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Redescription of the meiofaunal gastropod *Parhedyle cryptophthalma*, with focus on nervous system and sensory organs

(Acochlidia, Panpulmonata)

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Parhedyle cryptophthalma (Westheide & Wawra, 1974) is a poorly known meiofaunal slug from the Mediterranean, inhabiting intertidal sands under direct wave impact. The present study is the first redescription of the acochlid *P. cryptophthalma*, confirming original results on general morphology, integumental spicules, and aberrant radula morphology by light and scanning electron microscopy. Our focus was on the central nervous system and sensory organs, using 3D reconstruction based on serial semi-thin sections and immunocytochemistry (staining of FMRFamide and Tyrosine Hydroxylase) in conjunction with confocal laser scanning microscopy. There is a mass of, yet undifferentiated, accessory ganglia anterior to the cerebral ganglia, typical for microhedylacean acochlidians. Apart from the common setting of ganglia (paired rhinophoral, cerebral, pedal, pleural, and buccal ganglia and three distinct ganglia on the visceral nerve cord), we found a putative osphradial ganglion for the first time in the microhedylacean clade. No osphradium, no Hancock's organ and, in contrast to the original description, no pigmented eyes could be detected. Bundles of sensory cilia were found laterally on the head-foot complex and scattered cilia are present on the head appendages. FMRFamidergic immunoreactivity was detected in all cerebral ganglia but not in the accessory ganglia. Tyrosine Hydroxylase (TH) expression and aldehyde-induced fluorescence was observed in cerebral ganglia such as pedal ganglia, in labiotenctacular, rhinophoral, and pedal nerves and in single neurons in the anterior region of the foot sole. Central nervous and sensory features may greatly vary among acochlidians and other heterobranch taxa, and comprehensive comparative approaches are necessary to reveal their presence, function, homology, and evolution.

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Introduction

Acochlidia are the most diverse and abundant group of meiofaunal heterobranch gastropods. Inhabiting shallow subtidal sands worldwide some species may occur intertidally in protected places such as reef lagoons. In contrast, *Parhedyle cryptophthalma* (Westheide & Wawra, 1974) is known to occur on Mediterranean beaches with direct wave impact. Despite recent advances in acochlidian morphology and anatomy (Neusser et al. 2006, Neusser & Schrödl 2007, Jörger et al. 2008, Neusser et al. 2009a,b, Brenzinger et al. in press), very little is still known about the biology of these mainly marine mesopsammic slugs and the adaptation of the sensory and locomotory system to their interstitial habitat. Being one of the smallest acochlids with an adult size of only 1.6 mm the original description applying traditional light microscopy lacks considerable detail, especially regarding fine nervous structures. Moreover, a trend towards reduction within organ systems ("regressive evolution"), e. g. with aphallic males and reduced reproductive systems, is observed in the acochlidian Microhedylacea clade (Swedmark 1959, 1968, Neusser et al. 2009b, Schrödl & Neusser 2010). The small amount of distinguishing characters paired with a lack of morphological knowledge on some genera – in first place the genus *Parhedyle* – thus still results in partially unresolved relationships within Microhedylacea (for phylogeny, see Schrödl & Neusser 2010).

The genus *Parhedyle* Thiele, 1931 was characterized as a member of gonochoristic Microhedylidae having two pairs of highly mobile head tentacles and irregularly shaped spicule plates in the integument (Wawra 1987). The type species *Parhedyle tyrtowii* (Kowalevsky, 1901) (as *Hedyle*) from the Black Sea was described to have clearly visible pigmented eyes and a symmetric radula with formula 2.1.2 by Kowalevsky (1901), while the Mediterranean *P. cryptophthalma* (as *Microhedyle*) has cryptic eyes and, uniquely among microhedylids, an asymmetric radula with a formula of 1.1.2 was found by light microscopical examination (Westheide & Wawra 1974). No details on central nervous or sensory features are known.

Traditionally, Acochlidia formed an order of the "Opisthobranchia" (see e.g. Dayrat & Tillier 2003, Wägele & Klussmann-Kolb 2005), but recent multilocus molecular data revealed pulmonate relationships (Klussmann-Kolb et al. 2008, Jörger et al. 2010). These recently proposed relationships question traditional homology assumptions within Heterobranchia and require re-evaluation of problematic character sets such as the nervous system. Investigations characterizing serotonergic, FMRFamidergic, and/or cathecholaminergic parts of the nervous system have shown to provide phylogenetically useful characters across different taxa (Dickinson & Croll 2003, Croll & Dickinson 2004, Wanninger 2009, Heuer et al. 2010, Kristof & Klussmann-Kolb 2010, Worsaae & Rouse 2010). Moreover, spatial distribution and immunoreactivity of neurites are indicating their function (see Faller et al. 2008, and references therein). Among Acochlidia Hochberg (2007) provided the first detailed information on the chemistry of the nervous system in a species of microhedylacean *Asperspina*. He revealed the presence of an extensive serotoneric network in the central nervous system, with similarities to model heterobranchs such as *Aplysia,* in the presence of serotoneric pericarya in cerebral and pedal ganglia and absence from pleural ganglia (Hochberg 2007). Due to its minute body size *Parhedyle cryptophthalma* offers the possibility of immunocytochemical studies on whole-mount specimens for visualization by confocal laser scanning microscopy (cLSM).

