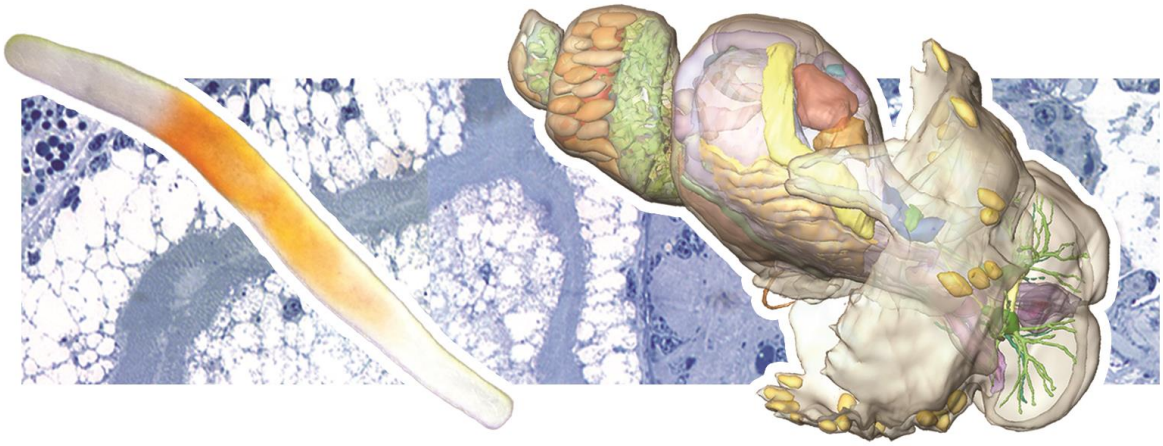

Insights from enigmatic clades – comparative microanatomy and evolution of the Heterobranchia (Mollusca, Gastropoda)

Dissertation

zur Erlangung des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

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vorgelegt von Dipl.-Biol. Bastian Brenzinger aus München

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Erstgutachter: Prof. Dr. Michael Schrödl

Zweitgutachter: Prof. Dr. Gerhard Haszprunar

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ERKLÄRUNG

Hiermit versichere ich an Eides statt, dass die vorgelegte Dissertation von mir selbständig und ohne unerlaubte Hilfsmittel angefertigt ist.

Die Dissertation wurde weder ganz, noch in wesentlichen Teilen, bei einer anderen Prüfungskommission vorgelegt.

Ich habe noch zu keinem früheren Zeitpunkt versucht, eine Dissertation einzureichen oder mich einer Doktorprüfung zu unterziehen.

München, den 8. Juli 2016

(Bastian Brenzinger)

Diese Dissertation wurde angefertigt
unter der Leitung von Prof. Dr. Michael Schrödl
an der Zoologischen Staatssammlung München
und der Fakultät für Biologie der Ludwig-Maximilians-Universität München

Angefertigt an der Zoologischen Staatssammlung München

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Titelbild: Links: Lebendfoto von *Rhodope* cf. *veranii* Kölliker, 1847 (links) aus Istrien, Kroatien. Rechts: 3D-Rekonstruktion von *Ringicula doliaris* Gould, 1860 aus Kagoshima, Japan. Hintergrund: Histologischer Semidünnschnitt (Nervensystem und Ösophagus von *Rhodope rousei*; aus Brenzinger et al. 2011b).

Für meine Mutter, Maria, und meinen Vater, Rolf.

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1 ABSTRACT

Heterobranchia comprise several ten thousand species of gastropod Mollusca ("non-prosobranchs"). They have evolved an enormous variety of forms of snails and slugs in almost all of the world's major ecosystems, ranging from marine interstitial, benthic, or pelagic to freshwater and terrestrial habitats. They also impact human lives through their ecological role as food or pests, disease vectors, objects for scientific study or simply their aesthetic and cultural value. In the last decade, mainly molecular phylogenetic studies have opened up radical new perspectives on heterobranch evolution. Understanding of these phylogenetic trees is, however, severely impaired by the lack of robust anatomical data on the majority of lineages, as many crucial taxa are small bodied, and hence hard to collect and examine.

The present thesis provides novel data on eight target taxa, focusing on phylogenetic placement and comparative anatomy of minuscule interstitial seaslug clades and their putative sister groups together with selected shelled species. These taxa are members of Rhodopemorpha and philinoglossid Cephalaspidea slugs, benthic Acochlidia (freshwater), Sacoglossa and Corambidae slugs, Murchisonellidae and Ringiculidae snails, and pelagic shelled Pteropoda. The herein included ten publications present data on phenotypes derived from scanning electron microscopy, live observations and comprehensive 3D-microanatomical reconstructions based on histology of serial semithin sections of whole animals, including complex central nervous systems and reproductive structures. Molecular phylogenetic hypotheses, based on Maximum-Likelihood and Bayesian analyses of multi-locus datasets, are provided for selected clades.

The discussion gives a review and graphical overviews over relevant morphologies found among heterobranch taxa. It classifies Heterobranchia on the basis of novel phylogenetic hypotheses and still unpublished data, most importantly including 1), a reorganization of lower heterobranchs with recognition of a monophylum of minute highspired snail families and Rhodopemorpha slugs (Micracicula new concept), 2), a new and more inclusive crown group containing five lineages with characteristically "bubble"-shelled members (Physotesta new concept) including 3), a new sister group to Euthyneura (Parvaplustra new concept) and 4), a new sister group to Nudipleura (Ringiculidae, together forming Ringipleura new taxon). In addition, new patterns of evolution within speciose Euopisthobranchia and Panpulmonata are highlighted. Based on outgroup comparison, potential synapomorphies within selected organ systems are proposed: the evolution of specialized cerebral nerves and sensory tentacles, a crucial change in the relationship of head and mantle coupled with a change in morphology of the mantle, the latter leading to the evolution of a characteristic "bubble" shell in the crown group. A new scenario explaining the occurrence of diverse morphotypes, including slugs, is described as "Heterochronic Pendulum". It proposes repeatedly convergent paedomorphosis and peramorphosis, each time starting with bubble-shelled ancestors, as a common theme throughout euthyneuran evolution.

The present thesis closes a large gap of knowledge at the interface between hitherto unresolved lower and derived higher Heterobranchia, and provides reinterpretation of several aspects on evolution, including that of shells or of interstitial seaslugs. Thereby it provides a testable framework and novel views for future anatomical and phylogenetic work on evolutionarily old clades of lower heterobranchs and highly diverse euthyneurans.

2 INTRODUCTION

2.1 Who are the Heterobranchia?

Gastropoda is the largest subgroup of the phylum Mollusca and contains among the most iconic animals. For enthusiasts, gastropods are astonishing by their diversity of their coiled shells and their colours (Abbott & Dance 1986, Gosliner et al. 2008). For scientists inside or outside the field of malacology, they are additionally one of the most diverse clades with respect to their morphology, fossil history, and ecology. This hints at a wealth of evolutionary history examinable by phylogenetics and palaeontology; current research identifies up to six Recent monophyletic gastropod clades stemming back to the Paleozoic (e.g. Ponder & Lindberg 2008, Kocot et al. 2011, Zapata et al. 2014, Vinther 2015).

The Heterobranchia Gray, 1840 comprise one of the two largest gastropod lineages with approximately 36,000 of the estimated 80,000 known species (Ponder & Lindberg 2008). Many heterobranchs are snails with translucent and thin shells; others outgrow a reduced shell or have lost it completely—the semislugs and slugs. Their body sizes range from less than 1 millimeter (among them some of the smallest gastropods—Omalogyridae G. O. Sars, 1878, and meiofaunal slugs; Jörger et al. 2014a) to several tens of centimeters, including the largest seaslugs (*Hexabranchus* Ehrenberg, 1828; *Aplysia* Linnaeus, 1767) and the largest land snails (*Achatina* Lamarck, 1799). Heterobranchs are originally marine, benthic snails living on hard or soft substrates, but have also newly colonized the marine interstitial (between the pore spaces of marine sands and gravel; Swedmark 1968, Arnaud et al. 1986), open water (the pelagial and neuston—Lalli & Gilmer 1989), or non-marine conditions such as freshwater (Strong et al. 2008), and air-exposed areas above the coastal splash zone (amphibious taxa), or fully terrestrial habitats (e.g. Solem 1978). Some diverse groups live as ectoparasites, closely associated with their invertebrate hosts (e.g. some nudibranchs, and the pyramidellids; Fretter & Graham 1949). Heterobranchs contain the majority of land snails (at least in temperate regions; Solem 1978) and the overwhelming majority of slugs, and comprise such celebrated groups as the colorful and toxic nudibranch seaslugs, the seahares and sea butterflies, photosynthetic seaslugs, and the iconic land snails with stalked eyes (Rudman & Willan 1998, Smith & Stanisic 1998).

Heterobranchs impact human lives negatively as pests in bivalve aquaculture (Pyramidellidae Gray, 1840; Cole & Hancock 1955) and in terrestrial crops, as invasive species in island ecosystems, and as intermediate vectors for human pathogens (many pulmonate taxa and limnic Hygrophila harbour schistosomiasis and other worms) (Smith & Stanisic 1998). On the other hand however, heterobranchs function as abundant food items for many economically and ecologically important vertebrates (e.g. Pteropoda for oceanic fish and whales, Hygrophila in wetlands and Stylommatophora on land for birds). Some heterobranchs are renowned for their aesthetic value; nudibranchs and other seaslugs act as flag species for tourism (Gosliner 2015), and colourful shells of land snails are prized collectors' items (e.g. *Polymita* Beck, 1837; see Guillén 2014). Scientifically, heterobranch seaslugs and land snails have yielded important discoveries in the field of neurobiology and learning (e.g. Jing et al. 2009, Nomaksteinsky et al. 2013); a Nobel Prize was given for the study of *Aplysia* (see Moroz 2010 for review). Other studies have advanced aspects of biochemistry and photosynthesis research (on nudibranchs and Sacoglossa; e.g. Wägele et al. 2006, 2010, Benkendorff

2010, Rumpho et al. 2011 for reviews). Furthermore, heterobranchs commonly function as models in other fields of biology such as evolution, ecology, and behavior (e.g. Hausdorf 2001, Lange et al. 2013).

2.2 History and State of the Art in the phylogenetics of Heterobranchia

Heterobranchia differ from other gastropods in some characteristic ways. Their name (meaning "different-gilled") stems from the fact that the heterobranch breathing organ is—if not lost completely—morphologically different from the "ctenidium" of remaining gastropods, the paraphyletic Prosobranchia (Salvini-Plawen & Haszprunar 1987, Haszprunar 1988). Originally, Heterobranchia were conceptualized to encompass two large groups of gastropods: the Opisthobranchia Milne-Edwards, 1848 contained all seaslugs and their relatives with more or less reduced shells, breathing with lateral or posterior gills. The Pulmonata Cuvier, 1817 comprised land snails, slugs and their coastal relatives breathing air with a lung. Opisthobranchia and Pulmonata were paired as Euthyneura Spengel, 1881, referring to a common loss of torsion of their posterior central nervous systems (the so-called "visceral loop"). The taxa Euthyneura and Heterobranchia were in many cases treated more or less synonymously until the discovery and recognition of "prosobranch" snails which had unequivocal heterobranch characters but did not fit within either opisthobranchs or pulmonates, and which were thus grouped outside of Euthyneura but still inside Heterobranchia (see Haszprunar 1985a; Fig. 1A).

Haszprunar (1985a, 1988) fundamentally refined the taxonomical concept of Heterobranchia by using morphological cladistics: his trees (Fig. 1A) assumed prosobranch-like Rissoelloidea Gray, 1850 as a first branch, followed by monophyletic Architectonicoidea Gray, 1850 and Pyramidellidae; Euthyneura were split into a grade of five opisthobranch monophyla (the first three shown as a polytomy), the last of which was sister to a clade of opisthobranch-like pulmonate slugs and "true" Pulmonata snails. Thus, "prosobranch" Caenogastropoda Cox, 1960 were established as sister group (later more precisely defined by Ponder & Lindberg 1997), and several families of prosobranch-like taxa were grouped as paraphyletic "lower" Heterobranchia (sometimes as taxa Heterostropha P. Fischer, 1885 or Allogastropoda Haszprunar, 1985a). Identifying euthyneury as convergent character, Haszprunar (1985a) also renamed Euthyneura to Pentaganglionata according to the hypothesized presence of (at least ontogenetically) five visceral loop ganglia (instead of only three). Haszprunar (1985a) identified several synapomorphies as characterizing Heterobranchia as a whole, including: 1) the presence of a sinistrally coiled larval shell (protoconch) on a generally dextrally coiled adult shell (teleoconch) – a phenomenon known as heterostrophy; 2) (secondary) gills in the mantle cavity being replaced or supported by the presence of paired ciliary strips creating a water current for respiration, 3) detorsion of the body as a common phenomenon, 4) mature sperm cells with a screw-shaped, spiral head, 5) and the kidney in the dorsal roof of the mantle cavity (as opposed to lying more ventrally).

In the early nineties, several taxa of minute snails were either newly discovered or reattributed to the assemblage of lower Heterobranchia, further expanding the idea of prosobranch-like snails that were nevertheless unequivocally heterobranchs by morphological characteristics: marine Valvatoidea Gray, 1840, Orbitestellidae Iredale, 1917, and *Tjaernoeia* Warén & Bouchet, 1988

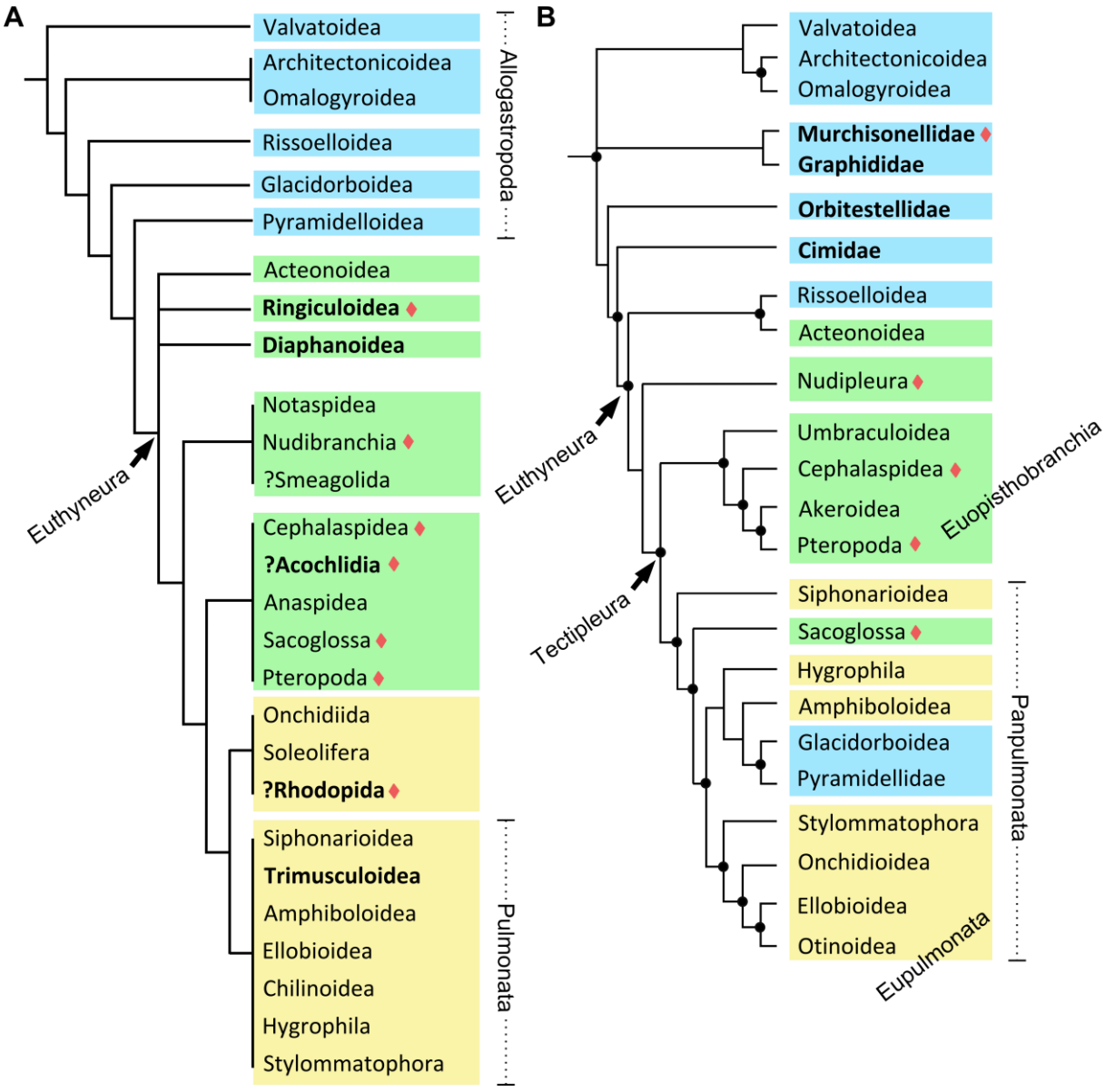

Figure 1. Comparison of two major phylogenetic hypotheses on Heterobranchia, based on morphological (A) and molecular (B) datasets.

