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Undersized and underestimated: 3D visualization of the Mediterranean interstitial acochlidian gastropod *Pontohedyle milaschewitchii* (Kowalevsky, 1901)

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Abstract

Pontohedyle milaschewitchii (Kowalevsky, 1901) is one of the most common mesopsammic opisthobranchs in the Mediterranean and Black Seas and has been considered as a comparably well-described acochlidian species. However, data on its complex internal anatomy were fragmentary and little detailed due to inadequate methodology available, and contradictory between different sources. The present study redescribes all major organ systems of *P. milaschewitchii* in full detail by three-dimensional reconstruction from serial semithin sections using AMIRA software. The prepharyngeal central nervous system (cns) of *P. milaschewitchii* is highly concentrated and shows a euthyneurous and epiathroid condition. Contrary to earlier reports, the cerebral and pleural ganglia are not fused. Aggregations of precerebral accessory ganglia can be grouped into three complexes supplied by distinct cerebral nerves. Rhinophoral ganglia with thin, double cerebro-rhinophoral connectives are described for the first time in acochlidians. A Hancock's organ is present in the form of a conspicuous, curved fold in the epidermis posterior to the oral tentacles. Cerebral nervous features and sensory structures are discussed comparatively. Our study confirms *P. milaschewitchii* as having the male genital opening in an unusual position above the mouth. Homology of the ciliated vas deferens of the gonochoristic and aphallic *P. milaschewitchii* with that of hermaphroditic acochlidian species with cephalic male genitals is discussed. The radula formula of *P. milaschewitchii* is 41–54 × 1-1-1, i.e. the single lateral teeth are broad and, contrary to previous descriptions, undivided. SEM examination of the body wall of entire specimens revealed a special and constant ciliary pattern. Providing a novel additional set of characters for taxonomic and phylogenetic purposes, external SEM examination is suggested as the standard method for describing acochlidian species in the future.
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Keywords: Mollusca; Gastropoda; Opisthobranchia; 3D reconstruction; Anatomy; Histology

Introduction

Only few gastropods are able to colonize the marine interstitial, a habitat with extreme ecological conditions (Swedmark 1968b). Within the opisthobranchs the

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Acochlidia are the most successful mesopsammic group, with currently 27 valid species (Wawra 1987; Sommerfeldt and Schrödl 2005). Among the most common species in the shallow subtidal sands of the Mediterranean is *Pontohedyle milaschewitchii* (Kowalevsky, 1901), with densities of more than 200 individuals per m^2 (Poizat 1984), reported from numerous collecting sites throughout the Mediterranean (e.g. Swedmark 1968b; Salvini-Plawen 1973) and from the Black Sea (Kowalevsky 1901). Correspondingly, *P. milaschewitchii* has been treated in several ecological papers (e.g. Hadl et al. 1969; Poizat 1984) and commonly considered a well-known acochlidian species (Arnaud et al. 1986). However, biological and anatomical knowledge was fragmentary, little detailed and hardly reliable. The original description by Kowalevsky (1901) mainly concentrated on external morphology and offered little data on the anatomy. Wawra (1986) supplied additional details of the reproductive system of this gonochoristic species. He described an intraepidermal vas deferens opening slightly dorsally of the mouth opening – which was the first report of a male genital opening between the oral tentacles in acochlidians – but he did not provide a complete revision of the male or female genital system. Up to now, the most detailed description of the anatomy of *P. milaschewitchii* was presented by Marcus and Marcus (1954), who examined a single male specimen from the coast of southern Brazil. On the basis of the latter description Rankin (1979) erected a new genus and species, *Gastrohedyle brasilensis*. However, Jörger et al. (2007) recently clarified the status of *G. brasilensis* as a junior synonym of *P. milaschewitchii* on a morphological basis. Molecular data will be necessary to determine whether or not Mediterranean, Black Sea and Atlantic *Pontohedyle* populations represent cryptic species.

The present study redescribes Mediterranean *Pontohedyle milaschewitchii* providing a detailed anatomical and histological revision of all major organ systems. Using computer-based three-dimensional (3D) reconstruction, we show how the anatomy of such diminutive yet complex animals can be accessed reliably and efficiently. We further discuss whether or not external SEM examination of entire specimens can provide an additional set of characters that may be useful for taxonomic and phylogenetic purposes.

Material and methods

Samples of coarse sand were taken by snorkeling in a depth range of 5–9 m at different collecting sites near Rovinj (Istria, Croatia) in June and September 2005. Specimens of *Pontohedyle milaschewitchii* were extracted from the samples following the method described by Schrödl (2006). Extracted specimens were slowly anaesthetized, using 7% isotonic $MgCl_2$ solution, to prevent them from retracting prior to and during fixation. Specimens used for semithin sectioning and SEM examination were fixed in 4% glutardialdehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2); specimens used for radula preparation were fixed in 75% ethanol. The glutardialdehyde-fixed specimens were rinsed in 0.2 M sodium cacodylate buffer (0.1 M NaCl and 0.35 M sucrose, pH 7.2), post-fixed in 1% OsO_4 buffered in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2) for 1.5 h, and again rinsed in 0.2 M cacodylate buffer (0.3 M NaCl, pH 7.2). The fixed specimens were decalcified in ascorbic acid, dehydrated by a graded acetone series, and embedded in Spurr's (1969) low-viscosity epoxy resin for sectioning. The epoxy resin blocks were cut at 1.5 µm intervals with a rotation microtome (Microtom HM 360; Zeiss), using glass knives and contact cement at the lower cutting edge (Henry 1977), to receive ribboned serial sections. Four complete series were prepared and stained with methylene blue-azure II (Richardson et al. 1960). Computer-based 3D reconstruction of all major organ systems was performed with the software AMIRA 3.0 (TGS Template Graphics Software, Inc., USA). All sections have been deposited in the Zoologische Staatssammlung München (ZSM), Mollusca Section (ZSM Mol 20060522–20060525).

For SEM examination 20 glutardialdehyde-fixed specimens were dehydrated through a graded ethanol series followed by a graded acetone series. The specimens were critical-point dried in 100% acetone in a Baltec CPD 030. After mounting on SEM stubs with self-adhesive carbon stickers, the dried specimens were coated with gold in a Polaron Sputter Coater for 120 s. Seven ethanol-fixed specimens were used for SEM analysis of the radula. They were macerated up to 24 h in 10% KOH to separate the radula from the surrounding tissue. Remaining tissue was removed mechanically under a stereo microscope. Prepared radulae were rinsed in Aqua bidest and transferred to SEM stubs with self-adhesive carbon stickers. The radulae were coated with gold for 120 s (Polaron Sputter Coater). Scanning electron microscopic examinations were conducted using a LEO 1430VP SEM at 10–15 kV.

Results

External morphology and spicules

(Figs. 1, 2)

The body of *Pontohedyle milaschewitchii* is divided into a cylindrical anterior part (head–foot complex) and a posterior sac-like, elongated and broadened visceral

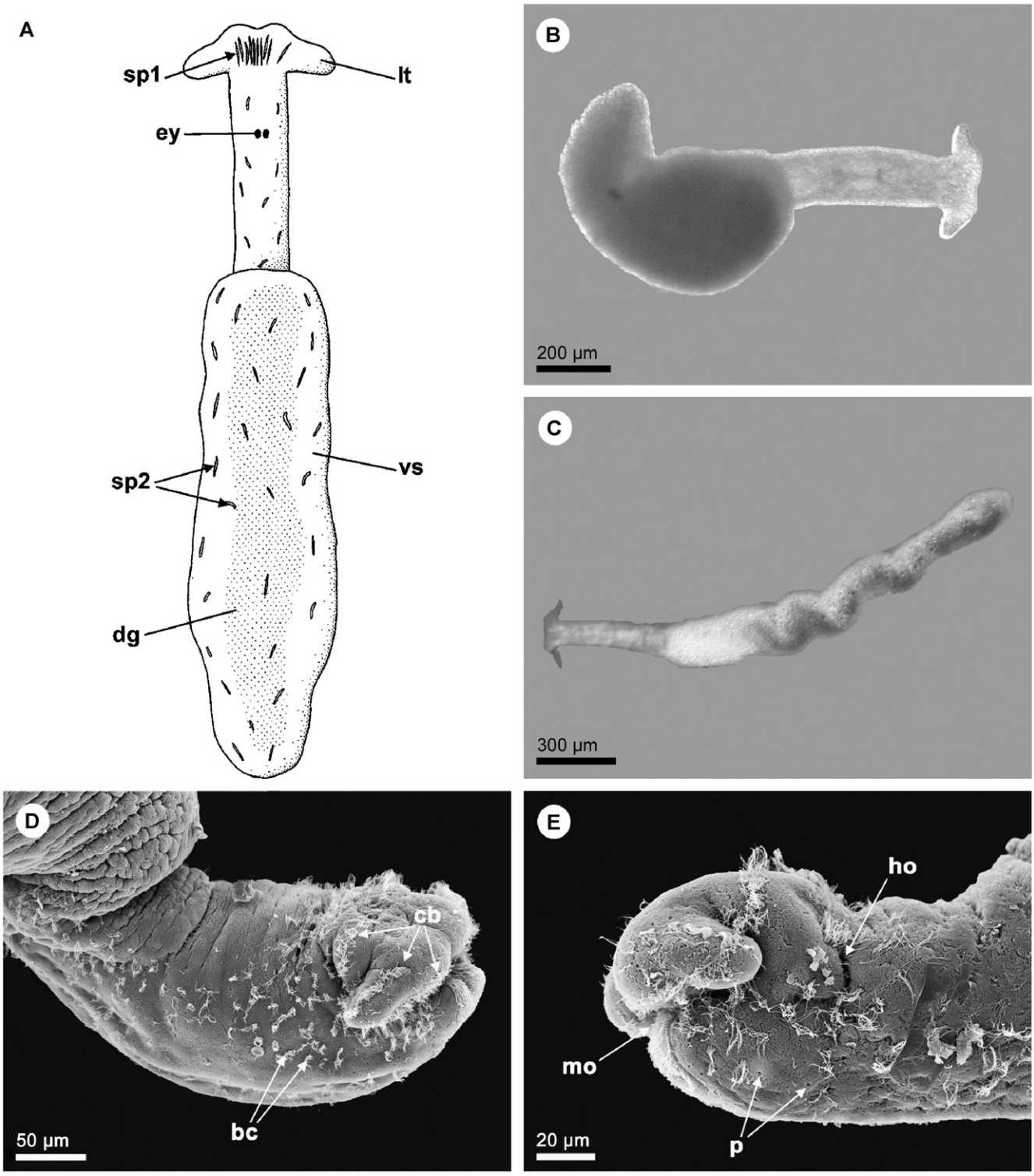

Fig. 1. External morphology of *Pontohedyle milaschewitchii*. (A) Semi-schematic drawing of an entire specimen, dorsal view. (B, C) Photographs of living specimens in dorsal view, showing range of variation in external morphology. (D, E) SEM micrographs of the head–foot complex. (D) Pattern of ciliation, dorsolateral view. (E) Hancock's organ, ventrolateral view. Abbreviations: bc = bundles of cilia, cb = ciliary band on head and tentacle, dg = digestive gland, ey = eye, ho = Hancock's organ, lt = labial tentacle, mo = mouth opening, p = pore of epidermal gland, sp1/2 = spicules of type I/II, vs = visceral sac.

