Taxonomy, systematics, and life cycles of Azygiids (Digenea: Azygiidae) of the Southeastern United States

by

Matthew Ryan Womble

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Approved by

Stephen Bullard, Chair, Associate Professor
Cova Arias, Member, Professor
Dennis DeVries, Member, Professor
Matthew Catalano, Member, Assistant Professor

ABSTRACT

Digeneans (Platyhelminthes: Trematoda: Digenea) comprise the vast majority of Trematoda, which is one of three entirely parasitic classes within the Platyhelminthes. They are characterized as having complex life cycles involving asexual reproduction in invertebrates, sexual reproduction in vertebrates, and free-living dispersal stages between/among those requisite hosts. In this thesis, using morphology and genetic data, I aimed to taxonomically characterize the life history stages of species of *Proterometra* Horsfall, 1933 and *Leuceruthrus* Marshall and Gilbert, 1905 (Digenea: Azygiidae), which undergo asexual reproduction in snails of Pleuroceridae*,* and mature in the buccal cavity (=*Proterometra* spp.) and stomach (=*Leuceruthrus* spp.) of freshwater fishes, primarily those of Centrarchidae. These flukes are unique among other digeneans by having furcocystocercous cercariae that are macroscopic, progenetic (i.e., *Proterometra* spp.), and when shed from the snail host seemingly mimic a fish prey item thus luring the fish host to consume it. This work has culminated in the taxonomic characterization of life history stages of 5 species of *Proterometra* and 4 species of *Leuceruthrus* obtained from 6 river drainages and 4 states, established geographic ranges and summarized all accounts for nominal species of both genera, and provided a taxonomic review/ updated diagnosis for both genera. Additionally, and comprising the largest phylogenetic taxon sampling for Azygiidae to date, molecular data from the ribosomal internal transcribed spacer 2 (ITS2) aided in confirming

conspecificity among cercariae and adults of both *Proterometra* and *Leuceruthrus*, and did not reject the generic assignments of species described herein, or monophyly of either *Proterometra* or *Leuceruthrus.*

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Authors: Matthew R. Womble, Raphael Orélis-Ribeiro, and Stephen A. Bullard

ABSTRACT

Proterometra epholkos sp. n. asexually reproduces in the stream dwelling prosobranch, *Elimia* cf. *modesta* (Cerithioidea: Pleuroceridae) and infects the buccal cavity epithelium of spotted bass, *Micropterus punctulatus* (Perciformes: Centrarchidae) in the Coosa River (Terrapin Creek; N33°51'36.56", W85°31'28.15"; Cleburne County, Alabama, USA). We characterize cercariae and adults of the new species using morphology and molecular sequence data and redescribe its morphologically similar congener *P. albacauda* based on the holotype and paratype (USNPC Nos. 61229-30). The new species can be distinguished most easily from *P. albacauda* by the combination of having cercariae with long mamillae (>100 μm) that encircle the tail stem anteriorly, that are restricted to 1 lateral column per body margin at midbody, and that are absent from the medial surface of the tail stem as well as by having adults with a partly extra-caecal uterus, a transverse metraterm occupying the space between the oral sucker and prostatic sac, and a vitellarium that is longer than the caeca and extends anteriad to the level of or beyond the posterior margin of the oral sucker. Sequence data from the ribosomal internal transcribed spacer 2 (ITS2; 251 bp) did not reject the notion that the cercariae and adults we collected

simultaneously from those infected, sympatric, individual snails and fish in Terrapin Creek were conspecific. Also provided herein for species of *Proterometra* are (i) taxonomic keys for cercariae and adults based on morphological and behavioral characteristics sourced from the published literature, (ii) updated lists of host records (prosobranchs and fishes) and geographic locality records for *Proterometra* spp., and (iii) synopses and assessments of the morphological features previously used to differentiate them. *Proterometra macrostoma* (type species), *P. melanophora*, and *P. hodgesiana* are *species inquirendae*; requiring new collections from type localities and hosts concomitant with neotype designations. *Proterometra macrostoma* seems a repository for conspicuous, furcocystocercous cercariae shed from freshwater prosobranchs in eastern North American rivers and streams. The specific epithet "*pinguis*" associated with specimens purportedly infecting *Esox lucius* and deposited by JF Mueller is a *nomen nudum*. *Proterometra guangzhouensis*, *P. sillagae*, *P. brachyuran*, and *P. lamellorchis* are *incertae sedis*. Significant barriers to characterizing biodiversity and distributions (host range and geographic distribution) of *Proterometra* spp. comprise a paucity of data on adult morphology, dubious species-level identification or a lack of information regarding prosobranch hosts, lack of molecular data for putative comparisons among fluke 'strains' and species as well as between cercariae and adults, lack of consistency in terminology, and indeterminate homology for key morphological features. Uncertainty about the providence and identity of, or absence of, accessioned museum materials of *P. macrostoma*, *P. catenaria*, and *P. hodgesiana* together represent another fundamental problem. The present study comprises the first description of a new species of *Proterometra* in

nearly 20 yrs, first report of a species of the genus from the Coosa River (Mobile-Tensaw River Basin) and from these host species, and first use of molecular sequence data to elucidate a life cycle for a species of *Proterometra*.

1. INTRODUCTION

Proterometra Horsfall, 1933 (Digenea: Azygiidae) is readily distinguished from the remaining 3 genera of Azygiidae, i.e., *Azygia* Looss 1899, *Otodistomum* Stafford 1904, and *Leuceruthrus* Marshall and Gilbert 1905, by having testes that are transverse, abreast, and positioned near the posterior extremity of the body as well as having a uterine field and vitellarium that each extend anteriad beyond the prostatic sac, occupying the space between it and the oral sucker [1,2]. No infection by an accepted species of *Proterometra* has been documented from beyond North America (in chronological order): *P. macrostoma* (Faust, 1918) Horsfall, 1933 (type species; *species inquirenda*), *P. melanophora* (Smith, 1932) Smith 1936 (herein treated as *species inquirenda* but considered a junior subjective synonym of *P. macrostoma* by other authors), *P. hodgesiana* (Smith, 1932) Smith, 1936 (*species inquirenda*), *P. catenaria* Smith, 1934, *P. sagittaria* Dickerman, 1946, *P. dickermani* Anderson, 1962, *P. albacauda* Anderson and Anderson, 1967, *P. septimae* Anderson and Anderson, 1967, *P. edneyi* Uglem and Aliff, 1984, and *P. autraini* Labeau and Peters, 1995 (Tables 1, 2). We concur with Labeau and Peters [29] and Gibson [38] in excluding *Proterometra guangzhouensis* Lu (1992), *P. sillagae* Wu (1997), *P. brachyuran* Wu (1997), and *P. lamellorchis* Wu (1997) from *Proterometra* and herein consider these

species *incertae sedis*. Species of *Proterometra* are endemic to North American freshwater environments, and only one report details an infection west of the main stem of the Mississippi River [30] (Table 2).

Since Dickerman [33], experimental infections of *Proterometra* spp. (8 of 10 species) have indicated that these flukes undergo asexual reproduction in freshwater prosobranch snails, primarily species of *Elimia* but also *Pleurocerca acuta* [1,6], *Lithasia obovata* [4; see Graff [39] for synonymy], and *Campeloma subsolidum* [3], that range in rivers and streams draining to the Great Lakes, Gulf of Mexico, and Atlantic Ocean (Table 1). Of note is that, in an abstract, Dickerman [40] stated that he succeeded in feeding cercariae to "fish and turtles;" however, no subsequent work, including Dickerman [13,24], mentions a turtle host for any species of *Proterometra*. These flukes have a macroscopic, 3–22 mm long, furcocystocercous cercaria that can be progenetic [25,29,41] and flamboyantly swims in the water column, perhaps luring the fish definitive host to bite and/or swallow it [22,35]. The cercarial body (= "distome") is typically withdrawn inside a cavity (= "tail cavity") within the anterior or midbody portion of the tail stem before cercarial emergence (= "shedding") from the gastropod. No encysted metacercaria has been reported for any species of *Proterometra*, leading some to regard the 2-host life cycle of these flukes as truncated [42]. Adults develop rapidly (if the cercaria is not already egg-bearing, progenetic) upon infecting the epithelial surfaces of the buccal cavity, esophagus, esophageal sphincter, and occasionally gut [22] of sunfishes (*Lepomis*) and black basses (*Micropterus*) (Perciformes: Centrarchidae) as well as representatives of five other families of primary division freshwater fishes in North America (Table 2).

Historically, these flukes have been differentiated based on primarily the habitus and behavior of their charismatic cercariae, which first were described by Faust [3] as "*conspicuous objects*" and which have likewise attracted aquatic biologists within and beyond parasitology since the early 20th century. Perhaps because few parasitologists specifically examine the buccal cavity and esophageal sphincter of fishes for digenean infections and perhaps because of the appeal of rather focusing on the charismatic free-living cercariae, adults of *Proterometra* spp. have not garnered the same level of anatomical scrutiny as their cercariae (Tables 3-6). As a result, relatively little information is available on diagnostic features of adults of *Proterometra* spp. Some authors consider adult specimens to be indistinguishable across species, e.g., Anderson and Anderson [22] stated that, *"adults [sic] of all seven* (species of *Proterometra*) *are very similar and their separation would be questionable were it not for the striking differences between their cercariae."* Another gap comprises the fact that molecular data have yet to be employed in differentiating species of *Proterometra*; one sequence (28S) exists in GenBank (*Proterometra* sp.) [43].

Towards contributing to the knowledge of *Proterometra* spp., the addition of morphological features of adults and cercariae, and the development of molecular taxonomic information, we herein use morphology and molecules to explore the biodiversity and life history of these azygiids in Alabama. Also provided herein for species of *Proterometra* are taxonomic keys for cercariae and adults based on morphological and behavioral characteristics sourced from their respective original taxonomic works and select museum materials, updated lists of hosts (prosobranchs

and fishes) and geographic localities for *Proterometra* spp., and synopses and assessments of the morphological features previously used to differentiate them.

2. MATERIALS AND METHODS

Prosobranch snails (hereafter “snails”) were collected by hand while snorkeling in Terrapin Creek (South Fork; N33°51’36.56”; W85° 31’28.15”; Cleburne County, Alabama, USA) on 22 May 2013 and immediately thereafter transported to the laboratory in 20-L plastic buckets filled with ambient stream water and aerated using battery-powered aerators and airstones. They were identified as *Elimia* cf. *modesta* (Lea, 1845) as per Thompson [44] by having (i) 4 rust colored bands visible through aperture, (ii) broadly conical shell, (iii) shoulder whorls smooth, and (iv) adults lacking carina. In the laboratory, snails were isolated individually in cell culture well plates modified with perforated lids and bottoms, allowing water flow through each well, and immersed in 40-L aquaria filled with aerated, filtered, and de-chlorinated tap water [9]. Snails were monitored for 36 h and indicated as infected by the presence of swimming, naturally-shed cercariae within their particular well. Infected snails immediately were relocated to 1-L glass jars filled with aerated aquarium water. The total volume of each container was changed semiweekly. Swimming cercariae were collected with a large bore plastic pipette and transferred to a glass jar wherein their behavior could be observed without aeration current before being preserved for morphology and molecular biology. Fluke specimens for morphology were wet-mounted on glass slides and heat killed under slight coverslip pressure with heat from

an EtOH burner flame or isolated and heat killed within a dish flooded with freshwater heated to 60°C. Killed specimens were then transferred to a vial of 10% neutral buffered formalin. Specimens for molecular biology were pipetted from the aquaria alive and immediately preserved in a vial of 95% EtOH and stored at -20°C. Germinal sacs and intramolluscan cercariae were separated from soft tissues of infected snails by transverse fracturing of each shell using a 150 mm Bessey workbench vice before teasing apart snail soft tissues from parasites in a petri dish filled with stream water and with the aid of a dissecting microscope. Tissue vouchers (snail soft tissue) were placed in a vial of 95% EtOH and stored at -20°C. Shell vouchers for each infected snail were placed in a vial of 70% EtOH and deposited in the United States National Museum (USNM), Smithsonian Institution, Washington DC, USA.

Fish hosts were collected with a cast net from Terrapin Creek on 22 May 2013 and 24 October 2013, maintained alive in a cooler filled with ambient stream water and aerated using battery-powered aerators and airstones, transported to the laboratory, killed by spinal severance, measured, and identified as spotted bass (*Micropterus punctulatus* [Rafinesque, 1819]) as per Boschung and Mayden [45] by having (i) black lateral stripe with bars or blotches extending along lateral line, (ii) >13 scale rows on cheek, (iii) tooth patch on tongue, (iv) pyloric ceca un-branched, and (v) rows of spots beneath level of lateral line. Shears were used to hemisect the jaw and the buccal cavity to reveal epithelial surfaces before inspection with the aid of a stereoscope at 50× magnification. Flukes were removed using fine forceps or brushes and preserved as cercariae were (see above).

All flukes, adults and cercariae, intended for morphology were left in fixative for at least 48 h and subsequently rinsed overnight in distilled water, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, made basic in 70% ethanol with lithium carbonate and butyl-amine, dehydrated, cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Measurements, and illustrations of stained, whole-mounted specimens were made with aid of a Leica DM-2500 equipped with differential interference contrast (DIC) optical components and a drawing tube. Measurements are herein reported in micrometers (μm) followed by the mean and number of specimens measured for that feature in parentheses.

Specimens for molecular biology comprised 1 adult and 2 cercariae. Total genomic DNA from collected specimens was extracted using a DNeasy™ Blood and Tissue kit (Qiagen) according to the manufacturer's protocol. PCR was carried out using the forward primer GA1" (5'-AGA ACA TCG ACA TCT TGA AC-3') (3' end of 5.8S rDNA) [46] and the reverse primer "ITS2.2" (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') (5' end of 28S rDNA) [47] and sequenced as per Nolan and Cribb [48]. The PCR products were purified using the QIAquick Gel Extraction Kit (QIAGEN) following the manufacturer's protocols. DNA sequencing was performed by Lucigen with an ABI 3730xl sequencer at Lucigen Corp. (Madison, WI) using the same primers as used in the PCR. Chromatograms were analyzed with BioNumerics® version 7.0 (Applied Maths, Saint-Martens-Latem, Belgium), and amplification primer sequences were trimmed after consensus sequences between both strands were generated. IUPAC ambiguity codes were used for coding polymorphic sites, i.e., M should be read as the

presence of A and C, rather than as an ambiguous reading between A or C. These positions were identified in the chromatogram by the occurrence of overlapping peaks, where both signals differ from each other by less than 20% [49,50]. To further rule out the inclusion of low quality reads as polymorphisms, we added the restriction that double peaks had to occur on the same position on both forward and reverse strands. The ITS2 region was identified using the annotation tool of “ITS2 Database” [51]. All sequences were deposited as Genbank accession numbers KM503118 and KM503119

Nomenclature of snails, fish, and digeneans is as follows. Common names for snails follows Johnson et al. [52]. Higher-level gastropod nomenclature and classification follows Graff [39], Johnson et al. [52], Burch and Tottenham [53], and Burch [54,55]. Pre-1995 works assigned species of *Elimia* to *Goniobasis*, but we herein treat the latter genus as a junior subjective synonym of *Elimia* [56]. Common names for fishes follows Boschung and Mayden [45], and higher-level fish classification follows Nelson [57]. Prior to Horsfall [1], wherein she proposed *Proterometra*, species of *Proterometra* were assigned to the collective group name *Cercaria* [3,17,20] (see International Code of Zoological Nomenclature [ICZN] page 70, article 67.14). Subsequent to the establishment of *Proterometra*, published works assigned the adults and cercariae separately, with the former being assigned to *Proterometra* and the latter to *Cercaria* [1,6,15,16,24,33]. To provide taxonomic resolution within *Proterometra* we discuss conspecificity of species assigned to *Cercaria* and treat those species as junior subjective synonyms of their conspecific adults assigned to *Proterometra*.

Type materials were borrowed from the United States National Parasite Collection (USNPC), Beltsville, Maryland, USA, courtesy of Dr. Eric Hoberg and Pat Pilitt, and type materials of the new species were deposited in the USNM.

3. RESULTS

3.1. Proterometra albacauda *Anderson & Anderson, 1967 (Figs. 1–6)*

3.1.1 Diagnosis of adult based on light microscopy of holotype (USNM No. 61229).

Body of adult 2160 long, 1240 wide or 1.7× longer than wide, anterior end narrower than posterior end; tegument approximately 10 thick; tegumental projections (not illustrated) dense anteriorly, sparse at level of and posterior to ventral sucker, not associated with rim of oral sucker or ventral sucker (Fig. 1). Excretory pore medial, terminal. Nervous system indistinct. Oral sucker subterminal, 160 or 7% of body length from anterior body end, 640 long or 31% of body length, 670 wide or 54% of body width, posterior margin 440 from anterior margin of ventral sucker. Ventral sucker in posterior half of body, with anterior margin 1240 or 57% of body length from anterior body end, 330 long or 15% of body length, 370 wide or 30% of body width, 52% of oral sucker length, 56% of oral sucker width. Mouth opening ventrally, 55 long, 238 wide, 4.3× wider than longer. Pharynx ovoid, muscular, immediately posterior to oral sucker, 105 long or 5% of body length, 125 wide or 1.2× wider than longer (Fig. 1). Oesophagus extending posteriad from mouth 470 before bifurcating immediately posterior to pharynx, with oesophageal branches arching posterolaterad before joining with intestinal caecae; each oesophageal branch extending posterolaterad 305

(dextral) and 245 (sinistral), constricted distally by a muscular sphincter; intestinal caecae confluent with oesophageal branches, appearing inverse U-shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caecae; dextral caecum 1240 long or 57% of body length, beginning 940 or 44% of body length from anterior end of body, post-caecal space, 110 or 5% of body length from posterior end; sinistral caecum 1340 long or 62% of body length, beginning 800 or 37% of body length from anterior end of body, post-caecal space 90 or 4% of body length.

Testes 2 in number, transverse, abreast, oblique, ovoid to pyriform; dextral testis 335 long or 15% of body length, 180 wide or 15% of body width, post testicular space 160 or 7% of body length; sinistral testis 225 or 10% of body length, 140 wide or 11% of body width, post testicular space 55 or 3% of body length (Fig. 1). Vasa efferentia approximately 5 wide, coalescing into 2 main collecting ducts extending anteriad from testes and lateral to ventral sucker before connecting with seminal vesicle (= a robust vas deferens enveloped by prostatic sac) dorsally and in posterior half of prostatic sac (Figs. 1, 2). Prostatic sac medial, anterior margin 90 from posterior margin of oral sucker, posterior margin 90 from anterior margin of ventral sucker, occupying space between pharynx and ventral sucker, 250 long, 280 wide, having a highly muscular wall incorporating myriad gland-like cells, enveloping seminal vesicle and pars prostatica. Seminal vesicle thin walled, highly convoluted, having swollen proximal region and narrow distal region; proximal region of seminal vesicle extending sinuously anteriad, S-shaped, 448 long, 60 in maximum width; distal region highly convoluted, 220 long, 23 in maximum width, connected to pars prostatica via a minute duct. Minute duct (= possibly “verschlussapparat” of Looss [58] in Horsfall [6], page

321) 25 long, thin-walled, appearing to pierce proximal surface of ejaculatory duct (= thick walled pars prostatica) (Fig. 2). Pars prostatica 263 long, 38 wide proximally, 13 wide distally, dorsolateral to seminal vesicle, arched, widest and thick-walled in proximal half, narrow and thin-walled distally, extending posteriad before exiting prostatic sac posteriorly. Ejaculatory duct (= continuation of pars prostatica external to prostatic sac) extending posteriad from prostatic sac and becoming confluent with hermaphroditic duct, thin walled for entire length (lacking gland-like cells or muscular wall), 60 long or 22% of pars prostatica length, 15 in maximum width. Confluence of male and female genitalia indistinct (Fig. 2). Sinus organ indistinct in holotype. Hermaphroditic pore sinistral, anterior to genital pore, occupying space between prostatic sac and ventral sucker, directing ventrally before opening into genital atrium (= “genital sinus” of Horsfall [1]) (Fig. 2). Genital atrium connecting hermaphroditic pore and genital pore. Genital pore immediately anterior to ventral sucker, medial, posterior to prostatic sac, opening ventrally in posterior half of body. Cervical groove indistinct.

Ovary medial, intercaecal for entire breadth, immediately anterior to testes, 140 long or 6% of body length, 230 wide or 19% of body width or 1.6 × wider than long; post-ovary space, 275 long or 13% of body length; germarium present (not illustrated), a chamber occupying center portion of ovary, becoming confluent with proximal portion of oviduct at level of muscular sphincter; oviduct thin-walled, lacking sperm in proximal portion, emanating from mid-dorsal surface of ovary, extensively convoluted and extending sinuously anteriad before curving dextrad and becoming confluent with Laurer’s canal, 225 long from confluence with Laurer’s canal to ootype (Fig. 1).

Laurer's canal narrow proximally, swollen medially with sperm (= perhaps functioning as a "rudimentary seminal receptacle", sensu [2]), indistinct for most of its course dorsal and ventral to ovary in holotype, opening dorsally and posterior to ventral sucker (Fig. 3). Ovovitelline duct indistinct. Ootype dorsal to ovary, directing sinistrad, anterior to testes, 73 long, 40 in maximum width; Mehlis gland indistinct. Uterus extensively convoluted, not extending lateral to caecae, occupying space between posterior margin of oral sucker and ovary, comprising a field 1000 long or 46% of body length and 1100 wide or 88% of body width, passing ventral sucker sinistrally, thin-walled for entire length, 80 in maximum width, with hundreds of eggs, with distal portion comprising a metraterm; uterine seminal receptacle indistinct. Metraterm 450 long or 20% of body length, 75 wide, confluence with uterus posterior to medial axis of ventral sucker, sinistral, extending anteriorly lateral to ventral sucker, muscular and thick-walled, becoming confluent with ejaculatory duct to form a common duct (= herein a 'hermaphroditic duct') within sinus organ (Figs. 1-3). Vitellarium follicular, ventral to caeca, distributing in 2 bilaterally symmetrical fields, distance between fields 870 wide or 70% of body width, extending from level of pharynx to mid-testes; dextral vitelline field 1100 long or 50% of body length, terminating 40% of body length from anterior end of body, terminating 11% of body length from posterior end of body, 88% of dextral caeca length; sinistral vitelline field 1220 long or 56% of body length, terminating 37% of body length from anterior body end, terminating 7% of body length from posterior body end, 91% of sinistral ceacum length; primary vitelline collecting ducts symmetrical, coursing between ovary and testes, post-uterine, extending posteromediad from respective vitelline field before becoming confluent medially and

forming vitelline reservoir; dextral vitelline collecting duct 650 long or 30% of body length, 15 wide near vitelline reservoir, proximal end branches from vitellarium at 47% of dextral vitelline field length; sinistral vitelline collecting duct 555 long or 25% of body length, 8 wide near vitelline reservoir, proximal end branches from vitellarium at 65% of sinistral vitelline field length; vitelline reservoir medial, transverse, post-ovarian, pre-testicular (Fig. 1). Uterine eggs ovoid or pyriform, enlarging from approximately 40 x 25 in proximal portion of uterus to approximately 70 x 40 in distal portion of uterus, with one polar surface of each egg bearing minute fimbria or papilla-like projections (Figs. 1, 3, 4).

3.1.2 Diagnosis of cercaria based on whole-mounted paratype (USNPC 61230).

Mamillae (= mound like tegumental protuberances, which are located in the anterior portion of the tail stem) 35 in maximum length, 100 in maximum width or 3.1 × wider than long (Fig. 5, 6). Tail stem cavity at anterior end of cercaria, thick walled, muscular. Distome residing within tail stem cavity, 1200 long, 760 wide or 1.6 × longer than wide. Eggs absent in paratype.

3.1.3. Taxonomic summary

Type host: Pumpkinseed sunfish, *Lepomis gibbosus* (experimental) (Linnaeus, 1758) Brag, 1949 (Perciformes: Centrarchidae).

Intermediate host: Gravel elimia, *Elimia catenaria* (as *Goniobasis c.*) Say, 1822 (Cerithioidea: Pleuroceridae).

Other hosts: see Tables 1 and 2.

Site of infection: Cardiac stomach (fish) and gonoducts (prosobranch snail).

Type locality: Blue Springs (GPS N30°47’23.18; W85°08’33.57), Marianna, Jackson County, Florida.

Other localities: see Tables 1 and 2.

Specimens Examined: USNM Nos. 61229 (holotype) and 61230 (paratype).

3.1.4. Remarks.

The original description of *P. albacauda* by Anderson and Anderson [22] comprised a general account of an unspecified number of stained, whole-mounted adult specimens as well as live and stained cercariae. That description was incomplete, omitting or inaccurately depicting anatomical details of the proximal and distal portions of the genitalia in the adult and largely omitting internal anatomical details of the distome. Details of the genitalia provided in the original description comprised an illustration of the adult that located the gonads, uterus, hermaphroditic pore (as “genital pore”), and a structure we confirmed as the metraterm. However, it did not provide high magnification views or fine details of their connections, orientations, and relative positions. In light of the fact that this is not atypical for descriptions comprising species of *Proterometra*, we are puzzled by previous authors who have dismissed adult morphology as taxonomically useful, given that no such comparisons have been published to our knowledge. Indeed, features associated with the genitalia of adult digeneans typically differentiate species. As such, documenting the presence/absence, dimensions, and relative proportions and positions of the genitalia among *Proterometra* spp. could likewise be helpful in reducing uncertainty about the identities of species, especially those having suspiciously wide geographic distributions and low host specificity, e.g., “*P. macrostoma*.” We accept that many of

these features have yet to be proved as diagnostic for species of *Proterometra* but as more detailed descriptions and re-descriptions are entered into the literature such assessments can be made; whereas, now they are impossible due to a lack of comparative data. For example, the redescription provided herein (based on the holotype [intact adult] and a paratype [partial cercaria, including only the anterior portion of the tail stem plus the distome]) provides the first fine-scale anatomical details of the male and female genitalia of *P. albacauda*. We emphasize that a complementary study of live cercariae and adults could help elucidate features of the genitalia as well as the excretory system and nervous system, which have yet to be adequately detailed in *Proterometra* spp.

No information was available previously on the vasa efferentia, verschlussapparat, ejaculatory duct, sinus organ, genital atrium, germarium, oviduct, Laurer's canal, and ootype of *P. albacauda*. We determined that the vasa efferentia extend anteriad from the testes, flanking the ventral sucker, before becoming confluent anterior to the ventral sucker at level of the prostatic sac (Fig. 2). The ejaculatory duct (external to the prostatic sac) extends from the pars prostatica and becomes confluent with the metraterm (Fig. 2), forming a short common hermaphroditic duct that connects with the hermaphroditic pore. We observed a hermaphroditic pore that opens into a large genital atrium (Fig. 2), which in the holotype contains two eggs. The genital atrium communicates with the genital pore, which opens anterior to the ventral sucker (Fig. 2). We infer from Fig. 1 of Anderson and Anderson [22] that they misinterpreted the hermaphroditic pore, considering it the genital pore while not providing detail of another pore associated with the genitalia. The ovary has an obvious germarium, the

lumen of which communicates with the oviduct wherein there is a muscular sphincter (Fig. 3). Although not detailed in the narrative of Anderson and Anderson [22], we infer from their Fig. 1 that they interpreted the oviduct as emanating from the sinistral margin of the ovary. However, we confirm that the oviduct courses dorsal to the ovary, where it is extensively convoluted, i.e., no portion of the holotype's oviduct was illustrated by Anderson and Anderson [22]. We also document that the Laurer's canal is present, that the ootype is dorsal to the ovary, and that the metraterm is present and illustrated as a hollow chamber arching anteromediad from the distal portion of the uterus (Fig. 1).

Details of additional genital structures need clarification and correction based on a comparison between the holotype and the original description by Anderson and Anderson [22]. Because no explanation of the following features was provided in the narrative portion of the original description, the following information is sourced from Fig. 1 of Anderson and Anderson [22]. Therein, the seminal vesicle appears as a comma-shaped structure that lacks convolutions within the prostatic sac; however, in the holotype we studied the seminal vesicle is extensively convoluted within the prostatic sac and comprises a swollen proximal portion and a narrow distal portion (Fig. 2). The narrow duct extending from the seminal vesicle and within the prostatic sac is undoubtedly the pars prostatica (Fig. 2); however, they did not identify it as such and their drawing of it shows a uniformly narrow and thin-walled tube. The pars prostatica of the holotype is proximally expanded and thick walled, becoming thin walled and narrow distally before connecting with the ejaculatory duct, (Fig. 2). The vitelline reservoir and collecting ducts were not described in detail originally but Fig. 1

of Anderson and Anderson [22] indicates the location of the reservoir between the ovary and testes. Our observations of the holotype confirm that arrangement (Fig. 3).

Regarding the cercaria, the original description of *P. albacauda* included an illustration of the distome within the tail stem plus a mamilla (as “papilla”) bearing spines. This arrangement of mamillae is evidently characteristic of *P. albacauda*. The partial specimen comprising the paratype has three aspinous mamillae only (Fig. 5). Anderson and Anderson [22] reported that the mamillae of the tail stem were distributed in “*four lateral rows of eight to ten* (mamillae) *each, bearing three to five radially arranged spines*”; however, we describe these “rows” as columns, since they are depicted in Fig. 1 of Anderson and Anderson [22] as longitudinal and extending posteriad in parallel with the body margin. The only type material comprising a cercaria is that of the damaged paratype we studied, making impossible a confirmation of that arrangement of mamillae. The paratype lacks eggs and its tail stem cavity, containing the distome, markedly differs from other described cercariae in that the cavity is thick-walled and muscular (Fig. 5), perhaps functioning to forcibly extrude the distome upon contraction.

3.2. Proterometra epholkos *sp. n. (Figs. 7–14)*

3.2.1 Diagnosis of adult based on light microscopy of 7 stained whole mounted specimens.

Body of adult orange (live coloration), 1980–2300 (2189, 7) long, 820–1280 (1169, 7) or 1.7–2.7 (1.9, 7) × longer than wide, ventrally concave; tegument approximately 10–20 (14, 7) thick; papillae loosely studding rim of the mouth, tightly encircling the

outside rim of the ventral sucker, pored, lacking sensory cilium; tegumental projections (not illustrated) distributed evenly from anterior to posterior end (Fig. 7). Excretory system difficult to trace, unites anterior to oral sucker, extends posteriad lateral to ventral sucker, unites with excretory bladder near ovary; excretory pore medial, terminal. Nervous system indistinct in fixed whole-mounted specimens. Oral sucker subterminal, 70–200 (146, 7) or 3–10% (7%, 7) of body length from anterior body end, 600–710 (669, 7) long or 25–36% (31%, 7) of body length, 550–750 (675, 7) wide or 53–67% (58%, 7) of body width, posterior margin 320–680 (441, 7) from anterior margin of ventral sucker (Fig. 7). Ventral sucker in posterior half of body, with anterior margin 1140–1360 (1263, 7) or 52–62% (58%, 7) of body length from anterior body end, 310–360 (339, 7) in length or 14–18% (16%, 7) of body length, 320–400 (357, 7) in width or 27–39% (31%, 7) of body width, consistently wider than longer, 44–58% (51%, 7) of oral sucker length, 49–59% (53%, 7) of oral sucker width. Mouth opening ventrally or anteroventrally, 125–160(130, 7) long, 135–380 (259,7) wide, 1.1–2.4 (1.8, 7) × wider than longer (Fig. 7). Pharynx ovoid, posterior to the oral sucker, 120–150 (131, 7) long or 5–7 % (6%, 7) of body length 125–150 (142, 7) wide or 1–1.2 (1.1, 7) × wider than longer. Oesophagus extending posteriad from mouth 360–720 (540, 7) before bifurcating 15–40 (26, 7) posterior to pharynx, with oesophageal branches arching posterolaterad before joining with intestinal caecae; dextral oesophageal branch 180–300 (225, 7) long; sinistral oesophageal branch 150–265 (181, 7) long; intestinal caecae confluent with oesophageal branches, appearing inverse U-shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caecae; dextral caecum 870–1260 (1125,6) or 44– 60% (52%, 6) of body length, beginning

810–1080 (932,6) or 38–49% (43%, 6) of body length from anterior end of body, post-caecal space, 70–320 (157, 6) or 3–14% (7%, 6) of body length from posterior end of body; sinistral caecum 950–1280 (1097, 6) or 44–61% (51%, 6) of body length, beginning 720-1000 (882, 6) or 36–46% (41%, 6) of body length from anterior end of body, post-caecal space, 120–330 (205, 6) or 6–15% (9%, 6) of body length from posterior end of body (Fig. 7).

Testes 2 in number, transverse, abreast, oblique, oval to elliptical in shape; dextral testis 250–335 (290, 7) or 11–17% (13%, 7) of body length, 130–215 (160, 12) or 11–26% (14%, 7) of body width, anterior margin 1660-2120 (1871, 7) from anterior end of body, post testis space, 50–200 (101,7) from posterior end; sinistral testis 260–380 (301, 7) or 11–19% (14%, 7) of body length, 100–215 (161, 7) or 8–26% (14%, 7) of body width, 1620–1900 (1777, 7) from anterior end, post testis space, 35–245 (140, 7) from posterior end (Fig. 7). Vasa efferentia approximately 8–10 (9, 7) wide, coalescing into 2 main collecting ducts that extend anteriad from testes lateral to ventral sucker before connecting dorsally and in posterior half of prostatic sac with seminal vesicle (= a robust vas deferens enveloped by prostatic sac) (Figs. 7–9). Prostatic sac dextrad or sinistrad, occupying space between oral sucker and ventral sucker, anterior margin 15–365 (15, 7) from posterior margin of oral sucker, posterior margin 55–130 (81, 7) from anterior margin of ventral sucker, 200–250 (227, 7) long, 218–305 (260, 7) wide (Figs. 8, 9). Seminal vesicle thin walled, highly convoluted, having swollen proximal region and narrow distal region; proximal region of seminal vesicle 305–710 (396, 5) long, 58–100 (91, 5) wide; distal region 95–260 (177, 5) long,

15–28 (24, 5) wide, connected to pars prostatica via a short minute duct. Minute duct (= possibly “verschlussapparat” of Looss [58] in Horsfall [6], page 321) 10–30 (19, 7) long, thin-walled, appearing to pierce proximal surface of ejaculatory duct (= thick walled pars prostatica) (Figs. 8, 9). Pars prostatica 190–262 (219, 7) long, 48–58 (53, 7) wide distally 18–21 (19, 7) wide proximally, arched, lined by prostatic gland cells, thick walled for entire length, extending posteriad before exiting prostatic sac posteriorly. Ejaculatory duct (= continuation of pars prostatica external to prostatic sac) extending posteriad from prostatic sac and becoming confluent with hermaphroditic duct, thick walled for entire length (lacking gland-like cells or muscle in wall), ventral to metraterm, 25–75 (44, 7) long or 11–34% (20%, 7) of pars prostatica length, 10–20 (15, 7) wide (Figs. 8, 9). Confluence of terminal male and female genitalia occurring within sinus organ. Sinus organ conical, medial, dorsal to genital atrium; papillae in probable region of sinus organ absent. Hermaphroditic pore posterior to prostatic sac, anterior or level with ventral sucker, 49–57% (52%, 7) of body length from anterior body end, directing ventrally before opening into genital atrium (Figs. 8, 9). Genital Atrium connecting hermaphroditic pore and genital pore, thick walled, comprised of a large anterior lobe and small posterior lobe; anterior atrium lobe 30–22 (26, 2) in length; posterior atrium lobe 16–13 (15, 2) in length (Figs. 8, 9). Genital pore immediately anterior to ventral sucker, medial, posterior to prostatic sac, opening ventrally in posterior half of body. Ventro-cervical groove transverse depression surrounding genital pore, extending horizontally then curving posteriorly around ventral sucker, length and width variable (Figs. 8, 9).

Ovary medial, intercaecal for entire length, anterior margin anterior to posterior margin of testes, 155–200 (172, 7) long or 7–9% (8%, 7) of body length, 190–290 (218, 11) wide or 15–25% (19%, 7) of body width or 1–1.8 (1.2, 7) × wider than long, post-ovary space 200–440 (275, 7) long or 10–19% (12%, 7) of body length; germarium present, a chamber occupying center portion of ovary, becoming confluent with proximal portion of oviduct where a muscular sphincter is present (Figs. 7, 10); sphincter 40–48 (44, 7) wide; oviduct thin-walled, dorsal and sometimes anterior to ovary, immediately extensively convoluted and extending sinuously anteriad, briefly lined by cuboidal epithelium proximally before becoming confluent with Laurer's canal, extending 160–173 (167, 2) from commissure with Laurer's canal to ootype (Figs. 7, 10). Laurer's canal wide proximally at commissure with oviduct, swollen medially with sperm (= perhaps functioning as a "rudimentary seminal receptacle" [2]), narrow distally, 45–106 (75, 2) long, 35–13 (24, 2) wide including thick glandular wall, opening dorsally and posterior to ventral sucker (Figs. 7, 10). Ovovitelline duct short, arched after emanation from yolk reservoir, becomes confluent with oviduct at distal end immediately prior to ootype. Ootype dorsal or anterior to ovary, directing anteriad, anterior to testes 70–115 (91, 3) long, 30–45 (38, 3) in maximum width, Mehlis gland indistinct. Uterus extensively convoluted, intercaecal posterior to ventral sucker, may extend lateral to caecae anterior to ventral sucker, occupying space between posterior third of oral sucker and ovary, comprising a field 1020–1500 (1191, 7) long or 46–65% (54%, 7) of body length and 460–1040 (890, 7) wide or 56–85% (75%, 7) of body width, passing ventral sucker dextrally or sinistrally, thin-walled for entire length 45–100 (76, 7) in maximum width typically with hundreds of eggs, with proximal

portion comprising a uterine seminal receptacle and distal portion comprising a metraterm; uterine seminal receptacle with sperm; metraterm 315–635 (471, 5) or 14–32% (22%, 5) of body length, 45–60 (54, 5) wide, confluence with uterus anterior to the medial axis of the ventral sucker, transverse, sinistral or dextral, extending slightly anteriad and transverse from distal end of uterus, becoming confluent with ejaculatory duct to form a common duct (= herein a ‘hermaphroditic duct’) within sinus organ (Figs. 9, 10). Vitellarium follicular, ventral to caeca, distributing in 2 bilaterally symmetrical fields, distance between fields 510–820 (656,7) or 43–68% (57%, 7) of body width, extending from near posterior margin of oral sucker to near posterior margin of body, dextral vitelline field, 1060–1520 (1369, 7) long or 54–66% (62%, 7) of body length, terminating anteriorly at 32–38% (34%, 7) of body length, terminating posteriorly at 92–98% (95%, 7) of body length, 1.1–1.3 (1.2, 6) x longer than dextral caecum; sinistral vitelline field 1070–1450 (1297, 7) long or 48–64% (59%, 7) of body length, terminating anteriorly at 28–39% (33%, 7) of body length, terminating posteriorly at 88–98% (92%, 7) of body length, 1–1.3 (1.2, 6) x longer than sinistral caecum; primary vitelline collecting ducts asymmetrical, dextral vitelline collecting duct 2.8 (2.8, 2) × longer than sinistral vitelline collecting duct, extending posteromediad from respective vitelline field before becoming confluent dextrally and forming vitelline reservoir; dextral vitelline collecting duct 145–285 (192, 4) long or 6–12% (9%, 4) of body length, 10–20 (17, 4) wide near yolk reservoir, proximal end branches from vitellarium at 71–78% (75%, 3) of dextral vitelline field length; sinistral vitelline collecting duct, 463–500 (481, 2) long or 21–25% (23%, 2) of body length, 10–20 (14,

4) wide near yolk reservoir, proximal end branches from vitellarium at 64–82% (74%, 3) of sinistral vitelline field length (Fig. 7). Vitelline reservoir slightly dextral, T-shaped, pre- or post-ovarian, pre-testicular. Uterine eggs typically filling entire lumen of uterus, ovoid to pyriform, enlarging from approximately 40–55 (51, 7) x 20–30 (26, 7) in proximal portion of uterus to approximately 68–95 (78, 7) x 40–50 (44, 7) in distal portion of uterus; well-developed eggs having minute fimbria or papillae at one pole (Figs. 7, 10, 11).

3.2.2 Diagnosis of cercaria based on light microscopy of 10 whole-mounted, naturally shed cercariae with withdrawn distome.

Cercaria furcocystocercous, khaki in color, 6300–8550 (7234,10) long, 1600–2640 (2040, 10) wide or 2.5–4.6 (3.6, 10) × longer than wide, comprising a tail stem and paired furcae (Figs. 12-14). Tail stem spindle shaped, laterally expanded at midbody, 4980–7300 (5950, 10) long or 76–86% (82%, 10) of cercaria length; comprised of a laterally compressed anterior region and dorsoventrally compressed posterior region (Fig. 13); anterior tail stem region, 3400–4940 (4070, 10) long or 52–62% (56%, 10) of cercaria length, maximum width same as reported for cercaria, cone shaped, containing distome, bearing mamillae, weakly muscular; posterior tail stem region, 1280–2400 (1880, 10) long or 20–30% (26%, 10) of cercaria length, 1000–1350 (1194, 10) wide or 2–4% (3%, 10) × longer than wide, lacking mamillae, strongly muscular. Furcae obocordate (= ends broadly rounded with slight medial notch), amber in color, dorsoventrally compressed, margin smooth to serrulate, paired; dorsal furcae, 1020–1340 (1215, 8) or 15–19%, (17%, 8) of cercaria length, 1420–1840 (1671, 9) or 1.1–1.6 (1.4, 8) × wider than longer (Fig. 13); ventral furcae 1100–1340

(1250, 10) or 14–20% (17%, 10) of cercaria length, 1420–1840 (1636, 10) or 1.2–1.5 (1.3, 10) × wider than longer. Tail stem cavity positioned in anterior portion of tail stem, thin walled, not strongly muscular. Withdrawn distome (= cercarial body) 1340–2040 (1604, 10) long or 17–28% (22%, 10) of cercaria length, 660–880 (760, 10) wide or 2–3 (2, 10) × longer than wider, specimens with 2–42 (20, 10) stage 1 eggs (see [35]) in proximal end of uterus near ootype. Mamillae usually bearing spines, maximum length 115–185 (147, 10), maximum width 185–290 (241, 10) or 1.2–2.5 × wider than longer, cercaria length with mamillae 3000–4600 (3748, 10) or 45–56% (52%, 10) of cercaria length, cercaria length without mamillae 2700–4000 (3217, 10) (Figs. 12, 13); mamillae restricted to anterior tail stem region, completely encircling tail at level of tail cavity, restricted to 1 lateral column (4 total), of 5–6 mamillae, per body margin at midbody, ending at synthesis of posterior tail stem region; mamilla spines numbering 0-6 per mamilla, erect, proximally expanded, distally sharply pointed. Excretory system with 2 paired primary excretory canals, extending posteriad along the medial axis, from the anterior tail stem region, through the posterior tail stem region, extending independently through furcae, opening via excretory pore in the medial notch of each furcae (Fig. 13).

3.2.3 Diagnosis of germinal sacs based on 4 whole mounted sporocysts collected from cracked snails (see section 2).

Sporocyst 2200–4600 (3145, 4) long, 900–1580 (1155, 4) wide, birth pore at one end, with 4–8 (6, 4) cercariae of varying sizes, 0–5 (2, 4) germ balls present per sporocyst, no pharynx, gut, or mouth observed (Fig. 15).

3.2.4. Taxonomic Summary

Type host: Spotted bass, *Micropterus punctulatus* Rafinesque, 1819 (Perciformes: Centrarchidae).

Intermediate host: *Elimia* cf. *modesta* Lea, 1845 (Cerithioidea: Pleuroceridae)

Site of infection: Oesophagus (fish); indeterminate site (prosobranch snail).

Type locality: Terrapin Creek (South Fork) (33°51'36.56"N, 85°31'28.15"W), Cleburne County, Alabama.

Prevalence of infection: 2 of 2 (100%) spotted bass had 75 and 17 flukes respectively (mean intensity = 46).

Specimens Deposited: Syntypes USNM Nos. 1251729, 1251730, 1251731 (cercariae), 1251732, 1251733, 1251734 (adults); host voucher USNM No. 1251735.

GenBank Accession numbers: KM503118 (ITS2, adult) and KM503119 (ITS2, cercaria).

Etymology: The Greek specific epithet *epholkos* (epholkos, alluring) refers to the alluring appearance and presumptive luring behavior of the cercariae while swimming.

3.2.5. Remarks.

Naturally shed, fully-developed cercariae of *Proterometra epholkos* sp. n. are most easily distinguished from comparable cercariae of its congeners by the combination of having i) a medially constricted tail stem not exceeding 10 mm long, ii) mamillae of tail stem > 0.1 mm in diameter, iii) mamillae distributed in 2 lateral columns on each tail stem surface and each comprising 5-6 mamillae per each body margin (i.e., 4 total columns, 20-24 total mamillae) (not arranged in transverse rows across surface of tail

stem), iv) furcae > 1 mm long and ≥ 1.4 mm wide, v) distome withdrawn in swimming cercariae, vi) withdrawn distome (hence also, tail stem cavity) positioned in anterior portion of tail stem (not medial portion of tail stem), and vii) uterine eggs in distome typically numbering < 200. In addition, cercariae of *P. epholkos* swim upon shedding.

Adults of *Proterometra epholkos* sp. n. can be distinguished from congeners by the combination of having i) an oral sucker diameter equal to at least twice that of the ventral sucker, ii) a uterine field that extends dorsal to or lateral to the caecae anterior to level of the ventral sucker (not intercecal for entire length), iii) a vitellarium that extends posteriad beyond the posterior margin of the ventral sucker and to near the posterior body end (vitellarium longer than caeca), and iv) a uterus that loops extensively in the space between the ovary and ventral sucker. *Proterometra epholkos* sp. n. most closely resembles *P. albacauda* but can be distinguished from it by the combination of having cercariae with a medially constricted tail stem, long mamillae (>100 μm) that encircle the tail stem anteriorly, that are restricted to 1 lateral column of 5-6 mamillae per body surface and margin, and that are absent from the medial surface of the tail stem as well as by having adults with a vitellarium longer than the caecae, a vitellarium that extends anteriad to the oral sucker, and a uterus that extends dorsal or laterad to the caecae (= extercaecal uterus). Although inadequate justification for differentiating it from the new species, *P. albacauda* reportedly infects *Elimia catenaria* in the Apalachicola River (Gulf of Mexico Basin) and Ogeechee River (Atlantic Basin) as well as *Lepomis* spp., *Noturus gyrinus*, and *Pomoxis annularis* in the Ogeechee River [22,27]. No report of *E. catenaria* in an Alabama river exists to our knowledge.