Bold terminals: taxa not included in the respective other study. Blue boxes: paraphyletic taxon "Allogastropoda", green: "Opisthobranchia", yellow: "pulmonate" taxa. Names of terminals emended according to current usage.

A. Schematized consensus of the morphocladistic trees by Haszprunar 1985 (green and yellow taxa including question marks) and 1988 (blue taxa). Note paraphyly of Opisthobranchia (green taxa). First three opisthobranch taxa constitute Architectibranchia Haszprunar, 1985a; Soleolifera and Onchidiida=Onchidioidea constitute Systellommatophora Pilsbry, 1948. **B**. Schematized version of the molecular tree by Dinapoli & Klussmann-Kolb (2010), based on Bayesian and Maximum Likelihood analyses of a concatenated four gene dataset. Robustly supported nodes (pp > 0.98 or BS > 98) are marked with a black dot. Note changed position of Sacoglossa, Glacidorboidea Ponder, 1986 and Pyramidellidae, leading to non-monophyly of "Pulmonata". Classification after Schrödl et al. (2011a), including emendation of Tectipleura Schrödl et al., 2011a, Euopisthobranchia Jörger et al., 2010 and Panpulmonata Jörger et al., 2010.

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(Ponder 1990a,b, 1991, Warén 1991a,b, 1993, Warén & Bouchet 1993, Warén et al. 1993) and freshwater Glacidorbidae Ponder, 1986 (all summarized in Ponder et al. 1998). Later morphocladistic studies added taxa and characters to their datasets, but results were confounded by low resolution yielding mainly polytomies (Dayrat et al. 2001), by concept bias (monophyletic Opisthobranchia assumed *a priori*; Salvini-Plawen 1990), or by uncertainty due to obvious convergencies (meiofaunal taxa as a dubious monophylum; Wägele & Klussmann-Kolb 2005).

Meanwhile, Heterobranchia were shown to be a robustly supported clade by molecular phylogenetic studies, with many subsequent studies focusing on establishing phylogenies based on consecutively larger sets of taxa and genes for inference of relationships (Tholleson 1999, Dayrat et al. 2001, Grande et al. 2004, Klussmann-Kolb et al. 2008). Several further studies focused on the internal phylogeny of subclades and their close relatives, e.g. Stylommatophora (Wade et al. 2001, 2006), Pteropoda (Klussmann-Kolb & Dinapoli 2006), Acteonoidea d'Orbigny, 1843 (Göbbeler & Klussmann-Kolb 2010a), Pleurobranchoidea Gray, 1827 (Göbbeler & Klussmann-Kolb 2010b) and Pyramidellidae (Dinapoli et al. 2011).

The largest sampling of *lower* heterobranch taxa to date remains published by Dinapoli and Klussmann-Kolb (2010; tree summarized in Fig. 1B); their analysis suggested or confirmed some surprising relationships: 1) "Opisthobranchia" were, again, paraphyletic with respect to pulmonates and formed four distinct lineages; 2) Pyramidellidae and freshwater *Glacidorbis* Iredale, 1943 were not lower heterobranchs but closer to pulmonates (as suggested earlier by Ponder 1986, Dinapoli & Klussmann-Kolb 2010 and Dinapoli et al. 2011); 3) the minute snails *Graphis* Jeffreys, 1867, *Ebala* Gray, 1847 and *Murchisonella* Mörch, 1875 were not pyramidellids but found as a clade of lower Heterobranchia with high-spired shells, while flatspired taxa formed another clade (Architectonicoidea, Omalogyroidea G. O. Sars, 1878 and Valvatoidea), and others again were closer to Euthyneura (albeit with low statistical support); finally, 4) *Rissoella* Gray, 1847 was not one of the lowermost lineages but sister to opisthobranch Acteonoidea.

Summarizing, two overarching themes were apparent from studies that sampled Heterobranchia as a whole: first, the long-standing concept of monophyletic Opisthobranchia was more and more questioned through opisthobranch and lower heterobranch taxa being found very close to or even intercalated between pulmonates (Vonnemann et al. 2005, Grande et al. 2004, Dinapoli et al. 2010). This, in consequence, led to radical reorganization of the tree and naming of novel major clades, Euopisthobranchia and Panpulmonata (Jörger et al. 2010). A basic backbone of four euthyneuran/derived taxa (Acteonacea, Nudipleura, and sister taxa Euopisthobranchia and Panpulmonata) was later found by comparable studies (Dayrat et al. 2011) and confirmed by phylogenomics using thousands of genes (Kocot et al. 2011, Zapata et al. 2014). Second, low resolution of "lower" heterobranch taxa was apparent in all of these studies, thus leaving many basic questions of euthyneuran evolution difficult to answer.

This topology, exemplified by the study of Dinapoli and Klussmann-Kolb (2010), will be the phylogenetic starting point for this thesis (see Fig. 1B).

2.3 Towards integrative systematics in Heterobranchia – microanatomy and "standard" marker phylogenies

In the last almost twenty years, the advent of molecular phylogenetics in biology reached malacology, followed by the establishment of commonly used "standard" DNA sequence markers (mitochondrial genes CO1, H3, 16S rNA, and nuclear genes 18S rRNA, 28S rRNA) and methods for the inference of phylogenetic trees (Bayesian and Maximum likelihood algorithms). Many taxa remained unsequenced, yet these methods led to a growing knowledge on the phylogeny of Heterobranchia which built upon the foundation of preceding morphocladistic studies and morphological studies that were based mainly on SEM- and paraffin histological-examination.

However, knowledge of anatomy barely kept up with the pace, and many taxa remained essentially unstudied since their original descriptions from often fifty or a hundred years ago. From a modern morphological standpoint, many of these original descriptions were dubious or unreliable (e.g. giving details of nervous systems of very small organisms derived from potentially inadequate methods such as dissections, or single histological sections only), or data were fully missing (such as information on soft internal organs in studies focusing on hard parts). This especially affected small-bodied and difficult-to-collect taxa, including meiofaunal taxa (e.g. Marcus 1953, Salvini-Plawen 1991) or lower heterobranchs (e.g. Haszprunar et al. 2011).

One method closing the gap between the accelerating appearance of phylogenetic reconstructions and the lack of comparative data on small-bodied organisms became computer-based three-dimensional (3D) reconstruction based on microscopic images using the software Amira (e.g. Neusser et al. 2006, Ruthensteiner 2008). These can be based on a number of image sources; one of them being photographs of semithin histological sections with a thickness 1-2 µm, a resolution near the approximate thickness of many nerves. By combining these into aligned image stacks, 3D reconstruction is capable to resolve the interconnection of tubular or convoluted organ parts, and display complex microanatomy of organs such as nervous systems or even entire organisms (daCosta et al. 2007). Together with the possibility to include digital 3D models with publications (e.g. Ruthensteiner & Heß 2008), and with additional histological information on staining properties or gross cellular organization (such as ciliation, vacuolization), this tool is capable of creating comprehensive and detailed datasets suitable for comparative anatomy. This is especially important in small organisms (which are hard to dissect), thick organisms (not translucent enough to view entirely under a microscope), or those lacking hard parts made up of a cuticle or mineralized components (ideal for examination under SEM, after dissection). All these problems are often encountered in microscopic gastropods, especially meiofaunal slugs (Geiger et al. 2007).

The combination of molecular tree reconstructions and comparative anatomy covering as many morphological aspects as possible presents a powerful way of filling a tree with life. This combination has been described as "integrative taxonomy" (e.g. Dayrat 2005; but see Padial et al. 2010, Riedel et al. 2013 for a different perspective on the term) or "evolutionary systematics" by some authors (see e.g. Schwentner et al. 2015).

Some integrative studies led to novel hypotheses about the origin and evolution of large or small subgroups of Heterobranchia. For example, the study of Acochlidia had great impact on phylogenetic research of heterobranchs, transferring them from "opisthobranchs" to the middle of Panpulmonata (Jörger et al. 2010, 2014b), while microanatomy placed these results in a larger context of

morphological evolution (Neusser & Schrödl 2007; Neusser et al. 2007, 2009, 2011a). This demonstrated that despite their apparent exoticness, minute meiofaunal taxa had the potential to yield important insights into understanding evolution and diversity of a much larger clade, and were not justified to be regarded as sideshows (Jörger et al. 2010). This led to interest in other meiofaunal slug taxa beyond Acochlidia, which was the taxonomic starting point of interest in this thesis.

2.4 Aims of the thesis

Three major aims are pursued in the present thesis:

1) To explore and describe anatomically unknown interstitial heterobranch slugs, providing microanatomical all-organ datasets derived from 3D reconstruction of histological semithin sections and other morphological methods and to place these taxa in a phylogenetic context, either by using morphological or own molecular phylogenetic hypotheses. These data are used to describe or redescribe enigmatic and potentially basal taxa (= members of low diversity clades, splitting early in the respective taxon) by comparing phylogenetically relevant organ systems (CNS, reproductive system). Thereby, they are used to inform molecular phylogenetic trees, giving genotypes (from trees) a phenotype.

2) To explore putative sister taxa and compare their anatomy, thereby revising higher-hierarchy taxa. Comparative morphology is performed on taxa that are morphologically divergent, but phylogenetically closely related. Hoping to find characters with phylogenetic signal (potential symplesiomorphies for rooting, or synapomorphies for defining derived clades), the studied species act as exemplars or clearly-cut outgroups to the rest of clade, thereby rooting taxa in larger parts of the tree and corroborating phylogenetic hypotheses. In all cases, size-range of the examined species is between 1 mm (*Rhodope* Kölliker, 1847*, Koloonella* Laseron, 1959) and 8 mm (*Strubellia* Odhner, 1937).

3) To discuss the new data in a framework of Heterobranchia *per se*, and revise evolutionary scenarios. Comparative anatomy, using the scaffold of existing phylogenetic hypotheses, is used to trace evolution of Heterobranchia anew. Testable hypotheses are devised on the evolutionary history of key organ systems and of major heterobranch groups (taxon Euthyneura, groups of interstitial taxa, lower heterobranchs), thereby filling trees with life.

The present thesis compiles microanatomical work on a number of minute Heterobranchia, all belonging to distinct clades previously unstudied using modern methods (Fig. 2) Prior to these studies, no data comparable in detail or comprehensiveness existed on either dorid nudibranchs, ringiculids, cephalaspid slugs, pteropods, rhodopids or any high-spired lower heterobranch. The papers presented herein can be divided in five main parts: The first question for my thesis was: how do you obtain samples? Referring to interstitial heterobranchs, **chapter 1** (Jörger et al. 2014a) summarizes techniques in collecting, sampling, and documenting, describes and reviews the diversity of interstitial slugs (exemplary taxa shown in Fig. 2A), together with a key to identify these groups when found in the field. **Chapters 2 to 4** form the second part of this thesis, redescribing in detail euthyneuran taxa that are close relatives to interstitial taxa, or are interstitial themselves: chapter 2 (Brenzinger et al. 2011a) is an integrative study describing a new species of *Strubellia* (Panpulmonata:

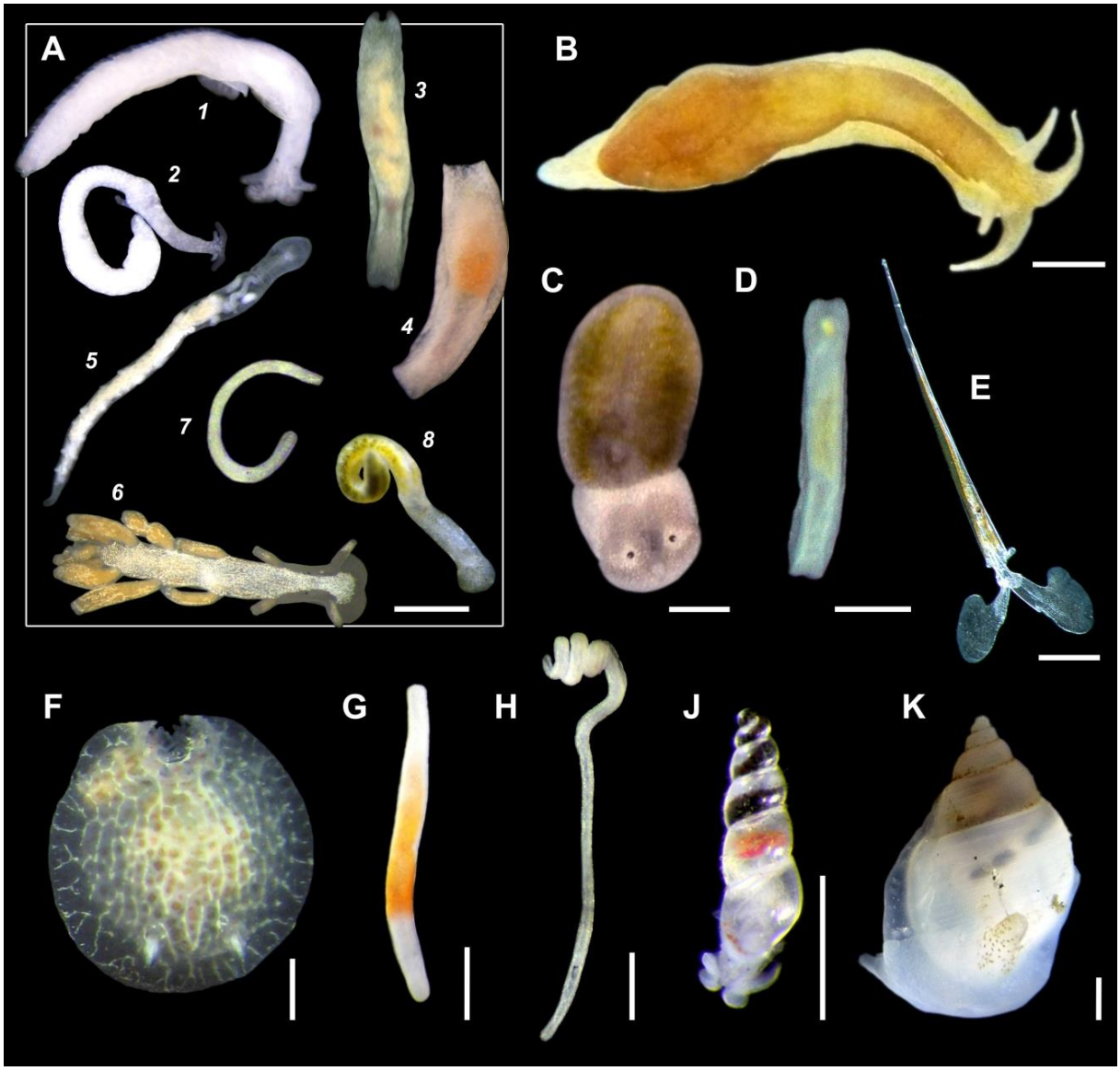

Figure 2. Examples of taxa examined in this thesis. Living specimens, dorsal view (C-K: dorsal end down).

