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Research paper

Phylogenetic position, complete larval development and larval sexual dimorphism in a rhizocephalan barnacle, *Lernaeodiscus rybakovi* sp. nov. (Cirripedia: Rhizocephala: Peltogastridae), parasitizing the crab *Pachycheles stevensii* Stimpson, 1858 (Decapoda: Anomura: Porcellanidae)

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ABSTRACT

Molecular and morphological methods are used to describe *Lernaeodiscus rybakovi*, a new rhizocephalan species parasitizing the porcellanid crab *Pachycheles stevensii* Stimpson, 1858, collected in Russian waters of the Sea of Japan. Molecular analysis of three species, including the new one, confirms the monophyly of the genus *Lernaeodicus* Müller, 1862 and its recent transfer to the family Peltogastridae. The main morphological features of the new species are also common with characters of the other species of the genus *Lernaeodiscus* Müller, 1862. Externa of *L. rybakovi* differs from the well studied *Lernaeodiscus porcellanae* from the E. Pacific by molecular markers, color and the absence of pronounced marginal lobes. Retinacula on the internal cuticle of *L. rybakovi* are found in the genus *Lernaeodiscus* for the first time. Some of the female hosts with adult externae were unusual in also carrying their own eggs on the pleopods, a rare situation among rhizocephalans. The complete larval development in the genus *Lernaeodiscus* is described here for the first time and includes five naupliar and one cypris instar. The main morphological features of *Lernaeodiscus* nauplii (the presence of flotation collar, morphology of the frontolateral horns and furcal spines, and the arrangement of dorsal setae on the shield head) are common with those of other known peltogastrid larvae, but also resemble nauplii of *Peltogasterella* in the presence of naupliar eyes and thin structure of the flotation collar. The sizes of male and female cyprids of *L. rybakovi* overlap slightly. In summer months, larval sex ratio is male-biased. We briefly review important larval characters in the Rhizocephala.

1. Introduction

Based on the phylogenetic analysis and taxonomic revision in Høeg et al. (2019a) the genus *Lernaeodiscus* is now part of an apparently monophyletic Peltogastridae. At present, the genus *Lernaeodiscus* includes eight species: *Lernaeodiscus ingolfi* Boschma, 1928, *L. okadai* Boschma, 1935, *L. porcellanae* Müller, 1862, *L. pusillus* Boschma, 1950, *L. schmitti* Reinhard, 1950, *L. squamiferae* Pérez, 1922, *L. tableta* Boyko & Harvey, 2000, and *L. triangularis* Lützen, 1985 (WoRMS 2018).

L. porcellanae Müller, 1862, the type species of the genus, is the best studied *Lernaeodiscus* species and a "model species" for reproductive biology, larval biology and life cycles in the Rhizocephala (Høeg & Lützen 1995), but some doubt exists whether the well studied specimens from S. California belong the same species as those originally described by Müller (1862) from the Atlantic

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(see Boyko & Harvey 2000). At present it is advisable to refer to Pacific specimens as *L.* aff. *porcellanae*.

We have collected numerous *Pachycheles stevensii* Stimpson, 1858 (Porcellanidae) infested with a species of *Lernaeodiscus* in Russian waters of the Sea of Japan. Detailed molecular, morphological and electron microscopic examinations revealed that this rhizocephalan belongs neither to *L. porcellanae* nor to *Lernaeodiscus* aff. *porcellanae* or to any other known species of the genus. Here we describe these specimens as a new species based on a detailed morphological description and molecular analysis of the new species and two other members of the genus *Lernaeodiscus*. Furthermore, we offer for the first time a description of the complete larval development in the genus. The cyprids are sexually dimorphic in size and antennular morphology and can occur in broods with mixed sex ratios as found for other species of rhizocephalans with a kentrogonid type host invasion. We also review important characters in rhizocephalan larval morphology.

2. Material and methods

2.1. Sampling

Specimens of *P. stevensii* Stimpson, 1858 infested with *Lernaeodiscus* sp. nov. were collected by SCUBA diving at a depth of 1.5–6 m. This species was found in Vostok Bay (Peter the Great Bay, Sea of Japan) in June–August 2005, in July 2017, in July 2018, and at Popov Island (Peter the Great Bay, Sea of Japan) in September 2019. The type material was fixed in 96° ethanol. The Holotype with rhizocephalan the externa undetached was deposited at the Museum of the National Scientific Center of Marine Biology, Vladivostok, Russia (catalogue number 39151) (Fig. 1A). Additional specimens were deposited as paratypes as specified below.

2.2. Molecular techniques

Høeg et al. (2019a, b) performed the hitherto most comprehensive molecular analyses of the Rhizocephala using molecular methods. Here we use subset of their species in new analysis with the specific focus of confirming our samples as a new species and testing its position within the Rhizocephala, including verification of *Lernaeodiscus* as a monophyletic genus.

2.3. DNA extraction and gene amplification

Total genomic DNA was extracted from ~ 1mm^3 of tissue from the mantle of individual externae for *Lernaeodiscus porcellanae* and *L. rybakovi* using the QiagenDNeasy Blood & Tissue Kit following the QiagenDNeasy Protocol for Animal Tissues 07/2006. Data for *L. ingolfi* was available from existing next-generation sequencing work (unpublished data) while, for the other two species, two nuclear and two mitochondrial genes were amplified and sequenced using the primers indicated in Table 1. Almost complete coverage (~1800bp, unaligned sequence) of the nuclear 18S ribosomal RNA gene was achieved together with a ~1400-1800bp fragment of nuclear 28S. For the mitochondrial 16S rDNA two primer pairs were utilised, yielding approximately 500bp and 360bp (H621/L12247L and 16Sar-L/16Sbr-H, respectively). Mitochondrial COI amplification was performed using standard DNA Barcoding protocols with Folmer primers (LCO1490 and HCO2198). All PCR reactions were carried out using a Bio-Rad C1000 Thermal Cycler in 25 μl volumes containing 1 μl of DNA extract, 2.5 μl 10× PCR buffer, 1.2 μl of dNTP mixture (2.5 μM each), 1 μl of each 10 μM primer and 0.75U of Takara polymerase. Conditions for all amplifications were as follows: initial denaturation at 94 °C for 5 min then 35 cycles of 30s denaturation at 94 °C, 1 min primer annealing at 52 °C and 1 min extension at 72 °C, with a final 7 min 72 °C extension. All PCR products were visualized on 1% agarose gels and stored at 4 °C prior to purification and sequencing. PCR products were cleaned by the addition of 0.1 μl (1U) Exonuclease I, 1 μl (1U) of Shrimp Alkaline Phosphatase and 0.9 μl of ddH_2O to 8ul of PCR product. This was followed by incubation at 37 °C for 30 min and deactivation of the enzymes at 85 °C for 15 min. Sequence reactions were performed using the BigDye v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc., Norwalk, CT, USA) with the same primers used for initial PCR amplification. Both strands of all PCR products were sequenced using an ABI 3730 capillary sequencer.

2.4. Sequence handling and phylogenetic analyses

All sample PCR products were sequenced for both heavy and light strands, in order to improve accuracy, and aligned using Clustal W (Thompson et al., 1994) implemented in Seaview (Castresana 2000; Gouy et al., 2010) for 16S and MUSCLE (via the EMBL-EBI web service: https://www.ebi.ac.uk/Tools/msa/muscle/) for 18S, 28S and COI. Additional data for outgroup and ingroup taxa were taken from GenBank; accession numbers for all taxa included

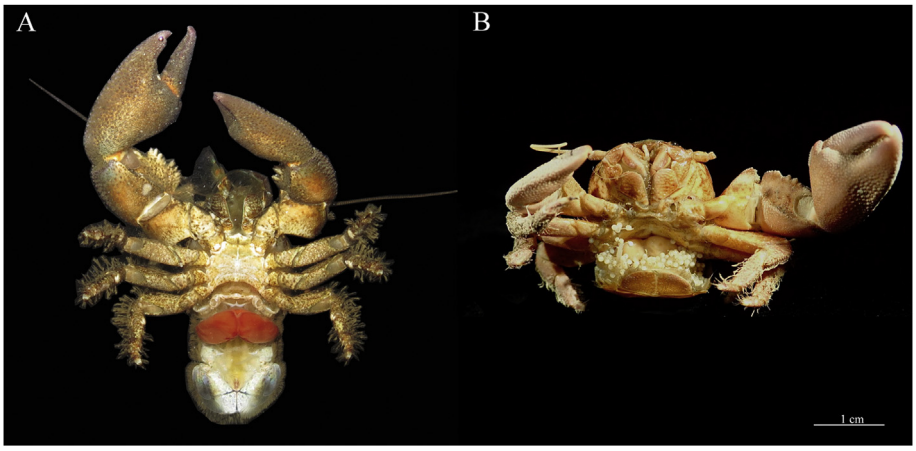

Fig. 1. The host crab, *Pachycheles stevensii*, infested by *Lernaeodiscus rybakovi* with one externa. (A) Male. (B) Female that also carries a large brood of its own embryos attached to the pleopods.

Table 1
Primer details for gene amplification (18S, 28S and 16S); all primers presented as 5' –3'. Primer directions are indicated in parentheses by F (forward) and R (reverse).

Gene	Primer	Sequence (5′ to 3′)	Reference
18S	329 (F)	TAATGATCCTTCCGCAGGTT	Spears et al. (1992)
	a- (R)	CAGCMGCCGCGGTAATWC	Spears et al. (1992)
	345+ (F)	GCATCGTTTAHGGTT	Spears et al. (1992)
	UnivF15 (R)	CTGCCAGTAGTCATATGC	Frischer et al. (2002)
28S	1274 (F)	GACCCGTCTTGAAACACGGA	Nunn et al., 1996 [= D3A]
	28S1a (F)	CCCSCGTAAYTTAAGCATAT	Friedrich & Tautz 1995 [= D1F]
	FF (R)	GGTGAGTTGTTACACACTCCTTAG	Modified from Hillis & Dixon 1991
	28S4 (R)	CCTTGGTCCGTGTTTCAAGAC	Crandall et al., 2000 [= rD4b]
16S	16Sar-L (F)	CGCCTGTTTATCAAAAACAT	Palumbi (1991)
	16Sbr-H (R)	CCGGTCTGAACTCAGATCACGT	Palumbi (1991)
	H621 (F)	CYGTGCAAAGGTAGCATA	Tsuchida et al. (2006)
	L12247L (R)	TTAATYCAACATCGAGGTCRC	Tsuchida et al. (2006)

in alignments and phylogenetic analyses can be found in Table 2. Following alignment, lengths of individual gene datasets were 1.895 bp for 18S, 1.903 bp for 28S, 640 bp for 16S and 703 bp for COI; the concatenated data set was 5.262 bp (see Table 2 for accession numbers). Best-fit nucleotide substitution models were inferred for each individual gene and the concatenated dataset using JModeltest v.2.1.4 (Darriba et al., 2012) and the Smart Model Selection (SMS) option of PhyML (Guindon et al., 2010) implemented through the South of France bioinformatics platform (http://www.atgc-montpellier.fr/phyml-sms/). AIC results from JModeltest indicated the best-fit models were: TVM+I+G for 16S and COI, and TIM+I+G for 18S and 28S. SMS indicated GTR+I+G as the best-fit model for the concatenated dataset. For Bayesian analyses where a specific model could not be implemented (i.e. TIM/TVM) it was substituted by the closest available over-parameterized model, applied independently to each gene partition. Phylogenetic analyses were performed on the full concatenated data set using Bayesian methods coupled with Markov chain Monte Carlo (MCMC) inference, as implemented in MrBayes v.3.2 (Ronquist et al., 2012). For these analyses, two independent runs were performed, each consisting of four chains and proceeding for 10 million generations sampling every 1000 generations. Results were visualized in Tracer v.1.5.0 (Rambaut & Drummond 2007), and proper mixing of the MCMC was assessed by calculating the effective sampling size (ESS) for each parameter. For each data set, the maximum clade credibility tree (MCC; the tree with the largest product of posterior clade probabilities) was selected from the posterior tree distribution (after removal of 25% burn-in). Maximum Likelihood analyses were performed using PhyML with default settings and the model selected by SMS. Clade support was assessed using the nonparametric bootstrap procedure (Felsenstein 1985) with 100 replicates.

2.5. Investigation of the externa

Ten externae of rhizocephalans collected in 2018 were detached from the host crabs and fixed in 4% formaldehyde. We measured the carapace width of the host crabs, drew outlines of the parasite externae and recorded their width (the greatest distance between lateral margins).

Two externae of rhizocephalans collected in 2018 were detached from the host crabs and fixed in Bouin solution, dehydrated through a gradient ethanol-xylene series and embedded in paraffin. Transverse and longitudinal sections, 6 μm thick, were stained with Ehrlich hematoxylin. The external cuticle of the mantle of two externae collected in 2018 was fixed in 70% ethanol and prepared for SEM as described below for larvae.

For describing the externae we follow the terminology of Høeg & Lützen (1995) and Øksnebjerg (2000). The orientation of

Table 2
Genbank accession details for all data used in phylogenetic analyses.