Here, we redescribe the nervous system and associated sensory structures of *P. cryptophthalma* by 3D reconstruction from histological semi-thin serial sections. To gain insights in the function of certain nervous structures, this data is supplemented by the characterization of FMRFamidergic and chatecholaminergic parts of the nervous system by means of immocytochemistry in conjunction with cLSM. The results are compared to other acochlidian nervous systems and previous homology assumptions are discussed.

Material and methods

Sampling

Specimens of *Parhedyle cryptophthalma* were recollected at Arco Felice, near Naples, Italy, a locality that was reported in the original description by Westheide & Wawra (1974). Approximately 70 individuals were extracted from sand samples collected in the high intertidal (wave zone) following the method described by Schrödl (2006). All specimens were anaesthetized with $MgCl_2$ prior to fixation to prevent retraction. Specimens were identified using light-microscopy and 10 individuals were fixed in 75 % ethanol for radulae preparation. For histology and scanning electron microscopy (SEM) specimens were fixed in 4 % glutardialdehyde.

Histology and 3D reconstruction

For serial semi-thin sectioning 20 glutardialdehyde-fixed specimens were embedded in Spurr's low viscosity epoxy resin (Spurr 1969). Specimens were rinsed three times in 0.2 M sodium cacodylate buffer (0.1 M NaCl and 0.35 M sucrose, pH 7.2), followed by post-fixation in buffered 1 % OsO_4 for 1.5 h in the dark. Sub-

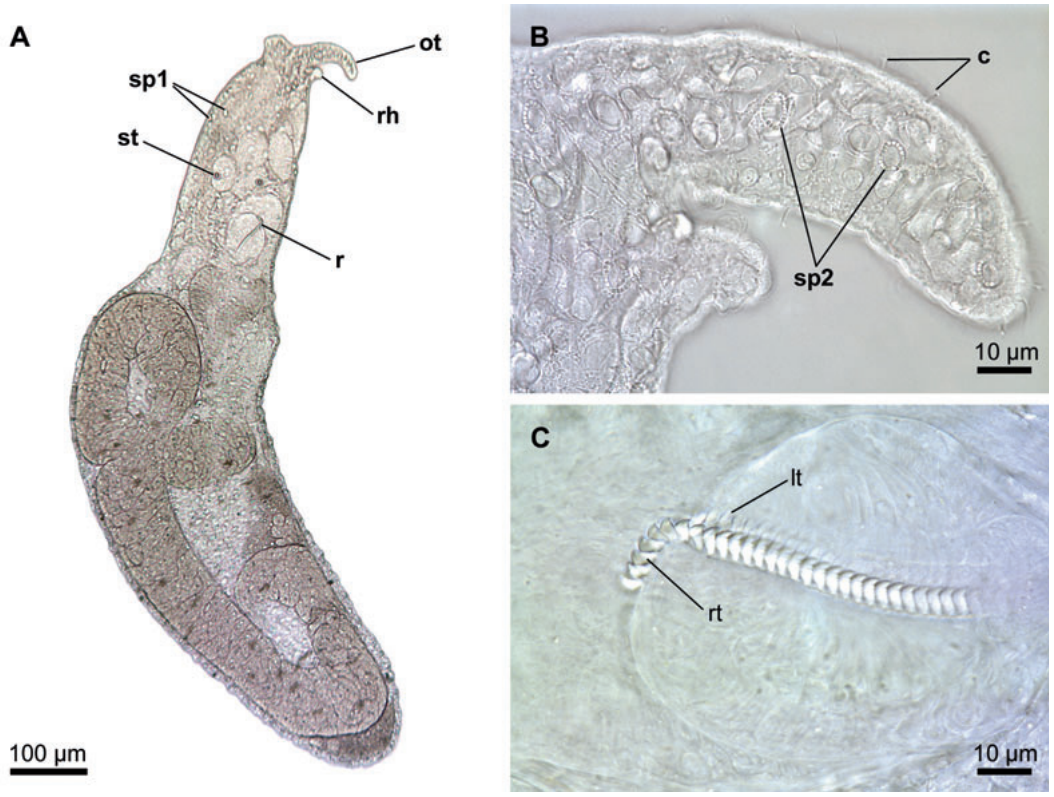

Fig. 1. Light microscopy. **A.** External morphology of a living specimen. **B.** Oral tentacle and rhinophore, with scattered sensory cilia and circular bead-chain spicules (type 2). **C.** Typical J-shaped radula with ascending and descending limb. Abbreviations: **c**, cilia; **lt**, lateral tooth; **ot**, oral tentacle; **r**, radula; **rh**, rhinophore; **rt**, rhachidian tooth; **sp1**, plate-like spicules (type 1); **sp2**, circular bead-chain spicules; **st**, statocyst.

