulf of Mexico, USA	Orélis-Ribeiro et al. 2017
Elopicola nolancribbi Bullard, 2014	lady fish, *Elops saurus* Linnaeus, 1766	Northern Gulf of Mexico, off Ship island, Mississippi sound,	Bullard 2014

		Mississippi, USA	
Gymnurahemecus bulbosus Warren et Bullard, 2019	smooth butterfly ray, *Gymnura micrura* (Bloch and Schneider, 1801)	Gulf of Mexico, Mobile, AL, USA	Warren et al. 2019
Hyperandrotrema walterboegeri Orélis-Ribeiro et Bullard, 2013	shortfin mako shark, *Isurus oxyrinchus* Rafinesque, 1810	Viosca Knoll, northern Gulf of Mexico, 123 km south/southwest of Dauphin Island, Alabama, USA	Orélis-Ribeiro et al. 2013
Littorellicola billhawkinsi Bullard, 2010	Florida pompano, *Trachinotus carolinus* (Linnaeus, 1766)	Northern Gulf of Mexico, off Ship island, Mississippi sound, Mississippi, USA	Bullard 2010
Myliobaticola richardheardi Bullard et Jensen, 2008	Atlantic stingray, *Hypanus sabinus* (Lesueur, 1824)	Deer Island, Mississippi Sound, Northern Gulf of Mexico off Biloxi, Mississippi, USA	Bullard and Jensen 2008