Alabama rivers harbor 3 species of *Proterometra*: *P. melanophora*, *P. hodgesiana*, and *P. catenaria* (see Smith [15,20,21]). Based on published descriptions of these species, the new species is most easily differentiated from these congeners by having (i) a vitellarium extending beyond anterior margin of testes, (ii) an oral sucker twice as wide as ventral sucker, (iii) obcordate furcae, (iv) a spindle shaped tail stem > 4.9 mm long and > 1.6 mm wide, (iv) and a swimming cercaria (*P. edneyi* and *P. hodgesiana* do not reportedly swim [15,28], Table 3).

After primer trimming, the sequence data obtained for 2 cercariae from *Elimia* cf. *modesta* and 1 adult from *Micropterus punctulatus* presented 49 bp of the 3'end of the 5.8S ribosomal RNA gene followed by the complete internal transcribed spacer 2 (ITS2) of 251 bp, and ending with 49 bp of the 5' end of 28S ribosomal RNA gene. All sequences were identical aside from an intra-individual, single-site polymorphism in position '128'; which showed overlapping double peaks of Adenosine and Cytosine. ITS2 sequence data has been routinely employed as a species-level barcode for digeneans [59], and we interpret the 100% sequence match obtained herein to reflect that all specimens were conspecific. This result comprises the first application of molecular sequence data to elucidate a life cycle for a species of *Proterometra*.

4. DISCUSSION

4.1. Diversity and distribution.

Comprising the first description of a new species of the genus since 1995, the present study brings the total number of species of *Proterometra* to 10 (Tables 1, 2).

Proterometra presently accommodates flukes that mature in primary division freshwater fishes of Centrarchidae (species of *Lepomis, Micropterus,* and *Pomoxis)* [1,35]*,* Cottidae (*Cottus* spp.) [28,29], Percidae (*Etheostoma* spp. and *Perca flavescens*).) [28,29]*,* Lotidae (*Lota lota*) [29], Ictaluridae (*Noturus gyrinus*) [27], and Characidae (*Astyanax mexicanus)* [30] (Table 2). Infections in snails are reported from *Elimia* spp. (earlier records as *Goniobasis*), *Pleurocera* spp. [1,6,13,18], *Lithasia obovata* [4] (see [39] for synonymy), and *Campeloma subsolidum* [3]. Infections have been documented in these hosts ranging in rivers, streams, and lakes in 12 states and 6 water resources regions [60] in North America. To date, no record of an accepted species of *Proterometra* exists from beyond North America, and only one species ranges to the west of the main stem of the Mississippi River (Tables 1, 2). Noteworthy is that members of Centrarchidae are endemic to North American rivers, and, similarly, species of *Elimia* are endemic to eastern North American freshwater environments; many of which are highly endemic to the southeastern United States [52]. Taken together, the flukes, fishes, and snails are all North American endemics, making this parasite-host system rather interesting from the perspective of biodiversity and conservation science in the Southeastern United States. The exception to high regional endemism is "*Proterometra macrostoma*" (herein regarded as *species inquirenda*), which reportedly infects 8 snail species and 15 fish species in myriad river basins (Tables 1, 2). Typically, a morphological diagnosis of "*P. macrostoma*" does not accompany these locality reports, and, as previously stated, no molecular data have been sourced from any specimen from which these records are based. As such, we suspect this broad host and geographic distribution may reflect poor taxonomic

resolution rather than the true geographic and ecological distribution of the named species. If *P. macrostoma* is a species complex, additional work is needed (see sections 4.2, 4.3).

Proterometra guangzhouensis, which was described by Lu [61] from rice eels, *Monopterus albus*, of the Zhujiang River (southeast China) is not an accepted species of *Proterometra*. Based on Lu's [61] figures, this species has several features that would exclude it from *Proterometra*: (i) the vitellarium and the uterus are posterior to the prostatic sac and (ii) the testes are level with the ovary. Moreover, the site of infection reported by Lu [61] was "intestine," not buccal cavity or esophagus, which are the typical sites of infection for adults of species of *Proterometra* infecting fishes. Of interest is that the rice eel is a highly invasive species throughout south Florida [62]. We examined several rice eels from south Florida canals but none was infected by an azygiid. We agree with Gibson [38] that *P. sillagae*, *P. brachyura*, and *P. lamellorchis*, which were described by Wu et al. [63] and collected from sillagos (*Sillago* spp.) captured in the Guangdong Province of China, are not species of *Proterometra.* Based on our observations of Figs. 1–3 of Wu et al. [63]), these species have features that exclude them from *Proterometra*: (i) the testes are tandem, and, at least in one species, lobed, (ii) an external seminal vesicle is present in all three species, and (iii) the female genitalia is medial or completely restricted to the forebody.

*4.2. Type specimens of "*Proterometra macrostoma*" et al.*

The holotype of *P. macrostoma* (as *Cercaria macrostoma*) has probably been lost or was never designated. This species, the type species of *Proterometra*, was

described from a single cercarial specimen taken from "*an aquarium in the Zoological Laboratory of the University of Illinois*" [3]. The collection locality for the cercaria was an aquarium, and no original collection locality for the snails in the aquarium was given; hence, no wild type locality was specified. Because multiple species of snails (i.e., *Campeloma subsolidum* and *Elimia semicarinata* [as *G.* pulchella]) were present in the single aquarium from where the cercaria was collected, no type host was specified. Because no fish were examined by Faust [3], no adult specimen was described. Finally, no holotype was specified by Faust [3]. Later, however, a holotype was mentioned [6] but not by Faust.

Horsfall [1] reported *Cercaria macrostoma* in snails identified as *Goniobasis livescens* from Salt Fork Branch Vermillion River, Illinois, and in *Pleurocera acuta* from Oconomowoc River, WI. She fed the cercariae to several fishes but only centrarchids became infected with adult flukes, which were used to propose the new genus *Proterometra* (*P. macrostoma*, type species). A year later, she [6] studied (i) whole-mounted specimens of *Cercaria fusca* of Pratt [17]; sent to her by HB Ward, (ii) mounted and vialed specimens of the forked tailed cercariae of Cahn [18], (iii) the "*holotype of Cercaria macrostoma which Faust kindly loaned*," which remains the only literary mention of a holotype of *P. macrostoma* (the present disposition of this specimen is indeterminate), (iv) cercariae shed in her laboratory from snails (*Elimia livescens* and *Pleurocera acuta*; perhaps sourced from the Des Plaines River) that were sent to her by Dickerman in summer 1933, and (v) photomicrographs, along with whole-mounted, and vialed specimens of *Cercaria melanophora* (of Smith [20]) from *Elimia opaca* in the Cahaba River, Alabama. After studying the aforementioned

material, she considered them as conspecific with *Cercaria macrostoma*, therein assigning them to the collective group name *Cercaria*. This was an odd taxonomic and nomenclatural decision since she had previously made available the genus group name *Proterometra* for the type species *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933. Horsfall [6] reported that "*[t]he typical adults of Proterometra macrostoma are deposited in the United States National Museum, number 8767, 8768 and in the collection of Dr. Henry B. Ward, number 34.10.*" According to the USNPC database USNPC Nos. 8767 and 8768 are extant but listed as vouchers, not types. At the time of submission of this manuscript, specimen number 34.10 from Ward's collection was a part of the USNPC under accession No. 51817 and was labeled as a "cotype." "Cotype" is a term not recognized by the ICZN but formerly was used for either a syntype or paratype but not a holotype (see Recommendation 73E, ICZN).

Like that for the type species of *Proterometra*, a perusal of the literature on *Proterometra* reveals that a lack of type material or uncertainty regarding the holotype is not unusual. Pratt [17] described *Cercaria fusca* from a snail identified as *Goniobasis livescens* in the Oneida River, New York, without deposition of type materials. Cahn [18] described "*a new forked tailed cercaria*" from *Pleurocera acuta* in the Oconomowoc River, Wisconsin. Adults were reported from "*the young of fish belonging to the family Centrarchidae*." As with Pratt [17], no type or voucher materials reportedly were deposited. Dickerman [19] reported "*a large number of cystocercous cercariae in the mirabilis group*" from snails identified as *Goniobasis livescens correcta* in the Des Plaines River, Illinois. No type or voucher materials were deposited. Smith [20], in an abstract, described *Cercaria melanophora* from *Elimia* spp. from Alabama

without mentioning type materials. The USNPC specimens of "*Proterometra pinguis"* from *Esox lucius* reportedly deposited by JF Mueller are apparently unaccompanied by a published description, and we herein consider it a *nomen nudum*. Also, The USNPC database indicates that the type materials for *P*. *albacauda* (USNPC Nos. 61229-30) and *P. septimae* (USNPC Nos. 61231-32) are crazed; however, we have studied the type materials for *P. albacauda* and they are in good condition. Regarding *P. hodgesiana*, we cannot locate type materials for this species, and none was reportedly accessioned nor resides in the USNPC.

4.3. Is P. macrostoma *a species complex?*

Dickerman [33] studied cercariae from *Elimia livescens* (as *Goniobasis livescens correcta*) in the Des Plains River, Illinois, and adults from *Pomoxis nigromaculatus* (as *Pomoxis spiroides*), *Micropterus salmoides* (as *Helioperca incisor*), and *Lepomis gibbosus* (as *Eupomotis gibbosus*). He considered these specimens conspecific with *P. macrostoma* but, again, no voucher materials reportedly were deposited. Smith [15] revisited the taxonomic treatment of *C. melanophora*, based on specimens collected from a prosobranch (as *Goniobasis opaca*), and determined that adults recovered from *Micropterus salmoides* (as *Huro floridana*) from Cooley Creek, Alabama, (Cahaba River) were conspecific with the adults of *P. macrostoma*, agreeing with Horsfall [6]. By doing so Smith [15] established a novel combination as *Proterometra melanophora*. Since then, *P. melanophora* has been typically considered a junior subjective synonym of *P. macrostoma*. Unfortunately, neither author, Smith nor Horsfall, provided what we consider to be a robust taxonomic justification for the

synonymy of *P*. *macrostoma* and *P. melanophora*. Dickerman [13] studied cercariae shed from unspecified species of *Elimia* (as *Goniobasis*) and *Pleurocera* in waters adjacent to the "*Great Lakes Region*." Therein, he initiated the concept of 3 types (= "kinds") of *P. macrostoma* (later indicated by Riley and Uglem [8] as "strains," which they further separated into 8 "morphotypes") based on the morphology of cercariae collected from unspecified species of *Goniobasis* (=*Elimia*) in the "*Bass Island region of Lake Erie*." This work, although providing a novel perspective on morphological variation among cercariae assigned to *Proterometra*, added a layer of intrigue to the taxonomy of the genus since it made precedent the use of morphological features of cercariae as diagnostic for strains. Since 1945, no author has described a species later to be considered a junior subjective synonym of *P. macrostoma***.** One species, *P. autraini*, was initially identified as *P. dickermani* [37] and later as *P. macrostoma* [26] before being described as a new species [29].

Aside from the nomenclatural and morphological cloud surrounding "*P. macrostoma*," based on the myriad snail and fish species reported as hosts for "*P. macrostoma*" as well as the fact that its geographic distribution encompasses that of all other named species of *Proterometra* (Tables 1, 2), it seems probable that *P. macrostoma* has become a repository for conspicuous, furcocystocercous cercariae shed from snails of *Elimia* in eastern North American rivers and streams. Noteworthy also is that the taxonomy of both the intermediate and definitive hosts have changed considerably since 1918, i.e., species of *Elimia* were previously assigned to *Goniobasis* and *Melania* (see section 4.8) [39,54,55], many new species of those gastropod genera have been described since the early 20th century, and *Micropterus*

now includes several cryptic species typically misidentified as or considered as "*Micropterus salmoides*" [64,65]. We think that continued morphological studies of cercariae and adults plus careful taxonomic identification of the snail hosts (specimens vouchered in curated invertebrate collections) and fish hosts coupled with molecular sequence data can help further resolve these taxonomic uncertainties.

4.4. Proterometra spp. from Alabama.

The present study brings the total number of species of *Proterometra* documented from Alabama rivers to four: *P. epholkos*, *P. melanophora*, *P. hodgesiana*, and *P. catenaria*. The latter three species were treated initially by Smith [15,12,21] and all of them require further taxonomic study considering the type materials and based on naturally shed cercariae as well as adults collected from infected, wild-caught fish hosts. Fundamentally problematic is that we could not locate or find reference to an extant type specimen for any species of *Proterometra* described from Alabama. In an abstract of a demonstration at the 8th Annual Meeting of the American Society of Parasitologists, Smith [20] provided scant details for "*[t]wo new cystocercous cercariae*," *P. melanophora* (as *Cercaria m.*) and *P. hodgesiana* (as *Cercaria h.*), based on cercariae shed from *Elimia* spp. *Proterometra melanophora* was subsequently treated by Horsfall [6] and Smith [15] as conspecific with *P. macrostoma* (see above). Smith gave Horsfall [6] "*information concerning host, locality, and distinguishing characteristics*" and cercariae of *P. hodgesiana* shed from a prosobranch (as *Goniobases* spp.) "*collected in the Warrior River, Alabama.*" Smith differentiated the cercariae of *P. hodgesiana* from that of *P*. *macrostoma* based on the

following cercarial characteristics (i) presence of presumed "*functioning genital organs*," (ii) tail stem "*globular anteriorly*" (i.e., circular vs. laterally compressed), (iii) mamillae aspinous, (iv) "*furcae small proportional to tail stem*,*"* and (v) "*cercaria small*." Four years after naming the species in the abstract, Smith [15] revisited the taxonomy of *P. melanophora* and *P. hodgesiana* by providing additional information on the cercariae of *P. hodgesiana* from a prosobranch (as *Goniobasis vicina*) in Big Sandy Creek (Black Warrior River) and Miller Springs (Cahaba River), Alabama. She emphasized that the time of emergence of cercariae occurred in the morning hours rather than at night (as in *P. macrostoma*) and that the cercariae were unable to swim (as opposed to swimming cercariae of *P. macrostoma*). Cercariae of *P. hodgesiana* made "*lashing movements*" that she speculated made for a "*conspicuously attractive object to fish*." Adult flukes were recovered from pumpkinseed sunfish (*L. gibbosus*; as *Eupomotis g.*) that were fed cercariae. No work [6,20,21] listed an accession number for a type specimen. Further noteworthy regarding nomenclature is that Smith [20,21] made available the names *melanophora* and *hodgesiana* in abstracts. Article 9 (pg. 8) of the ICZN defines forms of communication that do not constitute a "published work." Specifically Article 9.9 (pg. 8) of the ICZN states that "*abstracts of articles, papers, posters, texts of lectures, and similar material when issued primarily to participants at meetings, symposia, colloquia or congresses*" do not meet the criteria of published work. However, because these abstracts were published in volumes of their respective journals (i.e., The Journal of the Alabama Academy of Sciences and The Journal of Parasitology), we regard *melanophora* and *hodgesiana* as available names.

Smith [21] described *P. catenaria* based on cercariae from *Elimia catenaria* (as *Goniobasis c*.) in the Apalachicola, St. Johns, and Suwannee Rivers of Florida as well as from *Elimia doolyensis* from the Choctawhatchee River (Mobile River), Alabama. She demonstrated that it experimentally infected various centrarchids. She did not list an accession number for a type specimen nor state that any type specimen was deposited. Anderson and Anderson [22] provided supplemental observations on *P. catenaria* based on cercariae taken from *Elimia catenaria* from Blue Springs, Florida, that were experimentally fed to *Lepomis cyanellus* (sourced from Blue Springs) and *Lepomis gibbosus* (sourced from Douglas Lake, Michigan). Their report on the anatomy of *P. catenaria* lacked a specimen from Alabama, which unambiguously included Smith's [21] type locality. In addition, Anderson and Anderson's [22] work added few additional morphological features of the genitalia, and cercarial features were limited to color, surface features, and swimming behavior. That work is somewhat problematic also in that no diagnosis for any species treated therein was provided, including for that of *P. catenaria*. Hence, from that work it is unclear what diagnostic features made the Florida specimens of Anderson and Anderson [22] conspecific with the Alabama specimens of *P. catenaria* in Smith [21]. In comparing the illustrations in both works, the adults of *P. catenaria* seem indistinct; however, the depictions of the cercariae seem in general agreement, i.e., the distome resides at midbody, the tail stem is massive, and the furcae are somewhat lanceolate.

4.5. Molecular data.

In addition to issues related to type materials, another obstacle for taxonomy, systematics, and life history studies of *Proterometra* spp. is that molecular sequence data are lacking for all but one confirmed species (present study). This makes the assessment of operational taxonomic units based on cercarial morphology alone challenging, especially given the low number of reported diagnostic characteristics. Various workers have treated morphology, behavior, and host-parasite relationships of the cercariae [13,6–12,33,66–72], development and morphology of the eggs [35,73] and adults [1,6,15,21–25,28,33,35], and general attributes of the intra-molluscan stages, sporocyst and redia [11,14,74]. Moreover, 8 cercarial strains of *P. macrostoma* have been morphologically characterized from Lake Erie [13], Kentucky [8,75], Michigan [8], and Ohio [8]. Including the present study, molecular sequence data exist only for *Proterometra* sp. [43] and *P. epholkos*, despite marked advances in molecular systematics of trematodes [76]. Sequence data for the commonly applied markers (ITS2, 18S, 28S) can help throw light on the identity of species that have been reported in the literature. For instance, although low intra-individual and intra-specific variation is assumed because of the action of concerted evolution on rDNA arrays [77], some studies have shown that this phenomenon might be incomplete in some digeneans [78].

In the present study, sequences from the adult and cercarial specimens each presented divergent intra-genomic ITS2 copies comprising a double chromatogram peak in position 128. Noteworthy is that hybrids having different ITS2 variants from different species have been reported from species of *Schistosoma* [50,79] and

Fasciola [80]. While our sample size constraints further discussion of the matter, we consider Azygiidae as an interesting group to explore concerted evolution. Moreover, multiple ITS2 copies within individuals might have been overlooked in other digeneans, raising concerns about the use of this locus in phylogenetic analyses because the employment of different variants could mislead such estimations. Also important is that our own results demonstrate that universal primers for flukes are effective with species of *Proterometra*. Molecular approaches are especially needed in determining the level of intraspecific variation in cercariae towards testing strain hypotheses, matching cercariae and adult flukes towards documenting life cycles, and sequencing snail tissues to help confirm their taxonomic identities.

Scant sequence data exist for Azygiidae. Before the present study, GenBank held sequence data for 3 markers (CO1 [1 taxon], 18S [2 taxa], 28S [3 taxa]) representing 3 genera and 5 species: *Azygia angusticauda* (CO1 [526bp]) [81], *Azygia longa* (28s [1,406 bp]) [43], *Otodistomum veliporum* (18s [302 bp]) [82], *Otodistomum cestoides* (18s [1932 bp]) [83], *Otodistomum cestoides* (28s [1,303 bp]) [84], and *Proterometra* sp. (28s [1,399bp]) [43]. As additional studies incorporate and publish azygiid molecular sequence data, a better understanding of interrelationships of azygiid genera will likely emerge; however, at present, impactful phylogenetics is out of reach, simply for lack of available sequence data.

4.6. Differential morphological features.

Proterometra spp. are principally differentiated based on cercarial morphology and behavior [1,15,21,22,24,25,28,29] (Table 4). Based on direct observations of cercarial

specimens and a review of the published literature, the taxonomic validity of several cercarial characters is uncertain. Taxonomic descriptions of the cercaria of a species of *Proterometra* should derive from naturally shed cercariae only, not from cercariae taken from sporocysts, rediae, or “crushed” snails. Specimens sourced by the latter method are likely immature. Basing taxonomic decisions on immature cercariae has a high probably of causing taxonomic confusion. In addition, naturally shed cercariae should be heat killed (see section 2) so as to ensure reliable and repeatable morphometric comparisons across species. We have noted that most published descriptions make ambiguous the provenance of and fixation method associated with the studied specimens and/or type materials (when designated). Moreover, the distome can be extruded or withdrawn, and, therefore, the size of the cercaria as a whole can vary intraspecifically depending on the state of the specimen when fixed. Perhaps as a result of some of these different approaches, several morphological features of species of *Proterometra* need further clarification. Some seem inconsistently reported; others are likely reliably diagnostic; and some seem dubious.

4.6.1. Tail stem.

Tail stem length is the distance from the anterior end of the tail stem, i.e., the opening through which the distome extrudes, to the confluence of the furcae [22]. Tail stem maximum length and width have been used as diagnostic features for species of *Proterometra*, and, given the likelihood that swimming behavior of cercariae is under strong selection pressure, this feature is likely a useful taxonomic feature. However, several species reportedly have inconsistent ranges, wide ranges, or lack morphometric data altogether (e.g., *P. catenaria*, *P. macrostoma*, *P. hodgesiana*;

respectively). For example, reported tail stem length and width measurements for *P. macrostoma* are 2–9 mm x 0.59–1.7 mm [6,8]. These ranges encompass those measurements for all but one congener. As another example, Smith [15], for *P. catenaria*, did not report tail stem length but instead reported cercarial length (tail stem + furcae) as 9–16 mm. Supplemental observations of that species reported tail stem length as 9–16 mm and 5.2–8.2 mm in living and fixed specimens, respectively [22]. Thus, for example, clarification is needed regarding the actual tail stem dimensions for *P*. *macrostoma* and *P. catenaria*. Furthermore, based on our observations of living and fixed cercariae of *P. epholkos* and the published works treating cercariae of *Proterometra* spp., the cercarial tail stem is divided into a laterally expanded anterior portion, which seems less muscular, has mamillae, and is laterally compressed, and a relatively narrow posterior portion, which seems more muscular, lacks mamillae, and is dorso-ventrally flattened (Fig. 13). These features may be diagnostic for species of *Proterometra*, and histological study of the tail stem tegument would be helpful in further characterizing these attributes. In addition to more consistency being applied to how the tail stem is characterized, correlating the shape of the tail stem with swimming behavior and definitive host food habits could reveal an interesting story about cercarial mimicry in stream ecosystems. That is, tail shape relates to swimming behavior, which relates to the type of definitive host that attacks the putatively luring cercaria.

4.6.2. Mamillae and spines.

Smith [20] coined the term "*mammilations*" for the mound-like tegumental protuberances (spinous or not) of the cercarial tail stem (Figs. 6,12). Others have

referred to these tail stem protuberances as "*papillae*" [22] or "*wartose structures*" [33]. Herein, we refer to Lawrence [85] and call these protuberances on the tail stem 'mamillae.' We think that the application of "cercarial papillae" is ambiguous and potentially confusing because small papillae occur on the tegument of the cercarial distome as well as about the rim of the oral and ventral suckers. Hence, as we define them, mamillae are the protuberances associated with the tail stem, not the distome. These mamillae are not likely homologous to tegumental papillae of the distome, which for the new species are pored but lack a sensory cilium. The distribution, appearance, and number of mamillae in *P. epholkos* are useful as diagnostic features (see Description) but comparable information from congeners is predominantly indeterminate or lacking [1,15,21,22,24,25,28,29]. In some instances, and without the benefit of molecular sequence data, a high degree of variation seems present; leading some authors to diagnose subspecific taxa, i.e., "strains" or "types" [8,13,75]. In addition to the pattern of mamillae, the presence/absence, appearance, length, and number of spines ornamenting each mamilla are intriguing as potential taxonomic features. At least specimens identified as *P. macrostoma, P. catenaria*, *P. albacauda*, *P. septimae*, *P. edneyi*, *P. autraini*, and *P. epholkos* possess spinous mamillae in portions of the tail stem (Table 3).

To our knowledge, the function of these spined mamillae of the tail stem is indeterminate. Based on the site of infection in the definitive host (i.e., buccal cavity) and the ability of the distome to withdraw inside the tail stem, we speculate that the mamillae and their spines may act as cleat-like contact surfaces that facilitate affixing the tail stem, along with its withdrawn cercarial distome, to the soft tissues of the

buccal cavity epithelium and oesophagus upon being engulfed and taken into the buccal cavity by the fish host. Or, simply, these spines could increase the likelihood that engulfed cercaria entangle in the rugose esophageal sphincter of the fish host. This could permit the distome to emerge from the tail stem, attach to the apposed epithelial surface of the buccal cavity, and detach from the tail stem with low risk of being separated from the host surface.

4.6.3. Tail stem cavity.

The distome of *Proterometra* spp. can be withdrawn inside the tail stem cavity or extruded. Published observations indicate that whether or not the distome is withdrawn or extruded upon being shed from the snail is species-specific. Aside for *P. dickermani*, the distome of all species of *Proterometra* is withdrawn upon shedding from the snail host [26]. The relative position of the tail stem cavity may also be diagnostic for some species [22,24,25]. However, this feature should only be described from and compared among naturally shed cercariae because the distome in cercariae excised from crushed snails is typically extruded (MRW, personal observations), suggesting that the distome withdraws immediately before shedding.

4.6.4. Cercarial behavior.

As previously discussed, the cercariae of *Proterometra* spp. are unique among most digeneans because of their swimming behavior, which purportedly mimics a fish prey item that lures the definitive fish host to consume it. However, this unique behavioral attribute differs among species of *Proterometra*. *Proterometra hodgesiana* and *P. edenyi* reportedly lack the ability for coordinating swimming (Table 3) and, instead, lure the fish definitive host by rapidly lashing and contracting the tail stem

[15,28]. Three studies [28,66,68] have addressed the elements involved and mechanisms responsible for the unique swimming behavior displayed by cercariae of *Proterometra* spp. Consistency of swimming patterns in *Proterometra* spp. is the result of "*a pattern generating mechanism that is centrally, rather than reflexly, controlled*" [66], and inter-specific variation in swimming behavior purportedly could result from the number of myoneural junctions present in the tail stem [28,68]. The results of these studies support the fact that swimming behavior, and likely swimming duration, may reliably differentiate species. Swimming behavior may also reflect the diet of the fish definitive host, e.g., benthic fishes (darters, Percidae) that graze on invertebrates and algae [45] may have more opportunity to consume sedentary cercariae than swimming cercariae that remain higher in the water column. If cercariae are luring, then we assume that cercarial behavior is at least partly determined by host dietary preference and foraging ecology.

4.6.5. Progenesis.

"Progenesis" is defined herein as the maturation of gametes before completion of body growth [85,86]. The literature indicates that a specimen typically is regarded as "progenetic" if there is sufficient evidence of precocious egg development, i.e., morphologically viable/embryonated eggs (those enveloping ciliated, active miracidia) present in the uterus of the not yet fully developed individual (larva, cercaria). This evidence supports the classical definition of progenesis since it indicates that the individual fluke indeed has produced mature gametes requisite for fertilization and subsequent embryonic development. The matter can be made more complex when considering that not all fluke eggs necessarily are embryonated ('viable'), and, hence,

presence of a uterine egg does not necessarily prove that the individual produced a sperm and an ovum that preceded fertilization. The literature on *Proterometra* spp. seemingly defines a species as progenetic if an egg, of any stage of development, is observed to be present in the uterus of the cercaria: "*P. macrostoma*," *P. catenaria*, *P. sagittaria*, *P. dickermani*, *P. albacauda*, *P. edenyi*, *P. autraini*, and *P. epholkos* sp. n. fit that definition [1,21,22,24,25,28,29]. Interestingly, *P. dickermani* has yet to be collected from a wild fish, and, therefore, may have a single host life cycle [25,26,36]. Caution is needed here to differentiate the apparent lack of infections in a fish host as support for a single host life cycle, however. Perhaps giving support to the single host life cycle, *P. dickermani* is further unique in that it reportedly lacks a tail cavity [25,26]. Yet, cercariae of other species, e.g., *P. sagittaria* and *P. autraini*, have yet to be observed without uterine eggs but infect fishes [24,29] (see Table 2).

4.7. Genitalia as diagnostic.

The seminal works regarding *P. macrostoma* by Horsfall [1,6] and Dickerman [33] provided the template for describing the genitalia for species of *Proterometra*. Since these works, however, little attention has been given to the genitalia of adults or cercariae. As such, several features of the genitalia require clarification. Previous descriptions refer to the structure that surrounds the seminal vesicle and pars prostatica as the cirrus sac [1,6,24,29,33]. Herein, this structure is described as the prostatic sac (Figs. 1, 7) (sensu [87]). The portion of the male duct distal to the seminal vesicle is comprised of two structures, the pars prostatica and the ejaculatory duct (Figs. 2, 8) [24,33]. Previous descriptions described the former as a "*bulbous*

cavity of the prostate region," and the latter as the "*ductus ejaculatorius*" [1,6]. Light microscopy suggests that the thickness of the wall of these structures differs between *Proterometra* spp. The metraterm (distal, muscular portion of uterus) becomes confluent with the ejaculatory duct within the copulatory organ forming a hermaphroditic duct (Figs., 8, 9). Only one author [1] has described and noted a metraterm. Dickerman [24,33] called the metraterm a "vagina" in *P. macrostoma* and *P. sagittaria*. The copulatory structure, or sinus-organ (sensu [2]), has been described as a "*genital cone*" [33] or "*genital papilla*" [24,25,28,29,33]. We regard species of *Proterometra* as having a permanent sinus organ (Figs. 8, 9), and scant observations of this organ indicate it may or may not be papillate [29]. At the distal end of the sinus organ is a hermaphroditic pore that opens into a large, seemingly bi-lobed, genital atrium (Fig. 8) [2,22,25]. Previous accounts have described this area as a genital sinus [1,24,33]. We think that the anterior lobe is typically larger than the posterior lobe and that the atrium contains eggs in some specimens [24]. Because of the limited number of specimens examined, and since many of these features have not been previously described or detailed, additional observations and molecular data of congeneric species are needed for confirmation. Doing so, along with applying equal precedence to cercariae and adults, will help inform future taxonomic works.

4.8. Snail (Elimia spp.) identification is critical.

Species-level identification of snail hosts, especially those of *Elimia*, is a major concern regarding logistics of accurately documenting the host and geographic distribution of species of *Proterometra* as well as to testing hypotheses concerning the

specificity of these digeneans to their molluscan first intermediate hosts. The chronic lack of taxonomic resolution within Pleuroceridae, specifically regarding *Elimia*, is especially problematic because (i) most snail hosts of *Proterometra* spp. are presently assigned to *Elimia* and (ii) each nominal species of *Proterometra* has been reported to infect at least one species of *Elimia* as well as *Pleurocera acuta*, *Lithasia obovata,* and *Campeloma subsolidum* (Table 1). Illustrated field guides that treat species of *Elimia* are equivocal regarding differentiating species without geographic locality data, and, to our knowledge, no modern dichotomous key to *Elimia* spp. has been published in the peer-reviewed literature or exists as an unpublished field guide (Dr. Paul Johnson, Alabama Department of Conservation and Natural Resources, Marion, Alabama; personal communication). Because previous authors have not (i) given characters for which they used to identify snails, (ii) cited resources or personal communication with malacologists regarding snail identification, or (iii) co-deposited snail vouchers with the parasite type/voucher material, we doubt that most of the snail hosts for *Proterometra* spp. have been identified correctly. For example, *P. catenaria, P. albacauda* and *P. septimae* have been reported and described from the same snail host, *Elimia catenaria* (as *Goniobasis c.*), in the same river, Apalachicola River, Blue Springs, Florida. However, according to Johnson et al. [52], *E. catenaria* does not range in Florida nor any Gulf of Mexico drainage but rather is restricted to Atlantic drainages in Georgia, South Carolina, North Carolina, and Virginia. Issues such as this are problematic regarding progenetic digeneans *sensu lato* that may have evolved a higher degree of host specificity (fidelity) to the host in which they mature sexually, i.e., the molluscan first intermediate host, as opposed to non-progenetic digeneans

that seemingly exhibit a higher level of specificity to the craniate definitive host. In addition, we recommend that molecular markers be applied to assist in identifying the snail hosts as well as per Fukuda et al. [88] or Galindo et al. [89].

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Table 1. Snail hosts for cercariae of *Proterometra* spp. Horsfall, 1933[a] (Digenea: Azygiidae).

Species	Host[b]	Locality[c]	Author (yr.) [ref.]
P. macrostoma (Faust, 1918) Horsfall, 1933[d]	*Campeloma subsolidum*	Univ. IL zoological laboratory, Homer, IL	Faust [3]
	Lithasia obovata (as *Goniobasis depygis*)	McCormicks Creek, IN (OH)	Cable [4]
	Elimia floridensis	Wekiva River, FL (SAG)	Hunter & Wigington [5]
	Elimia livescens	Vermillion River, IL (GL)	Horsfall [1]
		Des Plaines River, IL (UM)	Horsfall [6]
		Indian River, MI (GL)	Lushbaugh [7]
		Sandusky River, OH (GL)	Lushbaugh [7]
		Carp Lake River, MI (GL)	Riley & Uglem [8]
		Olentangy River, OH (UM)	Riley & Uglem [8]
		Clear Creek, IN (OH)	Krist [9]
	Elimia semicarinata (as *Goniobasis pulchella*)	Univ. IL zoological laboratory, Homer, IL	Faust [3]
	Elimia semicarinata	Elkhorn Creek, KY (OH)	Lewis et al. [10]; Riley & Uglem [8]; Rosen et al. [11,12]
	Elimia spp.	Great Lakes (GL)	Dickerman [13]
		Elkhorn Creek, KY (OH)	Uglem & Lee [14]
	Pleurocera acuta	Oconomowoc River, WI (UM)	Horsfall [1]
		ns	Horsfall [6]
	Pleurocera spp.	Great Lakes (GL)	Dickerman [13]
	ns	Texas	Smith [15]
		Rutherford County, TN (TN [Stones River, Christmas Creek, Bushnell Creek, McKnight Brook]) (T)	Viyanant & Dunn [16]
Cercaria fusca Pratt, 1919[d, e]	*Elimia livescens*	Oneida River, NY (GL)	Pratt [17]
"Forked-Tailed Cercaria" Cahn, [illegible][e]	*Pleurocera acuta*	Oconomowoc River, WI (UM)	Cahn [18]

mirabilis group" Dickerman, 1931[e]	*Goniobasis livescens correcta*)		
P. melanophora (Smith, 1932) Smith, 1936 [d]	Prosobranch (as *Goniobasis opaca*)[f]	Cooley Creek, AL (SAG)[f]	Smith [15]
	Elimia spp. (as "*Goniobases* sp.")	"Alabama"	Smith (abstract) [20]
P. hodgesiana (Smith, 1932) Smith, 1936	Prosobranch (as *Goniobasis vicina*)[f]	Big Sandy Creek, AL (SAG)[f]	Smith [15]
		Miller Springs, AL (SAG)	Smith [15]
	Elimia spp. (as "*Goniobases* sp.")	"Alabama"	Smith (abstract) [20]
		Warrior River, AL (SAG)	Horsfall [6]
P. catenaria Smith, 1934[e]	*Elimia catenaria*	St. John's River, FL (SAG)	Smith [21]
		Suwannee River, FL (SAG)	Smith [21]
		Apalachicola River, FL (SAG)	Smith [21]
		Blue Springs, FL (SAG)	Anderson & Anderson [22]
	Elimia dooleyensis	Choctawhatchee River, AL (SAG)	Smith [21]
P. sagittaria Dickerman, 1946	*Elimia livescens*	Carp River, MI (GL)[g]	Anderson and Anderson [22]
	Elimia and *Pleurocera* snails	Lake Erie, OH (GL)[f]	Dickerman [24]
		Maumee River, OH (GL)	Dickerman [24]
		Sandusky River, OH (GL)	Dickerman [24]
P. dickermani Anderson, 1962	*Elimia livescens* [f]	Ocqueoc River, MI (GL)[f]	Anderson [25]; Lushbaugh [7]; Uglem et al. [26]
		Looking Glass River, MI (GL)	Lushbaugh [7]
P. septimae Anderson and Anderson, 1967	*Elimia catenaria*[f]	Blue Springs, FL (SAG)[f]	Anderson & Anderson [22]
P. albacauda Anderson and Anderson, 1967	*Elimia catenaria*[f]	Blue Springs, FL (SAG)[f]	Anderson & Anderson [22]

		Ogeechee River, GA (SAG)	Aliff et al. [27]
P. edneyi Uglem and Aliff, 1984	*Elimia semicarinata*[f]	Elkhorn Creek, KY (OH)[f]	Uglem & Aliff [28]; Lewis et al. [10]
P. autraini LaBeau and Peters, 1995	*Elimia livescens*[f]	Au Train River, MI (GL)[f]	LaBeau & Peters [29]
***P. epholkos* n. sp.**	*Elimia* cf. *modesta*[f]	Terrapin Creek, AL (SAG)[f]	present study

(a) "*Cercaria"* is a junior subjective synonym of *Proterometra*.
(b) *Goniobasis* is treated as a junior subjective synonym of *Elimia*; original host identifications in parentheses.
(c) Water resources regions (Seaber et al. 1987) for collection localities in parentheses. TG = Texas Gulf; GL= Great Lakes; UM= Upper Mississippi, OH= Ohio; T= Tennessee; SAG= South Atlantic Gulf.
(d) Type host or locality indeterminate or not specified by original author.
(e) Conspecific with *Proterometra macrostoma* sensu Horsfall (1934).
(f) Type host or type locality.
(g) Introduced population.
ns, not specified.

Table 1-2. Fish hosts for species of *Proterometra* Horsfall, 1933 (Digenea: Azygiidae).

Species	Host	Locality[a]	Author (yr.) (ref.)
P. macrostoma (Faust, 1918) Horsfall, 1933[b]	*Astyanax mexicanus*	Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Ambloplites rupestris	ns	Horsfall [1]
		Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
		Lake Superior, Lake Ontario (GL)	Dechtiar and Lawrie [31]; Dechtiar and Christie [32]
	Lepomis auritus	Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Lepomis cyanellus	ns	Horsfall [1]
	Lepomis gibbosus[c]	Des Plaines River, IL (UM)	Dickerman [33]
		Bass Lake, MI (GL)	Horsfall [6]
		ns	Dickerman [24]
		Lake Huron, Lake Ontario (GL)	Dechtiar et al. [34]; Dechtiar & Christie [32]
	Lepomis gulosus	ns	Horsfall [1]
		Elkhorn Creek, KY (OH)	Riley & Uglem [8]; Rosen et al. [35]
		Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Lepomis humilis	ns	Horsfall [1]
	Lepomis macrochirus	ns	Horsfall [1]
		Des Plaines River, IL (UM)	Dickerman [33]
		Elkhorn Creek, KY (OH)	Riley & Uglem [8]; Rosen et al.

			[35]
		Bass Lake, MI (GL)	Horsfall [6]
		ns	Krist [9]; Dickerman [33]
		Wekiva River, FL (SAG)	Hunter & Wigington [5]
		Upper San Marcos River, Texas (TG)	Underwood & Dronen [3]
	Lepomis megalotis	Elkhorn Creek, KY (OH)	Riley & Uglem [8]; Rosen et al. [35]
	Lepomis microlophus	Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Lepomis punctatus	Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Micropterus dolomeiu	ns	Horsfall [1]
	Micropterus salmoides	ns	Horsfall [1]
		Bass Lake, MI (GL)	Horsfall [6]
		Elkhorn Creek, KY (OH)	Riley & Uglem [8]
		Upper San Marcos River, Texas (TG)	Underwood & Dronen [30]
	Pomoxis annularis	ns	Horsfall [1]
	Pomoxis nigromaculatus	ns	Horsfall [1]
		Des Plaines River, IL (UM)	Dickerman [3]
	Centrarchidae spp.	ns	Smith [15]
“Forked-Tailed Cercaria”[d](= *P. macrostoma*)	Centrarchidae spp.	ns	Cahn [18]

P. melanophora[d]	*Micropterus salmoides*[e]	Cooley Creek, (SAG)[e]	Smith [15]
P. catenaria Smith, 1934[b]	Centrarchidae spp.[c]	ns	Smith [21]
	Lepomis cyanellus[c]	Blue Springs, FL (SAG)	Smith [21]; Anderson & Anderson [22]
	Lepomis gibbosus[c]	ns	Anderson & Anderson [22]
P. hodgesiana (Smith, 1932) Smith, 1936[b]	Centrarchidae spp.[c]	ns	Smith [15]
	Lepomis cyanellus[c]		Smith [15]
P. sagittaria Dickerman, 1946[b]	*Lepomis gibbosus*[c]	ns	Dickerman [24]
	"Fish"	Maumee River, OH (GL)	Dickerman [24]
P. dickermani Anderson, 1962[b, f]	*Lepomis gibbosus*[c]	ns	Anderson [25]; Anderson & Anderson [36]
	Lepomis macrochirus[c]	ns	Anderson [25]
	Micropterus salmoides[c]	ns	Anderson & Anderson [36]
P. septimae Anderson and Anderson, 1967	*Lepomis gibbosus*[c, e]	ns	Anderson & Anderson [22]
P. albacauda Anderson and Anderson, 1967	*Noturus gyrinus*	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Lepomis auritus	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Lepomis gibbosus[c, e]	Douglas Lake, MI	Anderson & Anderson [22]
		Ogeechee River, GA ((SAG)	Aliff et al. [27]

	Lepomis gulosus	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Lepomis macrochirus	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Lepomis megalotis	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Lepomis microlophus	Ogeechee River, GA (SAG)	Aliff et al. [27]
	Pomoxis annularis	Ogeechee River, GA (SAG)	Aliff et al. [27]
P. edneyi Uglem and Aliff, 1984	*Cottus carolinae*	Elkhorn Creek, KY (OH)	Uglem & Aliff [28]
	Etheostoma spectabile[e]	Elkhorn Creek, KY (OH)[e]	Uglem & Aliff [28]
	Etheostoma blennioides	Elkhorn Creek, KY (OH)	Uglem & Aliff [28]
	Etheostoma caeruleum	Elkhorn Creek, KY (OH)	Uglem & Aliff [28]
	Etheostoma flabellare	Elkhorn Creek, KY (OH)	Uglem & Aliff [28]
P. autraini LaBeau and Peters, 1995	*Micropterus salmoides*[c, g]	Au Train River, MI (GL)	Spence & Peters [37]
	Lota lota	Au Train River, MI (GL)	LaBeau & Peters [29]
	Cottus bairdi[c, e]	Au Train River, MI (GL)[e]	LaBeau & Peters [29]
		Dead River, MI (GL)	LaBeau & Peters [29]
	Ambloplites rupestris	Au Train River, MI (GL)	LaBeau & Peters [29]
	Lepomis macrochirus[c]	Redberry Lake, MI (GL)	LaBeau & Peters [29]
	Micropterus dolomieu	Au Train River, MI (GL)	LaBeau & Peters [29]
	Perca flavescens[c]	Au Train River, MI (GL)	LaBeau & Peters [29]

***Proterometra epholkos* n. sp.** Present study	*Micropterus punctulatus*[e]	Terrapin Creek, AL (SAG)[e]	Present study

(a) Water resources regions (Seaber et al. 1987) for published collection localities in parentheses; Texas Gulf (TG); Great Lakes (GL); Upper Mississippi UM), Ohio (OH); South Atlantic Gulf (SAG).
(b) Type host or locality not specified by original author.
(c) Experimental host.
(d) Conspecific with *Proterometra macrostoma* sensu Horsfall (1934).
(e) Type host or locality.
(f) *Proterometra dickermani* matures without a vertebrate host in an experimental setting; no infection from a wild-caught fish has been published.
(g) This record was originally identified as *P. dickermani* by Spence and Peters (1971) (see LaBeau and Peters, 1995).
ns = not specified.

Table 1-3. Cercarial morphology of *Proterometra* spp.

Species	Tail stem L×W[a]	Furcae L×W[a]	Color	Furcae shape	Shedding	Distome position in tail stem[b]	Spines per mamilla	Egg #[a]	Swim[c]	Author (yr.) (ref.)
P. epholkos n. sp.	4.9–7.3 (6) × 1.6–2.6 (2)	1.1–1.3 (1.3) × 1.4 –1.8 (1.6)	Amber	Obcordate	Late PM–early AM	A/+	0–6	2–42 (20)	□	Present study
P. macrostoma	2–6.8 × 0.6–1.7	0.3–1.5 × 0.5– 1.6	Yellow	Obcordate	PM	A-M/ +	0–5	max. 50	□	Horsfall [6]; Dickerman [33]
P. catenaria	9–16 long; 5.2–8.2 × 1.1–1.3[d]	1.0–1.3 × ns	White	Ovate	Early PM	A-M/ +	10–20	"a few ova"	□	Smith [21]; Anderson & Anderson [22]
P. hodgesiana	3.8 × ns	0.8 ×0 .7	Clear	Obcordate	AM	A	Aspinous	ns	×	Smith [15]
P. sagittaria	12–18 × 1–1.2	1–3 × 0.3–0.5	No pigment	Lanceolate	AM	A/ +	Aspinous	20–30	□	Dickerman [24]
P. dickermani	3.2 × 0.3	0.7 × 0.3	Brown/ yellow	Obcordate	Late PM–early AM	Ex./ ×	Aspinous	max. 293	□	Anderson [25]

P. albacauda	3.5–6.5 × 1–1.8	0.8 × 1.2	White	Obcordate	ns	A/ +	0–5	0–10		Anderson & Anderson [22]; present study
P. septimae	3.4–4.6 × 0.8–1.8	0.7–0.9 × ns	Brown w/ white spots	Ovate, ends incurled	ns	A/ +	6–7	ns		Anderson & Anderson [22]
P. edneyi	2.9–3.4 (3.2) × 0.4–0.5 (0.5)	0.7–0.8 (0.8) × 0.4–0.6 (0.5)	Amber	Obcordate	PM	M / +[e]	0–1	16–25	×	Uglem & Aliff [28]
P. autraini	4.3–5.5 (4.8) × 1.3–1.8 (1.5)	1.1–1.4 (1.2) × 0.8–1.43 (1)	Light gold	Obcordate	Diurnal	Ex.-A/ + -	0–8	200–300		LaBeau & Peters [29]

(a) All measurements in millimeters (mm); means in parentheses.
(b) Extruded (Ex.) from tail stem, within anterior portion (A) of tail stem, within middle portion (M) of tail stem; within a cavity (+), external to a cavity (-), cavity not reported or reported as absent (×).
(c) = swimming, × = not swimming.
(d) Smith's (1936) original description described length of the cercaria as 9–16 mm. Anderson and Anderson (1967) published supplemental observations for *P. catenaria* and described the fixed cercaria as 5.2–8.2 mm long.
(e) Based on figure 2 of Uglem and Aliff (1984).
ns = not specified.

