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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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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the anterior tail stem only, and (v) a proportionally small distome that has relatively few uterine eggs and remains withdrawn in the anterior tail stem region in actively swimming cercariae.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 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) and the second use of a molecular marker in concert with

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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]	Reference
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						
Transversotrema 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.

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 *Pleurocera vestitum* in Graf [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 the 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 10× objective magnification.

Fluke specimens for morphology were removed from the buccal cavity and epithelial surface of the esophagus 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 (19.32 g/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 50 μl 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 50 μl, 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 28 s 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 min at 95 °C for denaturation, followed by 35 repeating cycles of 95 °C for 30 s for denaturation, 57 °C for 30 s for annealing, and 72 °C for 45 s for extension, followed by a final 10 min 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 5.8 s, ITS2, and 28 s 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 1000 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], Graf [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).

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.

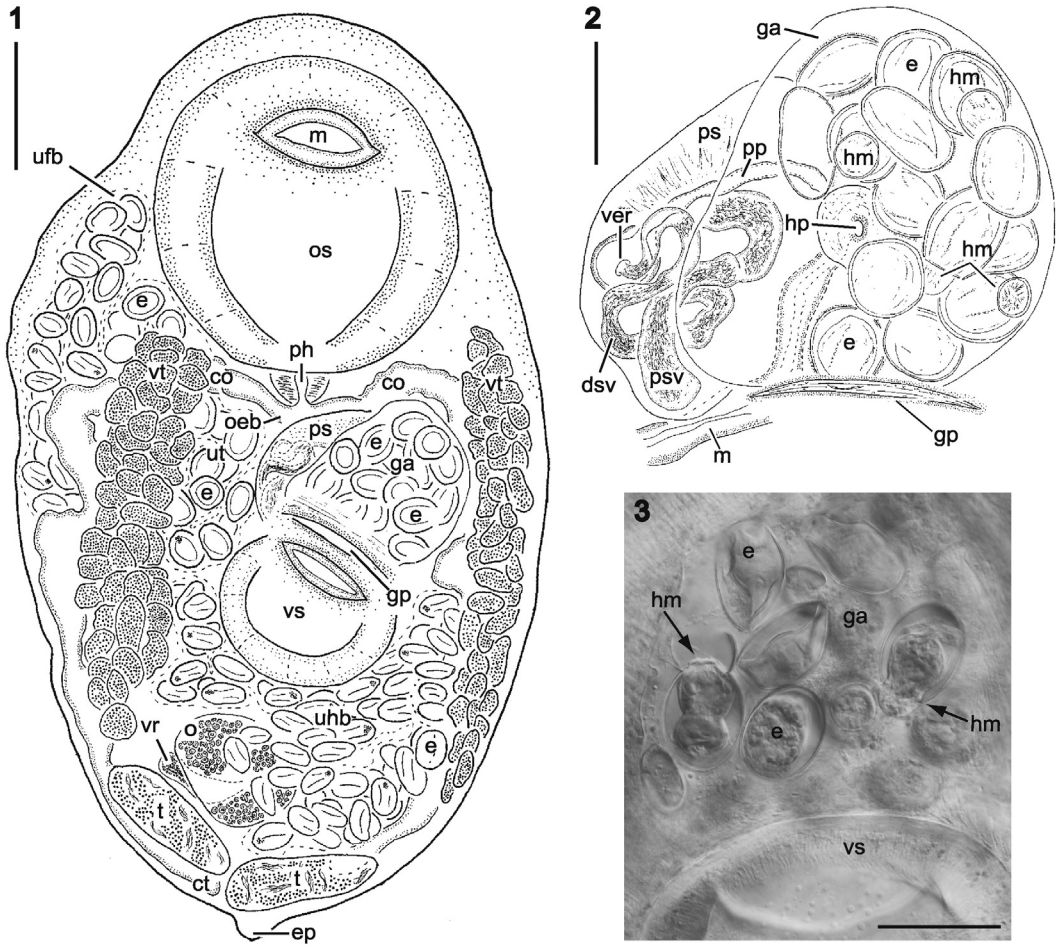
3. Results

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.

