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On the life cycle of *Hemiurus levinseni* Odhner, 1905 (Digenea: Hemiuridae)

D.Yu. Krupenko[1], A.G. Gonchar[1,2], G.A. Kremnev[1], A.A. Uryadova[1]

[1] *Saint Petersburg State University, Department of Invertebrate Zoology, Universitetskaia emb., 7-9, Saint Petersburg, 199034, Russia. E-mail: midnightcrabb@gmail.com, d.krupenko@spbu.ru*
[2] *Zoological Institute RAS, Universitetskaia emb., 1, Saint Petersburg, 199034, Russia.*

ABSTRACT: Daughter sporocysts and cystophorous cercariae were found in the gastropod *Cylichna alba* (Heterobranchia: Cephalaspidea) from the White Sea. By evidence from the rDNA sequences (partial 28S and 5.8S+ITS2) they match sexual adults identified as *Hemiurus levinseni* (Digenea: Hemiuroidea: Hemiuridae). We propose an outline of *H. levinseni* life cycle, describe morphology of its sporocysts and cercariae, and compare the latter with cercariae of other hemiuroideans. The position of the genus *Hemiurus* within the Hemiuridae is also discussed based on the molecular data.
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KEY WORDS: life cycle, Digenea, Hemiuroidea, Hemiuridae, cercariae, rDNA.

Жизненный цикл *Hemiurus levinseni* Odhner, 1905 (Digenea: Hemiuridae)

Д.Ю. Крупенко[1], А.Г. Гончар[1,2], Г.А. Кремнев[1], А.А. Урядова[1]

[1] *Санкт-Петербургский государственный университет, кафедра зоологии беспозвоночных, Университетская наб., 7-9, Санкт-Петербург, 199034, Россия.*
E-mail: midnightcrabb@gmail.com, d.krupenko@spbu.ru
[2] *Зоологический институт РАН, Университетская наб., 1, Санкт-Петербург, 199034, Россия.*

РЕЗЮМЕ: В беломорских моллюсках *Cylichna alba* (Heterobranchia: Cephalaspidea) были обнаружены дочерние спороцисты и цистофорные церкарии. По последовательностям рДНК (фрагменты 28S и 5.8S+ITS2) оказалось, что они совпадают с маритами *Hemiurus levinseni* (Digenea: Hemiuroidea: Hemiuridae). Мы предлагаем схему жизненного цикла *H. levinseni*, приводим описание спороцист и церкарий этого вида и сравниваем церкарий разных видов гемиуроидей. Также обсуждается положение рода *Hemiurus* в сем. Hemiuridae на основе молекулярных данных.
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КЛЮЧЕВЫЕ СЛОВА: жизненные циклы, Digenea, Hemiuroidea, Hemiuridae, церкарии, рДНК.

Paper is dedicated to the memory of A.A. Dobrovolsky.
Статья посвящена памяти А.А. Добровольского.

Introduction

Exploring life cycle patterns is one of the major clues for understanding the evolution of the Digenea. It is particularly so for the superfamily Hemiuroidea Looss, 1899, as its representatives are found in both marine and freshwater environments (Hunninen, Cable, 1943; Madhavi, 1978; Stunkard, 1980; Goater *et al.,* 1990) and possess different types of life cycles. Typical hemiuroidean life cycle includes three or four hosts (Cribb *et al.,* 2003), though some species demonstrate a tendency towards progenesis (Kulachkova, 1972; Køie, 1979). This tendency probably led to the emergence of one-host cycles in several different lineages of the Hemiuroidea (Poulin, Cribb, 2002). The definitive hosts of the hemiuroideans are usually teleost fishes, with several exceptions (Gibson, 2002). Crustaceans (frequently — planktonic copepods) act as the second intermediate hosts and set up the route of infection through food webs towards the definitive host (Køie, 1979, 1989; Marcogliese, 1995). Another fascinating feature of the hemiuroideans is an extremely wide range of the first intermediate hosts. They comprise the Caenogastropoda (Madhavi, 1978; Køie, 1979; Stunkard, 1980; Køie, Gibson, 1991), Vetigastropoda (Køie, 1990a), Heterobranchia — both “opisthobranchs” (Hunninen, Cable, 1943; Køie, 1989, 1990b, 1991, 1992, 1995) and “pulmonates” (Goater *et al.,* 1990); several species are also known from the Scaphopoda (Køie *et al.,* 2002), and one from the Bivalvia (Wardle, 1975). Collecting more life cycle data will give an excellent opportunity to explore coevolution and host-switch processes within this group of the Digenea.

All known cercariae of the Hemiuroidea are of cystophorous type[1]: their tail is highly modified to form a caudal cyst into which the cercarial body is withdrawn (Gibson, Bray, 1986). During the tail development, a delivery tube — structure providing infection of the second intermediate host — is formed as an appendage on the caudal cyst. Later the delivery tube is retracted into the caudal cyst. When copepod bites the caudal cyst with mandibles the delivery tube everts and injects cercarial body into the host haemocoel (Matthews, 1981a). In many hemiuroidean cercariae caudal cyst also bears a so-called excretory appendage of various shapes which can provide motility of the infective cercariae (Pelseneer, 1906; Chubrik, 1966; Køie, 1979, 1990b, 1992, 1995). The advent of cystophorous cercariae was crucial for the transmission strategy of the Hemiuroidea, thus its structure is another important character to analyze from the perspective of the hemiuroidean evolution.