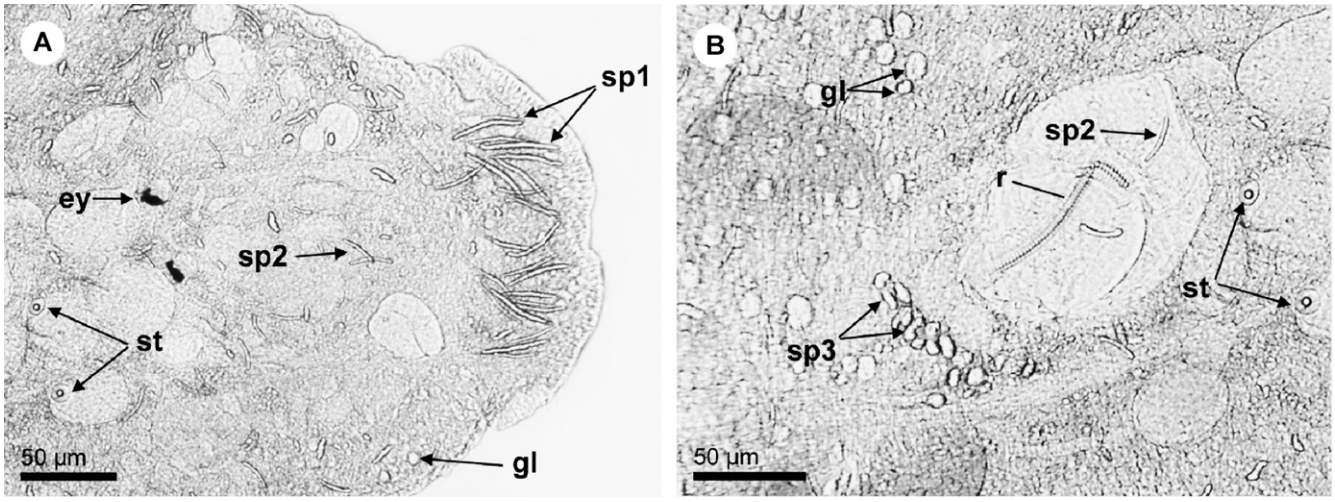

Fig. 2. Different types of spicules in *Pontohedyle milaschewitchii*. (A) Accumulation of large, needle-like spicules (type I) between oral tentacles. (B) Accumulation of small, oval spicules (type III) in posterior portion of pharynx. Abbreviations: ey = eye, gl = epidermal gland, r = radula, sp1–3 = spicules of types I–III, st = statocysts.

hump (Fig. 1A). The head–foot complex can be retracted into the visceral hump. Body length of extended mature specimens examined varied from 1.5 to 3.0 mm. Body coloration is whitish, transparent; the digestive gland is bright green to olive green. The ciliated foot is short (i.e. there is no free tail extending behind the head–foot complex); its posterior end is rounded. The head bears a pair of large, flattened oral tentacles. The shape of the oral tentacles is variable among specimens of a population, ranging from bow-shaped and curved to elongated and slightly triangular (Fig. 1B, C), and also varies depending on the stage of activity or contraction of the animal. Rhinophores are lacking completely. A pair of darkly pigmented eyes is located at approximately mid-length of the head–foot complex. An accumulation of parallel-oriented calcareous spicules occurs between the oral tentacles (Figs. 1A, 2A). The spicules are up to 40 µm long, 2 µm wide, and have a needle-like monoaxonic form (type I). Numerous monoaxonic spicules are also found irregularly distributed in the rest of the body, but smaller in size (length approximately 25 µm; type II). Spicules are embedded in the subepidermal mesenchyma. Oval to bean-shaped spicules (length about 10 µm; type III) are found in an aggregation in the posterior portion of the pharynx behind the radula (Fig. 2B).

SEM examination shows that the head–foot complex is covered laterally and in the anterior dorsal region with scattered bundles of cilia; the posterior dorsal region lacks cilia (Fig. 1D, E). On the dorsal and the anterior side of the oral tentacles run two 30 µm long and 3 µm wide ciliary bands. Another band with similar dimensions traverses the anterior dorsal region behind the oral tentacles (Fig. 1D). The visceral hump only bears a few scattered bundles in its anterior ventrolateral region; the remainder of the hump shows no ciliation. Overall, the density of cilia bundles varies among individuals, but the described pattern is always present.

Microanatomy

(Fig. 3)

The cavity of the head–foot complex contains the central nervous system (cns) and the anterior digestive organs (oral tube, pharynx, salivary glands and oesophagus). Ventral to the oral tube, around the central muscle strand, the large, bilobed anterior pedal gland extends. An unpaired duct connects the anterior pedal gland to the exterior, opening slightly ventral to the mouth opening (Fig. 3). Three strong muscle strands (one central and two lateral) extend through the head–foot complex, with the lateral strands leading far into the visceral hump. Additionally, a network of fine muscle fibres runs subepidermally in the body wall. A diaphragm separates the cavity of the head–foot complex from that of the visceral hump. The majority of the visceral hump cavity is filled with the digestive gland and the genital system (Fig. 3). Excretory and circulatory systems are located in the anterior right portion of the visceral hump. The anus opens on the right side of the visceral hump clearly behind the junction with the head–foot complex. Nephroporus and female gonopore open anterior to the anus on the right side of the head–foot complex, close to the junction with the visceral hump. The male genital system empties in the anterior-most region of the head–foot complex, just dorsal to the mouth opening.

Three different types of epidermal gland cells are present in *P. milaschewitchii*: (1) large (diameter about

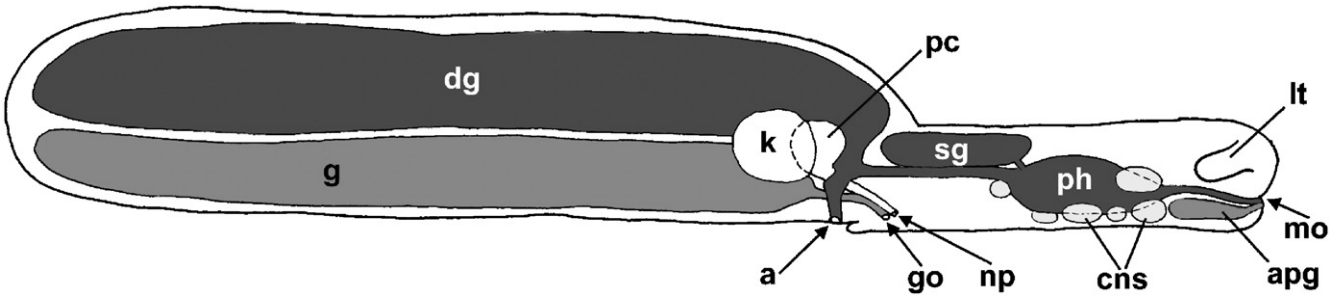

Fig. 3. Schematic overview of arrangement of internal organs in female *Pontohedyle milaschewitchii*, lateral view. White = excretory and circulatory systems, light grey = central nervous system, grey = genital system, dark grey = digestive system. Abbreviations: a = anal opening, apg = anterior pedal gland, cns = central nervous system, dg = digestive gland, g = genital system, go = genital opening, k = kidney, lt = labial tentacle, mo = mouth opening, np = nephropore, pc = pericardium, ph = pharynx, sg = salivary glands.

15 µm), spherical, whitish glandular cells (type I) forming a subepidermal sac and distributed on the head–foot complex and, in higher concentration, on the visceral hump (Fig. 4A); (2) small (5 µm), irregular-shaped ochre-colored glandular cells (type II); and (3) spherical cells (10 µm; type III) filled with dark blue-stained granules exclusively found in one row on the inner border of the visceral hump near the transition region to the head–foot complex (Fig. 4A). Dorsal to the ciliated foot sole numerous small (5–10 µm) pedal glands could be detected subepidermally, showing similar lilac staining properties as the anterior pedal gland (Fig. 4B).

Nervous system

(Figs. 5, 6)

The central nervous system (cns) of *P. milaschewitchii* consists of the paired cerebral, rhinophoral, pedal, pleural and buccal ganglia and three distinct unpaired ganglia on the short, euthyneurous visceral nerve cord (Fig. 5). Cerebral, rhinophoral and pedal ganglia are located prepharyngeally; the pleural ganglia in the anterior part of the pharynx, the ganglia of the visceral cord in its posterior part. Only the buccal ganglia are located postpharyngeally. The cns is epiathroid. The terms for the ganglia are used according to Schmekel (1985) and Haszprunar (1985), accessory ganglia are determined following Neusser et al. (2006).

Accessory ganglia

Many accessory ganglia in various sizes can be found in the anterior region of the cns of *P. milaschewitchii* (Fig. 6A). They are characterized as well-defined groups of cells showing homogenous distribution of nuclei (i.e. a lack of subdivision into cortex and medulla; see Fig. 6E), surrounded by relatively thin connective tissue. In *P. milaschewitchii* the accessory ganglia are arranged

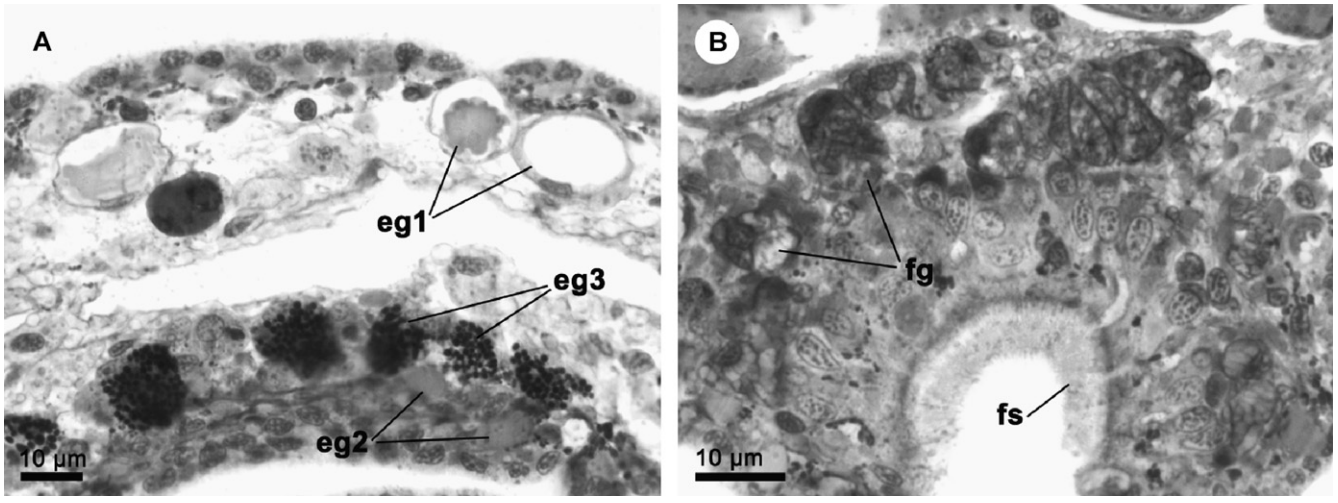

Fig. 4. Different types of glandular cells in *Pontohedyle milaschewitchii*. (A) Semithin cross-section of anterior region of visceral hump. (B) Semithin cross-section of foot. Abbreviations: eg1–3 = epidermal gland types I–III, fg = foot gland, fs = ciliated foot sole.