Table 1-4. Adult morphology of *Proterometra* spp.

Species	Body L×W[a]	Body color	OS:VS ratio[b]	Testes shape	Uterus[b]	Extent of vitellarium[b]	Eggs[c, d]	Author (yr.) (ref.)
P. epholkos n. sp.	1980–2300 (2189) × 820–1280 (1169)	Orange	2:1	Ovoid	Extercaecal, convoluted from posterior region of body to posterior third of OS	Lateral, posterior quarter of OS to posterior margin of body	51 × 26, 78 × 44	Present study
P. macrostoma	1800–2100 × 1000–1200	“Light tan /pink”	2:1	Ovoid	Intercaecal, loose irregular loops from ovary to posterior margin of OS	Lateral, posterior margin of OS to region of testes	65 × 34, 95 × 75	Horsfall [1,6]; Dickerman [33]
P. catenaria	1700–1830 × 1180–1300	Pale yellow	2.5:1	Pyriform	Extercaecal, anterior loops even with vitellaria	Lateral, between anterior & posterior 1/3 of OS to posterior margin of VS	52 × 28, 59 × 34	Smith [21]; Anderson & Anderson [22]
P. hodgesiana	ns	ns	ns	ns	ns	ns	ns	Smith [15]
P. sagittaria	1500–2000 × 900–1000	Orange	2:1	"Elongate pyriform"	Intercaecal, loose transverse coils from ovary to OS	Lateral, OS posterior margin to posterior region of testes	67 × 35, 79 × 44	Dickerman [24]
P. dickermani	1760–1870 × 1010–1180	“Tan w/ pink tinge”	1.7:1	Ovoid	Intercaecal, coils extend anterior to posterior margin of OS	Lateral, mid-level of OS to or beyond posterior margin of testes	74 × 47, 98 × 66	Anderson [25]

P. albacauda	1990–2200 × 1230–1380	Yellow/ Tan	2:1	Pyriform	Intercaecal, convoluted between ovary and posterior margin of OS	Lateral, posterior margin of OS to posterior fourth of testes	62 × 34, 75 × 42	Anderson & Anderson [22]
P. septimae	1610–1860 × 1150–1270	Pale orange	2.7:1	Ovoid	Extercaecal, extends to mid level of OS	Lateral, center of OS to center of VS	65 × 26, 73 × 36	Anderson & Anderson [22]
P. edneyi	550–1010 (737) × 330–610 (450)	ns	2:1	Elongate, elliptical	Intercaecal, one loop between VS and OS	Lateral, OS posterior margin to anterior one half of testes	71 × 40, 77 × 42	Uglem & Aliff [28]
P. autraini	1920–2160 (2030) × 1320–1540 (1430)	Light orange	2:1	Elongate elliptical	Intercaecal, convoluted, extends to posterior third of OS	Lateral, Posterior level of OS to anterior level of testes	75 × 42, 92 × 64	LeBeau & Peters [29]

(a) Measurements in micrometers (µm); mean in parentheses.
(b) OS = oral sucker; VS = ventral sucker
(c) Measurements in millimeters (mm).
(d) Smallest egg (LxW), largest egg (LxW).
ns = not specified.

Table 5. Key to *Proterometra* spp. (cercaria).

1a. Tail stem length exceeding 10 mm 2
1b. Tail stem length not exceeding 10 mm 3

2a. Furcae lanceolate, 1–3 mm long; mamillae aspinous ..... *P. sagittaria*
2b. Furcae ovate, 1–1.3 mm long; 10–20 spines/mamillae ..... *P. catenaria*

3a. Furcae obcordate, ends rounded, with medial notch 4
3b. Furcae ovate, ends incurled, lacking medial notch ..... *P. septimae*

4a. Many uterine eggs 5
4b. Few uterine eggs 6

5a. Distome extruded; tail cavity absent; tail stem < 3.5 mm long; mamillae aspinous ..... *P. dickermani*
5b. Distome extruded or withdrawn; tail cavity present; tail stem < 4 mm long; mamillae aspinous or with up to 8 spines/mamillae ..... *P. autraini*

6a. Mamillae arranged in transverse bands throughout tail stem; cercaria does not swim; tail stem ≤ 3.4 mm long; tail stem ≤ 0.6 mm wide ..... *P. edneyi*
6b. Mamillae in a lateral column per body margin posterior to the tail cavity (Fig. 12); cercaria swims; tail stem ≥ 3.5 mm long; tail stem ≥ 1 mm wide 7

7a. Tail stem medially constricted; mamillae > 0.1 mm, 5–6 per lateral column, aspinous or with up to 6 spines/mamillae; cercaria amber in color; furcae > 1mm long, ≥ 1.4 mm wide; distome > 1.3 mm long ..... *P. epholkos*
7b. Tail stem gradually sloping from medial axis to furcae synthesis; mamillae minute < 0.05 mm, 8-10 per lateral column, aspinous or with up to 5 spines/mamillae, cercaria white in color; furcae < 1 mm long, < 1.4 mm wide; distome < 1.3 mm long ..... *P. albacauda*

Table 6. Key to species of *Proterometra* (adults).

1a. Diameter of oral sucker / ventral sucker ≥ 2:1 2
1b. Diameter of oral sucker / ventral sucker < 2:1 ..... *P. dickermani*

2a. Vitellarium not or slightly extending posteriad beyond margin of ventral sucker; posterior uterus looping anterior or lateral to ventral sucker 3
2b. Vitellarium extending posteriad beyond ventral sucker; uterus looping between ovary and ventral sucker 4

3a. Uterus intercecal or dorsal to ceca; vitellarium not extending beyond level of mid ventral sucker; egg > 60 mm in diameter; testes nearly equal in size ... *P. septimae*
3b. Uterus extracecal; vitellarium extending to level of or slightly posterior to ventral sucker; egg ≤ 60 mm long; testes unequal ..... *P. catenaria*

4a. Body ≤ 1,100 µm long; body ≤ 650 µm wide ..... *P. edneyi*
4b. Body > 1,100 µm long; body > 700 µm wide 6

5a. Vitellarium longer than intestinal caecae 7
5b. Vitellarium shorter than intestinal caecae 8

6a. Ventral sucker > 270 µm long; oral sucker typically > 600 µm long; testes ovoid, not intercaecal, typically 0.3 × 0.16 mm ..... *P. epholkos*
6b. Ventral sucker < 270 µm long; oral sucker ≤ 600 µm long; testes elongate pyriform, intercaecal, typically 200 µm × 80 µm ..... *P. sagittaria*

7a. Anterior uterine loops not extending beyond pharynx; vitelline reservoir pretesticular; vitellarium not extending beyond anterior margin of testes ..... *P. autraini*
7b. Anterior uterine loops extending to posterior third of oral sucker; vitelline reservoir midtesticular; vitellarium extending beyond anterior margin of testes ..... *P. albacauda*

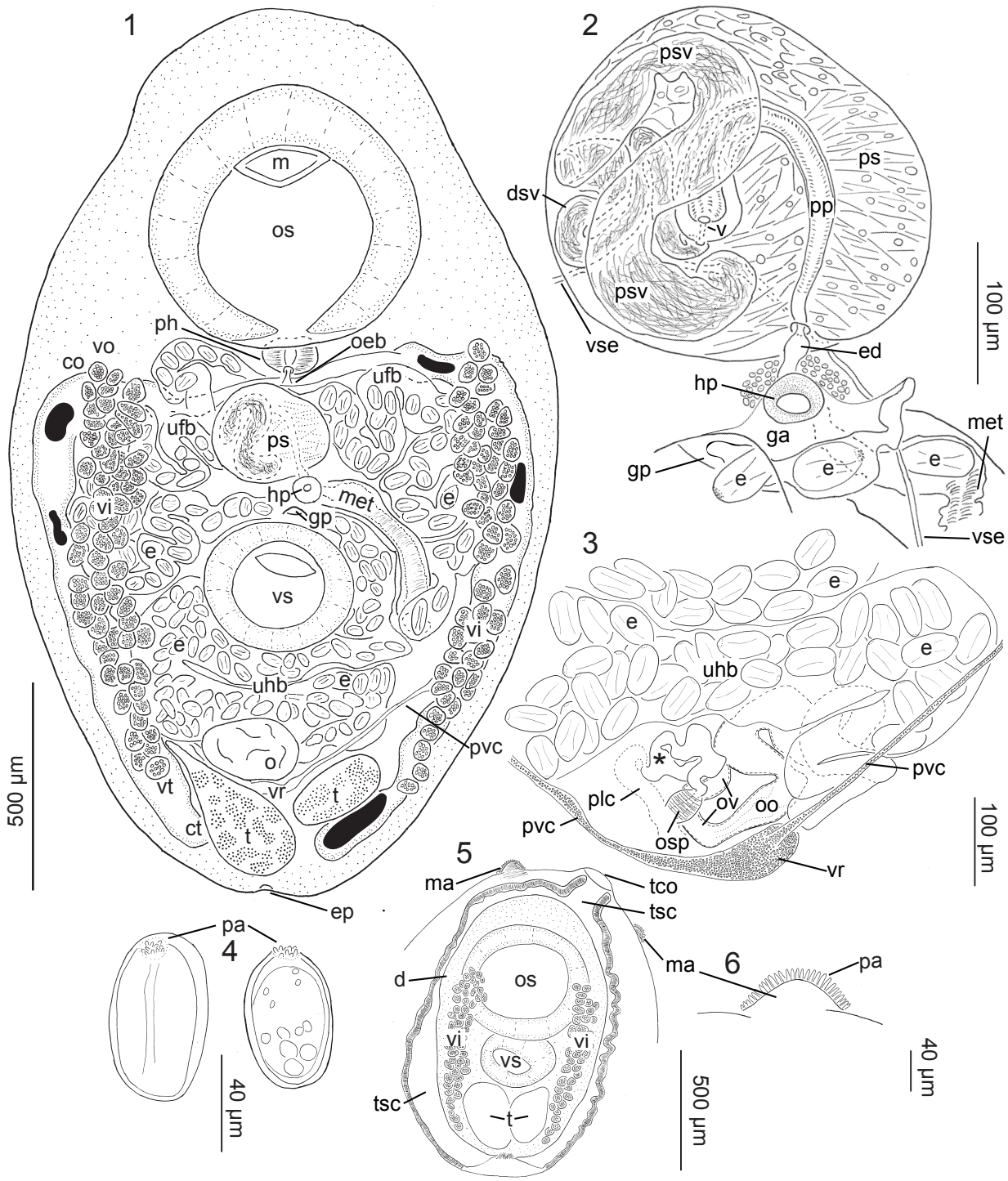
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Plate 1-1, Figs. 1–6: *Proterometra albacuada* Anderson and Anderson, 1967 (Digenea: Azygiidae). Figs. 1–4, adult (holotype, USNPC No. 61229) from cardiac stomach of pumpkinseed sunfish, *Lepomis gibbosus* (Linnaeus, 1758) Berg 1949 (Perciformes: Centrarchidae). Scale values aside each bar. (**1**) Ventral view of body of adult showing the mouth (m), oral sucker (os), pharynx (ph), oesophagus bifurcation (oeb), caecae (co) near origin, vitelline follicles (vo) near origin of vitellarium, uterus (ufb) convoluted in forebody, prostatic sac (ps), eggs (e), hermaphroditic pore (hp), metraterm (met), genital pore (gp), ventral sucker (vs), vitelline follicles (vi), uterus in hindbody (uhb) looping between ventral sucker and ovary (o), vitelline reservoir (vr), primary vitelline collecting ducts (pvc), vitelline follicles (vt) near termination of vitellarium, testes (t), caecae termination (ct), and excretory pore (ep). (**2**) Ventral view of the male genitalia showing the prostatic sac (ps), swollen proximal region of seminal vesicle (psv), distal tubular region of seminal vesicle (dsv), minute pore, possibly verschlussapparat (v) (see section 3.1), pars prostatica (pp), ejaculatory duct (ed), metraterm (met), vasa efferentia (vse), hermaphroditic pore (hp), genital atrium (ga), eggs (e), and genital pore (gp). (**3**) Ventral view of the female genitalia with ovary omitted from image, showing the uterus in hindbody (uhb) looping between ovary and ventral sucker, eggs (e), confluence of the oviduct and Laurer's canal (*), proximal end of laurers canal (plc), oviduct (ov), ootype (oo), oviduct sphincter (osp), vitelline reservoir (vr), and primary vitelline collecting ducts (pvc). (**4**) Egg from distal portion of the uterus (left) and proximal portion of the uterus (right) showing papilla-like projections (pa). Figs. 5–6, cercaria (paratype, USNPC No. 61230) from gonoducts of gravel elimia, *Elimia* catenaria (as *Goniobasis* catenaria) Say, 1822 (Cerithioidea: Pleuroceridae). (**5**) Tail cavity opening (tco), muscular tail stem cavity (tsc), tail stem mamilla (ma), distome (d), and positions of the oral sucker (os), vitelline follicles (vi), ventral sucker (vs), and testes (t) in distome. (**6**) Tail stem mamilla (ma) showing papilla-like projections (pa).

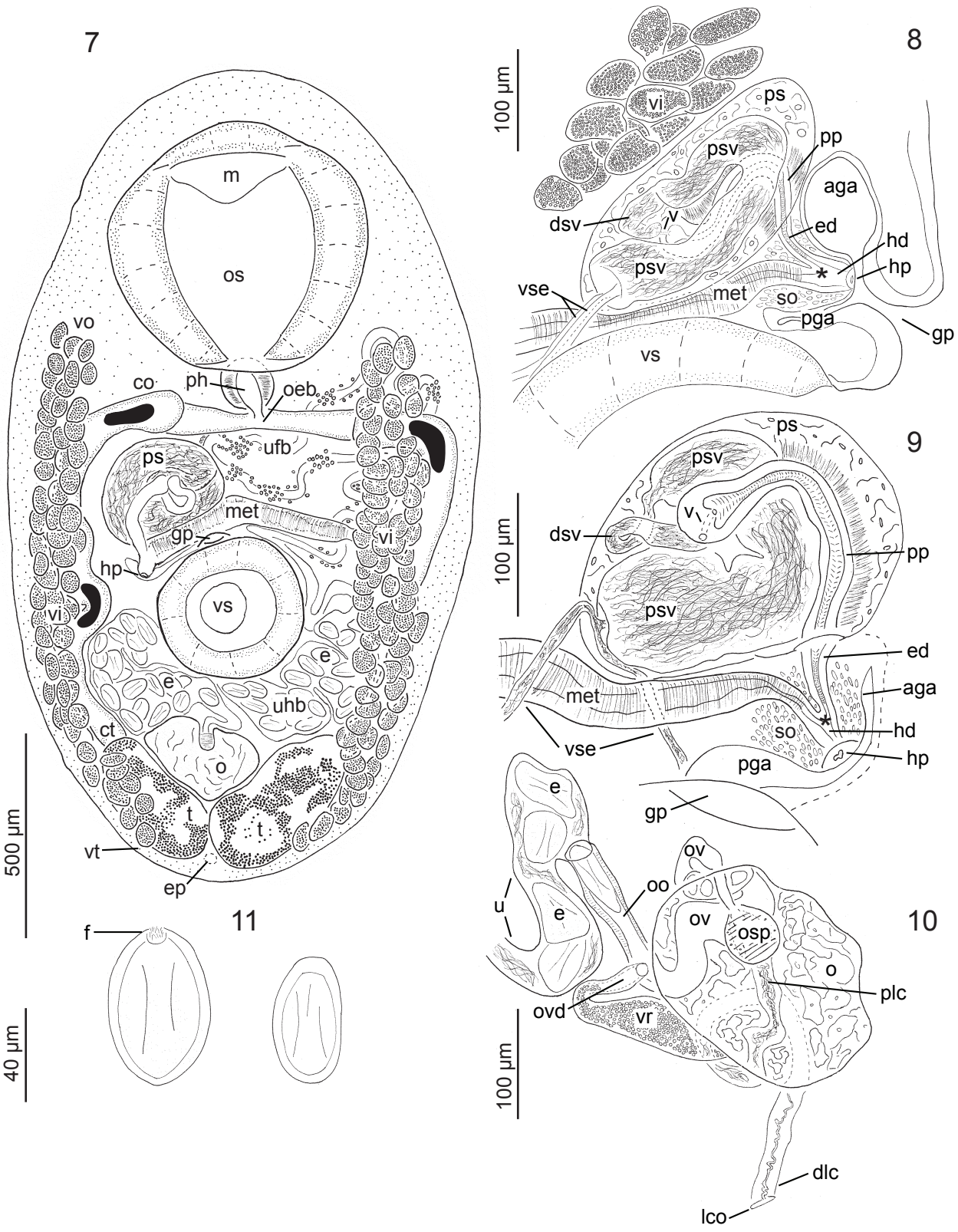
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Plate 1-2, Figs. 7–11: *Proterometra epholkos* sp. n. (Digenea: Azygiidae), adults from oesophagus of spotted bass, *Micropterus punctulatus* (Rafinesque, 1819) (Perciformes: Centrarchidae). Scale value aside each bar. (**7**) Ventral view of body of adult showing mouth (m), oral sucker (os), pharynx (ph), oesophagus bifurcation (oeb), caecae (co) near origin, vitelline follicles (vo) near origin of vitellarium, uterus (ufb) convoluted in forebody, prostatic sac (ps), hermaphroditic pore (hp), metraterm (met), genital pore (gp), ventral sucker (vs), vitelline follicles (vi), uterus in hindbody (uhb) looping between ventral sucker and ovary (o), eggs (e), caecae termination (ct), testes (t), vitelline follicles (vt) near termination of vitellarium, and excretory pore (ep). (**8**) Lateral view of male genitalia showing locations of vitelline follicles (vi), prostatic sac (ps), vasa efferentia (vse), swollen proximal region of seminal vesicle (psv), distal tubular region of seminal vesicle (dsv), minute pore, possibly verschlussapparat (v) (see section 3.1), pars prostatica (pp), ejaculatory duct (ed), metraterm (met), sinus organ (so), confluence of male and female terminal genitalia (*), hermaphroditic duct (hd), hermaphroditic pore (hp), anterior genital atrium lobe (aga), posterior genital atrium lobe (pga), genital pore (gp), and ventral sucker (vs). (**9**) Ventral view of male genitalia showing locations of the prostatic sac (ps), vasa efferentia (vse), swollen proximal region of seminal vesicle (psv), distal tubular region of seminal vesicle (dsv), minute pore, possibly verschlussapparat (v) (see section 3.1), pars prostatica (pp), ejaculatory duct (ed), metraterm (met), sinus organ (so), confluence of male and female terminal genitalia (*), hermaphroditic duct (hd), hermaphroditic pore (hp), anterior genital atrium lobe (aga), posterior genital atrium lobe (pga), and genital pore (gp). (**10**) Ventral view of the female genitalia showing ovary (o), oviduct sphincter (osp), oviduct (ov), proximal portion of laurers canal (plc), distal end of Laurer's canal (dlc), laurers canal opening (lco), vitelline reservoir (vr), ovovitelline duct (ovd), ootype (oo), and proximal end of uterus (u) with eggs (e) and sperm. (**11**) Eggs from distal (left) and proximal (right) portions of uterus showing fimbria (f) or papilla-like projections.

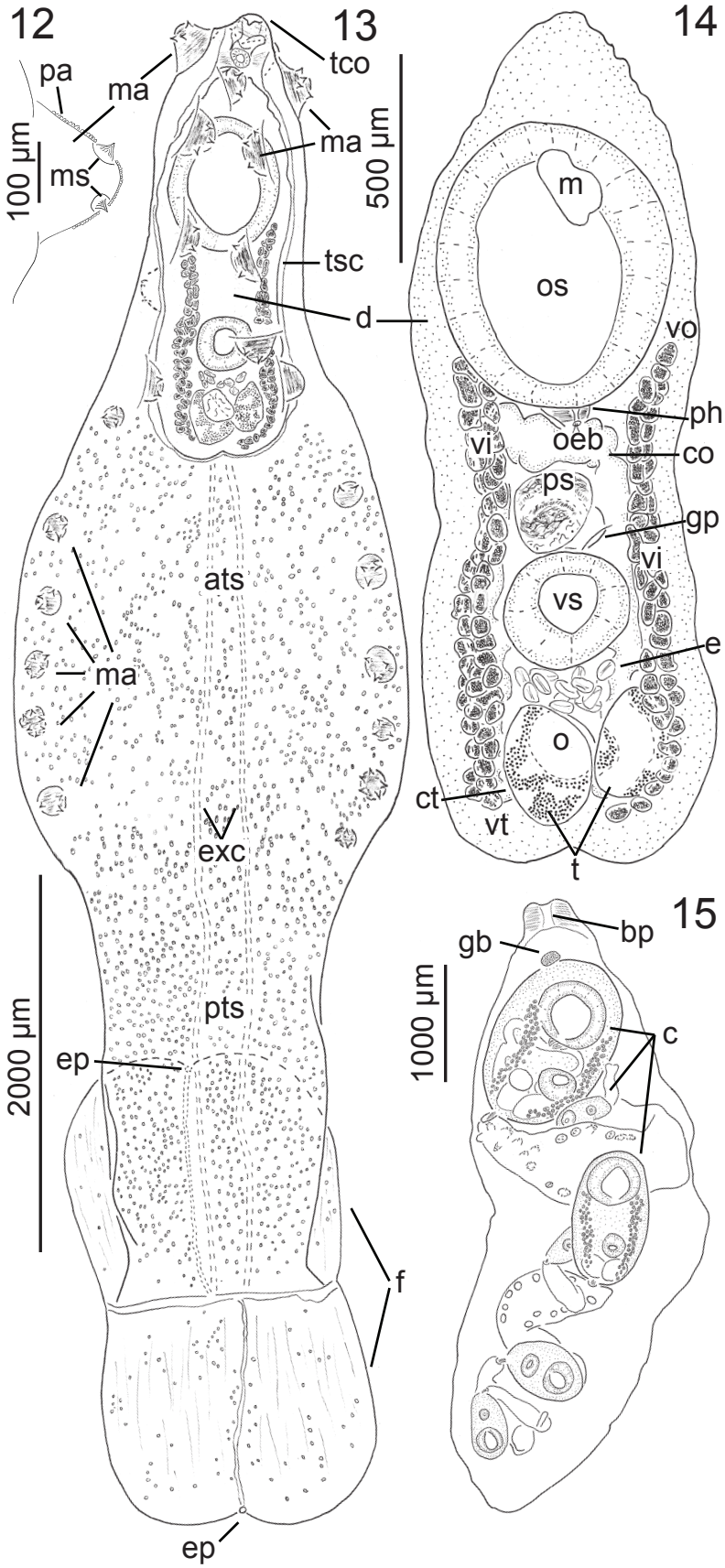
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Plate 1-3; Figures 12-15. *Proterometra epholkos* sp. n. (Digenea: Azygiidae), naturally shed cercariae and sporocysts from *Elimia* cf *modesta* (Lea, 1845) (Cerithioidea: Pleuroceridae). Scale values aside each bar. (**12**) Tail stem mamilla (ma) showing minute papilla-like projections (pa) and mamilla spines (ms) (**13**) Cercaria showing tail cavity opening (tco), mamillae (ma), tail stem cavity (tsc), distome (d), anterior tail stem region (ats), excretory canals (exc), posterior tail stem region (pts), furcae (f), and excretory pores (ep). (**14**) View of distome showing mouth (m), oral sucker (os), vitelline follicles (vo) near origin of vitellarium, pharynx (ph), oesophagus bifurcation (oeb), caecae (co) near origin, prostatic sac (ps), genital pore (gp), vitelline follicles (vi), ventral sucker (vs), eggs (e), ovary (o), caecae termination (ct), vitelline follicles (vt) near termination of vitellarium, and testes (t). (**15**) View of sporocyst showing the location of the birth pore (bp), cercariae (c), and a germ ball (gb).

CHAPTER 2: NEW SPECIES OF *PROTEROMETRA* (DIGENEA: AZYGIIDAE) AND ITS LIFE CYCLE IN THE CHICKASAWHAY RIVER, MISSISSIPPI USA, WITH SUPPLEMENTAL OBSERVATIONS OF *PROTEROMETRA AUTRAINI*

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Authors: Matthew R. Womble, Raphael Orélis-Ribeiro, Stephen A. Bullard

ABSTRACT

We describe *Proterometra ariasae* n. sp. based upon cercariae shed from a freshwater snail, *Pleurocera* sp., and adults infecting the buccal cavity of longear sunfish, *Lepomis megalotis,* captured from the Chickasawhay River, Mississippi, USA. We also provide supplemental observations of cercarial and adult paratypes of *Proterometra autraini* from the Au Train River, Michigan, USA. Sequence data for the ribosomal internal transcribed spacer 2 (ITS2) from adults and cercariae of the new species were identical. Adults of the new species differ from congeners by having (i) a markedly large body, (ii) a proportionally large oral sucker, (iii) ovoid testes, (iv) a strongly muscular and laterally expanded pars prostatica, (v) a uterus that is extensively convoluted between the ovary and ventral sucker (vi) and a vitellarium as long as the caeca and extending posteriad beyond the anterior margin of the testes. Cercariae of the new species differ from those of its congeners by having (i) a tail stem that is shorter than 10 mm and that lacks a medial constriction, (ii) obcordate furcae that are wider than long, (iii) mamillae distributed throughout the anterior tail stem only, and (v) a proportionally small distome that has relatively few uterine eggs and remains withdrawn in the anterior tail stem region in actively swimming cercariae. This is the first report of *Proterometra* from Mississippi, the second description to employ morphology and sequence data to

elucidate a life cycle for *Proterometra*, and the third species of *Proterometra* from an intermediate host not assigned to *Elimia*.

1. INTRODUCTION

Species of *Proterometra* Horsfall, 1933 (Digenea: Azygiidae Looss, 1899) exploit a diverse assemblage of primary division freshwater fishes and undergo asexual reproduction in freshwater prosobranch snails (Pleuroceridae) of high conservation value [1] (see Tables 1 & 2 of Womble et al. [2]). To date, no accepted species of *Proterometra* has been documented from beyond North America, and all but one record [3] sources from east of the main stem of the Mississippi River [2]. Given the reported geographic ranges for known hosts of *Proterometra* spp. [1,2,4] and considering that many closely related snails and fishes lack records of infection, many species of *Proterometra* likely remain unnamed in North American rivers and streams.

All species of *Proterometra* reportedly exhibit a 2-host life cycle (considered a truncated life cycle based on phylogenetic inference) wherein the macroscopic cercaria is progenetic and presumably, and flamboyantly, mimics its host's prey; thereby luring the definitive fish host to swallow it. Moreover, and taxonomically troublesome, adult flukes of *Proterometra* are subtly morphologically distinct and have garnered little taxonomic attention, with most publications treating cercarial morphology [5-17], life history [18-20], physiology and behavior [21-27], and host-parasite interactions [28-30]. Before 2014, no sequence data for any species of *Proterometra* was published. GenBank now holds sequence data for *Proterometra epholkos* Womble, Orélis-Ribeiro,

and Bullard, 2015 (ribosomal internal transcribed spacer 2 [ITS2], nuclear ribosomal DNA region [rDNA] [2]) and *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933 (*species inquirendae*) (18S rDNA & cytochrome c oxidase subunit 1 (CO1) [31]; see Womble et al. [2] for sorted issues concerning the taxonomic identity of *P. macrostoma*) plus *Proterometra* sp. from Florida (28S rDNA; [32]).

We herein (i) describe and elucidate the life cycle of a new species of *Proterometra* using morphology and phylogenetic inference (ITS2) and (ii) provide needed supplemental observations of its morphologically similar congener *Proterometra autraini* LaBeau and Peters, 1995. The present study comprises the first report of a species of *Proterometra* from Mississippi (Pascagoula River Drainage) USA and the second use of a molecular marker in concert with morphology to elucidate a life cycle for a species of *Proterometra.* The new species is only the third species of *Proterometra* reported from an intermediate host not assigned to *Elimia* Adams, 1854.

2. MATERIALS AND METHODS

2.1. Specimen collection, identification, and preparation

Prosobranch snails were collected by hand in the Chickasawhay River (31°57’04”N; 88°42’06”W; Clarke County, Mississippi, USA) on 13 October 2013. Snails were subsequently transported to the laboratory in 20-L plastic buckets filled with ambient stream water and aerated using battery powered aerators and airstones. In the laboratory, methods for snail husbandry as well as cercarial isolation and collection followed Womble et al. [2]. Cercarial specimens for morphology were isolated and heat

killed within a dish flooded with freshwater heated to 60°C. Killed specimens were then transferred to a vial of 10% neutral buffered formalin (nbf). Infected snails initially were identified as *Pleurocera* cf. *chakasahaense* Tyron, 1873 (considered a junior subjective synonym of *Pleurocera vestita* (Conrad, 1834) [1]; as *P. vestitum* in Graff [33]) based on shell shape and the collection locality. However, given that (i) a taxonomic key is lacking for Pleuroceridae, (ii) a taxonomically discrete description for *P. chakasahaense* or *P. vestita* is lacking, and (iii) the collection locality falls outside of the reported geographic range for *P. vestita* [1], we identified our snail specimens as "*Pleurocera* sp." Shell vouchers preserved in 70% ethanol and representative mantle tissue preserved in 95% ethanol were deposited in the collection of Auburn University Museum of Natural History (AUMNH).

Fish were cast-netted from the Chickasawhay River on 23 July 2014, maintained alive in ambient river water, transported to the laboratory, killed by spinal severance, and identified as longear sunfish, *Lepomis megalotis* (Rafinesque, 1820) (Perciformes: Centrarchidae), by the combination of having (i) 3 anal fin spines, (ii) a forked caudal fin, (iii) a deep body, (iv) no teeth on the tongue, (v) a short and rounded pectoral fin, (vi) "short and stubby" gill rakers, (vii) long, dark opercular tabs each with a white posterior border, and (viii) 14 pectoral fin rays [34]. Bone cutting shears were used to hemisect the jaw and the buccal cavity to reveal epithelial surfaces before inspection with the aid of a stereoscope at 10x objective magnification.

Fluke specimens for morphology were removed from the buccal cavity and epithelial surface of the oesophagus with fine forceps, wet-mounted on glass slides, heat-killed under slight coverslip pressure with heat from an ethanol burner flame, and preserved in

10% neutral buffered formalin (nbf). Specimens for light microscopy were held in 10% nbf for at least 48 h, rinsed overnight in distilled water, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, dehydrated in a graded series of ethanols, briefly immersed in xylene, further cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Measurements, photographs, and illustrations of stained, whole-mounted specimens were made with aid of a Leica DM-2500 equipped with differential interference contrast (DIC) optical components and a drawing tube. Specimens for scanning electron microscopy (SEM) were fixed in nbf as above, washed in de-ionized water, dehydrated through a graded ethanol series, critical point dried in liquid CO_2, mounted on standard aluminum SEM pin stubs with double-sided carbon tape, sputter coated with gold palladium (19.32g/cm^3; 25 mA), and viewed with a Zeiss EVO 50VP scanning electron microscope. Measurements are herein reported in micrometers (μm), unless otherwise noted, followed by the mean and number of specimens measured for that feature in parentheses.

2.2. DNA extraction, amplification, and sequencing

Three live cercariae for molecular biology were pipetted into separate vials of 95% EtOH and stored at -20°C. Four live adults for molecular biology were excised with fine forceps, placed into separate vials of 95% EtOH, and stored at -20°C. Total genomic DNA was extracted using a DNeasy™ Blood and Tissue Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions, except for the final elution step wherein only 50μl of elution buffer was used, in order to increase the final DNA concentration in

the eluate. DNA concentrations of samples were quantified (i.e., ng/ µl) using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Polymerase chain reaction (PCR) amplifications of the ITS2 rDNA region were performed in a total volume of 50µl, consisting of approximately 2.5 µl of template DNA, 10 µl of 5× Taq Buffer, 1 µl of dNTPs (Promega, Madison, WI), 1 µl of the forward primer "GA1" (5'-AGA ACA TCG ACA TCT TGA AC-3') (3' end of the 5.8S rDNA) [35], and 1 µl of the reverse primer "ITS2.2" (5'-CCT GGT TAG TTT CTT TTC CTC CGC-3') (5' end of 28s rDNA) [35], 0.3 µl of Taq polymerase (5 prime, Inc., Gaithersburg, MD) and 32 µl of molecular grade distilled water. PCR amplification was carried out with an amplification profile consisting of an initial 5 minutes at 95°C for denaturation, followed by 35 repeating cycles of 95°C for 30 seconds for denaturation, 57°C for 30 seconds for annealing, and 72°C for 45 seconds for extension, followed by a final 10 minutes at 72°C for extension. PCR products were viewed on a 1% agarose gel stained with ethidium bromide. Sequencing was performed by Lucigen Corp. (Madison, WI) using the same primers as used in the PCR. Sequence assembling and analysis of chromatograms was conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium). The Internal Transcribed Spacer 2 Ribosomal Database [36] was used to determine the borders of the 5.8s, ITS2, and 28s gene regions. IUPAC ambiguity codes were used for coding polymorphic sites, i.e., should be read as the presence of guanine and cytosine, rather than as an ambiguous reading between guanine or cytosine. These positions were identified in the chromatogram as per Womble et al. [2]. Representative sequences have been deposited in GenBank (see Table 1).

2.3. Phylogenetic methods

Reference specimens of *Azygia longa* (Leidy, 1851) Manter, 1926 (Digenea: Azygiidae) and *Leuceruthrus micropteri* Marshall and Gilbert, 1905 (Digenea: Azygiidae) were collected and preserved for morphological and molecular analyses. Briefly, specimens of *L. micropteri* were excised from the stomach of largemouth bass, *Micropterus salmoides* (Lacepède, 1802) (Perciformes: Centrarchidae), electroshocked in Wheeler Reservoir, Alabama (34°37'17.8" N; 86°49'51.47" W; Limestone County, Alabama, USA). Specimens of *A. longa* were collected on 27 February 2009 from the stomach of chain pickerel, *Esox niger* Lesueur, 1818 (Esociformes: Esocidae), in the Pascagoula River, Mississippi (30°36'40.22"N; 88°38'29.97"W; Jackson County, Mississippi, USA). Fish hosts were identified as per the features given by Boschung and Mayden [34]. Fluke specimens were stained and whole-mounted as described previously and identified as per Gibson [38]. A voucher specimen of *L. micropteri* was deposited in the USNM and a voucher specimen of *A. longa* was previously deposited (see [32]) in the University of Southern Mississippi, Gulf Coast Research Laboratory Museum (GCRL) (Table 1). Extraction, amplification, and sequencing of DNA from two specimens of *L. micropteri* were performed as detailed previously (see section 2.2), and the methodology for the single specimen of *A. longa* followed Calhoun et al. [32].

The resulting sequences were aligned using MEGA v.6.06 [39] with default ClustalW parameters. The resulting alignment was checked by eye and trimmed to match the shortest fragment, resulting in a total fragment length of 363 base pairs (bp) including gaps. Absolute site differences and sequence similarity percentages were calculated

using the “compute pairwise distances” function in MEGA v.6.06 [39] and are displayed in Table 2, for each analysis gaps were treated using the pairwise deletion function. The aligned sequences were analyzed using the neighbor-joining (NJ) [40] and maximum likelihood (ML) methods using MEGA v.6.06 [39]. The ML analysis was performed using the best-fit DNA model analysis estimated with MEGA v.6.06 [39] as Kimura’s 2-parameter model with gamma distributed rate variation among sites (K2 + G) [41] in combination with the Nearest-Neighbor-Interchange heuristic method. All sites including gaps were considered in the analysis. A bootstrap analysis based on 1,000 replicates was used to establish nodal support values. Branch support was considered as significant when bootstrap values were > 70%. Sequence data from the ITS2 for *Transversotrema borboleta* Hunter and Cribb, 2012 (Transversotrematidae: Digenea) (GenBank JF412524) was selected as an outgroup and included in the analyses based on its proposed phylogenetic relationship to Azygiidae [42] (Table 1).

2.4. Host and parasite nomenclature

Common names, scientific names, taxonomic authorities and dates, and higher-level gastropod classification follow Johnson et al. [1]. Nomenclature for *Pleurocera* follow Goodrich [43], Burch and Tottenham [44], Burch [45], Graff [33], and Johnson et al. [1]. Higher-level fish classification and nomenclature follow Nelson [46] and fish common names follow Boschung and Mayden [34]. Nomenclature for Azygiidae follow Gibson and Bray [47] and morphological terminology for *Proterometra* follow Womble et al. [2] and references therein. Type material of *P. autraini* was borrowed from the Harold W. Manter Laboratory of Parasitology (Lincoln, Nebraska: HWML)

3. RESULTS

3.1. Proterometra autraini *LaBeau and Peters, 1995 (Figs. 1-13)*

3.1.1. Diagnosis of adult based on light microscopy of 2 whole-mounted paratypes [HWML 37903-6, 7].

Body of adult, oval, 1780–1800 (1790, 2) long, 1040–1120 (1080, 2) wide or 1.6–1.7 (1.7, 2) × longer than wide, ventrally concave (Fig. 1); forebody 990–1000 (995, 2) long or 56% (56%, 2) of overall body length; hindbody 480–500 (490, 2) long or 27–28% (27%, 2) of overall body length, 48–50% (49%, 2) of forebody length; tegument unarmed, approximately 30–35 (33, 2) thick. Excretory system mostly indistinct in paratypes; excretory pore medial, terminal (Fig. 1). Nervous system indistinct in paratypes. Oral sucker subterminal, 90–100 (95, 2) or 5% (5%, 2) of body length from anterior body end, 1180–1100 (1140, 2) or 61–66% (64%, 2) of body length from posterior body end, 590–610 (600, 2) long or 33–34% (34%, 2) of body length, 590–600 (595, 2) wide or 54–57% (55%, 2) of body width, posterior margin 310 (310, 2) from anterior margin of ventral sucker (Fig 1). Ventral sucker in posterior half of body, with anterior margin 990–1000 (995, 2) or 56% (56%, 2) of body length from anterior body end, 300–330 (315, 2) long or 17–18% (18%, 2) of body length, 350 (350, 2) wide or 31–34% (32%, 2) of body width, wider than long, 51–54% (52%, 2) of oral sucker length, 58–59% (58%, 2) of oral sucker width (Fig. 1). Mouth, subterminal, opening ventrally. Pharynx ovoid, posterior to oral sucker, 115 (115, 2) long or 6 % (6%, 2) of body length, 100–115 (108, 2) wide (Fig 1). Oesophagus extending posteriad from mouth 315–325 (320, 2) before bifurcating 15 posterior to pharynx, oesophageal

branches extending laterad before synthesis with intestinal caeca (Fig. 1); dextral oesophageal branch 60–170 (115, 2) long, 35 (35, 2) at maximum width; sinistral oesophageal branch 105–125 (115, 2) long, 30 (30, 2) at maximum width; intestinal caeca confluent with oesophageal branches in forebody, appearing inverse U-shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caeca (Fig.1); dextral caecum 1275–1425 (1350, 2) long or 71–80% (75%, 2) of body length, 110–120 (115, 2) in maximum width, pre-caecal space 670-700 (685, 2) or 38% (38%, 2) of body length from anterior end of body, post-caecal space 15 (2) or <1% (2) of body length from posterior end of body; sinistral caecum 1340–1380 (1360, 2) long or 74–77% (76%, 2) of body length, 90–95 (93, 2) in maximum width, pre-caecal space 660-670 (665, 2) or 37% (37%, 2) of body length from anterior end of body, post-caecal space 25–50 (38, 2) or 1–3% (2%, 2) of body length from posterior end of body.

Testes 2 in number, oblique, obtuse or nearly parallel along lateral axis, elliptical in shape (Fig. 1); dextral testis 295–370 (333, 2) long or 17–21% (19%, 2) of body length, 90–115 (103, 2) wide or 8–11% (9%, 2) of body width, pre-testicular space, 1500–1520 (1510, 2) from anterior end of body or 84% (84%, 2) of total body length, post-testicular space, 45–100 (73, 2) from posterior end; sinistral 255–290 (273, 2) long or 14–16% (15%, 2) of body length, 105–125 (115, 2) wide or 11% (11%, 2) of maximum body width, pre-testicular space 1640–1680 (1660, 2) from anterior end of body or 92–93% (93%, 2) of total body length, post-testicular space 40–53 (47, 2) from posterior end. Vasa efferentia indistinct in paratypes. Prostatic sac medial, dorsal to ventral sucker, anterior margin 83–85 (84, 2) from posterior margin of oral sucker, posterior margin variable in relation to anterior margin of ventral sucker, 150–265 (207, 2) long, 265–320

(293, 2) wide (Figs. 1–3). Seminal vesicle thin walled, highly convoluted, occupying majority of space within prostatic sac, 806 (1) long, having swollen proximal region and narrow distal region (Figs. 1, 2); proximal region of seminal vesicle 406 (1) long or 50% (1) of total seminal vesicle length, 43 (1) wide (Fig. 2); distal region of seminal vesicle 400 (1) long or 50% (1) of total seminal vesicle length, 23 (1) wide (Fig. 2). Verschlussapparat (see Horsfall [6]) piercing proximal region of pars prostatica ventrally (Fig. 2). Pars prostatica 188–215 (202, 2) long, 55–60 (58, 2) wide proximally, 13 (2) wide distally, tapering 76–78% (77%, 2) in width from proximal to distal end, slightly arched, thin walled for entire length, exiting prostatic sac ventrally (Fig. 2). Ejaculatory duct (= continuation of pars prostatica outside of prostatic sac) extending ventrally from prostatic sac becoming confluent with hermaphroditic duct, lacking gland-like cells or muscle in wall, 38 (2) long or 18% (1) of pars prostatica length. Confluence of terminal male and female genitalia occurring within sinus organ. Sinus organ directed ventrally. Hermaphroditic pore anterior of ventral sucker, at 48–51% (50%, 2) of body length, directed ventrally communicating with genital atrium (Fig. 2). Genital atrium, circular in ventral view, communicating hermaphroditic pore and genital pore, filled with many fully developed/ hatching eggs, 325–375 (350, 2) in diameter or 29–36% (33%, 2) of body width, on average equal to ventral sucker width, occupying majority of area between oral and ventral suckers (Figs. 1–3). Genital pore immediately anterior to ventral sucker, medial, posterior to perpendicular midline of prostatic sac (Figs. 1, 2). Ventro-cervical groove indistinct in paratypes.

Ovary near medial, intercaecal, 133 (1) long or 7% (1) of body length, 200 (1) wide or 18% (1) of body width or 1.5 (1) × wider than long (Fig. 1); post-ovary space 150–180

(165, 2) or 8–10% (9%, 2) of body length; germarium indistinct in paratypes. Female genitalia inclusive of oviduct, laurers canal, ovovitelline duct, ootype and mehlis gland indistinct in paratypes. Uterus occupying space between posterior half of oral sucker and near posterior margin of body, comprising a field 1300–1360 (1330, 2) long or 73–76% (74%, 2) of body length and 820–860 (840, 2) wide or 77–79% (78%, 2) of body width, lateral to caeca, posterior to ventral sucker, extracaecal anterior to ventral sucker, extensively convoluted looping between testes and ventral sucker, passing ventral sucker dextrally or sinistrally, extending anteriad to near midline of oral sucker, arching posteriorly, and extending to near prostatic sac and ventral sucker, prior to synthesis with metraterm distally, with hundreds of eggs (Fig. 1); uterine seminal receptacle indistinct; metraterm thick walled, confluence with uterus anterior to medial axis of ventral sucker, extending laterad from distal end of uterus, becoming confluent with ejaculatory duct to form a common duct (= herein a ‘hermaphroditic duct’) within sinus organ (Figs. 1, 2). Vitellarium follicular, thick, ventral to caeca distributing in 2 bilaterally symmetrical fields, distance between fields 500–570 (535,2) or 45–55% (50%, 2) of body width, extending from near posterior margin of oral sucker to midline of ovary anterior to testes (Fig.1); dextral vitelline field 800–880 (1219, 2) long or 44–49% (47%, 2) of body length, terminating anteriorly at 34–39% (36%, 2) of body length, terminating posteriorly at 83–86% (85%, 2) of body length, 62–63% (62%, 2) of dextral caecum length; sinistral vitelline field 940–950 (945, 2) long or 53% (53%, 2) of body length, terminating anteriorly at 35–38% (37%, 2) of body length, terminating posteriorly at 80–83% (81%, 2) of body length, 68–71% (70%, 2) of sinistral caecum; primary vitelline collecting ducts nearly symmetrical, extending posteriad from respective

vitelline field before briefly extending laterad prior to joining vitelline reservoir; vitelline reservoir triangular shaped, dorsal to ovary (Fig.1). Uterine eggs densely distributed throughout uterus filling lumen, ovoid proximally, nearly circular distally, enlarging from approximately 55–60 (58, 2) x 35 (35, 2) in proximal portion of uterus, to approximately 90–95 (90, 12) x 70–75 (73, 2) in distal portion of uterus, some eggs within genital atrium with hatching miracidia (Figs. 1–3).