Pearsonellum lemusi Bullard, 2012	gag grouper, *Mycteroperca microlepis* (Goode and Bean, 1879)	North central Gulf of Mexico, Groper reef, Approx. 80 km south of Dauphin Island, Alabama, USA	Bullard 2012
Primisanguis caribbeanensis, Bullard, 2012	stoplight parrotfish, *Sparisoma viride* (Bonnaterre, 1788)	Caribbean Sea, off La Parguera, Puerto Rico	Bullard et al. 2012
Selachohemecus benzi Bullard, Overstreet et Carlson, 2006	blacktip shark, *Carcharhinus limbatus* (Valenciennes, 1839)	Apalachicola Bay, Florida, USA Northern Gulf of Mexico, off Mississippi, USA	Bullard et al. 2006
New genus and species Warren et Bullard	smalltooth sawfish, *Pristis pectinata* Latham, 1796	Central Florida, Gulf of Mexico, USA	present study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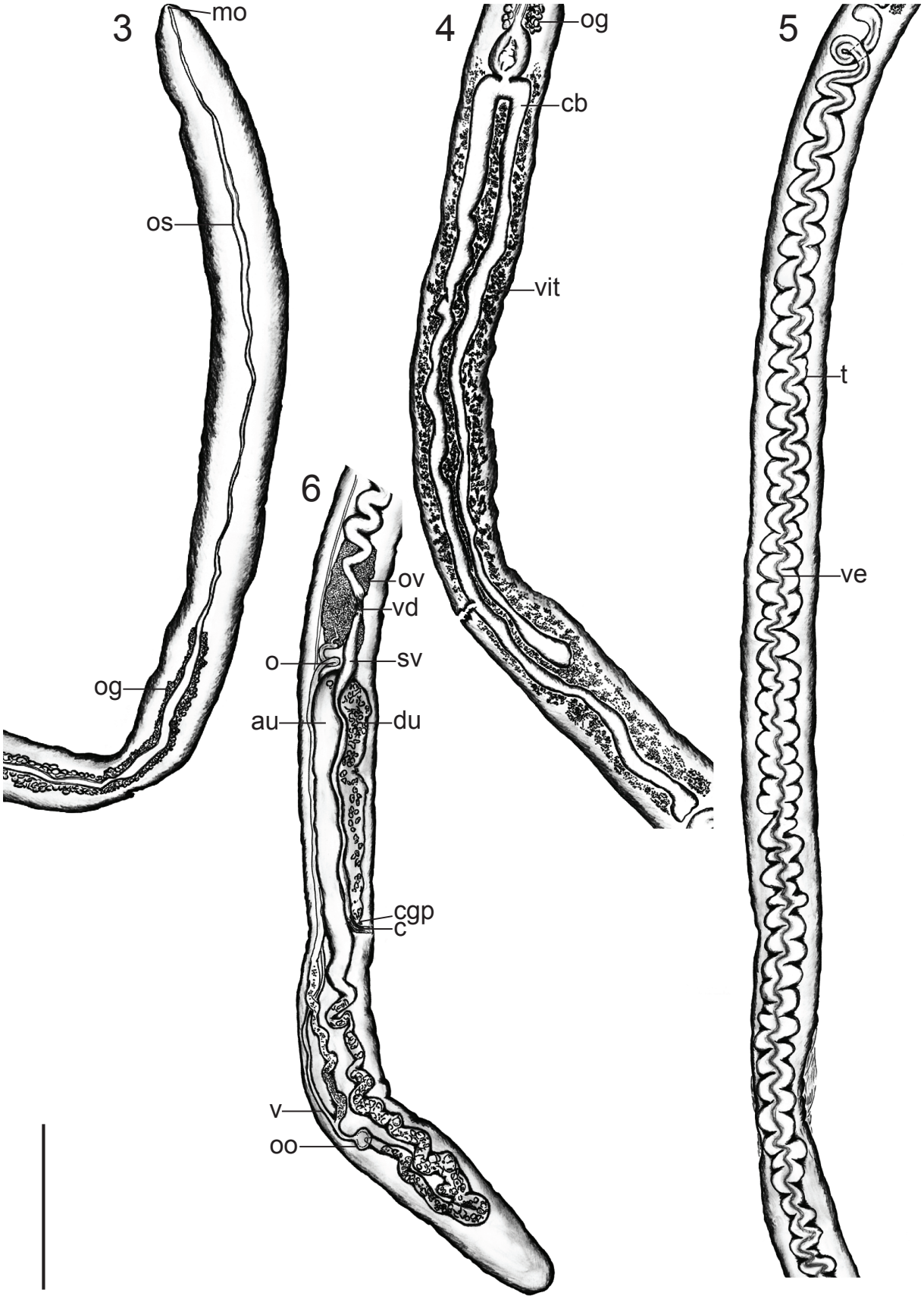
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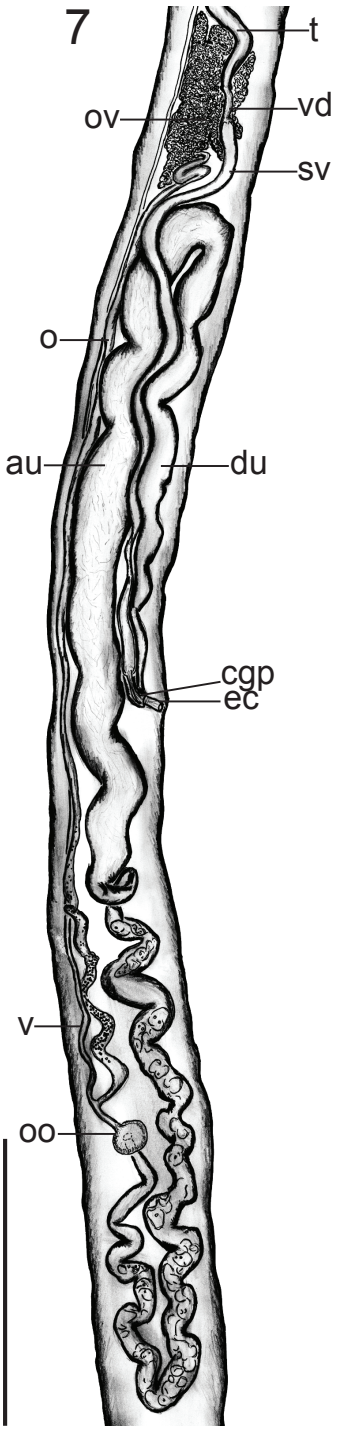
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