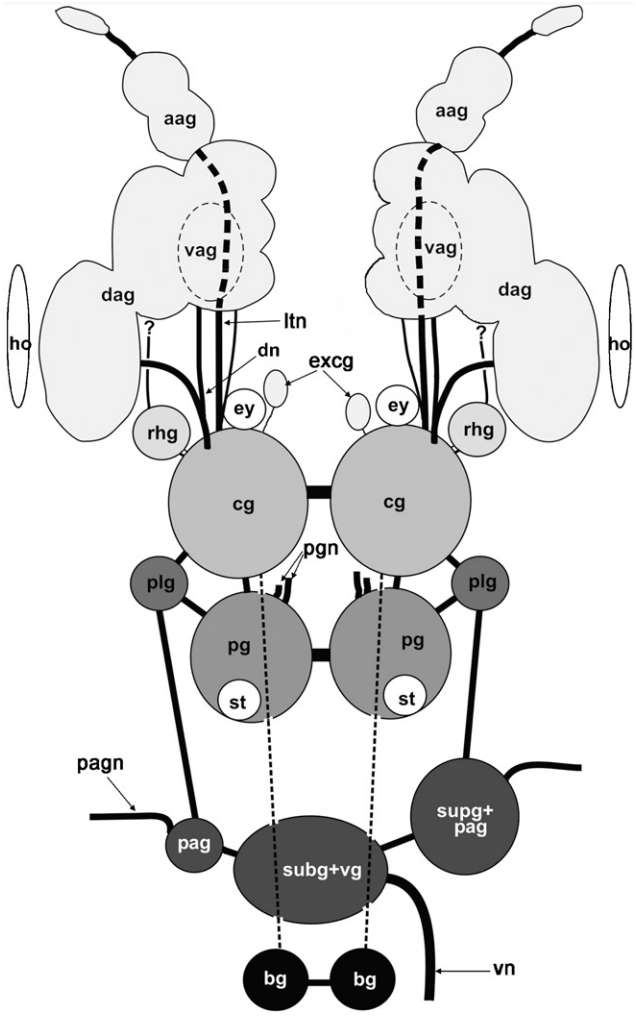

Fig. 5. Schematic of central nervous system (cns) in *Pontohedyle milaschewitchii*, dorsal view. Abbreviations: aag = anterior accessory ganglia complex, bg = buccal ganglion, cg = cerebral ganglion, dag = dorsolateral accessory ganglia complex, dn = dorsal nerve, excg = 'extra-cerebral accessory ganglion', ey = eye, ho = Hancock's organ, ltn = labiotentacular nerve, pag = parietal ganglion, pagn = nerve emerging from parietal ganglion, pg = pedal ganglion, pgn = nerve emerging from pedal ganglion, plg = pleural ganglion, rhg = rhinophoral ganglion, st = statocyst, subg = subintestinal ganglion, supg = supraintestinal ganglion, vag = ventral accessory ganglia complex, vg = visceral ganglion, vn = visceral nerve.

in three paired complexes: the anterior, the dorsolateral and the ventral accessory ganglia complex (Fig. 6C). Unfortunately, it remains unclear whether the dorsolateral accessory ganglia complex forms one continuous mass of accessory ganglia or should be subdivided into a dorsal and a lateral complex. Size and shape of the accessory ganglia complexes vary from individual to individual, and even within a single specimen between the right and left body sides.

The anterior accessory complex can be subdivided into the main complex, which is innervated by the strong labiotentacular nerve emerging ventrally from the cerebral ganglion, and a small accessory-ganglion-like swelling in the oral tentacle (Fig. 6C). A strong nerve connects the main complex with the swelling in the tentacle. At the cerebral base of the labiotentacular nerve a thinner nerve splits off and runs to the inner dorsal part of the dorsolateral accessory ganglion complex. Apart from this nerve the large dorsolateral accessory ganglia complex receives two more cerebral nerves, and most likely the nerve from the rhinophoral ganglion. The strong dorsal nerve emerges from an anterodorsal position of the cerebral ganglion. The nerve bifurcates at its cerebral base. The strong outer branch of the dorsal nerve innervates the lateral part, its thinner inner branch the dorsal part of the dorsolateral accessory ganglia complex (Figs. 5, 11A). No cerebral or other nerves innervating the ventral accessory ganglia complex could be detected. This comparatively small complex is located ventrally of the other accessory ganglia dorsolateral to the anterior pedal gland.

Additional very small (diameter 10 μm) nervous structures could be detected anterior to the cerebral ganglia. These swellings, in the following referred to as 'extra-cerebral accessory ganglia', vary in presence, number (one or two) and position from anterolateral to anterodorsal of the eyes (Fig. 6C). They are connected by a very short and thin connective to the cerebral ganglion. A thin nerve emerges from the 'extra-cerebral accessory ganglia' and runs anteriorly, probably leading to the dorsal part of the dorsolateral accessory ganglia complex.

Ganglia

A pair of large cerebral ganglia (diameter 70 μm) lies dorsally to the other ganglia and is connected by the strong and short cerebral commissure (Fig. 6B, D). Two pairs of connectives can be distinguished: Ventrally from the cerebral ganglia emerges the strong and relatively short cerebro-pedal connective. The very short cerebro-pleural connective emerges in the ventrodistal region of the cerebral ganglion. The cerebro-buccal connective could not be found.

Small rhinophoral ganglia (diameter 25 μm; for identification see Discussion below) are located anterolaterally on the cerebral ganglia. They are surrounded by a layer of connective tissue and by a second, thinner layer which they share with the cerebral ganglia (Fig. 6D). A clear division of the rhinophoral ganglia in cortex and medulla could not be detected, but on the basis of their general appearance (staining qualities, arrangement of nuclei and presence of a comparatively thick layer of connective tissue) they differ from accessory ganglia. The rhinophoral ganglia are connected to the cerebral ganglia by two extremely short

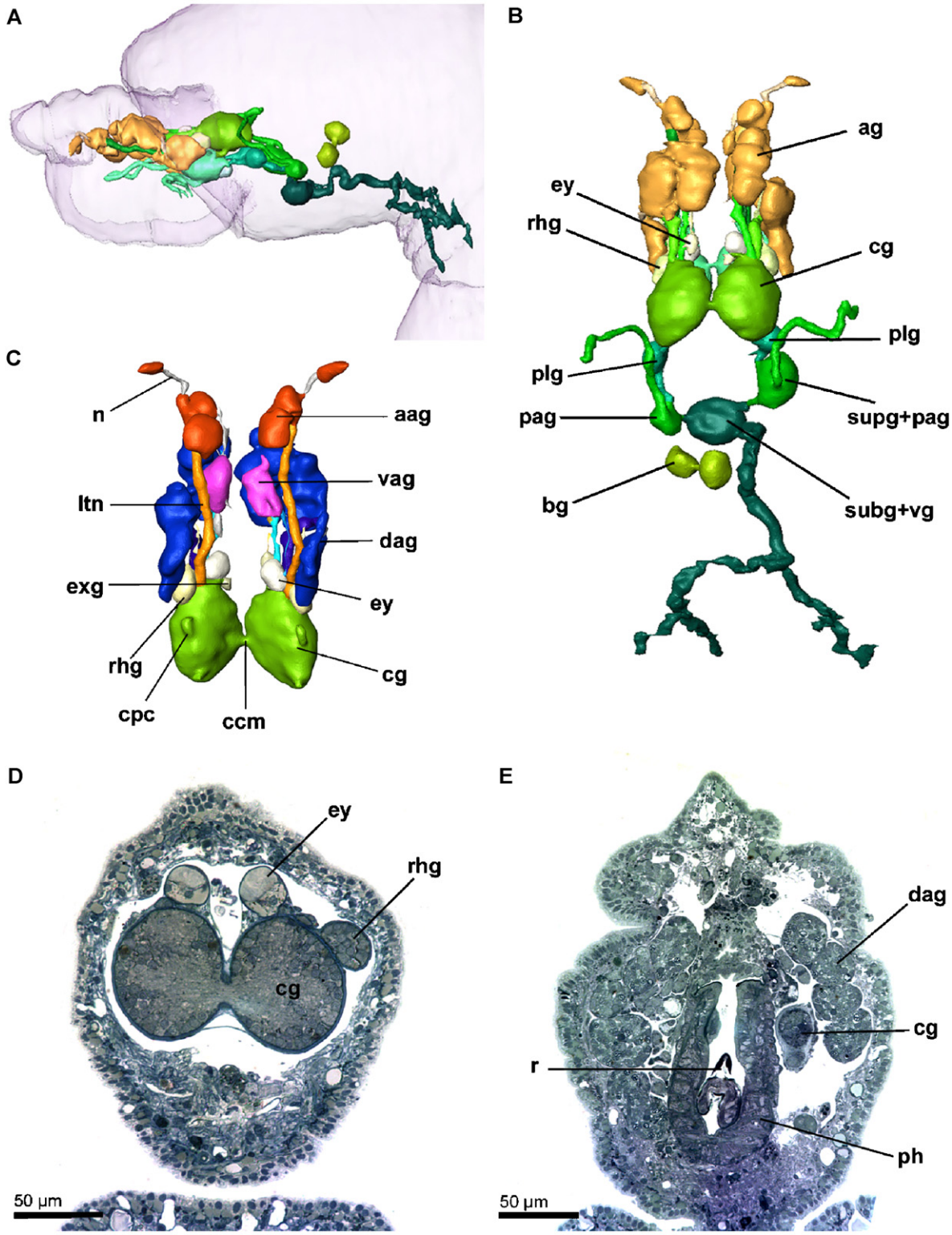

Fig. 6. Central nervous system (cns) in *Pontohedyle milaschewitchii*. (A) Overview of position of organ system in specimen, lateral view. (B) 3D reconstruction, dorsal view. (C) 3D reconstruction of innervation of accessory ganglia complexes, ventral view. (D, E) Horizontal semithin sections. (D) Cerebral ganglia with eyes. (E) Dorsolateral accessory ganglia complex. Abbreviations: aag = anterior accessory ganglia complex, ag = accessory ganglia, bg = buccal ganglion, ccm = cerebral commissure, cg = cerebral ganglion, cpc = cerebro-pedal connective, dag = dorsolateral accessory ganglia complex, exg = 'extra-cerebral accessory ganglion', ey = eye, ltn = labiotentacular nerve, n = nerve connecting parts of aag, pag = parietal ganglion, ph = pharynx, plg = pleural ganglion, r = radula, rhg = rhinophoral ganglion, subg = subintestinal ganglion, supg = supraintestinal ganglion, vag = ventral accessory ganglia complex, vg = visceral ganglion.

and thin connectives. A thin nerve leaves the rhinophoral ganglia anteriorly and runs along the dorsolateral accessory ganglia complex, most likely leading into the dorsal part of this complex.

The pedal ganglia are slightly smaller (diameter 60 μm) than the cerebral ganglia and located anteroventrally of those. They are connected by the strong and short pedal commissure. No parapedal commissure could be detected. The cerebro-pedal connective is clearly shorter than the pleuro-pedal connective. Two nerves leave each of the pedal ganglia: one runs in anterior direction and most probably innervates the anterior region of the foot, the other nerve runs to anterior as well, then twists to run backwards before turning to ventral and leading towards the posterior part of the foot.

The pleural ganglia are relatively small (diameter 25 μm) and are situated posteroventrally of the cerebral ganglia. The cerebro-pleural connectives are thin and very short; the pleuro-pedal connectives are slightly longer. Accordingly, the circumoral ring is quite narrow.

Three ganglia (for identification see Discussion) lie on the visceral cord: the right supraintestinal/parietal ganglion (40 μm), the subintestinal/visceral ganglion (55 μm), and the small left parietal ganglion (25 μm). The pleuro-supraintestinal/parietal connective is relatively short compared to the long pleuro-parietal connective on the left side of the visceral loop. A strong nerve emerges from each parietal ganglion dorsally, then passes laterally into the body wall of the visceral sac. The oval subintestinal/visceral ganglion is shifted slightly to the left side of the visceral cord. The supraintestinal/parietal-subintestinal/visceral connective is slightly longer than the parietal-subintestinal/visceral connective. The very strong, thick visceral nerve emerges laterally from the right side of the subintestinal/visceral ganglion and passes to posterior ventrally of the pharynx. Reaching the visceral sac the nerve bifurcates, with both parts running along the sides of the visceral sac.

Small buccal ganglia (diameter 25 μm) lie postpharyngeally, dorsolaterally of the pharynx-to-oesophagus transition. They are connected by a relatively long and thin commissure. One nerve leaves each of the buccal ganglia dorsolaterally, running laterally into the pharynx and passing through its epithelium in anterior direction. It is regarded as the cerebro-buccal connective. A radula nerve could not be detected. The presence of gastro-oesophagial ganglia could not be determined in the sectioned series.