3.1.2. Diagnosis of cercaria and distome (based on 2 whole-mounted paratypes [HWML 37904-1, 3]).

Cercaria, furcocystocercous, 5500–5580 (5540, 2) long, 1180–1420 (1300, 2) wide or 3.9–4.7 (4.3, 2) × longer than wide, comprised of a tail stem and paired furcae (Figs 3, 4). Tail stem slightly swollen posteriorly, appearing spindle-shaped, 4480–4560 (4520, 2) long or 82% (82%, 2) of cercariae length; comprised of a spindle-shaped anterior tail stem and dorsoventrally compressed posterior tail stem (Figs 3, 4); anterior tail stem, 2660–3020 (2840, 2) long or 48–55% (51%, 2) of cercariae length, maximum width 1160–1180 (1170, 2), containing distome, bearing mamillae (Figs 3, 4); posterior tail stem region dorsoventrally compressed, slightly swollen medially, 1460–1900 (1680, 2) long or 27–34% (30%, 2) of cercariae length, 1140–1280 (1210, 2) wide at anterior end, 960–1000 (980, 2) wide at posterior end, tapering posteriorly, 12–25% (19%, 2), from anterior to posterior end, devoid of mamillae (Figs 3, 4). Furcae cordate shaped (=broadly semi-circular with a pointed apex), with sucker comprising pointed apex (Figs. 10–12), longer than wide, dorsoventrally compressed, smooth to slightly crenate margin (Figs. 4,5); dorsal furca, 1040–1160 (1100, 2) long or 19–21%, (20%, 2) of cercariae length, 780–830 (805, 2) wide or 72–75% (73%, 2) of dorsal furca length, ventral furca,

1040–1220 (1130, 2) long or 19–22%, (20%, 2) of cercaria length, 790–850 (820, 2) wide or 70–76% (73%, 2) of ventral furca length. Tail cavity opening at anteriomedial end of cercaria, directing anteriad, a narrow heavily constricted pore surrounded by musculature extends posteromedially from tail cavity opening connecting with and opening to tail cavity (Figs. 4,5); tail stem cavity at anterior end of cercaria, within anterior tail stem region, thin walled, appearing non-muscular (Figs. 4, 5). Mamillae mound-like tegumental protuberances of the anterior tail stem region, usually bearing rounded spines (Figs. 6-9), maximum length 100–110 (105, 2), maximum width 120–250 (185, 2) or 1.1–2.5 × wider than longer, anterior mamillae wider than long, surface naked (Figs. 6, 7), posterior mamillae longer than wide, surface with minute spine-like fimbria (Figs. 8, 9), tail stem length with mamillae 2660–3020 (2840, 2) or 58–67% (62%, 2) of cercaria length, tail stem length without mamillae 1460–1900 (1680, 2); mamillae restricted to anterior tail stem region, tightly encircling anterior half of anterior tail stem near distome, irregularly distributed throughout tail stem, ending at synthesis of anterior and posterior tail stem (Fig. 4). Mamilla spines blunt, short, 0–7 per mamilla (Figs. 6–9). Excretory system with 2 paired primary excretory canals, extending posteriad along medial axis, from anterior tail stem region, through posterior tail stem region, bifurcating at synthesis of furcae, extending independently through furcae, opening via excretory pore in pointed apex of each furcae. Distome (= cercarial body) contained within tail cavity sac in paratypes, and varying in position within the tail stem, located anteriorly (Fig. 4) and medially (see Fig. 5), 1960–1980 (1970, 2) long or 35–36% (35%, 2) of cercaria length 720–840 (780, 2) wide or 2.3–2.8 (2.5, 2) × longer than wider, specimens with hundreds of eggs restricted to the uterus (Fig. 13).

3.1.3. Taxonomic summary

Type host: Mottled sculpin, *Cottus bairdi* Girard, 1850 (Scorpaeniformes: Cottidae).

Intermediate host: Liver elimia, *Elimia livescens* Menke, 1830 (Cerithioidea: Pleuroceridae).

Other hosts: Burbot, *Lota lota*; rock bass, *Ambloplites rupestris*; small mouth bass, *Micropterus dolomieui*; and yellow perch, *Perca flavescens* (experimental host).

Site of infection: oesophagus (fish) and “mantle area” (snail).

Type locality: Au Train River (GPS N46°25’; W86°50’), Alger County, Michigan.

Specimens Examined: HWML Nos. 37903-6 (adult), 37903-7 (adult), 37904-1 (cercaria), 37904-3 (cercaria).

3.1.4. Remarks

The original description of adult specimens of *P. autraini* given by LaBeau and Peters [15], hereafter “LP,” was based on 10 specimens. The 2 adult paratypes we studied were in good condition, well fixed (fully extended, not curled), and well stained. The original description includes morphometric data and a general account of the body, oral sucker, ventral sucker, testes, and uterine eggs as well as that of the shapes and positions of the caeca, sinus organ (as “genital papilla”), prostatic sac (as “cirrus sac”), seminal vesicle, uterus, vitellarium, vitelline reservoir, and Laurer’s canal.

We herein provided novel observations sourced from adult paratypes (i.e., HWML 37903-6 & 37903-7). No Information was available previously for the pars prostatica, ejaculatory duct, hermaphroditic duct, hermaphroditic pore, genital atrium, genital pore, ovary, and fine details of the female genitalia. We observed the pars prostatica to be broad and spheroid proximally before tapering, greater than 70% of its original width,

and remaining narrow and thin walled for nearly its entire length (Fig. 2). The ejaculatory duct emanates from the distal region of the pars prostatica and merges with the metraterm to form a short hermaphroditic duct that communicates with the hermaphroditic pore before opening within the large, circular genital atrium that is capable of holding a large number of eggs (Figs. 2–3) (Figs. 2, 3). The genital pore is ventral and at level of the posterior extremity of the genital atrium, opening posteriorly in the direction of the ventral sucker (Figs.1, 2). The vitellarium is distributed in two dense, non-dispersed, symmetrical fields, extending from near the oral sucker to the anterior margin of the testes. LP illustrated the vitellarium in two loosely dispersed fields having few follicles. The ovary is posteromedial and dorsal to the vitelline reservoir. The uterus was described as "*convoluted…. sometimes extending [sic] to the posterior third of the oral sucker*" and illustrated as entirely intercaecal (see fig. 1 of LP). However, in both paratypes the anterior margin of the uterus is extracaecal, anterior to the ventral sucker and extends anteriad to the level of the middle of the oral sucker (Fig. 1). Most fine features of the female genitalia were indistinct in both of the adult paratypes we studied. However, LP reported that the Laurer's canal pore was "*mid-dorsal at level of the ventral sucker*." We could not confirm this feature; however, the Laurer's canal pore is posterolateral to the ventral sucker in *Proterometra catenaria* Smith, 1934 (MRW, personal observations), *Proterometra albacauda* Anderson and Anderson, 1967, *P. epholkos* [2], and the new species described herein.

We confirm the presence of "*fine projecting filaments*" on eggs within the uterus of the paratypes we studied; however, eggs with projecting filaments (perhaps more appropriately called 'fimbria') occupied only the distal portion of the female genitalia, i.e.,

distal uterus, metraterm, and genital atrium. Noteworthy also is that we observed miracidia that were seemingly emerging from hatched, operculate eggs in the genital atrium (Figs. 2, 3). Because adjacent eggs as well as eggs located in the proximal portion of the female reproductive tract were not hatched (opercula were not detached from eggs) and because the paratypes of LP were in excellent condition, we doubt that the apparent en-utero hatching of miracidia was an artifact of fixation or mounting. Although only length and width of eggs have been used previously to differentiate species of *Proterometra*, we think that additional features associated with the eggs, including presence/absence, density, distribution, and size of fimbria ("filaments" of LP) or polar papillae [2] as well as their level of uterine development [48] may help differentiate species.

The original description of cercariae of *P. autraini* included measurements for tail stem length, tail stem width, furcae length, furcae width, distome length, and distome width coupled with observations of the tail stem, tail stem cavity (as "*saclike cavity*"), furcae, mamillae (as "*mammilations*"), mamillae spines, and the excretory canals. The description was accompanied by two illustrations of the cercariae (see figs. 2 and 3 of LP) comprising withdrawn and extruded distomes as well as an illustration of the tail stem mamillae, which have rounded mamillae spines (see fig. 4 of LP). We found few discrepancies between our measurements of the paratypes when compared with those provided in the original description. However, several features of the cercaria were not described: morphometric data for the cercaria, tail stem, anterior tail stem, posterior tail stem, furcae, mamillae, and distome. Based on previous works [2] and studying the

paratypes of *P. autraini*, we herein provide further description and clarification of some features of the cercaria of *P. autraini*.

Distome: In agreement with the measurements reported by LP, the distomes from the two cercarial paratypes we studied were greater in length, by approximately 200 micrometers, than the adult paratypes. In parallel with the unusually large size of the distome, the distome of *P. autraini* closely resembles the adult, by appearing well developed based on the position and development of reproductive and digestive structures. In addition, by the presence of hundreds (<250) of uterine eggs (Figs. 4, 5, 13) restricted to the uterus, and having no egg within the genital atrium as observed in adult specimens. The distome's size, degree of sexual development, and fecundity indicates that it develops completely within the tail stem.

Furcae: We observed a structure associated with the apex of the furcae that we interpret as a putatively functional sucker (Figs. 10–12). To our knowledge, this feature has not been described previously for any species of *Proterometra* or Azygiidae. Given that all published life cycles for species of *Proterometra* are trophically mediated (i.e., the definitive host eats the cercaria), except for that of *Proterometra dickermani* Anderson, 1962 [12,18], we have previously speculated (see Womble et al., [2]) that cercarial behavior may evolve in response to the definitive host's diet and foraging behavior. For example, and regarding *P. autraini*, perhaps the sucker at the apex of each furca (Figs. 10–12) facilitates cercarial attachment to a substratum and thereby increases the probability of encounter with a definitive host(s) (e.g., mottled sculpin, *Cottus bairdi*, and burbot, *Lota lota*) that consume the attached cercaria as they forage within the benthos.

In addition, the furcae of *P. autraini* are longer than wide (Figs. 4, 5). Of the species of *Proterometra* for which comparable morphometric data is available and excluding those with lanceolate furcae (i.e., *Proterometra sagittaria* Dickerman, 1946, and *P. catenaria*), only *Proterometra hodgesiana* (Smith, 1932) Smith, 1936, *P. dickermani*, and *Proterometra edneyi* Uglem and Aliff, 1984 reportedly have furcae that are longer than wide; although all have distinctively small furcae [2,8,11,14].

Mamilla spines: We confirmed blunt, short mamilla spines in specimens of *P. autraini* (Figs. 4-6, 8, 9; see also LP). In contrast, all other accepted species of *Proterometra* that have spinose mamillae (see Table 3 of Womble et al. [2]) have minaret shaped spines. We also observed minute spine-like fimbria (Figs. 8, 9) that cover the mamilla surface. This feature seemed restricted to mamillae of the posterior portion of the anterior tail stem (Figs. 6, 7). Womble et al. [2] speculated that mamillae spines function as cleats facilitating adherence of the infective larva (i.e., distome) to the soft epithelial tissues of the fish's buccal cavity. If so, the diversity of mamillae spine shapes may help differentiate species.

3.2. Proterometra ariasae *sp. n. Womble and Bullard, 2015 (Figs. 14–32)*

3.2.1. Diagnosis of adult (based on 13 stained, whole-mounted specimens).

Body of adult, 1560–2120 (1878, 13) long, 850–1220 (1041, 13) wide or 1.6–2.1 (1.8, 13) × longer than wide, ventrally concave (Fig. 14); forebody 890–1180 (1050, 12) long or 50–60% (56%, 12) of overall body length; hindbody 400–660 (519, 12) long or 24–32% (27%, 12) of overall body length, 41–65% (49%, 12) of forebody length; tegument approximately 5–20 (12, 12) thick; tegumental papillae minute, pored,

encircling ventral most surface of mouth and ventral sucker. Excretory system difficult to trace, uniting anterior to oral sucker, extending posteriad and lateral to ventral sucker, connection with excretory bladder indistinct; excretory pore medial, terminal (Fig. 14). Nervous system indistinct in fixed, whole-mounted specimens. Oral sucker subterminal, 520–740 (589, 13) long or 28–35% (31%, 13) of body length, 530–730 (601, 13) wide or 50–72% (58%, 13) of body width, 90–270 (150, 13) or 4–13% (8%, 13) of body length from anterior body end, 880–1390 (1117, 13) or 52–66% (59%, 13) of body length from posterior body end, posterior margin 180–550 (316, 12) from anterior margin of ventral sucker (Fig. 14). Ventral sucker 240–350 (291, 12) long or 13–18% (16%, 12) of body length, 295–390 (328, 12) wide or 28–38% (32%, 12) of body width, in posterior half of body, with anterior margin 890–1180 (1051, 12) or 50–60% (57%, 12) of body length from anterior body end, consistently wider than long, 43–59% (50%, 12) of oral sucker length, 48–60% (55%, 12) of oral sucker width (Fig. 14). Mouth opening ventrally (Fig. 14) or anteroventrally, Pharynx ovoid, dorsal to oral sucker, 75–150 (105, 13) long or 5–7 % (6%, 13) of body length, 110–155 (124, 13) wide or 1–1.7 (1.2, 13) × wider than longer (Fig. 14). Oesophagus extending posteriad from mouth 305–460 (359, 11) before bifurcating 15–50 (24, 11) posterior to pharynx, with oesophageal branches extending laterad before joining with intestinal caeca (Fig. 14); dextral oesophageal branch 80–225 (156, 9) long, 40–145 (97, 8) at maximum width; sinistral oesophageal branch 110–245 (166, 9) long, 65–140 (102, 8) at maximum width; intestinal caeca confluent with oesophageal branches, appearing inverse U-shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caeca extending from near posterior

margin of oral sucker to posterior of the midline of the testes (Fig. 14); dextral caecum 900–1505 (1214, 11) long or 53– 89% (65%, 11) of body length, 75–170 (126, 11) in maximum width, laterad caecum length 115–305 (215, 9) or 0.7–2.1 (1.4, 9) × dextral oesophageal branch, descending caecum length 760–1260 (992, 11) or 3.3–6.6 (4.6, 8) × longer than laterad caecum, pre-caecal space, 590-920 (773, 12) or 36–46% (41%, 12) of body length from anterior end of body, post-caecal space, 70–250 (142, 11) or 3–13% (8%, 11) of body length from posterior end of body; sinistral caecum 900–1375 (1164, 9) long or 56–75% (63%, 11) of body length, 70–165 (127, 11) in maximum width, laterad caecum length 150–295 (214, 10) or 0.8–1.4 (1.1, 10) × sinistral oesophageal branch, descending caecum length 630-1110 (932,11) or 2.9– 6.5 (4.4, 9) × longer than laterad caecum, pre-caecal space, 540-900 (735, 12) or 30–44% (39%, 11) of body length from anterior end of body, post-caecal space, 100–360 (176, 11) or 5–20% (10%, 11) of body length from posterior end of body.

Testes 2 in number, oblique, transverse, abreast, round to oval in outline (Fig. 14); dextral testis 225–435 (323, 13) or 13–22% (17%, 13) of body length, 185–290 (217, 12) or 17–25% (21%, 12) of body width; pre-testicular space, 1180-1880 (1499, 13) from anterior end of body or 71–89% (80%, 13) of total body length, post-testicular space 50–150 (90, 13) long; sinistral testis 245–350 (312, 11) or 14–21% (17%, 11) of body length, 135–280 (209, 11) or 13–28% (20%, 11) of body width, pre-testicular space, 1210–1700 (1403, 11) from anterior end of body or 70–86% (76%, 11) of total body length, post-testicular space, 50–380 (145, 11) from posterior end. Vasa efferentia and connection with seminal vesicle indistinct in whole-mounted specimens. Prostatic

sac typically medial, dorsal to ventral sucker, anterior margin 70–225 (121, 11) from posterior margin of oral sucker, posterior margin overlaps with anterior margin of ventral sucker (Fig. 14), 175–280 (226, 13) long, 155–325 (220, 13) wide. Seminal vesicle thin-walled, highly convoluted, nearly filling prostatic sac, 458–645 (561, 9) long, having proximal and distal regions (Figs. 15, 16); proximal region of seminal vesicle short, swollen, 105–255 (183, 9) or 16–46% (33%, 9) of total seminal vesicle length, 48–95 (70, 9) wide; distal region of seminal vesicle elongate, narrow, 245–540 (378, 9) or 54–84% (67%, 9) of total seminal vesicle length, 23–38 (31, 9) wide, connected to pars prostatica via a minute duct. Verschlussapparat (see Horsfall [6]) 5–25 (21, 5) long, thin-walled, proximal end opening within wide distal region of pars prostatica (Fig. 16). Pars prostatica 150–280 (190, 13) long, 38–55 (47, 13) wide proximally, 20–30 (24, 13) wide distally, tapering 38–58% (47%, 12) in width from proximal to distal end, slightly arched, extending anteriad distally and posteriad proximally lined by prostatic gland cells, thick-walled for entire length, exiting prostatic sac ventrally (Figs. 15, 16). Ejaculatory duct (= the continuation of the pars prostatica external to prostatic sac) extending ventrally from prostatic sac and becoming confluent with hermaphroditic duct, lacking gland-like cells or muscle in wall, ventral to metraterm, 40–73 (61, 12) long or 23–45% (33%, 11) of pars prostatica length (Figs. 15, 16). Confluence of terminal male and female genitalia occurring within sinus organ (Fig. 16). Sinus organ directed ventrally, appearing spheroid in outline, nearly medial, immediately dorsal to genital atrium, papillate (Figs. 15, 16). Hermaphroditic pore at level of or slightly posterior to prostatic sac, anterior to ventral sucker, pre-ventral sucker distance 48–55% (51%, 13) of body length, directed ventrally before opening into genital atrium (Figs. 15, 16). Genital atrium connecting

hermaphroditic pore and genital pore, thick-walled, non-lobed, funnel-shaped, comprised of a small area immediately ventral to hermaphroditic pore, with few uterine eggs (Fig. 15). Genital pore immediately anterior to ventral sucker, usually medial, posterior to perpendicular midline of prostatic sac; pre-genital pore distance 48–60% (55%, 13) of total body length (Fig. 14–16).

Ovary variable in relation to the body, typically dextral, intercaecal, 130–238 (188, 13) long or 7–13% (10%, 13) of body length, 100–265 (200, 13) wide or 10–25% (19%, 13) of body width or 0.6–1.6 (1.1, 13) × wider than long (Figs. 14, 17); post-ovarian space 145–350 (234, 11) long or 7–18% (13%, 11) of body length; oviduct emanating from anterior margin of ovary, extending anteromediad from anterior margin of ovary, lacking muscular sphincter (cf. *P. epholkos* [2]), thin-walled, dorsal and wholly or principally anterior to ovary, extensively convoluted before becoming confluent with Laurer's canal, extending 125–205 (156, 5) from commissure with Laurer's canal to ootype (Fig. 17). Laurer's canal swollen proximally with sperm immediately after commissure with oviduct, becoming convoluted, narrow and thick-walled distally, with slight swelling at proximal end immediately before pore, 173–278 (210, 8) long, 20–30 (27, 11) wide including thick glandular wall, opening dorsally and posterior to ventral sucker (Fig. 17), 225–350 (285, 11) from posterior margin of body. Ovo-vitelline duct short, synthesis with oviduct occurring immediately prior to ootype (Fig. 17), 70–225 (142, 4) in length. Ootype dorsal to ovary, directing sinistrad or dextrad, transverse, anterior to testes, 85–125 (112, 5) long, 40–75 (59, 5) in maximum width (Fig. 17). Mehlis' gland indistinct. Uterus occupying space between posterior half of oral sucker and ovary (Fig. 14), comprising a field 760–1200 (948, 12) long or 44–57% (50%, 12) of

body length and 550–930 (725, 12) wide or 61–82% (70%, 12) of body width, intercaecal posterior to ventral sucker, may extend lateral to caeca and esophageal branches anterior to ventral sucker, proximal portion comprising a uterine seminal receptacle, extensively convoluted between testes and ventral sucker, dextral or sinistral to ventral sucker, extending anteriad to near or beyond posterior margin of oral sucker, arching posteriad near pharynx, extending to near prostatic sac and ventral sucker; uterus of hindbody typically with greater than 100 eggs; uterine seminal receptacle with sperm (Fig. 17); metraterm thick-walled, 260–430 (359, 9) or 15–23% (19%, 9) of body length, 30–75 (51, 5) wide, confluence with uterus anterior to medial axis of ventral sucker (Fig. 14), extending slightly anteriad and transverse from distal end of uterus, sinistral or dextral to prostatic sac, becoming confluent with ejaculatory duct to form a common duct (= herein a 'hermaphroditic duct') within sinus organ (Figs. 15, 16). Vitellarium follicular, ventral to caeca, distributing in 2 bilaterally symmetrical fields, distance between fields 410–690 (547,11) or 47–60% (53%, 11) of body width, extending from oral sucker to near posterior body end (Fig. 14); dextral vitelline field 920–1540 (1219, 12) long or 57–78% (65%, 12) of body length, terminating anteriorly at 25–37% (31%, 12) of body length, terminating posteriorly at 91–98% (94%, 12) of body length, .74–1.2 (1, 12) x longer than dextral caecum; sinistral vitelline field 940–1480 (1203, 12) long or 57–70% (64%, 12) of body length, terminating anteriorly at 26–37% (31%, 12) of body length, terminating posteriorly at 92–98% (95%, 12) of body length, .85–1.2 (1, 12) x longer than sinistral caecum; primary vitelline collecting ducts nearly symmetrical, extending posteromediad from respective vitelline field before becoming confluent and forming vitelline reservoir; dextral vitelline collecting duct 205–255 (228,

4) long or 9–13% (12%, 4) of body length, 8–20 (114, 4) wide near vitelline reservoir, proximal end branches from vitellarium at 56–78% (66%, 4) of dextral vitelline field length; sinistral vitelline collecting duct 175–375 (251, 3) long or 11–18% (13%, 3) of body length,10–13 (12, 3) wide near vitelline reservoir, proximal end branches from vitellarium at 55–72% (66%, 3) of sinistral vitelline field length. Vitelline reservoir tetrahedral, dorsal to ovary; vitelline collecting ducts extending ventral to ovary and perpendicular to long axis of body. Uterine eggs typically filling lumen of uterus throughout its length, spheroid to oblong (Figs. 14–17), 53–68 (62, 12) x 23–35 (30, 12) and 85–95 (90, 12) x 45–53 (47, 12) in proximal and distal portions of uterus, respectively.

3.2.2. Diagnosis of cercaria (based on light microscopy of 10 whole-mounted, naturally shed cercariae having a withdrawn distome).

Cercaria furcocystocercous, beige, 4640–6080 (5344,10) long, 1180–1920 (1493, 9) wide or 3.2–3.9 (3.6, 9) × longer than wide, comprising a tail stem and paired furcae (Figs. 18, 24). Tail stem 3660–5000 (4316, 10) long or 78–84% (82%, 10) of cercariae length, comprising a spindle-shaped anterior tail stem and dorsoventrally-compressed posterior tail stem (Figs. 18, 19). Anterior tail stem 2540–3540 (3082, 10) long or 54–62% (58%, 10) of cercariae length, containing distome, with mamillae (Figs. 18–22). Posterior portion of tail stem flat 1040–1460 (1234, 10) long or 21–25% (23%, 10) of cercariae length, 1100–1800 (1407, 9) in maximum width anteriorly, 620–920 (798, 9) wide at posterior end, tapering 51–62% (60%, 9) from anterior to posterior end, lacking mamillae (Figs. 18, 19). Furcae obcordate (= broadly semi-circular with medial notch),

yellow, dorsoventrally flattened (Figs. 18, 19, 23, 24); dorsal furca 880–1060 (988, 10) long or 17–20%, (20%, 10) of cercariae length, 1120–1360 (1255, 9) wide or 1.2–1.5 (1.3, 9) × wider than longer; ventral furca 820–1100 (993, 9) long or 15–21%, (18%, 9) of cercariae length, 960–1340 (1217, 9) wide or 1–1.5 (1.2, 9) × wider than longer; furca margin bearing many protuberances and appearing serrate; protuberances minute, pored, irregularly placed, marginal and slightly submarginal (Figs. 18, 19, 23, 24, 28). Tail stem cavity at anterior end of cercaria, within anterior tail stem region, thin walled, seemingly amuscular (Fig. 18). Tail stem cavity opening at anteriomedial end of cercaria, directing anteriad, a narrow and heavily constricted pore surrounded by aspinous mamillae (Fig. 26). Mamillae comprising mound-like tegumental protuberances, of the anterior tail stem (Figs. 18–22, 25, 30), usually spinous (Figs. 20-22, 27), maximum length 70–110 (84, 7), maximum width 110–180 (155, 10) or 1.6–2.3 × wider than longer, anterior-most mamillae near region of distome with pores at the mamilla base (Figs. 19, 25), tail stem length with mamillae 2540–3540 (3082, 10) or 54–62% (58%, 10) of cercaria length, tail stem length without mamillae 1040–1460 (1234, 10). Mamillae distributed throughout anterior tail stem region (Figs. 18, 19), ending at proximal margin of posterior tail stem. Mamilla spines (Figs. 20–22, 27, 29) 0–7 per mamilla, small, minaret shaped, always erect. Primary excretory canals 2 in number, paired, extending posteromediad from anterior tail stem and coursing through posterior tail stem before bifurcating at synthesis of furcae, with each canal coursing through respective furca; excretory pore opening at medial notch of each furca (Fig. 18). Distome (= cercarial body) restricted to extreme anterior portion of tail stem (Fig. 18, 31, 32), 1220–1400 (1324, 10) long or 20–30% (25%, 10) of cercaria length, 670–760 (720,

10) wide or 1.7–2 (1.8, 10) × longer than wider, with large, prominent oral sucker (Figs. 31, 32), specimens with 0–12 (7, 10) eggs in proximal end of uterus (Fig. 31).

3.2.3. Taxonomic Summary

Type host: Longear sunfish, *Lepomis megalotis* Rafinesque, 1820, (Perciformes: Centrarchidae).

Intermediate host: *Pleurocera* sp. (Cerithioidea: Pleuroceridae).

Additional hosts: Bluegill, *Lepomis macrochirus* Rafinesque, 1819 (Perciformes: Centrarchidae).

Type locality: Chickasawhay River (N31°57’4.80”; W88° 42’9.19”), Clarke County, Mississippi USA.

Site in fish host: Oesophagus.

Site in molluscan host: Indeterminate.

Specimens Deposited: Syntypes (adults; USNM collection nos. 1283298, 1283299, 1283300), (cercariae; USNM collection nos. 1283301, 1283302, 1283303) and intermediate host vouchers in AUMNH.

Etymology: The specific epithet *ariasae* honors Dr. Cova R. Arias (Professor and Assistant Director, School of Fisheries, Aquaculture, and Aquatic Sciences, Auburn University) for her contributions to the study of symbiont biodiversity in Alabama and is in gratitude for her mentorship in molecular biology.

3.2.4. Remarks

Adults of *Proterometra ariasae* sp. n. are most easily distinguished from those of its congeners [2] by the combination of having adults with (i) a large body (>1100 µm x 650 µm), (ii) a proportionally large oral sucker (≥ 2x ventral sucker diameter), (iii) ovoid

testes, (iv) a strongly muscular and laterally expanded pars prostatica, (v) a uterus that is extensively convoluted between the ovary and ventral sucker (vi) and a vitellarium as long as the caeca and extending posteriad beyond the anterior margin of the testes. Adults of the new species most closely resemble those of *P. epholkos* but can be distinguished from them by the combination of having (i) a seminal vesicle with a short and swollen proximal region as well as a relatively elongate and dilated distal region (Figs. 15, 16), (ii) a vitellarium as long as the caeca (Fig. 14), (iii) a prostatic sac at level of the anterior margin of the ventral sucker (Figs. 14, 15), (iv) a proximal portion of oviduct lacking a sphincter, and (v) a papillate sinus organ (Fig. 16). Additionally, cercariae of *P. ariasae* differ from those of *P. epholkos* by the combination of having a tail stem that lacks a medial constriction and mamillae distributed throughout the anterior tail stem (Figs. 18, 19).

Cercariae of the new species differ from those of its congeners [2] by the combination of having (i) a tail stem that is shorter than 10 mm and that lacks a medial constriction, (ii) obcordate furcae that are wider than long (Figs. 18, 19), (iii) mamillae distributed throughout the anterior tail stem only, and (v) a proportionally small distome (≤ 30% of cercariae length) (Figs. 18, 31, 32) that has relatively few uterine eggs and remains withdrawn in the anterior tail stem region in actively swimming cercariae (Figs. 18, 19, 31). Cercariae of *P. ariasae* most closely resemble those of *P. autraini* but can be distinguished based on the dimensions of the furcae plus the shape of the mamillae spines. In addition, comparative study of the distomes of *P. ariasae* and *P. autraini* revealed several critical features: (i) the distome of *P. ariasae* is small (< 70% of adult length), (ii) has few uterine eggs (n=7), (iii) and is seemingly immature (or young) based

on the position and development of digestive and reproductive structures; i.e., (i) the proximal musculature of the oral sucker is dorsal to the prostatic sac; (ii) the sinus organ, hermaphroditic pore, and genital atrium are ventral to the musculature of the ventral sucker; (iii) the ovary and testes are dorsal to the proximal musculature of the ventral sucker; and (iv) the vitellarium extends to near the medial axis of the oral sucker (Fig. 31).

Using SEM, we observed pores (10-20 μm in diameter) at the base of mamillae at level of the distome in the anterior tail stem (Figs. 24–26). These pores have not been described previously for any species of *Proterometra*. The furcae of *P*. *ariasae* have minute, pored protuberances (approximately 8-10 μm in diameter) of indeterminate function. Each protuberance lacks a sensory cilium, and the protuberances are irregularly spaced along each furcal margin, which appears accordingly serrate (Figs. 23, 30, 31). The minute pored protuberances resemble adhesive gland pores [49,50]. Aside from our description of *P. epholkos* [2], no previous author has provided observations of the furcae margin.

The mamillae of *P. ariasae* have minaret-shaped spines (0-8 in number), each with a proximal base comprising an extensively ridged tegument (Figs. 23, 24). Some mamillae showed evidence of detached spines (Fig. 30), which should not be confused with the pores associated with the base of mamillae in the region of the distome (Figs. 19, 25). Mamillae throughout the anterior tail stem were spinose (Figs. 20-22), indicating that the spines are not restricted to mamillae in a particular region of the anterior tail stem. Finally, the distome of *P. ariasae* has a prominent oral sucker that, when compared to closely related congeners (i.e., *P. epholkos*; Fig. 33), appears bowl-

shaped. At present, comparison of this feature among species of *Proterometra* is not possible, however, future investigators should further explore the oral suckers of distomes as a potentially diagnostic feature.

ITS2 sequences from cercariae (n=4) and adults (n=3) of *P. ariasae* were 100% identical and interpreted as conspecific. Interestingly, and as previously reported for *P. epholkos* [2], we report an intra-individual single-site polymorphism in position '174,' which showed overlapping double peaks of cytosine and guanine, that was present in all specimens of *P. ariasae*. Noteworthy is that we have observed that the position of the intra-individual polymorphism differs interspecifically (i.e., between sequences of *P. ariasae* and *P.* epholkos) but always occurs in the same position intraspecifically (i.e., between adult and cercariae replicates). Additionally, Diaz et al., [51] also recently reported intra-individual polymorphic positions within ITS2 sequences of three species of *Paradiscogaster* (Digenea: Faustulidae). We agree with Diaz et al., [51] that this phenomenon may be more common in trematodes than what has been previously reported in the literature, but as previously reported, we add that such variation has been documented in rDNA for species in at least two other families of trematodes, i.e., species of *Schistosoma* and *Fasciola* (see [2]). Individual pairwise sequence comparisons between *P. ariasae* and *P. epholkos* revealed 19 polymorphisms resulting in a 5.7% sequence divergence (Table 2).

4. DISCUSSION

4.1. Host specificity & diversity.

Existing host records for *Proterometra* spp. [2] indicate that they have greater specificity for the intermediate host than for the definitive host. Species of *Proterometra* infect prosobranch snails assigned to 4 genera and 2 families (Viviparidae: *Campeloma*; Pleuroceridae: *Pleurocera*, *Elimia*, *Lithasia*). However, we infer that the single record of an infection in a species of *Campeloma* is dubious. Faust [52] listed it as a host because a snail identified as "*Campeloma subsolidum*" resided in the aquarium that harbored a cercaria of *P. macrostoma*. Nearly a century later, no species of *Campeloma*, nor any non-pleurocerid, has been confirmed as a host for a species of *Proterometra*. As such, *Proterometra* spp. are highly host-specific to pleurocerids of *Elimia*, *Pleurocera*, and, perhaps, *Lithasia*. Of the 11 accepted species of *Proterometra*, *P. ariasae* is the only species that has no known association with a species of *Elimia*, and one of only three species to have been reported from a pleurocerid not of *Elimia*: *P. macrostoma* from *Lithasia obovata* (as *Goniobasis depygis*) [53], *Pleurocera acuta* [5,6], and *Pleurocera* spp. [10,54]; *P. sagittaria* from *Pleurocera* spp. [11]; and *P. ariasae* from *Pleurocera* sp. [present study].

Of interest would be to explore specificity of cercarial infections among closely related, congeneric, snails as well as across genera and families of gastropods. However using published literature to guide this effort is challenging because most (9 of 11; 82%) species of *Proterometra* were described long before the taxonomic status of many pleurocerids (especially those of *Pleurocera* and *Elimia*) had begun to be

resolved. Our opinion on the matter is that no previously-reported snail host for a species of *Proterometra* can be corroborated in light of recent morphological and molecular pleurocerid taxonomy and systematics (except those for *P. epholkos* and *P. ariasae*). No previous worker listed a morphological feature used to identify the snail host nor deposited a snail voucher specimen (as a shell voucher or formalin-fixed whole specimen) in a curated museum collection. As such, testing specificity of these azygiids to their snail hosts will require focused effort on snail identification in future parasitological studies. Additionally, and of importance to future investigators, pleurocerid taxonomy still remains disputed within the malacological community. For example, we [2] deposited sequence data for a cercaria of *P. epholkos* (GenBank no. KM503119.1) sourced from *Elimia* cf. *modesta*. We were told by NCBI GenBank personnel that GenBank does not recognize the genus group name *Elimia*, despite the fact that this genus is unambiguously available, "valid," and widely accepted to include dozens of species [1]. We regard Johnson et al. [1] as authoritative and current, and those workers accepted 162 pleurocerid species in 7 genera, including *Pleurocera* and *Elimia*.

4.2. Phylogenetic analysis.

Comparisons between *P. epholkos, P. ariasae, L. micropteri*, and *A. longa* revealed a range of polymorphisms and corresponding sequence divergence percentages (Table 2). Phylogenetic trees reconstructed using Neighbor-Joining and Maximum-Likelihood analyses recovered the same topology. With *T. borboleta* as the outgroup, Azygiidae was monophyletic and grouped *P. ariasae* and *P. epholkos* as sister taxa comprising a

clade sister to *L. micropteri*, which together formed a clade sister to *A. longa* (Fig. 34). This is the first molecular phylogenetic study that tests the interrelationships of *Proterometra* spp. along with *A. longa* and *L. micropteri*. The low bootstrap support values for the *Proterometra* clade and *Proterometra*+*L. micropteri* clade suggested that these genera might need taxonomic revision. Sequences from additional taxa within these genera are needed to test monophyly of *Proterometra* but molecular data for members of the genus presently are limited. A significant barrier in defining *Proterometra* is the taxonomic identity of the type species (*P. macrostoma*), which clearly needs to be defined with the designation of a neotype concomitant with a description of those "*macrostoma*-like" specimens from the presumptive type locality and type hosts (see Womble et al. [2] for a discussion of these issues). Obtaining ITS2 sequences for putative specimens of *P. macrostoma* will identify *Proterometra* within the phylogeny of Azygiidae and allow for generic revisions, which may result in the proposal of new genera for species currently assigned to *Proterometra*.

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Table 1. Provenance of ITS2 sequence data for phylogenetic analysis.

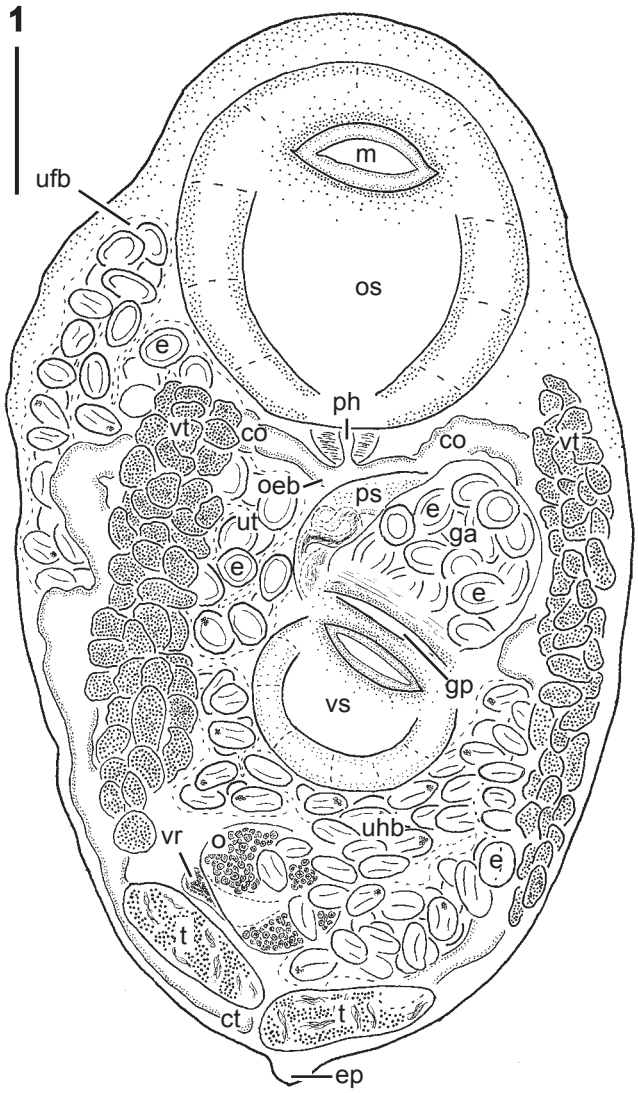
Species	Host	Locality	Specimen [a]	GenBank no(s).	Museum no. [b]	Ref.
Ingroup						
Azygia longa	*Esox niger*	Pascagoula River, Mississippi	1 adult	KT808319	GCRL 06511-12	Calhoun et al. [32]; Present study
Leuceruthrus micropteri	*Micropterus salmoides*	Wheeler Reservoir, Alabama	1 adult	KT808320	USNM 1283304	Present study
Proterometra epholkos	*Micropterus punctulatus*	Terrapin Creek, Alabama	1 adult	KM503118	USNM 121732-34	Womble et al. [2]
	Elimia cf. *modesta*	Terrapin Creek, Alabama	1 cercaria	KM503119	USNM 121729-31	Womble et al. [2]
Proterometra ariasae n. sp.	*Lepomis megalotis*	Chickasawhay River, Mississippi	1 adult	KT808317	USNM 1283298-300	Present study
	Pleurocera sp.	Chickasawhay River, Mississippi	1 cercaria	KT808318	USNM 1283301-03	Present study
Outgroup						
T. borboleta	*Chaetodon auriga*	Lizard Island, Australia	1 adult	JF412524	QM 238126	Hunter & Cribb [37]

[a] Number of sequences included in phylogenetic analyses.
[b] GCRL = Gulf Coast Research Laboratory Museum; USNM= Smithsonian National Museum of Natural History, Department of Invertebrate Zoology; QM = Queensland Museum, Queensland, Australia.

Table 2. Individual pairwise sequence comparisons and base pair polymorphisms[a]

	Azygia longa	*Leuceruthrus micropteri*	*Proterometra epholkos*	*Proterometra ariasae*
A. longa	- - - - - - - -	7.9% (26)	6.3% (21)	7.9% (26)
L. micropteri	- - - - - - - -	- - - - - - - -	7.6 %(25)	8.3% (27)
P. epholkos	- - - - - - - -	- - - - - - - -	- - - - - - - -	5.7% (19)
P. ariasae	- - - - - - - -	- - - - - - - -	- - - - - - - -	- - - - - - - -

[a]Percent sequence divergence (number of base pair polymorphisms), 363 total bases.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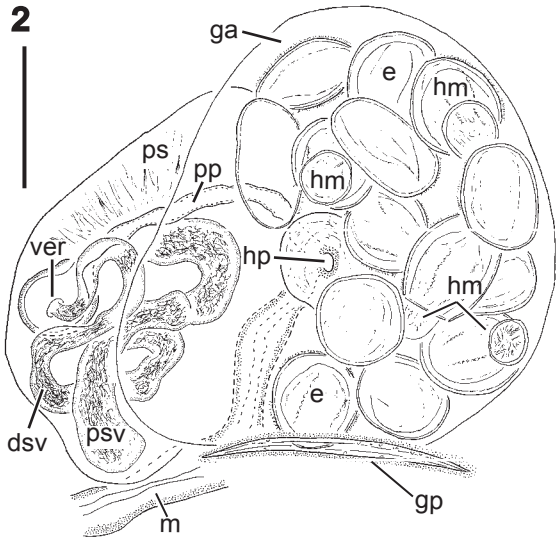
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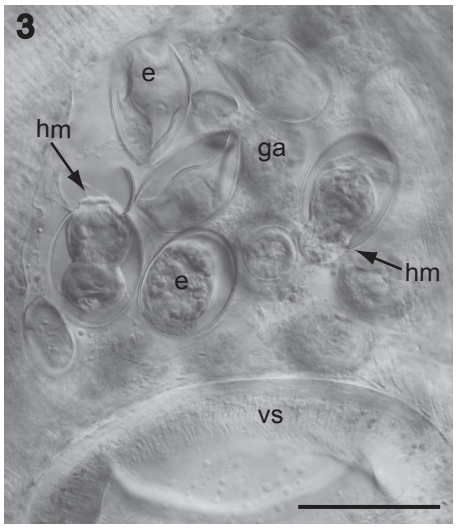
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Plate 2-1; Figures 1–3. Adults of *Proterometra autraini* from oesophagus of mottled sculpin, *Cottus bairdi* from the Au Train River, Michigan. **(1)** Body of adult (paratype HWML 37903-6) showing mouth (m), oral sucker (os), uterus in forebody (ufb), pharynx (ph), caeca (co) near origin, vitelline field (vt), uterus (ut) filled with eggs (e), prostatic sac (ps), genital atrium (ga), genital pore (gp), ventral sucker (vs), uterus in hindbody (uhb) looping between ventral sucker and ovary (o), vitelline reservoir (vr), testes (t), caeca termination (ct), and excretory pore (ep). Ventral view. Scale bar = 250 µm **(2)** Terminal male genitalia (paratype HWML 37903-6) showing comparable features as illustrated in Figure 1 plus swollen proximal region of seminal vesicle (psv), narrow distal region of seminal vesicle (dsv), verschlussapparat (ver), pars prostatica (pp), metraterm (m), hermaphroditic pore (hp), and hatching miracidia (hm). Some eggs omitted from drawing to show prostatic sac, seminal vesicle, and hermaphroditic pore. Ventral view. Scale bar = 100 µm. **(3)** Light micrograph of terminal male genitalia (paratype HWML 37903-6) showing genital atrium with eggs, some of which are hatching, and ventral sucker. Ventral view Scale bar = 100 µm.