A. Chapter 1: major groups of meiofaunal Heterobranchia, exemplified by Mediterranean species: 1, hedylopsacean acochlidian *Hedylopsis spiculifera* (Kowalewsky, 1901), 2, microhedylacean acochlidian *Microhedyle glandulifera* (Kowalewsky, 1901); 3-4, philinoglossid Cephalaspidea (*Philinoglossa praelongata* Salvini-Plawen, 1973 and *Abavopsis latosoleata* Salvini-Plawen, 1973); 5-6, cladobranch nudibranchs *Pseudovermis* Perejaslavtzeva, 1891 sp. and *Embletonia pulchra* (Alder & Hancock, 1844); 7, Rhodopemorpha (*Helminthope* Salvini-Plawen, 1991 sp.); 8, Sacoglossa (*Platyhedyle denudata* Salvini-Plawen, 1973). **B.** Chapter 2: the freshwater acochlidian *Strubellia wawrai* Brenzinger et al., 2011a from Solomon Islands. **C.** Chapter 3: the mudflat sacoglossan *Gascoignella* Jensen, 1985 (represented by photo of Thailand *G. nukuli* Swennen, 2001). **D.** Chapter 4: meiofaunal cephalaspidean *Pluscula cuica* Marcus, 1953 from Brazil. **E.** Chapter 5: pelagic thecosome pteropod *Creseis* cf. *acicula* (Rang, 1828)(exemplified by photo of north Pacific specimen). **F.** Chapter 6: kelp-dwelling corambid nudibranch *Corambe mancorensis* Martynov et al., 2011, tropical Peru. **G.** Chapter 7: phytal-dwelling rhodopemorph *Rhodope.* Exemplified by specimen of *Rh.* cf. *veranii* Kölliker, 1847, Istria, Adriatic Sea. **H.** Chapter 8: meiofaunal rhodopemorph *Helminthope*. Specimen from Madang, Papua New Guinea. (continued on next page)

Acochlidia; Fig. 2B), a genus imagined as a link between presumably plesiomorphic meiofaunal and derived freshwater acochlidians (minute *Pseudunela* Salvini-Plawen, 1973 and giant *Acochlidium* Strubell, 1892); chapter three (Kohnert, Brenzinger et al. 2013) redescribes *Gascoignella*, a mudflat sacoglossan slug from tropical eastern Asia (Fig. 2C) assumed to be sister to widely distributed meiofaunal *Platyhedyle* Salvini-Plawen, 1973 (Panpulmonata; see also Fig. 2A-8); chapter four (Brenzinger et al. 2013a) redescribes *Pluscula cuica*, regarded as most primitive member of the wholly interstitial family Philinoglossidae Hertling, 1932 (see Fig. 2A-3,4 for further examples) or in a family of its own, and at the time unplaced among euopisthobranch Cephalaspidea.

I coauthored papers presented in **chapters 5 and 6,** covering taxa with suspected evolutionary history of progenesis, placing them in a larger context: pelagic *Creseis* Rang, 1828 (Euopisthobranchia: Pteropoda: Thecosomata; Fig. 2E) (Kubilius et al. 2013), and benthic *Corambe* Bergh, 1869 (Nudipleura: Doridacea; Fig. 2F) (Martynov, Brenzinger et al. 2011).

Part 4 builds upon the surprising result by Wilson et al. (2010) that lower heterobranch Murchisonellidae Casey, 1904 (minute, highspired snails) are sister group to Rhodopemorpha Salvini-Plawen, 1991 (= Rhodopidae Ihering, 1876, = Rhodopida Minichev 1970 of Fig. 1A) , aberrant acoel-like slugs previously placed as *incertae sedis* either along nudibranch or pulmonate groups. In a series of three papers (**chapters 7 to 9**), I compare anatomies of species of the rhodopemorphs *Rhodope* (Brenzinger et al. 2011b; Fig. 2G) and interstitial *Helminthope* (Brenzinger et al. 2013b; Fig. 2A-7 & H) with the shell-bearing murchisonellid *Koloonella* (Brenzinger et al. 2014; Fig. 2J), exploring if this counterintuitive relationship is still traceable by morphological synapomorphies, and dwelling on questions of how to become a (meiofaunal) slug.

Finally, an integrative study (molecular and anatomical) was undertaken to elucidate the relationships of *Ringicula* Deshayes, 1838 (Ringiculoidea/Ringiculidae Philippi, 1853; Fig. 2K), a charismatic marine snail with shells known from an extensive fossil record, yet relationships were at best tentative. We provide both the first sequences for the group besides comparative microanatomy (**chapter 10:** Kano, Brenzinger, et al. in review).

These papers will be presented in the following section.

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Figure 2 (continued).

J. Chapter 9: estuarine murchisonellid *Koloonella minutissima* (Laseron, 1951) from southeast Australia. **K.** Chapter 10: mud-dwelling ringiculid *Ringicula doliaris* Gould, 1860 from southern Japan.

All scale bars approximately 500 µm. Photo credits: photos A6, A8, D & F – Michael Schrödl, Zoologische Staatssammlung München/Ludwig-Maximilians-Universität München. C – Somsak Buatip, University of Pattani. E – Russell Hopcroft, University of Fairbanks. J – image taken from video still. K – Yasunori Kano, University of Tokyo. All remaining photos by the author.

3 RESULTS - PAPERS PRESENTED IN THIS THESIS

Chapter 1. Jörger KM, Neusser TP, **Brenzinger B** & Schrödl M (2014): **Exploring the diversity of mesopsammic gastropods: How to collect, identify, and delimitate small and elusive sea slugs?** *American Malacological Bulletin*, **32**(2): 290-307.

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Exploring the diversity of mesopsammic gastropods: How to collect, identify, and delimitate small and elusive sea slugs?*

Katharina M. Jörger, Timea P. Neusser, Bastian Brenzinger, and Michael Schrödl

Mollusca Department, SNSB-Bavarian State Collection of Zoology, Münchhausenstr. 21, 81247 München, Germany and Department Biology II, BioZentrum, Ludwig-Maximilians-Universität, Großhadernerstr. 2, 82152 Planegg-Martinsried, Germany

Correspondence, Katharina Jörger: Katharina.Joerger@zsm.mwn.de

Abstract: Sediment-covered ocean floors constitute one of the largest and at the same time least explored habitats on Earth, still hiding an unknown level of species diversity. Coastal areas of this marine mesopsammic habitat harbor a variety of heterobranch snails and slugs. These gastropods long were puzzling due to their unclear phylogenetic positions, their aberrant morphologies and the lack of knowledge regarding their biology and diversity. Herein, we briefly review the advances of interstitial gastropod exploration, emphasizing that molecular approaches on formerly enigmatic mesopsammic groups like rhodopemorphs or acochlidians contributed to a drastic reconsideration of heterobranch systematics and evolution. We give an overview of the known diversity of mesopsammic heterobranchs and a list of type localities. In order to enhance surveys on the biodiversity of yet unexplored coasts, we then provide a suitable method to take samples of mesopsammic heterobranchs, and to extract and document slugs and snails from sands. A key based largely on externally-visible features allows for initial identification of already known taxa. Most mesopsammic gastropods show a "meiofaunal syndrome", *i.e.*, their morphology is constrained by the spatially-restricted interstitial environment, favoring rather uniform, worm-like body shapes and simple internal organization, which causes problems in conventional taxonomic approaches. Here, we present and discuss an integrative taxonomic workflow for delimiting potentially cryptic and elusive mesopsammic species, that also may be of use for other rare(ly) sampled invertebrates.

Key words: Acochlidia, field key, integrative taxonomy, Mollusca, Panpulmonata

THE MESOPSAMMON AND ITS INHABITANTS

Marine sediments and the interstices between sand grains, sometimes referred to as mesopsammon (Remane 1940), belong to the most ancient ecosystems of our planet (Rundell and Leander 2010). By the mid-19th and at the beginning of the 20th century, scientists discovered the water-filled interstitial space between the grains of coastal marine sands as a habitat for organisms (*e.g.*, Lovén 1844, Kowalevsky 1901a, 1901b, Giard 1904). Considerable progress has yet been achieved in different areas of meiofaunal research (*e.g.*, Remane 1952, Swedmark 1964, Ax 1969, Higgins and Thiel 1988a, Worsaae and Kristensen 2005, Giere 2009, Curini-Galletti *et al.* 2012, Worsaae *et al.* 2012). However, our knowledge of meiofaunal biodiversity, ecology and evolution is still limited and Rundell and Leander (2010) emphasized that the exploration of the meiofauna "remains among the most challenging, the most neglected and potentially the most enlightening frontiers of discovery in biology".

The interstitial milieu is characterized by extreme ecological conditions, such as faint light and limited amount of space, which restricts the body size and limits the interstitial fauna to minute, vermiform organisms suited to a lacunar environment (Swedmark 1964, 1968, Ax 1969, Higgins and Thiel 1988a). Currents and wave action transform the interstitial biotope by permanent restratification of the surface layer of the sand (Swedmark 1964). The continuous rearrangement of the particles contributes to a dynamic environment and makes the colonization by, *e.g.*, algae, difficult (Swedmark 1968). Furthermore, the living conditions in the intertidal zone or shallow water are complicated by diverse physical factors: the temperature varies with the time of day, seasons, and the rhythm of tides and, thus, fluctuates significantly in the surface layers of the sand layer; and, the salinity may increase by evaporation or decrease by rainfall or by the inflow of coastal freshwater (Giere *et al.* 1988). Organisms that successfully colonize the marine interstitial often develop special morphological and biological adaptations: body sizes are typically very small ranging from 0.5 mm to approx. 3 mm; flat

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and broad or vermiform-elongated body shapes are commonly favored. The body wall is often reinforced by subepidermal spicules or cuticle for mechanical protection. Members of the interstitial fauna frequently have a strong contractibility and a high adhesive capability by different glandular systems to avoid being washed away (Swedmark 1964, 1968, Botosaneanu 1986, Higgins and Thiel 1988a). Consequently, the study of mesopsammic taxa is challenging—species are small, hard to collect, problematic to distinguish externally and difficult to describe by means of traditional techniques.

Different terms are associated with the fauna inhabiting marine sediments (see Higgins and Thiel 1988b, for summary). Most commonly a practical size definition is applied, characterizing all fauna which passes through a 1 mm mesh and is retained by 42 μm mesh as meiofauna (Higgins and Thiel 1988b). While this definition provides no relationship to a specific ecology and is also controversial due to deviations depending on *e.g.*, anesthetized vs. living organisms, it is still of high practical value as directly related to extraction techniques in the field (Higgins and Thiel 1988b). Nearly all major metazoan taxa are represented in this size-defined marine meiofauna, *e.g.*, Cnidaria, Platyhelminthes, Nemertea, Gnathostomulida, Gastrotricha, Rotifera, Annelida, Priapulida, Loricifera, Kinorhyncha, Acari, crustacean taxa, and Mollusca (*e.g.*, Swedmark 1964, Botosaneanu 1986, Higgins and Thiel 1988a, Giere 2009, Rundell and Leander 2010). With the exception of Cephalopoda, all major molluscan clades include members that are at least temporarily meiofaunal (*i.e.*, in early stages of their life cycle). Juveniles of molluscan taxa that inhabit soft sediments as adults (as sand-dwellers or epibenthic) are frequently encountered in sediment samples. Infaunal Scaphopoda and sediment-burrowing Caudofoveata are temporarily meiofaunal; few prochaetodermatid Caudofoveata retain meiofaunal sizes as adults (Morse and Scheltema 1988). The same applies for several Polyplacophora (pers. obs.; E. Schwabe pers. comm.), with currently only one species—*Leptochiton intermedius* (Salvini-Plawen, 1968)—being described as permanent meiofauna (Salvini-Plawen 1968). Among infaunal Bivalvia and Solenogastres, however, several representatives are known with permanently minute body sizes (*e.g.*, bivalve Nuculidae and Mallettiidae (Poizat and Arnaud 1988) or neomeniomorph Meiomeniidae and Simrothiellidae (Morse and Scheltema 1988, García-Alvarez *et al.* 2000)). Among gastropod molluscs, we also frequently encounter temporarily meiofaunal forms in marine sediment samples. For example representatives of cephalaspidean *Chelidonura* A. Adams, 1850, nudibranchs *Gymnodoris* Stimpson, 1855, *Aegires* Lovén, 1844 and some chromodoridids, or some Runcinacea (*Runcina* Forbes [in Forbes and Hanley], 1851) were repeatedly observed in sand samples (pers. obs.). There are also several clades of shelled gastropods (*e.g.*, Pyramidellidae, Omalogyridae, Caecidae, Neritiliidae, Seguenziidae) with minute body sizes assigning them to permanent meiofauna. In most of these cases it is unknown, however, whether these snails lead an epibenthic or infaunal lifestyle.

The fauna inhabiting the interstices of sediment grains and which moves through its habitat with minimal disturbance (*i.e.*, in contrast to organisms digging through the sand) is defined as 'interstitial' (Nicholls 1935) or 'mesopsammic' (Remane 1940) fauna. Usually this mesopsammic or interstitial fauna also falls in the size-defined category of meiofauna but not necessarily so when inhabiting very coarse sediments and shell gravel. Some 'typical' meiofaunal snails (*sensu* Arnaud *et al.* 1986) that are frequently extracted from bulk sediment samples may not be mesopsammic; instead they are surface dwellers that never venture deep into the sand (*e.g.*, most *Caecum* Fleming, 1813 or *Embletonia* Alder and Hancock, 1851), or venture across the sand but live mostly on algae (*Omalogyra* Jeffreys, 1859, Runcinacea, large *Rhodope* Koelliker, 1847). In the present review we focus on meiofaunal and at least externally shell-less gastropods that show the characteristic adaptations of their body plan typical for interstitial taxa (see above) and which can, therefore, be considered as truly inhabiting the marine mesopsammon.

MESOPSAMMIC SLUGS PLACED IN A PHYLOGENY

Mesopsammic heterobranchs from different lineages (Fig. 1) often look quite similar to other meiofaunal "worms", having streamlined vermiform bodies and often lacking tentacles, body appendages or pigments, and even anatomically show similar organ reductions or special structures such as accessory ganglia or spicules. This phenomenon of particular morphoanatomical similarity caused by adaptation to a special environment was termed the "meiofaunal syndrome" by Brenzinger, Haszprunar *et al.* (2013). Multiple convergence in virtually all major organ systems causes problems not only for species identification and assignment of aberrant worm-like species to higher taxa, but especially for reconstructing their relationships in morphocladistic analyses (Schrödl and Neusser 2010).

Therefore, trials to recover the origin of mesopsammic heterobranch lineages using multi-locus sequence data appeared more promising and have, in hindsight, given invaluable contributions to heterobranch systematics. Vonnemann *et al.* (2005) first included three acochlidian species (representing both major subclades) into molecular analyses (18S and 28S rRNA), and failed to recover monophyletic opisthobranchs or pulmonates. Expanding the heterobranch taxon sampling and adding mitochondrial COI and 16S rRNA markers, Klussmann-Kolb *et al.* (2008) recovered a tree that

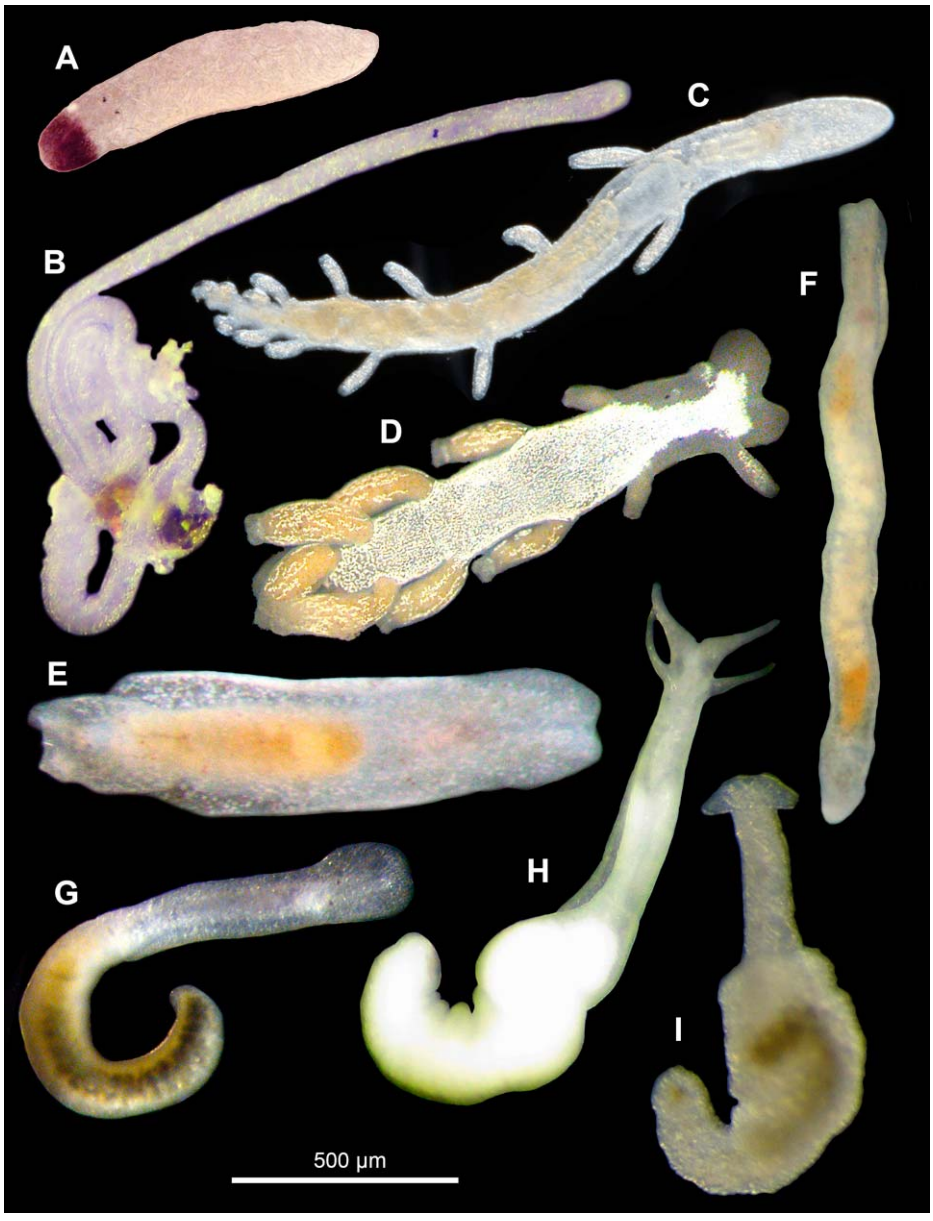

Figure 1. Living specimens of the major mesopsammic slug lineages. **A,** *Rhodope* sp. from Belize (Rhodopidae, Rhodopemorpha); **B,** *Helminthope* sp. from Papua (Rhodopidae, Rhodopemorpha); **C,** *Pseudovermis salamandrops* (Pseudovermidae, Aeolidoidea?); **D,** *Embletonia pulchra* (Embletoniidae, Aeolidoidea?); **E,** *Philine exigua* ('Philinidae', Cephalaspidea); **F,** *Philinoglossa marcusi* (Philinoglossidae, Cephalaspidea); **G,** *Platyhedyle denudata* (Platyhedylidae, Sacoglossa); **H,** *Pseudunela viatoris* (Pseudunelidae, Acochlidia); **I,** *Pontohedyle milaschewitchii* (Microhedylidae, Acochlidia). (Color shown in electronic version only).