sequently, specimens were decalcified in 1 % ascorbic acid overnight and dehydrated in a graded acetone series. Four series of serial semi-thin sections (1.0-1.5 µm thickness) were prepared using a diamond knife (Histo Jumbo, Diatome, Biel, Switzerland) and contact cement on the lower cutting edge to form ribbons (Ruthensteiner 2008). Sections finally were stained with methylene-azure II (Richardson et al. 1960) and are deposited at the Bavarian State Collection of Zoology (ZSM Mol 20071113, 20071923, 20071925, 20071931). Digital photographs were taken of every section with a CCD microscope camera (Spot Insight, Diagnostic Instruments, Sterling Heights, USA) mounted on a DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). A computer-based 3D-reconstruction of the nervous system of *Parhedyle cryptophthalma* was conducted with the software AMIRA 5.2 (Visage Imaging GmbH, Germany), following in principle the procedure explained by Neusser et al. (2006) and Ruthensteiner (2008).

Scanning electron microscopy (SEM)

10 entire specimens of *Parhedyle cryptophthalma* were stepwise dehydrated in graded acetone series and critical-point-dried in 100 % acetone in a Baltec CPD 030. After mounted on SEM stubs with self adhesive carbon stickers, the dried specimens were sputtered with gold in a Polaron Sputter Coater for 120 sec. SEM examination of the sensory organs of *P. cryptophthalma* were carried out using a LEO 1430VP SEM at 10-15 kV. For the preparation of radulae 10 specimens were macerated over night in 10 % KOH, remaining tissue was removed mechanically under a stereomicroscope. Radulae were rinsed in Aqua bidest., mounted on SEM stubs, sputtered, and examined as described above.

Immunolabelling and confocal laser scanning microscopy

Specimens were prepared basically following the protocol described by Kristof & Klussmann-Kolb (2010): 15 specimens of *P. cryptophthalma* were anaesthetized with 7 % $MgCl_2$ and fixed in 4 % paraformaldehyde (PFA) in

0.1 M phosphate-buffered saline (PBS, pH 7.3) for 5 hours at 4 °C. Subsequently, the specimens were rinsed three times (2 × 5 min, 1 × 60 min) in PBS (= PBS rinse). After decalcification (0.5 M EDTA for three hours), followed by another PBS rinse, specimens were permeabilized for 24 hours (4 % Triton X-100 in PBS) and incubated with the rabbit anti-FMRFamide (Immunostar, Hudson, Wisconsin, USA, diluted in PBS to 1:500 final working concentration) in blocking solution (1 % goat serum in PBT (= PBS with 1 % Triton X-100)) for 72 hours at 4 °C. After primary labelling, specimens were rinsed three times for 15 min in PBS and incubated for 24 hours in 1:50 dilution of goat anti-rabbit antibodies labelled either with fluorescein isothiocynate (FITC) or rhodamine (TRITC) (Jackson ImmunoResearch Laboratories, West Groove, PA, USA) at 4 °C. This was again followed by several rinses in PBS, before the specimens were mounted on glass slides in a 3:1 mixture of glycerol to TRIS-buffer (0.5 M) with 2 % propyl gallate added to prevent fading (Longin et al. 1993).

For Tyrosine Hydroxilase (TH) labelling (marks catecholaminergic cells) 15 specimens were anaesthetized (see above) and fixed in 99 % Methanol (+1 % acetic acid) for 30 min at −20 °C and subsequently transferred for 10 min each in a decreasing methanol series (70 %, 50 %, 30 %), followed by several PBS rinses. Specimens were decalcified, permeabilized (see above), and incubated with a monoclonal TH antibody (Immunostar, Inc. Hudson, WI, USA) (1:500 dilution in PBS) in blocking solution (1 % goat serum in PBT (= PBS with 1 % Triton X-100)) for 72 hours at 4 °C. This was followed by several rinses in PBS and incubation in secondary antibody (sheep anti-mouse) conjugated to TRITC for 24 hours at 4 °C. After several rinses in PBS the specimens were mounted on glass slides as described above.

Autofluorescence were excluded by negative controls, in which specimens were processed without incubation in primary antibody. Positive controls involved parallel processing of *Aeolidiella stephanieae* Valdés, 2005 and *Haminoea japonica* (Pilsbry, 1895) larvae with known staining patterns (Kristof & Klussmann-Kolb 2010, and C. Schulze (Zoological Museum Hamburg) pers. comm.).

The immunolabelling was viewed with a Leica TCS SP5 confocal laser-scanning microscope, generating optical sections at 0.1-0.5 µm intervals. All sections were digitally merged to maximum projections and edited with general imaging software.