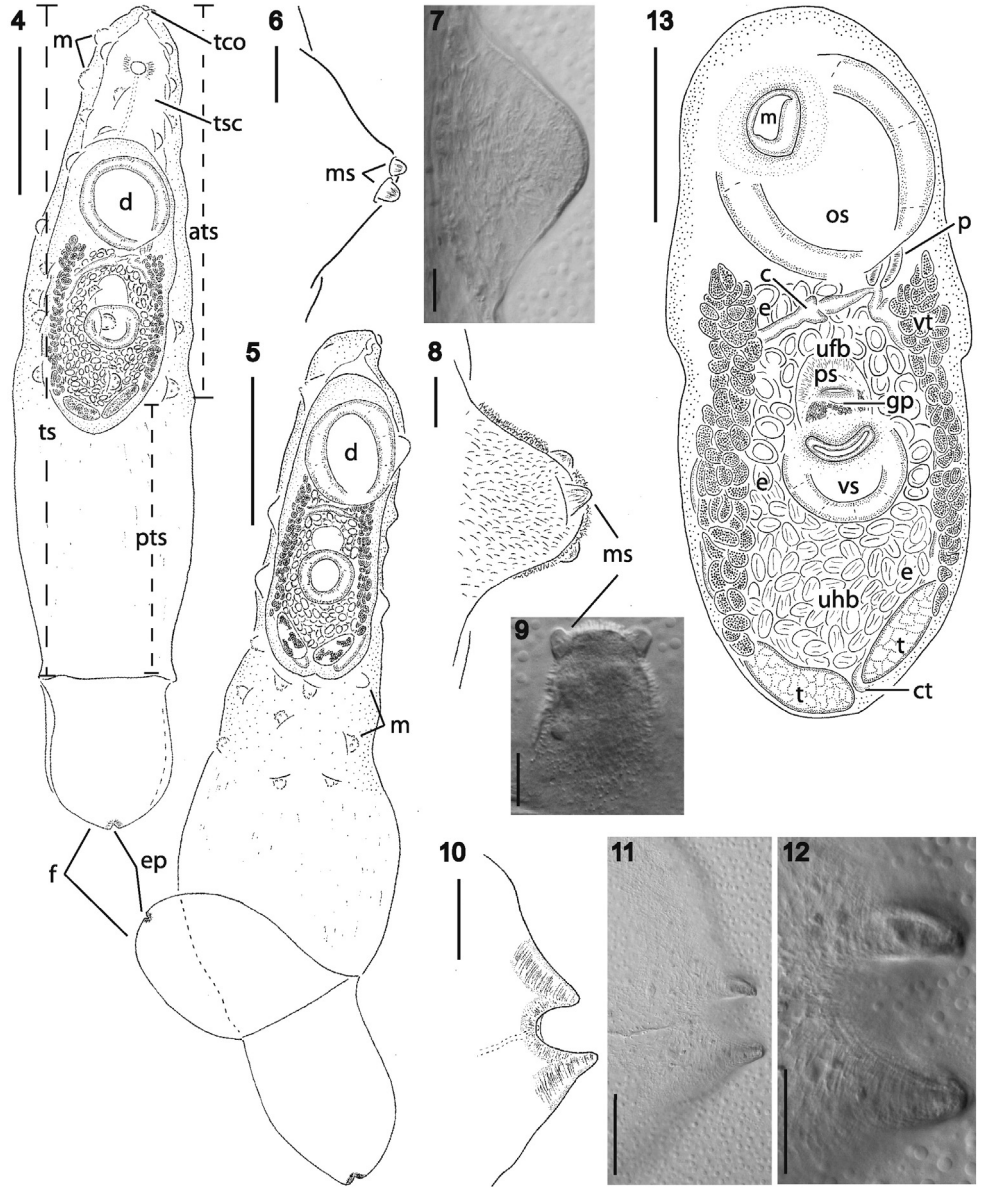
Testes 2 in number, oblique, obtuse or nearly parallel along lateral axis, elliptical in shape (Fig. 1); dextral testis 295–370 (333, 2) long or 17–21% (19%, 2) of body length, 90–115 (103, 2) wide or 8–11% (9%, 2) of body width, pre-testicular space, 1500–1520 (1510, 2) from anterior end of body or 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