clearly contradicted Milne Edwards' (1848) classical division of the Euthyneura into Opisthobranchia and Pulmonata. Jörger, Stöger *et al.* (2010) reviewed potential morphological evidence for Pulmonata or Opisthobranchia, and could not find any. Retrospectively, Opisthobranchia or Pulmonata have never been well-supported monophyla (Haszprunar 1985, Dayrat and Tillier 2002), but the scientific community has long kept to a traditional concept of the two euthyneuran subtaxa, particularly because it conveniently bears a rough resemblance to the ecological division between sea slugs on the one hand and limnic and terrestrial slugs and snails on the other (Wägele *et al.* 2014). Based on multilocus data on a heterobranch taxon sampling including mesopsammic Philinidae, Philinoglossidae, Platyhedylidae, and six of seven acochlidian families, Jörger, Stöger *et al.* (2010) formally reclassified the Euthyneura. Major novelties were the exclusion of Acteonoidea, and the basal position of Nudipleura (with unsampled mesopsammic Pseudovermidae and Embletoniidae) sister to a clade with all other euthyneurans, termed Tectipleura (Schrödl *et al.* 2011). The latter divides into Euopisthobranchia, including Cephalaspidea *sensu stricto* with interstitial philinids and philinoglossids, and Panpulmonata, comprising sacoglossans (with mesopsammic *Platyhedyle* Salvini-Plawen, 1973) and Acochlidia related to siphonariids, pyramidellids, glacidorbids, amphibolids, and typical pulmonate groups (Fig. 2). Mesopsammic, extremely worm-like rhodopemorphs (Brenzinger, Wilson *et al.* 2011, Brenzinger, Haszprunar *et al.* 2013) surprisingly clustered with shelled and long-spired Murchisonellidae, which are basal heterobranchs (Brenzinger *et al.* 2014) in initial molecular analyses (Wilson *et al.* 2010). The new heterobranch tree is shown and discussed by Wägele *et al.* (2014).

From the perspective of interstitial fauna, heterobranchs invaded the mesopsammon at least eight times independently (Fig. 2), and in conclusion adapted to the environment convergently. Interestingly, and so far unique among interstitial gastropods, several acochlidian lineages reversed 'regressive evolution' characteristic for the mesopsammic fauna (Swedmark 1968; Westheide 1987) and

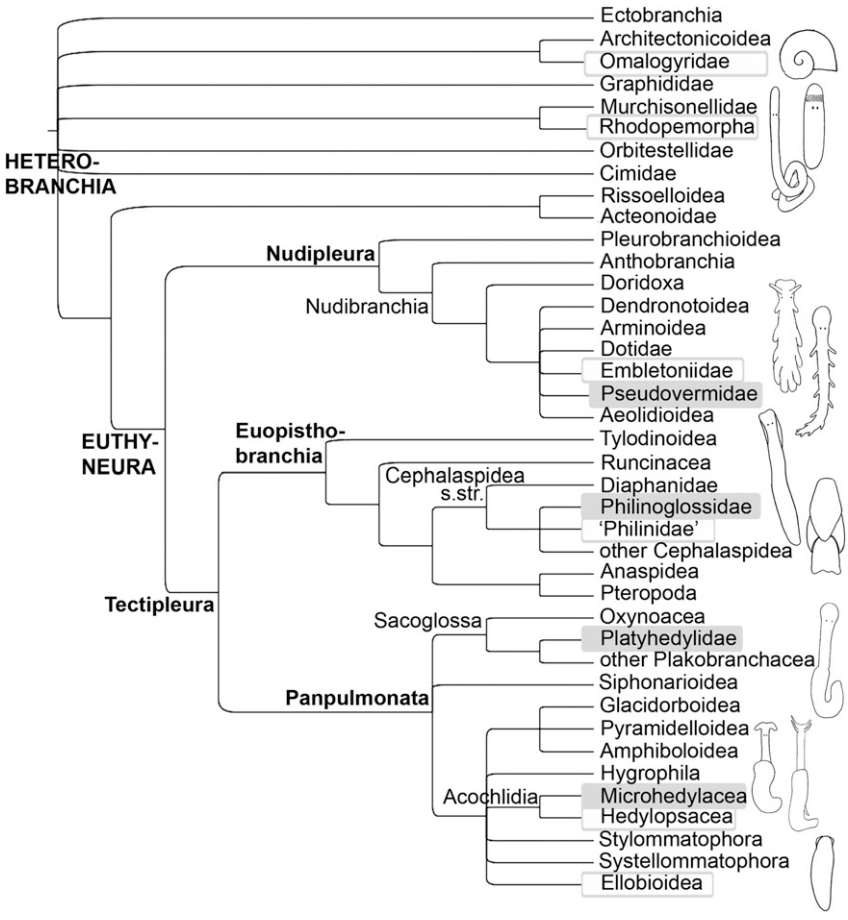

Figure 2. Cladogram of Heterobranchia modified after Jörger *et al.* (2010b), combined with the consensus topology presented in Wägele *et al.* (2014), showing mesopsammic lineages in grey boxes (clades not entirely mesopsammic with grey outline only).

reestablished an epibenthic lifestyle. Intertidal to supratidal Aitengidae adapted to semiterrestrial conditions (Neusser, Fukuda *et al.* 2011), the brackish-water adapted *Pseudunela espiritusanta* Neusser and Schrödl, 2009 lives under intertidal stones (Neusser and Schrödl 2009), and Acochlidiidae inhabit freshwater (*e.g.*, Wawra 1974, 1979, Brenzinger, Neusser *et al.* 2011).

DIVERSITY OF MESOPSAMMIC SLUGS

Compared to other meiofaunal taxa such as nematodes, polychaete annelids or copepods, all meiofaunal slug lineages are small taxonomic groups with few described species (Table 1). At current stage of research the Acochlidia are the largest clade of sea slugs inhabiting the mesopsammon with currently 26 valid species of Microhedylacea (an exclusively mesopsammic clade) and six Hedylopsacean species described from the interstices of sand grains (see Table 1, remaining Hedylopsacea live epibenthic in marine, limnic, and (semi)terrestrial systems) (Schrödl and Neusser 2010; unpublished data). Pseudovermidae are entirely mesopsammic and currently comprise 16 valid species (Urgorri *et al.* 1991, Jörger *et al.* 2014). Seven mesopsammic species of cephalaspidean Philinoglossidae are currently valid and a single species is described from the mesopsammon for cephalaspidean Philinidae and Sacoglossa (Arnaud *et al.* 1986). In Rhodopemorpha it remains unclear whether the five species of *Rhodope* truly inhabit the mesopsammon or lead an epibenthic lifestyle; the monotypic *Helmithope* is considered a truly interstitial species based on its morphological adaptations (Brenzinger, Haszprunar *et al.* 2013). Five species of Smeagolidae are reported to inhabit cobble beaches (Tillier and Ponder 1992); given the large interstitial spaces of this habitat it probably differs considerably from the mesopsammic environment. Usually, mesopsammic slugs are rare and there are only few taxa in which higher local densities (> 100 individuals / 0.05 m³) are reported (*e.g.*, some microhedylacean Acochlidia or philinoglossid Cephalaspidea (Poizat 1991)). The Acochlidia show worldwide distribution and present the only known lineage of mesopsammic sea slugs which also inhabit cold waters (*Asperspina murmanica* (Kudinskaya and Minichev, 1978) (see Neusser, Martynov *et al.* 2009)). All remaining lineages are restricted to tropical and temperate sands (Fig. 3); a compilation of type localities from all valid mesopsammic slugs is provided for future research in Appendix 1 (http://www.bioone.org/doi/suppl/10.4003/006.032.0205/suppl_file/Jorger_2014_suppl.PDF). The comparably high diversity in European waters is likely a sampling artifact with a major effort of meiofaunal research focusing in Europe for decades (Coull and Giere 1988) and relatively little and isolated sampling effort in tropical zones (especially the Indian Ocean and large parts of the Indo-Pacific).

The sampling effort of our workgroup supported by a series of international collaborators have significantly raised the diversity in each of the lineages recovering a wealth of putative new species, which raises the number of species by up to ten fold (see Table 1). The presumably low reproductive output and dispersal abilities of meiofaunal slugs including Acochlidia suggests a high degree of endemism, which is supported by the detection of rather narrow ranges of distribution in many meiofaunal slugs and deep genetic divergence

Table 1. Status of diversity of known meiofaunal slugs (Heterobranchia). ? No data available. *According to own unpublished data. **Hedylopsacea contain at least three secondarily non-meiofaunal lineages (Aitengidae, Acochlidiidae, and *Pseudunela espiritusanta*). *Rhodope* and *Embletonia* might be epibenthic and *Smeagol* inhabits cobble beaches. Anatomical characters do not necessarily fit the 'meiofaunal syndrome'.

Taxon	Described meiofaunal species/ Estimated total number including undescribed species*	References
RHODOPEMORPHA		
Rhodope Kölliker, 1847	6 / 15*	Haszprunar and Heß 2005; Wilson *et al.* 2010
Helminthope Salvini-Plawen, 1991	1 / 8*	Brenzinger, Haszprunar *et al.* 2013
NUDIBRANCHIA		
Pseudovermis Perejaslavtzeva, 1891	16 / 20*	Urgorri *et al.* 1991
Embletonia cf. *pulchra* Alder and Hancock, 1844)	1 / 10*	Miller and Willan 1992; Martynov 2007
CEPHALASPIDEA		
Philinoglossidae	7 / 15+*	Salvini-Plawen 1973; Brenzinger, Padula *et al.* 2013
Philine exigua Challis, 1969 and similar	1 / 5-10+*	Challis 1969
SACOGLOSSA		
Platyhedyle Salvini-Plawen 1973	1 / 4+*	Salvini-Plawen 1973; Rückert *et al.* 2008
ACOCHLIDIA		
Microhedylacea	26 / 40+*	Schrödl and Neusser 2010; Jörger and Schrödl 2013
Meiofaunal Hedylopsacea**	6 / 16+*	
OTINOIDEA		
Smeagol Climo, 1980	5 / 8?	Tillier and Ponder 1992; Fukuda and Ueshima 2010

in globally distributed lineages (Jörger *et al.* 2012). Moreover, the patchy occurrence typical for meiofaunal animals (*e.g.*, Poizat and Arnaud 1988, Andrade *et al.* 2011) can easily cause species to go undiscovered even in densely sampled areas (Curini-Galletti *et al.* 2012; pers. obs.). Considering the fact that the vast majority of marine sands, worldwide and in all varying depth ranges, are still virgin soil to meiofaunal research, the currently known diversity of mesopsammic slugs and probably also the reported unpublished findings in Table 1 still severely underrepresents true diversity. Overall, the contribution of meiofauna to marine biodiversity surveys has doubtlessly been underestimated, leaving this important ecosystem largely neglected in conservation approaches. We need fast, efficient and reliable means of species delineation in meiofaunal taxa to address this taxonomic impediment—means that are capable of dealing with the putatively high degree of cryptic speciation likely to be the rule for meiofauna (Jörger *et al.* 2012). Accounting for the still exploratory stage of meiofaunal research, we aim to contribute in the following to exploring worldwide mesopsammic sea slugs by providing guidelines on how to extract specimens from sediments and a key for initial determination of mesopsammic sea slugs into major taxa at least, updating Arnaud *et al.* (1986). Moreover, we discuss the pitfalls of species delimitation in meiofaunal slugs, evaluate the pros and cons of various species delimitation methods, and propose an integrative work flow that especially addresses the needs of mesopsammic and other taxa with only few samples available.

INSTRUCTIONS TO STUDY MESOPSAMMIC SLUGS IN THE FIELD

Searching and extracting mesopsammic sea slugs

In the course of our studies we conducted sampling trips to different biogeographic zones for initial exploration of the interstitial malacofauna, and experienced very heterogeneous

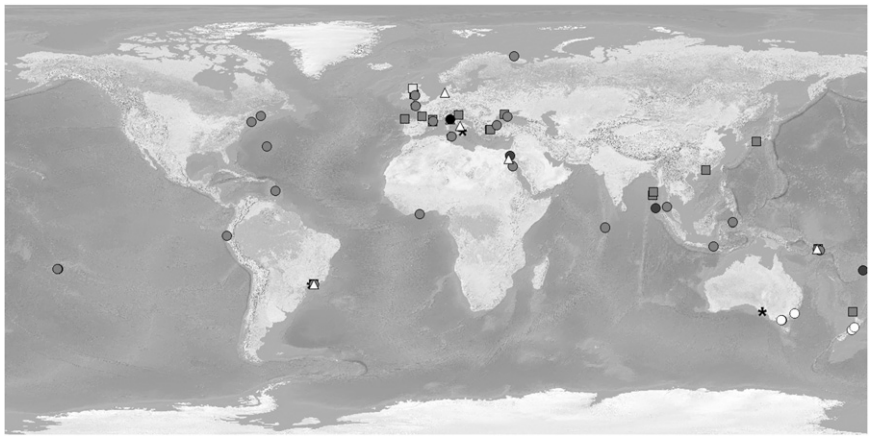

Figure 3. Overview of the type localities of the different mesopsammic microslugs (for details see also Table 1). 'Lower heterobranch' Rhodopemorpha marked with black star. Nudipleuran taxa marked with squares (dark grey: Pseudovermidae, light grey: Embletoniidae). Euopisthobranch Cephalaspidea shown in triangles (light grey: Philinoglossidae, dark grey: Philinidae). Panpulmonate clades marked as dots (dark grey: hedylopsacean Acochlidia, light grey: microhedylacean Acochlidia, white: Sacoglossa, black: Smeagolidae).

regional density of specimens and species diversity. In general, subtropical or tropical coasts seem much more species-rich than cold waters (compare in Fig. 3), and not all depths or sediment types host the same or an equally rich gastropod meiofauna. For example, in our experience lava sands are generally poorer with respect to meiofaunal gastropod diversity then coral sands. Usually, coarse oxygenated subtidal substrates with steady water currents appear privileged in species diversity relative to intertidal, wave-exposed fine sandy beaches or fine sediments with much organic content (Poizat and Arnaud 1988; pers. obs.). We now know of specialized acochlidians from all continents but Antarctica, from many islands regardless their distances to continental coasts, and even from high energy beaches or brackish water-influenced estuaries. Other mesopsammic groups such as cephalaspideans also occur in finer, detritus-rich sediments, often sporadically or seasonally (Poizat 1984). Freshwater-influenced sediments may host acochlidians and sacoglossans, and, so far, a single known acochlidian species (*Tantulum elegans* Rankin, 1979 from the Carribbean) even occurs in sediments of a swampy mountain spring (however, our own recollecting attempts at the type locality in 2009 failed).