Results

Taxonomic identification of *Parhedyle cryptophthalma* conducted in the field was based on the locality and special type of habitat (described in the original literature, see Westheide & Warwra 1974), and general morphology (Fig. 1A-C). Light microscopy on living specimens revealed irregular, plate-like calcareous spicules and circular bead-chain spicules (see Fig. 1A,B) as described by Westheide & Wawra (1974). Preliminary identification was confirmed by SEM examination of the unusual radulae. The radula is asymmetric, with formula 1-1-2; the first right lateral is narrow and the second lateral is broad (Fig. 2A), what is unique among known acochlidians. Identification as *P. cryptophthalma* is further supported by clear genetic distinction to the only other described congener *Parhedyle tyrtowii* (Kowalevsky, 1901) that was collected at the type locality in the Black Sea (unpublished data).

All specimens available for the present study were sub-adult juveniles with the genital system not yet (fully) developed.

Central nervous system (CNS)

The CNS of (sub-adult) *Parhedyle cryptophthalma* confirms the general setting within Acochlidia and ganglia are identified following previous species (re-)descriptions (see e. g. Neusser et al. 2006). The prepharyngeal CNS consists of the paired cerebral, rhinophoral, pedal, pleural and buccal ganglia and three distinct ganglia on the visceral nerve cord (respectively the left parietal ganglion, the fused visceral/subintestinal ganglion and the fused right parietal/supraintestinal ganglion), see Fig. 3. An additional unpaired, putative osphradial ganglion (Fig. 3A,B) was found attached to the right parietal/supraintestinal ganglion (Fig. 3B). Anterior to the cerebral and rhinophoral ganglia there is an undifferentiated mass of supposedly nervous tissue, interpreted as accessory ganglia (Fig. 3D). Cerebral and rhinophoral ganglia are located pre-pharyngeally, pedal and pleural ganglia in the anterior region of the pharynx, and the ganglia of the visceral nerve cord near the posterior end of the pharynx; the buccal ganglia lie post-pharyngeal. All ganglia are situated very close together. Cerebral as well as pedal ganglia are connected via short, strong commissures; short cerebro-pedal, cerebro-pleural, and pleuro-pedal connectives could be detected. The rhinophoral ganglion was identified as such due to its position anterolateral of the cerebral ganglion and a thin connective with the cerebral ganglion, even though no nerve could be detected emerging from this small ganglion and the putative rhinophoral nerve emerges dorsally from the cerebral ganglion (see below).

Three cerebral nerves could be detected emerging from the cerebral ganglion (for identification see previous redescriptions e.g. Jörger et al. 2008): 1) slightly ventrally the labiotentacular nerve, 2) more dorsally the dorsal nerve, herein interpreted as the rhinophoral nerve, and 3) the thin static nerve.

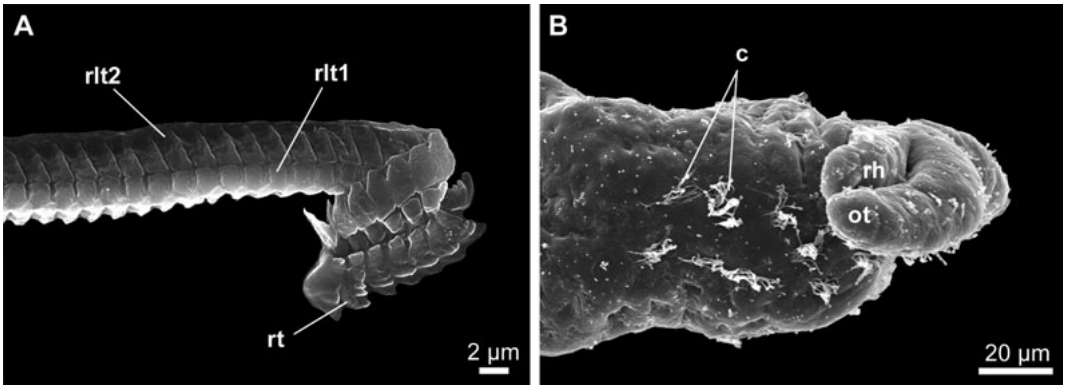

Fig. 2. Scanning electron micrographs. **A.** Right lateral view of radula with rhachidian tooth (with one central and three lateral cusps) and two lateral teeth on the right side. **B.** Lateral view of the head-foot complex showing scattered bundles of cilia. Abbreviations: **c**, cilia; **ot**, oral tentacle; **rh**, rhinophore; **rlt1**, right lateral tooth 1; **rlt2**, right lateral tooth 2; **rt**, rhachidian tooth.