One of the common hemiuroidean species in arctic-boreal waters is *Hemiurus levinseni* Odhner, 1905 (Hemiuridae) (Gibson, Bray, 1986; Hemmingsen, MacKenzie, 2001; Køie, 2009). It is the only species of this genus in the White Sea (Shulman, Shulman-Albova, 1953). The life cycle of *H. levinseni* remains unknown: cystophorous cercariae from *Euspira pallida* (Broderip et Sowerby, 1829) which were supposed to belong to *H. levinseni* (Køie, 1990c) later were shown to be some unidentified representative of the family Derogenidae (Køie, 1995, 2000). Heterobranch gastropods from the order Cephalaspidea should be considered when searching for the first intermediate host of *H. levinseni* as these molluscs are utilized by several other species of the family Hemiuridae (Køie, 1990b, 1991, 1992, 1995). In the White Sea these gastropods have never been examined for digeneans. As *Cylichna alba* (Brown, 1827) is the most abundant representative of the Cephalaspidea in shallow waters of the White Sea, we supposed it to be the most likely candidate for the first intermediate host of *H. levinseni*.

In this paper we describe daughter sporocysts and cystophorous cercariae found in *C. alba* which by molecular evidence match sexual adults of *H. levinseni* from fish. Thus, the life cycle for this species is outlined.

Material and methods

Hosts were sampled in the White Sea, Kandalaksha Bay, Keret Archipelago. Gastropods

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[1] Ptychogonomidae may be the only exception, but their systematic position is uncertain for now.

Cylichna alba were collected in the upper subtidal (2–5 m) near the island Vichennaya luda in June (n = 34) and August (n = 82) 2019. Eight species of fish were collected near the islands Vichennaya luda, Malyi Gorelyi and Matrenin in summer 2018 and summer–autumn 2019. These were Atlantic cod *Gadus morhua* Linnaeus, 1758, navaga *Eleginus nawaga* (Walbaum, 1792), shorthorn sculpin *Myoxocephalus scorpius* (Linnaeus, 1758), herring *Clupea pallasii* Valenciennes, 1847, European flounder *Platichthys flesus* (Linnaeus, 1758), Arctic flounder *Liopsetta glacialis* (Pallas, 1776), common dab *Limanda limanda* (Linnaeus, 1758), and wolffish *Anarhichas lupus* Linnaeus, 1758.

Hosts were dissected, tissues of *C. alba* were examined in seawater and guts of fish — in physiological solution (0.9% NaCl). Adults of *Hemiurus levinseni* from fish were identified according to the keys in Gibson & Bray (1979, 1986) also considering Bray & Cribb (2005). Sporocysts and cercariae from *C. alba* and sexual adults of *H. levinseni* from fish were preserved in 96% ethanol for molecular analysis and preparation of whole mounts. Also, cercariae and daughter sporocysts from *C. alba* were fixed with 2.5% glutaraldehyde in seawater and with 4% paraformaldehyde in 0.01 M phosphate buffered saline. The sexual adults of *H. levinseni* from fish were mounted in glycerol unstained to double-check the morphological identification and to make photographs. Glutaraldehyde-fixed cercariae and sporocysts had a better-preserved shape, so they were temporarily mounted to make photographs and line drawings. Photographs of the whole-mounted worms were made using a compound microscope Leica DM 2500 (Leica Microsystems) and a camera Nikon DS Fi1 with differential interference contrast microscopy (DIC). Measurements were made using Fiji software (Schindelin *et al.,* 2012). All measurements are in micrometres. Measurements of daughter sporocysts are based on four young and four mature specimens, of pre-infective cercariae — on nine fixed specimens, of infective cercariae — on 21 fixed specimens; not all specimens contributed a data point to all metrical variables. For SEM glutaraldehyde-fixed cercariae were dehydrated in ethanol and acetone, dried in critical point dryer, coated with platinum, and examined with Quanta 250 at 15 kV. Additional information on the cercariae inner structure was obtained by means of TRITC-phalloidin labelling and confocal laser scanning microscopy (CLSM) following protocol previously described by Krupenko & Gonchar (2017); this way f-actin in the flame-cell collars and musculature in the distal part of excretory vesicle were visualized.

For molecular analysis we used sporocysts from three different specimens of *C. alba* and parts of the sexual adults of *H. levinseni* from different fish species (Table 1). They were taken from 96% ethanol, dried completely, and DNA was extracted from each of them separately by incubation in 200 μL 5% Chelex® 100 resin (Bio-Rad, USA) solution with 0.2 mg/mL proteinase K at 56 °C overnight; 8 min at 90 °C; and centrifugation at 16,000 g for 10 min. Supernatant containing DNA was then transferred to a new tube and stored at –20 °C. To amplify a ~500 base pairs (bp) long D2 domain of the 28S rRNA gene (LSU) we used a forward C2'B (GAAAAGTACTTTGRARAGAGA, Bayssade-Dufour *et al*., 2006) and a reverse D2 primer (TCCGTGTTTCAAGACGGG, Vân Le *et al.,* 1993) with the following thermocycling conditions: initial denaturation for 5 min at 95 °C; 35 cycles with 30 s at 95 °C, 30 s at 53 °C and 1 min at 72 °C; final elongation for 10 min at 72 °C; and cooling to 4 °C. To amplify a ~1200 bp long D1–D3 LSU domains region we used a digl2 forward (AAGCATATCACTAAGCGG, Tkach *et al.,* 1999) and a 1500R reverse primer (GCTATCCTGAGGGAAACTTCG, Olson *et al*., 2003) with the following thermocycling conditions: 3 min at 95 °C; 40 cycles with 30 s at 95 °C, 30 s at 54 °C and 2 min at 72 °C; 10 min at 72 °C; and cooling to 4 °C. To amplify a ~600 bp fragment comprising 5.8S+ITS2, we used a forward 3S (GGTACCGGTTCACGTGGCTAGTG) and a reverse ITS2.2 primer (CCTGGTTAGTTTCTTTTCCTCCGC) (Morgan, Blair, 1995) with thermocycling conditions described by Shimazu *et al*. (2014). Amplifications were performed in

Table 1. Isolates, their origin, and GenBank accession numbers for sequences.
Таблица 1. Изоляты, их происхождение и номера последовательностей в базе данных GenBank.