Taxon	COI	16S	18S	28S
Amphibalanus amphitrite	KM974415	JQ035493	KM974369	KM217530
Heterosaccus californicus	–	AY520756	AY265359	AY520623
Heterosaccus dollfusi	AY117691	FJ481949	EU082413	EU082333
Heterosaccus lunatus	DQ059778	FJ481947	EU082414	EU082334
Lepas anatifera	GU993603	GU993670	FJ906773	KU052603
Lernaeodiscus porcellanae	MN605965	MN625174	MN625166	MN625171
Lernaeodiscus ingolfi	MN605966	MN625175	MN625167	MN625172
Lernaeodiscus rybakovi	MN605964	MN625173	MN625165	MN625170
Loxothylacus panopaei	KU905819	FJ481956	AY265364	–
Loxothylacus texanus	HQ848069	–	L26517	–
Mycetomorpha vancouverensis	–	MH974513	MH974514	MH974515
Peltogaster paguri	KT209453	FJ481958	DQ826570	EU082335
Peltogasterella sulcata	–	FJ481955	DQ826572	EU082336
Polyascus gregarius	–	JN616263	AY265363	GU190705
Polyascus planus	–	FJ481954	AY265368	GU190698
Polyascus polygeneus	–	–	AY265362	GU190704
Sacculina carcini	KT208581	FJ481957	AY265366	AY520622
Parasacculina leptodiae	–	FJ481952	AY265365	–
Parasacculina sinensis	–	–	AY265360	GU190707
Sacculina upogebiae	–	KF539762	KF539758	KF539760
Parasacculina yatsui	AB197809	MG822656	MG604305	MG604305
Scalpellum scalpellum	KT209468	–	EU082388	EU082307
Semibalanus balanoides	MG314454	AM497883	DQ777622	AY520592
Septosaccus rodriguezii	–	–	DQ826571	–

rhizocephalan externae cannot be compared to other crustaceans in any meaningful way. We follow the convention that the side with the stalk (facing the host) is considered dorsal and the opposite ventral, while the position of the mantle opening marks the anterior end.

2.6. Investigation of the larvae

Infested crabs collected in 2005 and 2018 were maintained in 2 L glass vessels with aerated seawater at a temperature of 22–23 °C and a salinity of 32‰ until the larvae hatched. Newly hatched nauplii were concentrated using a point light source, collected by a pipette and transferred to 1 L glass vessels with filtered and ultraviolet (UV) sterilized sea water of the same temperature and salinity. The water in the vessels was changed daily. Nauplii were reared during 4 days to the cypris stage.

The larvae were fixed in 4% formaldehyde immediately after hatching, after 1 h passed, and later with 12 h intervals. Before fixation, cyprids were transferred into 0.3M $MgCl_2$ solution for 2 h to induce extension of their antennules. The relaxed male and female cypris larvae were separated by the structure of the antennules following Glenner et al. (1989). The outlines of the larvae were drawn under Olympus CX41 microscope with a *camera lucida*. The length of 100 cyprids, between the anterior and posterior carapace margins, was measured using an ocular micrometer.

From eight larval broods obtained in 2018, one was males only, seven were mixed with variable sex ratios. Although rhizocephalans have Genetyic Sex Determination they become sexually dimorphic only at the cypris stage (Høeg & Lützen 1995; Rybakov et al., 2003). The total length of 20 nauplii of each stage, from the anterior margin to the tips of the furcal spines, was measured using an ocular micrometer. Since none of our broods contained females only, it was accordingly impossible to determine the range of female naupliar sizes. Therefore, the size dimensions of nauplii reported here are for male larvae only.

Specimens of larval stages and the spent female were deposited at the Museum of the National Scientific Center of Marine Biology, FEB RAS, Vladivostok (catalogue number 36145).

2.7. SEM analysis

For SEM, the fixed larvae of two broods were carefully rinsed, postfixed in OsO_4.

Externa cuticle and larvae were dehydrated in an alcohol series and acetone, and critically point dried in CO_2. They were observed in either a JEOL JSM-840 SEM (Zoological Museum, University of Copenhagen) or a Zeiss Sigma 300 VP microscope (National Scientific Center of Marine Biology FEB RAS, Vladivostok).

3. Results

3.1. Molecular analysis

The molecular analysis showed the new material to be separate from both *Lernaeodiscus* aff. *porcellanae* and *L. ingolfi*, confirming its status as a new species (Fig. 2). Furthermore, all three species were recovered as a monophyletic clade, *Lernaeodiscus*, nested within the Peltogastridae, thus confirming the phylogenetic position of the genus already argued by Høeg et al. (2019a).

3.2. Taxonomy

Superorder Rhizocephala Müller, 1862.

Family Peltogastridae Lilljeborg, 1861; amended by Høeg et al., 2019a.

Genus *Lernaeodiscus* Müller, 1862.

Lernaeodiscus rybakovi sp. nov.

3.2.1. Diagnosis

The externa is large, red-orange and without marginal lobes. It is mostly single and attached on the ventral surface of the second abdominal segment of the host crab. The externa is bilobed, flattened dorsoventrally and bilaterally symmetrical. The mantle opening and the stalk are placed in the median plane and directed dorsally. The external cuticle is wrinkled but without excrescences. The internal cuticle of the mantle cavity carries with retinacula of lamp-brush type. The visceral sac has a bilobed ovary placed dorsally in the posterior part of the mantle cavity. The colleteric glands are simple and placed symmetrically about median plane in the central part of visceral sac. The paired receptacles are almost symmetrical, placed dorsally in the posterior part of the visceral sac and have a spiral shape forming nearly two turns.

3.2.2. Material examined

The holotype and five paratypes were collected in Vostok Bay (Peter the Great Bay, the Sea of Japan) at a depth of 1–4 m, 1 July 2017.

Holotype. Male *P. stevensii,* 16.2 mm in width, with externa, 11.0 mm in width, was collected on 1 July 2017 and deposited at the Museum of the National Scientific Center of Marine Biology, FEB RAS, Vladivostok (catalogue number 39151).

Paratypes. After DNA extraction, eight paratypes were deposited at the Museum of the University of Bergen, Bergen, Norway (catalogue numbers ZMBN 132207–132214).

Non-type species. Three crab specimens with externae were collected at Popov Island (Peter the Great Bay, the Sea of Japan) at a depth of 1–4 m, 10 September 2019, and deposited at the Museum of the National Scientific Center of Marine Biology, FEB RAS, Vladivostok: female *P. stevensii,* 15.0 mm in width, with an externa, 9.5 mm in width (catalogue number 39152); female *P. stevensii,* 14.6 mm in width, with an externa, 10 mm in width (catalogue number 39153); female *P. stevensii,* 12.0 mm in width, with an externa, 6 mm in width (catalogue number 39154).

3.2.3. Type locality

Vostok Bay, Peter the Great Bay, the Sea of Japan, Russia.

3.2.4. Host

L. rybakovi, sp. nov. was found on the porcelain crab *P. stevensii* Stimpson, 1858 (Anomura: Porcellanidae). Among 208 crabs, 19 specimens (9.1%) were infested by this rhizocephalan. The carapace width of the infested crabs ranged from 11 to 16 mm. Most singly infested specimens had the externa attached on the ventral surface of the second abdominal segment. The externa may also appear on the board between the first and second abdominal segment, on the first or third segments. A single *P. stevensii* carried two small externae located on the second and the third abdominal segments.

3.2.5. Distribution

The distribution of *L. rybakovi* outside Peter the Great Bay is unknown. The host crab *P. stevensii* also occurs along Japan Islands from Hokkaido to Honshu (Marin 2013).

3.2.6. Bathymetrical range

Crab *P. stevensii* occurs from 0 to 15 m depth, infested crabs are found at 1.5–6 m depth.

3.2.7. Etymology

The name of a new species is given in honour of Dr. Aleksey Rybakov (1959–2013), who devoted his life to the study of the

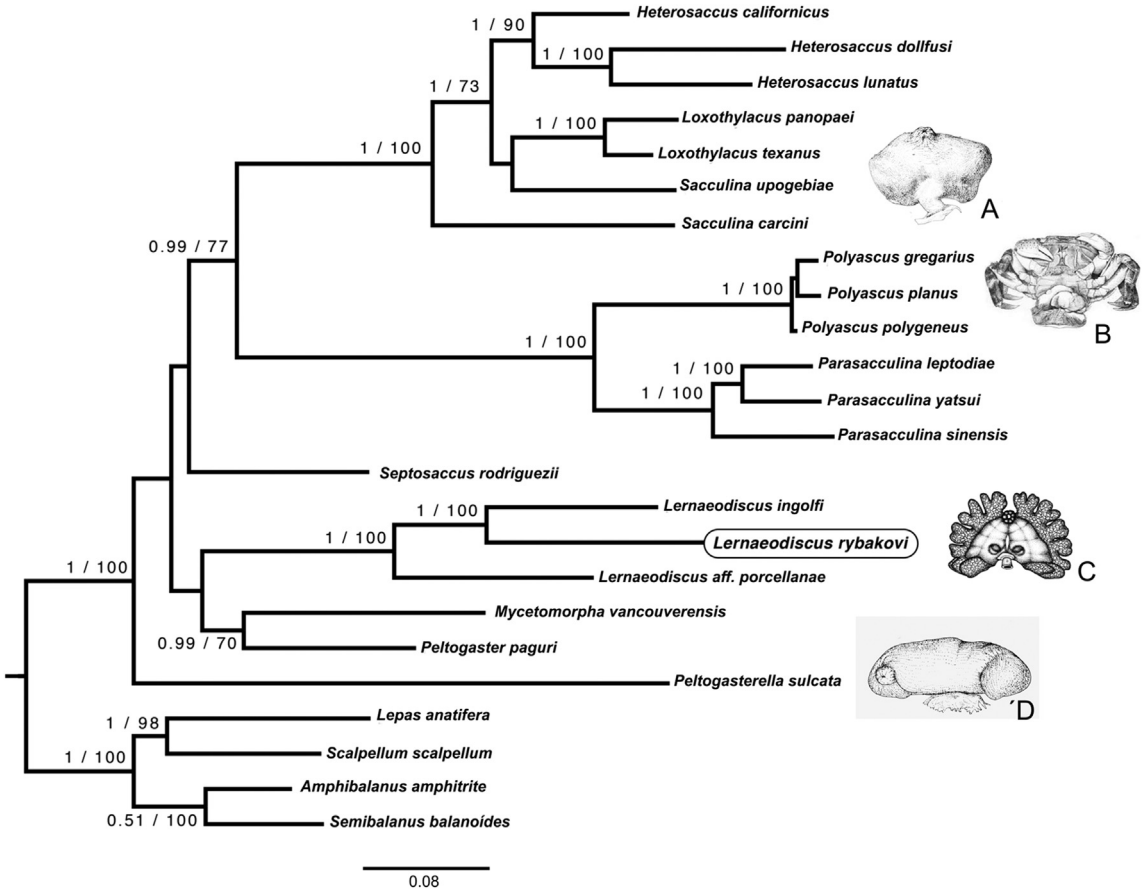

Fig. 2. Bayesian phylogenetic tree for the three-gene concatenated dataset (18S, 28S and COI). The phylogeny that shows the position of *Lernaeodiscus rybakovi* within a monophyletic *Lernaeodiscus* genus. Nodal support is indicated in the form of Bayesian posterior probabilities (PP) and maximum likelihood bootstrap values; nodes with PP values ≤ 50 have been collapsed. The thoracican barnacles *Lepas anatifera, Scapellum scalpellum*, *Amphibalanus amphitrite*, and *Semibalanus balanoides* have been used as out group in the analysis. (A) *Sacculina carcini*. (B) *Polyascus polygeneus*. (C) *Lernaeodiscus* aff. *porcellanae*. (D) *Peltogaster paguri*.

diversity of marine parasitic invertebrates, and published several bench mark papers on the biology of rhizocephalan cirripedes.

3.3. *Externa morphology*

The externa of *L. rybakovi* is large. Their size ranged from 4 to 13 mm in width. Coloration in life of immature externa is red, mature externa (with mature oocytes and advanced embryos) is red-orange (the eggs of the host crab have the same color). The externa is bilobed, flattened dorsoventrally, bilaterally symmetrical. Immature externa is trapeziform, mature externa is more oval. Marginal lobes (lappet-like extensions of the mantle) are absent (Fig. 3A). After naupliar release, the mantle becomes wrinkled but true lobes are absent as before. The mantle opening and the stalk are placed in median plane, both directed dorsally. The externa is enveloped by a mantle that is significantly thicker near the mantle opening and the stalk. The external cuticle is wrinkled but lack any papillae or excrescens (Fig. 4A). The cuticle of the mantle cavity is covered with numerous retinacula of lamp-brush type (for terminology see Rybakov & Høeg 2002). Barbed spindles can be single or grouped up to five ones (Fig. 4B and C).

Visceral sac is placed dorsally in the posterior part of the mantle cavity. The dorsal mesentery is broad and extends along almost the whole dorsal surface of visceral sac from the stalk to the mantle opening. The ventral mesentery is much narrower and shorter. In both ovary and receptacles (see below) the germ cells are generally all at virtually the same stage of development, but this stage varies between parasites according to the reproductive cycle. The bilobed ovary occupies the most part of visceral sac, and contains oocytes at various stages of development according to the stage in the reproductive cycle (Fig. 5A and B). The paired oviducts (colleteric glands) originate from the lobes of the ovary, are simple and placed symmetrically about median plane in the central part of visceral sac (Fig. 5C). Their walls are lined with glandular epithelial cells and the duct lumen is filled with secreted substance. The largest diameter of a colleteric gland duct is about 70–160 μm. The embryos develop up to the naupliar stage in the mantle cavity. In June–July, all externae were ovigerous, with embryos in different parasites being at different stages of development (Fig. 5D). In the latter stages of embryonic development, a new generation of mature oocytes are ready in the ovary to be released and fertilized by sperm from the receptacles. This happens soon after the release of the preceding brood as free-swimming nauplii.