Sensory structures

Parhedyle cryptophthalma possesses two pairs of head appendages (Figs 1A, 2B). Ventrally located are the curved, roundish and only slightly tapered oral tentacles. Rhinophores are slightly shorter than the oral tentacles, and are pointed upwards in living, crawling specimens. Using light microscopy, scattered cilia, supposedly with sensory function are found on oral tentacles and rhinophores (Fig. 1B). SEM examination of the head-foot complex reveals additional bundles of cilia in the anterior region of the head-foot complex (Fig. 2B).

Surprisingly, the eponymous "cryptic eyes" could not be detected in our material from Italy. No pigment anterior to the cerebral ganglia was found on histological sections or by light microscopy of living specimens. However, minute unpigmented globules are identified nestling anterior to the cerebral ganglia. Those might be interpreted as remainder or ontogenetic precursor of eyes (see discussion).

Each pedal ganglion bears a comparably large statocyst (Fig. 3A,E; containing one statolith each), which is attached dorsally near the posterior end of the ganglion (also clearly visible using light microscopy, see Fig. 1A).

No Hancock's organ (i. e. paired ciliated grooves posterior to the head appendages, commonly innervated by part of the rhinophoral nerve) could be detected with SEM examination of the head-foot or on the histological sections.

Immunolabelling and cLSM

Successful labelling against FMRFamides was only achieved in completely retracted specimens, where allocation of labelled nuclei within the concentrated CNS was difficult. FMRFamide immunoreactivity could be detected in all ganglia, but no reactivity was observed in the accessory ganglia anterior to the cerebral ganglia (see Fig. 4A).

TH-like immunoreactivity could be detected in the anterior region of the cerebral ganglion and the labiotentacular and rhinophoral nerves (Fig. 4B). Additionally, TH-immunoreactivity was observed in the centre of the pedal ganglia, within a bifurcating pedal nerve leading ventrally to the anterior part of the foot and in a series of single neurons bordering the anterior region of the foot near the mouth opening (Fig. 4C).

Discussion

Taxonomy

The genus *Parhedyle* is among the most ill-defined acochlidian taxa, comprising two (or three) species that are only known by their original descriptions (Wawra 1987, Schrödl & Neusser 2010). While not resolved as a monophylum in the cladistic analysis by Schrödl & Neusser (2010), *Parhedyle tyrtowii* and *Parhedyle cryptophthalma* are quite similar. Both are agile slender microhedylids with two pairs of slender, highly mobile tentacles. Irregular calcareous spicule plates and pearl-chain like aggregations were known from *P. tyrtowii* and *P. cryptophthalma* (see Kowalevsky 1901, Westheide & Wawra 1974). The re-examination of *P. cryptophthalma* by SEM confirms the presence of an asymmetric radula with a formula of 1.1.2. *P. cryptophthalma* thus is the only member of the gonochoristic Microhedylidae sensu lato with an asymmetric radula. Interestingly, the first right lateral is a much narrower plate than the