			GenBank accession number	
ID	Stage	Host species	28S rDNA	5.8S rDNA+ITS2
30.48.c	Sporocysts and cercariae	*Cylichna alba* (Brown, 1827)	MN962990	MT644465
294.48.c	Sporocysts and cercariae	*Cylichna alba* (Brown, 1827)	MN962991	MT644463
295.48.c	Sporocysts and cercariae	*Cylichna alba* (Brown, 1827)	MN962992	MT644466
2.45.c	Sexual adult	*Liopsetta glacialis* (Pallas, 1776)	MN962993	MT644462
4.45.c	Sexual adult	*Platichthys flesus* (Linnaeus, 1758)	MN962994	MT644470
10.45.c	Sexual adult	*Eleginus nawaga* (Walbaum, 1792)	MN962995	MT644471
305.48.c	Sexual adult	*Myoxocephalus scorpius* (Linnaeus, 1758)	MN962996	MT644464
284.48.c	Sexual adult	*Gadus morhua* Linnaeus, 1758	MN962997	MT644467
1.49.c	Sexual adult	*Gadus morhua* Linnaeus, 1758	MN962998	MT644469
3.49.c	Sexual adult	*Myoxocephalus scorpius* (Linnaeus, 1758)	MN962999	MT644468

25 µL reaction mixtures containing 16 µL Milli-Q® water, 5 µL ScreenMix-HS reaction mix (Evrogen, Russia), 1 µL of both F and R primers, and 2 µL DNA template. PCR products were visualized on a 1% agarose gel with SYBR® Green (Invitrogen, MA, USA), and sequenced on an ABI PRISM 3500xl (Applied Biosystems, MA, USA) with PCR primers. Sequence data were processed using Geneious® 11.1.5 (https://www.geneious.com). To annotate the ITS region, we used the sequence of *Echinostoma revolutum* (U58102; Morgan, Blair, 1995) as a reference. Dataset for phylogenetic reconstructions was based on that from Sokolov *et al*. (2019) and included part of the taxa from the Clade B: Hemiuridae, Lecithasteridae and *Merlucciotrema praeclarum* (Manter, 1934) Yamaguti, 1971. Among these sequences the one named "*Hemiurus luehei* Odhner, 1905" (MH628316) appeared to be misidentified in the original paper (personal communication of one of the coauthors I.I. Gordeev); that is why we will use this species name in quotation marks[2]. Additionally our dataset included *Hemiurus appendiculatus* (Rudolphi, 1802) Looss, 1899 (KR349118 and KR349121) sequences from Bao *et al.,* 2015. *Bunocotyle progenetica* (Markowski, 1936) Chabaud et Buttner, 1959 (DQ354365) (family Bunocotylidae) from Pankov *et al*. (2006) served as an outgroup. Phylogeny was inferred with maximum likelihood (ML) and Bayesian inference (BI) approaches. The best substitution model was estimated as GTR+G using jModelTest 2.1.10 (Guindon, Gascuel, 2003; Darriba *et al.,* 2012). The ML tree was constructed in PhyML

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[2] According to the personal communication of Dr Ilya I. Gordeev, the authors had at their disposal a fragment of a trematode body with a label "*Hemiurus luehei* ex *Ophidion rochei*, stomach; the Black Sea near Sevastopol" provided to them by the colleagues from the A.O. Kovalevsky Institute of Biology of the Southern Seas (Sevastopol). The researchers were not able to verify the species affiliation of this partial specimen. Dr Gordeev suggests that in fact this fragment belongs to a representative of the genus *Lecithochirium*.

Fig. 1. Stages of *Hemiurus levinseni* life cycle (DIC). A — sexual adult; B–E — daughter sporocyst; B — anterior part of body; C — posterior part of body; D — anterior end with birth pore; E — infective cercariae inside sporocyst.

Abbreviations: bp — birth pore; ec — ecsoma; os — oral sucker; ov — ovary; pp — pars prostatica; ss — sinus-sac; sv — seminal vesicle; te — testes; ut — uterus; vi — vitellarium; vs — ventral sucker. Scale bars: A–C — 100 µm; D, E — 50 µm.

Рис. 1. Стадии жизненного цикла *Hemiurus levinseni* (DIC). A — марита; B–E — дочерние спороцисты; B — передняя часть тела; C — задняя часть тела; D — передний конец тела с родильной порой; E — инвазионные церкарии внутри спороцисты.

Обозначения: bp — родильная пора; ec — эксома; os — ротовая присоска; ov — яичник; pp — простатическая часть; ss — бурса синуса; sv — семенной пузырёк; te — семенники; ut — матка; vi — желточники; vs — брюшная присоска. Масштаб: A–C — 100 мкм; D, E — 50 мкм.

3.0 (Guindon *et al.*, 2010) with 1000 bootstrap replicates. The BI tree was constructed in MrBayes v.3.2.6 with 10 000 000 generations (Huelsenbeck, Ronquist, 2001; Ronquist, Huelsenbeck, 2003).