In summary, almost all our sampling trips resulted in finding a variety of mesopsammic sea slugs and snails, often including lineages new to science (see above). To encourage and support sampling of mesopsammic slugs, we here provide a suitable and detailed step-by-step procedure to extract mesopsammic molluscs from sand samples (modified from Pfannkuche and Thiel (1988) and updated from Schrödl (2006) and Neusser (2011)) and list suggestions based on our experience on how to anesthetize and fix encountered specimens for various purposes (Appendix 2) (http://www.bioone.org/doi/suppl/10.4003/006.032.0205/suppl_file/Jorger_2014_suppl.PDF).

Documenting mesopsammic slugs in the field

During specimen collection, researchers should consider that samples have to be treated and selected wisely, and prepared differently according to later, various uses. In absence of an external shell, the taxonomy of meiofaunal slugs or snails with reduced internal shell was mainly based on external morphology of living or preserved specimens, presence and type of calcareous spicules and radula characteristics (Kowalevsky 1901b, Arnaud *et al.* 1986, Wawra 1987). Snails and slugs may distort or retract during fixation if not relaxed very carefully, pigments may fade especially in ethanol, and calcareous spicules will disappear quickly in any calcium carbonate undersaturated or acidic fixative (Poizat and Arnaud 1988). Live documentation of ephemeral morphological features and of behavior and movement, thus, is a unique opportunity to study phenotypes and essential to preliminarily identify new findings and to correlate them with existing taxonomy. Of special interest are—next to lucky occasional observations on, *e.g.*, feeding or reproduction—the behavior during disturbance (*e.g.*, ability to retract) and the behavior in motion (*e.g.*, the ability to adhere to the substrate). Important taxonomic characters in meiofaunal slugs are the general body shape, shape and relative size of head appendages (*i.e.*, oral tentacles, rhinophores), body appendages (*i.e.*, cerata in nudibranch *Pseudovermis* and *Embletonia*), and relative length and width of the foot; all of which should preferably be documented on living material to avoid fixation artifacts. Observations of internal anatomy (*e.g.*, (different types of) spicules or pigments (including eyes)) are most informative using carefully squeezed, anesthetized specimens under transmission light microscopy, preferably with differential interference contrast (DIC). Whenever possible, photographs and high-resolution videos of living animals should be used to later identify and describe internal taxonomic characters (*e.g.*, minute, thin-walled structures like the heart can easily collapse during preparation for histology and detection might be easier on living material than on histological sections). Radulae of meiofaunal species are resistant to fixation and preservation, but very small and structural details may not be adequately revealed using light microscopy requiring further analyses via scanning

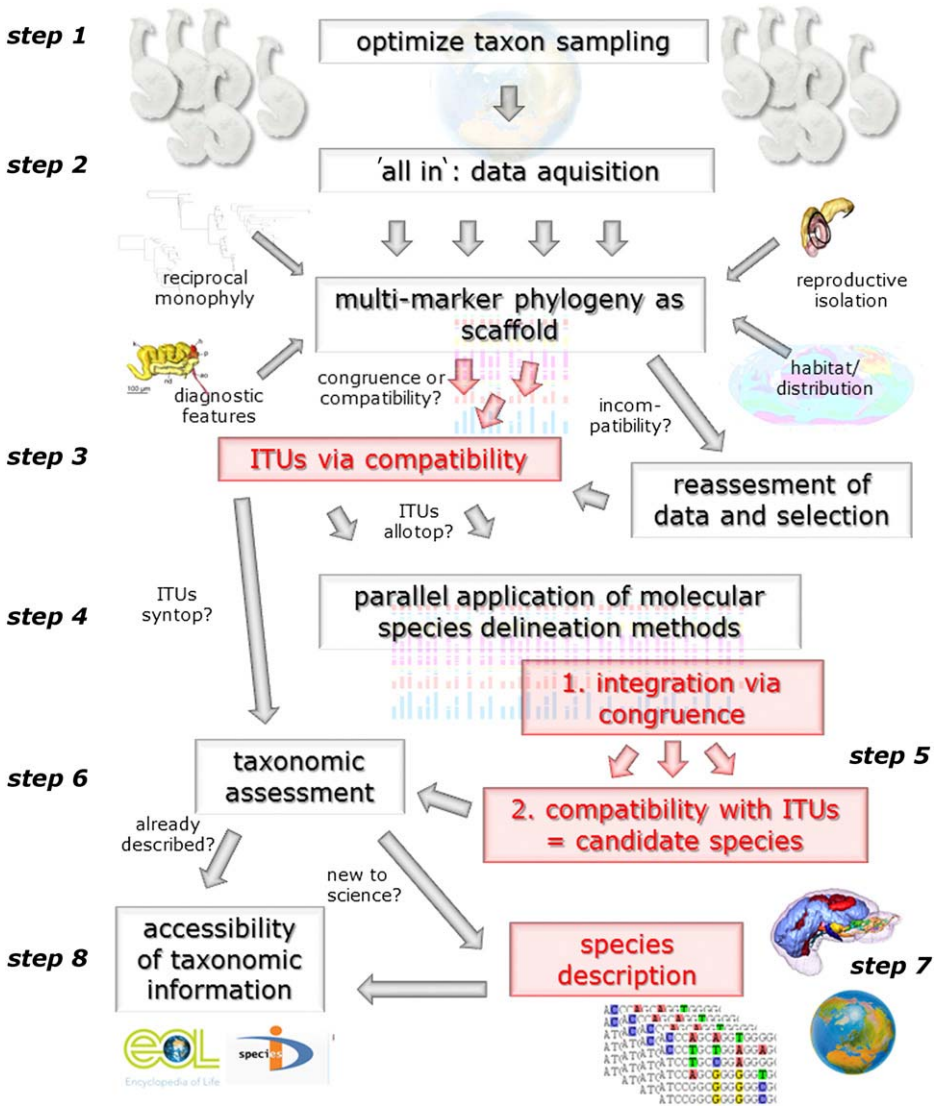

Figure 4. Flowchart on the proposed workflow on species delineation in elusive taxa, modified after the approach by Jörger *et. al.* (2012). (Color shown in electronic version only).

electron microscopy (SEM). Since preparation of minute radulae is challenging (Geiger *et al.* 2007), prior adequate documentation via light microscopy is indispensable. Rather than preparing whole mounted specimens for soft part anatomy, we recommend recovering the documented individuals and fix them for molecular studies or advanced morphological techniques (see Appendix 1, http://www.bioone.org/doi/suppl/10.4003/006.032.0205/suppl_file/Jorger_2014_suppl.PDF) for different fixatives successfully applied).

A key to identify mesopsammic slugs

There are ongoing efforts to investigate in detail the microanatomical and morphological diversity of described mesopsammic lineages and their descendants and close relatives (*e.g.*, Neusser *et al.* 2006, 2007, Jörger *et al.* 2008, Rückert *et al.* 2008, Jörger *et al.* 2009, Neusser, Heß *et al.* 2009, Neusser, Martynov *et al.* 2009, Brenzinger, Wilson *et al.* 2011, Kohnert *et al.* 2011, Brenzinger, Haszprunar *et al.* 2013, Brenzinger, Padula *et al.* 2013, Kohnert *et al.* 2013, Jörger *et al.* 2014) and to reconstruct the evolution of phenotypes and biology from molecular approaches. This contributes to a better knowledge of the diversity in the mesopsammon and an evaluation of the diagnostic characters for identification of these taxa. As reviewed above, sampling efforts in the past years have discovered a series of species potentially new to science, which partially differ unequivocally from all described mesopsammic slug lineages by characters of the external morphology (see *e.g.*, Fig. 1A). On the other hand, the new material from the mesopsammon largely comprises cryptic lineages (especially within morphologically static Microhedylacea), which could only be revealed as novel evolutionary entities through the use of integrative approaches employing 3D-microanatomical descriptions and molecular data (Neusser, Jörger *et al.* 2011, Jörger *et al.* 2012, Jörger and Schrödl 2013), see discussion on species delimitation below. Nevertheless, many sea slug lineages inhabiting the interstitial—known and still unknown ones—may be tentatively identifiable to higher taxonomic categories such as family or genus-level in the field using the key presented herein (Appendix 3, http://www.bioone.org/doi/suppl/10.4003/006.032.0205/suppl_file/Jorger_2014_suppl.PDF). For observing external features a dissecting microscope is necessary and internal features such as presence and structure of shell, spicules or radulae requires a transmission light microscope.

SPECIES DELIMITATION IN MEIOFAUNAL GASTROPODS – AND OTHER RARE AND ELUSIVE TAXA

Morphological species delineation

Traditionally, the taxonomy of gastropods relies on external morphological characters; even in 2006, approximately 80% of new gastropod species descriptions were based solely on shell characters combining the advantages of unproblematic preservation in natural history collections and potential for *post mortem* identification (Bouchet and Strong 2010). However, several studies with largely molecular scope have demonstrated the potentially high intraspecific variability of both shell morphology (*e.g.*, Hauswald *et al.* 2008, Bouchet and Strong 2010, Puillandre, Modica *et al.* 2012) and external characters in general, such as color variation in slugs (Nitz *et al.* 2009). When representatives of meiofaunal slug lineages were first discovered, their aberrant external morphology was in many cases sufficient for species delimitation (*e.g.*, Kowalevsky 1901a, 1901b). This changed with every new discovery of other closely related meiofaunal gastropods, making further characteristics of radulae and spicules obligatory for species delineation within clades (Salvini-Plawen 1973, Arnaud *et al.* 1986, Wawra 1987). In restricted geographic areas, a combination of these characters might still be sufficient to diagnose mesopsammic slugs (Eder *et al.* 2011), but on a broader scale these characters become insufficient (Neusser, Jörger *et al.* 2011, Jörger *et al.* 2012). External features in mesopsammic slugs are heavily constrained by the requirements of the spatially restricted habitat and provide little variation. Other features—such as the presence of externally visible eyes—show high intraspecific plasticity (Neusser, Jörger *et al.* 2011, Jörger *et al.* 2012).

For most gastropods the radula morphology is of major importance for taxonomy, in meiofaunal slugs diagnostic characters are, however, often limited to minute details (*e.g.*, the number of lateral denticles on the rhachidian tooth in Pseudovermidae (Urgorri *et al.* 1991)). Therefore, light-microscopic investigation might be insufficient to reliably analyze the minute radulae of meiofaunal gastropods and re-investigation by SEM is needed for reliable comparative analyses. Due to the partially minor interspecific variation, intraspecific and intraindividual variation requires special attention and in some cases radula characteristics may be insufficient to diagnose species (*e.g.*, Jörger *et al.* 2014).

Anatomical data included in species descriptions were traditionally based on morphological data from squeezed whole mounts (Kirsteuer 1973), whole mount or crush preparation of the radula (*e.g.*, Doe 1974) and/or the examination of histological sections of up to 10 μm thickness, which were frequently paraffin-based and distorted (*e.g.*, Odhner 1937, Marcus 1953, Challis 1968, Challis 1970, Morse 1976), and, therefore, not always reliable considering modern standards. Traditional manual reconstruction techniques from semithin histological sections (Sommerfeldt and Schrödl 2005) are time-consuming and challenging. We currently consider a 3D-based microanatomical approach most powerful: because modern 3D reconstructions based on μCT and synchrotron microtomography data currently do not allow for detailed microanatomical investigation in micromolluscs (Kunze 2013), we favor an approach using AMIRA software to reconstruct 3D models of all major organ systems based on serial semithin histological sections of 1–1.5 μm thickness (method after Ruthensteiner (2008); for methodological discussion see also DaCosta *et al.* (2007)).

Redescriptions of all major meiofaunal slug lineages based on advanced 3D-microanatomy in conjunction with ultrastructural data from, *e.g.*, sperm have compiled microanatomical characters across all organ systems, and these characters have proven reliable for taxonomic purposes (Neusser *et al.* 2006, Neusser and Schrödl 2007, Jörger *et al.* 2008, Neusser, Martynov *et al.* 2009, Jörger, Kristof *et al.* 2010, Martin *et al.* 2010, Brenzinger, Wilson *et al.* 2011, Eder *et al.* 2011, Kohnert *et al.* 2011, Brenzinger, Padula *et al.* 2013). These studies demonstrated the high quality of modern morphological approaches, which provide reliable, highly detailed diagnostic characters for taxonomic and systematic studies.

But even high-end morphological study reached its limits when confronted with the extraordinary degree of convergent adaptation that gastropods are notorious for (Ponder and Lindberg 1997, Dayrat and Tillier 2002, Wägele *et al.* 2014); and this adaptation is carried to an extreme in taxa that inhabit environments such as the mesopsammon, which selects for certain morphological and anatomical adaptations. Moreover, 3D-microanatomical approaches can be very time-consuming and taxonomists are faced with a trade-off between detailed accounts on a small number of specimens and estimations of the intraspecific vs. interspecific variability of characters. Therefore, in cases with ambiguous morphological data—which is the rule rather than the exception in mesopsammic slugs—only integrative approaches that combine evidence from morphology and molecules represent a viable method for tackling their diversity (Neusser, Jörger *et al.* 2011, Jörger *et al.* 2012). Given the putative high degree of cryptic speciation in meiofaunal taxa with supposedly low dispersal abilities (*e.g.*, Westheide and Schmidt 2003, Casu *et al.* 2009, Fontaneto *et al.* 2009, Leasi and Todaro 2009, Andrade *et al.* 2011, Jörger *et al.* 2012, Tulchinsky *et al.* 2012), it currently seems most efficient to reverse the traditional taxonomic workflows and initiate species delineation in meiofauna with barcoding and molecular species delineation approaches and integrate morphoanatomical and other data (rather than initiating with morphoanatomical lines of evidence and integrate molecular data).

Molecular species identification and delineation

DNA barcoding and molecular species delineation have been broadly advocated as fast and efficient means for dealing with the taxonomic impediment in times of biodiversity crisis (Blaxter *et al.* 2004, Blaxter *et al.* 2005, Hebert and Gregory 2005, Markmann and Tautz 2005, Hajibabaei *et al.* 2007). DNA-barcoding in its similarity-based form, which uses genetic distances, is a tool of species (re-)identification and not species discovery (DeSalle *et al.* 2005, DeSalle 2006). Lacking a predictive component, DNA-barcoding fails when no identical sequences are deposited in public databases (like Barcode of Life Data System http://www.boldsystems.org/ or GenBank http://www.ncbi.nlm.nih.gov/genbank/). Ongoing efforts of the workgroup include depositing barcodes of all valid mesopsammic slugs to public databases to allow for identification via barcodes (current coverage approx. 60%, partially still unpublished). However, as discussed above the vast majority of marine meiofauna have yet to be explored (Curini-Galletti *et al.* 2012), not to mention identified and sequenced, identical matches of newly collected material with deposited sequences will be the exception for meiofaunal taxa for decades to come (Jörger *et al.* 2012). Meiofaunal biodiversity assessments, therefore, will not be focused in typical DNA barcoding identification approaches, but require advanced methods of molecular species discovery.

Most of the numerous emerging programs and algorithms that have recently become available for molecular species delineation either rely on the comparison of genetic distances or use phylogenetic trees to estimate support under different model assumptions. To cluster sequences based on genetic distances, programs either use fixed or relative thresholds between intraspecific and interspecific variation (*e.g.*, Hebert *et al.* 2004, Jones *et al.* 2011, Ratnasingham and Hebert 2013) or aim to detect breaks in patterns of distance distribution, *i.e.*, a 'barcoding gap' (Meyer and Paulay 2005, Puillandre, Lambert *et al.* 2012). Distance-based approaches are usually based on mitochondrial cytochrome *c* oxidase subunit I (COI); this standard barcoding marker presents unique species-specific diagnostics in approximately 95% of all tested species. Moreover, the interspecific differences clearly exceed the intraspecific variability in most of these cases (Hebert, Cywinska *et al.* 2003, Hebert, Ratnasigham *et al.* 2003, Ratnasingham and Hebert 2013). Despite high success rates in praxis, the use of thresholds as proxy of species delimitation has been criticized for their arbitrariness; and criticism has been underscored by empirical studies demonstrating the disappearance or absence of a 'barcoding gap' (*i.e.*, intraspecific exceeds interspecific variability) with increased sample size (Moritz and Cicero 2004, Meyer and Paulay 2005, Wiemers and Fiedler 2007, Astrin *et al.* 2012). Model-based approaches such as the General-Mixed-Yule-Coalescent (GMYC) model infer evolutionary entities by evaluating likelihood values under speciation vs. population genetic processes on phylogenetic trees (Pons *et al.* 2006, Monaghan *et al.* 2009). But the accuracy of this and other model-based approaches also relies on sampling coverage (Lohse 2009). A dense sampling coverage is usually utopic when it comes to elusive taxa, whose sampling records frequently include a high degree of singletons. The rarity of taxa in undersampled datasets hampers reliable estimation of intraspecific vs. interspecific variation, and constitutes the primary obstacle of successful molecular species delineation in elusive taxa. Currently, a Bayesian approach evaluating for differences among gene trees is potentially best capable of dealing with singletons, provided that data from several independent markers are combined (Yang and Rannala 2010, Zhang *et al.* 2011). In the absence of a 'gold standard' for evaluating the performance of different species delineation approaches, and in view of high degrees of rarity in putatively undersampled datasets, an integrative approach of species delineation is needed for elusive taxa, one which allows for thorough cross-validation between approaches.