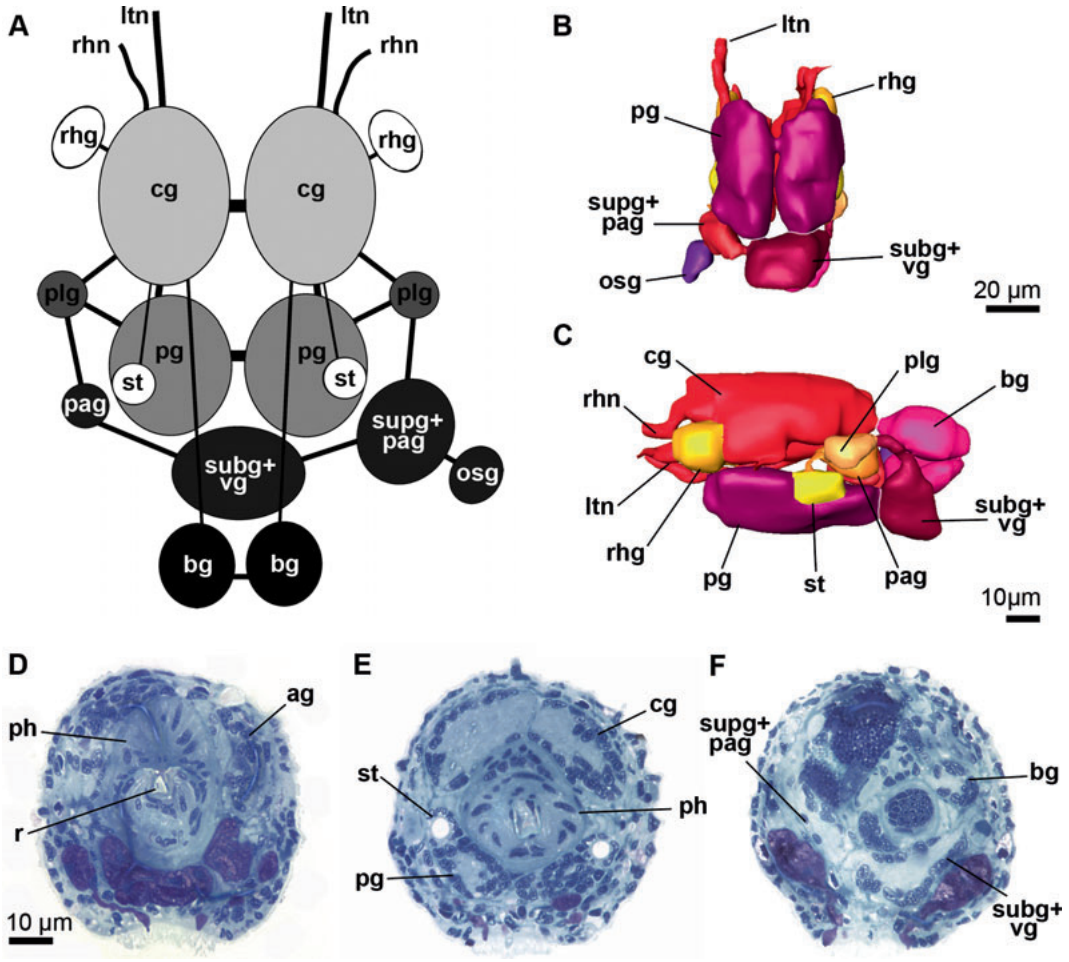

Fig. 3. Central nervous system (CNS). **A.** Schematic overview, dorsal view. **B-C.** 3D reconstruction of the CNS based on histological semi-thin sections; ventral view (**B**), lateral left view (**C**). **D-F.** Histological semi-thin sections (1 µm); undifferentiated mass of accessory ganglia anterior to cerebral ganglia (**D**), cerebral and pedal ganglia with statocysts (**E**), ganglia of visceral nerve cord (**F**). Abbreviations: **ag**, accessory ganglia; **bg**, buccal ganglion; **cg**, cerebral ganglion; **ltn**, labiotentacular nerve; **osg**, osphradial ganglion; **pag**, parietal ganglion; **pg**, pedal ganglion; **ph**, pharynx; **plg**, pleural ganglion; **r**, radula; **rhn**, rhinophoral nerve; **rhg**, rhinophoral ganglion; **st**, statocyst; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion.

second, rectangular one. This is in contrast to all hedylopsacean and *Asperspina* species with an asymmetric radula; here, the first right lateral is a broad rectangular plate and the second tooth a narrow plate or spine-like (Schrödl & Neusser 2010). According to Kowalevsky (1901: figs 38, 40), *P. tyrtowii* has a narrow first lateral and a broad second one as well; the originally described symmetric condition in the latter species needs to be re-examined. While the presence versus absence of well-visible, pigmented eyes clearly distinguish the species, morphological and genetic evidences may finally confirm *P. tyrtowii* and *P. cryptophthalma* as belonging to a monophyletic genus *Parhedyle*. The just rudimentarily known *Parhedyle gerlachi* (Marcus & Marcus, 1959) from the Maldives (Indian Ocean) was tentatively related to *Parhedyle* (see Wawra 1987). However, flat oral tentacles and a radula with single lateral teeth (formula 1.1.1) do not support a closer relationship to *Parhedyle*. *P. gerlachi* needs re-examination before conclusions on its relationships can be made.