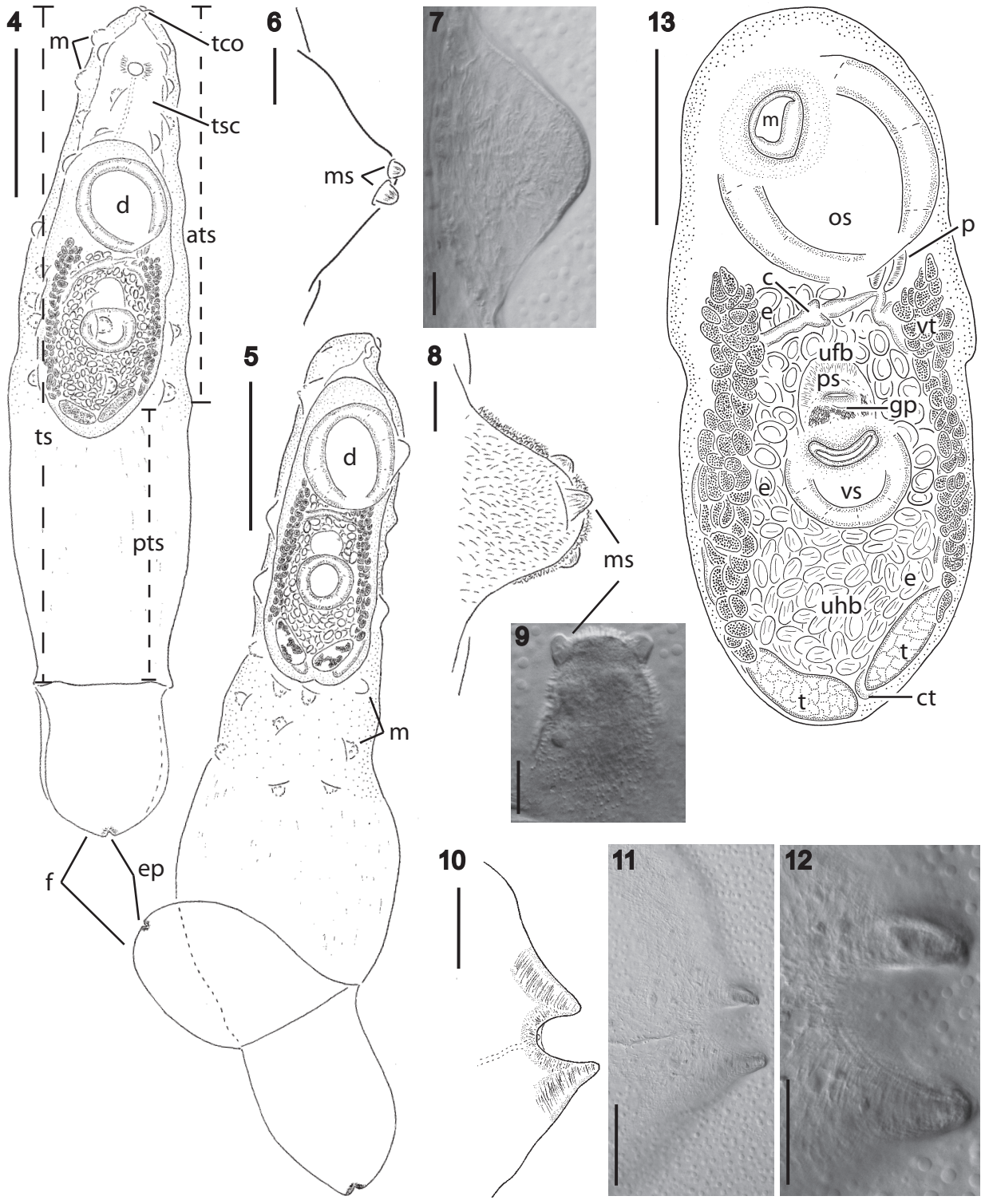
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Plate 2-2; Figures 4–13. Cercariae of *Proterometra autraini* LaBeau and Peters, 1995 (Digenea: Azygiidae) from mantle area of liver elimia, *Elimia livescens* Menke, 1830 (Cerithioidea: Pleuroceridae) from the Au Train River, Michigan **(4)** Cercaria (paratype HWML 37904-1) showing tail stem (ts), anterior tail stem (ats), posterior tail stem (pts), furcae (f), tail cavity opening (tco), tail stem cavity (tsc), distome (d), mamillae (m), and excretory pore (ep). Ventral view. Scale bar = 1 mm. **(5)** Cercaria (paratype HWML 37904-3) showing slightly contracted tail stem with anteriorly positioned distome and similar details as Fig. 5. Ventral view. Scale bar = 1 mm. **(6)** Anterior tail stem mamilla (paratype HWML 37904-3) with 2 blunt mamilla spines (ms). Lateral view. Scale bar = 25 µm. **(7)** Anterior tail stem mamilla (light micrograph, paratype HWML 37904-3). Lateral view. Scale bar = 30 µm. **(8)** Posterior tail stem mamilla (paratype HWML 37904-3) with 3 blunt mamilla spines (ms) and minute fimbria. Lateral view. Scale bar = 25 µm. **(9)** Posterior tail stem mamilla (light micrograph, paratype HWML 37904-3) showing similar details as Fig. 8. Lateral view. Scale bar = 25 µm. **(10)** Illustration of distal apex of furca showing sucker. Ventral view. Scale bar = 100 µm. **(11)** Distal end of furca with similar details as Fig. 10 (light micrograph, paratype HWML 37904-1). Ventral view. Scale bar = 100 µm. **(12)** High magnification view of distal end of furcae with sucker (light micrograph, paratype HWML 37904-1). Ventral view. Scale bar = 50 µm. **(13)** Distome: mouth (m), oral sucker (os), pharynx (p), caeca (c), vitelline field (vt), uterus in forebody (ufb) filled with eggs (e), prostatic sac (ps), genital pore (gp), ventral sucker (vs), uterus in hindbody (uhb) filled with eggs (e), testes (t), and caeca near termination (ct). Ventral view. Scale bar = 500 µm.

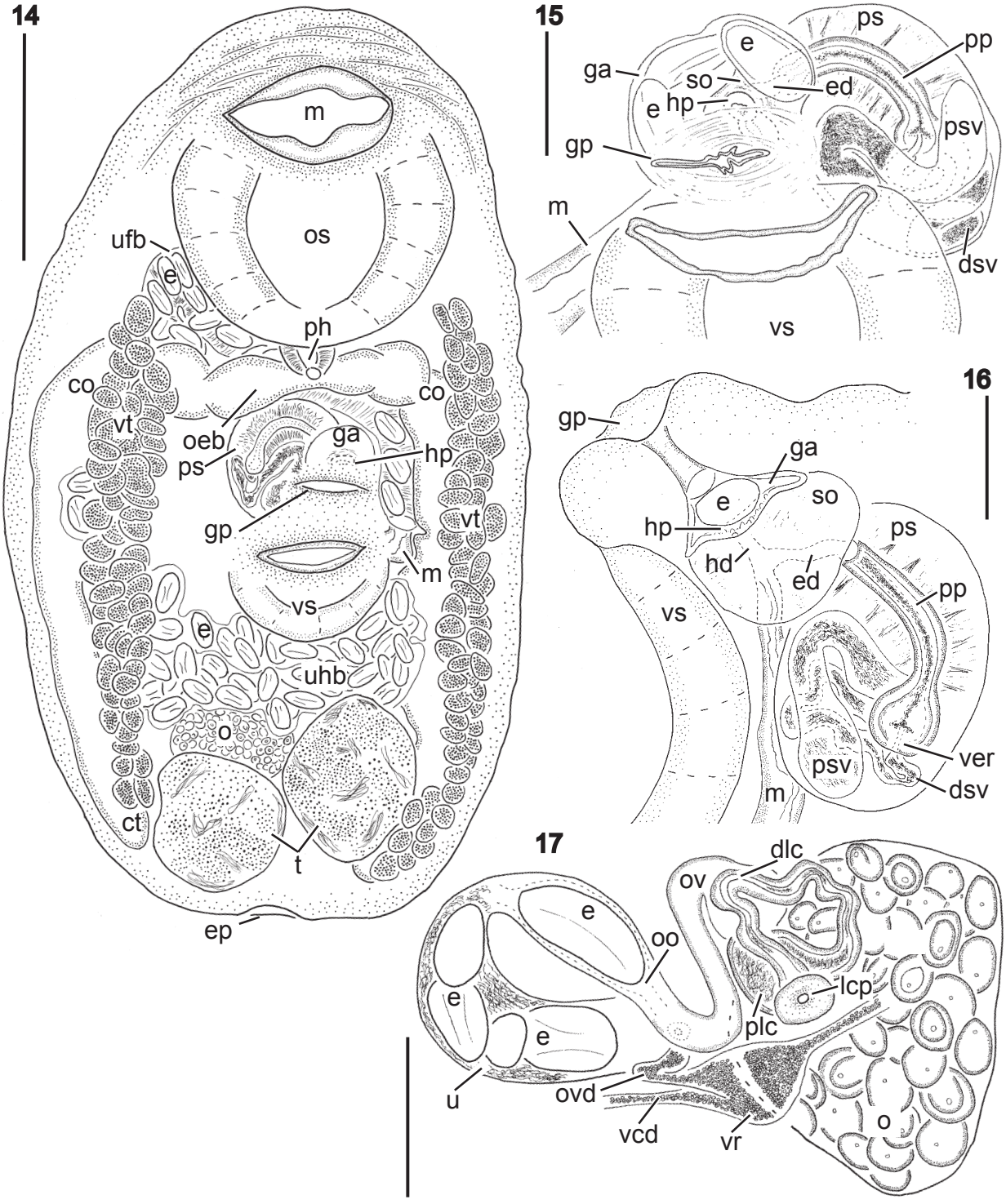
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Plate 2-3; Figures 14–17. Adults of *Proterometra ariasae* n. sp. (Digenea: Azygiidae) from oesophagus of longear sunfish, *Lepomis megalotis* Rafinesque, 1820 (Perciformes: Centrarchidae) from the Chickasawhay River, Mississippi. **(14)** Adult (USNM coll. no. 1283298) showing mouth (m), oral sucker (os), pharynx (ph), oesophagus bifurcation (oeb), caeca (co) near origin, vitellarium (vt), uterus in forebody (ufb), prostatic sac (ps), genital atrium (ga), hermaphroditic pore (hp), metraterm (m), genital pore (gp), ventral sucker (vs), uterus in hindbody (uhb) looping between ventral sucker and ovary (o), eggs (e**)**, testes (t), caeca termination (ct), and excretory pore (ep). Ventral view. Scale bar = 500 µm. **(15)** Terminal male genitalia (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figure 14 plus swollen proximal region of seminal vesicle (psv), narrow distal region of seminal vesicle (dsv), pars prostatica (pp), ejaculatory duct (ed), and sinus organ (so). Ventral view Scale bar = 100 µm. **(16)** Male genitalia (USNM coll. no. 1283300) showing comparable features as illustrated in Figures 14 and 15 plus the hermaphroditic duct (hd), and verschlussapparat (ver). Lateral view. Scale bar = 100 µm. **(17)** Female genitalia showing ovary (o), oviduct (ov), proximal portion of Laurer's canal (plc), distal end of Laurer's canal (dlc), Laurer's canal pore (lcp), vitelline reservoir (vr), vitelline collecting duct (vcd), ovovitelline duct (ovd), ootype (oo), and proximal end of uterus (u) with eggs (e) and sperm surrounding eggs. Dorsal view. Scale bar = 100 µm.

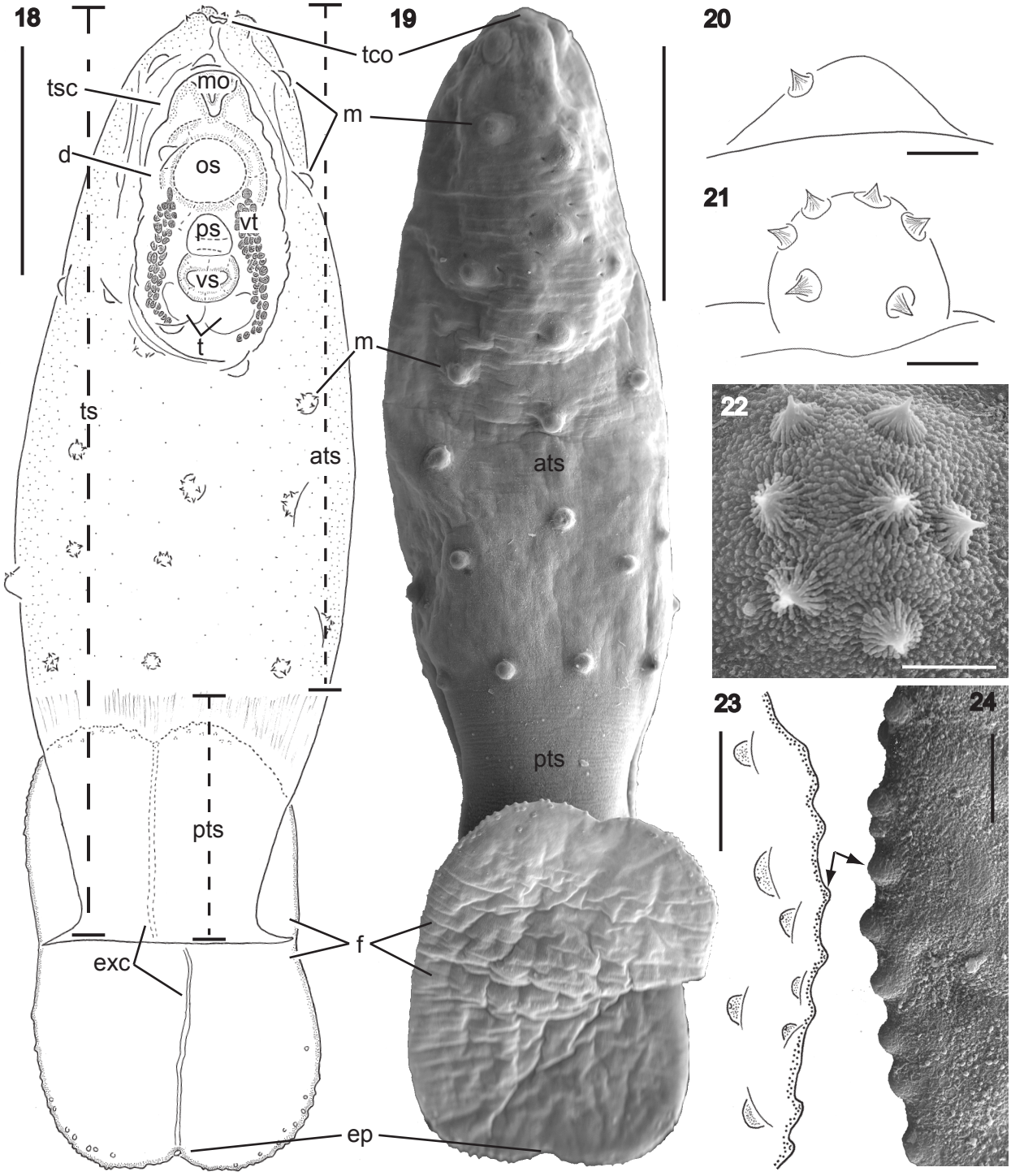
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Plate 2-4; Figures. 18–24. Cercariae of *Proterometra ariasae* n. sp. (Digenea: Azygiidae) from *Pleurocera* sp. (Cerithioidea: Pleuroceridae) from the Chickasawhay River, Mississippi. **(18)** Cercaria (USNM coll. no. 1283302) showing tail stem (ts), anterior tail stem (ats), posterior tail stem (pts), furcae (f) with medial notch, tail cavity opening (tco), tail stem cavity (tsc), mamillae (m), excretory canals (exc), and excretory pore (ep), distome (d), mouth (mo), oral sucker (os), vitellarium (vt), prostatic sac (ps), ventral sucker (vs), and testes (t). Ventral view. Scale bar = 1 mm. **(19)** Cercaria, with distome withdrawn, and similar details as Fig. 18. SEM. Scale bar = 1 mm. **(20)** Anterior mamilla with a spine. Lateral view. Scale bar = 50 µm. **(21)** Medial mamilla with spines. Lateral view. Scale bar = 50 µm. **(22)** Posterior mamilla with spines. SEM. Dorsal view. Scale bar = 25 µm. **(23)** Crenate margin of furca. Scale bar = 100 µm. **(24)** Crenate margin of furca. SEM. Scale bar = 25 µm.

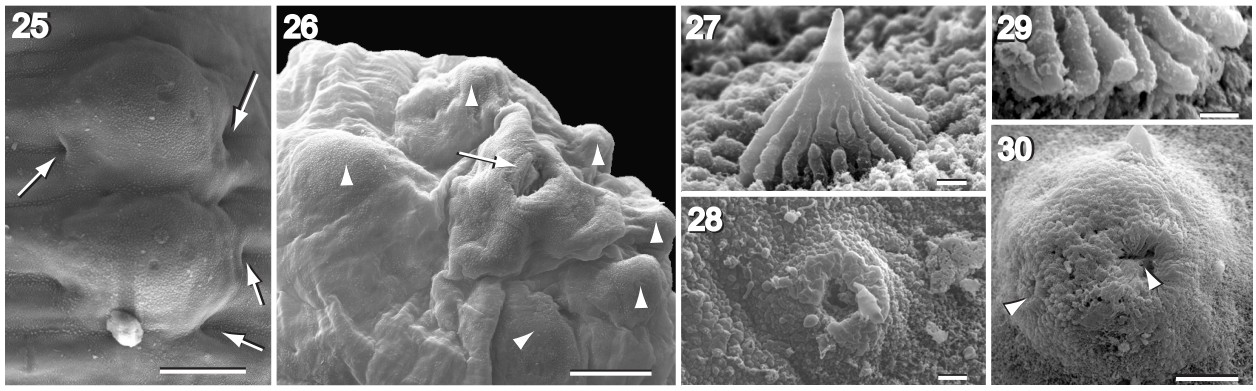

Plate 2-5; Figures 25–30: Cercariae of *Proterometra ariasae* n. sp. (Digenea: Azygiidae) from *Pleurocera* sp. (Cerithioidea: Pleuroceridae) from the Chickasawhay River, Mississippi. SEM. **(25)** Anterior tail stem at level of withdrawn distome showing pores (arrows) at the base of two mamillae. Scale bar = 100 µm. **(26)** Anterior end of tail stem showing tail stem cavity pore (arrow) with flanking mamilla (arrowheads), in a crowning fashion. Scale bar = 100 µm **(27)** Mamilla spine. Scale bar = 2 µm. **(28)** Pored protuberance associated with furca. Scale bar = 2 µm. **(29)** Lateral margin of mamilla spine, projecting from the tegument of the cercaria. Scale bar = 2 µm. **(30)** Tail stem mamilla showing putative site of detached spines (arrowheads). Scale bar = 20 µ m.

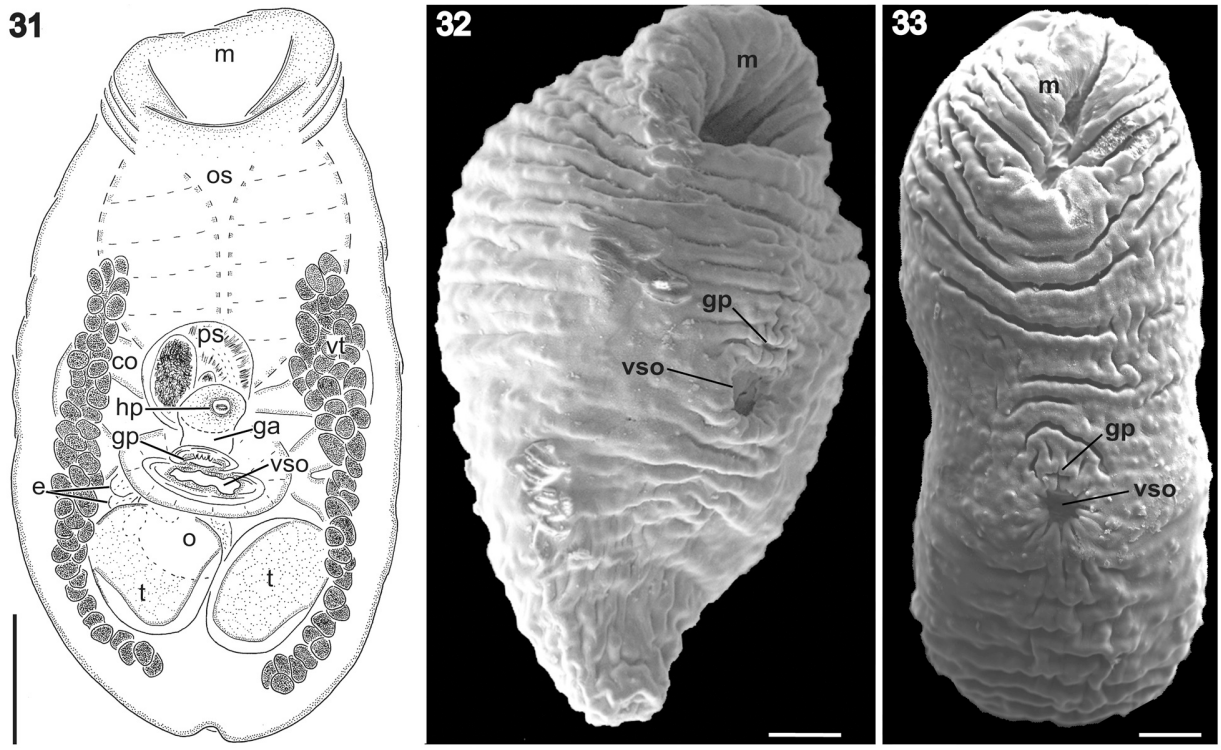

Plate 2-7; Figures 31–33: Distome of *Proterometra ariasae* from *Pleurocera* sp. from the Chickasawhay River, Mississippi. **(31)** Distome (USNM coll. no. XXXXXX) showing mouth (m), oral sucker (os), vitellarium (vt), caeca near origin (co), prostatic sac (ps), hermaphroditic pore (hp), genital atrium (ga), genital pore (gp), ventral sucker opening (vso), eggs (e), ovary (o), testes (t). Ventral view. Scale bar = 300 µm. **(32)** Distome showing prominent bowl-shaped oral sucker and positions of the mouth (m), genital pore (gp), and ventral sucker opening (vso). SEM. Lateral view. Scale bar = 100 µm. **(33)** Distome, of *Proterometra epholkos* Womble, Orélis-Ribeiro, and Bullard 2015 (from free-swimming, naturally shed cercariae) from *Elimia modesta* from Terrapin Creek, Alabama USA, showing similar details as Fig. 32. SEM. Ventral view. Scale bar = 100 µm.

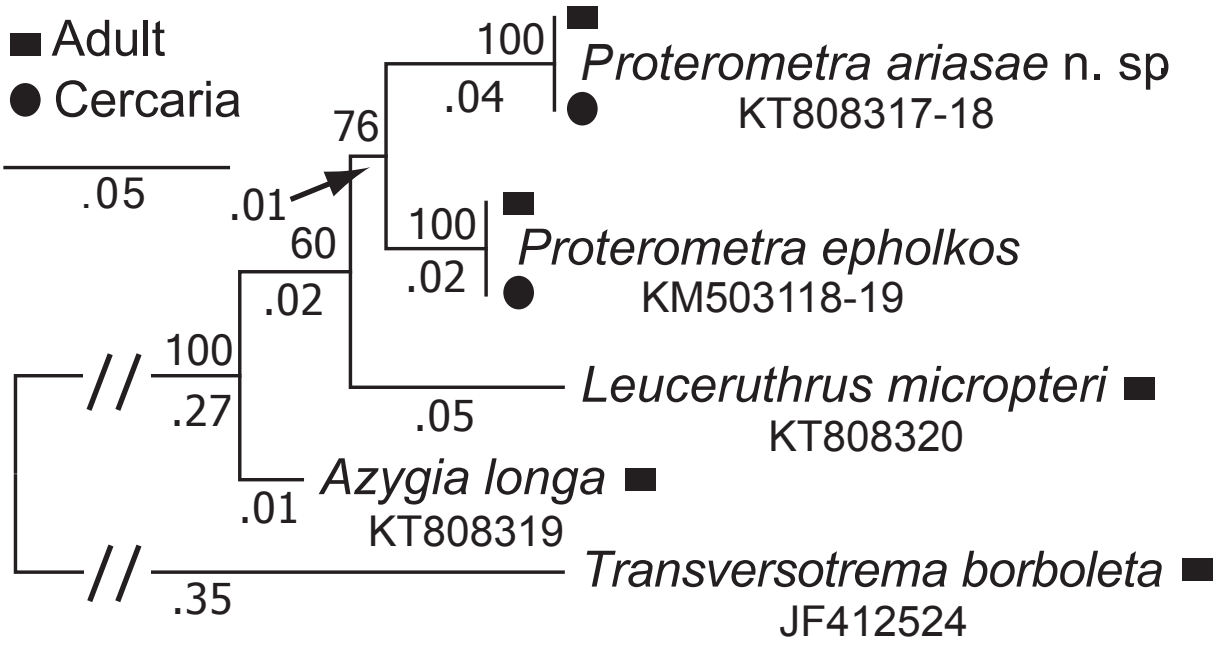

Figure 34: Maximum likelihood tree (inclusive of all sites) including two species of *Proterometra*, *Leuceruthrus micropteri*, and *Azygia longa*, (cercariae = circles; adults = squares), inferred from the ribosomal internal transcribed spacer 2 (ITS2; 363 bp). Bootstrap support values, based on 1,000-replicates, are reported aside each node and branch lengths are given below each branch.

CHAPTER 3: TAXONOMIC REDESCRIPTION, MORPHOLOGICAL AND MOLECULAR DIAGNOSIS, AND LIFE CYCLE OF *PROTEROMETRA CATERNARIA* SMITH, 1934 (DIGENEA: AZYGIIDAE) FROM THE CHOCTAWHATCHEE RIVER, FLORIDA, U.S.A.

***In Review for publication in Comparative Parasitology (Submitted 24 Sept. 2015)**

Authors: Matthew R. Womble, and Stephen A. Bullard

ABSTRACT

Light and scanning electron microscopy plus molecular data from the ribosomal internal transcribed spacer 2 (ITS2) confirmed that *Proterometra catenaria* Smith, 1934 (Digenea: Azygiidae) undergoes asexual reproduction in rasp elimia, *Elimia floridensis* (Reeve 1860), (Cerithioidea: Pleuroceridae) and sheds a furcocystocercous cercariae that matures in the esophagus of redspotted sunfish, *Lepomis miniatus* (Jordan 1877), (Perciformes: Centrarchidae) and longear sunfish, *Lepomis megalotis* (Rafinesque 1820), in Holmes Creek (Choctawhatchee River), Florida. Adults of *P. catenaria* differ from those of congeners by having an oral sucker ≥2.4× ventral sucker length, a pharynx ventral to the posterior musculature of oral sucker, a short, robust (thick-walled) pars prostatica, an ovary abutting the ventral sucker, a uterus lacking convolutions between the ovary and ventral sucker and that extends beyond the lateral margins of the forebody ceca, and paired, pre-testicular vitelline fields that rarely extend beyond the posterior margin of the ventral sucker. Cercariae of *P. catenaria* differ from those of congeners by having an elongate body (=tail stem+furcae) 9–16 mm long (7mm in fixed specimens), paired, ovate furcae that are longer than wide and taper posteriorly, 2 distinct anterior fields of tail stem mamillae, and a distome located

in the middle of the tail stem. A preliminary phylogenetic analysis, including ITS2 sequences of that species plus *Proterometra ariasae* Womble and Bullard, 2015, *Proterometra epholkos* Womble, Orélis-Ribiero, and Bullard, 2015, *Azygia longa* (Leidy, 1851) Manter, 1926, and *Leuceruthrus micropteri* Marshall and Gilbert, 1905 (type species) had low support but did not reject assignment of *P. catenaria* to *Proterometra*, conspecificity of the azygiid adults and cercariae from Holmes Creek, monophyly of *Proterometra*, or the sister-taxa relationship between azygiids (*L. micropteri* and *Proterometra* spp.) that infect North American basses and sunfishes (Centrarchidae). The redspotted sunfish is a new host record for *Proterometra*. We also clarify the taxonomic identity of prosobranchs previously reported as hosts for *P. catenaria*.

1. INTRODUCTION

Species of *Proterometra* Horsfall, 1933 (Digenea: Azygiidae) have a trophically-mediated, two-host ("truncated") life cycle wherein macroscopic, progenetic, furcocystocercous cercariae are shed from a prosobranch snail (typically species of *Elimia* Adams, 1854) and flamboyantly swim before being partially swallowed by the definitive fish host. Therein, as ectoparasites, they infect the buccal cavity epithelium and/or esophagus (Womble et al. [1]). *Proterometra* currently includes 12 species, making it the most species rich azygiid genus. These azygiids are among the most common parasites of several ubiquitous and commonly encountered eastern United States freshwater fishes, e.g., black basses (*Micropterus* spp.) and sunfishes (*Lepomis*

spp.) [1]. However, because several species of *Proterometra* were originally inadequately described and their hosts were likely misidentified, taxonomic revision that includes analyses of morphological characters and molecular sequence data is needed for several species. This is true even for the type species *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933 (*species inquirenda*) [1]. Among other congeners needing redescription, *Proterometra catenaria* Smith, 1934 stands out as a morphologically unique species of *Proterometra*; perhaps warranting the proposal of a new genus [2]. However, no detailed morphological treatment of this rather unique species had been published to date, and its placement within *Proterometra* was uncertain. This taxonomic conundrum is further entrenched by the fact that apparently no extant type materials exist for *P. catenaria*, and no author(s) has collected specimens of this azygiid from the type host (presumed) or type locality since Smith [2].

Herein, we redescribe *P. catenaria* based on adults and cercariae collected from the esophagus of redspotted sunfish, *Lepomis miniatus* (Jordan 1877), longear sunfish, *Lepomis megalotis* (Rafinesque 1820), and rasp elimia, *Elimia floridensis* (Reeve 1860) relatively nearby its type locality in Florida. This is the first record of a species of *Proterometra* infecting *L. miniatus*, and *P. catenaria* is the second species of *Proterometra* to be reported from *E. floridensis* [1,3]. The present study provides new morphometric data for the adult and cercaria of *P. catenaria*, details the male and female genitalia plus previously ignored tegumental features of its cercaria, and provides novel sequence data from the ribosomal internal transcribed spacer 2 (ITS2). We also present a preliminary phylogenetic analysis that includes *P. catenaria*, *Proterometra epholkos* Womble, Orélis-Ribiero and Bullard, 2015, and *Proterometra*

ariasae Womble and Bullard, 2015 along with *Azygia longa* (Leidy, 1851) Manter, 1926 and *Leuceruthrus micropteri* Marshall and Gilbert, 1905; comprising the largest phylogenetic taxon sampling to date for Azygiidae.

2. MATERIALS AND METHODS

Prosobranch snails were collected from Holmes Creek (Choctawhatchee River) (30°36’25”N; 85°44’49”W), near Vernon, Florida, U.S.A, on 29 June 2014. Fish were collected with a cast-net from the aforementioned site on 25 March 2015. In the field and laboratory, methods for transport and husbandry of snails and fish as well as collection, isolation, fixation, and preparation for study by light microscopy and scanning electron microscopy (SEM) of cercariae and flukes follows Womble et al. [4].

Snails were identified as *Elimia floridensis* by having (i) a unicolor shell with (ii) distinct vertical sculpture and (iii) strongly developed vertical ribs (iv) that are serrate at the periphery plus (v) robust spiral threads (iv) forming small sharp spines where they intersect the vertical ribs [5-7]. The fish specimens unambiguously keyed to *L. miniatus* as per Boschung and Mayden [8]; however, this geographic area comprises an intergrade region for hybrid *L. miniatus* × *L. punctatus* (“spotted sunfish”) [8].

Cercariae and adults for molecular biology were pipetted into and preserved in separate vials of 95% EtOH and stored at -20°C. Methods for total genomic DNA extractions, polymerase chain reaction (PCR) amplifications, and sequencing were performed as per Womble et al. [4]. Sequence assembling and analysis of chromatograms was conducted using BioNumerics version 7.0 (Applied Maths, Sint-

Martens-Latem, Belgium). The Internal Transcribed Spacer 2 Ribosomal Database [9] was used to determine the borders of the 5.8s, ITS2, and 28s gene regions. Representative sequences have been deposited in GenBank (XXXXXXXX).

Sequences for *P. epholkos* (GenBank: KM503118-19), *P. ariasae* (KT808317-18), *A. longa* (KT808319), and *L. micropteri* (KT808320) were obtained from GenBank. All sequences were aligned using MEGAv.6.06 [10] with default ClustalW parameters, and trimmed at the border of the ITS2, and 28s gene regions. Alignment curation, i.e., removal of ambiguous regions (i.e., those containing gaps and/or poorly aligned), was performed using Gblocks (v0.91b); resulting in a total fragment length of 306 base pairs (bp) including gaps [11,12]. Absolute site differences, and sequence similarity percentages, were calculated using MEGAv.6.06 using the same methods, parameters and models specified in Womble et al [4]. Sequences were analyzed using the Maximum Likelihood algorithm in MEGAv.6.06 according to best-fit DNA model analysis estimated with MEGAv.6.06 as Kimura's 2-parameter model with gamma distributed rate variation among sites (K2+G) [13] in combination with the Nearest-Neighbor-Interchange heuristic method. All sites, including gaps, were included in the analyses. A bootstrap analysis based off 1,000 replicates was used to establish nodal support values. Branch support was considered as significant when bootstrap values were > 70%. *Bivesicula unexpectata* Cribb, Bray, and Barker, 1994 (GenBank KR092222) was selected as the outgroup based on its proposed relationship to Azygiidae (see [14]).

Common names, scientific names, taxonomic authorities and dates, and higher-level gastropod classification follow Johnson et al. [15]. Nomenclature for *Elimia* follows

Clench and Turner [5], Burch [16], Chambers [7], and Johnson et al. [15]. Higher-level fish classification and nomenclature follows Nelson [17] and fish common names follow Boschung and Mayden [8]. Nomenclature for Azygiidae follows Gibson and Bray [18] and morphological terminology for *Proterometra* follows Womble et al. [1] and references therein.

3. RESULTS

3.1. Proterometra catenaria *Smith, 1934 (Figs. 1–11)*

Synonyms: *Cercaria catenaria* Smith, 1934.

3.1.1 Diagnosis of adult based on results from light microscopy of 9 stained whole mounted specimens.

Body sub-ovate, with anterior end more broadly rounded than posterior end, 1,220–2,180 (1,564, 9) long, 700–1,420 (952, 9) or 1.5–1.8 (1.7, 9) × longer than wide, widest at level of ventral sucker (Fig 1); forebody 790–1,520 (1,024, 9) long or 63–70% (65%, 9) of body length; hindbody 220–420 (328, 9) long or 18–23% (21%, 9) of overall body length, 27–36% (32%, 9) of forebody length; tegument smooth, lacking projections, approximately 10–20 (16, 9) thick. Nervous system not evident. Oral sucker subterminal, 15–160 (61, 9) or 1–8% (4%, 9) of body length from anterior body end, 700–1,340 (930, 9) or 56–62% (59%, 9) of body length from posterior body end, 475–690 (572, 9) long or 32–40% (37%, 9) of body length, 480–720 (570, 9) wide or 50–69% (61%, 9) of body width, posterior margin of oral sucker 285–680 (396, 9) from anterior margin of ventral sucker (Fig 1). Ventral sucker in posterior half of body,

145–255 (198, 9) long or 11–15% (13%, 9) of body length, 210–275 (231, 9) wide or 19–32% (25%, 9) of body width, wider than long, 31–41% (35%, 9) of oral sucker length, 33–47% (41%, 9) of oral sucker width (Fig 1).

Mouth anteroventral (Fig 1). Pharynx ovoid, ventral and partly posterior to the oral sucker (Fig 1), 100–160 (121, 9) long or 6–9 % (8%, 9) of body length, 110–160 (133, 9) wide or 1–1.4 (1.1, 9) × wider than longer. Esophagus extending posteriad from mouth 325–485 (388, 9) before bifurcating 15–25 (20, 9) posterior to pharynx, with esophageal branches arching posterolaterad before joining with intestinal ceca; dextral esophageal branch 60–150 (87, 9) long, sinistral esophageal branch 50–160 (90, 9) long (Fig 1); intestinal ceca confluent with esophageal branches, appearing inverse U-shaped inclusive of esophageal branches, comprising paired dextral and sinistral ceca (Fig 1); dextral cecum 625–1,260 (906, 9) or 52–68% (57%, 9) of body length, 80–205 (145, 9) in max width; pre-cecal space 540–890 (673, 9) or 41–47% (43%, 9) of body length from anterior end of body; post-cecal space 45–150 (96, 9) or 3–10% (6%, 9) of body length from posterior end of body; sinistral cecum 620–1,310 (943, 9) or 44–57% (50%, 9) of body length, 75–260 (150, 8) in max width; pre-cecal space 530-940 (666, 9) or 38–45% (43%, 9) of body length from anterior end of body; post-cecal space, 95–165 (129, 8) or 5–12% (8%, 8) of body length from posterior end of body.

Testes 2 in number, sub-terminal, abreast, abutting posterior margin of ovary, ovoid (Fig 1); dextral testis 200–325 (250, 9) long or 12–19% (15%, 9) of body length, 130–225 (168, 9) wide or 13–26% (18%, 9) of body width, pre-testicular space 900-1,680 (1,231, 9) or 71–84% (79%, 9) of body length; post testicular space 35–175 (103,

9) or 3–10% (6%, 9) of body length from posterior end; sinistral testis 193–325 (255, 9) or 15–21% (16%, 9) of body length, 125–223 (164, 9) or 12–23% (18%, 9) of body width; pre testicular space 900–1,860 (1,239, 9) from anterior end of body or 74–85% (79%, 9) of total body length; post testicular space 50–160 (99, 9) or 3–10% (7%, 9) of body length from posterior end. Vasa efferentia not evident. Prostatic sac medial, with anterior margin ventral to posterior margin of esophageal branches (Fig 1), 95–300 (182, 9) from posterior margin of oral sucker, with posterior margin anterior to or level with genital pore (Figs 1, 2), 20–180 (59, 9) from anterior margin of ventral sucker, 143–225 (172, 9) long, 160–240 (197, 9) wide. Seminal vesicle thin-walled, highly convoluted, occupying majority of space within prostatic sac (Fig 2), 270–658 (443, 6) long, swollen for entire length (Fig 2), distal end connecting to pars prostatica via verschlussapparat [1]. Verschlussapparat 15–38 (29, 8) long, thin-walled, proximal end opening within wide distal region of pars prostatica (Fig 2). Pars prostatica 100–163 (131, 9) long, 40–55 (49, 9) wide proximally, 18–30 (26, 9) wide distally, tapering 38–54% (45%, 9) in width from proximal to distal end, slightly arched, seemingly muscular, lined by prostatic gland cells, with wall 5–10 (8, 8) for entire length, exiting prostatic sac ventrally (Fig 2). Ejaculatory duct (= diminutive continuation of pars prostatica outside of prostatic sac) extending ventrally from prostatic sac becoming confluent with hermaphroditic duct, 33–53 (43, 9) long or 20–47% (34%, 9) of pars prostatica length. Male and female terminal genitalia becoming confluent within sinus organ. Sinus organ directed ventrally. Hermaphroditic pore nearly level with posterior margin of prostatic sac (Fig 2), anterior of ventral sucker, at 60–62% (61%, 9) of body

length, directing ventrally before opening into genital atrium (Fig 1, 2). Genital atrium connecting hermaphroditic pore and genital pore, thick walled, circular in outline (Figs 1, 2), 95–260 (139, 8) in diameter, 3 of 9 (33%) specimens containing 7–25 (15, 3) uterine eggs. Genital Pore anterior and ventral to anterior musculature of ventral sucker, usually medial, posterior of prostatic sac, opening ventrally at 61–67% (64%, 9) of total body length (Fig 1, 2).

Ovary nearly medial, intercecal, anterior margin abut with posterior musculature of ventral sucker (Fig 1, 3), level with anterior margin of testes (Fig 1), 95–150 (121, 9) long or 6–10% (8%, 9) of body length, 123–255 (166, 9) wide or 14–22% (17%, 9) of body width or 1–1.7 (1.4, 9) × wider than long, post-ovarian space 180–300 (243, 9) long or 14–20% (16%, 9) of body length. Oviduct thin-walled, typically emanating dorso-anteriorly from ovary, where a muscular sphincter 38–48 (40, 9) wide is present, extensively convoluted before confluence with Laurer's canal, extending 150–183 (167, 2) from commissure with Laurer's canal to ootype (Fig 3). Laurer's canal swollen proximally with sperm after commissure with oviduct (= possible oviducal ampulla [19]) (Fig 3), becoming narrow and thick walled, extending 128–175 (154, 3) to Laurer's canal pore (Fig 3), pore opening dorsally and posterior to ventral sucker, 225–355 (268, 8) from posterior margin of body. Ovo-vitelline duct indistinct. Ootype dorsal to ovary, 63–68 (64, 5) long, 25–30 (28, 5) in maximum width (Fig 3). Mehlis gland indistinct. Uterus occupying space between posterior fourth of oral sucker and ovary (Fig 1), comprising a field 415–1440 (755, 8) long or 32–66% (45%, 8) of body length; without convolutions between ovary and ventral sucker proximally, immediately passing ventral sucker dextrally or sinistrally, sometimes looping between ventral

sucker and prostatic sac, proceeding dextrally beyond lateral margins of dextral ceca and vitellaria, arching posteromedial near level with pharynx, extending sinistrally across body, posterior to oral sucker, dorsal to esophageal branches, to beyond lateral margins of sinistral ceca and vitellarium, extending posteromedial to near prostatic sac, synthesis with metraterm occurring distally near prostatic sac, typically with many eggs; uterine seminal receptacle present. Metraterm thick walled, 163–355 (236, 8) or 10–20% (15%, 8) of body length, 30–60 (39, 5) wide, becoming confluent with uterus anterior to ventral sucker, becoming confluent with ejaculatory duct to form a common duct (= herein a 'hermaphroditic duct') within sinus organ (Fig 2). Vitellarium follicular, ventral to ceca, distributing in 2 bilaterally symmetrical fields, distance between fields 220–550 (372, 9) or 31–44% (39%, 9) of body width, extending from slightly anterior of posterior musculature of oral sucker to near midline of ventral sucker (Fig 1), rarely extending (if so only slightly) beyond posterior musculature of ventral sucker; dextral vitelline field 450–1,000 (661, 9) long or 36–46% (42%, 9) of body length, terminating anteriorly at 27–40% (34%, 9) of body length,

terminating posteriorly at 73–84% (77%, 9) of body length, shorter than cecum or 76–90% (85%, 8) of dextral cecum length (Fig 1); sinistral vitelline field 425–1,140 (679, 9) long or 35–52% (43%, 9) of body length, terminating anteriorly at 27–41% (32%, 9) of body length, terminating posteriorly at 71–82% (75%, 9) of body length, always shorter than cecum or 76–94% (85%, 7) of sinistral cecum length (Fig 1); primary vitelline collecting ducts nearly symmetrical, extending posteromediad from respective vitelline field before becoming confluent and forming vitelline reservoir; dextral vitelline collecting duct 120–310 (229, 7) long, 15–25 (21, 7) wide near yolk

reservoir, proximal end branches from vitellarium at 81–97% (92%, 7) of dextral vitelline field length; sinistral vitelline collecting duct 175–440 (265, 5) long, 15–25 (20, 5) wide near yolk reservoir, proximal end branches from vitellarium at 81–89% (85%, 5) of sinistral vitelline field length. Vitelline reservoir typically in-between testes and ovary (Fig 2). Uterine eggs typically densely distributed throughout uterus filling lumen, pyriform, enlarging from approximately 45–55 (49, 8) x 20–30 (26, 8) in proximal portion of uterus to approximately 60–75 (69, 8) x 30–45 (38, 8) in distal portion of uterus (Fig 1), no papilla-like projections evident on polar surface of eggs. Excretory system largely indistinct; excretory bladder filling area dorsal and posterior to testes, with diminutive excretory duct extending 50–75 (62, 6) to terminal excretory pore.

3.1.2 Diagnosis of cercaria based on light microscopy of 8 whole-mounted, naturally-shed cercariae [distome withdrawn] plus SEM of 2 naturally-shed cercariae.

Furcocystocercous, 5,560–8,020 (6,852, 8) long, 720–980 (886, 8) wide or 5.8–8.9 (7.7, 8) × longer than wide, comprised of a tail stem and paired furcae (Fig 4). Tail stem 4,800–6,740 (5,740, 8) long or 75–89% (84%, 8) of cercariae length; having anterior and posterior regions (Fig 4); anterior tail stem (ATS) cylindrical, narrow and elongate anteriorly, swollen posteriorly in vicinity of distome, 2,900–4,670 (3,769, 8) long or 4–5.6 (4.9, 8) × longer than wide, 45–59% (54%, 8) of cercariae length, anterior width 440–630 (520, 8), width at distome 670–940 (800, 7), bearing mamillae (Figs 4–7); posterior tail stem dorsoventrally compressed (Fig 4), 1,740–2,330 (1,959, 8) long or 26–33% (29%, 8) of cercariae length, 660–980 (819, 8) wide at anterior end, 420–590 (503, 8) wide at posterior end, tapering 24–52% (38%, 8) from anterior to posterior

end, devoid of mamillae, irregularly studded with minute projections along lateral margins (Figs 8, 9). Mamillae comprising

mound-like tegumental protuberances of the anterior tail stem (Figs 4–7), 32–42 (37, 8) per cercaria, usually bearing spines; tail stem mamillae distributing in 2 separate fields in anterior, and posterior segments of the anterior tail stem; anterior field (Figs 4, 5) 270–500 (421, 8) long, comprising 6–10 (8, 8) mamillae about tail cavity opening (Figs 4, 5); mamillae of anterior field 40–60 (51, 6) in maximum length, 80–140 (111, 6) in maximum width; posterior field (Fig 4) encircling distome for entire distome length, terminating at posterior margin of anterior tail stem, 1,320–2,000 (1638, 8) long, comprising 25–34 (29, 8) mamillae (Fig 4); mamillae of posterior field 55–90 (68, 8) in maximum length, 65–110 (91, 8) in maximum width. Mamilla spines (Figs 6, 7) absent or comprising up to 13 per mamilla, minaret-shaped. Furcae paired, ovoid, dorsoventrally flattened (Figs 4, 10); furcae margin, serrate (Figs 4, 10, 11), having numerous minute protuberances irregularly placed on furcae margins (Figs 10, 11), approximately 8–10 long. Dorsal furcae 950–1,240 (1081, 7) long or 14–18%, (16%, 7) of cercariae length, 420–800 (642, 9) wide or 43–71% (59%, 9) of dorsal furcae length; ventral furcae 980–1,400 (1163, 8) long or 15–19%, (17%, 8) of cercariae length, 500–780 (645, 8) wide or 38–66% (55%, 8) of ventral furcae length. Tail cavity opening terminal, anteromedial, appearing as a constricted and muscular sphincter (Figs 4, 5), communicating with tail stem cavity via narrow duct (Fig 5); tail stem cavity extending throughout anterior tail stem region (Fig 4), thin-walled anteriorly, swelling posteriorly to accommodate distome (Fig 4). Distome (= cercarial body) (Fig 4) contained within tail cavity sac when withdrawn, restricted to posterior portion of the anterior tail stem,

anterior margin 1,340–2,950 (2,155, 8) of cercaria length from tail cavity opening; body of distome (Fig 4) oblong, 1,200–1,480 (1,354, 8) long or 17–22% (19%, 8) of cercaria length 540–860 (693, 8) wide or 1.5–2.6 (2, 8)× longer than wide, 10–40 (26, 4) eggs in uterus; forebody 770–890 (838, 8) long or 56–68% (62%, 8) of overall body length; hindbody 380–610 (513, 8) long or 32–44% (38%, 8) of overall body length. Nervous system not evident. Oral sucker subterminal (Fig 4), 120–210 (154, 8) of body length from anterior body end, 480–710 (587, 8) of body length from posterior body end, 550–750 (605, 8) long or 40–54% (45%, 8) of body length, 370–550 (446, 8) wide. Ventral sucker in posterior half of body (Fig 4), 150–200 (175, 8) long, 150–240 (209, 8) wide, 20–36% (29%, 8) of oral sucker length, 38–58% (48%, 8) of oral sucker width. Intestinal ceca confluent with esophageal branches, appearing inverse U-shaped inclusive of esophageal branches, comprising paired dextral and sinistral ceca; dextral cecum 750–970 (851, 4) long or 54–66% (60%, 4) of distome length, with pre-cecal space 600–680 (645, 4) long or 43–49% (46%, 4) of distome length, with post-cecal space 95–130 (116, 4) long or 6–9% (8%, 4) of distome length; sinistral cecum 650–920 (797, 4) long or 46–67% (57%, 4) of distome length, with pre-cecal space 600–720 (665, 4) long or 43–51% (47%, 4) of distome length, with post-cecal space 120–140 (129, 4) long or 9–10% (9%, 4) of distome length.