Workflow of integrative species delineation

Herein, we present a workflow capable of dealing with the above-mentioned problems, which are likely symptomatic for most meiofaunal taxa and other little explored and rare taxa such as, *e.g.*, many deep-sea clades, or many invertebrates in general. Due to the putative high degree of cryptic speciation and intraspecific variability on morphological character sets, the workflow is founded on molecular data. Faced with incomplete datasets and rarity, however, it is designed to make best use of the taxonomic information scattered across different lines of evidence. The proposed workflow (see Fig. 4) is based on the approach described in Jörger *et al.* (2012).

Step 1: Optimize taxon sampling and character sampling

In concordance with previous species delineation workflows (*e.g.*, Puillandre, Modica *et al.* 2012), this approach emphasizes the importance of dense taxon sampling to ensure reliable species delineation and to avoid overestimating interspecific variability (Hebert *et al.* 2004). In elusive taxa, taxonomists frequently lack knowledge on biology, dispersal abilities and geographic distribution, which requires an even stronger emphasis on targeted taxon sampling with regard to both geography and phylogenetic relationships. This includes collecting and analyzing several individuals from populations covering the potential geographic range of a taxon. Hence this workflow requires an *a priori* survey of the described species of a lineage; and whenever possible, material of valid species derived from type material or specimens re-collected at type localities should be included.

Under the unified species concept, species are defined as independently evolving metapopulation lineages, with the former secondary species criteria of competing concepts serving as equal operational criteria, *i.e.*, lines of evidence (de Queiroz 2005b, 2007). As central operational criteria serve intrinsic reproductive isolation, monophyly, exclusive coalescence, diagnosibility and deficits of genetic intermediates; the reliability of proposed species hypotheses increases with the number of supporting lines of evidence (de Queiroz 2005b, 2007). Entities discovered in molecular species delineation approaches should, therefore, be supported by a minimum of one line of evidence. Consequently, the workflow requires the grouping of all characters sets which were evaluated and selected by the taxonomist as contributions to one of these operational criteria (*e.g.*, molecular data, morphological and anatomical characters with special emphasis on reproductive features, geographic distribution, behavioral data, ecological niches). Therein, the workflow strongly encourages to initially give equal priority to all putatively useful character sets.

Step 2: 'All-in' – plotting of data onto a molecular phylogeny

Studies in species delineation of elusive taxa such as mesopsammic slugs should aim to gather as much putatively relevant information from as many different sources as possible (*i.e.*, morphoanatomical, ecological, molecular, and so on). Despite all efforts to compile 'complete' data matrices, in reality some populations will provide exhaustive information, while others will be represented by singletons only. Furthermore, amplification success may vary among samples, species and individuals, as bulk fixation of sediment samples usually degrades DNA. And finally, immature specimens can prevent the exploration of reproductive features and body retraction or damages often occur. In typical broad-scale barcoding approaches for biodiversity assessments, amplification problems of the COI-barcoding marker can result in incomplete, ambiguous sequences that are unable to pass quality filters on automated pipelines on Barcode of Life Data systems (BOLD) (Ratnasingham and Hebert 2013), thus, causing a potentially 'correct' lineage to be irretrievably lost for further assessments. To ensure the inclusion of all available lineages in this species delineation approach in spite of missing data, the scattered information on individual lines of evidence must be compensated by the amount of (self-contained) lines of evidence. Therefore, and to account for problems of incompatibility between species and gene trees (caused mainly by incomplete lineage sorting, pseudogenes, or introgression (Bensasson *et al.* 2001, Funk and Omland 2003, Song *et al.* 2008), this approach is based on 'multi-barcoding', including independently evolving markers (ideally from mitochondria and nucleus) (Jörger *et al.* 2012). As this approach is based on phylogenetic theory via the criterion of monophyly, single gene trees are calculated from each individual marker. The risk of producing artificial topologies is minimized by using Bayesian and/or Maximum Likelihood algorithms, rather than rapid distance based methods. Each gene should be checked for reticulate evolution (*e.g.,* using Dendroscope 3 (Huson and Scornavacca 2012)). Then, a concatenated all-marker phylogeny serves as scaffold for plotting other sources of data (focusing on those that putatively serve as operational criteria).

There are two major advantages of this unfortunately time-consuming initial step: 1) The unvalued objective plotting of the available characters of all terminals (or at least populations, if there is no doubt on conspecificity) in the dataset without biased pre-selection is based either on initial (single-gene) molecular data or on taxonomic intuition relying on, *e.g.*, morphological criteria. A prefiltering of available data into morphotypes (Riedel, Sagata, Suhardjono *et al.* 2013) leaves potential cryptic species undiscovered and is, therefore, not advisable for meiofaunal taxa. 2) The potential for quality checks and cross-validation between different lines of evidence. This critical reassessment of the primary data can help to reveal problematic molecular markers or potential homoplasies on morphological characters.

Step 3: 'Wild cards' and selection of integrative taxonomic units (ITUs) via compatibility.

Based on the plotted data, integrative taxonomic units (ITUs) are defined: Integrative taxonomy is commonly considered best practice (Dayrat 2005, Will *et al.* 2005, Valdecasas *et al.* 2008, Padial and de la Riva 2010, Padial *et al.* 2010, Astrin *et al.* 2012, Riedel, Sagata, Suhardjono *et al.* 2013), but approaches differ considerably in how exactly they integrate their data, with far-reaching consequences for the resulting species hypothesis. Typical large-scale barcoding workflows cluster COI sequences into operational taxonomic units (OTUs) based on genetic distances via different algorithms, and they encourage the addition of accessory data from other molecular markers or *e.g.*, morphology (Jones *et al.* 2011, Puillandre, Modica *et al.* 2012, Ratnasingham and Hebert 2013). When more data is added, the species diagnosis becomes integrative, whereas species delineation, which has led to the discovery of the OTU, remains based on a single line of evidence and will not be questioned or critically revised by additional data.

When truly integrating data into the process of species delineation, there is debate on the degree of congruence that different characters need to provide (Padial *et al.* 2010). Integrating via congruence requires a minimum of two selected lines of evidence to support the proposed species hypothesis, while in 'integration via cumulation' approaches the divergence of any character can justify the designation of species (Padial *et al.* 2010). The former approach promotes taxonomic stability, but implies the risk of underestimating species numbers. Integrative taxonomy via cumulation, on the other hand, tends to overestimate species, but is thereby likely best

suited to discover recent lineages (Padial *et al.* 2010). Ideally, ITUs can be selected across the integrative scaffold established in this workflow via congruence across all lines of evidence, *i.e.*, reciprocal monophyly supported by morphological features and geographic and habitat boundaries. For small datasets, concordance between operational criteria can be evaluated by eye, but especially in larger datasets, the use of statistical methods for testing of support values as developed by Cardoso *et al.* (2009) is advisable. The herein presented workflow suggests a less stringent application of congruence, promoting integration via compatibility, *i.e.,* allowing for entities supported by some and uncontradicted by other lines of evidence (see the 'minimum consensus' approach in Jörger *et al.* (2012)). This accommodates the fact that the process of speciation does not necessarily implement changes on all different levels of characters (Padial and de la Riva 2010, Padial *et al.* 2010) and that methods of detection have different sensitivities, but the approach remains conservative in relying solely on uncontradicted support for a particular species hypothesis.

The effects of speciation patterns may, however, result in incongruent datasets (Padial and de la Riva 2010, Padial *et al.* 2010). Integrative taxonomy, thus, must not be misunderstood as simply adding more and more data, rather it urges cautious selection of the appropriate character set for the species under investigation (Valdecasas *et al.* 2008). Faced with incongruence, the debate on which character set is best suited to species delineation cannot be solved universally, but has to be decided in each individual case with regard to the specifics of each taxon.

At this point the proposed workflow offers a potential short-cut to species assignment: reciprocally monophyletic clades occurring in syntopy, *i.e.*, in the same biotope, can be considered species under the unifying species concept, combining the operational criteria of intrinsic reproductive isolation with reciprocal monophyly (de Queiroz 2005a, 2007). The evaluation of syntopy in meiofauna is problematic, however, due to the largely unknown ecological interactions and potentially small-scale microhabitats.

Although less conservative than integrative taxonomy via (strict) congruence, the compatibility approach will nevertheless tend to lump species. Because it relies on the criterion of reciprocal monophyly, this initial step is likely not suited for detecting recently evolved lineages (Knowles and Carstens 2007, Sauer and Hausdorf 2012). It, therefore, needs to be refined in Step 5 in order to uncover any potential lumping of species.

Step 5: Parallel application of available methods of molecular species delineation

As discussed above, the accuracy of all available algorithms of molecular species delineation suffers from undersampled datasets and the inclusion of singletons (Jörger *et al.* 2012) and also tends to oversplit datasets in empirical studies (*e.g.*, Sauer and Hausdorf 2012). In the absence of a 'gold-standard' for comparing the performance of each analysis on the respective dataset, our workflow suggests an unbiased parallel application of available molecular species delineation methods across all markers. Special emphasis should be given to model-based approaches such as GMYC (Pons *et al.* 2006, Monaghan *et al.* 2009)—provided that minimum requirements, *e.g.,* on sampling density, are fulfilled—and algorithms capable of dealing with rarity, such as Birky's (2013) simple coalescence theory based approach. The inference of genetic connectivity via haplotype networks applying statistical parsimony (Clement *et al.* 2000) is merely an indirect method of estimating species boundaries (Pons *et al.* 2006); nevertheless, it visualizes the genetic structure in the dataset, which is valuable for cross-validating molecular entities revealed by other approaches. Even though the performance of distance-based approaches is conceptually disputed and practically hindered in lineages that putatively suffer from incomplete sampling (Meyer and Paulay 2005, Hickerson *et al.* 2006, Meier *et al.* 2006, Wiemers and Fiedler 2007, Meier *et al.* 2008), the parallel application of Refined Single Linkage analysis (RESL) (Ratnasingham and Hebert 2013) or the Automatic Barcode Gap Discovery (ABGD) (Puillandre, Lambert *et al.* 2012) contributes to the empirical evaluation of the efficiency of standard barcoding approaches on elusive taxa. All the above methods are applied to single genes, and single gene histories may differ and, thus, results need to be compared and their significance evaluated. Using concatenated markers is not appropriate since information from single loci may be dominant and mask potential conflict. A powerful approach based on multilocus markers is the Bayesian Species delineation (Yang and Rannala 2010, Zhang *et al.* 2011); the more independent markers are available for combination, the better it can deal with rarity of samples.

Step 6: Congruence in molecular species delineation and compatibility of ITUs to determine candidate species

To exploit the potential of molecular species delineation methods for revealing prior lumping of species, integration via compatibility is inapplicable in this step, as it would directly transfer over-splitting of each individual method to the identification of the candidate species. The workflow aims for a cross-validation between the different approaches achieved by integrating the results via congruence. Only molecular entities supported by all molecular species delineation approaches are then further integrated via compatibility to the formerly identified ITUs, in order to lead to the final determination of the candidate species.

Step 7: Assessment of the taxonomic history

With regard to ecological studies or biodiversity assessments, an advantage of rare or elusive taxa compared to common or

hyperdiverse clades is the fact that the former usually lack an exhaustive history of available descriptions and putative synonyms, with the majority of lineages being still undescribed. To avoid the creation of synonyms, it is crucial to clarify whether the discovered candidate species already bears a valid name, *i.e.*, a thorough literature review on all potential names available. If several names are available, the oldest name has priority according to nomenclatural rules. If old descriptions do not permit unambiguous assignment, we recommend using the oldest name that can be reliably assigned to the newly delimitated species (Ornelas-Gatdula *et al.* 2012). Alternatives would be to resurrect unused or dubious older names, which is a rather arbitrary procedure, or to deliberately establish and name a "new" species despite the existence of already available names, thus, creating a junior synonym.

Step 8: Species description

Independently evolving lineages discovered as candidate species, but which cannot be assigned to valid species, should be described to receive formal recognition. Molecular species delineation approaches frequently terminate their efforts with the discovery of independently evolving lineages (*e.g.*, Fontaneto *et al.* 2009, Monaghan *et al.* 2009, Astrin *et al.* 2012). The BIN system (Barcode Index Numbering on BOLD) even propagates the use of OTUs as an alternative taxonomic reference system (Ratnasingham and Hebert 2013), drawing on initial proposals on DNA taxonomy (Tautz *et al.* 2003). The amount of deposited sequences, which are unidentified at the species level and bear other unique identifiers, has grown tremendously in the course of the barcoding endeavor over the past few years (see http://iphylo.blogspot.de/2011/04/dark-taxa-genbank-in-post-taxonomic.html). Clustering sequences into OTUs may be sufficient for further applications such as biodiversity assessments, while unidentified sequences can still contribute valuable information in the absence of species assignment. The name of a species is entirely extrinsic, it could, thus, be replaced by any alternative identifier such as a BIN. However, the use of OTUs not merely as a source of taxonomic characters, but also as a taxonomic reference system, can be problematic when it comes to establishing novel unique identifiers, as these identifiers create parallel taxonomies flagged with new acronyms and classification systems in competition with traditional taxonomy (Jörger and Schrödl 2013). The Linnaean name anchors the species to its taxonomic history and all available biological and morphological data (Polaszek *et al.* 2008, Patterson *et al.* 2010). Moreover, the genus name includes a hypothesis on phylogenetic relationships. A species name can be linked to life science identifiers via ZooBank (http://zoobank.org/), capable of uniquely linking content on this species through different computational platforms (Polaszek *et al.* 2008). In order to reduce and not enhance impediments in taxonomy by parallel yet inconsistent identifiers, discovered lineages should be connected to the taxonomic history of a clade by providing formal descriptions (Jörger and Schrödl 2013). Depending on the chosen operational criteria of species delimitation, this set of characters will form the basis for the diagnoses of species. Jörger and Schrödl (2013) illustrated how molecular diagnostic characters can be extracted via character-based barcoding approaches (Sarkar *et al.* 2008, Bergmann *et al.* 2009) and used for taxonomic description. Future efforts should aim to automate the extraction of diagnostic molecular characters to facilitate and accelerate species description, as has already been achieved in 'turbo-taxonomic' approaches for morphological data (Butcher *et al.* 2012, Riedel, Sagata, Suhardjono *et al.* 2013, Riedel, Sagata, Surbakti *et al.* 2013). However, the fair option to diagnose species entirely based on molecular characters, morphological data should nevertheless be included in the species description (Jörger and Schrödl 2013).

Step 9: Ensure accessibility of all data

Digital technologies provide powerful methods for making taxonomic data more accessible to the research communities via, *e.g.*, virtual access to museum collections, digitalized biodiversity libraries, online registration systems for zoological names and infrastructure for biogeographic assessments (*e.g.*, Wheeler 2008, Padial and de la Riva 2010, Padial *et al.* 2010). Next to the obligatory voucher deposition in museum collections (including vouchers of extracted DNA) and of genetic sequences in public databases, data from species descriptions can now be deposited in online platforms (*e.g.*, the Encyclopedia of Life http://eol.org/). This increases the accessibility of taxonomic knowledge and allows for dynamic expansion of species diagnoses through future studies (Riedel, Sagata, Suhardjono *et al.* 2013), ideal for gradually augmenting knowledge on enigmatic taxa.