Sensory organs

The subepidermal eyes nestle directly on the anterior surface of the cerebral ganglia (Fig. 6D). Each eye is approximately 20 μm long, oval, and forms a pigmented cup with a clear lens. The innervation of the eyes could not be detected using light microscopy. The eyes are surrounded by a thin layer of connective tissue which also surrounds cerebral and rhinophoral ganglia. The pair of statocysts is attached to the pedal ganglia at their posterior ends. The oval statocysts have a diameter of about 20 μm and contain one statolith each. The Hancock's organ is a pair of conspicuous folds in the epidermis just posterior to the oral tentacles (Fig. 1E). This organ is straight to bow-shaped, 60 μm long and 5 μm wide. The cells are non-glandular; some bear short cilia. A nerve innervating the Hancock's organ could not be fully ascertained, but innervation of the closely associated dorsal part of the dorsolateral accessory ganglia complex is likely.

Digestive system

(Fig. 7)

The mouth is located subterminally between the oral tentacles and leads into the oral tube. In its anterior part the thin epithelium of the oral tube is ciliated. The pharynx is bulbous and muscular; its tissue appears dark blue (in methylene blue-stained semithin sections) and folded. The entire radula lies in a radula sac in the center of the pharynx towards its posterior end (Fig. 7D). The radula is approximately 90–110 μm long, 20 μm wide, and bent to ventral in the anterior part. The dorsal part is about 2.5 times as long as the ventral part, which bears the older teeth. The number of rows in adult specimens varies between 41 and 54, 31–38 of them located on the dorsal ramus, 8–18 on the ventral one. The radula is symmetrical: each row consists of a central rhachidian tooth and one lateral plate on each side. Thus, the radula formula of *P. milaschewitchii* is 41–54 × 1-1-1. The rhachidian tooth consists of the central cusp and three lateral denticles on each side (Fig. 7C). The central cusp and the lateral denticles are triangular and slightly recurved. The lateral plates are thin, wide and slightly curved rectangular plates, each bearing one central triangular denticle (Fig. 7C). Each lateral plate has a matching notch on the anterior surface margin into which the denticle of the posterior plate fits. Jaws are absent.

The salivary glands are well developed and form a fused mass on the left side of the body (Fig. 7B). The mass fills large parts of the posterodorsal portion of the head–foot complex. Two ciliated ducts connect the salivary glands with the buccal mass laterally on the left and the right side of the transition between pharynx and oesophagus (Fig. 7D). The tube-like, ciliated oesophagus leaves the pharynx posterodorsally, connecting to the digestive gland and the intestine on the right side of the anterior part of the visceral hump. A histologically or anatomically differentiated stomach could not be detected. The digestive gland is holohepatic; it has an elongated sac-like shape with a number of bends and folds and extends over the entire length of the visceral hump (Fig. 7A). In adult specimens the digestive gland

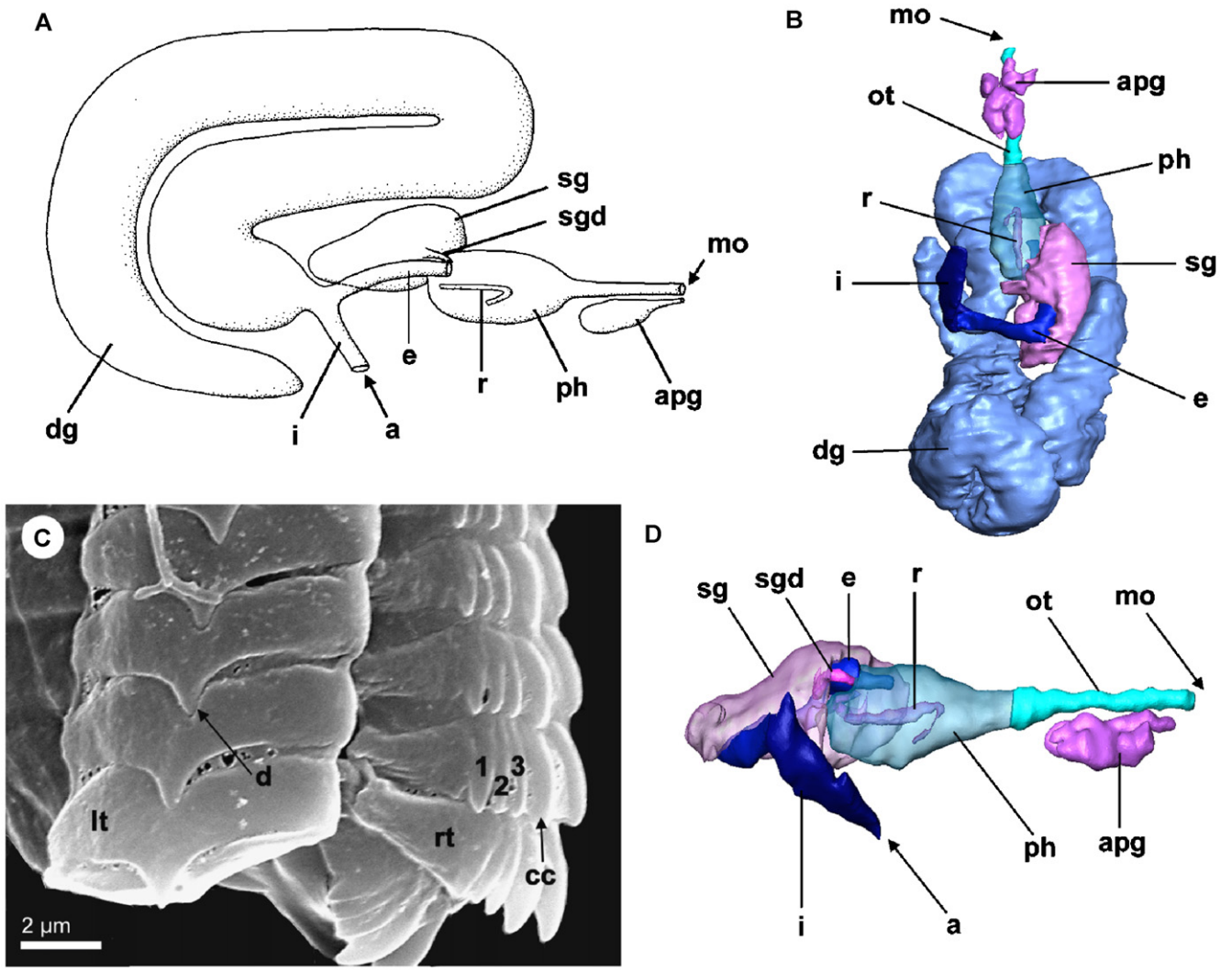

Fig. 7. Digestive system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) 3D reconstruction, ventral view. (C) SEM micrograph of right lateral and rhachidian tooth of radula. (D) 3D reconstruction, lateral view, digestive gland omitted. Abbreviations: a = anal opening, apg = anterior pedal gland, cc = central cusp, d = denticle, dg = digestive gland, e = oesophagus, i = intestine, lt = lateral tooth, mo = mouth opening, ot = oral tube, ph = pharynx, r = radula, rt = rhachidian tooth, sg = salivary glands, sgd = salivary gland duct, 1–3 = lateral denticles of rhachidian tooth.

is coiled around the gonad; its position seems to depend on the development of the gonads and the stage of contraction of the animal. The intestine is a relatively short, strongly ciliated tube. It leads to the anal opening, located on the right side of the visceral hump, clearly behind the transition from the head–foot complex to the visceral hump, and posterior to the female genital opening and the nephropore (Fig. 3).

Excretory and circulatory systems (Fig. 8)

Excretory and circulatory organs are located on the anterior right side of the visceral hump (Fig. 8A); they comprise a reduced heart enclosed in a thin but relatively spacious pericardium, and a spherical kidney. The pericardium lies anteriorly to the kidney; in its posterior region it encloses the heart. The latter is a $40 \times 10 \times 10\,\mu m^3$ chamber (Fig. 8D); no subdivision into ventricle and auricle could be detected. Pericardium and kidney are connected via the very short but relatively wide renopericardial duct (Fig. 8C). No cilia could be detected in the duct's lumen. The renopericardial duct emerges laterally from the posterior end of the pericardium and enters laterally the lumen of the kidney. The slightly spherical kidney encloses the posterior third of the pericardium (Fig. 8B). The kidney (= nephridium, emunctorium) is characterized by a glandular and vacuolated epithelium. The nephroduct emerges ventrally and runs closely adjacent to the gonoduct. The nephropore opens anteriorly to the anus on the right side of the head–foot complex, close to the junction with the visceral hump. The position of

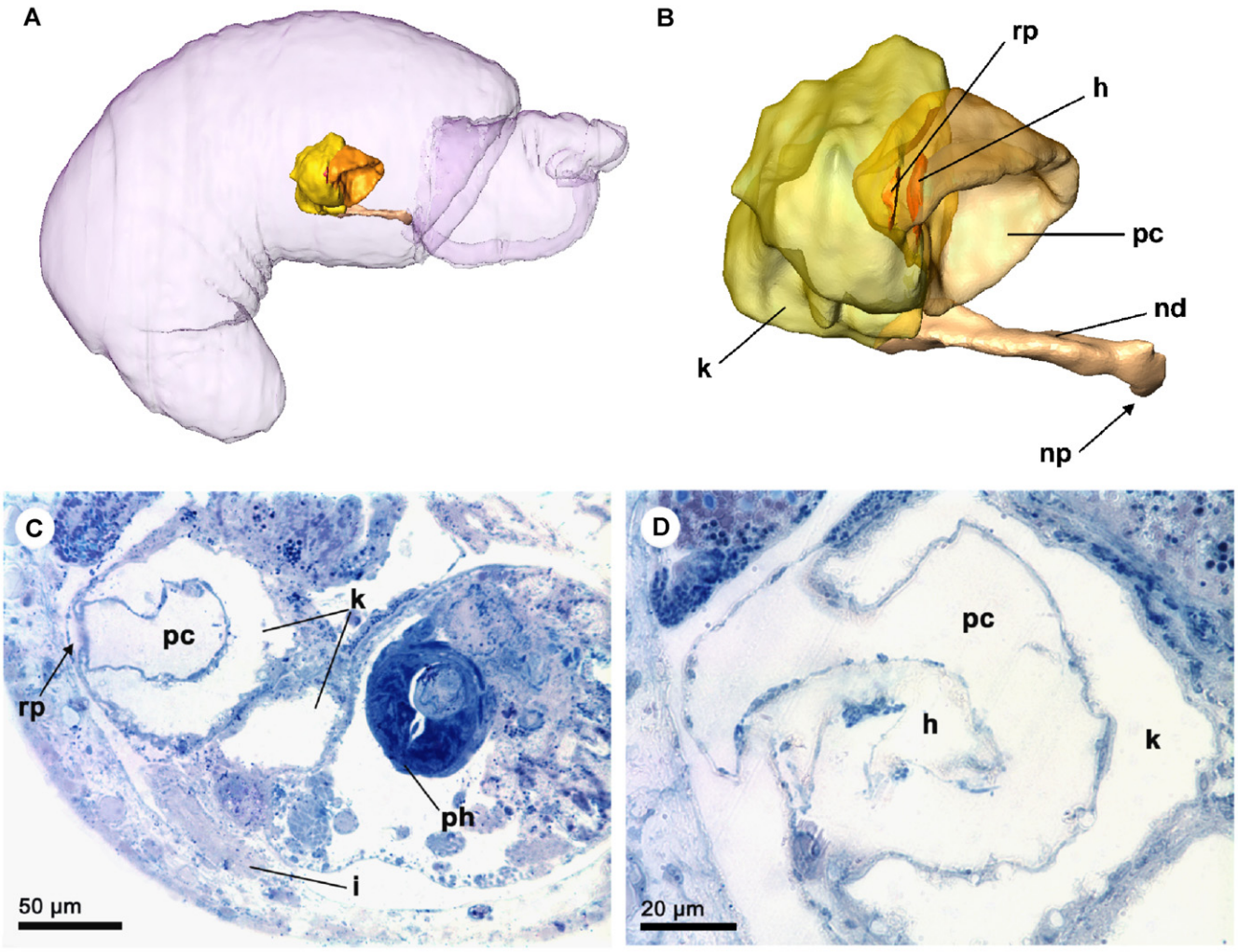

Fig. 8. Excretory and circulatory systems in *Pontohedyle milaschewitchii*. (A) Overview of positions of organ systems in specimen of 1.5 mm body length, lateral view. (B) 3D reconstruction, right-lateral view. (C, D) Semithin cross-sections. (C) Kidney and pericardium. (D) Heart. Abbreviations: h = heart, i = intestine, k = kidney, nd = nephroduct, np = nephropore, pc = pericardium, ph = pharynx, rp = renopericardial duct.

the nephropore relative to the female gonopore could not be determined, due to their close association and to poor histology in the sectioned series.