The paired receptacles are placed dorsally in the posterior part of the visceral sac. They are almost symmetrical relative to the median plane and have a spiral shape forming nearly two turns. The receptacles are enveloped with a multilayered wall of female cells with large nuclei that presumably perform a trophic function. Male cells, originally introduced by male larvae, are situated in the central part of the receptacles and can be at different stages of development, again depending on the stage of the reproductive cycle (Fig. 5E and F). The receptacle ducts run from receptacles to

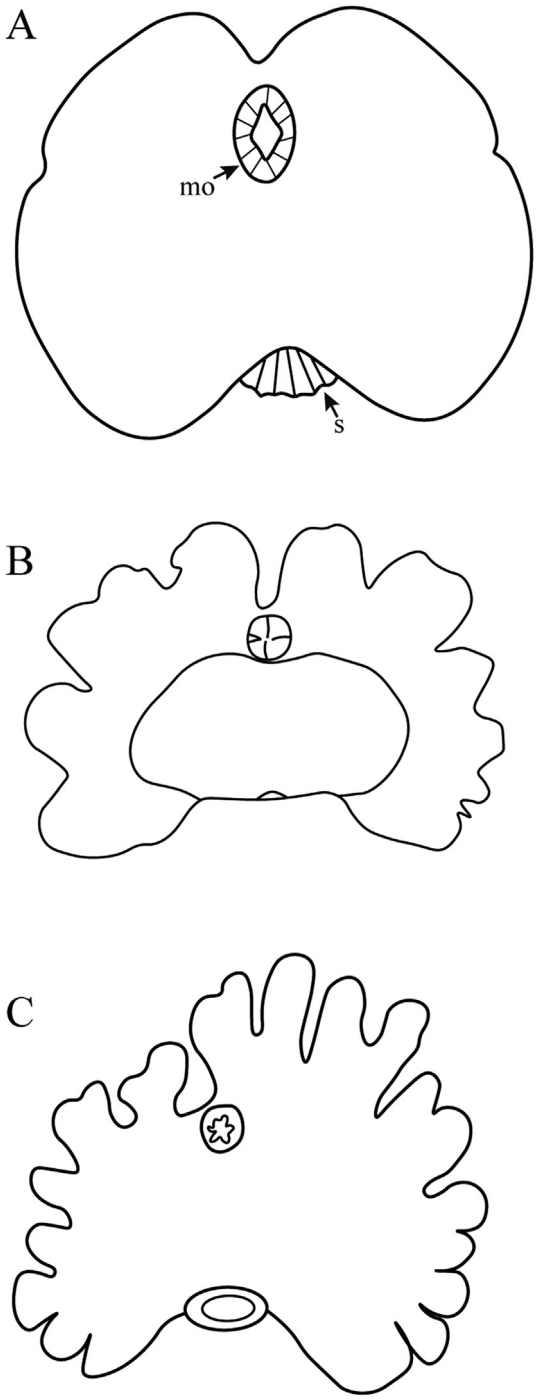

Fig. 3. Body outlines of externae (dorsal view). (A) *Lernaeodiscus rybakovi*. (B) *L. porcellanae* (after Boyko & Harvey 2000). (C) *L.* aff. *porcellanae* (after Boschma 1969). Mo, mantle opening; s, stalk.

the stalk along the dorsal surface of visceral sac, then turn to the ventral surface and open into the mantle cavity on the sides of the ventral mesentery (Fig. 5G and I). The diameter of receptacles is about 220–380 μm. The ducts are approximately two times less in diameter than receptacles themselves. As far as can be ascertained, sperm release and oviposition are near simultaneous events. A new cycle of spermatogenesis begins immediately after sperm release.

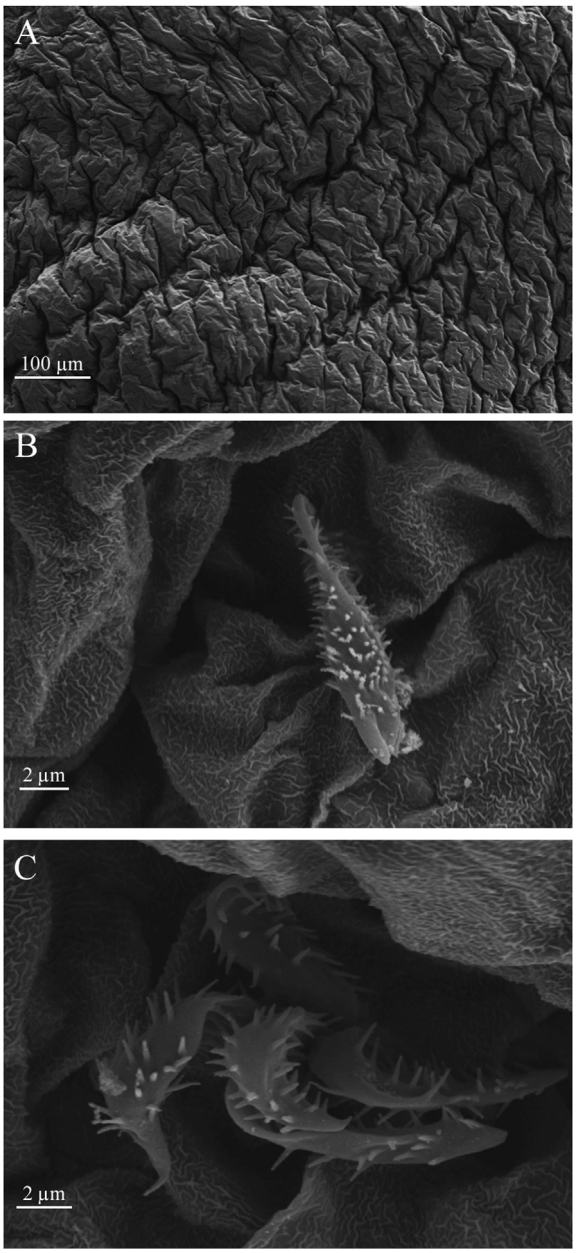

Fig. 4. SEM showing cuticle structure of the externa of *Lernaeodiscus rybakovi*. (A) External cuticle. (B) A single retinaculum. (C) A group of five retinacula.

3.4. Larval development

Larvae of *L. rybakovi* are lecithotrophic with distinct lipid globules inside the body. The larval development includes five naupliar and one cypris stage. The development to the cypris stage took 3.5–4 days at a water temperature of 22–23 °C.

Nauplii only slightly increase in size during the development, after nauplius-cyprid moult they decrease. Male larvae are slightly larger than female ones (Figs. 6 and 7). Male cyprids varies from 220 to 250 μm in length and females from 200 to 220 μm, whence the size ranges of the two sexes show some slight overlap (Fig. 7). Most likely that the sizes of male and female nauplii are also overlapped. In summer, larval sex ratio of *L. rybakovi* is male-biased (nearly 75% of male larvae in broods).

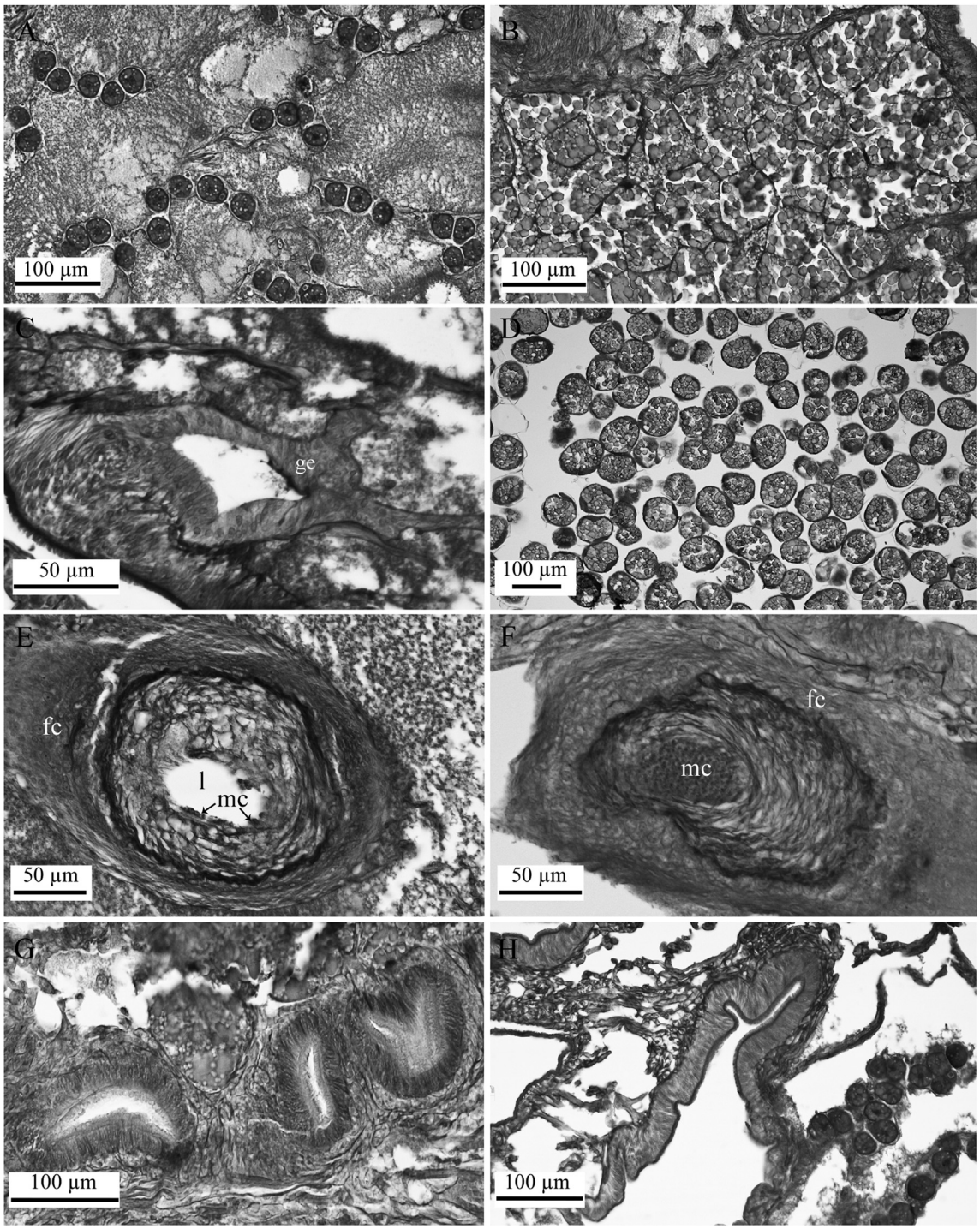

Fig. 5. Histology of the externa of *Lernaeodiscus rybakovi*. (A) Immature ovary after spawning. (B) Mature ovary before spawning. (C) Colleteric gland. (D) Embryos. (E) Receptacle after spawning. (F) Receptacle before spawning. (G) Receptacle duct. (H) Receptacle duct before outlet in the mantle cavity. Abbreviations: fc, female cells; ge, glandular epithelium; l, lumen; mc, spermatogenic cells. A–F, H, transversal sections; G, longitudinal section.

3.4.1. Nauplius I

The body shape is ovoid (Figs. 6 and 8A). A nauplius eye is present in this and all following naupliar stages including the cyprid. A floatation collar and its attachment ridge are absent. The head shield lacks setae but a single pore is present in the middle of the anterior region (Fig. 8B). The frontolateral horns are short, curve slightly backward, and terminate with the large opening of the frontal horn gland. The horns have no terminal fringes, but carry a short anterior subterminal seta with a terminal pore (Fig. 8C). A conical rudimentary labrum has small terminal opening, presumably a gland pore. The ventral side of hind body and the short furcal spines are irregularly covered with denticles. The segments of the pairs of appendages lack any hairs or spinules. The uniramous antennule consists of three segments with five plumose setae; setae 1, 2, and 5 being shorter than setae 3 and 4; setae 1–4 arise from the distal antennular segment, seta 5 from the middle one

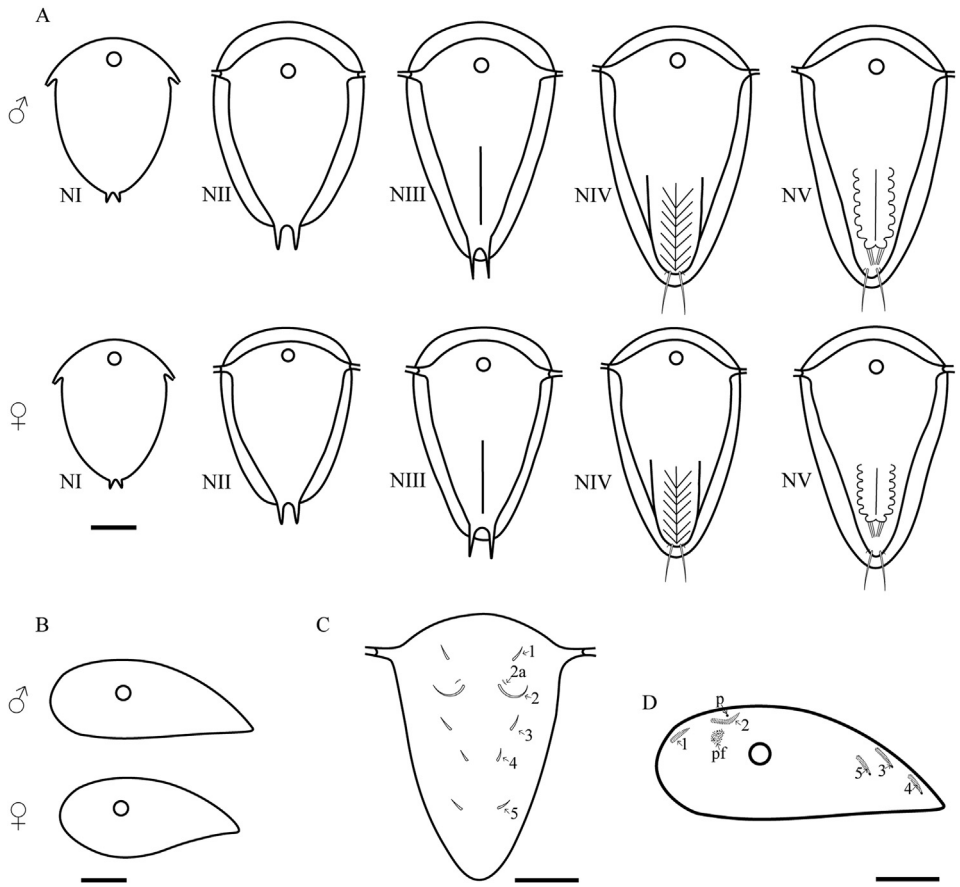

Fig. 6. Body outlines (ventral view) of male and female larvae of *Lernaeodiscus rybakovi*. (A) NI–NV, naupliar stages. (B) Cypris stages. (C) Head shield of nauplius IV (dorsal view with setation). (D) Carapace of cyprid (lateral view with lattice organs). Abbreviations: 1–5, head shield setae (C), lattice organs (D); p, pore; pf, porefield. Scale bar = 100 μm.