Testes 2 in number, abreast, ovoid (Fig 4), posterior of ventral sucker; dextral testis 210–240 (225, 4) long or 1.4–2 (1.6, 4) × longer than wide, 110–170 (140, 4) wide, with post testicular space 105–220 (168, 4) long or 8–15% (12%, 4) of total body length; sinistral testis 205–240 (223, 4) long or 1.3–1.7 (1.5, 4) × longer than wide, 135–160

(150, 4) wide, with post-testicular space 100–220 (160, 4) long or 7–16% (11%. 4) of total body length. Prostatic sac between oral sucker and ventral sucker. Fine features of terminal male genitalia not evident.

Ovary medial, intercecal, between ventral sucker and testes, near posterior end of body or 270–320 (303, 5) of body length from posterior end of body, 85–125 (108, 5) long, 100–145 (130, 5) wide. Fine features of terminal female genitalia not evident. Uterus difficult to discern, appearing convoluted in region dorsal of ventral sucker, metraterm not evident. Vitellarium follicular, ventral to ceca distributing in 2 bilaterally symmetrical fields, extending from near midline of oral sucker to near posterior margin of ventral sucker (Fig 5); dextral vitelline field 460–570 (518, 7) long or 34–41% (38%, 7) of body length, terminating anteriorly at 31–38% (35%, 7) of body length, terminating posteriorly at 70–76% (74%, 7) of body length; sinistral vitelline field 450–640 (546, 7) long or 32–45% (40%, 7) of body length, terminating anteriorly at 28–43% (36%, 7) of body length, terminating posteriorly at 70–81% (75%, 7) of body length. Primary excretory canal difficult to trace, extending from distome through entire length of posterior tail stem, bifurcating at synthesis of furcae and extending through each furca; excretory pore opening at distal end of each furca.

3.1.3 Molecular results.

ITS2 sequences from 1 cercaria and 2 adult flukes from Holmes Creek, Florida, were 100% identical, indicating conspecificity of all sequenced specimens.

Interspecific comparison between sequences of *P. catenaria* and *P. ariasae* resulted in a total difference of 23 base pairs (23/306 = 92 % similarity), and comparison with sequences of *P. epholkos* resulted in a total difference of 24 base

pairs (= 92 % similarity). Intergeneric comparison between sequences of *P. catenaria* and *A. longa* resulted in a total difference of 32 base pairs (= 90 % similarity), and comparison with *L. micropteri* resulted in a total difference of 28 base pairs (= 91 % similarity). As indicated by morphology, *P. catenaria* was molecularly most similar to *P. epholkos* and *P. ariasae*.

3.1.3 Taxonomic Summary

Type host: green sunfish, *Lepomis cyanellus* Rafinesque, 1819 (as *Apomotis c.*) (type host, experimental host) [2].

Prosobranch host: rasp elimia, *Elimia floridensis* (Reeve 1860) (as "*Goniobasis catenaria* Say" [2]) (Cerithioidea: Pleuroceridae) (type host).

Type locality: Apalachicola River, St. Johns River, and Suwanee River [2].

Site of infection: esophagus (fish); indeterminate (prosobranch).

Other hosts and localities: **adult**: Fishes of "Centrarchidae" in "Northern and Central Florida" and Choctawhatchee River, Alabama [2]; pumpkinseed, *Lepomis gibbosus* (L.) (experimental host) [20] (USNM 61233); redspotted sunfish, *Lepomis miniatus* (Jordan 1877), Holmes Creek, Florida (present study); longear sunfish, *Lepomis megalotis* (Rafinesque 1820), Holmes Creek, Florida (present study). **cercaria**: rasp elimia, *Elimia floridensis* (Reeve 1860) (as "*Goniobasis catenaria* Say" [20]), Blue Springs, Marianna, Jackson County, Florida (USNM 61234); graphite elimia, *Elimia dooleyensis* (Lea 1862) (as "*Goniobasis doolyensis* Lea" [2]), Choctawhatchee River, Alabama; rasp elimia, *Elimia floridensis* (Reeve 1860), Holmes Creek, Florida (present study).

Specimens deposited: 4 vouchers (USNM Nos. XXXXX-X, adults; USNM Nos. XXXXXXX-X, cercariae).

4. DISCUSSION

4.1. Taxonomic remarks

Adults of *P. catenaria* can be most easily differentiated from congeners by having the following combination of features: (i) an oral sucker ≥2.4× ventral sucker length (Fig1), (ii) a pharynx ventral to posterior musculature of oral sucker (Fig 1), (iii) a short, robust (thick-walled) pars prostatica (Fig 2), (iv) an ovary abutting the ventral sucker (Figs 1, 3), (v) a uterus lacking convolutions between the ovary and ventral sucker and that extends beyond the lateral margins of the forebody ceca (Fig 1), and (vii) paired, pre-testicular vitelline fields that extend beyond the posterior margin of the ventral sucker (Fig 1) [2,20] (present study). Cercaria of *P. catenaria* differ from those of congeners by having (i) an elongate body (=tail stem+furcae) 9–16 mm long (7 mm long in fixed specimens), (ii) paired, ovate furcae that are longer than wide and taper posteriorly (Fig 4), (iii) 2 distinct anterior fields of tail stem mamillae, and (iv) a distome located near the posterior end of the anterior tail stem, i.e., in the middle of the tail stem (Fig 4) [2,20] (present study).

Our specimens differed from previously published descriptions of adults and cercariae of *P. catenaria* in several taxonomically important ways [2,20]. Our measurements for adult body length and width, forebody length, oral sucker length and width, ventral sucker length and width, and uterine egg size for adults of *P. catenaria* differed from that provided by Anderson and Anderson [20] and Smith [2]. We interpreted these differences as oversights or resulting from the disposition of the materials being studied, i.e., living vs. fixed specimens, and differences in terminology

rather than species-level taxonomic differences between our specimens and previous descriptions of *P. catenaria* [2,20].

We deduced herein that Smith [2] measured live specimens only: she stated that cercariae were 9–16 mm long, adults were 2.4×1.4 mm, the oral sucker was 0.64 mm long, and the ventral sucker was 0.26 mm long. Anderson and Anderson [20] measured live and fixed specimens. Their measurements for live specimens matched those of Smith [2] regarding cercariae length (as “tail stem length”; 9–16 mm), adult length and width (2.6 × 1.05 mm), oral sucker length (0.62 –0.71mm), and ventral sucker length (0.22–0.28 mm). The measurements provided by Anderson and Anderson [20] for fixed material and those provided herein were similar but less than the measurements of live specimens provided by Anderson and Anderson [20]. We measured fixed, whole-mounted materials only and compared those measurements with those of Anderson and Anderson [20] for fixed material only. Smith [2] provided no information concerning type material deposition, and we were unable to locate any record of such material lodged at the USNM or the Harold W. Manter Laboratory of Parasitology.

4.2. Clarification of morphological features of adults of P. catenaria

Regarding the alimentary system, Anderson and Anderson [20] illustrated the pharynx dorsal to the posterior musculature of the oral sucker and the esophageal branches as narrow, short tubes (see Fig. 8 of Anderson and Anderson [20]). We observed the pharynx as ventral to the posterior musculature of the oral sucker (Fig 1). This has not been previously described in any species of *Proterometra.* We also

observed considerable variation in the appearance of the esophageal branches in our specimens: some were broad (Fig 1) and others were narrow, as depicted by Anderson and Anderson [20] (see Fig 8 of Anderson and Anderson [20]). Regarding the male genitalia, the pars prostatica of *P. catenaria* is unlike that of its congeners [1,4,21-23] in that it is short (~131 μm) and tapers approximately 45% of its original width distally (Fig 2) while remaining thick-walled (robust) for its entire length. Regarding the female genitalia, the vitellarium rarely extended beyond the posterior margin of the ventral sucker, was entirely pre-testicular, and was shorter than the corresponding cecum (see Fig 8 of [20]). Only *Proterometra septimae* Anderson and Anderson, 1967 has a similar distribution of the vitellarium; whereas, other *Proterometra* spp., i.e., *P. macrostoma* (type species; *species inquirenda*), *Proterometra melanophora* (Smith, 1932) Smith 1936 (*species inquirenda*), *Proterometra sagittaria* Dickerman, 1946, *Proterometra dickermani* Anderson, 1962, *Proterometra albacauda* Anderson and Anderson, 1967, *Proterometra edneyi* Uglem and Aliff, 1984, *Proterometra autraini* Labeau and Peters, 1995, *P. epholkos*, and *P. ariasae*, have paired vitelline fields that extend posteriorly beyond the ventral sucker to near the posterior body margin. Information is unavailable for the vitellarium distribution in *Proterometra hodgesiana* (Smith, 1932) Smith, 1936 (*species inquirenda*). Additionally, in specimens of *P. catenaria* the Laurer's canal immediately posterior to the commissure with the oviduct is swollen with sperm; perhaps indicative of an oviducal ampulla [19]. We have previously described such a proximal swelling of the Laurer's canal in *P. ariasae* but in *P. albacauda* and *P. epholkos* the proximal portion of the Laurer's canal is narrow and sinuous [1,4].

4.3. Clarification of morphological features of cercariae of P. catenaria

Tail stem length differentiates cercariae of *Proterometra* spp.; however, it can differ markedly between living and fixed specimens. Smith [2], presumably from live material (see above), reported cercarial length as 9–16 mm. Anderson and Anderson [20], based on live and fixed material, reported measurements for “tail stem” length only (see [1] for definition) but not cercarial length. However, the ranges reported herein for cercarial length (= 5.6–8.0 mm) were nearly identical with those reported by Anderson and Anderson [20] for fixed “tail stem” length (= 5.2–8.2 mm). Likewise, Anderson and Anderson [20] reported tail stem length as being 9–16 mm in living specimens, which is identical to the range reported for cercaria length by Smith [2]. Although based on comparison with our measurements and those provided by Smith [2], we speculate that it reflects cercaria length. This is important because the taxonomic keys for cercariae of *Proterometra* spp. [1,24] use Smith’s [2] and Anderson and Anderson’s [20] observations of *P. catenaria* regarding tail stem length (i.e., >9 mm). Our results herein indicated that tail stem length and cercarial total length for fixed specimens of *P. catenaria* is <9 mm. Future workers should treat measurements of fixed and live specimens separately.

The mamillae about the anterior portion of the tail stem and medial region of the tail stem were not detailed by Smith [2] but described by Anderson and Anderson [20] as spinous “slightly elevated areas.” Both noted the unique arrangement of mamillae. In other species of *Proterometra* the mamillae form a contiguous field that distributes over the surface of the tail stem (*P. sagittaria*, *P. dickermani*, *P. septimae, P. edneyi*, *P. autraini*, *P. ariasae*) or that encircles the anterior portion of the anterior tail stem and

distributes in two lateral columns (*P. albacauda*, *P. epholkos*). Information for mamillae distribution is unavailable or ambiguous for *P. macrostoma*, *P. melanophora*, and *P. hodgesiana*. The distribution of the posterior mamillae in *P. catenaria* corresponds with the position of the withdrawn distome. Regarding mamilla spines, Anderson and Anderson [20] reported that the anterior tail stem mamillae had 12–20 spines each; whereas, the mamillae about the distome had 10–20 spines. We observed a maximum of 13 spines per mamilla, regardless of position on the tail stem, with mamillae of the anterior field having 0–10 spines and those of the posterior field having 0–13 spines.

Regarding the furcae, our specimens had ovate furcae. Smith [2] described them as "sharply pointed," and Anderson and Anderson [20] described them as "long" and "tapering." *Proterometra sagittaria* and *P. septimae* are the only species of the genus that have elongated and pointed furcae that resemble those of *P. catenaria* [20,23]. Anderson and Anderson [20] noted that the furcae of *P. catenaria* had "numerous small papillae." We confirmed this feature in our specimens and have previously described putatively homologous features in cercariae of other species of *Proterometra* (Fig 10, 11) [1,4]. Regarding the distome, we herein provided the first morphometric data for the withdrawn distome of *P. catenaria*, which is unique in that it is restricted to the posterior end of the anterior tail stem (Fig 4), i.e., the middle of the tail stem. The distome of most *Proterometra* spp. is typically positioned near the tail stem opening, i.e., the extreme anterior part of the tail stem [1].

4.4. Life cycle of P. catenaria

The life cycle of *P. catenaria* has been studied previously and we confirmed those details herein using morphology and molecular data, including a particular emphasis on the identity of the snail host. Smith [2] described cercariae and adults of *P. catenaria* from prosobranchs and freshwater fishes in the Apalachicola, St. Johns, and Suwanee rivers (Florida panhandle) as well as experimentally elucidated its life cycle. Anderson and Anderson [20] repeated the life cycle and added supplemental observations of adults and cercariae based on specimens collected from Blue Springs (Marianna, Florida). Yet, as is the case for most *Proterometra* spp., the identity of the snail host(s) remains in question [1,4]. We suspect that the snails collected and identified by Smith [2] and Anderson and Anderson [20] were most likely specimens of *E. floridensis* and not *Elimia catenaria* (Say, 1822) as specified therein. Smith [2] and Anderson and Anderson [20] collected cercariae of *P. catenaria* from *E. catenaria* (as *Goniobasis catenaria*) [2,20] and *E. dooleyensis* (as *G. doolyensis*) [2]. Clench and Turner (1956) asserted that "*Goniobasis catenaria*," considered by Pilsbry [25] and Goodrich [26,27] as a senior subjective synonym of the widespread Floridian prosobranch *E. floridensis*, was erroneously applied to *E. floridensis*. Subsequent workers have accepted both *E. catenaria* (Atlantic Ocean drainages) and *E. floridensis* (Gulf of Mexico drainages) [7,15].

4.5. Molecular phylogeny and taxonomy of azygiids

Azygiidae is a phylogenetically undersampled taxon within the Digenea. Our phylogenetic results (including taxa all having a 2-host life cycle), while preliminary,

having low nodal support, and incorporating information from ITS2 only, are a step toward working out interrelationships of several genera within the family (Fig 12). Below, we throw a light on some of the alluring aspects of this preliminary phylogeny. Aside from these points of interest, and as alluded to in previous works [1,4], obtaining ITS2 sequences for putative specimens of the type species of *Proterometa*, *P. macrostoma*, is required to place *Proterometra* within the phylogeny of Azygiidae and to allow for a revision of *Proterometra*. Regarding molecular taxonomy, ITS2 seems to be a useful marker for indicating conspecificity of adults and cercariae of Azygiidae, which is consistent with results derived from this marker in other digenean groups [28]. However, additional taxa and evidence from additional molecular markers are needed to evaluate its usefulness for informing phylogenetic interrelationships of azygiids. Regarding molecular systematics, using all publically available sequences for *Proterometra* spp., monophyly of *Proterometra* is not rejected. The ecologically- and morphologically-similar *Proterometra* spp. are sister to *Leuceruthrus micropteri*. At present, the former genus is monotypic; however, we have specimens representing species that differ morphologically and molecularly from *Leuceruthrus micropteri* (Fig 12; unpublished data MRW). *Bivesicula unexpectata* apparently comprise a good outgroup for polarizing azygiids. If *Transversotrema borboleta* Hunter and Cribb, 2012 (GenBank: JF412524) is used as an outgroup, nodal support decreases for all ingroup taxa, *Azygia longa* is sister to *Leuceruthrus micropteri*, and *Proterometra* is paraphyletic (unpublished results).

Regarding phylogenetic inferences concerning co-diversification with their snail and fish hosts as well as their niche in the fish host, we note several aspects worthy of

future investigation. Monophyly of *Leuceruthrus micropteri* and *Proterometra* spp. and their relationship as sister taxa (Fig 12) is interesting because the former infects the alimentary canal (stomach) posterior to the esophageal sphincter; whereas, the latter comprise de facto ectoparasites that are seemingly restricted to the alimentary canal (buccal cavity) anterior to the esophageal sphincter. Both infect black basses (*Micropterus* spp.), among other centrarchid hosts [1,4], and concurrent infections of species assigned to these genera occur (personal observations MRW and SAB). Perhaps indicative of niche partitioning that has led to some level of morphological and molecular differences between these lineages. Further, species of *Proterometra* and *L. micropteri* infect first intermediate hosts of Pleuroceridae (i.e., *Elimia, Pleurocera, Lithasia, Leptoxis* [1,21,29]) and primary division freshwater fishes of North America only. The sister relationship of *A. longa* to the *Proterometra+Leuceruthrus* clade was not unexpected. Species of *Azygia* undergo asexual development in snails of 3 genera assigned to 3 families (Viviparidae: *Campeloma*; Amnicolidae: *Amnicola*; Lymnaeidae: *Lymnaea*) [30-32] and mature in a diverse assemblage of freshwater fishes in North America and Europe. The life cycle of some species of *Azygia* and *Leuceruthrus micropteri* involves a “paratenic” host. The planarian *Dugesia tigrinum* has been documented as a host for pre-vitellogenic adults of at least one species of *Azygia* [30,31,33], and catfishes (Siluriformes) and sunfishes can host pre-vitellogenic adults of *Azygia* spp. and *L. micropteri* (personal observations MRW, SAB; personal communication Stephen S. Curran). In contrast, the life cycles for 9 of 11 species of *Proterometra* have been elucidated by either experimental studies or molecular data, and, with exception to *P. dickermani*, which has not yet been reported from a

vertebrate host collected in the wild [34,35], all involve an intermediate snail host and a definitive fish host only.

ACKNOWLEDGEMENTS

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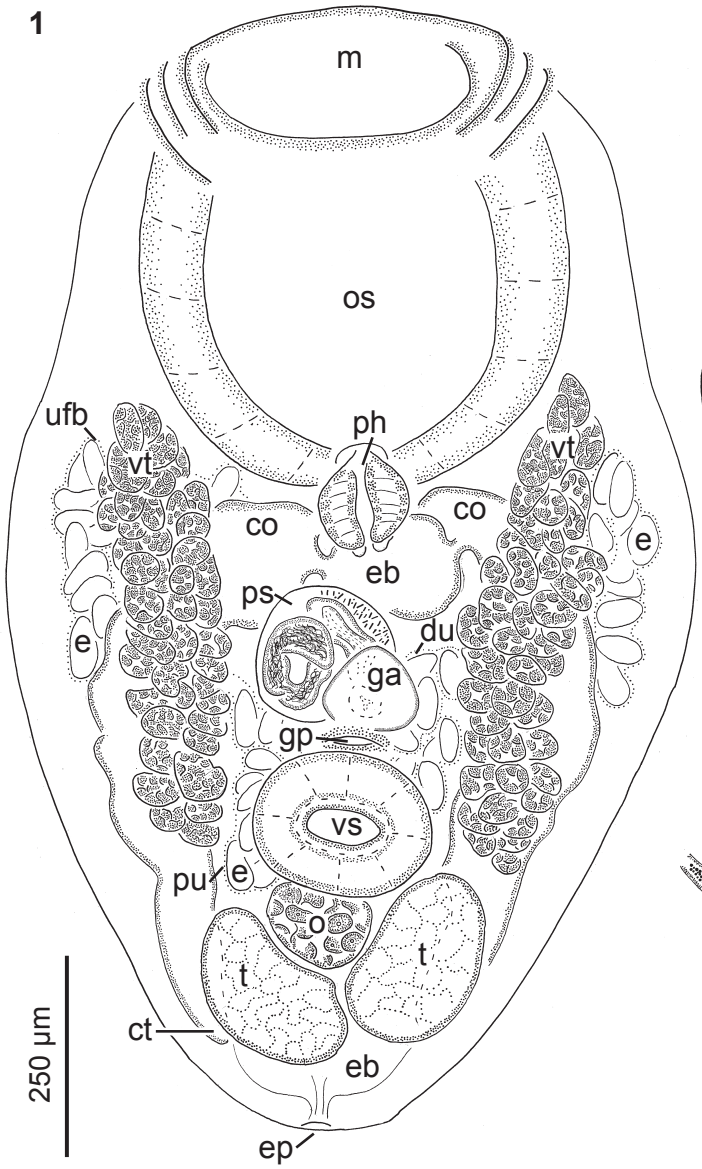
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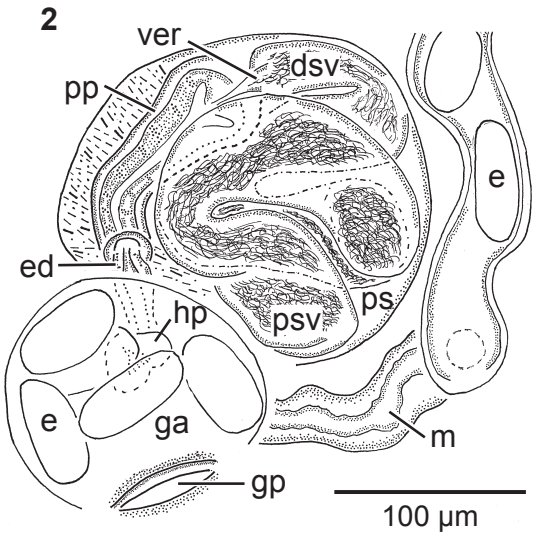
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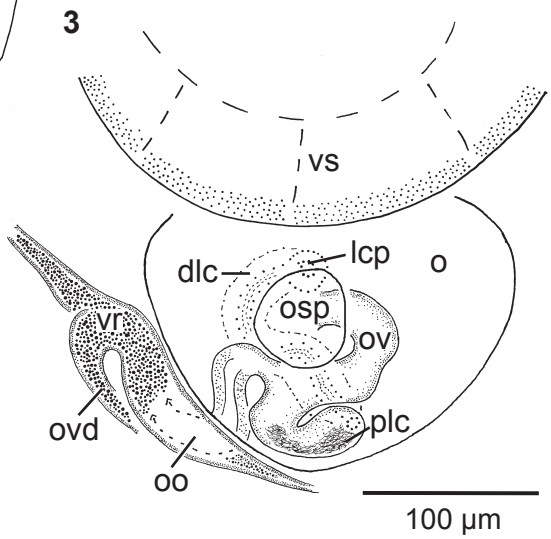
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Plate 3-1; Figures 1–3 Adults of *Proterometra catenaria* Smith, 1934 (Digenea: Azygiidae) from redspotted sunfish, *Lepomis miniatus* (Jordan, 1877) (Perciformes: Centrarchidae) from Holmes Creek, Florida. **(1)** Adult (USNM coll. no. XXXXXX) showing mouth (m), oral sucker (os), pharynx (ph), esophagus bifurcation (eb), ceca (co) near origin, vitellarium (vt), prostatic sac (ps), genital atrium (ga), genital pore (gp), ventral sucker (vs), proximal end of uterus (pu) emanating near ventral sucker and ovary (o) extending to forebody (ufb) and terminating distally (du) at synthesis with metraterm, eggs (e**)**, testes (t), ceca termination (ct), excretory bladder (eb) and excretory pore (ep). Ventral view. **(2)** Terminal male genitalia (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figure 1 plus proximal region of seminal vesicle (psv), distal region of seminal vesicle (dsv), verschlussapparat (ver), pars prostatica (pp), ejaculatory duct (ed), metraterm (m), and hermaphroditic pore (hp). Ventral view. **(3)** Female genitalia showing ventral sucker (vs), and outline of ovary (o), which should be interpreted as being ventral to, oviductal sphincter (osp), oviduct (ov), proximal portion of Laurer's canal (plc), distal end of Laurer's canal (dlc), Laurer's canal pore (lcp), and ootype (oo), also showing vitelline reservoir (vr), and ovovitelline duct (ovd). Ventral view.

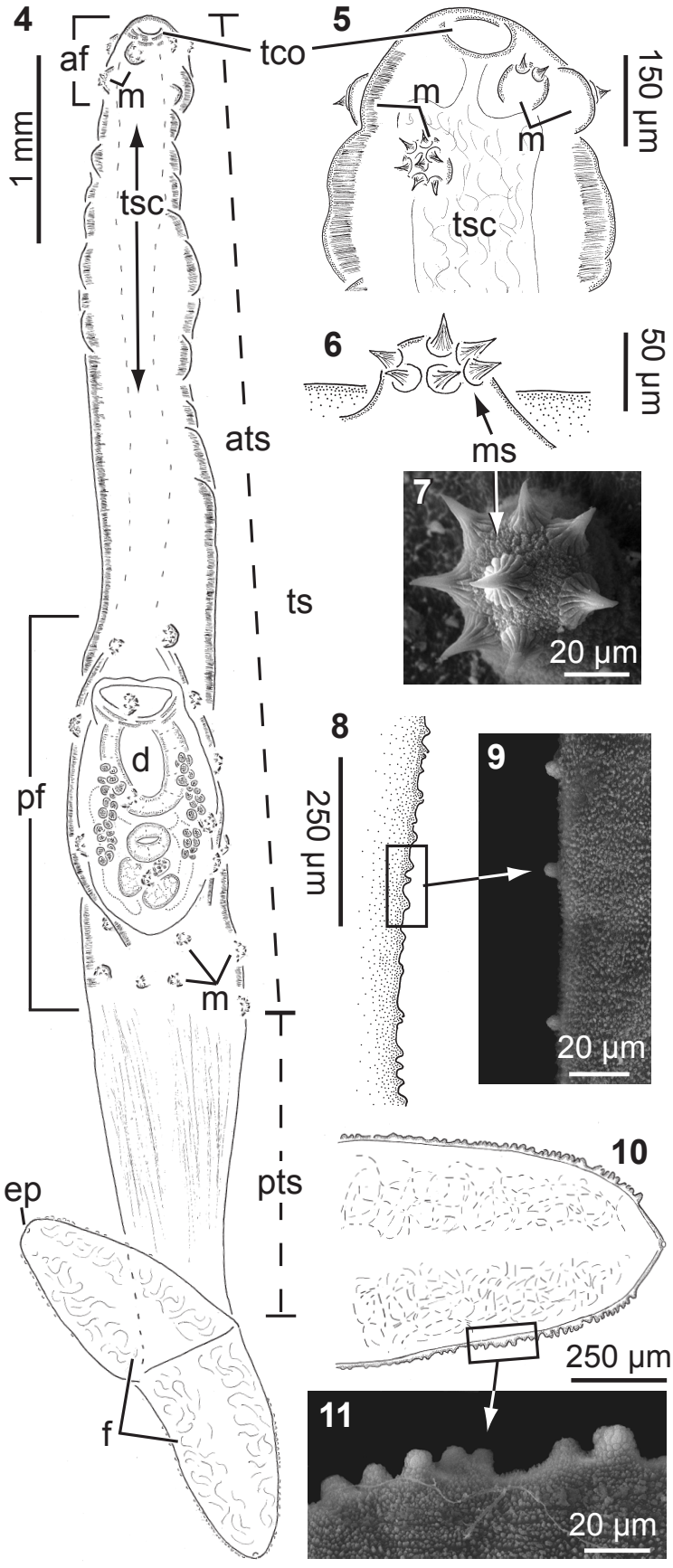
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Plate 3-2; Figures 4–11. Naturally shed cercaria of *Proterometra catenaria* from rasp elimia, *Elimia floridensis* from Holmes Creek, Florida **(4)** Cercaria (USNM coll. no. XXXXXX) showing tail cavity opening (tco), position of the anterior mamillae field (af), and associated mamillae (m), entire tail stem (ts), plus anterior (ats) and posterior tail stem (pts) regions, position of posterior mamillae field (pf), and associated mamillae (m), which are mirroring the position of the distome (d), that is within the tail stem cavity (tsc), and paired furcae (f) plus position of excretory pores (ep). Ventral view. **(5)** Anterior tail crown showing tail cavity opening (tco), tail stem cavity (tsc) and single row of mamillae (m) with spines. Ventral view. **(6)** Posterior mamilla with spines (ms). Lateral view. **(7)** Posterior mamilla with spines (ms). SEM. **(8)** Posterior tail stem with tegumental projections. Ventral view. **(9)** High magnification view of tegumental projections of posterior tail stem. SEM. **(10)** Anterior and medial region of furca showing serrate margin. **(11)** High magnification view of serrate furca margin showing minute protuberances. SEM.

12

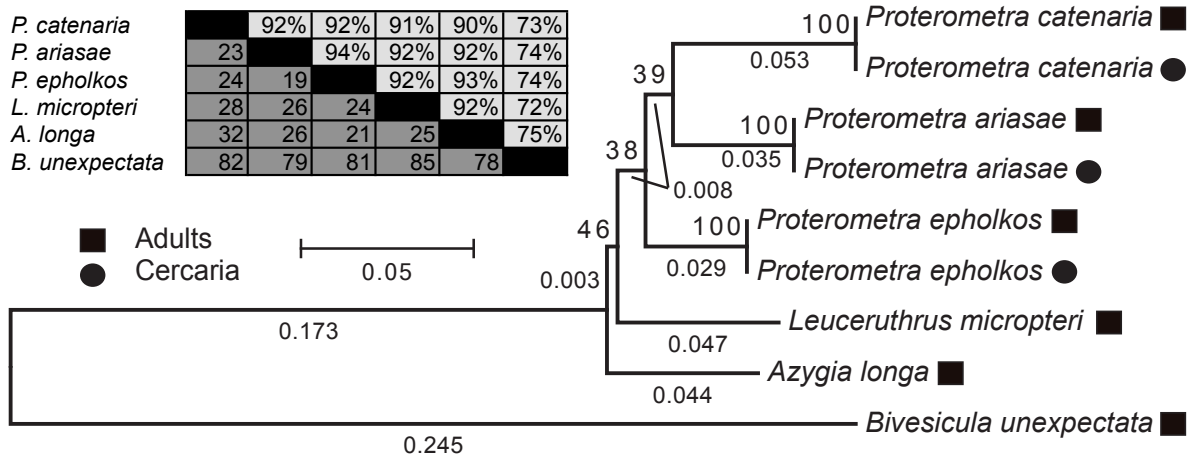

Figure 12: Maximum likelihood phylogenetic tree based on analyses of sequence data from the ribosomal internal transcribed spacer 2 using MEGA V.6.06. Tree includes sequences from 3 species of *Proterometra* plus *Leuceruthrus micropteri* and *Azygia longa. Bivesicula unexpectata* was used as the outgroup. Bootstrap support values (1,000-replicates) are reported aside each node, and branch lengths are below each branch. Adult sequences = squares, cercarial sequences = circles. In the shaded table sequence similarity is presented as a percentage and placed above the diagonal with base pair polymorphisms placed below the diagonal.

CHAPTER 4: REVISION OF *LEUCERUTHRUS* MARSHALL AND GILBERT, 1905 (DIGENEA: AZYGIIDAE), PARASITES OF NORTH AMERICAN FRESHWATER ENDEMIC FISHES (CENTRARCHIDAE) AND SNAILS (PLEUROCERIDAE)

***Prepared for publication in Parasitology International**

Authors: Matthew R. Womble, and Stephen A. Bullard

ABSTRACT

Herein, we (i) emend the diagnosis of and revise the taxonomy of formerly monotypic *Leuceruthrus* Marshall and Gilbert, 1905 (Azygiidae), (ii) provide the first molecular phylogenetic analysis of species within the genus based on novel ITS2 sequence data, (iii) redescribe *Leuceruthrus micropteri* Marshall and Gilbert, 1905 (type species) based on type materials and newly collected adult specimens infecting stomach of largemouth bass (*Micropterus salmoides*) captured in Wheeler Reservoir, Alabama (Tennessee River), (iv) provide the first description of adults of *Leuceruthrus ocalana* (Smith, 1935) n. comb. based on specimens infecting stomach of largemouth bass from Holmes Creek, Florida (Choctawhatchee River), and (vii) describe cercariae of *Leuceruthrus stephanocauda* (Faust, 1921) n. comb., *L. ocalana*, and *Leuceruthrus* sp. from pleurocerid snails. The freshwater azygiids (*Leuceruthrus, Azygia,* and *Proterometra*) differ from the only marine/estuarine azygiid genus (*Otodistomum*) by having (i) a bilateral vitellarium, (ii) a genital pore at level of or posterior to the prostatic sac, and (iii) a short ejaculatory duct. *Leuceruthrus* is unique by the combination of having (i) oblique, pre-ovarian testes, (ii) paired vitelline fields not extending into the forebody, and (iii) a uterus filling the intercecal space between the ovary and ventral sucker. Phylogenetic analysis including ITS2 sequences from *Azygia longa*, *Proterometra ariasae*,

Proterometra epholkos, the aforementioned *Leuceruthrus* spp. plus the outgroup *Bivesicula unexpecta* (Bivesiculidae) did not reject monophyly of *Leuceruthrus* and recovered *Proterometra* and *Leuceruthrus* as sister taxa (i.e., azygiids infecting North American pleurocerid snails were monophyletic) with *L. ocalana*+*Leuceruthrus* sp. and *L. micropteri*+*L. stephanocauda* each sharing a recent common ancestor. The identities of cercaria of *L. micropteri* and adults of *L. stephanocauda* and *Leuceruthrus* sp. remain indeterminate and require additional sampling and taxonomic study. The present study comprises the first detailed morphological treatment of *Leuceruthrus* in over a century as well as including the largest number of azygiid taxa in a molecular phylogenetic analysis to date.

1. INTRODUCTION

Collectively, Azygiidae (Platyhelminthes: Digenea) is an ecologically diverse group of digeneans that undergo asexual reproduction in snails (Pleuroceridae, Viviparidae, Amnicolidae, Lymnaeidae, Planorbidae), produce furcocystocercous cercariae that can be “progenetic” (egg-bearing upon emergence of cercariae from the snail host; e.g., Womble et al., [1]), are ectoparasites as well as endoparasites, and infect a phylogenetic spectrum of definitive hosts from the most basal extant gnathostomes (Chondrichthyes) to the most highly derived extant aquatic vertebrates (Euteleostei). Azygiidae includes 25 accepted species assigned to 4 genera: *Azygia* Looss 1899, *Otodistomum* Stafford 1904, *Leuceruthrus* Marshall and Gilbert 1905 (see below), and *Proterometra* Horsfall 1933.

Morphological taxonomy based on adult azygiids can be challenging because, unlike those of many other digenean groups, cercariae show remarkable interspecific variation but adults can be exceedingly difficult to distinguish [1–5]. This seems to be especially true among progenetic species (i.e., *Proterometra* spp.) and those with a 2-host life cycle (i.e., species of *Azygia*, *Leuceruthrus*, and *Proterometra*), perhaps because among these lineages natural selection exerts the most effect on the transmission bottleneck (i.e., cercarial behavior and functional morphology) rather than on the adult fluke-definitive host relationship; such that the most obvious morphological differences are manifested in the cercaria not the adult. However, typically, digenean cercariae lack genitalia and gonads that comprise key systems with which to diagnose genera and species [6–7]. Hence, digenean and platyhelminth taxonomy has historically and continues to rely predominantly on adult fluke morphology and their associated molecular sequences. Perhaps this is also because adult flukes are larger and relatively easier to work with than cercariae for many flukes, are less delicate regarding preparation techniques for light and scanning electron microscopy, and typically infect vertebrates that are more widely sampled and more easily obtained/captured than are mollusks. Moreover snails can be exceedingly difficult to identify (for lack of useful dichotomous keys) and collect (many are highly endemic and exhibit high specificity to a particular aquatic niche). Regarding azygiid taxonomy, excluding *Proterometra*, few snails have been examined specifically for infections, despite the remarkable variation among cercariae, thus the diversity of cercarial forms and behaviors apparently is underexplored. These gaps in knowledge comprise a significant barrier to azygiid species discovery, especially within *Leuceruthrus*.

Within Azygiidae, all but *Leuceruthrus* have been the focus of detailed taxonomic works. For *Azygia* (type genus), Manter [2] reviewed and re-described all “forms” of *Azygia* from North America, of which he recognized three species (*Azygia acuminata* Goldberger, 1911; *Azygia angusticauda* (Stafford, 1904) Manter, 1926; *Azygia longa* (Leidy, 1851) Manter, 1926), and summarized the available information regarding the identities of several European species. He considered all of them likely conspecific with *Azygia lucii* (Mueller, 1776) Lühe, 1909. Stunkard [3] provided a chronological synopsis for *Azygia*, accepted *Azygia sebago* Ward, 1910, demonstrated its life cycle, and concluded that *A. lucii*, long considered endemic to Europe, likely ranged in North America. Wootton [8] reviewed all available information regarding cercariae of *Azygia* spp. and circumscribed the life cycle of *A. acuminata*. Manter [2] provided a detailed study of *Otodistomum cestoides* (van Beneden, 1871) Odhner, 1911 from the Atlantic Ocean. Gibson and Bray [4] reviewed *Otodistomum*, redescribed *O. cestoides* and *Otodistomum veliporum* (Creplin, 1837) Stafford, 1904, detailed *Otodistomum plunketi* Fyfe, 1953, described metacercariae of these species from a variety of hosts, and summarized the available information and previous reports regarding metacercariae of *Otodistomum* spp. For *Proterometra*, Horsfall [9] and Dickerman [10] described adults and cercariae of the type species *Proterometra macrostoma* (Faust, 1918) Horsfall, 1933. Womble et al. [1,5] characterized the life history stages of 4 species of *Proterometra* and provided the first molecular sequence data for members of the genus, updated lists of hosts and geographic locality records for all nominal species, and synopses and assessments of morphological features used to differentiate species.

Relative to the number of nominal species, published works, cercarial and adult descriptions, available molecular sequence data, geographic distributions studied, and life cycles elucidated, monotypic *Leuceruthrus* (type species, *Leuceruthrus micropteri* Marshall and Gilbert 1905) is certainly the least explored. This is surprising considering that adult flukes are large (macroscopic) and that their fish hosts are among the most commonly captured North American inland fishes, i.e., sunfishes and black basses (Perciformes: Centrarchidae). In fact, adult specimens of *L. micropteri* (type and only accepted species) were last described 104 years ago (1911) and only once before that, in 1905.

Marshall and Gilbert [11] described *L. micropteri* based on adult specimens that infected the stomach of largemouth bass, *Micropterus salmoides* (Lacepède 1802) (considered herein the type host because it was the first host listed by Marshall and Gilbert [11]) and smallmouth bass, *Micropterus dolomieu* Lacepède 1802 from the Great Lakes Basin (Mendota, Monona, and Wingra lakes near Madison, Wisconsin, U.S.A.). Goldberger [12] diagnosed *Leuceruthrus* and supplemented the original description of *L. micropteri* from observations of the original type materials as well as new specimens collected from bowfin, *Amia calva* Linnaeus 1766 (Amiiformes: Amiidae), *M. salmoides*, and *M. dolomieu* captured in Lake Maxinkuckee, Indiana, U.S.A, plus one specimen from *Micropterus* sp. from an unspecified locality. Since these initial reports, *L. micropteri* (*sensu lato*) has been reported in the literature primarily from the stomach of black basses (i.e., *Micropterus* spp.), throughout much of eastern North America (Table 1). Similar to that described for species of *Proterometra* (see Womble et al. [1]), *L. micropteri* (*sensu lato*) reportedly undergoes asexual reproduction in freshwater

prosobranch snails of Pleuroceridae (i.e., *Elimia, Pleurocera*, *Lithasia*) (Table 2) [9,18,26,27], after which a macroscopic (=2-4 mm) furcocystocercous cercariae is shed from the snail and actively swims in a flamboyant, and perhaps luring, manner before being swallowed by the fish host [18,27]. However, unlike that of species of *Proterometra*, no cercaria are reportedly progenetic (Womble et al. [1]).

The taxonomic history of *Leuceruthrus* is confusing (e.g., [9,18,26,27]) because the identity of cercarial specimens is indeterminate and uncoupled from its adult form, type materials seldom informed published records, voucher materials were infrequently deposited for comparative purposes, and all records for the various cercarial forms applied the collective genus group name "*Cercaria*" and/or applied sub-specific names [9,18,26,27]. Additionally, and critical to the understanding of how *Leuceruthrus* biodiversity has been likely underestimated, morphological studies and experimental infections [9,18,27] demonstrated that morphologically distinctive cercariae developed pre-ovarian testes; which is characteristic of *L. micropteri*. The consensus of these earlier reports was that the relative position of the testes and ovary indicated *L. micropteri* (if pre-ovarian testes were present) or a species of *Proterometra* or *Azygia* (if pre-testicular ovary was present); however, gonad position is defensible as a generic feature (present study), perhaps less so as a species level characteristic, among azygiids (present study; [1,5]). As indicated previously, congeneric azygiids indeed are subtly morphologically distinct [2,4] and cercarial features have been widely used to differentiate species of *Proterometra* [1]. Moreover, prior to 2015 (GenBank no. KT808320, internal transcribed spacer 2 [ITS2] for *L. micropteri*; [5]), molecular sequence data for *Leuceruthrus* was not publically available, and, obviously therefore,

analysis of molecular sequence data has yet to be used in taxonomy of the group. As such, the literature indicates that unnamed species of *Leuceruthrus* exist and could be diagnosed by circumscribing their distinctive cercariae, close examination of adult specimens, and combining morphology with molecular sequence data to match larvae and adults infecting snails and fish from various rivers and lakes in North America.

To address these knowledge gaps, we herein circumscribe the identities and detail the morphological features of cercarial and adult specimens of *Leuceruthrus* based on type materials and new specimens that we collected from freshwater environments in Alabama and adjacent waters. This is the first detailed morphological treatment of any species of the genus in over 100 years.

2. MATERIALS AND METHODS

Prosobranch snails were collected by hand from Big Canoe Creek (33°48’21”N; 86°28’57”W; St. Clair County, Alabama, U.S.A.) on 23 May 2013, Holmes Creek (30°36’25”N; 85°44’49”W; Jackson County, Florida, U.S.A.) on 29 June 2014, and Simmons Creek (31°20’59”N; 86°44’10”W; Conecuh County, Florida, U.S.A. on 23 May 2015. Snails were subsequently transported to the laboratory in 20-L plastic buckets filled with ambient stream water and aerated using battery powered aerators and airstones. In the laboratory, snails from Big Canoe Creek, AL, were maintained in 40-L aquaria filled with aerated, filtered, and de-chlorinated tap water. Free swimming cercariae were observed alive in aquaria before being transferred to a glass jar wherein their behavior could be observed without aeration current before being preserved for

morphology and molecular biology. Two snail species were present in the aquaria in which the free-swimming cercariae were collected; they were identified as *Elimia* cf. *modesta* as per the features given by Thompson [28] and Womble et al. [1] and *Elimia* cf. *carinifera* as per Burch [29] and Thompson [28]. Methods for isolation, and collection of cercariae from Holmes Creek follow those provided above and in Womble et al. [5]. Snails from Holmes Creek, FL were identified as *Elimia floridiensis* as per Womble and Bullard ([30, *in review*]; also see Clench and Turner [31]; Thompson [32]; Chambers [33]). Intramolluscan cercariae collected from Simmons Creek, AL were separated from soft tissues of infected snails (identified as *Elimia* sp. as per Burch [34]) following transverse fracturing of snail shells using a vice. Liberated cercariae were subsequently maintained in a petri dish with stream water where their behavior was observed prior to being preserved for morphology and molecular biology. Shell vouchers of all snail hosts preserved in 70% EtOH were deposited in the Auburn University Museum of Natural History (AUMNH).

Fish were collected using a boat electrofisher in Wheeler Reservoir, Alabama (34°37’17.8” N; 86°49’51.47” W; Limestone County, Alabama, USA) on 13 August 2013 and 24 September 2013, transported to the laboratory, and identified as *Micropterus salmoides* as per the key provided in Boschung and Mayden [35]. Fish from Holmes Creek, FL, were caught by hook and line on 25 March 2015 maintained alive in ambient river water, transported to the laboratory, killed by spinal severance, and identified as *Micropterus salmoides* [35]. Bone cutting shears were used to hemisect the jaw and the buccal cavity to reveal epithelial surfaces, and stomachs were removed and immersed

in saline before inspection with the aid of a stereoscope capable of 50x objective magnification.

Flukes for morphology were isolated and heat killed within a dish flooded with freshwater heated to 60°C. Killed specimens were then transferred to a vial of 10% neutral buffered formalin (nbf). Specimens for light microscopy were left in 10% nbf for at least 48 h, rinsed overnight in distilled water, stained overnight in Van Cleave's hematoxylin with several additional drops of Ehrlich's hematoxylin, dehydrated in a graded series of ethanols, briefly immersed in xylene, further cleared in clove oil, and permanently mounted on glass slides using Canada balsam. Measurements, photographs, and illustrations of stained, whole-mounted specimens were made with aid of a Leica DM-2500 equipped with differential interference contrast (DIC) optical components and a drawing tube. Measurements are herein reported in micrometers (μm), unless otherwise noted, followed by the mean and number of specimens measured for that feature in parentheses. Specimens for scanning electron microscopy (SEM) were fixed in nbf as above, washed in de-ionized water, dehydrated through a graded ethanol series, critical point dried in liquid CO_2, mounted on standard aluminum SEM pin stubs with double-sided carbon tape, sputter coated with gold palladium (19.32g/cm^3; 25 mA), and viewed with a Zeiss EVO 50VP scanning electron microscope.

To identify the material we collected herein, and also to gain insights on potential species-level differences between putative species of *Leuceruthrus*, we made comparisons with and studied specimens of *L. micropteri* sourced from the United States National Museum, Smithsonian Institution, accessioned under the following

numbers: USNM 10678 (ventrally sectioned specimen), USNM 51685 (whole mounted specimen). All of the specimens were labeled and or listed as a “cotype” (see Recommendation 73E, ICZN; or pg. 64 of Womble et al. [1] for information regarding specimens listed as a “cotype”). The sectioned specimen under the number 10678 were deposited by Goldberger [12] and loaned to him by Professor Marshall. The specimen accessioned under the number 51685 has a slide label from M.C. Marshall and also Henry B. Ward and is labeled as a “cotype from author.” Presumably this specimen was sent to Ward by Marshall where it remained in the collection of Ward, prior to being deposited in the U.S. national collection.