CONCLUSIONS AND OUTLOOK

The marine mesopsammon is one of the largest, yet least explored habitats on Earth, and the taxonomic deficit is correspondingly high. To date, the mesopsammic fauna has been largely neglected in conservation approaches, despite their doubtless important role, *e.g.*, in the marine food web. The comparably low reproductive output and the poor dispersal abilities of mesopsammic slugs indicate small ranges of distribution and high degrees of endemism. The threat to their diversity by habitat destruction is consequently high. By providing practical sampling instructions on how to explore the mesopsammic malacofauna in the field, we aim to encourage the inclusion of this fauna into biodiversity assessments. A boost in sampling efforts world-wide—but with special emphasis to the numerous unsampled tropical regions—is urgently needed to get reliable estimations on the diversity of

these enigmatic taxa still hidden in global sands. Currently, the micromorphological diversity of known microslugs is comparatively investigated and the inclusion of minute mesopsammic sea slugs into multi-locus analyses on Heterobranchia has demonstrated how theses enigmas can help to understand the phylogenetic relationships and evolution of larger clades. We now need to fill in the gaps in existing taxon samplings with remaining elusive taxa (*e.g.*, Pseudovermidae) in order to supplement the complex picture of heterobranch evolution step by step and to understand the evolutionary pathways which led into the mesopsammon.

We emphasize that next to the initial exploration in the field, there is also a theoretical debate needed on how to efficiently and reliably delineate meiofaunal species—a task which usually presents a struggle with rarity and uniformity. Despite all valuable advances in accelerating the rates of taxonomic descriptions to face the 'taxonomic impediment' in times of biodiversity crisis, here we promote a form of 'deep taxonomy' in cases where the evolutionary history of species requires a thorough integrative workflow. This will ensure that these small clades do not slip through automated 'turbo-taxonomy' pipelines.

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Appendix 1: Type localities of marine, interstitial microslugs. * marks species in which it is unclear if they inhabit the mesopsammon. *Rhodope* and *Embletonia* might be epibenthic and *Smeagol* inhabits cobble beaches. + marks type localities in which the original description provides GPS-coordinates.

Taxon	Type locality	Original description
RHODOPEMORPHA		
Rhodopidae		
Rhodope crucispiculata	'Tunisia'. Mediterranean	Salvini-Plawen 1991b
*Rhodope marcusi**	Bay of Santos, Ilha das Palmas, São Paulo, Brazil. Atlantic Ocean	Salvini-Plawen 1991b
Rhodope roskoi	Roscoff, Bretagne, France. English Channel (+)	Haszprunar and Heß 2005
Rhodope rousei	Edithburgh Jetty, South Australia. Gulf St. Vincent	Brenzinger, Wilson *et al.* 2011
*Rhodope transtrosa**	unknown (? tropical Indo-Pacific)	Salvini-Plawen 1991b
*Rhodope veranii**	Messina, Sicily, Italy. Mediterranean	Kölliker 1847
Helminthope psammobionta	North rock reef/ Tobacco Bay, Bermuda. Atlantic Ocean	Salvini-Plawen 1991b
NUDIBRANCHIA		
Pseudovermidae		
Pseudovermis artabrensis	Ria de Ferrol, Galicia, Spain. Bay of Biskaya	Urgorri *et al.* 1991
Pseudovermis axi	Banyuls-sur-Mer, France. Mediterranean	Marcus and Marcus 1955
Pseudovermis boadeni	Anglesey, Great Britain. Irish Sea	Salvini-Plawen and Sterrer 1968
Pseudovermis chinensis	Kung Chau, N.W. tip, Hong Kong, Round Island, South Gau. South China Sea	Hughes 1991
Pseudovermis hancocki	Urupukapuka Island, Bay of Islands, New Zealand. Pacific Ocean	Challis 1969b
Pseudovermis indicus	Walthair Coast, Laccadive Archipelago, Andaman Islands, India. Bay of Bengal	Salvini-Plawen and Rao 1973
Pseudovermis japonicus	Seto Marine Laboratory, Kii, Japan. Pacific Ocean	Hamatani and Nunomura 1973
Pseudovermis kowalevskyi	Mytilini, Greece. Mediterranean	Salvini-Plawen and Sterrer 1968
Pseudovermis mortoni	Maraunibina Island, Solomon Islands. Solomon Sea	Challis 1969b
Pseudovermis papillifer	Mytilini, Greece. Mediterranean	Kowalevsky 1901a
Pseudovermis paradoxus	Cape Fiolent, Sebastopol, Krim, Ukraine. Black Sea	Kowalevsky 1901a
Pseudovermis salamandrops	São Sebastião, Brazil. Atlantic Ocean	Marcus 1953
Pseudovermis schulzi	Arcachon, France. Bay of Biskaya	Marcus and Marcus 1955
Pseudovermis setensis	Cote Languedocienne, France. Gulf of Lion	Fize 1961
Pseudovermis soleatus	Sound Island, North Andaman, India. Bay of Bengal	Salvini-Plawen and Rao 1973
Pseudovermis thompsoni	Punta Croce, Rovinj, Croatia. Mediterranean.	Salvini-Plawen 1991a
Embletoniidae		
Embletonia cf. *pulchra**	Rothesay Bay, Isle of Bute, Scotland, United Kingdom. North Atlantic	Alder and Hancock 1844
CEPHALASPIDEA		
Philinidae		
Philine exigua	Komimbo Bay, West Guadalcanal, Solomon Islands. Central Indo-West Pacific	Challis 1969
Philinoglossidae		
Philinoglossa helgolandica	Wittekliff, Heligoland, Germany. Northern Sea	Hertling 1932
Philinoglossa marcusi	Komimbo Bay, West Guadalcanal, Solomon Islands. Central Indo-West Pacific	Challis 1969a
Philinoglossa praelongata	Secche de la Meloria, off Livorno, Italy. Mediterranean.	Salvini-Plawen 1973
Philinoglossa remanei	Naples, Italy, Mediterranean.	Marcus 1954
Abavopsis latosoleata	Secche de la Meloria, off Livorno, Italy. Mediterranean.	Salvini-Plawen 1973
Sapha amicorum	Abomingar, Hurghada, Egypt. Red Sea	Marcus 1959
Pluscula cuica	Ilhabela, São Sebastião, Brazil. Atlantic Ocean	Marcus 1953a
SACOGLOSSA		
Platyhedylidae		
Platyhedyle denudata	Secche de la Meloria, off Livorno, Italy. Mediterranean.	Salvini-Plawen 1973

Appendix 1. (Continued)

Taxon	Type locality	Original description
ACOCHLIDIA		
Hedylopsidae		
Hedylopsis spiculifera	Prince Islands, Turkey, Sea of Marmara	Kowalevsky 1901b
Hedylopsis ballantinei	Dahab, Sinai, Egypt. Red Sea	Sommerfeldt and Schrödl 2005
Pseudunelidae		
Pseudunela eirene	Nicobar Islands, India. Andaman Sea	Wawra 1988
Pseudunela cornuta	Maraunibina Island, Solomon Islands. Central Indo-West Pacific	Challis 1970
Pseudunela viatoris	Laucala Bay, Nukumbutho Island, Viti Levu, Fiji. South Pacific	Neusser, Jörger *et al.* 2011
Pseudunela marteli	Honiara, Guadalcanal, Solomon Islands. Central Indo-West Pacific	Neusser, Jörger *et al.* 2011
Asperspinidae		
Asperspina brambelli	Menai Strait, Anglesey, Wales, United Kingdom. Irish Sea	Swedmark 1968
Asperspina riseri	Crowe Neck, North Trescott, Maine, USA. Bay of Fundy, North-West Atlantic	Morse 1976
Asperspina loricata	Trezen ar Skoden, near Roscoff, France. English Channel	Swedmark 1968
Asperspina murmanica	Dalniye Zelentsy, Murmanskaya obl., Russia. Barents Sea	Kudinskaya and Minichev 1978
Asperspina rhopalotecta	Secche della Meloria, Livorno, Italy. Mediterranean Sea	Salvini-Plawen 1973
Microhedylidae s.l.		
Microhedyle glandulifera	Prince Islands, Turkey. Sea of Marmara	Kowalevsky 1901b
Microhedyle remanei	off Castle Roads, Bermuda. Atlantic Ocean	Marcus E. 1953
Parhedyle cryptophthalma	Nabeul, Tunisia. Mediterranean Sea	Westheide and Wawra 1974
Parhedyle odhneri	Canet-Plage, Languedoc-Roussillon, France. Mediterranean Sea	Marcus and Marcus 1955
Parhedyle gerlachi	Addu Atoll, Gan Channel, Maldives. Indian Ocean	Marcus and Marcus 1959
Parhedyle tyrtowii	Cape Fiolent, Sebastopol, Krim, Ukraine. Black Sea	Kowalevsky 1901b
Parhedyle nahantensis	Canoe Beach, Nahant, Massachusetts, USA. Atlantic Ocean	Doe 1974
Pontohedyle milaschewitchii	Chersones, Cape Fiolent, Sebastopol, Krim, Ukraine. Black Sea	Kowalevsky 1901b
Pontohedyle brasilensis	Vila, Ilhabela, Brazil. Western Atlantic	Rankin 1979
Pontohedyle verrucosa	Maraunibina Island, Solomon Islands. Central Indo-West Pacific	Challis 1970
Pontohedyle kepii	Pulau Moyo, Nusa Tenggara, Indonesia, Flores Sea (+)	Jörger and Schrödl 2013
Pontohedyle joni	Bay of 'Windjammer Landing', St. Lucia, Caribbean Sea (+)	Jörger and Schrödl 2013
Pontohedyle neridae	Motu Iti, Moorea, French Polynesia, Central Pacific (+)	Jörger and Schrödl 2013
Pontohedyle liliae	Sha'ab Malahi, Egypt. Red Sea (+)	Jörger and Schrödl 2013
Pontohedyle wiggi	Raja Island, Phuket, Thailand. Andaman Sea (+)	Jörger and Schrödl 2013
Pontohedyle wenzli	Lembeh Strait, Sulawesi, Indonesia. Celebes Sea (+)	Jörger and Schrödl 2013
Pontohedyle peteryalli	Mia Mia, Ghana. Atlantic Ocean (+)	Jörger and Schrödl 2013
Pontohedyle martynovi	Cook's Bay Pass, Moorea, French Polynesia. Central Pacific (+)	Jörger and Schrödl 2013
Pontohedyle yurihookeri	Punta Sal, Tumbes, Peru. Pacific Ocean (+)	Jörger and Schrödl 2013
Ganitus evelinae	Vila, Ilhabela, Brazil, Western Atlantic	Marcus E. 1953
Paraganitus ellynnae	Komimbo Bay, Tambea, Guadalcanal, Solomons. South Pacific	Challis 1968
OTINOIDEA		
Smeagolidae		
*Smeagol manneringi**	Rhino Horns Point, Kaikoura Peninsula, South Island, New Zealand. Tasman Sea (+)	Climo 1980
*Smeagol climoi**	East of Houghton Bay, Wellington, South Island, New Zealand. Tasman Sea (+)	Tillier and Ponder 1992
*Smeagol hilaris**	Merry Beach, south of Ulladulla, New South Wales, Australia. Tasman Sea (+)	Tillier and Ponder 1992
*Smeagol phillipensis**	Sunderland Bay, Phillip Island, Victoria, Australia. Bass Strait (+)	Tillier and Ponder 1992
*Smeagol parvulus**	Kitty Miller Bay, Phillip Island, Victoria, Australia, Bass Strait (+)	Tillier and Ponder 1992

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Appendix 2: Step-by-step procedure to take samples of mesopsammic heterobranchs, and to extract and document slugs and snails from sands.

Selecting localities and substrates

- try all available places and habitats (you never know)
- prefer coarse, subtidal sand from places with continuous currents, and/or from the lower intertidal at places with modest wave action
- take just the surface layer (subtidal: a few centimeters, intertidal: slugs may inhabit greater depth; but never dig into dark anoxic layers)

Accumulation phase

- put the sample in a bucket/box, with sea water just covering (parts of) the sand surface (!); top layers remain oxygenated just by diffusion
- put the bucket in a cool place and let it rest for at least 1 day; depending on grain sizes, specimens will move to the oxygenated surface and accumulate just below the sand surface. Clean, coarse coral sands work better than finer sediments (specimens need more time to migrate); especially if there is much organic matter, deeper layers will become anoxic quickly: never disturb or mix layers. Oxic surface layers may contain living specimens even after weeks (if the sand stays humid)

Qualitative extraction: anesthetization-decantation technique modified after Pfannkuche and Thiel (1988)

- take some sand (a few spoons) from the surface layer
- put the sand in a 1 l beaker (or in a half plastic bottle)
- add at least the fivefold volume of an isotonic $MgCl_2$ solution (which should be mixed 1:1 with seawater to avoid stress)
- carefully mix sediment and fluid by rotating the jar
- wait a few minutes for anesthetization (depending on grain size; penetration of fine sediments may take up to 10 min)
- shake the beaker gently to suspend particles (*i.e.*, soft-bodied fauna), then wait an instant letting the heaviest particles settle
- quickly decant the liquid through a 63–100 µm sieve (try to avoid to pour sand)
- flush the filtrate of the sieve into a petri-dish using seawater
- repeat the suspending procedure more and more forcefully (collecting the different fractions in separate dishes), until abundance of extracted organic substance/specimens gets notably less. A final forceful shaking may be the only means to remove specimens glued to sand (but risking mechanical damage)

Analysis

- wait 5 min for anesthetized animals to become active again
- look for snails and slugs under a dissecting microscope; slugs may be contracted to "balls" and not identifiable as such before actively moving
- pay attention to all slowly (!) gliding creatures that 1) do not swim (if touched) or glide backwards, 2) do not have chaetae, 3) do not invert or evert large parts of the body
- most gastropod species either have a head with tentacles, a ventral foot, and/or a dorsal shell; specimens with shell can usually be well recognized as snails
- shell-less heterobranchs usually show at least one of the following features: a clearly defined foot, one or two pairs of cephalic tentacles, a pair of eyes, one pair of statocysts containing one statolith each a visceral sac separated from the head-foot complex, calcareous spicules.
- transfer specimens to small containers such as block dishes or similar for further examination. When using pipettes, quick transfer of specimens is suggested to prevent them from attaching to the inside of the pipette. Specimens sticking to the pipette can be elegantly removed using $MgCl_2$ solution rather than by force. Containers used for photography of specimens should be clean (*e.g.,* without detritus that adheres to the specimens); filtered seawater and/or $MgCl_2$ solution is recommended.
- taking photographs and high-resolution videos focusing up and down of living animals is essential for identifying and later description of valuable taxonomic characters! Note the presence of calcareous spicules (since they may easily dissolve in any preservation fluids). If possible, use transmission light microscopy for analysis and documentation of inner structures. Most mesopsammic slugs are small and transparent enough to see many relevant structures even without squeezing (and thereby killing) the specimen under study. The specimen is placed between slide and cover slip with sea water and blotting paper; water should be removed until the animal is paralyzed without being squashed. Thus, the animal and its internal structures can be observed and photographed in vivo, preferably under optical microscope with DIC.
- all specimens, except for those to be fixed and used for molecular analysis, must be relaxed before fixation by very slow and careful (avoid disturbance by drops!) addition of $MgCl_2$ solution until their bodies no more react to water movement, by *e.g.,* slightly to abruptly shaking the dish, or direct contact, *e.g.*, using a fine brush. If specimens contract, reanimate them in seawater and start relaxation procedure again. In our experience any premature addition of fixatives will cause irreversible retraction (in acochlidians) or contraction of specimens. Slow addition of fixatives may be beneficial.

Fixation

- after sorting into morphospecies and preidentification please think wisely about how to fix material: if only single specimens are available you may want to fix them in 70–80 % ethanol (as a compromise to allow for DNA, anatomy, radula and spicule preparation and histology). If several specimens per species are available better split into subsamples and apply special fixations. These may include, but of course are not limited to, the following suggestions.
- all specimens for molecular analysis (after photographic documentation!) should be fixed immediately in > 95 % ethanol; after fixation shake jars with samples several times during the first hour and after a day (avoids dilution artefacts); keep samples cool; do not store or send ethanol samples together with aldehyde-fixed samples (vapors penetrate plastic jars, lids and bags, especially at high temperatures). According to our experience, DNA of tiny specimens degrades quickly to a level which is problematic for routine work such as barcoding. So make sure to keep samples cold and extract DNA within a year at most.
- additionally, specimens for molecular analyses may be fixed in RNAlater® minimizing the need to immediately process or freeze samples. Store samples in RNAlater® at 4 °C overnight (to allow the solution to penetrate the tissue properly), for indefinite long-term storage keep the samples at -20 °C or -80 °C. Beware of the low pH of RNAlater® which will lead calcareous structures to dissolve. A detailed protocol for whole mount *in situ* hybridization is given *e.g.*, in Giusti *et al.* (2000) (on abalone larvae, not yet tested on mesopsammic slugs)
- specimens intended for examining hard parts (radula or potential spicules) should be fixed in ethanol rather than formalin; the latter, even if buffered, may dissolve or alter delicate spicules quickly. Remember that absence of (certain types of) spicules in preserved specimens may always be artificial.
- specimens for histology or ultrastructural research should be fixed in 4 % glutardialdehyde buffered in cacodylate buffer (*e.g.*, Neusser *et al.* 2006). If no ultrastructural research is intended, formalin (4 %) in seawater, later exchanged with 80 % ethanol, will do also.
- specimens prepared for immunocytochemistry must be fixed in freshly prepared 4 % paraformaldehyde solution (*e.g.,* Electron Microscopy Sciences, Hatfield, PA, U.S.A., www.emsdiasum.com, Catalog N° 157-4) for approx. 2 hours and then transferred to buffer solution. Storage at 4 °C is limited to a few weeks.