(Figs. 8D and 9). The antenna is biramous with unsegmented endopod and an 8-segmented exopod, including a terminal small element carrying the distal seta. The exopod carries five long and plumose setae. The endopod carries three long and plumose setae. The basis of antenna has a plumose seta equal in length to the endopod (Figs. 8E and 9). The mandible is also biramous with an unsegmented endopod and a 6-segmented exopod, again including a small segment carrying the distal seta. The exopod carries four long plumose setae, while the endopod carries two long plumose setae (Figs. 8E and 9). The mandible remains unchanged during the naupliar part of development.

3.4.2. Nauplius II

Compared to nauplius I, the body has increased slightly in size. It is now more elongated and surrounded by a hollow floatation collar (Figs. 6 and 10A). In male larvae the anterior margin of the head shield is slightly more convex than in females. In ordinary LM, the flotation collar looks transparent, while SEM shows that it has a fine net-like structure (Fig. 10B). Nauplii that have accidently lost the collar reveal that this device is attached only by a narrow ridge encircling the entire body (Fig. 13A). The surface of the head shield bears six pairs of setae (1, 2, 2a, 3, 4, 5); seta 2 is the longest, straight or slightly curved (Figs. 6C and 10C); all setae, except 2a, have pores in a slightly subterminal position (Fig. 13D). This setation pattern of the dorsal surface remains unchanged during the naupliar development, except that setae 2 that gradually becomes U-shaped, and seta 2a becomes shorter (Fig. 13C and E). The frontolateral horns become longer, directed laterally, and are now armed with terminal cuticular fringes and two thick subterminal setae (anterior and posterior). The horns are now subdivided into three portions by two sutures, with the proximal suture being rather weak (Fig. 10D). This horn morphology remains almost unchanged during the ensuing naupliar development. Patterns of denticles on hind body now resemble transversal and longitudinal stripes (Fig. 10A). The furcal spines are longer and covered with denticles in the distal part (Fig. 10E). The second (middle) antennular segment is now more swollen (Fig. 10F). The distal margins of antennular, antennal and mandibular segments are clearly defined by rows of denticles and the surface of the segments is covered with single denticles (Fig. 10F and G).

3.4.3. Nauplius III

The body has increased in size, with the hind body being more elongated (Figs. 6 and 11A). From this stage, head shield seta 2 remains curved. In LM, the hind body is marked with a median longitudinal line (Fig. 6), which in SEM appears to be a median strip of denticles (Fig. 11B). The furcal spines are somewhat longer, diverge and are now completely covered with denticles (Fig. 11C). Antennular seta 1 is reduced in size and a short, seta 6 with a subterminal pore appears near the base of seta 5 (Figs. 9 and 12C).

3.4.4. Nauplius IV

In LM, the prospective segmentation of the hind body appears as oblique lines (Fig. 6) which in SEM appear as oblique strips of denticles, indicating the location of the developing thoracic appendages of future cypris larva (Fig. 12A). The furcal spines become thinner and are set into cuticular sockets; one or two denticles are appear near the base of each ramus (Fig. 12A). The polygonous, net-

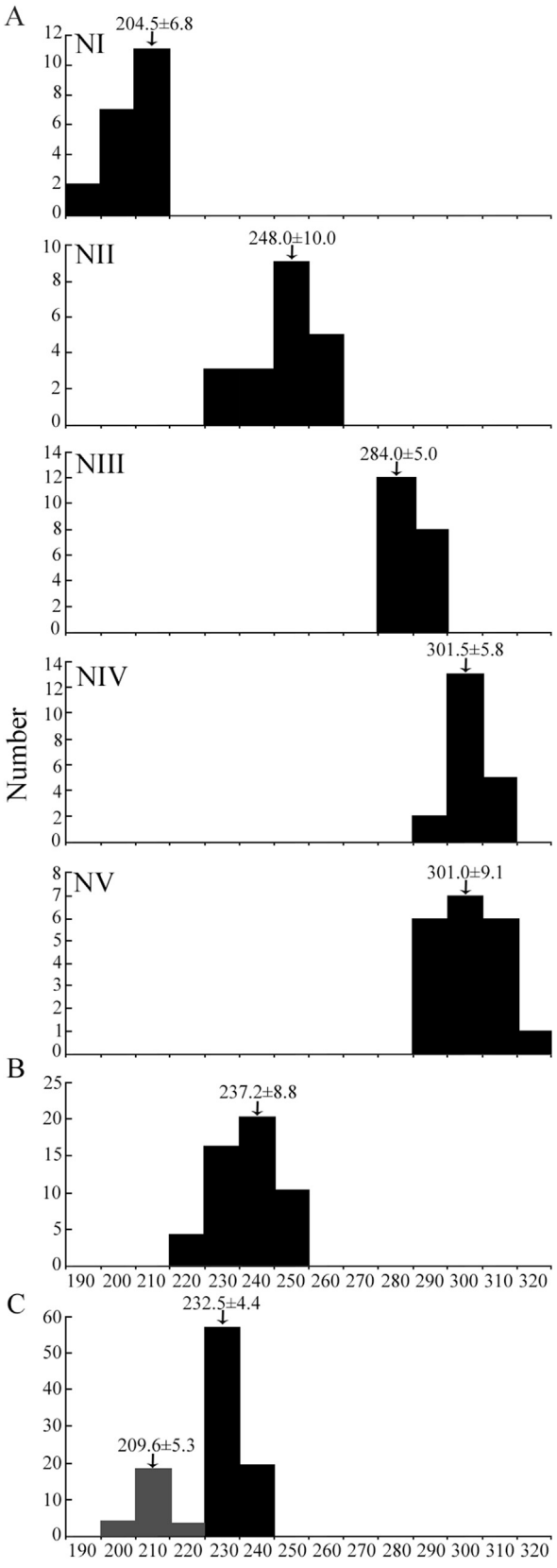

Fig. 7. Size-frequency distribution of larvae of *Lernaeodiscus rybakovi*. (A) Male nauplii (NI–NV) and (B) cyprids in male brood. (C) Male (black bars) and female (grey bars) cyprids in mixed brood with the prevalence of males (larval length in μm).

like structure of the flotation collar becomes more conspicuous (Fig. 12B). The antennule has only five setae, with seta 1 being much reduced; setae 5 and 6 arise from the distal antennular segment and the second antennular segment is more swollen, already heralding its future shape as an attachment organ in the cypris (Figs. 9 and 12C). The antennal basipod seta is now at half-length of the endopod (Fig. 9). The bases of the long setae on the naupliar appendages set into cuticular sockets (Fig. 12C and D). All the long setae of the appendages are covered with spinules and long slender setulae (Fig. 12E), while the short antennular setae 2 and 5, and the basipod seta of the antenna are covered with spinules, only.

3.4.5. *Nauplius V*

The larva is generally similar to the nauplius IV (Fig. 6). The head shield is more convex and more prominent in lateral view (Fig. 13A). The frontolateral horns are directed ventrally and backwards (Fig. 13B). On the head shield, setae 2 is more curved and setae 2a shorter (Fig. 13C and E). The hind body is narrower and in LM the developing thoracic appendages of the future cypris are clearly visible under the cuticle of the ventral side (Fig. 6). Denticles at the base of the furcal spines are more conspicuous (Fig. 13F). Seta 5 is inserted in the distal antennular segment; a short spine at the base of seta 5 remains; the second antennular segment is bulbous (Fig. 9).

3.4.6. *Cypris larva*

The cyprid is smaller than nauplius V (Figs. 6 and 14A). It has a smooth carapace surface, covered with long setae and scattered pores (Fig. 14B). Anterio-ventrally on each side of the carapace, the frontolateral horn glands exit as two separate and slit-like pores (Fig. 14F). Five pairs of lattice organs straddle the dorsal surface of the carapace, organized as two anterior (LO1–2) and three posterior (LO3–5) pairs. LO1–4 pairs are placed along the midline with LO5 lateral to LO4. The second pair (LO2) is crescent-shaped, with the convex side facing laterally, and an associated porefield is located posteriorly (Fig. 14C). The remaining lattice organs (LO1 and LO3–5) are nearly straight (Fig. 14B and D). There are no large terminal pores visible in LO1 and 2, although a single pore placed at the concave side of LO2, might represent a displaced terminal pore (Fig. 14C); LO3–5 have small terminal pores situated posteriorly in the organ (Fig. 14E). In addition, no less than five pairs of porefields superficially resembling like lattice organs are located on the lateral sides of the carapace.

There are four antennular segments (AS1–4). The morphology differs between male and female cyprids in the features specified below. AS2 is subterminally encircled by a breakage (abscission) zone (Fig. 14G). A postaxial seta 2 sits ventrodistally on AS2 (Fig. 14G). AS3 forms nearly rectangular attachment disc (AD), somewhat sparsely covered with cuticular villi and scattered pores, the latter most likely representing openings of the small unicellular antennular glands. The perimeter of the disc is bordered by a cuticular skirt (Fig. 14I). In female larvae, the antennular attachment disc has a characteristic flap-like extension at the posterior margin lacking in the male cyprids (Fig. 14I). A hard to see open-ended seta sits at the anterior (distal) part of the AD in male cyprids (Fig. 14G and H). We were unable to find it in females of *L. rybakovi*, but due to its small size we could easily have missed it. It is present in both male and female cyprids of *L.* aff. *porcellanae*. Therefore, we probably simply missed it in females of *L. rybakovi*. The postaxial sense seta 1 sits at the posterior (proximal) part of the

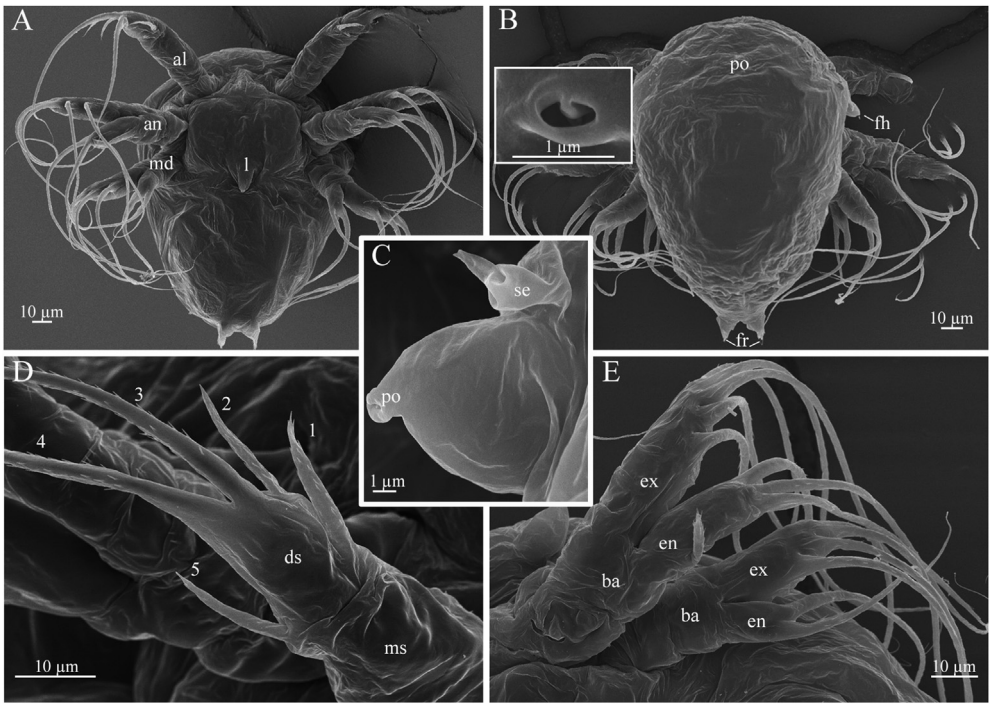

Fig. 8. *Lernaeodiscus rybakovi*, nauplius I. (A) Ventral view. (B) Dorsal view. (C) Frontolateral horn. (D) Antennule. (E) Antenna and mandible. Abbreviations: a, antennule; an, antenna; ba, basipod; ds, distal segment; en, endopod; ex, exopod; fh, frontolateral horn; fr, furcal rami; l, labrum; md, mandible; ms, middle segment; po, pore; se, subterminal seta; 1–5, antennular setae.

disc. There is no distally placed spinous process on the AD in either male or female cyprids.