Reproductive systems

The sexes are separate in *P. milaschewitchii*. In adult specimens the reproductive system extends over the entire length of the visceral hump. The terminology used in the following description of the female and the male genital systems follows Ghiselin (1965) and Klussmann-Kolb (2001).

Female genital system

(Fig. 9)

The female reproductive system includes ovary, oviduct and nidamental glands. The sac-like ovary is closely associated with the digestive gland and extends over the entire length of the visceral sac. The ovary is loosely packed with oocytes in different stages of development: various large, vitellogenic oocytes (stage III) with a diameter of 60 µm, a series of smaller oocytes (20–30 µm; stage II), and oocytes in follicles with vitellus aggregating around them (stage I). Developing oocytes in stage II sometimes contained more than one nucleolus per nucleus (up to three nucleoli). All stage III oocytes observed had one nucleolus per nucleus only. No consistent pattern of distribution of eggs in various stages of development within the ovary could be determined. Exogenous sperm was not found in any of the sectioned females.

There are three nidamental glands connected directly to each other and apparently showing a continuous lumen throughout (Fig. 9A). No histologically or anatomically defined proximal oviduct or adhesive

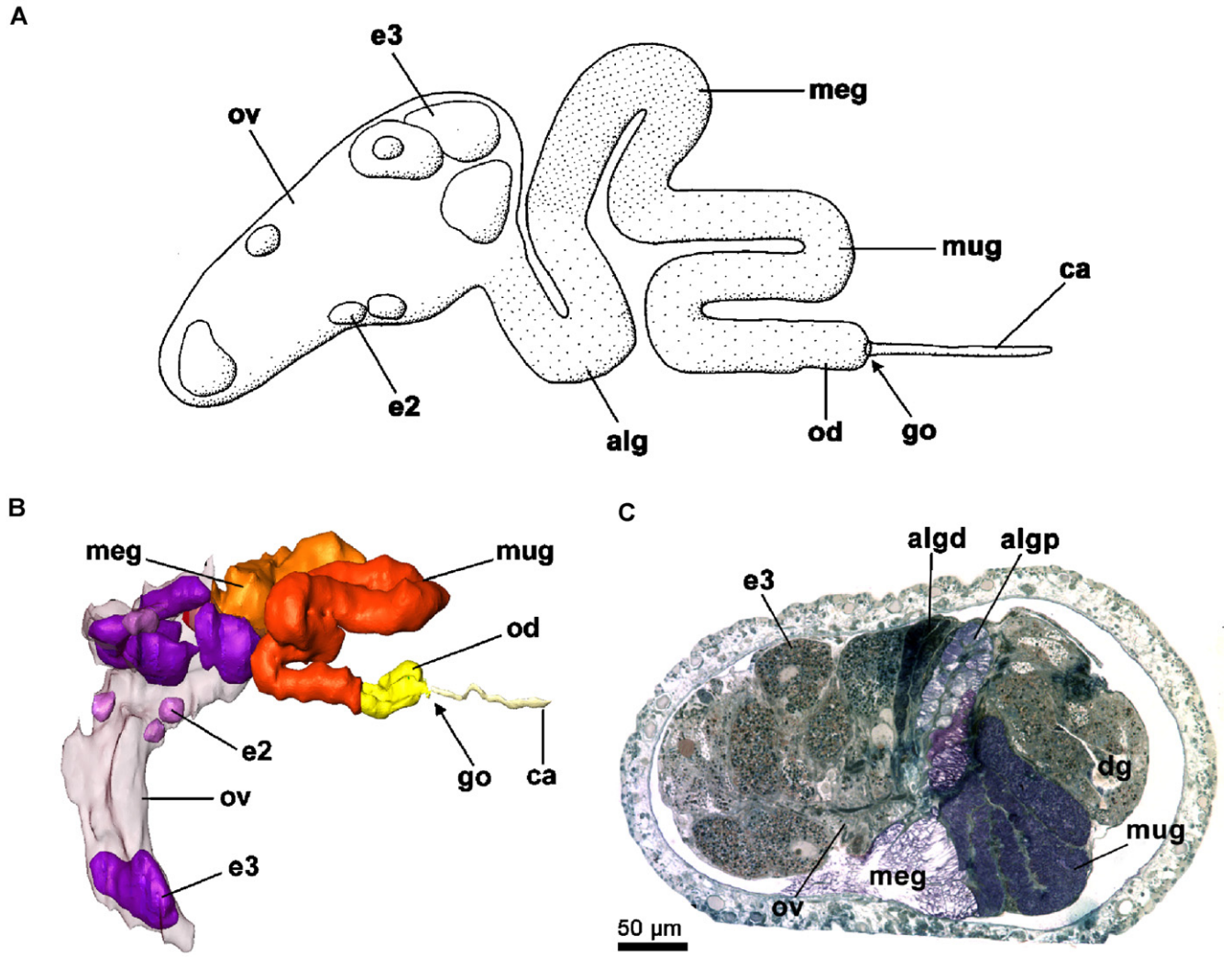

Fig. 9. Female genital system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) 3D reconstruction, right-lateral view. (C) Semithin cross-section of nidamental glands and ovary. Abbreviations: alg = albumen gland, algd = albumen gland distal part, algp = albumen gland proximal part, ca = ciliated area, dg = digestive gland, e2/3 = egg in stage II/III, go = genital opening, meg = membrane gland, mug = mucus gland, od = oviduct, ov = ovary.

region could be detected. The tube-like albumen gland is the smallest of the three nidamental glands. Histologically, it can be divided into a proximal and a distal part: the proximal part comprises elongated wedge-shaped secretory cells which contain a dense mass of very dark blue-stained granules; in the distal region the cells are of similar shape but do not contain granules and stain slightly purple (Fig. 9C). Over the entire gland, the epithelium bears relatively long cilia. The slightly larger membrane gland is also tube-like. Its secretory cells stain pinkish-purplish, have a glandular appearance, and contain large vacuoles. The epithelium of the membrane gland bears comparatively short cilia. The tube-like mucus gland is the largest of the nidamental glands and winds through the anterior part of the visceral hump (Fig. 9B). Its cells stain purple, contain few dark purple-stained granules, and are elongate oval in shape. The lumen partially widens to a size of $20 \times 40\,\mu m^2$; the epithelium is heavily ciliated, bearing long cilia. The distal oviduct emerges posteriorly from the mucus gland and at its beginning shows similar histology. The epithelium of the distal oviduct is ciliated. In its further course the distal oviduct loses the glandular appearance, as well as the purple staining. The duct runs ventrally of the kidney, closely adjacent to the nephroduct, and leads to the genital opening. In female *P. milaschewitchii* the genital opening is located anteriorly to the anus on the right side of the head–foot complex, close to the transition to the visceral hump. A ciliated band originates from the genital opening and runs along the right side of the head–foot complex (Fig. 9A). The band is about 15 µm wide and extends for

approximately one third of the length of the head–foot complex.

Male genital system

(Fig. 10)

The male genital system comprises the gonad and the gonoduct. The gonoduct can be divided from posterior to anterior into the ampulla, the prostatic vas deferens and the ciliated vas deferens (Fig. 10A).

The sac-like gonad is found closely associated with the digestive gland and extends over the entire length of the visceral sac (Fig. 10B). The gonad comprises various irregularly distributed groups of spermatozoids (Fig. 10E). The spermatozoids are elongated; their nuclei stain dark blue. The ampulla emerges from the anterior part of the gonad; no histologically or anatomically differentiated preampullary gonoduct could be detected. The tube-like ampulla has a diameter of about 50 µm and is bulging with sperm lying in disorder within the ampulla (Fig. 10E). A short post-ampullary gonoduct exists terminally of the ampulla. The epithelium of the post-ampullary gonoduct is thin, ciliated and bears a small lumen.

The vas deferens can be divided histologically and anatomically into a prostatic and a non-glandular section. The prostatic part has tube-like shape. Near its posterior end the diameter is about 25 µm; in its further course the prostatic part narrows to approximately 20 µm diameter. The prostatic vas deferens has elongate oval glandular cells which contain deeply pink-staining granules (Fig. 10E). Its epithelium bears long cilia. In the narrower part of the prostatic section no ciliation could be detected. The prostatic vas deferens passes to anterior on the right side of the body. In the posterior region of the head–foot complex it transforms into the non-glandular section of the vas deferens.

The non-glandular section of the vas deferens passes to anterior on the right side of the head–foot complex, slightly subepidermally. The duct has a diameter of about 10 µm; its epithelium is heavily ciliated. Before reaching the level of the oral tentacles it turns to dorsal. After passing the right oral tentacle it turns towards the midline of the head–foot complex. There it continues to the anterior tip of the head–foot complex (Fig. 10B, D). The male genital opening is located dorsally of the mouth opening (Fig. 10C).

Discussion

External morphology and spicules

All adult Acochlidia (as defined by Wawra 1987) are externally characterized by the absence of a shell and the presence of a visceral hump, which is axially elongated and clearly distinct from the head–foot complex, and into which the latter can be retracted at least partially. *Pontohedyle milaschewitchii* conforms to these general characteristics, but differs from the majority of other acochlidians in the lack of rhinophores.

We show that the shape of the oral tentacles is variable in *P. milaschewitchii*. This intraspecific variation caused some confusion in the past. Kowalevsky (1901) illustrated the oral tentacles of his Black Sea specimen with a curved bow-like shape. Marcus and Marcus (1954) described a *P. milaschewitchii* from Brazil with the oral tentacles "flat, triangular, the margins straight and angular." Challis (1970) suggested that because of the differences in the shape of the head (among others), the Brazilian specimen possibly represented a species different from Kowalevsky's. Rankin (1979) took this one step further by erecting the genus and species *Gastrohedyle brasilensis* based solely on the description by Marcus and Marcus (1954). Unfortunately, the type specimen of *Gastrohedyle brasilensis* from the Marcus collection seems to be lost, thus has not been available for re-examination (Jörger et al. 2007). The variation in the oral tentacles reported above, within our Mediterranean populations and even within single specimens, shows that this character by itself does not justify species separation. Flattened oral tentacles also occur in *Pontohedyle verrucosa* (see Challis 1970), and in the genera *Ganitus* (Marcus 1953) and *Hedylopsis* (e.g. Odhner 1937; Sommerfeldt and Schrödl 2005). Members of *Hedylopsis*, however, have significantly broader oral tentacles than those of *Pontohedyle*; in *Ganitus* the oral tentacles are not tapered towards the tip (Jörger et al. 2007). Thus, the quoted authors considered the flat, elongated to bow-shaped oral tentacles as a diagnostic feature for the genus *Pontohedyle*.