Figs. 1–3. Adults of *Proterometra autraini* from esophagus 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 Fig. 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.

(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). 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, Laurer's 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



Figs. 4–13. Cercariae of *Proterometra autraini* 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.

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) × 35 (35, 2) in proximal portion of uterus, to approximately 90–95 (90, 12) × 70–75 (73, 2) in distal portion of uterus, some eggs within genital atrium with hatching miracidia (Figs. 1).

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 (Perciformes: Cottidae).

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

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

Site of infection: esophagus (fish) and "mantle area" (snail).

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

Specimens Examined: HWML Nos. 37,903–6 (adult), 37,903–7 (adult), 37,904–1 (cercaria), 37,904–3 (cercaria).

3.1.4. Remarks

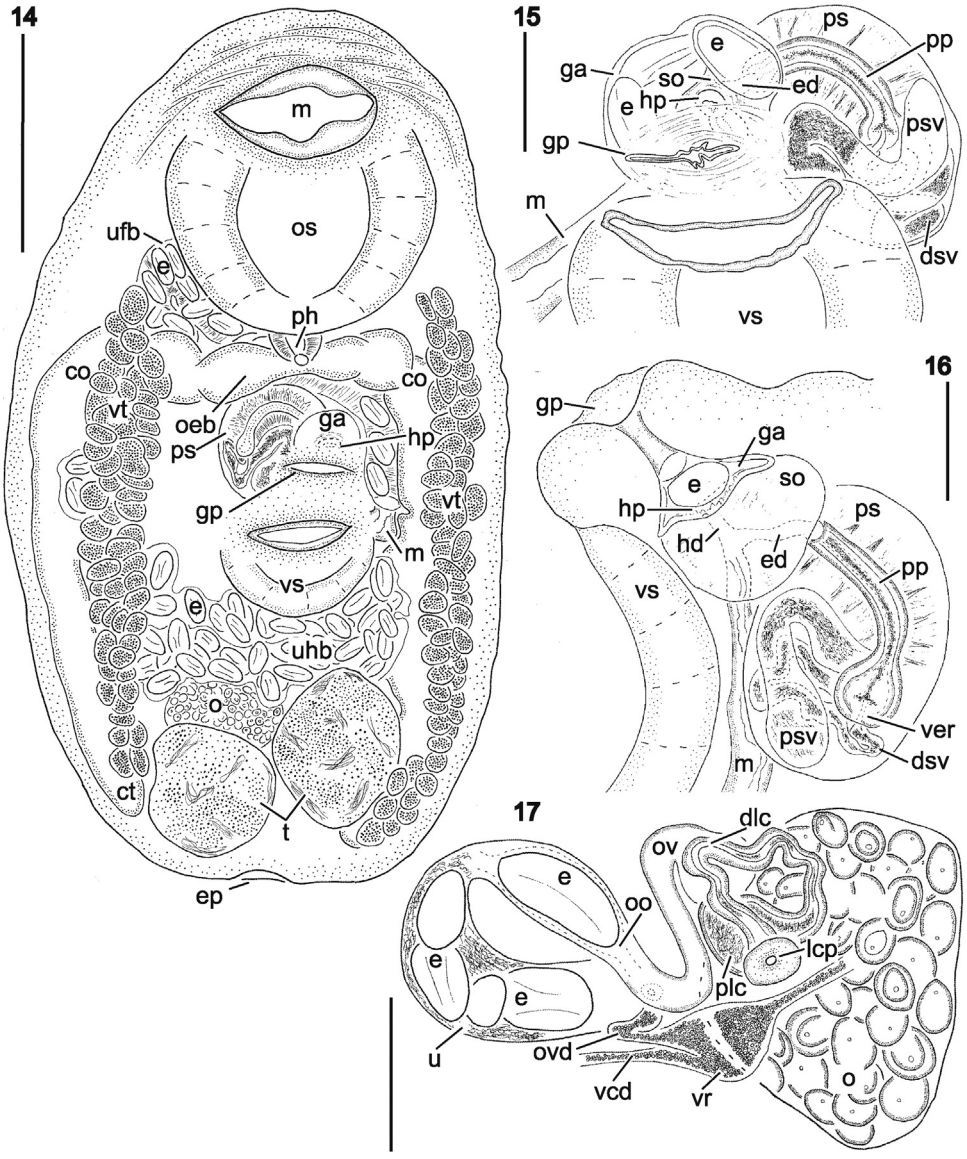
The original description of adult specimens of *P. autraini* given by LaBeau and Peters [15], hereafter "LP," was based on 10 specimens. The 2 adult paratypes we studied were in good condition, well fixed (fully extended, not curled), and well stained. The original description includes morphometric data 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 & 37,903–7). 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 the 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 μm, 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,