Male and female cyprids differ in the number and size of aesthetasc setae. A large male specific aesthetasc sits at the proximal margin of attachment disc. The short fourth segment bears similar but slightly shorter aesthetasc is situated subterminally. Terminally, the fourth segment carries three open-ended setae and a bag-shaped seta superficially resembling an aesthetasc and only half as long as the other three setae. Male specific aesthetasc on AS3 is slightly longer than subterminal aesthetasc on segment 4 (Fig. 14G). In female cyprids have no aesthetasc on AS3, but carry a short subterminally sited aesthetasc on AS4 (Fig. 14I). There are six pairs of biramous, natatory thoracopods and paired caudal appendages (Fig. 14J). The thoracopod morphology is not described here because details of segmentation and setation is difficult to see, especially with respect to the endopod rami.

4. Discussion

4.1. Systematic position

Høeg et al. (2019a) revised the entire taxonomy of the Rhizocephala on a phylogenetic basis. This entailed that the family Lernaeodiscidae is considered polyphyletic. Species of *Triangulus*, formerly in this abandoned family, do not form a monophyletic genus and none of them is closely related to *Lernaeodiscus*. Instead, species of *Triangulus* are now allocated to either a new family, Triangulidae, or a rediagnosed Peltogastridae. The Høeg et al. (2019a) analysis, included only two species of *Lernaeodiscus* (*L.* aff. *porcellanae* and *L. ingolfi*), which were recovered as sister groups and nested within the re-diagnosed Peltogastridae. The present analysis of three species of *Lernaeodiscus* supports the monophyly of the genus *Lernaeodiscus* and its position within the Peltogastridae.

Further verification must come from molecular analysis of the 8 additional members of the genus (WoRMS 2018) for which molecular data is not yet available. Last years, species composition of peltogastrid rhizocephalans was studies in Japan, Taiwan and Korea using morphological and molecular methods (Yoshida et al., 2011, 2012; 2014; 2015; 2016; Jung et al., 2019). Unfortunately, these investigations did not include Asian *Lernaeodiscus* species, eg *L. okadai*.

The taxonomic status of *L. porcellanae* is in need of re-investigation, especially because of its importance within the Rhizocephala for the study of larval development, life cycles and host-parasite relationships. It has been studied in terms of the entire life cycle, the details of cypris metamorphosis and host invasion, sexual biology and host-parasite relations (Ritchie & Høeg 1981; Høeg 1985; Høeg & Ritchie 1985, 1987; Glenner et al., 1989; Fleischer et al., 1992; Høeg 1995; Høeg & Lützen 1995; Høeg et al., 2005). Within the genus, *L. porcellanae* has the widest area of distribution; in the Atlantic from North Carolina (Boyko & Harvey 2000) to Brazil (Müller 1862) and in the Pacific from California to Mexico (Alvarez et al., 2001). Boyko & Harvey (2000) supposed that Atlantic and Pacific specimens of *L. porcellanae* represent two different species and referred the Pacific specimens to *L.* aff. *porcellanae*. Boyko & Harvey (2000) based their decision on the distribution, different hosts and on the shape of the visceral mass. All information on life cycle and other biological aspects come from Pacific specimens, while the Atlantic representatives are known only from their gross morphology and from separate samplings at either end of the supposed distributional range. Unfortunately, molecular data is not available for the Atlantic specimens. Using morphology Boschma (1969) insisted on the identity of specimens from the two areas. Obviously, neither zoogeography nor host species can be true criteria of species separation. It must also be mentioned that Atlantic specimens are unstudied except for sheer morphology, so there is at present little chance of damaging confusion due to the

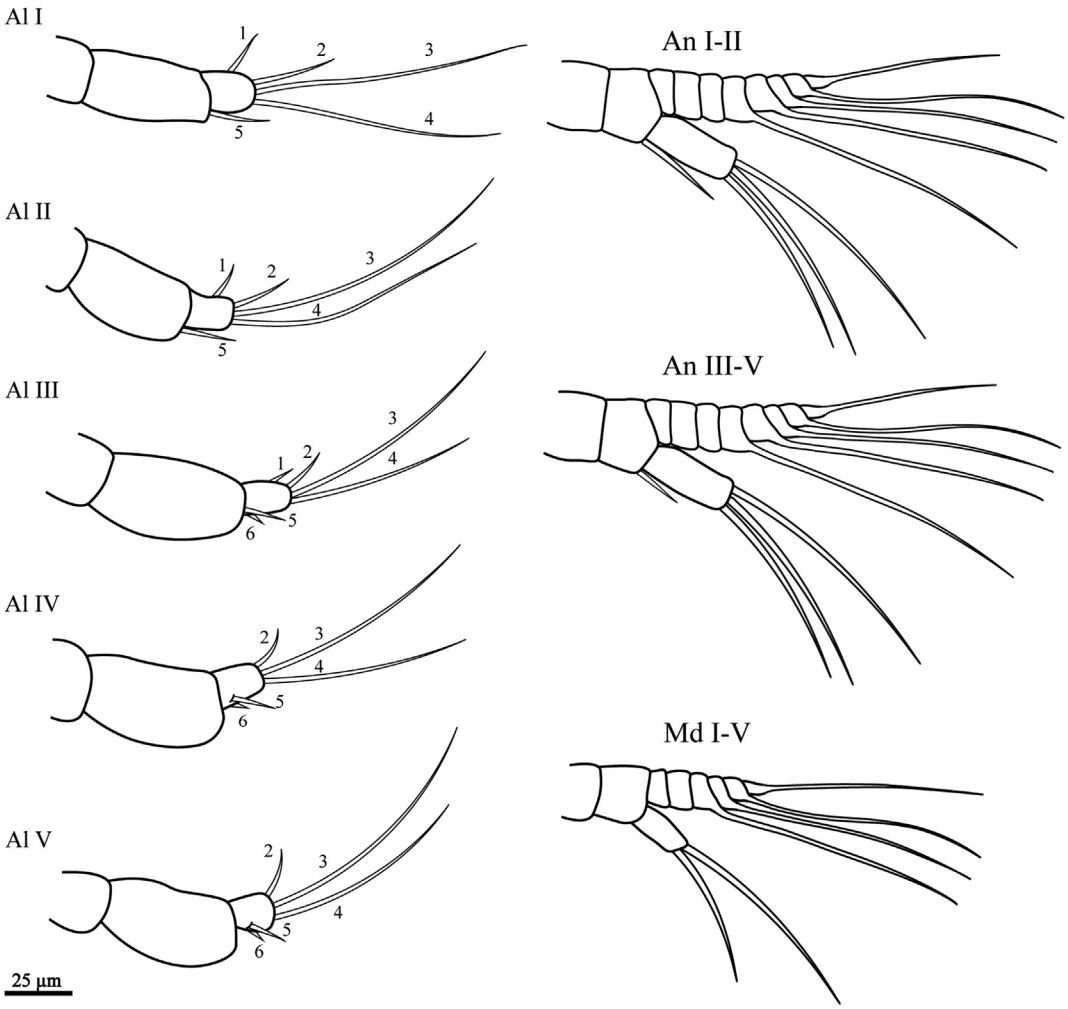

Fig. 9. Outlines of antennules (Al), antennae (An) and mandibles (Md) of the naupliar stages I–V of *Lernaeodiscus rybakovi*. Abbreviation: 1–6, antennular setae.

taxonomic problem, and it is hardly desirable to have well studied species change their name except when it truly signifies a very different phylogenetic position. Nevertheless, for the moment the use of *L.* aff. *porcellanae* for the Pacific specimens seems advisable.

4.2. Externa morphology

Our molecular analysis indicates that *L. rybakovi* is well separated from the two other species. For morphology based taxonomy of the Rhizocephala, the main characters concern the shape and colour of the externa, ornaments on the outer and inner mantle cuticles, the disposition and histology of the reproductive organs and the SEM based morphology of the larvae, especially the cyprids (Høeg & Lützen 1985; Øksnebjerg 2000; Høeg et al., 2019b).

The main morphological features of the new species, *L. rybakovi*, agree with all characters of the genus *Lernaeodiscus* as summarized by Høeg & Lützen (1985), Boyko & Harvey (2000) and Øksnebjerg (2000). Externae are usually single, bilaterally symmetrical, the stalk and the mantle opening are situated in the median plane. The mantle opening is directed dorsally near the anterior margin. Dorsal mesentery is very broad, extending from the stalk to the mantle opening. A much narrower and shorter ventral mesentery is placed in the posterior part of the body. Colleteric glands and receptacles are placed symmetrically to the median plane. The two receptacle ducts open at the ventral surface of the visceral mass, on the sides of the ventral mesentery. However, new species differs from each of the known *Lernaeodiscus* species by a number of peculiar features (Table 3).

Species of *Lernaeodiscus* infest anomuran crustaceans of the genera *Munida, Galathea, Petrolisthes, Munidopsis, Pisidia,* and *Aliaporcellana* (Boyko & Harvey 2000; Øksnebjerg 2000). Until now there was no record of any *Lernaeodiscus* species from *Pachycheles.*

Shape, sizes and color of mature externa are among the few morphological characters available for taxonomy in the Rhizocephala. The large red-orange externa of *L. rybakovi* is similar in size to the yellowish externae of *L. porcellanae* and *L.* aff. *porcellanae*, but differs in shape (Fig. 3B and C) and in color (Müller 1862; Boschma 1969; Boyko & Harvey 2000). For *L.* aff. *porcellanae* we have a very extensive dataset on externa sizes, and in this species the externa has a maximum width of 10 mm. Externae of some *Lernaeodiscus* species are small, not exceeding 5 mm in size, which for *L.* aff. *porcellanae* and *L. rybakovi* is within the size range of the juvenile, sexually immature externae (Høeg & Ritchie 1985; Høeg & Lützen 1995; present data). Both *L. ingolfi* and *L. triangularis* have large externae, with a trapeziform or triangular in outline and devoid of marginal lobes. However, these rhizocephalans are deep-water species (Boschma 1928; Lützen 1985; Høeg & Lützen 1985; Boyko & Harvey 2000; Øksnebjerg 2000), whereas *L. rybakovi* is not found deeper than 6 m. *L. tableta* differs from *L. rybakovi* by having

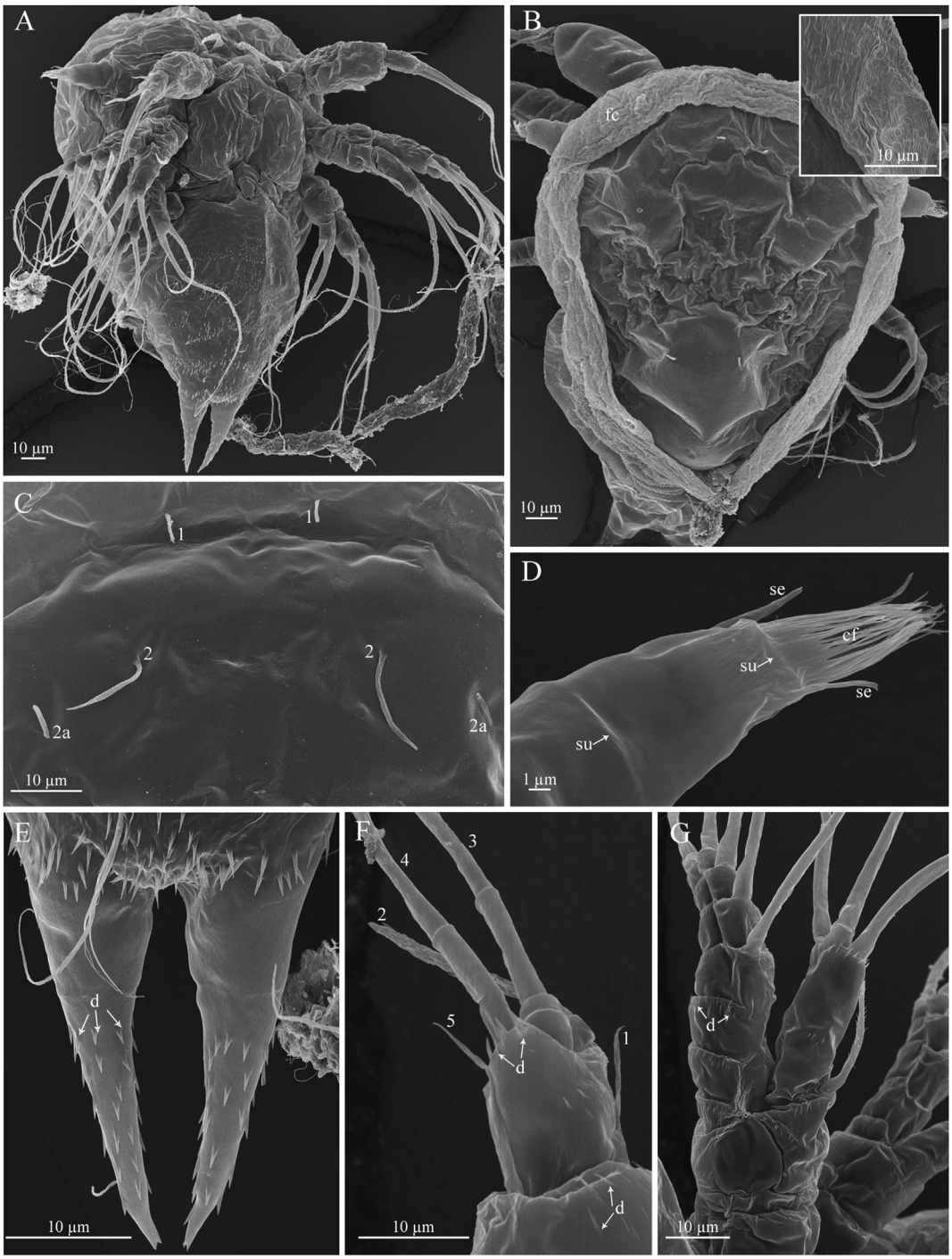

Fig. 10. *Lernaeodiscus rybakovi*, nauplius II. (A) Ventral view. (B) Dorsal view. (C) Head shield setae. (D) Frontolateral horn. (E) Furca. (F) Antennule. (G) Antenna. Abbreviations: cf, cuticular fringes; d, denticles; fc, flotation collar; se, subterminal setae; su, suture; 1, 2, 2a, anterior head shield setae; 1–5, antennular setae.

numerous marginal lobes (Boyko & Harvey 2000), externa of *L. squamiferae* is divided with transversal grooves into broad lobes (Høeg & Lützen 1985; Øksnebjerg 2000). Presence or absence of marginal lobes seems to be a reliable character. In *L.* aff. *porcellanae* a scrutiny of thousands of externae never revealed any without distinct lobes, but the details of the marginal lobes vary greatly between specimens. One explanation for the marginal lobes is that they increase the surface area and thus assist in keeping an oxygen rich environment for the developing embryos in the mantle cavity. A similar morphology has evolved completely independently within the monogeneric family Mycetomorphidae (Høeg & Rybakov 1996; Høeg et al., 2019a, b).