Subepidermal, calcareous spicules are a characteristic feature in many interstitial opisthobranchs, i.e. in *Rhodope, Helminthope* and most Acochlidia (Rieger and Sterrer 1975; Arnaud et al. 1986; Salvini-Plawen 1991). In *Asperspina* and *Hedylopsis* elongated and fairly long (up to 250 µm) needle-like spicules occur in high densities, forming dense covers and giving the visceral hump a stiff shape (e.g. Odhner 1937; Swedmark 1968a; Salvini-Plawen 1973; Morse 1976; Kudinskaya and Minichev 1978; Sommerfeldt and Schrödl 2005). In other acochlidians, only significantly smaller spicules (up to max. 40 µm) of various shapes (e.g. oval, plate-, star- or needle-like) occur randomly distributed in the tissue (e.g. Marcus 1953; Marcus and Marcus 1954; Challis 1968; Westheide and Wawra 1974; Neusser and Schrödl 2007), or spicules are lacking completely (Challis 1970; Neusser et al. 2006). Swedmark (1968b) assumed that densely arranged spicules as in *Hedylopsis* and *Asperspina* might serve the same protective purpose

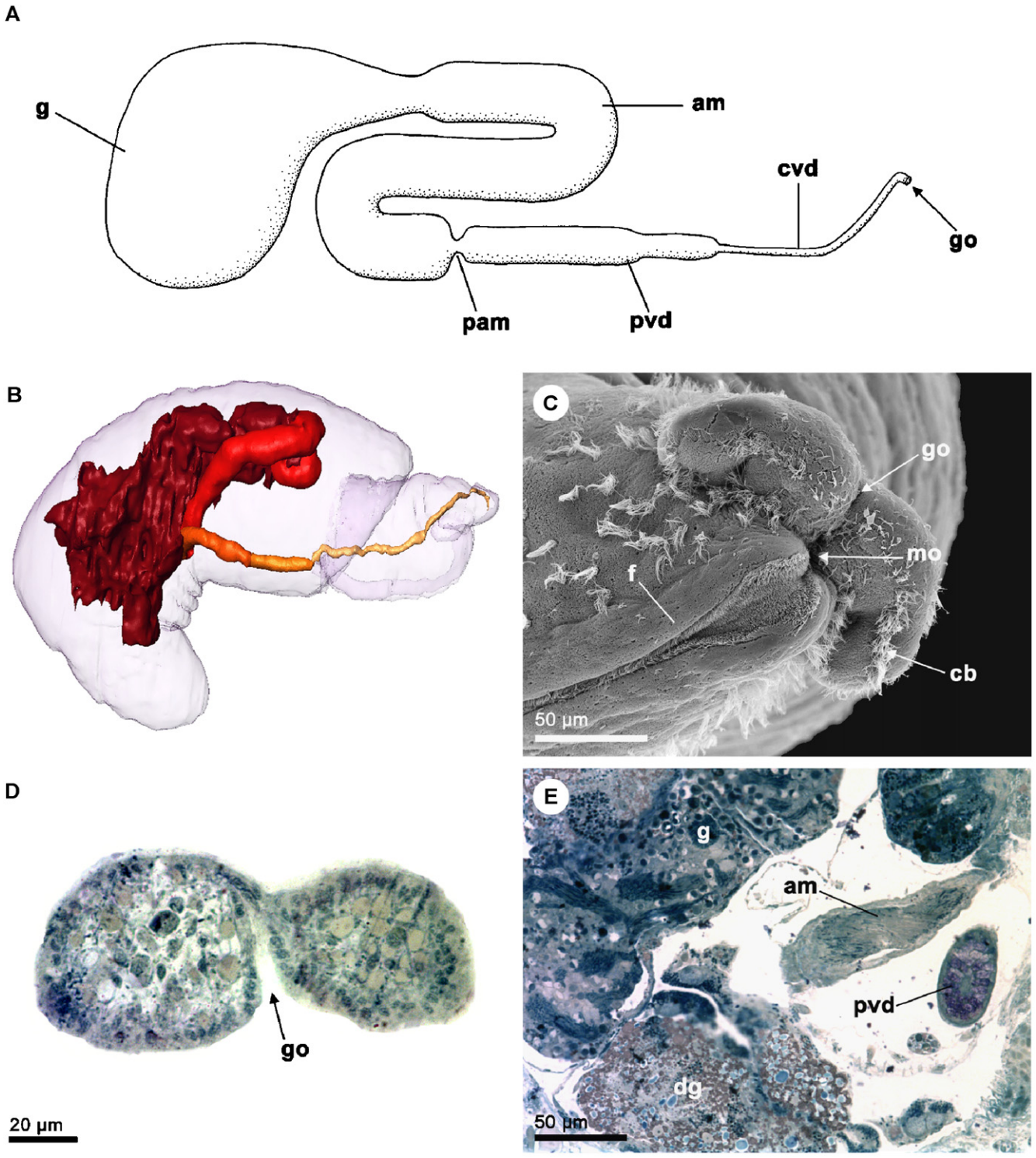

Fig. 10. Male genital system in *Pontohedyle milaschewitchii*. (A) Schematic overview, lateral view. (B) Overview of position of organ system in specimen of 1.5 mm body length, lateral view. (C) SEM micrograph of head showing male genital opening dorsally of mouth opening. (D) Semithin cross-section of male genital opening between oral tentacles. (E) Semithin cross-section of gonad and ampulla. Abbreviations: am = ampulla, cb = ciliary band on oral tentacle, cvd = ciliated vas deferens, dg = digestive gland, f = foot, g = gonad, go = genital opening, mo = mouth opening, pam = post-ampullary gonoduct, pvd = prostatic vas deferens.

as a shell. It is, however, unlikely that such a 'secondary spicule-shell' could resist direct wave action without being ground or smashed by the sand. Rigidly armored species also lose flexibility and might therefore prefer coarser sand and gravel habitats with larger interstices; in fact, most armored species are known from coarse subtidal sands. An exception is the polar *Asperspina murmanica*, which occurs in the intertidal (Kudinskaya and Minichev 1978), but this species' biological preferences and population densities in deeper sands are unknown. Spicule armor might offer some protection against potential interstitial predators such as polychaetes, considering that the head–foot complex can be retracted completely into the protected visceral hump. In contrast, loosely distributed, small spicules are unlikely to provide any special mechanical protection (Swedmark 1968b; Rieger and Sterrer 1975), but they allow higher flexibility and deformability of the body. 'Unprotected', flexible Microhedylidae, Ganitidae, and *Pseudunela* thus might be able to colonize finer sands, with higher mechanical energy, than their stiff counterparts. In addition to the poorly investigated, rigid *Asperspina murmanica*, the flexible *Parhedyle cryptophthalma*, *Ganitus evelinae*, *Paraganitus ellynae,* and *Pseudunela cornuta* (Westheide and Wawra 1974; Morse 1987; MS, pers. obs.) are the only acochlidians that occur in, prefer or even exclusively inhabit intertidal high-energy zones (i.e. sands directly exposed to wave action). The role of potential predators remains to be investigated.

What, then, are spicules good for in flexible species? Even small spicules may serve to stabilize the surrounding tissue or body region, especially when arranged in clusters (Rieger and Sterrer 1975). The aggregation of needle-like spicules between the oral tentacles of *P. milaschewitchii*, for example, might give the head additional stabilization while the animal is moving and digging between sand granules in the interstitial environment. Another conspicuous cluster of small oval or bean-shaped spicules (length around 10 µm) is found in *P. milaschewitchii* near the posterior end of the pharynx. Morse (1976) reported similar aggregations of irregular, amorphous spicules (measuring 15–38 × 9–12 µm) at the base of the buccal mass in *Asperspina riseri*. The function of these accumulations requires further investigation.

External SEM examination showed a conspicuous distribution of bundles of cilia in *P. milaschewitchii*. Although the density of the bundles varied between the specimens, a constant pattern could be detected, conforming to SEM micrographs of *P. milaschewitchii* published by Wawra (1986). Our preliminary SEM examinations of *Asperspina murmanica, Hedylopsis spiculifera* and *Paraganitus ellynnae* have revealed a unique overall ciliary pattern for each species. *Asperspina murmanica* shows constant ciliation over the entire head–foot complex and the anterior region of the visceral hump, with slightly more dense concentration of ciliary bundles on the rhinophores and oral tentacles. A similar pattern of cilia distribution was reported for *A. riseri* by Morse (1976). *H. spiculifera* shows an extremely dense ciliation over the entire head-foot complex, and randomly distributed cilia on the entire visceral hump, whereas *Paraganitus ellynnae* has only very few, scattered bundles of cilia in the anterior region of the head–foot complex.

Aside from the overall pattern, acochlidian species can be distinguished by special ciliated structures on the head appendages (two bands on the oral tentacles and one transverse band in *P. milaschewitchii*) or by ciliated areas originating from the gonopore. Differences occur also in the density and size of pores of epidermal gland cells. While the visceral hump of *P. ellynnae* is densely covered with large pores, *P. milaschewitchii* has fewer pores of various sizes, and *H. spiculifera* only some small pores.

These observations show that SEM examination can offer an additional set of external characters for taxonomic purposes that might also be of phylogenetic value. Thus, the method is recommended as the standard technique for describing acochlidian species. Suggested diagnostic characters for species are: (1) the general distribution pattern of cilia bundles on the head–foot complex and visceral hump; (2) the presence/absence, number and development of ciliary bands on the head appendages; (3) ciliated areas associated with the gonopore; and (4) the distribution, size and amount of pores of epidermal gland cells.

Microanatomy

The large anterior pedal gland in *P. milaschewitchii* accompanies the oral tube ventrally and discharges its mucus to the exterior via an opening slightly anterior of the mouth opening. Frequently in the past, similar glands in acochlidians have been termed "oral" or "vestibular" glands, e.g. by Challis (1970) in *Pontohedyle verrucosa* and *Hedylopsis cornuta* or by Doe (1974) in *Microhedyle nahantensis* (as *Unela*). However, the 'vestibular gland' of *M. nahantensis* has histochemical properties identical to those of the small pedal glands, shows no connection to the oral tube but instead an opening to the exterior ventral of the mouth opening, and thus was reinterpreted as an anterior pedal gland by Robinson and Morse (1976). Concerning the histology of the gland cells and the position of the gland, *P. milaschewitchii* closely resembles *M. nahantensis*. Robinson and Morse (1976) suggested that the mucous substances of the pedal glands in *M. nahantensis* play a role as a lubricant, aiding in locomotion and/or contributing to the ability to adhere to sand grains. A potential, perhaps additional role during feeding can

neither be suggested nor excluded in the absence of any knowledge on the food and feeding habits of acochlidians.

Nervous system

The cns of *Pontohedyle milaschewitchii* conforms to what has been shown recently for other acochlidian species (Sommerfeldt and Schrödl 2005; Neusser et al. 2006; Neusser and Schrödl 2007) concerning the high concentration, prepharyngeal position, and the euthyneurous and epiathroid condition.

Accessory ganglia

Neusser et al. (2006) defined accessory ganglia as distinct cell groups displaying a homogenous distribution of nuclei (i.e. without subdivision into cortex and medulla). Additionally, accessory ganglia can be characterized here as being surrounded by connective tissue that appears to be thinner than the one surrounding true ganglia. Several accessory ganglia on the anterior cerebral nerves are found in members of the Asperspinidae, Microhedylidae, Ganitidae, i.e. in 3 out of 6 families according to the classification of Wawra (1987). The latter author considered the lack of accessory ganglia as diagnostic for Hedylopsidae, Acochlidiidae and Tantulidae. Recently published data on *Hedylopsis ballantinei* and *H. spiculifera* (see Sommerfeldt and Schrödl 2005) agree with this assessment for the genus *Hedylopsis*. However, there are "some" accessory ganglia in the, according to Wawra, hedylopsid *Pseudunela cornuta* (see Challis 1970). Neusser and Schrödl (2007) reported aggregations of accessory ganglia in one examined specimen of *Tantulum elegans*, while there were no detectable accessory ganglia in other specimens. *Tantulum* was shown to be a truly sequential hermaphrodite, and the development or reduction of accessory ganglia may be related to preceding reorganizations at least of the reproductive organs. However, in the gonochoristic *P. milaschewitschii* accessory ganglia were present in all sections series (also in an early juvenile stage); therefore, their presence seems to be independent of the ontogenetic stage. No detailed data are available on cns features of the limnic *Strubellia*, also a sequential hermaphrodite, nor on any other Acochlidiidae.