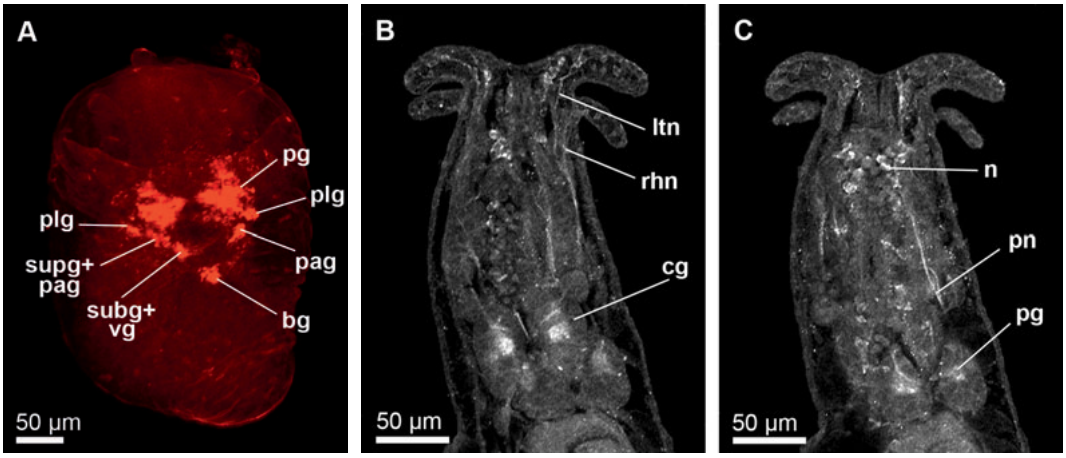

Fig. 4. Expression of neurotransmitters. **A.** FMRF-amidergic expression in ganglia, labelled in a completely retracted specimen (ventral view). **B-C.** TH-immunoreactivity, in cerebral ganglia, labiotentacular and rhinophoral nerves (**B**), in pedal ganglia, pedal nerves and scattered neurons at the foot sole (**C**). Abbreviations: **bg**, buccal ganglion; **cg**, cerebral ganglion; **ltn**, labiotentacular nerve; **n**, neurons in anterior part of the foot; **pag**, parietal ganglion; **pg**, pedal ganglion; **plg**, pleural ganglion; **pn**, pedal nerve; **rhn**, rhinophoral nerve; **subg**, subintestinal ganglion; **supg**, supraintestinal ganglion; **vg**, visceral ganglion.

Central nervous system

The condition of the prepharyngeal nervous system observed in *Parhedyle cryptophthalma* confirms the general setting of ganglia in microhedylid Acochlidia (Neusser et al. 2006, Jörger et al. 2008, Neusser et al. 2009b). Additionally, we found an unpaired ganglion attached to the right parietal/supraintestinal ganglion for the first time in Microhedylacea. Such unpaired ganglia are present in all hedylopsacean Acochlidia described in detail (Wawra 1989, Sommerfeldt & Schrödl 2005, Neusser & Schrödl 2007, 2009, Neusser et al. 2009a, Brenzinger et al. in press) and were regarded as osphradial ganglia even in the absence of detected osphradia. For the first time in Acochlidia, B. Brenzinger (pers. comm.) detected an osphradium in a comparably large, limnic hedylopsacean *Strubellia* species from the Solomon Islands. While the osphradium is well-visible as a bright spot on dark-pigmented living specimens, its histological detection is difficult (B. Brenzinger pers. comm.). Thus, this small unpaired ciliated groove, similar to the Hancock's organ, might have been overlooked in previous histological studies on tiny meiofaunal acochlids. The non-detection of an osphradium in the preliminary immunocytochemical approach herein does not necessarily mean its absence. The osphradial ganglion in hedylopsaceans and *P. cryptophthalma* may be a mere relic in species without (well-) developed osphradium. However, new functions – potentially related to copulation – are indicated by a thick penial sheath nerve leaving this ganglion in *Tantulum elegans* (see Neusser & Schrödl 2007).

Accessory ganglia

Accessory ganglia are aggregations of nervous tissue, closely associated to the cerebral ganglia and differentiated from "true" ganglia by the lack of division into cortex and medulla (Neusser et al. 2006). They are characteristic for Microhedylacea but were also found occasionally in some hedylopsacean acochlids (Wawra 1987, Schrödl & Neusser 2010). The present study on subadult *Parhedyle cryptophthalma* suggests that accessory ganglia develop later in ontogeny, after the development of the "true" ganglia. This supports earlier observations on juvenile *Pontohedyle milaschewitchii* (Kowalevsky 1901) with developing accessory ganglia (see Jörger et al. 2008). The function of accessory ganglia is still a matter of speculation, e. g. as adaptation to minute body sizes (Haszprunar & Huber 1990) or involved in reorganization of the reproductive system in sequential hermaphrodites (Neusser & Schrödl 2007). In contrast to "true" ganglia, no FMRFamide immunoreactivity could be detected in the accessory ganglia investigated herein. This might, however, be a result of the not fully developed stage of the accessory ganglia, which might have no expression of neurotransmitters, yet. Comparable studies on adult specimens are needed for further conclusions. TH-expression is observed in the cerebral ganglia and cerebral nerves inner-

vating the head appendages (labiotentacular and rhinophoral nerve, respectively). The presence in nerves leading to the sensory head appendages and the accumulation of TH neurites near the mouth opening suggests an involvement in the transmission of sensory stimuli, confirming general consideration that catecholamines are involved in contact chemoreception and mechanoreception (Croll et al. 2003, Faller et al. 2008). Considering the interstitial lifestyle of acochlidians the accessory ganglia might be necessary to be able processing all the sensory input from the surrounding cathecholaminergic neurites. In a Caribbean *Asperspina* species Hochberg (2007) found serotonergic expression in cerebral and pedal ganglia and in one ventral "accessory ganglion" connected to the pedal ganglion (with similar expression pattern as the pedal ganglion). As pointed out by the author this is rather surprising, since accessory ganglia are normally associated to cerebral ganglia in all Acochlidia studied in detail. Most of Hochberg's specimens were mounted left-side down, so observations were concentrated on the right lateral view, lacking information on the left body part. Assuming that the laser scanner could have penetrated more than half of the whole body width, or that a slight dislocation of left side ganglia occurred in the fixed specimens, there is a certain possibility that the "accessory ganglion" of *Asperspina* sp. refers to the left pedal ganglion. Reinvestigation on the potential serotonergic expression in accessory ganglia is needed.