Specimens for molecular biology comprised 2 adults from Wheeler Reservoir, AL, 1 cercaria from Big Canoe Creek, AL, 2 adults and 1 cercaria from Holmes Creek, FL, and 2 cercaria from Simmons Creek, AL. All specimens were individually preserved in separate vials of 95% EtOH and stored at -20°C. Methods for total genomic DNA extractions, polymerase chain reaction (PCR) amplifications, and sequencing were performed as per Womble et al. [5]. Sequence assembling and analysis of chromatograms was conducted using BioNumerics version 7.0 (Applied Maths, Sint-Martens-Latem, Belgium. Where applicable IUPAC ambiguity codes were used for coding polymorphic sites, briefly these sites were identified as per Womble et al. [1] and excluded from all subsequent phylogenetic analyses. The Internal Transcribed Spacer 2 Ribosomal Database [36] was used to determine the borders of the 5.8s, ITS2, and 28s gene regions. Representative sequences have been deposited in GenBank (XXXXXXXX).

Additional ITS2 sequences for *Proterometra epholkos* (GenBank: KM503118), *Proterometra ariasae* (KT808317), *Azygia longa* (KT808319), and *Bivesicula unexpectata* (outgroup) (KR092222) were obtained from GenBank. All sequences were aligned using MEGAv.6.06 [37] with default ClustalW parameters. Alignment curation (i.e., removal of ambiguous regions [= those containing gaps, and/or poorly aligned]) was performed using Gblocks (v0.91b) [38,39]. Absolute site differences and sequence similarity percentages were calculated as specified in Womble et al. [5]. Sequences were analyzed using the Maximum likelihood algorithm in MEGAv.6.06 according to the best-fit DNA model analysis estimated with MEGAv.6.06 as Kimura's 2 parameter model with equal distributed rate variation among sites (K2) [40]. A bootstrap analysis based off 1,000 replicates was used to establish nodal support values. Branch support was considered significant when bootstrap values were > 70.

Common names, scientific names, taxonomic authorities and dates, and higher-level gastropod classification follow Johnson et al. [41]. Higher-level fish classification and nomenclature follows Nelson [42] and fish common names follow Boschung and Mayden [34]. Nomenclature for Azygiidae follows Gibson and Bray [43] and morphological terminology follows Womble et al. [1] and references therein.

3. RESULTS

3.1. Leuceruthrus *Marshall and Gilbert, 1905, emended (Figs 1-18)*

3.1.1. Diagnosis

Body of adult large, lingulate, forebody shorter than hindbody. Excretory system Y shaped, with 2 branches joining in hindbody. Oral sucker subterminal. Ventral sucker smaller than oral sucker. Mouth anteroventral, subterminal. Pharynx near posterior margin of oral sucker; oesophagus bifurcating soon after pharynx; oesophageal branches joining with intestinal caecae; intestinal caecae U-shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caecae, extending along lateral margins of body. Testes 2 in number, inter-caecal, pre-ovarian, near posterior musculature of ventral sucker. Prostatic sac medial, anterior to ventral sucker. Seminal vesicle convoluted, entirely within prostatic sac. Pars prostatica present. Ejaculatory duct straight. Terminal genitalia joining within sinus organ; hermaphroditic pore opening into genital atrium; genital atrium communicating with hermaphroditic pore and genital pore; genital pore medial, immediately anterior to ventral sucker, posterior to anterior margin of prostatic sac. Ovary medial, inter-ceacal, post-testicular. Laurer's canal present. Uterus distributing primarily within hindbody, occupying nearly all inter-caecal space between ovary and ventral sucker, originating posterior to testes, extending between testes distally. Metraterm present. Vitellarium follicular, ventral to caeca, distributing in 2 bilaterally symmetrical fields, extending along lateral margins of body ventral to caecae. Uterine eggs densely distributed throughout uterus. Parasitic in stomach of freshwater fishes.

Cercaria furcocystocercous, comprising distome ('cercarial body,' the individual that develops into the adult) and tail (tail stem and paired furcae). Distome oblong. Oral sucker subterminal. Ventral sucker smaller than oral sucker. Alimentary system (mouth, pharynx, oesophagus, caecae) as in adult. Position of testes and prostatic sac as in

adult. Female genitalia present as anlagen only. Ovary post-testicular. Uterus originating posterior to testes, passing between testes distally. Vitellarium absent. Uterine eggs absent. Parasitic in freshwater snails of Pleuroceridae.

3.1.2. Differential diagnosis

Body lingulate. Testes pre-ovarian, immediately posterior to ventral sucker. Ejaculatory duct straight. Ovary post-testicular. Uterus originating post-testicular, extending between testes distally. Vitellarium bilateral, remaining in two distinct lateral fields. Genital pore immediately anterior of ventral sucker, posterior to anterior margin of prostatic sac.

3.1.3. Taxonomic Summary

Type and only nominal species: Leuceruthrus micropteri Marshall and Gilbert, 1905.

3.1.4. Remarks

Leuceruthrus, *Azygia,* and *Proterometra* comprise azygiids that infect freshwater fishes and that have (1) a bilateral vitellarium that remains in two distinct fields, (2) a genital pore immediately anterior to the ventral sucker, and (3) a straight ejaculatory duct [1–3,11,44]. *Otodistomum*, the remaining azygiid genus, comprises marine/estuarine species that infect sharks, rays, and chimaeras (Chondrichthyes) [4]. It differs from the freshwater azygiid genera by having (1) a vitellarium that is confluent posteriorly, (2) a genital pore near the pharynx and anterior to the prostatic sac, and (3) a coiled ejaculatory duct [1–4,8,11,12,45,46,47]. *Leuceruthrus* and *Azygia* reportedly are not progenetic and mature in the stomach and intestine of fishes [3,8, present study]. Morphologically, they are similar in having (1) distinctive, paired vitelline fields not extending into the forebody and (2) a uterus filling the intercecal space between the

ovary and ventral sucker. *Leuceruthrus* has oblique and pre-ovarian testes; whereas, *Azygia* has tandem and post-ovarian testes [1– 3,11,12,44,47]. *Proterometra* includes progenetic cercariae, comprises de facto ectoparasites of the buccal cavity [1], and differs from *Azygia* and *Leuceruthrus* by having (1) a sub-oval body with broadly rounded ends, (2) testes abreast in the posterior body extremity, and (3) a uterine field and vitellarium extending well within the forebody [1,44].

Marshall and Gilbert [11] made *Leuceruthrus* available to accommodate *L. micropteri* but did not diagnose the genus nor provide insights on generic vs. specific features of the type species or compare it with other azygiids. Goldberger [12] supplemented the description of *L. micropteri* and diagnosed *Leuceruthrus*; however, neither Marshall and Gilbert [11] nor Goldberger [12] assigned the genus to a family. Goldberger [12] speculated that, "*Leuceruthrus* will be found to represent the type of at least a new subfamily... and probably of a new family." Odhner [48] assigned *Leuceruthrus* to Azygiidae, based on similarities of the male genitalia between *Azygia* spp. and *L. micropteri* (see [2]). Yamaguti [49] provided the only diagnosis of the genus that considered features in other azygiid genera. Yamaguti [50], Gibson and Bray [43], and Gibson [44] provided diagnoses for the subfamily Leuceruthrinae Goldberger, 1911. Herein the diagnosis of *Leuceruthrus* is emended to include features of the body, alimentary system, genitalia, and cercaria.

3.1.5. Molecular Results

The biodiversity of *Leuceruthrus* has been underestimated and accommodates at least 3 nominal species (Fig 19); *L. micropteri*, *Leuceruthrus stephanocauda* (Faust, 1918) Womble and Bullard n. comb. (sec. 3.3), and *Leuceruthrus ocalana* (Smith, 1935)

Womble and Bullard n. comb. (Sec 3.4), and points to the existence of a 4th species (i.e., *Leuceruthrus* sp. [Sec. 3.5]). Specifically, comparison between sequences of *L. micropteri* and *L. stephanocauda* revealed a total of 5 polymorphisms (1.4% sequence divergence), comparison between sequences of *L. micropteri* and *L. ocalana* revealed a total of 8 polymorphisms (2.3% sequence divergence), and comparison between sequences of *L. stephanocauda* and *L. ocalana* revealed a total of 7 polymorphisms (2% sequence divergence) (Fig 19). Additionally, comparison between sequences of *Leuceruthrus* sp. and *L. ocalana* resulted in a total of 2 polymorphisms (0.6% sequence divergence), and indicated that these taxa share a recent common ancestor and form a clade sister to *L. micropteri* + *L. stephanocauda* (Fig. 19). Morphological diagnoses are provided for each species in the subsequent sections.

3.2. Leuceruthrus micropteri *Marshall and Gilbert, 1905 (Figs 1, 2)*

3.2.1. Morphological diagnosis of adult based on light microscopy of 14 stained whole mounted specimens. For comparative purposes, and because it was an outlier, values for the largest collected specimen are reported in brackets.

Body 3,480–5,440 (4,448, 13) [8,180] long or 2.3–3.4 (1.7, 9) [3.8] × longer than wide, width at level of oral sucker 1,280–2,060 (1,508, 12) [2,180], width at level of ventral sucker 1,240–2,060 (1,485, 12) [2,200]; forebody (= distance from anterior end of body to the anterior most margin of ventral sucker musculature) 1,380–1,960 (1,643, 13) [2,780] long or 35–41% (37%, 13) [34%] of overall body length; hindbody (= region of body posterior to the ventral sucker) 1,620–2,970 (2,272, 13) [4,520] long or 47–55% (51%, 13) [55%] of overall body length, 1.2–1.6 (1.4%, 13) [1.6] × greater than forebody

length; tegument unarmed, smooth, approximately 20–50 (33, 13) [60] thick. Excretory system difficult to trace anteriorly, appearing Y shaped in hindbody, branches joining at 82–91% (88%, 9) [91%] of body length from anterior body end forming excretory bladder; excretory bladder 185–465 (318, 9) [690] long, 30–85 (61, 9) [230] wide, becoming confluent with diminutive excretory duct; excretory duct extending 110–195 (154, 9) [110], communicating excretory bladder and terminal excretory pore. Nervous system not evident. Oral sucker 690–910 (801, 13) [1,260] long or 17–21% (18%, 13) [15%] of body length or 43–54% (49%, 13) [45%] of forebody length, 685–920 (814, 13) [1,280] wide or 44–60% (54%, 13) [59%] of body width, anterior margin 2–5% (3%, 13) [3%] of body length from anterior body end, posterior margin 520–850 (690, 13) [1,280] from anterior margin of ventral sucker (Fig 1). Ventral sucker in anterior half of body, 480–600 (533, 13) [880] long or 10–14% (12%, 13) [11%] of body length, 470–670 (573, 13) [820] wide or 33–45% (38%, 13) [38%] of body width, 62–75% (67%, 13) [69%] of oral sucker length 65–74% (70%, 13) [64%] of oral sucker width (Fig 1). Mouth opening ventrally. Pharynx ovoid, 185–280 (232, 13) [370] long or 4–6% (5%, 13) [4%] of body length 215–285 (251, 13) [400] wide (Fig 1). Oesophagus extending posteriad from mouth approximately 175–435 (341, 13) [310] before bifurcating 25–70 (40, 13) [40] posterior to pharynx; dextral oesophageal branch 130–255 (172, 13) [230] long, sinistral oesophageal branch 110–215 (164, 13) [210] long (Fig 1); dextral caecum 2,555–4,090 (3,555, 13) [7,020] long or 69– 93% (80%, 13) [86%] of body length, pre-caecal space, 820-1,140 (949, 13) [1,500] or 19–24% (21%, 13) [18%] of body length from anterior end of body, post-caecal space,100–360 (239, 13) [240] or 2–7% (5%, 13) [3%] of body

length from posterior end of body; sinistral caecum 2,525–4,035 (3,535, 13) [7,100] long or 70–93% (80%, 13) [87%] of body length, pre-caecal space 820-1,140 (954, 13) [1,500] or 19–24% (22%, 13) [18%] of body length from anterior end of body, post-caecal space 100–420 (264, 13) [400] or 2–8% (6%, 13) [5%] of body length from posterior end of body.

Testes oblique to askew, sub-oval, typically asymmetrical (Fig 1); dextral testis 220–425 (333, 13) [550] long or 5–10% (8%, 13) [7%] of body length, 215–425 (282, 13) [600] wide or 14–22% (19%, 13) [28%] of body width, pre testis space 1,740–2,820 (2,194, 13) [4,220] from anterior end of body or 42–59% (50%, 13) [51%] of total body length, post testis space 1,395–2,780 (1,927, 13) [3,410] from posterior end of body or 33–51% (43%, 13) [42%] of total body length; sinistral testis 250–405 (334, 13) [500] long or 6–10% (8%, 13) [6%] of body length, 220–415 (288, 13) [470] wide or 14–26% (19%, 13) [22%] of body width, pre testis space 1,840–2,680 (2,186, 13) [3,520] from anterior end of body or 43–56% (49%, 13) [51%] of total body length, post testis space 1,260–2,520 (1,925, 13) [4,160] from posterior end of body or 36–50% (43%, 13) [43%] of total body length. Vasa efferentia indistinct in whole mounted specimens. Prostatic sac anterior margin 295–590 (448, 13) [890] from posterior margin of oral sucker, 220–370 (275, 13) [450] long, 200–295 (245, 13) [300] wide (Fig 1). Seminal vesicle thin-walled, highly convoluted, filled with sperm, swollen for entire length, occupying most of prostatic sac, 395–940 (695, 13) [1,065] long (Fig 2), distal end connected to pars prostatica via verschlussapparat (see Womble et al. [1]). Pars prostatica diminutive, spherical, 125–185 (163, 10) [215] long, 40–65 (51, 9) [85] wide proximally,

20–40 (27, 9) [40] wide distally, exiting prostatic sac ventrally (Fig 2). Ejaculatory duct (= continuation of pars prostatica outside of prostatic sac) straight, extending ventrally from prostatic sac becoming confluent with hermaphroditic duct, 40–105 (63, 6) long or 23–60% (39%, 6) of pars prostatica length. Sinus organ difficult to trace in ventral orientation, directed ventrally. Hermaphroditic pore level with midline of prostatic sac, anterior of ventral sucker, at 32–37% (34%, 11) [31%] of body length from anterior end of body (Fig 2). Genital atrium circular in outline, 130–260 (186, 12) [415] in diameter, 4 of 13 (33%) specimens containing 4–5 (4.3, 4) uterine eggs (Fig 2). Genital Pore opening ventrally at 32–41% (35%, 13) [32%] of total body length from anterior end of body (Fig 1, 2).

Ovary 105–315 (200, 13) [325] long or 2–7% (5%, 13) [4%] of body length, 180–375 (275, 13) [420] wide or 13–23% (18%, 13) [19%] of body width, post-ovary space 600–1,070 (764, 13) [1,240] or 13–21% (17%, 9) [15%] of body length (Fig 1). Fine details of the female genitalia (i.e., oviduct, Laurer's canal, ovovitelline duct, and ootype) not evident, putative highly glandular mehlis complex present immediately anterior to ovary. Uterus comprising a field 1,040–2,000 (1,718, 12) [4,060] long or 30–45% (39%, 12) [50%] of body length, proximal end briefly extending anteriad from ovary and mehlis complex, looping laterally between caecae posterior to testes, becoming narrow and extending between testes, passing dorsal to musculature of ventral sucker, synthesis with metraterm distally near prostatic sac,
typically with hundreds of eggs (Fig 1); uterine seminal receptacle present, detected in observations of live specimens; metraterm thick walled, 200–495 (331, 10) [340] or 4–12% (8%, 9) [4%] of body length,

35–60 (51, 9) [55] wide, extending anteriad from commissure with uterus, becoming confluent with distal portion of ejaculatory duct, forming a short common duct (= herein a ‘hermaphroditic duct’) within sinus organ (Fig 2). Vitellarium extending from near posterior margin of ventral sucker to near posterior end of body (Fig 1), maximum distance between fields 720–1,280 (889,13) [1,360] or 54–66% (59%, 13) [62%] of body width; dextral vitelline field 1,160–2,240 (1,788, 13) [3,440] long or 33–50% (40%, 13) [42%] of body length, terminating anteriorly at 46–57% (50%, 13) [48%] of body length, terminating posteriorly at 87–97% (91%, 13) [90%] of body length, 43–62% (50%, 13) [49%] of dextral caecum length; sinistral vitelline field 1,180–2,380 (1,756, 13) [3,480] long or 34–48% (39%, 13) [43%] of body length, terminating anteriorly at 46–56% (51%, 13) [53%] of body length, terminating posteriorly at 89–95% (91%, 9) [94%] of body length, 40–62% (50%, 13) [49%] of sinistral caecum length; primary vitelline collecting ducts nearly symmetrical, extending posteromediad from respective vitelline field before becoming confluent and forming vitelline reservoir; dextral vitelline collecting duct 325–605 (470, 8) [840] long, 15–60 (30, 8) [30] wide near yolk reservoir, proximal end branches from vitellarium at 43–64% (53%, 8) [50%] of dextral vitelline field length; sinistral vitelline collecting duct 275–550 (439, 8) [1,100] long, 20–35 (27, 8) [50] wide near yolk reservoir, proximal end branches from vitellarium at 44–59% (53%, 8) [66%] of sinistral vitelline field length. Vitelline reservoir near posterior margin of ovary (Fig 1). Uterine eggs pyriform, varying in size from approximately 50–65 (59, 8) [50] × 25–35 (29, 8) [25] to approximately 70–95 (79, 8) [95] × 35–45 (39, 8) [40] (Fig 1).

3.2.2. Taxonomic summary

Type Host: Adults infecting largemouth bass, *Micropterus salmoides* (Lacepède, 1802)

Type Locality: Lake Mendota, Lake Monona and Lake Wingra Wisconsin (Dane County, Wisconsin, USA) (43°06'17"N; 89°25'09"W)

Site in fish host: Stomach

Other hosts and localities: See Tables 1, 2

Specimens examined: USNM Nos. 10678, 51685

Specimens Deposited: Vouchers USNM Nos. XXXXX

Prevalence: 82%; 14 of 17 fish infected. Data from specimens collected from largemouth bass, *Micropterus salmoides* in Wheeler Reservoir, Alabama (34°37'17. 8" N; 86°49'51. 47" W; Limestone County, Alabama, USA) (Impoundment of the Tennessee River System).

Mean Intensity: 3.4. Data from specimens collected from largemouth bass, *Micropterus salmoides* in Wheeler Reservoir, Alabama (34°37'17. 8" N; 86°49'51. 47" W; Limestone County, Alabama, USA) (Impoundment of the Tennessee River System).

Materials examined: 1 whole-mounted adult specimen comprising a cotype (USNM Coll. No. 51685) and a series of slides comprising sections of a single adult specimen (USNM Coll. No. 10678).

3.2.3. *Remarks*

Vouchers of *L. micropteri* (USNM 10678 & 51685) were 3,900–5,420 long × 1,100–1,800 long and had proportionally smaller caecae ($\bar{x}$ = 64% of body length) that extended farther anterior within the forebody (pre-caecal length = 680–820). In the

whole mounted voucher specimen USNM 51685 the uterus extended beyond the interior margins of the caecae, whereas in the specimens collected from Alabama (Fig 1) and the sectioned voucher specimen (i.e., USNM 10678) the uterus remained entirely intercaecal for its entire length. We considered this difference to be artifact likely as a result of excessive fixation pressure. Notably, although the majority of our specimens of *L. micropteri* were of a consistent length ($\bar{x}$ = 4.4 mm ± .5 mm), adults of *L. micropteri* are capable of growing to large lengths within the definitive host (i.e., up to 10mm). Measurements from one large specimen (8.2 mm long) provided herein were largest, although proportionally remained within the range of other specimens.

The morphological features of the cercaria of *L. micropteri* is uncertain. Horsfall [9], Smith [27], and Patton [18] identified cercariae as *L. micropteri* (as *Cercaria stephanocauda* Faust, 1921) but did not provide sufficient morphological details that justified these identifications nor that collectively support the notion that all of these accounts represent conspecific specimens. Further, unfortunately no voucher specimen from any of these studies is extant to our knowledge. Horsfall's [9] cercariae from *Pleurocera acuta* in the Oconomowoc River, Wisconsin, had a small body with cordate furcae that were approximately 1.5× longer than wide and that lacked tail stem spines but instead had "protrusible papillae" about the anterior tail stem; Plate 35, Fig 7 of Horsfall [9], which makes them distinct from those of *L. stephanocauda* (see below). We cannot know the identity of this cercaria but deduce that it is either a new species or *L. micropteri*. Smith's [27] cercariae may be conspecific with *L. stephanocauda*. These specimens infected *Elimia* spp. (as *Goniobasis* spp.) from north Alabama, as were the cercariae we described herein (see below) (Table 2). Smith's [27] photomicrograph of

the furcae and tail stem are identical to those of our specimens of *L. stephanocauda* (see below; Figs 5–10); however, she did not specify if spines or protuberances were present or absent on the tail stem (none are visible in her figure). Patton [18] took cercariae that infected *Elimia laqueata* (as *Goniobasis laqueata*) and *Pleurocera canaliculatum* from Elkhorn Creek, Kentucky, and fed them to fish (i.e., *Lepomis macrochirus*, *Micropterus punctulatus*, *M. salmoides*). She likened the cercariae to "*Cercaria stephanocauda*" sensu Faust [26] and Horsfall [9] based on morphometric similarities to "the type specimen" reported in Horsfall [9], i.e., presence of tail stem "annuli," and presence of a post-testicular ovary. Additionally, and although without providing a measurement, illustration, photomicrograph, or voucher specimen, Patton [18] reportedly could not distinguish adults obtained through feeding experiments from those of *L. micropteri*. Noteworthy also is that Patton [18] observed precocious development in the cercariae from Elkhorn Creek. If she was referring to the genitalia, this perhaps suggests that these cercariae were a progenetic species of *Proterometra* (see [1]). Faust [26] and Horsfall [9] did not observe precocious development in *Leuceruthrus*. Neither did we (present study).

3.3. Leuceruthrus stephanocauda *(Faust, 1921) Womble and Bullard n. comb. (Figs 5-10)*

Synonym: *Cercaria stephanocauda* Faust, 1921

3.3.1. Morphological diagnosis of cercaria based on light microscopy of 2 whole-mounted naturally shed cercariae with withdrawn distome.

Cercaria 3,320–3,580 (3,450, 2) long (Fig 5). Tail stem 2,600–2,900 (2,750, 2) long or 78–81% (80%, 2) of cercariae length, 510–660 (585, 2) wide or 4.4–5.1 (4.7, 2) × longer than wide, comprised of an anterior and posterior region (Fig 5); anterior tail stem region (ATS) oblong,1,320–1,460 (1,390, 2) long or 40–41% (40%, 2) of cercariae length, maximum width same as reported for tail stem, containing distome, tapering anteriorly, devoid of mamillae, armed with two rows of tail stem spines (Fig 5-9); posterior tail stem region (PTS) dorsoventrally compressed, 1,280–1,440 (1,360, 2) long or 39–40% (39%, 2) of cercariae length, 360–340 (350, 2) wide, nearly uniform in width anteriorly to posteriorly, devoid of mamillae, lateral margins bearing many minute protuberances (Figs 5, 10); PTS protuberances, minute, lacking pores, marginal (Fig 10). Furcae lanceolate, dorsoventrally compressed, margin bearing many protuberances, serrate (Fig 5); furcal protuberances minute, pored, marginal; dorsal furca 660–700 (680, 2) long or 18–21% (19%, 2) of cercariae length, 320–380 (350, 2) wide or 1.7–2.2 × (2, 2) longer than wide; ventral furca 620–720 (670, 2) long or 17–22% (20%, 2) of cercariae length, 320–340 (330, 2) wide or 1.8–2.3 × (2, 2) longer than wide (Fig 5). Tail cavity opening at anteriomedial end of cercaria, directing anteriad (Fig 5); tail stem cavity not evident. Mamillae none evident. Tail stem spines maximum length 40, maximum width 35 (Figs 7, 8), restricted to the anterior tail stem region, distributed in two concentric rows, anterior row encircling area near tail cavity opening (Figs 5, 6), posterior row encircling area near synthesis of posterior tail stem (Figs 5, 9). Excretory system with 1 primary excretory canals, extending posteriad along the medial axis of the posterior tail stem, bifurcating at the synthesis of the furcae, extending

independently through each furca, opening via excretory pore at the distal end of each furcae (Fig 5).

Body of distome (= cercarial body) (Fig 5) 1,220–1,780 (1,500, 2) long or 37–50% (43%, 2) of cercaria length, 470–580 (525, 2) wide or 2.6–3 (2.8, 2) × longer than wider, anterior margin 30–40 (35, 2) from tail cavity opening; forebody 630–900 (765, 2) long or 51–52% (51, 2) of overall body length; hindbody 350–600 (475, 2) long or 29–34% (31%, 2) of overall body length, 56–67% (61%, 2) of forebody length; tegument unarmed. Excretory system not evident. Nervous system not evident. Oral sucker 360–370 (365, 2) long or 21–30% (25%, 2) of body length, 340–370 (355, 2) wide or 64–72% (68%, 2) of body width, 60–30 (45, 2) or 2–3% (3%, 2) of body length from anterior body end, 850–1,360 (1,105, 2) or 70–76% (73%, 2) of body length from posterior body end (Fig 5). Ventral sucker in posterior half of body, with anterior margin 630–900 (765, 2) or 51–52% (51%, 2) of body length from anterior body end (Fig 5), 290–300 (295, 2) long or 24–25% (24%, 2) of body length, 290–300 (295, 2) wide or 50–64% (57%, 2) of body width, 78–83% (81%, 2) of oral sucker length, 78–88% (83%, 2) of oral sucker width (Fig 5). Pharynx ovoid, 100–140 (120, 2) long or 6–11 % (9%, 2) of body length, 110–135 (123, 2) wide (Fig 5). Oesophagus extending posteriad from mouth before bifurcating posterior to pharynx, oesophageal branches arching posterolaterad before joining with intestinal caecae (Fig 5); dextral cecum 1,050–1,518 (1,284, 2) or 82– 85% (84%, 2) of body length, laterad caecum length 150–163 (157, 2), descending caecum length 900–1,355 (1,128, 2), pre-caecal space 375–550 (463, 2) or 29–31% (30%, 2) of body length from anterior end of body, post-caecal space 20–50

(35, 2) or 2% (2%, 2) of body length from posterior end of body; sinistral caecum 1,000–1,620 (1,310, 2) or 78–91% (85%, 2) of body length, laterad caecum length 150 (150, 2), descending caecum length 850-1,470 (1,160, 2), pre-caecal space 375–510 (443, 2) or 29–30% (29%, 2) of body length from anterior end of body, post-caecal space 25–50 (38, 2) or 2–3% (2%, 2) of body length from posterior end of body. Testes abreast, round to oval (Fig 5); dextral testis 50 (50, 2) long or 2–4% (3%, 2) of body length, 65–90 (78, 2) wide or 1.3–1.8 (1.6, 2) × wider than long; sinistral testis 60 (60, 2) long or 3–5% (4%, 2) of body length, 60–75 (68, 2) wide or 1–1.3 (1.1, 2) × wider than long; pre testicular space 810–1,180 (995, 2) from anterior end of body or 63–66% (65%, 2) of total body length; post testicular space 300–540 (420, 2) from posterior end of body or 23–30% (27%. 2) of total body length. Vasa efferentia not evident. Prostatic sac 55–75 (65, 2) long, 90–110 (100, 2) wide or 1.5–1.6 (1.5, 2) × wider than long (Fig 5). Genital atrium circular in ventral view, 25–30 (28, 2) in diameter. Genital pore 650–840 (745, 2) of body length from anterior end of body or at 47–50% (49%, 2) of body length. Fine features of terminal male genitalia (i.e., seminal vesicle, pars prostatica, ejaculatory duct, sinus organ) not evident.

Ovary 140–210 (175, 2) of body length from posterior end of body, 65–70 (68, 2) long or 1.2–1.3 (1.2, 2) × longer than wide, 55 (55, 2) wide (Fig 5). Fine features of terminal female genitalia (i.e., oviduct, Laurer's canal, ovovitelline duct, ootype, and mehlis gland) not evident. Uterus 430–655 (543, 2) long or 34–37% (35%, 2) of body length, 13–20 (17, 2) wide. Metraterm not evident. Vitellarium not developed in distome (Fig 5).

3.3.2. Taxonomic Summary

Type Host: *Leptoxis carinata* (as *Anculosa carinata)* and/or *Elimia carinifera* (as *Goniobasis carinifera*); Faust, 1921 did not designate the type host but only examined cercariae from the two previously mentioned snails.

Type Locality: Rome, Georgia

Site in host: Undetermined

Other hosts and localities: see Table 2

Specimens Deposited: Vouchers USNM Nos. XXXXX-XXX (cercariae)

3.3.3. Remarks

Adults of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard n. comb. have not been characterized in the literature and no adult specimen has been deposited in a curated museum. However, ITS2 sequences differentiate cercariae of *L. stephanocauda* from adult *L. micropteri* (Fig. 19). All earlier workers [9,18,27,49–51] considered "*Cercariae stephanocauda*" a junior subjective synonym of *L. micropteri*. Our cercarial specimens of *L. stephanocauda* differed from the published descriptions of cercariae assigned to *Leuceruthrus* of Horsfall [9] and Patton [18] by having (1) lanceolate furcae rather than cordate furcae (as reported by Horsfall [9]), (2) two concentric rows of spines that encircle the anterior tail stem anteriorly and posteriorly, and by having (3) a pre-vitellogenic distome rather than a progenetic distome (as reported by Patton [18]). As previously mentioned, the cercariae that Smith [27] identified as *C. stephanocauda* may be conspecific with the cercariae we identify as *L. stephanocauda* but additional information on the presence/absence and distribution, if present, of spines and papillae on the tail stem are required.

Some features, both novel and previously reported, for the cercaria of *L. stephanocauda* require clarification. Our observations of cercariae of *L. stephanocauda* differ from those of Faust [26] and Horsfall [9]. These differences are likely attributable to intraspecific variation, perhaps from studying live vs. preserved and immature vs. mature cercariae. Faust [26] measured live specimens only; whereas, Horsfall [9] apparently studied whole-mounts only. Morphometric differences attributable to specimen disposition are demonstrated by studies of azygiid cercariae, e.g., *Proterometra catenaria* Smith, 1934 (see Womble and Bullard [*in review*]). Also important, and as evidenced by the extruded distome (see Plate 3, Fig 6 of Faust [26]; Plate 35, Fig 9 of Horsfall [9]), Faust [26] and Horsfall [9] evidently studied cercariae excised from crushed snails rather than free-swimming, naturally-shed cercariae. Consequently, the cercariae comprising the cotype series were not fully-developed and may have been preserved under extreme coverslip pressure (forcibly everting the distome from the tailstem) or damaged. Herein, we studied only naturally-shed cercariae, all of which had a withdrawn distome (Fig 5). Distome position within the tail stem depends on age of the cercaria [1]: immature specimens have an extruded distome; whereas, mature specimens have a withdrawn distome. Hence, the appearance of the anterior tail stem differs between those specimens described by Faust [26] and Horsfall [9] and those described herein.

Tail stem spines: The cercaria of *L. stephanocauda* has two concentric rows of spines that encircle the anterior tail stem anteriorly and posteriorly (Figs 5–9). Faust [26] called these “tubercules” and stated that they only occurred at the posterior end of the anterior tail stem. Horsfall [9] confirmed tail stem spines (“pointed cuticular

protuberances") in Faust's specimens and added that the spines were in a single row near the anterior portion of the anterior tail stem also. She noted that these spines were absent in some of Faust's specimens and speculated that they were a variable feature, only present in some specimens. Our specimens had tail stem spines. In one specimen (USNM XXXXX) the spines were few and nearly indistinct but the tegumental bases into which the spines insert were clearly visible. We suspect that some spines become dislodged during specimen preparation.

Posterior tail stem protuberances: The posterior tail stem has numerous minute protuberances that are restricted to the lateral margins and with light microscopy appear to lack pores (Fig 10).

Furcae: The furcae of *L. stephanocauda* are lanceolate and marginally studded with minute protuberances making them appear serrate (Fig 5). We described similar features in cercariae of several species of *Proterometra* (see [1,5]). Further study inclusive of SEM is needed to assess homology of this feature across azygiid genera.

Distome: The distome of *L. stephanocauda* resembles that of the adult: pre-ovarian testes near the posterior margin of the ventral sucker and inverse U shaped intestine (Fig 5). Noteworthy is that the distome of *L. stephanocauda* lacks a vitellarium and the genitalia are developing.

3.4. Leuceruthrus ocalana *(Smith, 1935) Womble and Bullard n. comb. (Figs 3, 4, 11-15)*

Synonym: *Cercaria stephanocauda ocalana* Smith, 1935

3.4.1. Diagnosis of adults based on light microscopy of 5 stained whole mounted specimens (Fig 3).

Body of adult, 3,840–6,260 (4,904, 5) long or 1.7–3.0 (2.4, 5) × longer than wide, width at level of oral sucker 1,800–2,060 (1,928, 5) width at level of ventral sucker 1,800–2,500 (2,064, 5) (Fig 3); forebody 1,400–2,460 (1,888, 5) long or 33–42% (38%, 5) of overall body length; hindbody 1,550–3,004 (2,381, 5) long or 38–58% (48%, 5) of overall body length, .90–1.6 (1.3, 5) × greater than forebody length; tegument unarmed, smooth, approximately 20–40 (28, 5) thick. Excretory system difficult to trace anteriorly branches joining at 81–91% (87%, 5) of body length from anterior body margin, forming excretory bladder (Fig 3); excretory bladder 280–360 (340, 5) long, 70–160 (98, 5) wide, becoming confluent with diminutive excretory duct posteriorly; excretory duct 110–280 (184, 5) long, communicating excretory bladder and terminal excretory pore (Fig 3). Nervous system not evident. Oral sucker 1,030–1,340 (1,150, 5) long or 21–27% (24%, 5) of body length or 54–74% (62%, 5) of forebody length (Fig 3), 1,110–1,340 (1,222, 5) wide or 55–67% (62%, 5) of body width, anterior margin 4–8% (5%, 5) of body length from anterior body end, posterior margin 160–920 (494, 5) from anterior margin of ventral sucker (Fig 3). Ventral sucker
in anterior half of body, 690–860 (772, 5) long or 14–20% (16%, 5) of body length, 740–880 (797, 5) wide or 36–43% (40%, 5) of body width at level of ventral sucker, 64–74% (67%, 5) of oral sucker length, 60–69% (65%, 5) of oral sucker width (Fig 3). Mouth opening ventrally (Fig 3). Pharynx ovoid, 300–370 (334, 5) long or 5–9% (7%, 5) of body length, 230–350 (316, 5) wide (Fig 3). Oesophagus extending posteriad from

mouth approximately 175–435 (341, 13), oesophagus bifurcation and oesophageal branches difficult to discern from surrounding tissue (stylized in illustration [Fig 3]); dextral caecum 3,760–4,160 (3,950, 3) or 78– 91% (84%, 3) of body length, pre-caecal space, 860-1,260 (1,138, 4) or 22–31% (25%, 4) [18%] of body length from anterior end of body, post-caecal space, 270–500 (384, 5) or 5–12% (8%, 5) of body length from posterior end of body; sinistral caecum 3,900–4,050 (3,975, 3) or 77–96% (84%, 3) of body length, pre-caecal space 1,000-1,300 (1,163, 4) or 23–31% (26%, 4) of body length from anterior end of body, post-caecal space 300–480 (374, 5) or 5–10% 8%, 5) of body length from posterior end of body.

Testes oblique to askew, always posterior of horizontal midline of ventral sucker, oval to sub-oval (Fig 3); dextral testis 350–400 (375, 2) long or 8% (2) of body length, 370–380 (375, 2) wide or 18% (2) of body width, pre testis space, 2,040-2,200 (2,120, 2) from anterior end of body or 40–53% (47%, 2) of total body length, post testis space, 2,560–2,640 (2,600, 2) from posterior end of body or 52–62% (57%, 2) of total body length; sinistral testis 350–470 (393, 3) long or 7–9% (8%, 3) of body length, 320–410 (353, 3) wide or 15–20% (18%, 3) of body width, pre testis space 2,060–2,340 (2,163, 3) from anterior end of body or 41–50% (45%, 3) of total body length, post testis space 2,520–2,760 (2,603, 3) from posterior end of body or 48–67% (55%, 3) of total body length. Vasa efferentia not evident. Prostatic sac oval appearing densely filled with glandular cells, anterior margin 0–550 (232, 5) from posterior margin of oral sucker, 250–320 (286, 5) long, 290–330 (264, 5) wide (Fig 3). Fine features of terminal male genitalia (i.e., seminal vesicle, pars prostatica, ejaculatory duct, sinus organ) difficult to

delineate within prostatic sac; seminal vesicle present. Hermaphroditic pore directed ventrally, at 33–39% (36%, 3) of body length from anterior end of body. Genital Atrium circular in outline, with seemingly strongly muscularized rim, 90–280 (186, 4) in diameter, 1 of 5 (20%) specimens containing ~35 uterine eggs (Fig 14). Genital pore near level with, or slightly posterior of midline of prostatic sac, opening ventrally at 34–41% (37%, 4) of total body length from anterior end of body (Fig 3).

Ovary 200–360 (298, 5) long or 5–9% (6%, 5) of body length, 200–390 (313, 4) wide or 11–19% (16%, 4) of body width, post-ovary space 880–1,100 (1,028, 5) or 17–27% (17%, 9) [22%] of body length (Fig 3). Fine features of the female genitalia (i.e., oviduct, Laurer's canal, ovovitelline duct, and ootype) difficult to discern, putative highly glandular mehlis complex present anterior to ovary (Fig 3). Uterus comprising a field 1,840–1,940 (1,890, 2) long or 36–47% (42%, 2) of body length, proximal end extending anteriad from ovary, looping laterally between caecae posterior to testes, extending through space between testes (Fig 3), distal portion of uterus difficult to delineate from surrounding tissue, synthesis with metraterm not evident; uterine seminal receptacle not evident; metraterm not evident. Vitellarium extending from near level of, or slightly posterior of, horizontal midline of ventral sucker, to near posterior end of body (Fig 3), maximum distance between fields 1,000–1,440 (1,214, 5) or 53–70% (62%, 5) of body width; dextral vitelline field 1,910–2,920 (2,378, 5) long or 44–57% (49%, 5) of body length, terminating anteriorly at 41–54% (49%, 4) of body length, terminating posteriorly at 87–95% (90%, 4) of body length, 58–62% (60%, 3) of dextral caecum length; sinistral vitelline field 1,730–3,200 (2,492, 5) long or 45–66% (51%, 5) of body length, terminating anteriorly at 42–54% (47%, 5) of body length, terminating posteriorly at

88–96% (92%, 5) of body length, 58–68% (63%, 5) of sinistral caecum length; primary vitelline collecting ducts nearly symmetrical, extending posteromediad from respective vitelline field before becoming confluent and forming vitelline reservoir; dextral vitelline collecting duct 350–720 (535, 2) long, proximal end branches from vitellarium at 38–57% (47%, 2) of dextral vitelline field length; sinistral vitelline collecting duct 450–550 (500, 2) long, proximal end branches from vitellarium at 50–54% (52%, 2) of sinistral vitelline field length; vitelline reservoir dorsal to ovary (Fig 3). Uterine eggs present in specimens greater than 4 (mm) in total body length, pyriform, varying in size from approximately 50–60 (55, 5) × 30–40 (37, 5) to approximately 70 (5) × 30–40 (37, 5) (Fig X).

3.4.2. Diagnosis of post-cercarial juveniles based on light microscopy of 6 stained whole mounted specimens (Fig 4).

Body 2,260–3,340 (2,960, 6) long or 2.2–3.2 (2.9, 6) × longer than wide, width at level of oral sucker 1,040–1,300 (1,156, 6) wide, width at level of ventral sucker 820–1,180 (1,040, 6) (Fig 4); forebody 1,060–1,560 (1,322, 6) long or 39–50% (45%, 6) of overall body length; hindbody 770–1,455 (1,223, 6) long or 34–45% (40%, 6) of overall body length, typically less than forebody length, .69–1.2% (.89%, 6) of forebody length; tegument unarmed, smooth, approximately 20–60 (34, 6) thick, 2 of 6 specimens enveloped with a thicker, yet distinct layer of tegument (= possibly tail stem cavity of cercaria). Excretory system not evident anteriorly, branches joining at 87–93% (90%, 6) of body length from anterior body end, forming excretory bladder (Fig 4); excretory bladder 155–250 (185, 5) long, 30–50 (40, 4) wide, becoming confluent with diminutive excretory duct posteriorly; excretory duct extending 105–140 (122, 4) from posterior

margin of excretory bladder to terminal excretory pore (Fig 4). Nervous system not evident. Oral sucker 560–790 (712, 6) long or 22–29% (24%, 6) of body length, 540–820 (735, 6) wide or 66–76% (71%, 6) of body width, anterior margin 2–3% (2%, 6) of body length from anterior body end, posterior margin 260–665 (476, 6) from anterior margin of ventral sucker (Fig 4). Ventral sucker near horizontal midline of body, 370–535 (474, 6) or 14–20% (16%, 6) of body length, 400–530 (498, 6) or 45–50% (48%, 6) of body width, 62–68% (66%, 6) of oral sucker length 65–74% (68%, 6) of oral sucker width (Fig 4). Mouth opening ventrally. Pharynx ovoid, 150–230 (195, 6) long or 6–9% (7%, 6) of body length 170–230 (212, 6) wide (Fig 4). Oesophagus extending posteriad from mouth approximately 295–450 (388, 6), before bifurcating 20–30 (25, 6) posterior to pharynx; dextral oesophageal branch 100–230 (154, 6) long, sinistral oesophageal branch 90–200 (144, 6) long (Fig 4); dextral caecum 1,640–2,800 (2,368, 6) or 72– 84% (77%, 6) of body length, pre-caecal space 660–890 (762, 6) or 22–29% (25%, 6) of body length from anterior end of body, post-caecal space 100–300 (200, 6) or 4–9% (6%, 6) of body length from posterior end of body; sinistral caecum 1,710–2,750 (2,354, 6) or 72–82% (76%, 6) of body length, pre-caecal space 650–860 (757, 6) or 22–29% (26%, 6) of body length from anterior end of body, post-caecal space 75–330 (194, 6) or 3–10% (6%, 6) of body length from posterior end of body (Fig 4).

Testes abreast, always posterior of horizontal midline of ventral sucker, oval (Fig 4); dextral testis 150–270 (223, 6) long or 6–10% (8%, 6) of body length, 100–190 (161, 6) wide or 12–18% (15%, 6) of body width, pre testis space 1,420–1,950 (1,734, 6) from

anterior end of body or 51–63% (57%, 6) of total body length, post testis space 660–1,380 (1085, 6) from posterior end of body or 29–43% (36%, 6) of total body length; sinistral testis 140–300 (222, 6) long or 5–9% (7%, 6) of body length, 95–190 (155, 6) wide or 12–18% (15%, 6) of body width, pre testis space 1,420–1,950 (1,762, 6) from anterior end of body or 51–63% (58%, 6) of total body length, post testis space 680–1,320 (1,081, 6) from posterior end of body or 30–41% (36%, 6) of total body length. Vasa efferentia indistinct in whole mounted specimens. Prostatic sac 95–210 (155, 6) long, 60–165 (141, 6) wide, anterior margin 105–440 (319, 6) from posterior margin of oral sucker. Features of terminal male genitalia (i.e., seminal vesicle, pars prostatica, ejaculatory duct, sinus organ) appearing as anlagen, difficult to discern. Genital Pore near level with, or slightly posterior of midline of prostatic sac, opening ventrally at 39–56% (46%, 6) of total body length from anterior end of body (Fig 4).

Ovary 95–180 (139, 6) long or 4–6% (5%, 6) of body length, 70–120 (100, 6) wide or 8–12% (10%, 6) of body width, post-ovary space 250–850 (640, 6) or 11–26% (21%, 6) of body length (Fig 4). Fine details of the female genitalia (i.e., oviduct, Laurer's canal, ovovitelline duct, ootype, and mehlis gland) appearing as anlagen, difficult to discern. Uterus extending in straight line anteriorly, from near anterior margin of ovary (Fig 4), passing between testes, becoming inevident and difficult to discern distally; uterine seminal receptacle not evident; metraterm not evident. Vitellarium not developed. Uterine eggs none present.

3.4.3. Diagnosis of cercaria based on light microscopy of 5 whole-mounted naturally shed cercariae with withdrawn distome.

Cercaria 3,600–4,140 (3,944, 5) long. Tail stem 2,980–3,460 (3,228, 5) long or 77–84% (82%, 5) of cercariae length, maximum width 560–740 (652, 2) or 4.3–5.7 (6, 2) × longer than wide, comprised of an anterior and posterior region (Fig 11); anterior tail stem region (ATS) 1,480–1,800 (1,668, 5) long or 39–46% (42%, 5) of cercariae length, with anterior ridge, and posterior collar, maximum width same as reported for tail stem, primarily cylindrical, tapering anteriorly, containing distome; ATS ridge near anterior end of tail stem (Figs 11,12), preceding tail cavity opening, 320–490 (446, 5) wide, 120–220 (168, 5) of cercaria length from anterior end of cercaria; ATS collar 620–740 (675, 4) wide or 1.3–2.2 (1.6, 4) × wider than anterior tail stem ridge, appearing as a laterally expanded “flange like” tegumental ring, at confluence of anterior and posterior tail stems (Figs 11, 12); posterior tail stem region (PTS) dorsoventrally compressed,1,400–1,820 (1,560, 5) long or 35–44% (39%, 5) of cercariae length, anterior width 520–600 (548, 4), medial width 380–580 (473, 4) posterior width 320–540 (408), nearly uniform in width anteriorly to posteriorly (Fig 11), bearing many minute protuberances (Figs 11, 13, 14); PTS protuberances, minute, marginally distributed throughout entire length of PTS, also encircling anterior third of PTS (Figs 11, 13, 14). Furcae with conspicuous black markings along margins when alive, oblong, dorsoventrally compressed, bearing many minute pored protuberances; furcal protuberances, minute, distributed marginally, and occasionally sub-marginally (Fig 11); dorsal furca, 690–790 (724, 5) or 17–19% (18%, 5) of cercariae length, 600–760 (678, 5) or 85–104% (94%, 5) of furca length, ventral furca, 680–860 (752, 5) or 17–20% (19%, 5) of cercariae length, 600–790 (684, 5) or 80–97% (91%, 5) of furca length. Tail cavity opening at anteromedial end of cercaria, directing anteriad (Fig X). Tail stem

spines none evident. Excretory system with 1 primary excretory canal, extending posteriad along medial axis of the posterior tail stem, bifurcating at synthesis of furcae, extending independently through each furcae, opening via excretory pore at distal end of each furcae (Fig 11).