In the present study the accessory ganglia of *P. milaschewitchii* could be grouped into three highly variable complexes. Marcus and Marcus (1954) recognized two groups of accessory ganglia in the Brazilian *P. milaschewitchii* ("tentacle ganglia" and "ganglia-like anterolateral groups of sensory neurons"), possibly referring to the anterior and the dorsolateral accessory ganglia complexes determined here. The ventral accessory ganglia complex is comparatively small and might have been overlooked by the earlier authors. The precerebral positions and cerebral innervation show obvious association of the accessory ganglia complexes to the cerebral ganglia, but the function of the accessory ganglia is still a matter of speculation. Haszprunar and Huber (1990) suspected the development of accessory ganglia in small opisthobranchs (e.g. *Platyhedyle* (Sacoglossa), *Philinoglossa* (Philinoidea)) to be a reaction to a lack of space for the neuronal tissue due to the small size of the animals, and to be a special adaptation to the interstitial environment. However, this does not explain why some similarly small acochlidians lack accessory ganglia, whereas the benthic runcinids, for example, also show precerebral nervous structures similar to accessory ganglia (Huber 1993). Immunocytochemical investigation and labeling against different neurotransmitters will be necessary to draw conclusions on the function of accessory ganglia and to determine whether certain groups of accessory ganglia are associated with certain sensory organs. Such an association can be suspected, e.g., for the anterior accessory ganglia complex in *P. milaschewitchii* with the oral tentacles and their associated ciliary bands, as well as for the accessory tentacle and rhinophoral ganglia in *Microhedyle remanei* (see Neusser et al. 2006). A possible neurosecretory function of the accessory ganglia should be investigated by TEM or ICC.

Cerebral nerves

The present examination of the cerebral nerves of *P. milaschewitchii* shows two strong bifurcating cerebral nerves (one emerging dorsally, one ventrally) and another thin nerve emerging from the rhinophoral ganglion (Fig. 11A). The static nerve could not be detected in *P. milaschewitchii*, but since statocysts are present, static nerves are assumed to be present as well. Edlinger (1980b) described three cerebral nerves for *P. milaschewitchii*: nerve 1 and 2 emerging anteriorly, nerve 3 laterally from the cerebral ganglion (Fig. 11B). A static nerve was assumed to be present, too. The most striking differences between the present study and Edlinger's (1980b) concern our findings of fully separate (rather than fused) cerebral and pleural ganglia and of a rhinophoral ganglion anterolateral of the cerebral ganglion. Instead, Edlinger (1980b) reported "nerve 3" in a posterolateral position and "with a lobe-like broadening" (see Fig. 11B). Since there is no such large, broadened nerve in this position, the "broadened nerve 3" of Edlinger (1980b) might correspond to the rhinophoral ganglion detected in the present study. The thin nerve emerging from the rhinophoral ganglion, however, does not resemble the splitting of "nerve 3" into several nerves illustrated by Edlinger. "Nerve 1" in Edlinger (1980b) clearly corresponds to the labiotentacular nerve of the present study (emerging ventrally). "Nerve 2" in Edlinger (1980b) corresponds to the strong bifurcating nerve emerging dorsally, here interpreted as

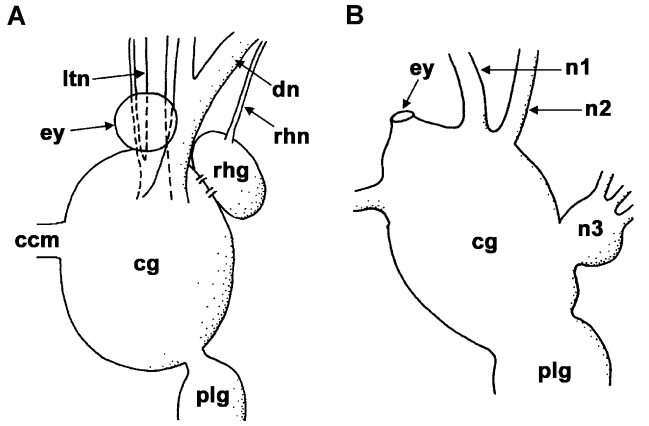

Fig. 11. Schematic drawings of cerebral nerve setting in *Pontohedyle milaschewitchii*; static nerve not shown. (A) Specimen from present study. (B) According to Edlinger (1980b, Fig. 5; as *Microhedyle milaschewitchii*). Abbreviations: ccm = cerebral commissure, cg = cerebral ganglion, dn = dorsal nerve, ey = eye, ltn = labiotentacular nerve, n1–3 = cerebral nerves 1–3, plg = pleural ganglion, rhg = rhinophoral ganglion, rhn = rhinophoral nerve.

a bifurcating oral nerve. Huber (1993) assumed a reduced number of cerebral nerves (labiotentacular, dorsal = fused rhinophoral/oral, and static nerves) as characteristic for Acochlidia, but overlooked a true rhinophoral ganglion in *Hedylopsis spiculifera* from which the dorsal nerve emerged (Sommerfeldt and Schrödl 2005). All acochlidians in which the cerebral nerves have been examined in detail share a strong ventral nerve that innervates the labial tentacles and thus is considered as the labiotentacular nerve (Sommerfeldt and Schrödl 2005; Neusser et al. 2006; Neusser and Schrödl 2007). Additionally, *Hedylopsis spiculifera*, *H. ballantinei* and *Tantulum elegans* possess true rhinophoral ganglia from which the strong rhinophoral nerve arises (Sommerfeldt and Schrödl 2005; Neusser and Schrödl, 2007). In *Hedylopsis* the rhinophoral nerve is joined with the thin optic nerve (Sommerfeldt and Schrödl, 2005), whereas in *Tantulum* the optic nerve emerges from an additional small optic ganglion and Hancock's nerve splits of from the rhinophoral nerve (Neusser and Schrödl, 2007). In *M. remanei* no true rhinophoral ganglion is present: the rhinophoral nerve emerges dorsally from the cerebral ganglion leading into an accessory ganglion (Neusser et al. 2006). An oral nerve could not be detected in any of these species. The nerve configuration in *P. milaschewitchii* is complicated by the numerous accessory ganglia, into which the cerebral nerves lead, making it difficult to determine which organs the cerebral nerves innervate. The (reduced) rhinophoral nerve in *P. milaschewitchii* probably leads into the dorsolateral accessory ganglion complex and might be involved with the innervation of Hancock's organ. However, the outer branch of the dorsal nerve also leads into this complex, and might therefore also innervate Hancock's organ. This would agree with Edlinger's (1980b) claim that "nerve 2" innervates Hancock's organ in *P. milaschewitchii*. But the author also stated that the situation in *Microhedyle glandulifera* is "similar" to *P. milaschewitchii*, with "nerve 2" also innervating the rhinophores. This seems questionable due to the presence of a true rhinophoral ganglion in *P. milaschewitchii*; reinvestigation of *M. glandulifera* is required. In general the settings and homologies of acochlidian cerebral features are far from being fully understood; comparative analyses of further acochlidians, related opisthobranchs and also pulmonates (see Neusser et al. 2007) could be facilitated by special histochemical or immunocytochemical techniques.

Ganglia

Rhinophoral ganglia in Acochlidia can be determined by their positions anterior to the cerebral ganglia, the cerebral innervation and by bearing the nerve innervating the rhinophores (Neusser et al. 2007). *Pontohedyle milaschewitchii* lacks rhinophores, but rhinophoral ganglia were recognized as such from their positions anterolateral to the cerebral ganglia and their cerebral innervation. Additionally, the rhinophoral ganglia of *P. milaschewitchii* are located in a second layer of connective tissue shared with the cerebral ganglia, as reported for the rhinophore-bearing *Tantulum elegans* (see Neusser and Schrödl 2007). In *P. milaschewitchii* the rhinophoral ganglia lack a clear division into cortex and medulla, but due to their general appearance (staining properties, arrangement of nuclei, and possession of relatively thick connective tissue) they are considered as ganglia rather than as accessory ganglia here. Accessory rhinophoral ganglia are known from the rhinophore-bearing *Microhedyle remanei* (see Neusser et al. 2006). More data are needed on different ontogenetic stages in *P. milaschewitchii*, and on related acochlidian species bearing rhinophores, in order to finally clarify the situation. A thin, double cerebro-rhinophoral connective has been detected for the first time in acochlidians. Neusser et al. (2007) found another double cerebro-rhinophoral connective in *Tantulum elegans* and pointed out that these tiny nerves can be overlooked easily or misinterpreted, thus might be present in other acochlidian species after all. Haszprunar and Huber (1990) also described a double cerebro-rhinophoral connective for *Rhodope veranii*; Huber (1993, figs. 9C and 10) showed a similar situation for, e.g. *Runcina adriatica* and *Philinoglossa praelongata*. With the double cerebro-rhinophoral connection the acochlidian rhinophoral ganglion and those of other opisthobranch groups show a condition similar to that in the pulmonate procerebrum (Van Mol 1967). Therefore, further study addressing the possibility of homology is needed.

Rankin (1979) concluded from the small, semi-schematic drawings by Kowalevsky (1901, figs. 46, 48) of an entire specimen of *P. milaschewitchii* that the pleural ganglia are fused with the cerebral ganglia. Edlinger (1980b) also illustrated these ganglia to be fused in his investigation of the cerebral nerve setting of *P. milaschewitchii* (Fig. 11B). However, the results of the present study clearly show that the pleural ganglia in *P. milaschewitchii* are fully separate from the cerebral ganglia. Cobo Gradin (1984) reported fused cerebro-pleural ganglia for *Asperspina loricata*; no pleural ganglia whatsoever had been mentioned in the original description by Swedmark (1968a). Huber (1993) considered the non-fused pleural ganglia as a characteristic feature in acochlidians and, indeed, all well-described acochlidian species show non-fused pleural ganglia (Neusser and Schrödl 2007). Accordingly, the cns of *A. loricata* requires reinvestigation.

Pontohedyle milaschewitchii has three distinct ganglia on the visceral cord. According to the pentaganglionate hypothesis of the nervous system of euthyneurans (Haszprunar 1985; Schmekel 1985), the basal condition shows five ganglia on the visceral cord: left parietal, right parietal, subintestinal, visceral, and supraintestinal ganglion. Following this hypothesis, two of the five ganglia must have either undergone fusion or been lost in *P. milaschewitchii*. While the left ganglion on the visceral cord of *P. milaschewitchii* reaches only about the size of the pleural ganglia, the median ganglion on the loop attains about double that size, and the right ganglion is only slightly smaller than the median one. Therefore, it can be assumed that the right parietal ganglion has fused with the supraintestinal ganglion, and the visceral ganglion with the subintestinal ganglion. Thus, the ganglion arrangement on the visceral cord in *P. milaschewitchii* resembles the one reported from *Microhedyle remanei* (see Neusser et al. 2006). It is also similar to those of *Hedylopsis ballantinei* and *H. spiculifera* (see Sommerfeldt and Schrödl 2005), with the only difference that the *Hedylopsis* species have an additional, 'osphradial' ganglion connected to the supraintestinal/parietal ganglion. Pattern differences exist mainly with the limnic *Tantulum elegans*, described with four separate ganglia on the visceral cord and an additional (probably penial) ganglion attached to the fused supraintestinal/parietal ganglion (Neusser and Schrödl 2007).

The cns of *P. milaschewitchii* reported here resembles the one described by Marcus and Marcus (1954) from their Brazilian specimen, except as follows: Marcus and Marcus (1954) detected only two ganglia on the visceral loop (determined as the median subintestinal/visceral ganglion and the supraintestinal ganglion on the right side), but a third one is indicated in their plate 26, fig. 13. It can be assumed that the authors overlooked the left parietal ganglion due to its small size and vicinity to the pedal ganglia, just like it probably had happened before in *Microhedyle remanei* (Marcus 1953 versus Neusser et al. 2006). Moreover, Marcus and Marcus (1954) indicated that the ganglia of the visceral cord are located close to the entrance of the pharynx rather than in the posterior region of the pharynx. However, in their plate 26, fig. 13, these ganglia seem to be located near the midline of the pharynx. Possibly, this slight shift to anterior is due to retraction or bending of the animal.