Figs. 14–17. Adults of *Proterometra ariasae* from oesophagus of longear sunfish, *Lepomis megalotis* from the Chickasawhay River, Mississippi. **(14)** Adult (USNM 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 no. 1283300) showing comparable features as illustrated in Fig. 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 showing comparable features as illustrated in Figs. 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.

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 n. sp. Womble and Bullard, 2015 (Figs. 14–32)

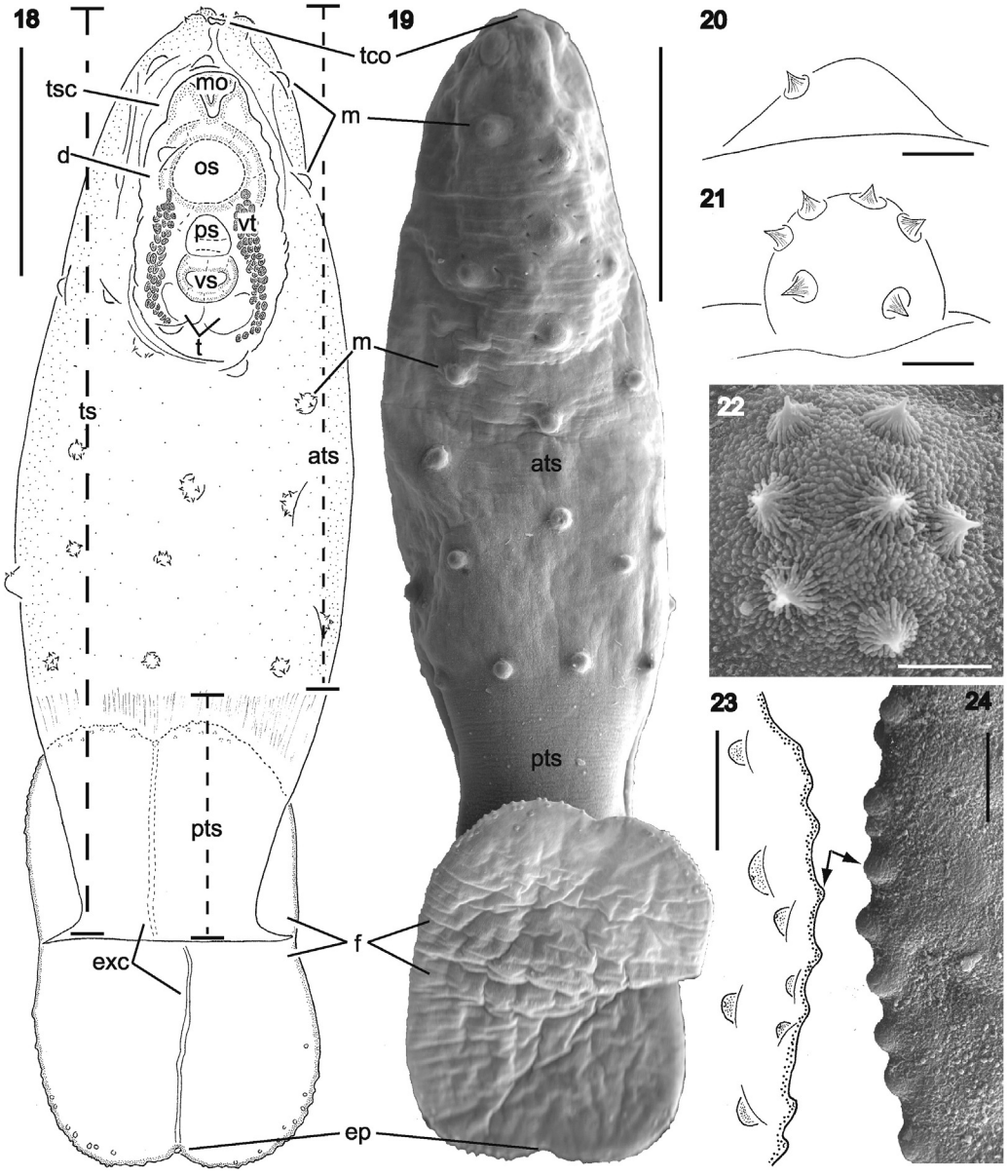
3.2.1. Diagnosis of adult (based on 13 stained, whole-mounted specimens)