The externa of *L. rybakovi* is similar in shape to those of *L. okadai* infesting *Petrolisthes japonicus* (Boschma 1935; Shiino 1943; Boyko & Harvey 2000). *P. stevensii*, the host of *L. rybakovi*, is known from Hokkaido and eastern Honshu (Japan) and *P. japonicus*, the host of *L. okadai*, from eastern Honshu. The distribution of these two rhizocephalan species, both devoid of marginal lobes, may overlap, but the *Petrolisthes* with a parasite was found at up to 81 m depth whereas *P. stevensii* is a shallow-water species, with infested

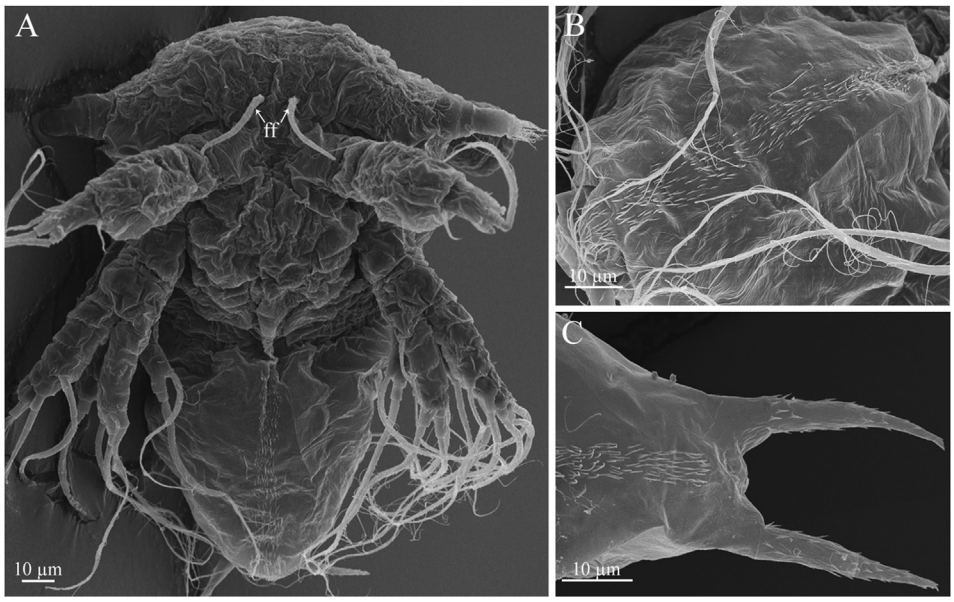

Fig. 11. *Lernaeodiscus rybakovi*, nauplius III. (A) Ventral view. (B) Hind body. (C) Furca. Abbreviation: ff, frontal filaments.

specimens being found only at a depth of 1.5–6 m. Moreover, Shiino (1943) noted that interna of *L. okadai* is greenisch, whereas in *L. rybakovi* it is light colored.

The position and shape of the receptacles are traditionally also important taxonomic characters. In *L. rybakovi* the receptacles have spiral a shape forming nearly two turns, whereas they form only one turn in *L. pusillus* (Boschma 1950; Boyko & Harvey 2000) and *L.* aff. *porcellanae*.

In the most rhizocephalans, the cuticle of externa bears microscopically discernible structures called retinacula located on the internal mantle cuticle. Some species have also papillae or excrescences on the external mantle cuticle. These characters have been much used in rhizocephalan taxonomy (Rybakov & Høeg 2002). Until now, it was considered that external mantle cuticle in the genus *Lernaeodiscus* has no papillae or excrescences and that the internal cuticle lacks retinacula (Boschma 1928, 1935; 1969; Boyko & Harvey 2000). Retinacula of lamp-brush type on the internal cuticle of *L. rybakovi* is the first finding of this character in *Lernaeodiscus*. Retinacula of the same type were described in *Peltogaster reticulata, Peltogaster paguri, Peltogasterella sulcata, Peltogasterella gracilis, Sacculina carcini,* and *Polyascus planus* (Rybakov & Høeg 2002).

4.3. Relation to the host

Many rhizocephalans exert a very considerable control over the biology of their hosts (Høeg 1995; Høeg & Lützen 1996; Kristensen et al., 2012). Normally, the rhizocephalan prevents both male and female host from reproducing and often male hosts are morphologically and behaviorally feminized. Our material is too limited to investigate the extent to which *L. rybakovi* morphologically affects its host, but the observation of several infected female hosts that also carried their own eggs is surprising (Fig. 1B). Normally rhizocephalans either arrest the function of the ovary or even cause its partial degeneration, the purpose probably being of redirecting reproductive efforts into the parasite itself (O'Brien 1999; Høeg 1995; Høeg et al., 2005). In *L.* aff. *porcellanae* a survey of thousands of infested hosts confirmed that female infested crabs are invariable sterilized and female hosts therefore never carry eggs. The same is true for the more than 10.000 *Carcinus maenas* specimens infested with *Sacculina carcini* recently surveyed by Mouritsen et al. (2018). Nevertheless, Høeg & Lützen (1985) confirmed Brinkmann's (1936) observation that female hosts of *Triangulus munida* can sometimes also carry their own eggs. Within the akentrogonid rhizocephalans host control may be at times somewhat relaxed and barnacles hosts of the Chthamalophilidae can often continue to reproduce even if with less success. Within the akentrogonid rhizocephalans of the Clistosaccidae, caridean hosts of *Sylon* can similarly be berried quite often (Glenner, personal observation), while within the same family female pagurids hosting *Clistosaccus paguri* are completely infertile. In the family Chthamalophilidae infested hosts can apparently continue to breed although probably with a somewhat reduced clutch size compared to uninfested specimens. Clearly, the degree to which rhizocephalan parasites control their hosts is subject to variation, not only between families, but also between closely related species. Such patterns may reflect continued coevolution between host and parasite including invasions into new areas or onto new species.

4.4. Larval development

In the early larval papers, it was assumed that Rhizocephala have an abbreviated, lecithotrophic development with only four naupliar instars (Hawkes et al., 1985; Walker 1988; Collis & Walker 1994; Walker & Clare 1994; Walker & Lester 1998). In early studies, one of naupliar stages (the third or the fourth) was missed because the larvae were sampled from the culture only daily, but the actual duration of an instar in temperate-water rhizocephalans ranges from 5 to 16 h (Kashenko & Korn 2002a, b, 2003; Kashenko et al., 2002). Walossek et al. (1996), by a very careful analysis, revealed that the peltogastrid species *Briarosaccus tenellus* has five instars. Using LM and SEM methods, the presence of five naupliar stages in the genera *Peltogasterella* (Korn et al., 1999; Rybakov et al., 2002), *Peltogaster* (Kashenko & Korn 2003), *Sacculina* (Korn & Rybakov 2001), *Polyascus* (Korn et al., 2000), and *Heterosaccus* (Ponomarenko et al., 2005) was revealed. In *P. gracilis, Polyascus polygeneus* and *P. reticulata,* the exact number of naupliar instars was determined by counting shed exuviae during each molt (Kashenko & Korn 2002a, b, 2003;

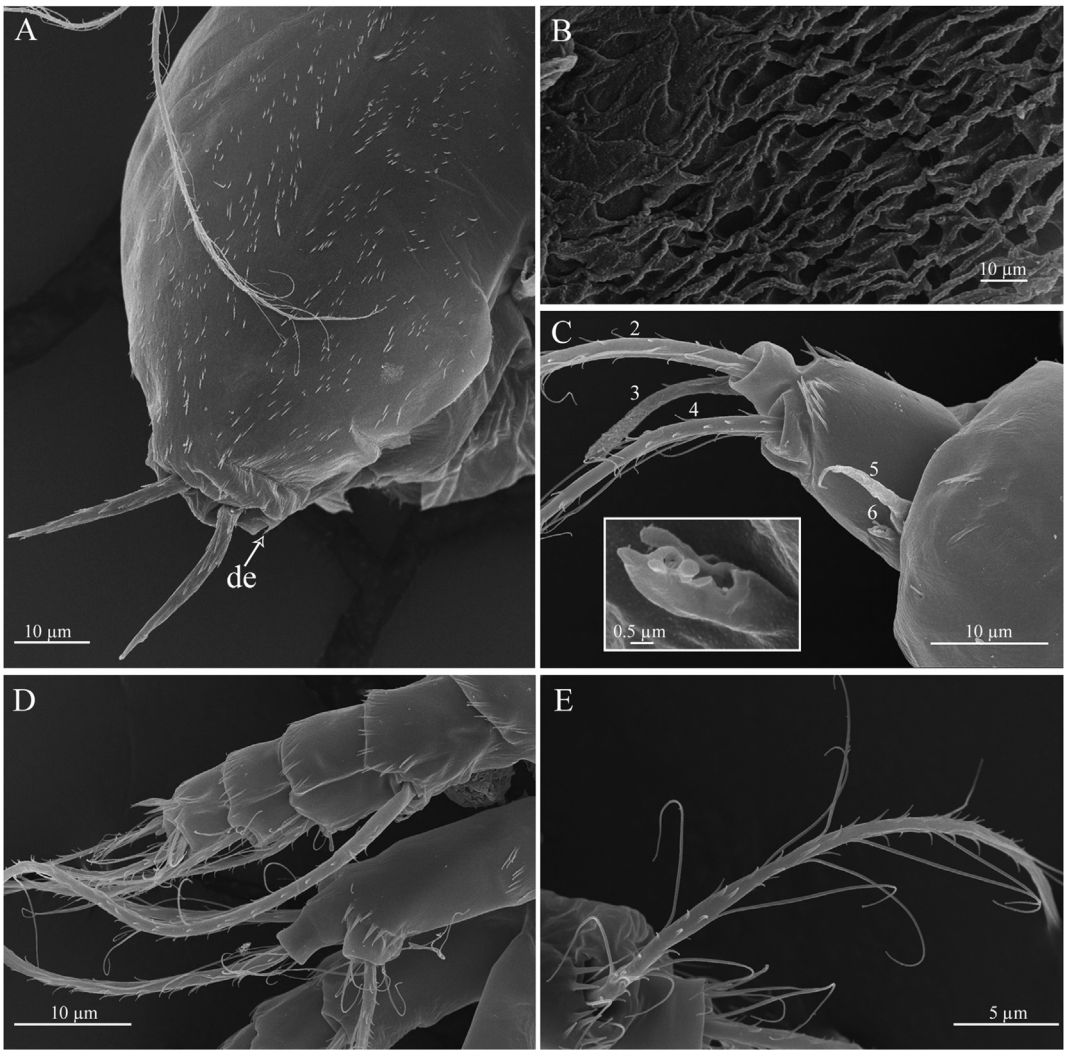

Fig. 12. *Lernaeodiscus rybakovi*, nauplius IV. (A) Hind body. (B). Flotation collar. (C) Antennule. (D) Antenna. (E) Long antennal seta. Abbreviations: de, denticles; 2–6, antennular setae.

Kashenko et al., 2002). In 2016, using a precise monitoring of broods every 4 h until the cypris stage, it was shown that the life cycle of *Sacculina carcini* also includes five naupliar stages (Trédez et al., 2016) instead of four as previously thought (Walker 1988; Collis & Walker 1994). Only four naupliar stages have been found in *Parasacculina sinensis* (Chan et al., 2005) and *P. planus* (Tu et al., 2009), despite the fact that the larvae of the latter species were collected at 6-hr intervals. This could indicate that some rhizocephalan species do in fact abbreviate naupliar development by loss of instars. The climax of this trend is of course the several species that hatch as cyprids (Høeg & Lützen 1995; Høeg et al., 2019b).