Results

We found sexual adults of *Hemiurus levinseni* (Fig. 1A) in Atlantic cod, navaga, shorthorn sculpin, herring, common dab, European flounder, and Arctic flounder.

Of 34 *Cylichna alba* collected in June 2019 one (3%) was infected with sporocysts containing infective cystophorous cercariae (Fig. 1B–E). Of 82 *C. alba* collected in August 2019 two (2%) were infected with similar sporocysts though these contained underdeveloped cystophorous cercariae.

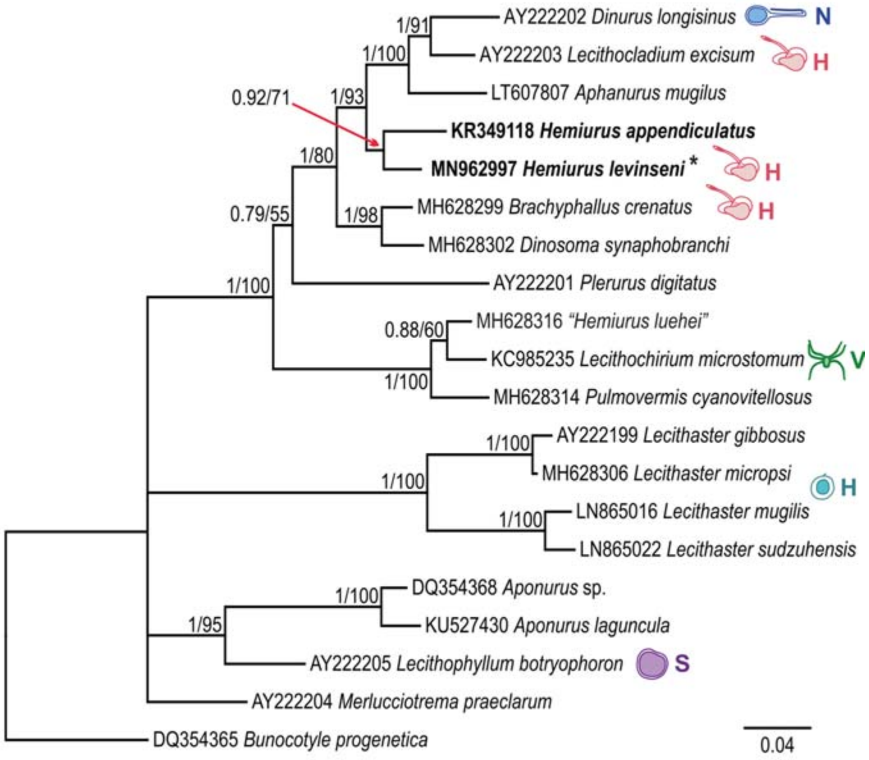

Fig. 2. Phylogenetic tree highlighting the position of *Hemiurus* based on 28S rDNA sequences, built with Bayesian inference (BI). Posterior probabilities are printed at nodes, supplemented with bootstrap support values computed for the same nodes with the ML method (BI/ML). Species names are preceded by the GenBank accession numbers. Asterisk indicates the sequence obtained in this study. Representatives of the genus *Hemiurus* are in bold (MH628316 does not belong to this genus (see "Material and Methods")). Available information on cercariae morphology (icons, see text for details) and taxonomic affinities of the first intermediate hosts (H — Heterobranchia, N — Neogastropoda, V — Vetigastropoda, S — Scaphopoda) is mapped on the tree next to relevant genera (except for *Dinurus longisinus*, where data on the representative of a closely related genus (*Tubulovesicula pinguis*) are mapped). Scale bar shows substitutions per site.

Рис. 2. Филогенетическое древо, построенное методом Байеса (BI) на основе последовательностей 28S рДНК и показывающее положение рода *Hemiurus*. В узлах отмечены апостериорные вероятности и значения бутстреп-поддержки (ML), полученные для того же набора данных методом максимального правдоподобия (BI/ML). Названия видов приведены с номерами из GenBank. Звёздочкой отмечена последовательность, полученная в этом исследовании. Представители рода *Hemiurus* выделены полужирным шрифтом (MH628316 не относится к данному роду (см. «Материал и методы»)). Имеющиеся данные по морфологии церкарий (пояснения значков см. в тексте) и таксономической принадлежности первых промежуточных хозяев (H — Heterobranchia, N — Neogastropoda, V — Vetigastropoda, S — Scaphopoda) отмечены на дереве рядом с соответствующими родами (кроме *Dinurus longisinus*, где использованы данные по представителю близкого рода — *Tubulovesicula pinguis*). Масштабная линейка показывает количество замен на одну позицию последовательности.