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.

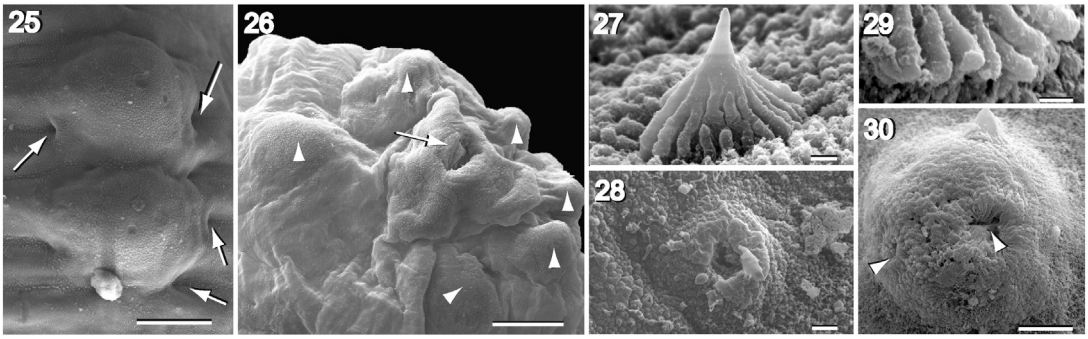
Testes 2 in number, oblique, transverse, abreast, round to oval in outline (Fig. 14); dextral testis 225–435 (323, 13) or 13–22% (17%, 13) of body length, 185–290 (217, 12) or 17–25% (21%, 12) of body width; pre-testicular space, 1180–1880 (1499, 13) from anterior end of body or 71–89% (80%, 13) of total 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 (Figs. 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) × 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) × 23–35 (30, 12) and 85–95 (90, 12) × 45–53 (47, 12) in proximal and distal portions of uterus, respectively.



Figs. 18–24. Cercariae of *Proterometra ariasae* from *Pleurocera* sp. from the Chickasawhay River, Mississippi. **(18)** Cercaria (USNM 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.



Figs. 25–30. Cercariae of *Proterometra ariasae* from *Pleurocera* sp. 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 μm.

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 (Figs. 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: Esophagus.