Sensory structures

Eyes. Surprisingly, we were unable to detect the eponymous cryptic eyes in our *Parhedyle cryptophthalma*. Westheide & Wawra (1974) reported the eyes to be invisible in squeezed material, alive or fixed. Only within brightened, fixed material the describing authors were able to detect two "tiny spots of pigment" anterior to the cerebral ganglia. We were unable to detect any pigment anterior to the cerebral ganglia in the sectioned series and also on observed living material. Since our study specimens were sub-adult juveniles, the observed unpigmented nervous globules might develop into eyes within fully developed adults. However, available data from other microhedylid species such as juvenile *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* (Kowalevsky 1901) suggest that fully developed, pigmented eyes are already present in an early juvenile stage (unpublished data). The unpigmented structures found in *P. cryptophthalma* might thus rather be interpreted a remainder of eyes developed in an earlier ontogenetic stage. In general, the presence and amount of pigmentation within the eyes of Acochlidia seems to be variable between and even within one population, e.g. observed in *Pseudunela* sp. from Indonesia (T. Neusser pers. comm.).

Hancock's organ. Hancock's organs (i.e. paired ciliated grooves behind the oral tentacles with supposedly chemosensory function) have been reported from acochlidians in three different families: in the small interstitial *Tantulum elegans* Rankin, 1979 (Tantulidae) (Neusser & Schrödl 2007), *Pontohedyle milaschewitchii* and *Microhedyle glandulifera* (both Microhedylidae) (Edlinger 1980a,b, Jörger et al. 2008) and the large benthic *Strubellia paradoxa* (Strubell, 1892) (Acochlidiidae) (Brenzinger et al. in press). However they were not detected in several other acochlidians examined in detail (Neusser & Schrödl 2009, Neusser et al. 2009a,b), among them other representatives of the family Microhedylidae (Neusser et al. 2007). We were also unable to detect Hancock's organs in *Parhedyle cryptophthalma*. Small ciliated grooves can easily be overlooked or misinterpreted as epidermal folds on retracted specimens or badly preserved material, thus Hancock's organs might have been overlooked in previous studies or even within the present one. The presence might also be related to a certain function corresponding e.g. to differences in habitat. However, this seems unlikely at the present stage of knowledge with Hancock's organs present/absent from marine interstitial species and present in (some?) limnic ones. Earlier authors suggest a potential homology of Hancock's organs within "Opisthobranchia"; i.e. of the epidermal grooves in Acochlidia with Hancock's organs of Acteonoidea and within Cephalaspidea sensu stricto, based on the similar position and innervations by a split of the rhinophoral nerve (Edlinger 1980a,b, Neusser et al. 2007). The recently revealed panpulmonate relationship of Acochlidia (Jörger et al. 2010) at first glance may contradict such an assumption due to phylogenetic distance, implying several independent losses in intermediate panpulmonate lineages. However, assuming homology of opisthobranch rhinophoral ganglia and pulmonate procerebrum (Jörger et al. 2010), at least parts of the ancestral, lower heterobranch actenoidean-like Hancock's organs could be homologous to the more or less elaborated epidermal folds and rhinophores, i.e. all sensory structures and tentacles innervated by the rhinophoral nerve, throughout the Euthyneura.

There is increasing knowledge on acochlidian microanatomy, greatly benefiting from a wide range of light microscopical, ultrastructural, histological and immunocytochemical techniques that are applied to a variety of formerly poorly or unknown taxa. Con-

firming aberrant features such as the special setting and shape of radula teeth in *Parhedyle cryptophthalma* is as important for future phylogenetic and evolutionary analyses as the revealing of new structures, such as the osphradial ganglion. Regarding central nervous and sensory organs, our understanding is still fragmentary. Latest anatomical redescriptions of acochlidian nervous systems underline the variability of nervous structures, especially the setting of cerebral nerves and the presence of sensory structures even within closely related species (see Sommerfeldt & Schrödl 2005, Neusser et al. 2006, Neusser & Schrödl 2007, Neusser et al. 2007, Jörger et al. 2008, Neusser & Schrödl 2009, Neusser et al. 2009a,b, Brenzinger et al. in press, present study). The presence/absence of rhinophoral or optic ganglia, the number and location of cerebral nerves, the innervation pattern of sensory structures and head appendages are highly variable and not entirely congruent with phylogenetic hypotheses no matter if based on morphological characters (Schrödl & Neusser 2010) or on molecular markers (Jörger et al. 2010). Thus, homology assumptions and generalization of reductions or inventions of cerebral features within Acochlidia, and even more across different heterobranch taxa, is difficult at the present stage of knowledge. A comprehensive comparative approach using a combination of detailed histological reexamination combined with immunocytochemical labelling against different neurotransmitters is needed to gain further insights into this quite variable character set within Heterobranchia.

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