Molecular data

We obtained LSU sequences for each sample: four D2 fragments (490–511 bp long) and six D1–D3 fragments (1167–1168 bp long). Pairwise identity of these sequences was 99.9%, with two ambiguities and two possible substitutions: G/A in 305.48.c and 284.48.c (position 438) and A/G in 294.48.c and 295.48.c (position 697). The closest BLAST hit was *Hemiurus appendiculatus*, a congener that differed from

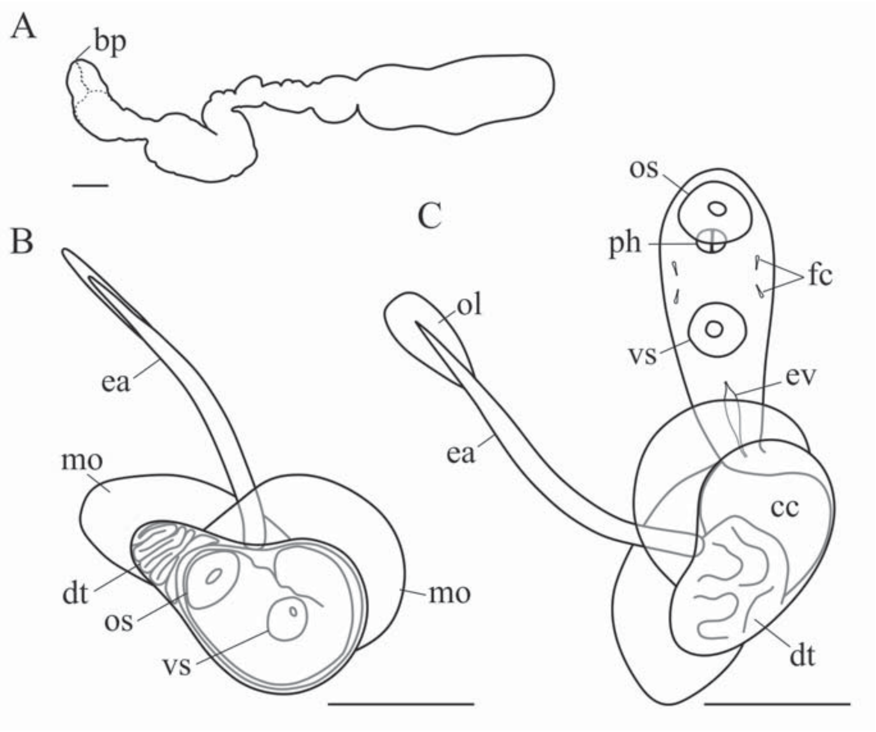

Fig. 3. Intramolluscan stages of *Hemiurus levinseni* life cycle. A — daughter sporocyst; B — infective cercaria; C — pre-infective cercaria.

Abbreviations: bp — birth pore; cc — caudal cyst; dt — delivery tube; ea — excretory appendage; ev — excretory vesicle (distal part); fc — flame cells; mo — membranous outgrowths; ol — oar-shaped lobe; os — oral sucker; ph — pharynx; vs — ventral sucker. Scale bars: A — 100 μm; B, C — 50 μm.

Рис. 3. Стадии жизненного цикла *Hemiurus levinseni* внутри первого промежуточного хозяина. A — дочерняя спороциста; B — инвазионная церкария; C — преинвазионная церкария.

Обозначения: bp — родильная пора; cc — хвостовая капсула; dt — извергательная трубка; ea — хвостовой придаток; ev — мочевой пузырёк (дистальная часть); fc — циртоциты; mo — гребневидные выросты капсулы; ol — веслообразная лопасть; os — ротовая присоска; ph — глотка; vs — брюшная присоска. Масштаб: A — 100 мкм; B, C — 50 мкм.

our sequences by 47–48 nucleotide substitutions (907 bp alignment). Two isolates of *H. appendiculatus* from *Alosa alosa* (KR349118) and *A. fallax* (KR349121) in western Spain had identical sequences.

We also obtained 612–667 bp sequences of the ITS region for each sample. They covered the partial 5.8S rDNA (140 bp), the complete ITS2 (451 bp) and 21–76 bp corresponding to the start of the 28S rDNA. The alignment trimmed to the shortest sequence was 612 bp long and had a 99.9% pairwise identity. Single ambiguous positions were found in sequences from three isolates: 1.49.c, 284.48.c and 3.49.c. Sequence from isolate 295.48.c had two substitutions: T/C in the position 295 and A/T in the position 513. Among the Hemiuroidea, replicate sequences of the ITS region are available for two species of the Lecithasteridae. Sequences from three isolates of *Hysterolecithoides epinepheli* were identical. In *Lecithaster confusus*, the pairwise identity was 99% among 7 isolates for a 643 bp alignment.

Phylogenetic trees inferred with ML and BI revealed congruent topology. The Bayesian tree is presented in Figure 2; the posterior probabilities are supplemented with the support values computed for the same nodes with the ML method. Our results were generally consistent with data from Sokolov *et al*. (2019) but had lower resolution. The composition of the Hemiuridae and relationships within this family on our tree agree with previous findings. Our sample of *H. levinseni* groups together with *H. appendiculatus*, but not with "*H. luehei*" (MH628316) apparently due to the misidentification of this sample in Sokolov *et al*. (2019) (see "Material and Methods").

Sporocysts

Each infected *C. alba* contained 30–40 daughter sporocysts. Sporocysts filled with cercariae at various stages of development, ~1500 long, 83–199 (126) wide, white, with constrictions (Fig. 1B–E, 3A). Only the specimen collected in June 2019 contained fully formed cercariae with body withdrawn into caudal cyst. Young daughter sporocysts containing only early embryos of cercariae 616–754 (690) long, 46–92 (71) wide, constrictions not prominent.

Cercariae

Early developmental stages of the cercariae are represented by germinal balls which later become divided into two parts: the cercarial body and the tail primordium. Next, the latter is divided into three lobes: the posterior one will become the motile excretory appendage, and two lateral lobes — the delivery tube and the caudal cyst itself (Fig. 4A). Due to asymmetrical growth of the caudal cyst, the base of the motile appendage moves to a side, and the base of the delivery tube primordium submerges into the cyst (Fig. 4B–F). The delivery tube develops as a column of cells, later their nuclei degenerate, the tube becomes hollow and withdraws into the cyst.