Site in molluscan host: Indeterminate.

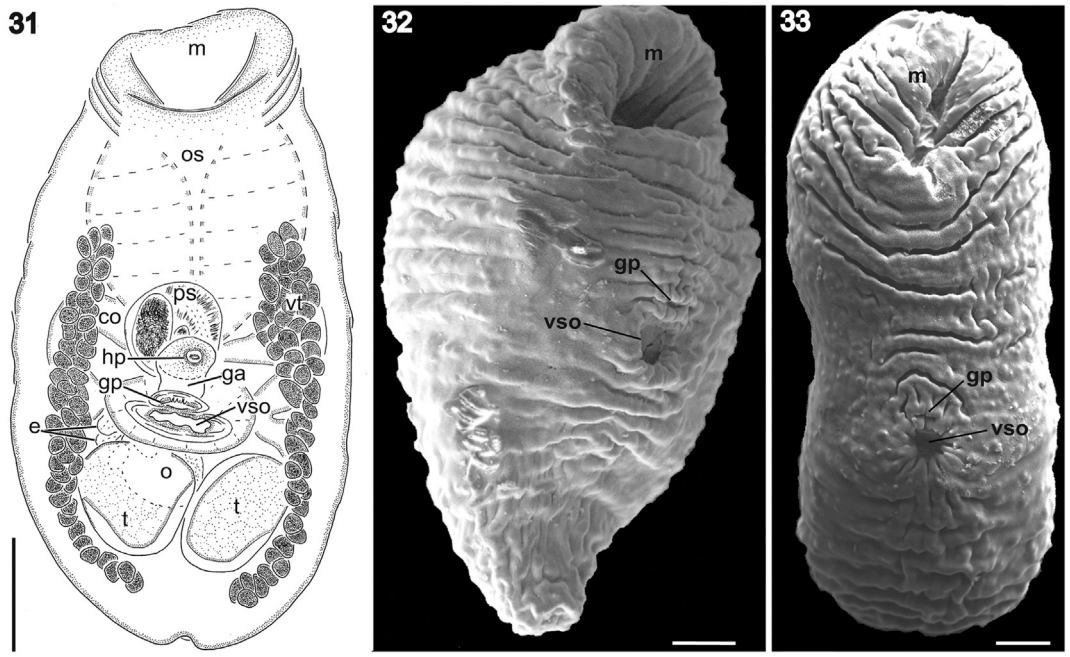
Specimens Deposited: Syntypes (adults; USNM collection nos. 1283298, 1283299, 1283300), (cercariae; USNM collection nos. 1283301, 1283302, 1283303) and intermediate host vouchers in AUMNH.

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



Figs. 31–33. Distome of *Proterometra ariasae* from *Pleurocera* sp. from the Chickasawhay River, Mississippi. **(31)** Distome 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.

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 posterior 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 posterior 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*.

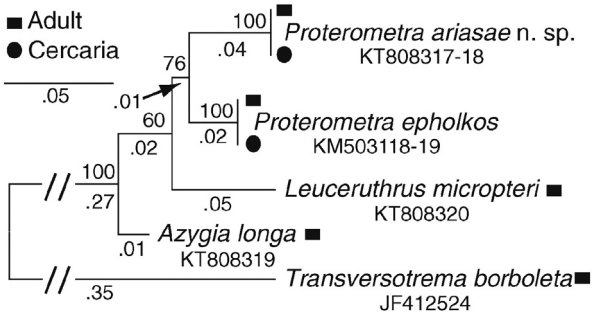


Fig. 34. Maximum likelihood tree (inclusive of all sites) including two species of *Proterometra*, *Leuceruthrus micropteri*, and *Azygia longa*, (cercariae = circles; adults = squares), inferred from the ribosomal internal transcribed spacer 2 (ITS2; 363 bp). Bootstrap support values, based on 1000-replicates, are reported aside each node and branch lengths are given below each branch.

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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