Body of distome (= cercarial body) (Fig 11) 1,540–1,630 (1,592, 5) long or 37–45% (41%, 5) of cercaria length, 490–550 (510, 5) wide or 2.8–3.3 (3.1, 5) × longer than wider, anterior margin 30–155 (88, 5) from tail cavity opening; forebody 880–980 (918, 5) long or 54–60% (57%, 5) of overall body length; hindbody 330–360 (350, 5) long or 21–22% (22%, 5) of overall body length, 37–40% (38%, 5) of forebody length, posterior end incurled; tegument unarmed. Excretory system not evident. Nervous system not evident. Oral sucker 420–500 (467, 5) long or 26–32% (29%, 5) of body length, 310–365 (341, 5) wide or 64–75% (67%, 5) of body width, 50–100 (75, 5) or 3–6% (4%, 5) of body length from anterior body end, 840–1,040 (976, 5) or 52–66% (61%, 5) of body length from posterior body end, posterior margin 325– 440 (373, 5) from anterior margin of ventral sucker (Fig 11). Ventral sucker in posterior half of body, 260–330 (296, 5) or 16–21% (18%, 5) of body length, 270–320 (294, 5) or 51–63% (58%, 5) of body width, 55–71% (64%, 5) of oral sucker length, 79–94% (86%, 5) of oral sucker width (Fig 11). Pharynx ovoid, 100–150 (125, 5) long or 6–9 % (8%, 5) of body length, 110–125 (112, 5) wide (Fig 11). Oesophagus extending posteriad from mouth before bifurcating posterior to pharynx, oesophageal branches arching posterolaterad before joining with intestinal caecae; dextral caecum 1,005–1,290 (1,173, 5) or 62– 84% (74%, 5) of body length, laterad caecum length 245–360 (288, 5), descending caecum length

740–960 (885, 5), pre-caecal space, 450–650 (526, 5) or 28–40% (33%, 5) of body length from anterior end of body, post-caecal space, 55–120 (74, 4) or 3–7% (5%, 4) of body length from posterior end of body; sinistral caecum 1,050–1,330 (1,190, 5) or 64–82% (75%, 5) of body length, laterad caecum length 230–340 (266, 5), descending caecum length 775-1,000 (924, 5), pre-caecal space 430–710 (532, 5) or 27–44% (33%, 5) of body length from anterior end of body, post-caecal space, 50–100 (74, 4) or 3–6% (5%, 4) of body length from posterior end of body (Fig 11).

Testes abreast, round to oval (Fig 11); dextral testis 55–65 (60, 4) or 3–4% (4%, 4) of body length, 50–70 (58, 4) or .85–1.2 (1, 4) × wider than long, sinistral testis 50–80 (61, 4) or 3–5% (4%, 4) of body length, 65–70 (66, 4) or .77–1.1 (.92, 4) × wider than long, pre testicular space 1,000–1,160 (1,075, 4) from anterior end of body or 61–75% (67%, 4) of total body length, post testicular space 300–420 (3489, 4) from posterior end of body or 19–26% (22%. 4) of total body length. Prostatic sac 65–75 (71, 5) long, 60–85 (100, 5) or .85–1.3 (1, 5) × wider than long (Fig 11). Genital atrium circular in ventral view, 35–45 (28, 2) in diameter. Genital pore 835–960 (877, 5) of body length from anterior end of body or at 52–59% (55%, 5) of body length. Features of terminal male genitalia (i.e., seminal vesicle, pars prostatica, ejaculatory duct, sinus organ) appearing as anlagen, difficult to discern.

Ovary near posterior end of body or 160–310 (210, 5) of body length from posterior end of body, 50–75 (59, 5) long or .8–1.5 (1, 5) × longer than wide, 50–60 (55, 5) wide (Fig 11). Fine details of the female genitalia (i.e., oviduct, Laurer’s canal, ovovitelline duct, ootype, and mehlis gland) difficult to discern. Uterus 430–655 (543, 2) long or

34–37% (35%, 2) of body length, 13–20 (17, 2) wide, extending in a straight line from anterior margin of ovary, passing between testes, difficult to discern distally; metraterm not evident. Vitellarium lacking in distome.

3.4.4. Taxonomic summary

Fish host: Largemouth bass, *Micropterus salmoides* (Lacepède 1802) (Perciformes: Centrarchidae)

Intermediate host: rasp elimia, *Elimia floridensis* (Reeve 1860) (Cerithioidea: Pleuroceridae) (type host).

Collection locality: Holmes Creek (30°36'25"N; 85°44'49"W) near Vernon, Florida

Site in fish host: Stomach

Site in intermediate host: Undetermined

Specimens Deposited: Syntypes USNM Nos. XXXXX (adults), Nos. XXXXX (cercariae); intermediate host vouchers USNM No. XXXXX.

Prevalence and Intensity in fish host = 100% (6/6); 2.8

3.4.5. Remarks

Adults of *L. ocalana* differ from those of *L. micropteri* by having (1) an oral sucker 24% of total body length and 62% of forebody length, and (2) a ventral sucker 16% of body length (Fig 3). Whereas, Adults of *L. micropteri* have (1) an oral sucker 18% of body length and 49% of forebody length, and (2) a ventral sucker 12% of body length (Fig 1).

Naturally-shed, fully-developed cercariae of *L. ocalana* differ from those of *L. stephanocauda* by having (1) a cylindrical anterior tail stem, (2) an anterior ridge posterior to the tail cavity opening (Figs 11, 12), (3) a flange-like collar separating the

anterior and posterior portions of the tail stem (Figs 11, 12), (4) posterior tail stem protuberances encircling the anterior third of the posterior tail stem and that extend along the lateral margins of the posterior tail stem (Figs 11, 13, 14), and (5) oblong, serrate furcae (Fig 11) that are nearly equal in length and width. Whereas, naturally-shed, fully-developed cercariae of *L. stephanocauda* (Fig 5) have (1) an ovoid anterior tail stem, (2) no anterior ridge posterior to the tail stem cavity opening, (3) no collar dividing the tail stem, (4) no medial tail stem protuberances, (5) two spine rows encircling both ends of the anterior tail stem, and (6) lanceolate serrate furcae that are twice as long as wide.

Because adults of *Leuceruthrus* spp. grow on the definitive host [12, present study] and because post-cercarial juveniles infect fish [27, present study], we compared values in 1 large adult of *L. micropteri* (8.2 mm long) and 6 post-cercarial juveniles (2.3–3.3 mm long) of *L. ocalana* (Fig 4). Proportional sucker lengths: body length were comparable to those for their respective congeners. However, oral sucker length: forebody length (54%) among juveniles of *L. ocalana* was less than that of the adult flukes (62%) but greater than that of adult *L. micropteri* (49%). Adults of *L. ocalana* had a greater mean width anteriorly than those of *L. micropteri* (Figs 1, 2). Morphologically, the ‘post-cercarial’ juveniles resembled adults except that they lacked a vitellarium and developed genitalia (Fig 4). Smith [27], based on feeding experiments, indicated that *L. ocalana* developed genital anlagen 38 days post-infection and ova 6 mos. post-infection. This suggests that the juvenile flukes we collected herein had colonized the host 40 days prior.

Smith [27] described the cercaria of *L. ocalana* (as *Cercaria stephanocauda ocalana*) based on specimens collected from *Elimia floridensis* (as *Goniobasis catenaria*; see [1]) from near Marianna, Florida. Smith's [27] choice to consider *L. ocalana* as a subspecies of *L. stephanocauda* appears to be nonsense because it was based on adults only and the two cercarial types (*C. stephanocauda* and C. *s. ocalana*) had "constant and characteristic differences." We concur that these cercariae are markedly morphologically distinct (see above). Smith [27] did not provide a measurement for an adult specimen nor did she deposit a voucher specimen.

ITS2 sequences from a cercaria and 2 adults of *L. ocalana* were 100% identical, aside from a single intra-individual single site polymorphism in position '304,' which presented overlapping double peaks of cytosine and thymine, that was present only in the cercarial specimen. In both adult specimens position '304' presented a single peak of thymine. As specified above this site was coded in the cercarial sequence using IUPAC ambiguity codes, i.e., Y should be read as the presence of C or T, rather than as an ambiguous reading between C or T. Given that this site was conserved among all other taxa used herein it was excluded from the analyses performed and further cercarial replicates are needed to inform the correct base for this position among cercaria of *L. ocalana*.

3.5. Leuceruthrus *sp.*

3.5.1. Diagnosis of cercaria based on light microscopy of 3 stained whole mounted specimens.

Cercaria 2,060–2,590 (2,337, 3) long, comprised of a tail stem and paired furcae (Fig 16). Tail stem 1,970–2,500 (2,237, 3) long or 95–97% (96%, 3) of cercariae length, maximum width 420–480 (447, 3) or 4.7–5.6 (5, 3) × longer than wide, comprised of an anterior and posterior region (Fig 16) bearing many unarmed papillae like tegumental projections (Figs 16, 17); anterior tail stem region (ATS) globular, tapering slightly anteriorly, widest medially, heavily tapering posteriorly, 990–1,320 (1,117, 3) long or 44–51% (48%, 3) of cercariae length, maximum width same as reported for tail stem, containing distome; posterior tail stem region (PTS) dorsoventrally compressed, 980–1200 (1120, 3) long or 46–51% (48%, 3) of cercariae length, widest medially, 250–360 (300, 3) wide, tapering anteriorly and posteriorly. Furcae paired, small, oblong, dorsoventrally compressed (Figs 16, 18); dorsal furca, 90–100 (93, 3) or 3–4% (4%, 3) of cercariae length, 70–140 (100, 3) wide; ventral furca 70–90 (80, 3) or 3–4% (3%, 3) of cercariae length, 70–130 (93, 3) wide. Tail cavity opening at anteriomedial end of cercaria, directing anteriad. Tail stem projections irregularly distributed throughout tail stem, densely distributed near tail cavity opening, and posterior end of posterior tail stem. Tail stem spines none evident. Excretory system with 1 primary excretory canal, extending posteriad along medial axis of posterior tail stem, bifurcating at synthesis of furcae, extending independently through each furcae, opening via excretory pore at the distal end of each furcae.

Body of distome (= cercarial body) (Fig 16) oblong, 790–990 (893, 3) long or 38% (3) of cercaria length 340–440 (380, 3) wide at level oral sucker or 2.3–2.5(2.4, 3) × longer than wider, contained within anterior tail stem, anterior margin 100–180 (150, 3) from tail cavity opening; forebody 370–470 (433, 3) long or 47–51% (48%, 3) of overall

distome length; hindbody 240–310 (273, 3) long or 30–31% (31%, 3) of overall distome length, 59–66% (63%, 3) of forebody length; tegument unarmed. Excretory system not evident. Nervous system not evident. Oral sucker subterminal, anterior end 40–50 (43, 3) from anterior end of distome, posterior end 510–605 (572, 3) from posterior end of distome, 230–285 (258, 3) long or 29% (3) of distome length, 260–310 (277, 3) wide or 70–76% (73%, 3) of distome width, posterior margin 90–175 (138, 3) from anterior margin of ventral sucker (Fig 16). Ventral sucker nearly entirely positioned in posterior half of distome, 180–215 (192, 3) or 20–23% (22%, 3) of distome length, 200–250 (222, 3) or 57–60% (58%, 3) of distome width, 69–78% (74%, 3) of oral sucker length 77–83% (80%, 3) of oral sucker width (Fig 16). Pharynx ovoid, dorsal to oral sucker, 85–100 (92, 3) long, 60–75 (68, 3) wide (Fig 16). Oesophagus extending posteriad from mouth before bifurcating posterior to pharynx, oesophageal branches arching posterolaterad before joining with intestinal caecae. Intestinal caecae confluent with oesophageal branches, appearing inverse U shaped inclusive of oesophageal branches, comprising paired dextral and sinistral caecae (Fig 16); dextral caecum 655–865 (768, 3) long or 83– 87% (85%, 3) of distome length, laterad/ascending caecum length 105–175 (132, 3), descending caecum length 550–690 (637, 3), pre-caecal space 250–330 (292, 3) or 32–33% (33%, 3) of body length from anterior end of distome, post-caecal space 50–55 (53, 3) from posterior end of distome; sinistral caecum 635–785 (720, 3) or 79–82% (81%, 3) of distome length, laterad/ascending caecum length 90–115 (102, 3), descending caecum length 545–670 (618, 3), pre-

caecal space, 235–295 (265, 3) or 29% (3) of distome length from anterior end of distome, post-caecal space 55–85 (68, 3) from posterior end of distome.

Testes 2 in number, abreast, round to oval (Fig 16), pre-ovarian, near posterior margin of ventral sucker; dextral testis 40–70 (53, 3) long, 30–50 (38, 3) wide; sinistral testis 45–75 (55, 3) long, 30–55 (40, 3) wide, pre testicular space, 490–610 (563, 3) from anterior end of distome or 60–68% (63%, 3) of total body length, post testicular space 240–270 (250, 3) from posterior end of distome or 27–30% (28%, 3) of total body length. Prostatic sac immediately anterior of ventral sucker. Genital atrium circular in ventral view, 35–45 (28, 2) in diameter. Genital pore anterior to ventral sucker, medial, 835–960 (877, 5) of body length from anterior end of body or at 52–59% (55%, 5) of body length. Features of terminal male genitalia (i.e., seminal vesicle, pars prostatica, ejaculatory duct, sinus organ) appearing as anlagen, difficult to discern.

Ovary medial, intercaecal, posterior to testes, near posterior end of body or 150–200 (173, 3) of body length from posterior end of distome, 55–65 (60, 3) long or 1.4–1.8 (1.6, 3) × longer than wide, 30–45 (38, 3) wide. Fine features of terminal female genitalia (i.e., oviduct, Laurer's canal, ovovitelline duct, ootype, and mehlis gland) difficult to discern. Uterus 155–250 (210, 3) long or 20–25% (23%, 2) of body length, narrow, sinuous, extending in a near straight line from ovary, passing between testes, difficult to discern distally. Metraterm not evident. Vitellarium not developed in distome.

3.5.2 Taxonomic Summary

Intermediate host: *Elimia* sp.

Collection locality: Simmons Creek (31°20'59"N; 86°44'10"W) near Paul, Alabama.

Site in intermediate host: Undetermined

Specimens Deposited: Vouchers USNM Nos. XXXXX (cercariae).

3.5.3 Remarks

Cercariae of *Leuceruthrus* sp. differ from those of its congeners by the combination of having (1) small (less than maximum tail stem width), stubby furcae, (2) numerous, minute projections throughout the tail stem surface, and (3) a post-distome cavity tail stem constriction, all of which are apparently lacking in all of the previously described cercariae of species of *Leuceruthrus*.

The specimens we studied herein were sourced from crushed snails but the distome of each cercaria was withdrawn and seemingly fully-developed (Fig 16). We did not maintain snails alive to await cercarial shedding. Without having observed free-swimming cercariae, we cannot be certain that these specimens were fully-developed, nor can we confirm if they resided within a sporocyst or if they had been ejected by the sporocyst within the snail. With certainty, however, our specimens morphologically differed from all comparable-staged (developing and free-swimming) cercariae previously described [9,26,27, present study]. As a result, we suspect that these cercariae represent a new species of *Leuceruthrus*, but we choose to obtain conspecific adults before naming the species.

The minute furcae of *Leuceruthrus* sp. indicated that it is a poor swimmer. Locomotion of azygiid cercariae, as indicated by tail stem and furcae morphology, may be linked to definitive host feeding habits and diet [1,5]. The definitive host for this fluke is indeterminate but we predict that it grazes on epibenthic insect larvae.

4. DISCUSSION

4.1. *Distribution and diversity*

The diversity, taxonomy, and life cycles of freshwater fish parasites in North America are underexplored [52], and *Leuceruthrus* is a prime example (present study). Despite these flukes infecting common fishes and ubiquitous snails, no description of an adult or cercaria of *Leuceruthrus* had been published in 104 years and 80 years, respectively. The present study brings the total number of nominal species of *Leuceruthrus* to 3 and suggests the existence of a 4th species. In North America, infections previously identified as *L. micropteri* (*sensu lato*) have been documented from 10 states and Lake Erie (Tables 1, 2). *Leuceruthrus* spp. are apparently endemic to North America, as are all of their snail and fish hosts. *Leuceruthrus* spp. infect snails of Pleuroceridae (*Elimia, Pleurocera*, *Leptoxis*), which comprises a family endemic to North America (from east of the continental divide and north into portions of Canada; [41]) and is the second most diverse group of North American freshwater gastropods. Likewise, all known fish hosts are North American endemics, which includes fishes of Centrarchidae (7 fish species of 3 genera), Amiidae (*A. calva*), and Moronidae (white bass, *Morone chrysops* [Rafinesque, 1820]) (Table 1).

4.2. *Geographic differences in azygiids*

Many parasites and hosts share a close evolutionary relationship resulting in high levels of specificity to some or all of their requisite hosts. Species of *Leuceruthrus* and *Proterometra* appear to exhibit greater specificity to their intermediate hosts than their

definitive hosts [1,5,53]. Species of *Proterometra* infect 26 species of primary division freshwater fishes assigned to 10 genera, 6 families, and 5 orders; whereas, they reportedly infect 8 species of freshwater snails assigned to 3 genera and 1 family [1]. Likewise species of *Leuceruthrus* infect 9 species of primary division freshwater fishes assigned to 5 genera, 2 families, and 2 orders (Table 1) whereas, they reportedly infect 6 species of freshwater snails assigned to 3 genera and 1 family. Typically, parasitological sampling bias is slanted towards definitive hosts (fishes), but this apparently is not the case here: most taxonomic papers for these genera treat cercariae [1,5], not adults.

Our results indicate that azygiid biodiversity in fishes may be a reliable indicator of snail diversity in rivers and streams, which may be reflective of habitat quality [41,54]. Conversely, fishes sampled from lakes, where snail diversity is lower, tended to have fewer azygiid species. Although speculative, this phenomenon would explain why so few species of *Leuceruthrus* had been described to date: most fish hosts were captured from areas of low or no snail diversity (i.e., artificial lakes or impoundments) (Table 1). Yet, *Leuceruthrus* biodiversity among centrarchids captured from rivers and streams remains little explored. Future workers may make exciting discoveries by seeking out stream sites with the highest snail diversity. Stated differently, alpha diversity of *Leuceruthrus* and *Proterometra* could potentially assist malacologists in obtaining valuable biological information regarding the presence/absence, biodiversity, and density of pleurocerids.

4.3. *Molecular phylogeny of Azygiids*

Our results did not reject any previous finding regarding generic interrelationships of azygiids [5, 30 *in review*]. Monophyly of *Leuceruthrus* was supported, and its placement within Azygiidae as sister to the ecologically similar *Proterometra* was confirmed (Fig. 19). This tree and our data (see Sec. 4.2) indicated specificity to intermediate hosts, not to definitive hosts. Species of *Proterometra* and *Leuceruthrus* (parasites of pleurocerids) formed a clade sister to that of *A. longa*, which infects *Amnicola limosus* (Amnicolidae: Rissooidea: Caenogastropoda) [53] (Fig 19). Adding more taxa, especially *Azygia* spp.*,* is needed to further test these relationships. Of particular interest would be the addition of the palearctic *A. lucii*, which infects pulmonate snails (i.e., Planorbidae, Lymnaeidae) only [55,56] and differs from that of the freshwater nearctic azygiids, which infect prosobranch snails only [1,8,53]. If freshwater azygiids have co-evolved with their intermediate hosts, *Azygia* spp. of palearctic pulmonates would likely share a recent common ancestor with nearctic *Azygia* spp. of prosobranchs. Together these would be sister to *Leuceruthrus* and *Proterometra*.

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Table 1. Definitive host and localities for *Leuceruthrus micropteri* (*sensu lato*)

Host	Locality	Reference
Amia calva	Lake Maxinkuckee, IN	Goldberger [12]
	Lake Erie	Bangham [13]
	Marshall, IN	Buckner et al. [14]
Morone chrysops	WI	Pearse [15,16]
	Lake Erie	Dechtiar & Nepszy [17]
Ambloplites rupestris	Elkhorn Creek, KY	Patton [18]
Lepomis gulosus	Marrowbone Lake, KY	Patton [18], experimental study
Lepomis macrochirus	Lexington, KY	Patton [18]
	KY	Aliff [19]
Lepomis megalotis	KY	Aliff [19]
Micropterus dolomieui	Madison, WI (Lakes Mendota, Monona, and Wingra)	Marshall & Gilbert [11]
	Round Lake, Washburn Co., WI	Marshall & Gilbert [11]
	Lake Maxinkuckee, IN	Goldberger [12]
	WI	Pearse [15,16]
	Lake Erie	Bangham [13]
	White River, AR	Becker et al. [20]
	Pickwick Reservoir	Hubert & Werner [21]
	Bushnell Creek, KY	Patton [18]
	AR	Kilambi & Becker [22]
	KY	Aliff [19]
	Marshall, IN	Buckner et al. [14]
	Lake Erie	Dechtiar & Nepszy [17]
M. punctulatus	Beaver Reservoir (White River), AR	Becker et al. [20]
	Lexington, KY	Patton [18], experimental study
	KY	Aliff [19]
M. salmoides (type host)	Madison, WI (Lakes Mendota, Monona, and Wingra) (type locality)	Marshall & Gilbert [11]
	Round Lake, Washburn Co., WI	Marshall & Gilbert [11]
	Lake Maxinkuckee, IN	Goldberger [12]
	Lake Erie	Bangham [13,23]
	White River (Drainage), AR	Becker et al. [20]
	Pickwick Reservoir	Hubert & Werner [21]
	Elkhorn Creek, KY	Patton [18]
	Marrowbone Lake, KY	Patton [18]
	Lexington, KY	Patton [18], experimental study
	KY	Aliff [19]
	Marshall, IN	Buckner et al. [14]
	Lake Ontario	Dechtiar & Christie [24]
	Wheeler Reservoir, AL	Present Study
Micropterus spp.	TN	Hubert & [21]
Micropterus sp.	AL	Sullivan's 1977 dissertation[a]
"black bass"	"Ohio Lakes"	Bangham [25]
	Lake Erie	Bangham [25]
not specified	Madison, WI	Horsfall [9]
	Round Lake, WI	Horsfall [9]
	Oconomowoc River, WI	Horsfall [9]
	Oconomowoc Lake, WI	Horsfall [9]
	Illinois River, IL	Horsfall [9]

a = Sullivan JR. The trematode parasites of the Black Basses (*Micropterus* spp.) of the Southeastern United States with some seasonal and locational variations of digenean populations in largemouth bass (*Micropterus salmoides*) (unpublished doctoral dissertation). 1977; Auburn University, Auburn, Alabama.

Table 2. Intermediate hosts for *Leuceruthrus* spp.

Parasite	Host	Locality	Reference
L. stephanocauda n. comb.	*Leptoxis carinata* (as *Anculosa c.)*	Rome, GA	Faust [26]
	Elimia carinifera (as *Goniobasis c.*)	Rome, GA	Faust [26]
	Elimia carinifera	Big Canoe Creek, AL	Present study
	Elimia modesta	Big Canoe Creek, AL	Present study
L. ocalana n. sp.	*Elimia* sp. (as *Goniobasis catenaria*)	Merritt's Mill Pond, FL	Smith [27]
		Northern FL	Smith [27]
		Southern GA	Smith [27]
	Elimia floridensis	Holmes Creek, FL	Present Study
	Elimia sp.	Blue Springs, AL	Present Study
Leuceruthrus sp.	*Elimia* sp.	Simmons Creek, AL	Present Study
	Pleurocera acuta	Oconomowoc River, WI	Horsfall [9]
	Elimia spp.	Northern AL	Smith [27]
	Pleurocera canaliculatum	Marrowbone Lake, TN	Patton [18]
		Bushnell Creek, TN	Patton [18]
	not specified	Oconomowoc River, WI	Smith [27]

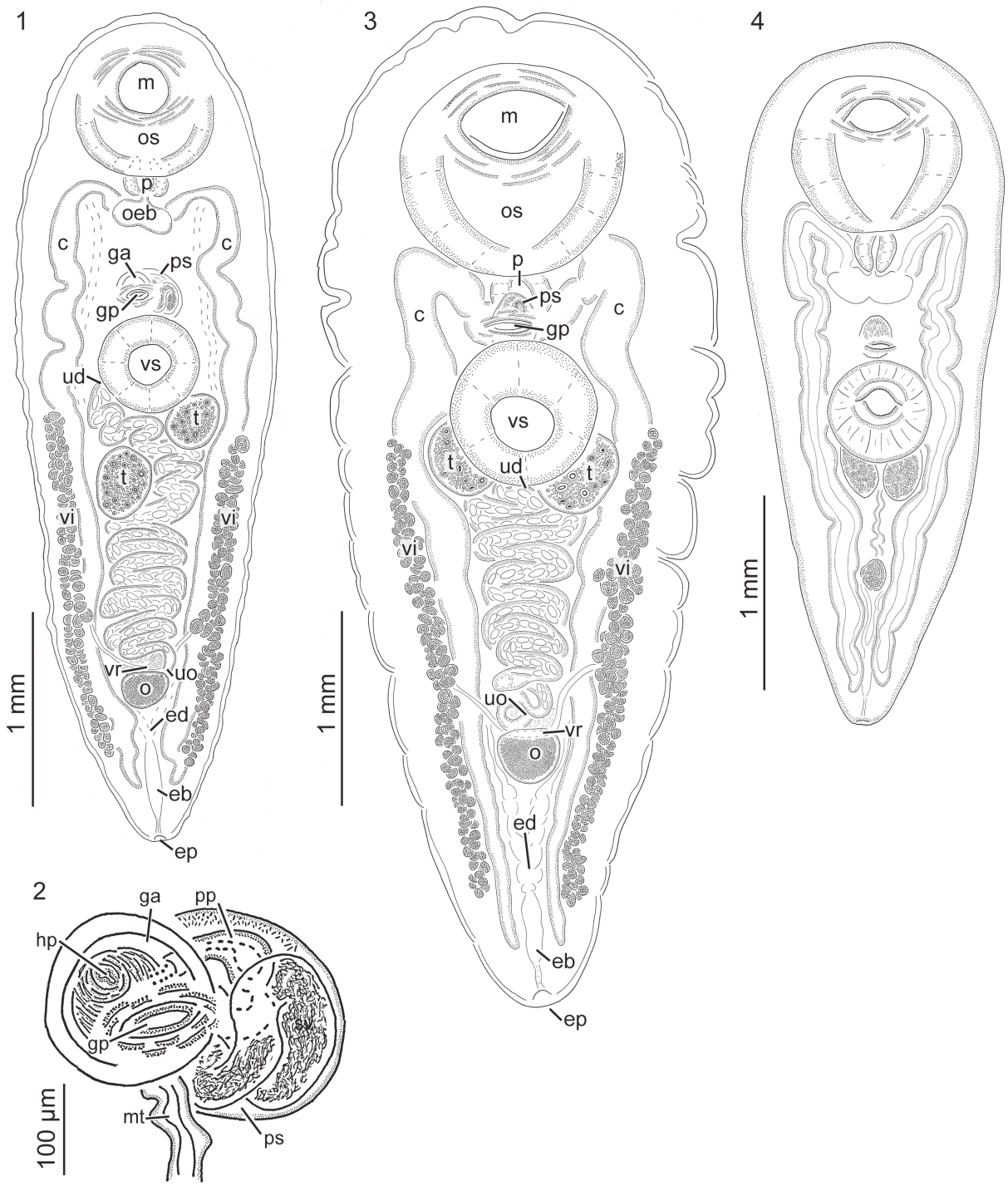
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Plate4-1; Figures 1–4. Flukes of *Leuceruthrus micropteri* Marshall and Gilbert, 1905 and *Leuceruthrus ocalana* (Smith, 1935) n. comb. from largemouth bass, *Micropterus salmoides* from Wheeler Reservoir, Alabama and Holmes Creek, Florida **(1)** Adult of *L. micropteri* (USNM coll. no. XXXXXX) showing mouth (m), oral sucker (os), pharynx (p), oesophagus bifurcation (oeb), caecae (c) near origin, prostatic sac (ps), genital atrium (ga), genital pore (gp), ventral sucker (vs), distal end of uterus (ud), testes (t), vitellarium (vi), uterus origin (uo) near ovary (o), vitelline reservoir (vr), confluence of excretory ducts (ed), excretory bladder (eb) and excretory pore (ep). Ventral view. (**2**) Terminal male genitalia of *L. micropteri* (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figure 1 plus seminal vesicle (sv), pars prostatica (pp), metraterm (mt), and hermaphroditic pore (hp). Ventral view. (**3**) Adult of *L. ocalana* (Smith, 1935) Womble and Bullard n. comb (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figure 1. Ventral view. (**4**) Post cercarial juvenile of *L. ocalana* (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figure 3.

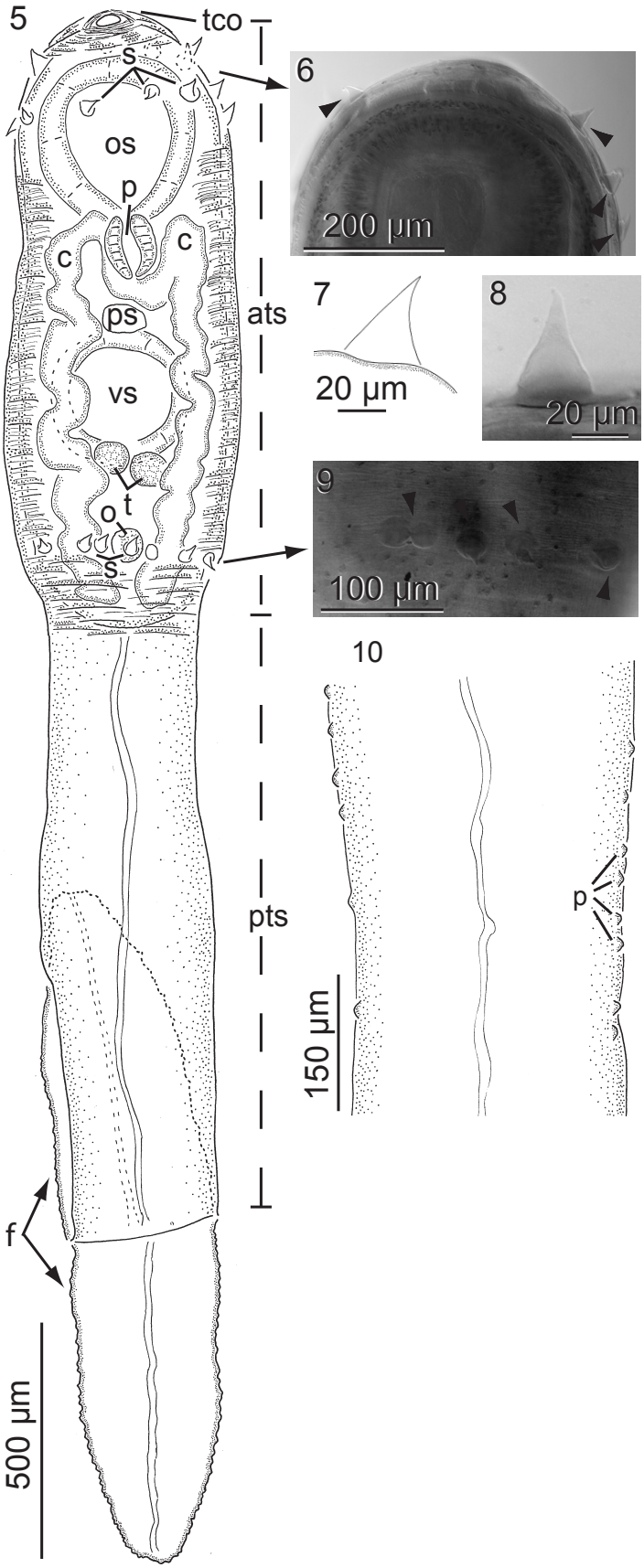
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Plate 4-2; Figures 5–10. Naturally shed cercaria of *Leuceruthrus stephanocauda* (Faust, 1921) Womble and Bullard n. comb. from *Elimia* spp. from Big Canoe Creek, Alabama. (**5**) Cercaria (USNM coll. no. XXXXXX) showing tail cavity opening (tco), position of tail stem spines (s), anterior (ats) and posterior tail stem (pts) regions, location of the distome, showing comparable features as illustrated in Figure 1, and paired lanceolate furcae (f). Dorsal view. (**6**) Anterior tail stem showing anterior row of tail stem spines (arrowheads). (**7**) Illustration of tail stem spine. (**8**) Photomicrograph of tail stem spine. (9) Anterior tail stem showing posterior row of tail stem spines (arrowheads). (10) Posterior tail stem showing marginal placement of associated protuberances (p).

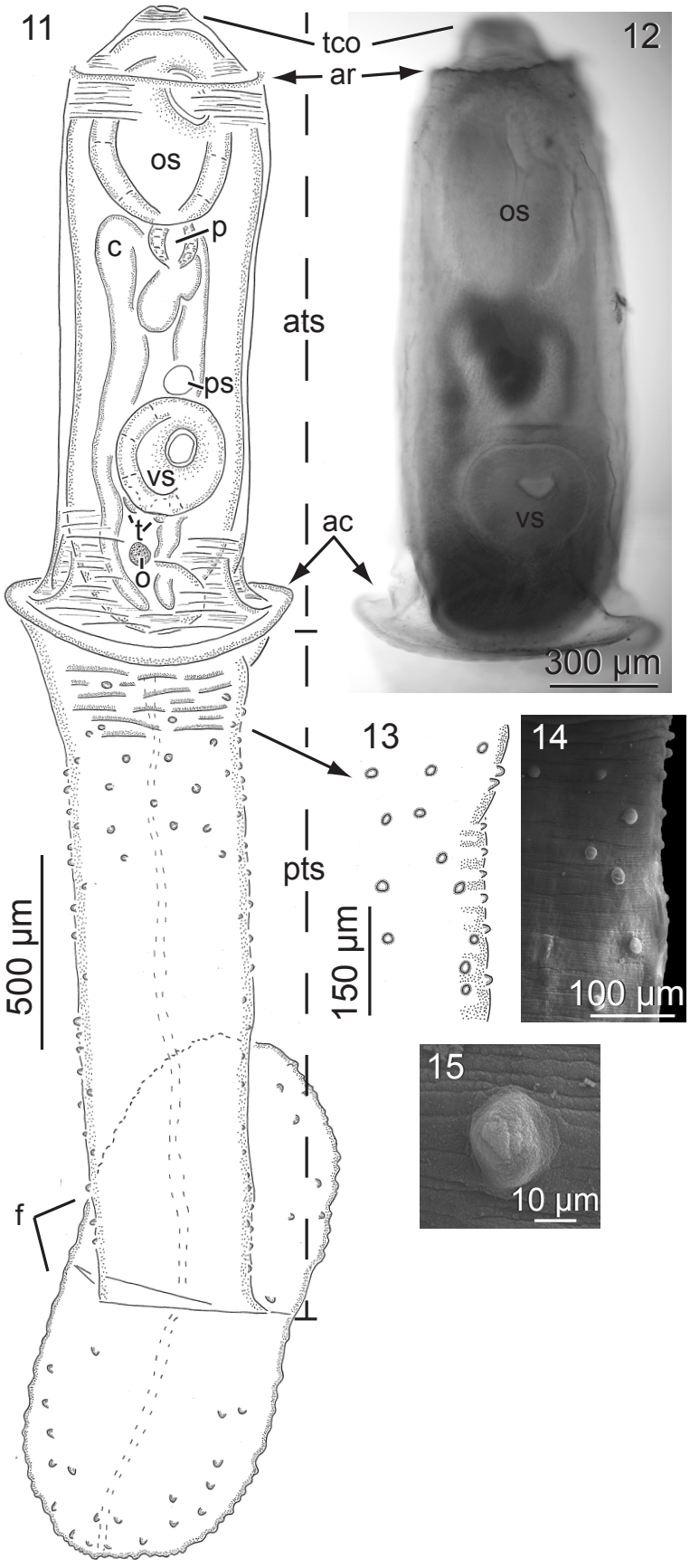
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Plate 4-3; Figures 11–15. **Figures 11–15**. Naturally shed cercaria of *Leuceruthrus ocalana* (Smith, 1935) Womble and Bullard n. comb. from *Elimia floridensis* from Holmes Creek, Florida. (**11**) Cercaria (USNM coll. no. XXXXXX) showing tail cavity opening (tco), position of anterior ridge (ar), and anterior collar (ac), anterior (ats) and posterior tail stem (pts) regions, location of the distome, showing comparable features as illustrated in Figure 1, and paired oblong furcae (f). Dorsal view. (**12**) Anterior tail stem showing comparable features as illustrated in Figure 11. (**13**) Illustration of posterior tail stem showing position of associated protuberances. (**14**) Posterior tail stem showing position of associated protuberances. SEM. (**15**) High magnification view of a posterior tail stem protuberance. SEM.

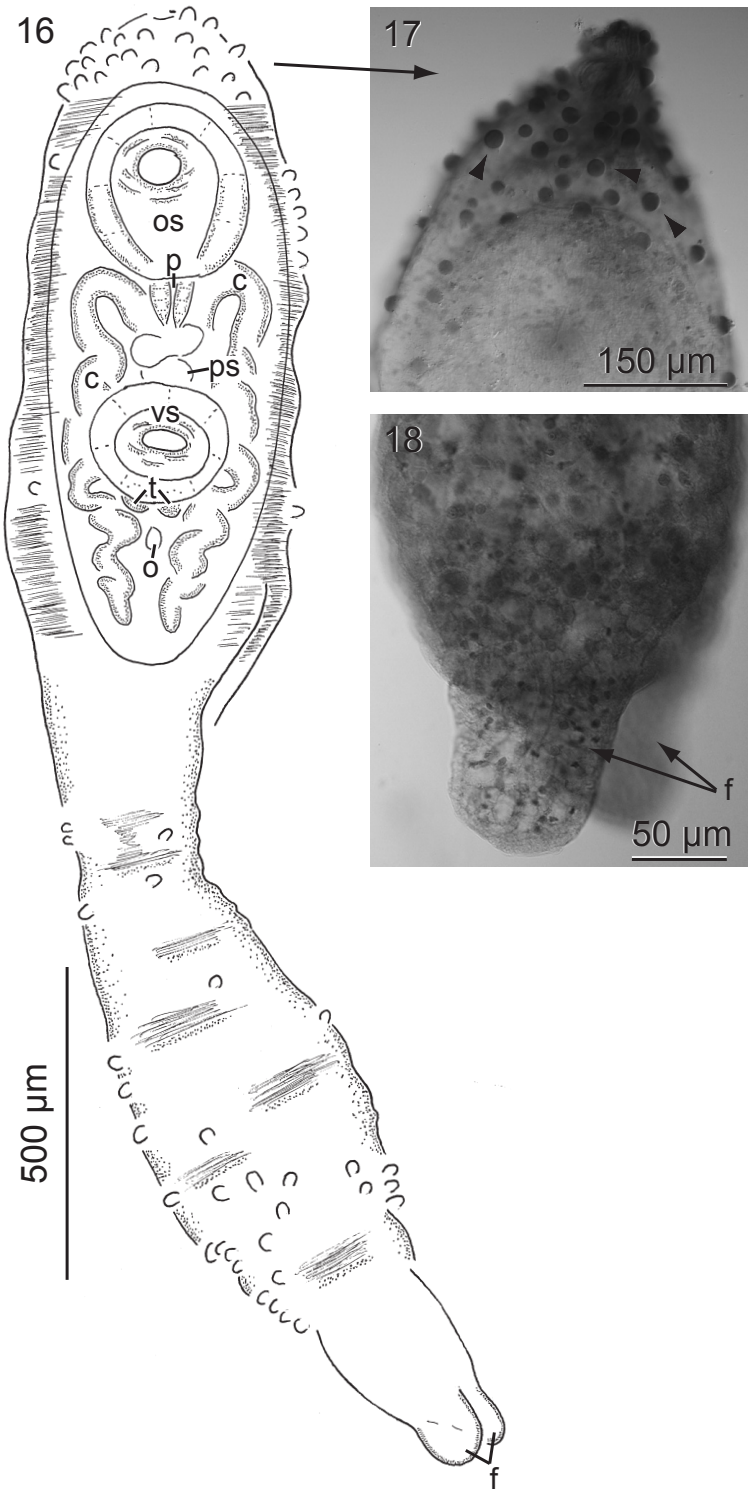

Plate 4-4; Figures 16–18. Naturally shed cercaria of *Leuceruthrus* sp. from *Elimia* sp. from Simmons Creek, Alabama. (**16**) Cercaria (USNM coll. no. XXXXXX) showing comparable features as illustrated in Figures 5 and 11. Dorsal view. (**17**) Anterior tail stem showing tegumental protuberances densely distributed near tail cavity opening (arrowheads). (**18**) Photomicrograph of short furcae (f).

19

L. oclana		0.6%	2.3%	2%	5.9%	6.5%	6.8%
Leuceruthrus sp.	2		2.8%	2.5%	6.5%	6.8%	7.3%
L. micropteri	8	10		1.4%	7%	7.6%	7.3%
L. stephanocauda	7	9	5		7.3%	7.9%	7.6%
P. epholkos	21	23	25	26		5.4%	5.9%
P. ariasae	23	24	27	28	19		7.3%
A. longa	24	26	26	27	21	26	

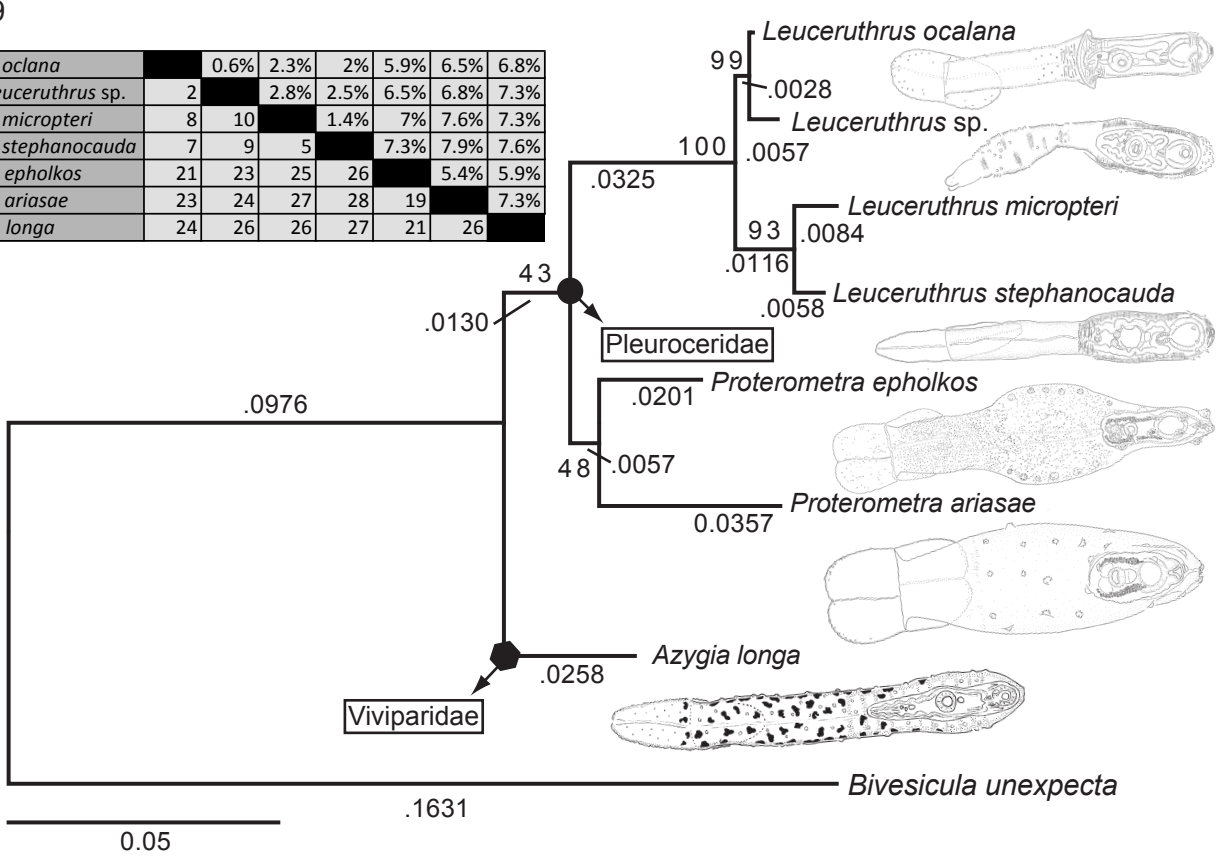

Figure 19: Maximum likelihood phylogenetic tree based on analyses of sequence data from the ribosomal internal transcribed spacer 2 using MEGA V.6.06. Tree includes sequences from 3 species of *Leuceruthrus*, 2 species of *Proterometra* and *Azygia longa. Bivesicula unexpectata* was used as the outgroup. Bootstrap support values (1,000-replicates) are reported aside each node, and branch lengths are below each branch. Illustrations for each species cercaria, aside for *L. micropteri*, are presented below their respective nodes. In the shaded table sequence dissimilarity is presented as a percentage and placed above the diagonal with base pair polymorphisms placed below the diagonal