Pre-infective cercariae have the delivery tube coiled inside the cyst, but the cercarial body is still outside (Fig. 3C, 4G). Body 99–134 (110) long, 31–38 (35) wide. Oral sucker 19–22 × 21–23 (21 × 22), ventral sucker 16–19 × 18–21 (18 × 20), pharynx 8–10 × 9–12 (9 × 11). Excretory formula 2(1+1) = 4.

The caudal cyst of infective cercariae is pear-shaped, with one side concave, 80–86 (83) long, 49–54 (52) wide (Fig. 3B, 4H, 5A). Its surface is finely folded. The aperture is located at the wide part of the cyst, near the concave surface (Fig. 5B). Two asymmetrical membranous outgrowths go along the concave surface. The motile excretory appendage is attached in the middle of the concave surface, between the outgrowths (Fig. 5A). It is 124–150 (134) long, the flattened oar-shaped distal part is 42–52 (48) long, 14–22 (19) wide, serrated apically (Fig. 5C). The stem is oval in cross-section, 10–11 × 6–7 (11 × 6), annulated (Fig. 5A, C).

In infective cercariae the narrow part of the caudal cyst is occupied by the tightly packed delivery tube. The cercarial body is withdrawn into the cyst and already detached from it; it lies with the anterior end close to the delivery tube.

Discussion

Using morphological and molecular data, we have partially elucidated the life cycle of *Hemiurus levinseni*. The identification of this species was based on the morphology of the sexual adults: all of them comply with the diagnosis of *H. levinseni*. It is also the only species of *Hemiurus* previously found in the White Sea (Shulman, Shulman-Albova, 1953), but three other species occur in the north-east Atlantic: *Hemiurus communis* Odhner, 1905, *H. appendiculatus*, and *H. luehei*. *H. levinseni* is readily distinguished through morphology from all of them. The most apparent character is the sucker ratio which is 1 : < 1.1 in *H. levinseni* and 1 : >1.43 in the other species (Gibson, Bray, 1986; Bray, Cribb, 2005). To match the sexual adults of *H. levinseni* and its intramolluscan stages, we used DNA sequencing.

Sequences of the partial 28S rDNA and 5.8S rDNA+ITS2 suggest that all our samples belong to a single species, and thus constitute the life cycle stages of *H. levinseni*. The only possible concern is that both fragments showed intraspecific variability, although very low. We believe that this is not sufficient to question the conspecificity of our material at this point. We have sequenced ten replicates for the species, and larger sample size increases the chances to