Sensory organs

Edlinger (1980a) first mentioned the presence of a paired Hancock's organ for *P. milaschewitchii* and described it as an "irregular system of folds" situated laterally at the anterior head–foot complex. In a second publication, Edlinger (1980b) referred to Hancock's organ in *P. milaschewitchii* as an "irregular system of folds, lying in a lateral furrow". No system of folds could be detected in the present study; but judging from the described position it can be assumed that Edlinger referred to a conspicuous fold in the epidermis just posterior to the oral tentacles. Edlinger (1980b) described the cerebral nerves 2 and 3 (Fig. 11B) as innervating Hancock's organ. In the specimens we examined, no nerves could be detected as leading directly to the potential Hancock organ. However, an innervation by the closely associated dorsal part of the dorsolateral accessory ganglia complex is likely.

The ciliary bands on the oral tentacles and across the head of *P. milaschewitchii* most likely also have a sensory function. No distinct nerves could be detected, but an innervation by the anterior accessory ganglia complex (which innervates the tentacles) is likely for the bands on the oral tentacles. Due to the more posterior position of the transverse ciliary band, the latter could be innervated by either the anterior or the dorsolateral accessory ganglia complex. Because of its rhinophore-like position and probable sensory function this band might be interpreted either as a (homologous) relic of the rhinophores or as a convergently developed substitute. Such a transverse ciliary band is absent in the examined rhinophore-bearing *Paraganitus ellynnae*, which only bears ciliary bands on oral tentacles and rhinophores. Additional SEM examination of other rhinophore-lacking species, such as *Pontohedyle verrucosa* and *Ganitus evelinae*, is necessary.

Digestive system

According to Marcus and Marcus (1954), the radula of their Brazilian specimen of *P. milaschewitchii* was symmetrical, with the radula formula $44 \times 2\text{-}1\text{-}2$. However, their drawings (op. cit., pl. 26, figs. 16, 17) show an almost identical radula configuration as the present

SEM examination. Thus, it can be assumed that the authors only misinterpreted the central denticle of the lateral plate as separation in the lateral plates. This has probably also been assumed by Wawra (1987), who defined the genus *Pontohedyle* with a radula formula of 1-1-1. The radula of *P. verrucosa* closely resembles the one of *P. milaschewitchii*, concerning both the radula formula (43 × 1-1-1) and the assemblage of the rhachidian tooth bearing three lateral denticles (Challis 1970). It differs, however, in the lack of a central denticle on the lateral plate (for comparison of the different *Pontohedyle* species, see Jörger et al. 2007). In sacoglossans the tooth size frequently increases with age (Jensen 1997), unlike in *P. milaschewitchii* where tooth size is uniform throughout. In *P. milaschewitchii* the entire radula lies in a radula sac in the pharynx, a condition also differing from the sacoglossan-typical ascus containing the descending limp (Jensen 1997).

A histologically and anatomically differentiated stomach could not be detected in specimens of *P. milaschewitchii* studied here. Marcus and Marcus (1954) described a spacious, spherical stomach in their Brazilian specimen, but this can be interpreted as an artefact resulting from fermenting stomach contents (Jörger et al. 2007).

The digestive gland in acochlidians is usually sac-like in shape (Rankin 1979). In some species the digestive gland reaches a length which makes internal folding within the visceral hump necessary for the digestive gland to fit into the cavity, as described for, e.g., *Microhedyle glandulifera* (see Salvini-Plawen 1973; as *M. glomerans*). A similar long, holohepatic digestive gland with internal folding has been observed for *P. milaschewitchii*. However, in some living specimens a conspicuously elongated visceral hump could be detected; in these cases the digestive gland could be observed as an unfolded sac (see Fig. 1C). Therefore, it can be assumed that folds of the digestive gland highly depend on the stage of contraction of the animals and cannot be seen as a constant character. This is supported by Marcus' (1953) observations of folded as well as unfolded digestive glands in *Ganitus evelinae* and *Microhedyle remanei*.

Excretory and circulatory systems

The reduced single-chambered heart of *P. milaschewitchii* found here was overlooked in previous studies (Kowalevsky 1901; Marcus and Marcus 1954). Similar hearts have been reported for *Hedylopsis spiculifera* (see Kowalevsky 1901), *Pseudunela cornuta* (see Challis 1970; as *Hedylopsis*) and *Tantulum elegans* (see Rankin 1979). However, the revision of *T. elegans* by Neusser and Schrödl (2007) detected a two-chamber heart, as also reported for *Microhedyle remanei* (see Neusser et al. 2006) and *Hedylopsis ballantinei* (see Fahrner and Haszprunar 2002; as *Hedylopsis* sp.). The detection and determination of the assemblage of the thin-walled hearts is difficult using light microscopy. Therefore, TEM re-examination of single-chambered hearts, and especially of acochlidians originally described as heartless, e.g. of *Ganitus evelinae* (see Marcus 1953) or *Parhedyle tyrtowii* (see Kowalevsky 1901), should be attempted. A grouping of the acochlidians based on the development of the excretory and circulatory systems, as globally stated by Rankin (1979), remains doubtful until reliable data exist.

Reproductive system

Most opisthobranchs are simultaneous or protandric hermaphrodites (Schmekel 1985). Uniquely within the opisthobranchs some gonochoristic species occur within the Acochlidia (among others *P. milaschewitchii*).

Female genital system

The female genital system in *P. milaschewitchii* basically resembles the ancestral form of the female genital system in Opisthobranchia as hypothesized by Ghiselin (1965), but differs in the lack of any sperm-storing structures (bursa copulatrix or receptaculum seminis).

Due to the similar histological, histochemical and ultrastructural characteristics, Klussmann-Kolb (2001) supposed the three nidamental glands to be homologous throughout the opisthobranchs, although the albumen gland might be modified into a capsule gland. Following this hypothesis and studies on other acochlidian species (Neusser et al. 2006; Neusser and Schrödl 2007), the nidamental glands in *P. milaschewitchii* were identified from proximal to distal as albumen, membrane and mucus gland. The albumen gland was termed as such due to its proximal position, its tube-like shape and the lack of internal folding (Klussmann-Kolb 2001). In contrast to *M. remanei* with a sac-like albumen and mucus gland (Neusser et al. 2006), the nidamental glands of *P. milaschewitchii* are all tube-like and show a continuous lumen throughout. The pattern of ciliation (albumen gland: relatively long cilia; membrane gland: short cilia; mucus gland: long cilia) also differs from *M. remanei*, with long cilia in the membrane gland but no cilia in the mucus gland (Neusser et al. 2006). However, the positions of the nidamental glands, their staining properties and histology (e.g. no internal folding in the albumen gland) are similar in the two species.

The genital pore in female *P. milaschewitchii* is located anteriorly of the anus, at the posterior end of the head–foot complex close to the transition to the visceral hump (Wawra 1986; present study). In contrast,

Kowalevsky (1901) originally described the female genital pore in *P. milaschewitchii* as located in the anterior region of the pharynx. Wawra (1986) suggested that this difference probably results from mobility of the internal organs (i.e. their positions depending on the stage of retraction). This is very unlikely, however, with regard to the relative positions of the genital (and other) openings. Wawra (1986) described a ciliated band in the female specimens of *P. milaschewitchii* originating from the genital pore, extending anteriorly to about one third of the length of the head-foot complex. This observation could be confirmed here from serial sections of female specimens. Similar ciliated areas have been reported from *Ganitus evelinae* (see Marcus 1953), *Paraganitus ellynnae* (see Challis 1968), *Hedylopsis ballantinei* (see Sommerfeldt and Schrödl 2005), and from *M. nahantensis* where such an area extends from the genital opening to the right rhinophore (Morse 1994). A transport function during egg deposition seems to be likely for the ciliated area (Wawra 1986), but observations *in vivo* are lacking.

Male genital system

The male genital system of *P. milaschewitchii* basically conforms to the hypothetic ancestral form of male portions of hermaphroditic opisthobranch genital systems according to Ghiselin (1965). Differences concern the reduction of the anterior genital organs in *P. milaschewitchii*, the absence of a copulatory apparatus, and sperm transfer taking place via spermatophores (Wawra 1992). While copulatory organs are present in many hermaphroditic acochlidians, e.g. in *Acochlidium fijiense* (see Haase and Wawra 1996), *Pseudunela cornuta* (see Challis 1970) and *Tantulum elegans* (see Neusser and Schrödl 2007), a reduction of the male anterior genital organs is common in gonochoristic species, e.g. in *Parhedyle cryptophthalma* (see Westheide and Wawra 1974), *Ganitus evelinae* (see Marcus 1953) and *Microhedyle remanei* (see Neusser et al. 2006), as well as in some hermaphrodites such as *Hedylopsis ballantinei* (see Sommerfeldt and Schrödl 2005).

In contrast to the unusual, frontal male genital pore observed in the present study, Marcus and Marcus (1954) described the genital pore in their Brazilian specimen as located on the right side of the head–foot complex close to the transition to the visceral hump. A ciliated vas deferens was not described, but the anterior part of the animal could not be sectioned because it was used for radula preparation. Thus, the authors had no possibility to detect the ciliated vas deferens. Either they observed an ontogenetic stage with a posterior genital opening, or they may have simply assumed the presence of a male genital opening in its usual posterior position in microhedylids (Jörger et al. 2007).

According to Ghiselin (1965) and Haszprunar (1985) the ancestral male reproductive system in opisthobranchs includes an open, ciliated seminal groove, which connects the pallial gonoduct with the copulatory apparatus. Sommerfeldt and Schrödl (2005) considered the open ciliary sperm groove as a plesiomorphic condition within the Acochlidia, even though the copulatory apparatus can be reduced. It may be assumed that a ciliated vas deferens evolved from the ciliated sperm groove by submerging into the epidermis and forming a closed tube (Ghiselin 1965). The ciliated part of the vas deferens has been described first in *P. milaschewitchii* by Wawra (1986), who termed it the "intraepidermal duct". However, it is a fully closed subepidermal duct attached to the epidermis and running towards the right side of the head. Similar ciliated male sperm ducts with a cephalic male genital opening have been described for the hermaphroditic *Pseudunela cornuta* by Challis (1970), and recently for *Tantulum elegans* by Neusser and Schrödl (2007). In both species the vas deferens opens on the level of the right rhinophore, and both bear a cephalic penis. Because of similar position and structure, homology between the ciliated vas deferens in the gonochoristic *P. milaschewitchii* and the hermaphroditic species is likely. The anterior part of the vas deferens in *P. milaschewitchii* entering the head cavity may be homologous to the backwards-leading part of the vas deferens in hermaphroditic species as well; like all aphallic acochlidians *P. milaschewitchii* can be assumed to have lost the associated glands. The unique anterior position of the male genital opening in *P. milaschewitchii* may be an adaptation to a more rapid and better-directed spermatophore transfer to a mate; frontal sperm transfer might be an advantage over a more lateral one, especially in an interstitial environment. The sensory oral tentacles might play a role in positioning of the spermatophore or in recognition of a potential spermatophore receiver. It can also be speculated that the mucous substances from the anterior pedal gland might be involved in attaching the spermatophore to other specimens.

Recent redescriptions of tiny acochlidian species (Neusser et al. 2006; Neusser and Schrödl 2007) have underscored the need for close and careful revision of primary data in order to gain a reliable and rich data set for phylogenetic analysis. The present study shows that even in a common and putatively well-known species, such as *Pontohedyle milaschewitchii*, reinvestigation of the anatomy with computer-based 3D reconstruction is rewarded with new and detailed results. To put these in a broader perspective the virtually unknown biology and ontogeny of this enigmatic opisthobranch group need to be revealed.

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