The larval development of *L. rybakovi* also comprises of five naupliar and one cypris stages. The larval period in *L. rybakovi* is similar to most of temperate-water species described before (Walker 1988; Korn et al., 1999, 2000; Korn & Rybakov 2001; Rybakov et al., 2002; Chan et al., 2005; Ponomarenko et al., 2005; Tu et al., 2009; Trédez et al., 2016), while in cold water species development lasts up to one month, just as observed in deep water species of the Cirripedia Thoracica (Hawkes et al., 1985; Walossek et al., 1996; Yorisue et al., 2012). Høeg & Lützen (1985) observed how a quickly elevated temperature could boost development from the last nauplius instar into cyprids in *P. paguri*. This is in line with current theory that the speed of development in invertebrate larvae depends on the ambient temperature by a fixed relation (Yorisue et al., 2012).

The complete larval development in the genus *Lernaeodiscus* is described here for the first time. Only partial data on shield setae in the naupliar dorsal head shield and the flotation collar of *L. porcellanae* are now available (Rybakov et al., 2003; Høeg et al., 2004b). Therefore, we have no opportunity to compare *L. rybakovi* nauplii with the larvae of the same genus. The cypris larvae of *L. porcellanae* are described in great detail by Høeg (1985), Jensen et al. (1994a), and Rybakov et al. (2003). The morphological features of male and female cyprids were described by Glenner et al. (1989). All descriptions *L. porcellanae* larvae (Ritchie & Høeg 1981; Høeg 1985; Høeg & Ritchie 1985; Glenner et al., 1989; Jensen et al., 1994a; Rybakov et al., 2003; Høeg et al., 2004b) are based on the material obtained in California, i.e. from *Lernaeodiscus* aff. *porcellanae* and may refer to another species.

General morphology of naupliar stages of *L. rybakovi* corresponds to common rhizocephalan pattern. The nauplius eye is visible during whole larval development as in all rhizocephalans except species of the genus *Peltogaster* (Høeg & Lützen 1995). All rhizocephalan nauplii are lecithotrophic and accordingly they do not exhibit many features of adaptive or taxonomic importance.

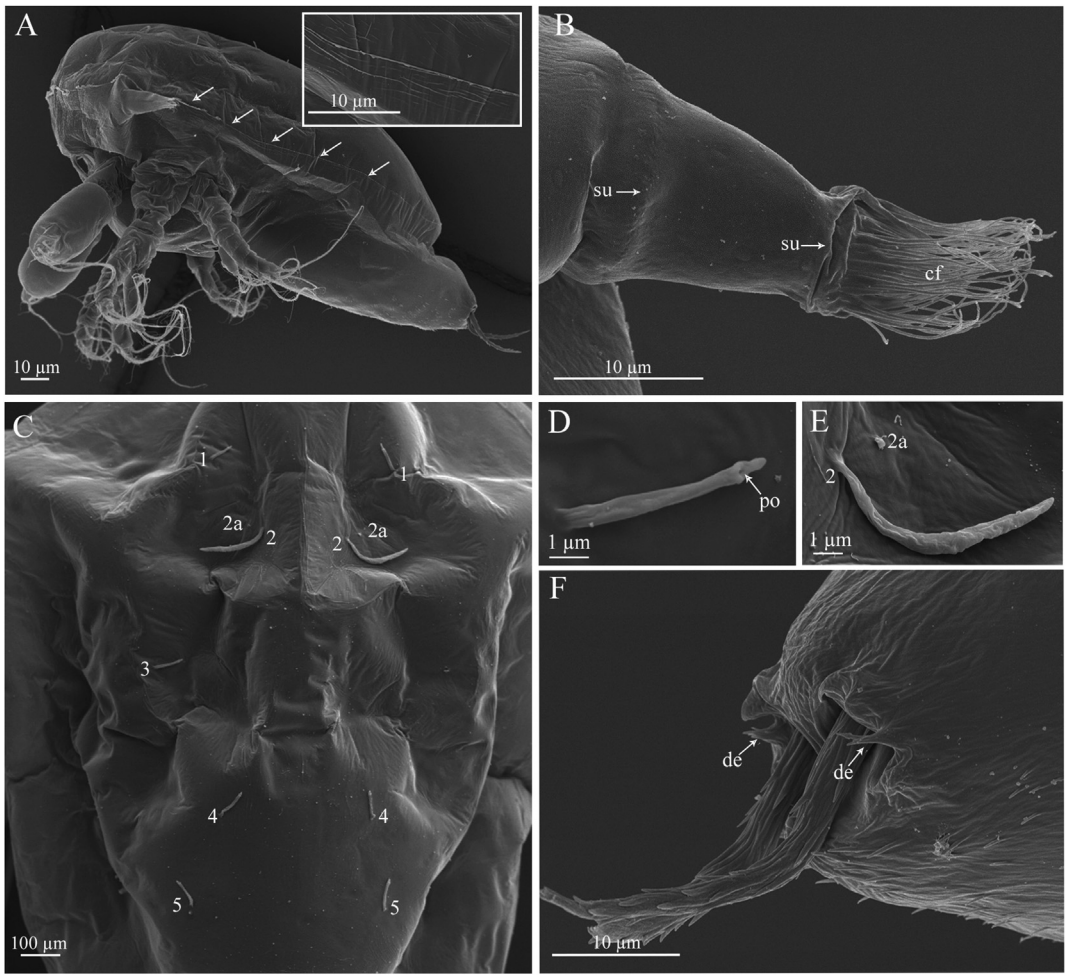

Fig. 13. *Lernaeodiscus rybakovi*, nauplius V. (A) Lateral view, arrows point to attachment ridge. (B) Frontolateral horn. (C) Head shield surface. (D) Seta 3. (E) Setae 2 and 2a. (F) Furca. Abbreviations: 1–5, head shield setae; cf, cuticular fringes; de, denticles; po, pore; su, suture.

The principal interesting feature is the structure and function of the flotation collar that occurs present in all rhizocephalan nauplii except those of the Sacculinidae and Polyascidae. It would also be interesting to compare the reductions due to lecithotrophy compared to lecithotrophic nauplii elsewhere in cirripedes, such as in the Scalpellidae and Neolepadidae. Generally, lecithotrophy entails a reduction in the setation associated with feeding, the appendages being used for swimming only. Lecithotrophy also implicates that the morphology of the mouth area (labrum, setae, glands, atrium oris) become affected as discussed in Walossek et al. (1996) and in rhizocephalan nauplii this trend seems to have reached a climax, with the labrum being a mere rudiment.

4.4.1. Dorsal setae

Localization, number and development of dorsal setae on the naupliar head shield are useful in species separation and phylogeny. Studies on larval ontogeny indicates that some of the dorsal setae are likelihood precursors lattice organs in the cyprid (Jensen et al., 1994a; Rybakov et al., 2003). The head shield of the late rhizocephalan nauplii more often comprises a set of six pairs of setae (Rybakov et al., 2003). Six pairs of dorsal setae are also present in nauplii of *L. rybakovi*. However, rhizocephalan nauplii can sport another number of dorsal setae which makes it a difficult task to make a pair by pair match of setae with the five pairs of lattice organs that occur in the ground pattern of cirripede cyprids (Rybakov et al., 2003). Nauplii of *P. planus* 5 pairs dorsal setae (Tu et al., 2009), *P. polygeneus* 4 pairs (Rybakov et al., 2003), and *P. sinensis* only 2 pairs (Chan et al., 2005). In Peltogastridae, setae of pair 2 are U-shaped (Rybakov et al., 2003) just as is mostly the case for the second pair of lattice organs in the cyprids, and both this and position in the setal series supports a homology. In some species (e.g. *P. gracilis*), setae of pair 2 appear already in the first naupliar stage, but in *L. rybakovi*, only in the second naupliar stage – no seta has been found in nauplius I. Setae of pairs 2 in the late naupliar stages of *L. rybakovi* are less curved than those of *L. porcellanae* (Rybakov et al., 2003).

The lattice organs are unquestionably chemosensory devices that originally evolved from setae (Høeg et al., 1998). In agreement, the naupilar precursor setae always have a terminal pore such as is expected in chemosensory sensilla other than aesthetascs and that probably corresponds to the large terminal pore that is present in the ground pattern of lattice organ morphology. Until experimental evidence is available, we can only speculate what is the specific function of these chemosensory organs, but the very fact that they occur in both nauplii and cyprids could indicate that they function during swimming, rather than during surface exploration as is the case for most or all of the antennular sensilla. Furthermore, lattice organs are omnipresent not only in the Cirripedia, but in the entire Thecostraca, and this strongly indicates that they fulfill crucial role (Høeg et al., 2009, Høeg et al., 2009b). This is again underlined by

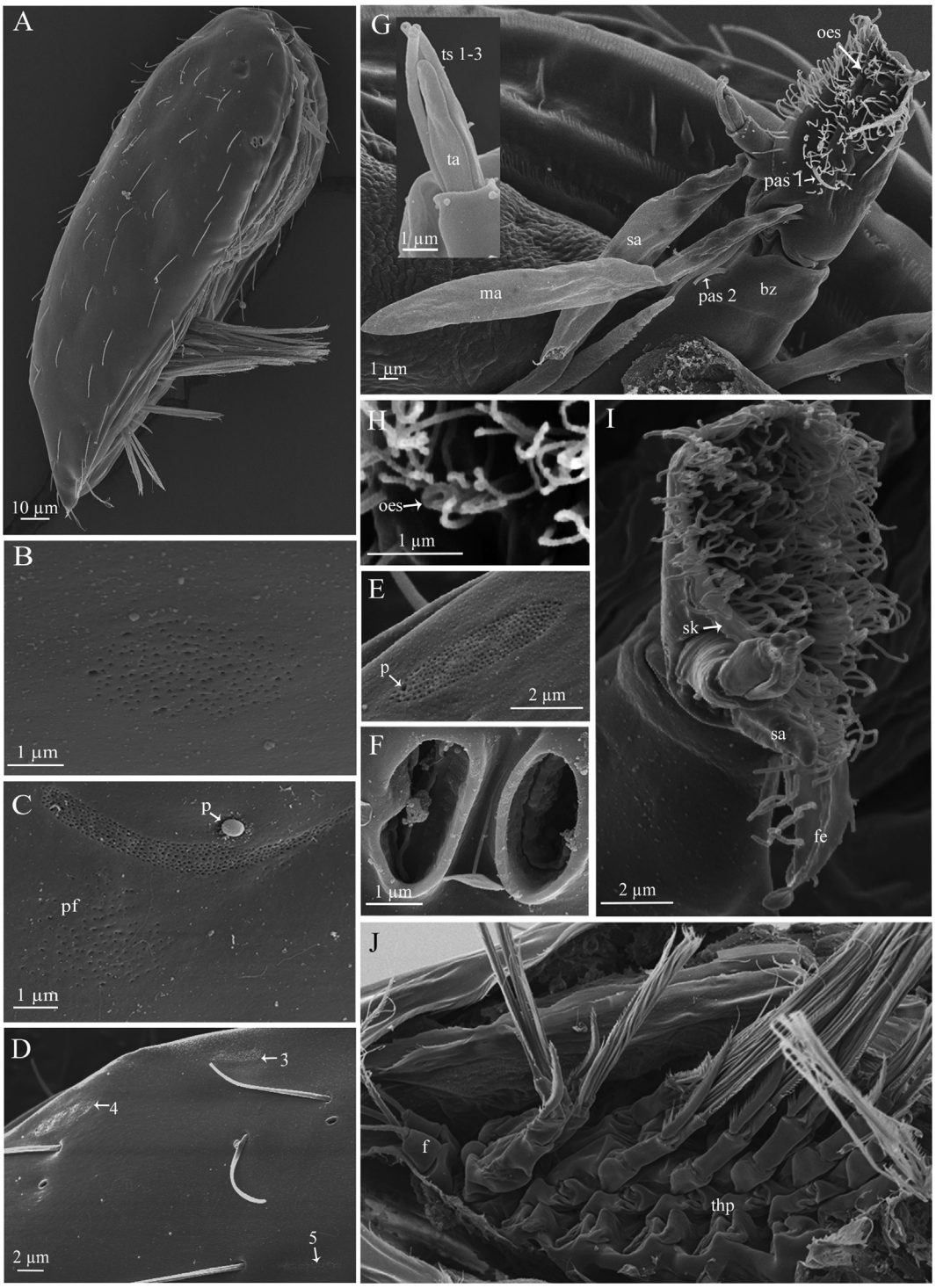

Fig. 14. *Lernaeodiscus rybakovi*, cypris larva. (A) Lateral view. (B) Anterior lattice organs 1 and 2. (C) Anterior lattice organ 2. (D) Posterior lattice organs 3–5. (E) Lattice organ 4. (F) Pores of frontolateral horn glands. (G) Male antennula. (H) Open-ended seta. (I) Female antennular attachment disc. (J) Thoracopods and furca. Abbreviations: fe, flap-like extension; ma, male aesthetasc; oes, open-ended seta; p, pore; pas 1, postaxial sense seta 1; pas 2, postaxial sense seta 2; pf, porefield; sa, subterminal aesthetasc; sk, skirt; ta, terminal aesthetasc; ts, 1–3, terminal setae.

the omnipresence of lattice organs in cyprids of the many cirripede species that lack naupliar stages.

4.4.2. Flotation collar

Floatation collar is a character of the nauplii of the genera *Peltogaster, Ommatogaster*, *Briarosaccus, Septosaccus*, *Lernaeodiscus*, *Peltogasterella,* and *Parthenopea*. In fact, except for species of the Sacculinidae and Polyascidae it seems that all rhizocephalans with nauplii sport such a collar and it may therefore represent a plesiomorphic feature within the entire taxon. The large collars in the larvae of the genera *Peltogaster* and *Briarosaccus* are ornamented by reticulated pattern of ridges, whereas collars in the nauplii of

Table 3
Main characters of the *Lernaeodiscus* species.