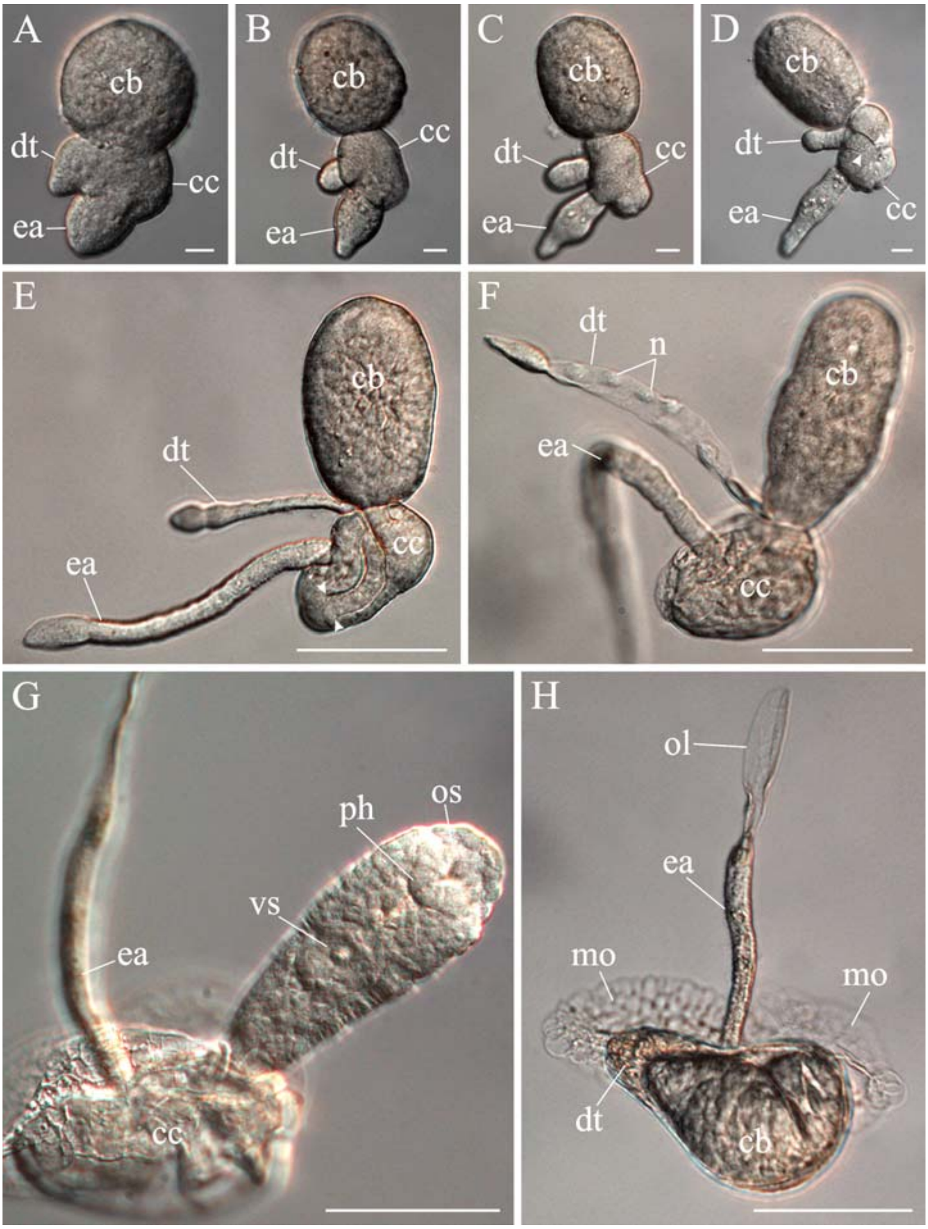

Fig. 4. Development of *Hemiurus levinseni* cercariae (DIC). A–F — sequential stages of cercariae development, arrowheads on D and E mark the base of delivery tube; G — pre-infective cercaria; H — infective cercaria.

Abbreviations: cb — cercarial body; cc — caudal cyst; dt — delivery tube; ea — excretory appendage; mo — membranous outgrowths; n — nuclei; ol — oar-shaped lobe; os — oral sucker; ph — pharynx; vs — ventral sucker. Scale bars: A–D — 10 µm; E–H — 50 µm.

Рис. 4. Развитие церкарий *Hemiurus levinseni* (DIC). A–F — последовательные стадии развития церкарий, стрелками на D и E отмечено основание извергательной трубки; G — преинвазионная церкария; H — инвазионная церкария.

Обозначения: cb — тело церкарии; cc — хвостовая капсула; dt — извергательная трубка; ea — хвостовой придаток; mo — гребневидные выросты капсулы; n — ядра; ol — веслообразная лопасть; os — ротовая присоска; ph — глотка; vs — брюшная присоска. Масштаб: A–D — 10 мкм; E–H — 50 мкм.

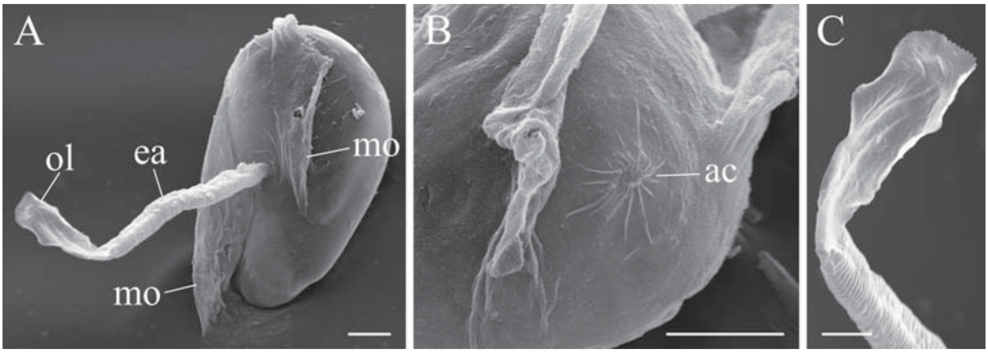

Fig. 5. Infective cercariae of *Hemiurus levinseni* (SEM). A — general view; B — aperture of caudal cyst; C — oar-shaped lobe on excretory appendage.

Abbreviations: ac — aperture of caudal cyst; ea — excretory appendage; mo — membranous outgrowths; ol — oar-shaped lobe. Scale bars: A, B — 10 µm; C — 5 µm.

Рис. 5. Инвазионные церкарии *Hemiurus levinseni* (SEM). A — общий вид; B — отверстие хвостовой капсулы; C — веслообразная лопасть на хвостовом придатке.

Обозначения: ac — отверстие хвостовой капсулы; ea — хвостовой придаток; mo — гребневидные выросты капсулы; ol — веслообразная лопасть. Масштаб: A, B — 10 мкм; C — 5 мкм.

detect intraspecific variability. Also, this is not a unique case: for example, there is variation within another hemiuroidean *Lecithaster confusus* (Atopkin *et al.,* 2018). Importantly, the few detected substitutions and ambiguities in the 28S rDNA and in the ITS2 are not consistent with each other. The reasons for the genetic variation that we observed within *H. levinseni* may get clarified in future.

In the White Sea two types of cystophorous cercariae have been described from the first intermediate hosts so far (Chubrik, 1966). The cercariae from *Cryptonatica affinis* (Gmelin, 1791) were proposed to belong to *Derogenes varicus* (Müller, 1784) Looss, 1901 and *Lecithaster* sp. (Chubrik, 1966; Timofeeva, 1976; Køie, 1979). Meanwhile, it is not *D. varicus* or *Lecithaster* sp. but two species of the family Hemiuridae (*Hemiurus levinseni* and *Brachyphallus crenatus* (Rudolphi, 1802) Odhner, 1905) which demonstrate the highest intensity of fish infection comparing to the other hemiuroideans in the White Sea (Shulman, Shulman-Albova, 1953). Thus, the knowledge of their life cycles is important to estimate the impact of digeneans on the White Sea communities. The life cycle of *B. crenatus* was established by M. Køie (1992), whereas data on *H. levinseni* in intermediate hosts are fragmentary. Metacercariae of *H. levinseni* were recovered from chaetognaths *Parasagitta elegans* (Verrill, 1873) in the White Sea (Kulachkova, 1972) and in the Gulf of Saint Lawrence (Weinstein, 1966, 1967). In chaetognaths many metacercariae become sexually mature and produce eggs (Kulachkova, 1972). As *H. levinseni* is the only species of this genus reported from the White Sea, the records of Walter *et al*. (1979) on *Hemiurus* sp. metacercariae from several copepod species probably also refer to *H. levinseni*.