Species	Host	Externa		Distribution	Bathymetrical range, m	References
		Maximal size, mm	Shape, color			
Lernaeodiscus rybakovi	*Pachycheles stevensii*	13	nearly trapeziform, without marginal lobes, red-orange	Pacific (Sea of Japan)	1.5–6 m	present data
L. porcellanae	*Petrolisthes galathinus, P. armatus*	11	with marginal lobes, yellowish	Atlantic (from North Carolina to Brazil)	intertidal	Müller 1862; Reinhard 1950; Boyko & Harvey 2000
L. aff. *porcellanae*	*Petrolisthes cabrilloi, Clastotoechus diffractus*	7.5	with marginal lobes, yellowish	Pacific (California)	intertidal	Boschma 1969; Høeg & Ritchie 1985; Boyko & Harvey 2000; Alvarez et al., 2001
L. pusillus	*Pisidia serratifrons*	2	with inconspicuous marginal lobes	Red Sea	unknown	Boschma 1950; Boyko & Harvey 2000
L. schmitti	*Munida iris*	17	without marginal lobes	Florida	247–285	Reinhard 1950; Boyko & Harvey 2000
L. squamiferae	*Galathea squamifera*	5	with transversal grooves, wine red or pinkish-orange	Norway, French Mediterranean coast, Canary Islands	30	Høeg & Lützen 1985; Øksnebjerg 2000
L. tableta	*Aliaporcellana suluensis*	2.6	with marginal lobes	Indonesia	20–35	Boyko & Harvey 2000
L. triangularis	*Munidopsis granosa*	10.9	triangular, without marginal lobes	Bay of Bengal	2600	Boschma 1928; Lützen 1985; Boyko & Harvey 2000
L. ingolfi	*Munida intermedia, M. tenuimana, M. sarsi, M. rugosa*	17	trapeziform, without marginal lobes, whitish to colorless, visceral mass with a shade of wine red	from Iceland and western Norway to Mediterranean	34–1438	Boschma 1928; Høeg & Lützen 1985; Boyko & Harvey 2000; Øksnebjerg 2000
L. okadai	*Petrolisthes japonicus, P. hastatus, P. lamarckii, P. militaris*	5	trapeziform, without marginal lobes, red	Honshu (Japan), Indonesia, Philippines	up to 81	Boschma 1935; Shiino 1943; Boyko & Harvey 2000

L. porcellanae, P. gracilis, *P. sulcata*, *Septosaccus rodriguezii,* and *Ommatogaster nana* are small, inconspicuous and almost smooth (Hawkes et al., 1985; Walossek et al., 1996; Rybakov et al., 2002; Høeg et al., 2004b; Yoshida et al., 2011). *L. rybakovi* also possess a small naupliar collar of second type. In LM this collar looks transparent, devoid of reticular ornamentation and can easily be missed. SEM micrographs of nauplii show a fine net-like structure of collar, more conspicuous in later naupliar stages, but this pattern is partly caused by shrinkage during processing.

4.4.3. Frontolateral horns

Frontolateral horns are considered to be a synapomorphy for the nauplii of all Cirripedia (Walossek et al., 1996; Martin et al., 2014). Internally, the horns are quite complex structures. Their tips serve as the exit pore for two large unicellular glands (Høeg et al., 2004a) and there are supplied with nerves (Semmler et al., 2008). The details of their arrangement are useful for deciding on relationships among rhizocephalan taxa. In the nauplii of *L. rybakovi* as well as in the larvae of *B. tenellus, P. gracilis,* and *P. sulcata,* frontolateral horns are subdivided into three portions by two sutures, although the distal part with a fringe is shorter in *Peltogasterella* and *Lernaeodiscus* than in *Briarosaccus* and *Peltogaster* (Walossek et al., 1996; Rybakov et al., 2002). Nauplii of *Peltogaster* and *Lernaeodiscus* have two setae sited subterminally on the horns, while *Peltogasterella* sports only one such seta (Walossek et al., 1996; Rybakov et al., 2002). It was earlier believed that frontolateral horns in the Sacculinidae and Polyascidae are short and unsegmented (Collis & Walker 1994; Ponomarenko et al., 2005), but in these families the horns also have a proximal suture and are therefore subdivided into two portions. Trédez et al. (2016) assume that frontolateral horns in *S. carcini* are subdivided into three portions, but their micrographs show that they have no distal suture and the same is true for nauplii of *Heterosaccus papillosus* (Ponomarenko et al., 2005), *P. sinensis* (Chan et al., 2005), and *P. planus* (Tu et al., 2009). The subterminal setae on the frontolateral horns of sacculinid and polyascid nauplii are absent.

4.4.4. Hind body

Cirripede nauplii always have an unsegmented hind body (the postcephalic body part, Walossek et al., 1996; Høeg & Møller 2006). The segmentation of hind body observed by LM, here also in rhizocephalan nauplii, indicates the formation of the thoracic segments of future cypris larva. From third nauplius, the emerging segmentation pattern becomes regular and allows to distinguish naupliar stages. In the fifth naupliar stage, six thoracic segments with paired appendages are well noticeable through the cuticle. In SEM, the arrangement of small denticles on the hind body (longitudinal strip in the third nauplius and oblique strips in the fourth and fifth larvae) marks the orientation of thoracic segments. In rhizocephalans, the time of the appearance of segmentation in different species slightly varies (Walossek et al., 1996; Korn et al., 2000; Korn & Rybakov 2001; Rybakov et al., 2002; Ponomarenko et al., 2005). In *L. rybakovi,* segmentation appears in the third naupliar stage as in the most rhizocephalans.

Development of furcal spines in *L. rybakovi* is almost the same as in *P. gracilis* and resembles those in *B. tenellus* (Walossek et al., 1996; Rybakov et al., 2002). Late nauplii of peltogastrids lack of a small tubercle which appear between furcal rami in some sacculinids and polyascids (Korn et al., 2000; Ponomarenko et al., 2005; Tu et al., 2009; Trédez et al., 2016).

4.4.5. Appendages

Antennule of rhizocephalan nauplii bears five plumose setae. In addition, a small spine adjacent to seta 5 has been described on the antennules of *B. tenellus* (Walossek et al., 1996), *P. gracilis, P. sulcata* (Rybakov et al., 2002), and *H. papillosus* (Ponomarenko et al., 2005).

Rybakov et al. (2002) assumed that this spine might represent a rudimentary seta 6. In *L. rybakovi*, this unusual serrated seta appears only in the third naupliar stage. In *S. carcini*, not all examined specimens carried this seta (Trédez et al., 2016) and the authors believe that this variable character could not be interpreted as a vestigial seta 6.

In *L. rybakovi* and *H. papillosus* (Ponomarenko et al., 2005), the fifth antennular seta inserted on the middle segment in early naupliar stages, gradually shifts to the distal segment in late naupliar stages. The same relocation was shown on the antennule of *S. carcini* (Trédez et al., 2016). These authors noted that this "jump" of setae across the antennular segment boundaries after molting has been previously described for cirripede larvae (Grygier 1994). Nevertheless, such a jump over segment boundaries could signify a violation of the strict criteria for setal homology laid down by Huys & Boxshall (1991). On the other hand, as elaborated by Walossek et al. (1996), the "portions" of the naupliar antennule may, unlike the remaining appendages, not represent a true segmentation (or articulation).

Antenna of *L. rybakovi* nauplii and apparently of all rhizocephalans has 8-segmented exopod but not 7-segmented as stated earlier. Several studies did not take into account either a small distal segment (Rybakov et al., 2002) or the most proximal eighth segment (Ponomarenko et al., 2005), which gradually increased from the first to the fifth naupliar stage. A long antennal basipod seta in the early naupliar stages of *L. rybakovi* is similar that of *Peltogaster* and *Briarosaccus* (Hawkes et al., 1985; Walossek et al., 1996), being considerably longer than basipod seta of *Peltogasterella* (Rybakov et al., 2002). In sacculinids and polyascids, the antennal basipod is unarmed.

Thus, the main morphological features of *Lernaeodiscus* nauplii (the presence of flotation collar, morphology of frontolateral horns and furcal spines and the arrangement of dorsal setae on the shield head) are common with those of known peltogastrid larvae. However, they are also similar to *Peltogasterella* nauplii (Peltogasterellidae) in the presence of naupliar eyes and thin structure of the flotation collar.

4.4.6. *Cypris larva*

In kentrogonid type Rhizocephala the cyprids are sexually dimorphic in several distinct structural features, most notably in the antennules (Walker 1985; Høeg 1987; Glenner et al., 1989; Kobayashi et al., 2018) and male cyprids are generally larger than females. Sometimes as in *P. gracilis* male and female cyprids are distinct also by size alone, but mostly there seems to be an overlap whence size alone is not a safe criterion for determining sex among the cyprids (Høeg 1984; Høeg & Lützen 1995). The cypris larvae of *L. rybakovi* and *L. porcellanae* are similar in size range. Ritchie & Hoeg (1981) originally reported that male and female cyprids of *L. porcellanae* do not overlap in size, but our unpublished observations of numerous additional broods show that a considerable overlap exists just as in *L. rybakovi*. The larval sex ratio of both *Lernaeodiscus* species is variable seasonally. In California, male cyprids of *L. porcellanae* predominate during winter months but female cyprids during summer (Ritchie & Hoeg 1981), whereas in the Sea of Japan, male cyprids of *L. rybakovi* predominate in summer months. In *Sacculina carcini* from the English Channel male larvae predominate in the late spring and early summer when virginal externae are very common, while female larvae are most abundant later in the season (Høeg 1985; Høeg & Lützen 1995). This still preliminary dataset seems to suggest that rhizocephalan species tune their sex ration to the ratio of virgin externae in the population.

Cypris head shield of *L. rybakovi* is covered with long setae as it has been previously described for *L. porcellanae*, *B. tenellus*, *P. gracilis*, and *P. paguri* (Glenner et al., 1989; Walossek et al., 1996; Rybakov et al., 2002). Five pairs of lattice organs are usually placed on the carapace surface. Lattice organ 2 is U-shaped as in all known peltogastrid species (Walossek et al., 1996; Rybakov et al., 2002, 2003). LO2 is less curved in *L. rybakovi* than in *L. porcellanae*, and have the convex side in lateral position, but LO2 in *L. porcellanae* is in posterior position (Jensen et al., 1994a). In most rhizocephalans, a terminal pore is found at the end of lattice organ, anterior in pairs 1–2 and posterior in pairs 3–5 (Jensen et al., 1994a). In *L. rybakovi*, LO3–5 have small terminal pores in a posterior position. In *L. porcellanae*, terminal pores are not found, with the exception of a single pore near the concave side of LO2 (Jensen et al., 1994a). In *L. rybakovi*, this pore is placed closer to the central part of LO2. Such reduction or absence of terminal pores is the rule among akentrogonid type rhizocephalan species such as the Clistosaccidae and Thompsoniidae (Jensen et al., 1994a, b).

Flap-like extensions of the attachment disc, found in female cyprids of *L. rybakovi*, were earlier noted in male larvae of *Heterosaccus lunatus* (Walker 1999), *H. papillosus* (Ponomarenko et al., 2005), and *Polysaccus mediterraneus* (Høeg & Rybakov 2007). The subterminal aesthetascs on the attachment disc is longer and a cuticular collar surrounding aesthetasc basis in *L. rybakovi* is some larger than in *L. porcellanae* (Glenner et al., 1989).

Male cypris larvae of some Rhizocephala have a so-called spinous process at the distal end of the attachment disc, unknown from thoracican cyprids. It is located within the exit pore of a large unicellular gland (LUG). TEM shows that it is a simple spine, but not a seta (Høeg 1987; Moyse et al., 1995). The spinous process is found in *Sacculina carcini, H. lunatus, Heterosaccus californicus, H. papillosus, P. sinensis, P. mediterraneus, Mycetomorpha vancouverensis, C. paguri,* and *Sylon hippolytes* (Glenner et al., 1989; Moyse et al., 1995; Walker 1999; Chan et al., 2005; Ponomarenko et al., 2005; Høeg and Rybakov, 2007). The LUG is illustrated in *P. planus* (Tu et al., 2009). Male cyprids of the Peltogastridae lack the spinous process (Glenner et al., 1989; Moyse et al., 1995; Walossek et al., 1996; Rybakov et al., 2002; present study). An open-ended seta in the anterior part of the attachment disc in *P. sulcata* and *P. paguri* was erroneously named as spinous process (Glenner et al., 1989). The simultaneous presence of the open-ended seta and the spinous process in *H. lunatus* (Walker 1999) and *M. vancouverensis* (Høeg & Rybakov 2007) supports that they are separate structures. Both the LUG gland and the spinous process are characters exclusive to the Rhizocephala (Glenner et al., 1989).

5. Conclusions

Our study has supported the monophyly and position of the genus *Lernaeodiscus*. Although data is limited, we also provide new perspective to host-parasite relations in terms of sterilization of the infested crab. The morphological differences between rhizocephalan naupliar instars furnish little interesting information, but the number of instars is important for interpreting larger phylogenetic patterns. The same is true for naupliar morphology in terms of the effect inherent in evolution of a lecithotrophic development. Finally, the generally recognized patterns in cypris rhizocephalan morphology are confirmed, while also showing that scrutiny at SEM levels can reveal interesting differences with as yet unknown adaptive significance such as presence or absence of the large unicellular gland and associated spinous process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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