Based on the previous data and our results, the life cycle of *H. levinseni* proceeds as follows. The gastropods *Cylichna alba* serve as first intermediate hosts; daughter sporocysts producing cystophorous cercariae develop in their visceral mass. After emergence, cystophorous cercariae should be grabbed by planktonic copepods, which become infected by means of the delivery tube injection mechanism. Metacercariae develop in the haemocoel of the copepods. Chaetognaths probably acquire metacercariae by ingesting copepods. Fishes get infected by feeding on copepods, chaetognaths, or smaller fish. The sexual maturation of metacercariae may occur both in fishes and chaetognaths. Therefore, the life cycle of *H. levinseni* in outline is similar to those of *H. luehei* and *H. communis* (Køie, 1990b, 1995). However, the first intermediate hosts highlight the distinctness of our material from *H. luehei* and *H. communis*: all of them belong to the order Cephalaspidea, but the superfamilies (Philinoidea and Bulloidea) are different from what we have shown for *H. levinseni* (Cylichnoidea).

The early development of cystophorous cercariae is quite uniform in all the cases studied (Hunninen, Cable, 1943; Matthews, 1981b; Køie, Gibson, 1991; etc.). In *H. levinseni* it goes almost the same way as previously described for *H. communis* (Køie, 1995). The main difference is that cercariae of *H. communis* become infective shortly after emergence from the sporocyst when the cercarial body retracts into the caudal cyst (Køie, 1995), while we found infective cercariae inside the sporocysts of *H. levinseni*. The body size in pre-infective cercariae and the size of the caudal cyst of *H. levinseni* are similar to *H. luehei* and *H. communis* (Køie, 1990b, 1995). However, the motile appendage of *H. levinseni* is substantially shorter than those of *H. luehei* and *H. communis*. Unfortunately, we did not have a chance to observe eversion of the delivery tube in infective cercariae of *H. levinseni*, thus the number and shape of extensions on the fully formed delivery tube — one of the characters Køie (1995) used to differentiate cercariae of *Hemiurus* — is unknown.

In our phylogenetic tree *H. levinseni* forms a clade with *H. appendiculatus*, the type species of the genus. The position of the genus *Hemiurus* on our LSU-based tree (*H. levinseni* + *H. appendiculatus* clade) together with genera *Dinurus* and *Lecithocladium* is the same as on the 18S rDNA (SSU)-based trees in Pankov *et al.,* 2006 (which included *H. communis*) and Bao *et al.,* 2015 (which included *H. communis* and *H. appendiculatus*). Erroneous labeling of MH628316 (Sokolov *et al.,* 2019) as "*H. luehei*" (see "Material and Methods") explains its grouping with *Lecithochirium* and *Pulmovermis*. Taking this into account, we do not consider *H. luehei* in the following discussion on the life cycles.

All the available data on the taxonomic affinity of the first intermediate host and cercariae morphology for the hemiuroideans included in our phylogenetic analysis are depicted in Figure 2. This information refers to exact genera except for *Dinurus longisinus* Looss, 1907 — here we used data on a representative of a closely related genus (according to Gibson, 2002) *Tubulovesicula pinguis* (Linton, 1940) Manter, 1947. The pattern shows that both the first intermediate hosts (ord. Cephalaspidea) and the morphology of cystophorous cercariae (pyriform caudal cyst with two membranous outgrowths, motile excretory appendage with oar-shaped distal part) bring together *Hemiurus*, *Brachyphallus crenatus* and *Lecithocladium excisum* (Rudolphi, 1819) Lühe, 1901 (Køie, 1990b, 1991, 1992, 1995). Other studied hemiurids and their close relatives lecithasterids have immotile cercariae which lose excretory appendage during development (Hunninen, Cable, 1943; Stunkard, 1980; Matthews, Matthews, 1988; Køie, 1989; Køie, 1990a; Køie *et al.,* 2002). However, all of them are quite different in morphology. To discuss which state is plesiomorphic, a broader context is needed.

Among the rest of the taxa from Clade B of Sokolov et al. (2019), the Bunocotylidae have a slightly motile caudal appendage (Chabaud, Buttner, 1954) and cercariae of the Isoparorchiidae are immotile (Ito, 1953; Shimazu *et al.,* 2014). Outside Clade B of Sokolov *et al*. (2019) there are many taxa with unknown cercariae (Hirudinellidae, Didymozoidae, Gonocercidae, Accacoeliidae). Only for the Derogenidae cercariae are described: both immotile (Goater *et al.,* 1990) and ones which possess motile excretory appendage, though not with an oar-shaped lobe, but furcate (Køie, 1979). This makes us suppose that the loss of an excretory appendage is probably a secondary event which occurred in the evolution of the Hemiuroidea multiple times: this would let them save limited energy resources and "make a bet" on the longevity of cercariae.

The first intermediate hosts of hemiurids and lecithasterids are of very different taxonomic affinity, including the Heterobranchia, Neogastropoda, Vetigastropoda, and even Scaphopoda. This makes one suspect several host-switch events in the evolution of their life cycles. We may also assume that heterobranchs from the order Cephalaspidea were parasitized by a common ancestor of a large branch of the Hemiuridae including *Brachyphallus*, *Lecithocladium* and *Hemiurus*. Evolution of life cycle traits within the Hemiuroidea will become more intelligible as new data on various genera of this diverse group appear.

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