Suggestion: interesting, unexplored beaches or coastal sandy bottoms are everywhere, but on suitable microscopic methods may not be available for watching and sorting meiofaunal gastropods on all trips. It is very easy and convenient to take/send subsamples to a (home) lab: After some phase of accumulation, take the surface layer sand, fill the wet sand into closable jars (without adding any additional water!). Make sure to remove excess water, the sample should be a moist but air-filled clump of sand sticking together; this is the crucial step avoiding deeper layers getting anoxic and permanent mixing of sediments by water currents. Allow a big body of air (or oxygen; half the jar volume at least, depending on temperatures and organic contents in the sediment). Avoid exposure to sun or heat. We got healthy wet sand samples that traveled around half the planet for 3 days (but make sure to cope with collecting and export legislation)!

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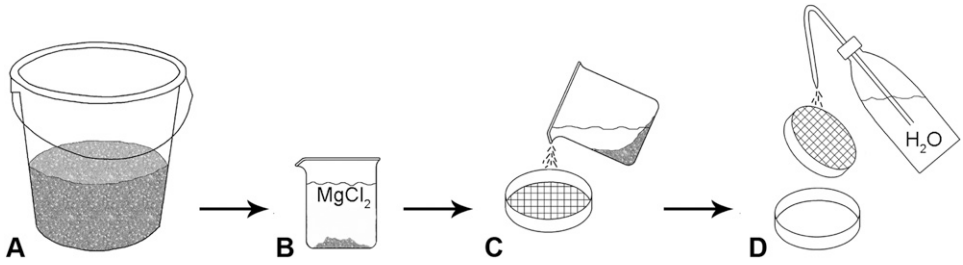

Figure to Appendix 2. Method for collecting interstitial molluscs. A: Collect sand sample with few seawater, let the sand rest in the shade for 1–2 days. B: Take few spoons of sand, add isotonic $MgCl_2$ solution, mix gently and wait at least 10 minutes. C: Shake gently to suspend soft-bodied meiofauna, decant quickly through a 63–100 µm sieve. D: Flip the sieve over, flush content into dish with seawater. Finally look for organisms under a binocular microscope. Modified after Neusser (2011) in Bouchet *et al.* (2011). © Publications Scientifiques du Muséum national d'Histoire naturelles, Paris.

Appendix 3: Key for the identification of mesopsammic slugs (to family and genus level) in the field (see Fig. S1 in the main document for photographs of living specimens)

Specimen found in marine sand sample (presumably belonging to meiofauna), not exceeding a few mm in body length, lack of shell in which the body can be at least partially retracted (internal shell or minute external shell might be present).....**try this key!**
Not found in marine sand sample and/ or larger than a few mm and/ or with external shell in which the body can be at least partially retracted.....**please find another key!**
I. Body dorso-ventrally flattened, notum undivided, notum overhangs the subpallial groove; small gill at posterior right end. A minute reduced shell may be present at the posterior end of the notum (externally or internally). Four gizzard plates in middle of body. Foot usually broad and longer than notum, no parapodia. Body brown, or speckled orange, red, or green.....**Runcinacea**
II. Body cylindrical or dorsally flattened, divided into a large cephalic shield and a visceral hump with posterior lappets (body shield). Mantle cavity with opposing ciliated strips. Shell lacking or fragile, external or at least partially covered by mantle. Shell ovate to elongate in shape, of very few whorls and with greatly dilated aperture, often with striated sculpture. Foot not longer than body, parapodia may cover sides of head and body shields. Three gizzard plates (one may be smaller) in middle of body. Body white, or with red to orange speckles (Fig. 1E).....**Cephalaspidea: Philinidae:** *Philine*
III. Body more or less rectangular shaped (three to six times longer than wide). Posterior end of dorsum extending past tip of foot. Tail end rounded/spoon-shaped or horizontal, fin-like. Foot and dorsum separated by lateral grooves at least along the anterior part of the body, parapodia reduced, limited to anterior head, radula 3-0-3, no gizzard plates (Fig. 1F).....**Cephalaspidea: Philinoglossidae**

- with small internal shell (may not be visible) in tail end, lateral groove between foot and notum extending along the entire length of the animal. Body white. Digestive gland yellow/orange, terminates in middle of body. No eyes.....*Pluscula*
- without internal shell, notum without separation between cephalic shield and visceral hump. Body dirty white to brownish, sometimes with black specks. Digestive gland almost reaches tail end.....*Philinoglossa/ Sapha*
- without internal shell, cephalic shield indicated by lateral cuts, narrow parapodia present. Body white .....*Abavopsis*

IV. Typical aeolid body slightly tapered towards the ends, head roundish with flattened lateral lobes and one pair of rhinophores, body with several elongated cerata (Fig. 1D).....**Nudibranchia: Embletoniidae:** *Embletonia*
V. Body vermiform, slightly tapered towards the end, lacking head appendages, often with some cerata. Head acorn-shaped, with subterminal mouth (Fig. 1C).....**Nudibranchia: Pseudovermidae:** *Pseudovermis*
VI. Body vermiform, not tapered towards the end, thickest at anterior third of body, round in cross-section all over. No appendages, distinct foot, mantle cavity, radula, or shell, or external grooves of any kind. Head rounded, slightly retractable. Tail end narrower and slightly flattened, corners of tail end with adhesive glands. Eyes and statocysts at anterior third of body. Dense layer of refracting spicules below epidermis.....**Rhodopemorpha**

- short and stout, resembling some meiofaunal flatworms (approx. length/width ratio 3–9, contracted vs. crawling). Spicules boomerang-shaped, with or without median notch, sorted at 45° to longitudinal body axis. Body whitish, or with distinct orange/ reddish bands around middle of body and dorsum, and/or purple color pattern at front end (Fig. 1A).....*Rhodope*
- elongated, resembling some meiofaunal nemerteans thin like a spaghetti (approx. length/width ratio 8–25, contracted vs. crawling). Spicules either boomerang-shaped, or in the form of crosses. Slow movement, tail end often slightly coiled. Coloration whitish, translucent (Fig. 1B).....*Helminthope*

VII. Body separated into an anterior head-foot complex and an elongated posterior visceral hump

- Head rounded, lacking any head appendages, broad head-foot, short, rounded free end of the foot, curls up in case of disturbance (Fig. 1G).....**Sacoglossa: Platyhedylidae:** *Platyhedyle*
- Head with one or two head appendages, in case of disturbance head-foot complex can (at least partially) be retracted into visceral hump (Fig. 1H, I).....**Acochlidia**

Key to the genera of Acochlidia
a) Head with one pair of appendages (oral/ labial tentacles).....**1.**
b) Head with two pairs of appendages (oral/ labial tentacles and rhinophores).....**2.**

1.

a) Head with bow-shaped, flattened oral tentacles tapered towards the ends, radula 1-1-1, with monaxone spicules (see Fig. 1 in Appendix 3 on different spicule types), digestive gland frequently with green color, very short free foot end (tail).....*Pontohedyle*

b) Head with flattened, broad oral tentacles not tapered towards the ends, short foot/tail, tip of foot pointed, dagger-shaped radula 0-1-0 (see Fig. 2 in Appendix 3 for different radulae types), bean-shaped (Fig. 1C Appendix 3) and/or very small "pearl chain spicules" (see Fig. 1D Appendix), digestive gland frequently orange colored.....*Ganitus*

2.

a) Head with flattened broad oral tentacles, clearly larger than finger-like rhinophores, fusiform spicules especially concentrated in visceral hump forming a secondary "spicule shell".....*Hedylopsis*

b) Oral tentacles not flattened.....**3.**

3.

a) Oral tentacles and rhinophores short finger-like, round in diameter more or less of equal length and quite immobile, visceral hump with densely arranged large, monaxone spicules forming a secondary "spicule shell".....*Asperspina*

b) Head appendages rather thin and slender, spicules (if present) only randomly distributed in visceral hump and head-foot complex, not forming a secondary "spicule shell" .....**4.**

4.

a) Oral tentacles slightly thicker and longer than rhinophores, both tapering towards the ends and rounded in sections, radula 1-1-1 (Fig. 2 Appendix 3), spicules lacking or scattered fusiform ones. Hermaphrodites, large copulatory organ with stylet(s) located behind pharynx.....*Pseudunela*

b) Head appendages similar to a) but species with separate sexes, lack of copulatory organs and sperm transfer occurs via spermatophores applied to the skin.....**5.**

5.

a) Oral tentacles slightly curved, thicker and longer than rhinophores; radula 1-1-1; variety of different spicules: monaxone, triaxial (Fig. 1A Appendix 3), "pearl-chain" or lacking.....*Microhedyle*

b) General body shape and head appendages like in *Microhedyle* (but more elongate), but with dagger-shaped radular teeth 0-1-0 (Fig. 2B Appendix 3), bean-shaped or thick, curved spicules (Fig. 1C Appendix 3).....*Paraganitus*

c) General body shape and head appendages similar to *Microhedyle* (but frequently smaller) comparably thin and slender oral tentacles and rhinophores, oral tentacles cylindrical, only very small "pearl-chain spicules" (Fig. 1D Appendix 3), radula formula 1-1-2 (Fig. 2D Appendix 3) with the small inner lateral tooth difficult to detect using light microscopy.....*Parhedyle*

Figures to Appendix 3

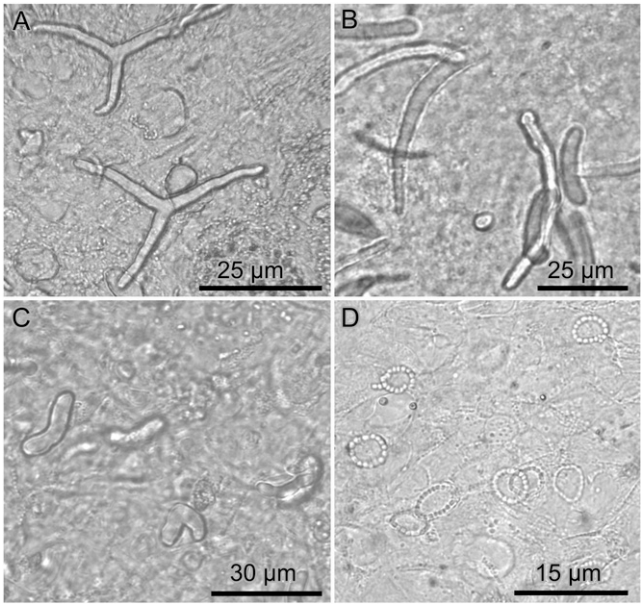

Figure 1. Different types of spicules in Acochlidia. **A,** triaxial spicules; **B,** monaxone spicules; **C,** bean-shaped spicules; **D,** 'pearl chain' spicules.

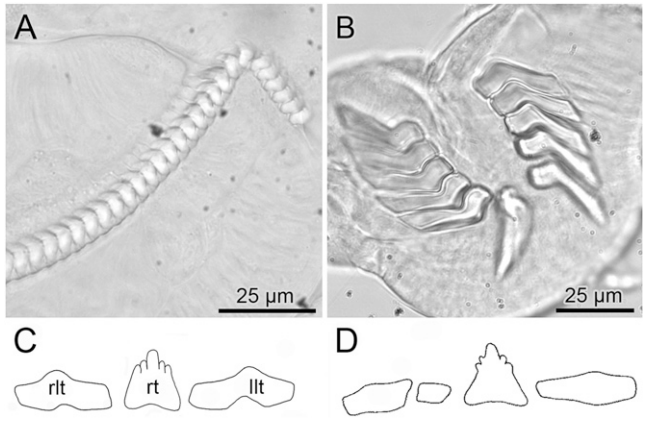

Figure 2. Different types of radulae in Acochlidia. **A,** Typical J-shaped acochlidian radula, with denticulate teeth; **B,** Dagger-shaped teeth with smooth margins and short radula of Ganitidae; **C,** Acochlidian radula with the formula 1-1-1; **D,** Radula of *Parhedyle* 1-1-2. Abbreviations: **llt,** left lateral tooth; **rlt,** right lateral tooth; **rt,** rhachidian tooth.

Chapter 2. **Brenzinger B**, Neusser TP, Jörger KM & Schrödl M (2011a) **Integrating 3D-microanatomy and molecules: natural history of the Pacific acochlidian freshwater slug *Strubellia* Odhner, 1937, with description of a new species.** *Journal of Molluscan Studies*, **77:** 351-374.

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INTEGRATING 3D MICROANATOMY AND MOLECULES: NATURAL HISTORY OF THE PACIFIC FRESHWATER SLUG *STRUBELLIA* ODHNER, 1937 (HETEROBRANCHIA: ACOCHLIDIA), WITH DESCRIPTION OF A NEW SPECIES

BASTIAN BRENZINGER, TIMEA P. NEUSSER, KATHARINA M. JÖRGER AND MICHAEL SCHRÖDL

Bavarian State Collection of Zoology, Münchhausenstr. 21, D-81247 Munich, Germany

Correspondence: B. Brenzinger; e-mail: bastian.brenzinger@arcor.de

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ABSTRACT

Forming a small group of mainly marine meiofaunal slugs, the Acochlidia have recently been separated from the traditional opisthobranch gastropods and placed within a mixed clade of pulmonates, Sacoglossa and Pyramidelloidea on the basis of molecular data. In the light of this new phylogenetic framework, we examined several populations of a comparatively giant *Strubellia* (Acochlidiidae *s. l.*) found in rivers of the Solomon Islands and Vanuatu, combining microanatomical and molecular methods (interactive three-dimensional models are given in the online version). Novel features include an extended set of nerves, a 'cephalic gland' of unknown function and an osphradium, all detected here for the first time in Acochlidia. The protandric genital system is characterized by three receptacles in the male phase, a possibly secondary open seminal groove and a complete reduction of the elaborate cephalic copulatory apparatus during ontogeny. Combined evidence from copulatory features and DNA sequences indicate a specific separation between the type species *S. paradoxa* (Strubell, 1892) from Ambon and the eastern Melanesian *Strubellia wawrai* n. sp. Live observations show the species to feed on the highly mineralized egg capsules of limnic Neritidae using a special piercing radula. Limnic Pacific acochlidians are suggested to be amphidromic, as are their prey organisms. A unique type of adhesive larva, observed in an *Acochlidium* species, indicates a possible dispersive stage in Acochlidiidae. Molecular phylogeny confirms the morphology-based placement of *Strubellia* as sister taxon to other Acochlidiidae.

INTRODUCTION

The Acochlidia consist of about 30 described species of heterobranch slugs that are characterized by a rather uniform external morphology, showing a freely projecting and uncurled visceral sac (giving the order its name) and one or two pairs of head appendages. For long time considered as one of the classic orders of the 'Opisthobranchia', morphological studies have repeatedly failed to place the taxon conclusively (e.g. Dayrat & Tillier, 2002; Wägele & Klussmann-Kolb, 2005) and molecular studies of Heterobranchia have cast further doubt on this classification (Klussmann-Kolb *et al.*, 2008). The most recent molecular studies with a direct focus on the group have consistently retrieved Acochlidia in a new monophylum comprising the Sacoglossa, Pyramidelloidea and the 'pulmonate' groups (all together called Panpulmonata), with acochlidians (including the recently described Aitengidae; Swennen & Buatip, 2009; Neusser *et al.*, 2011a) as sister group to Eupulmonata (Jörger *et al.*, 2010a). However, morphological synapomorphies of the panpulmonate group have not yet been identified.

Most acochlidian species are tiny inhabitants of worldwide marine interstitial sand habitats (Arnaud, Poizat & Salvini-Plawen, 1986). Internal phylogenetic relationships derived from morphology indicate a basal split into the completely meiofaunal Microhedylacea and partially meiofaunal Hedylopsacea, a relationship that has been confirmed by recent molecular approaches (Wawra, 1987; Jörger *et al.*, 2010a; Schrödl & Neusser, 2010). The hedylopsaceans also contain—uniquely among shell-less Gastropoda—two independent lineages that have colonized freshwater streams of tropical volcanic islands: the minute Caribbean *Tantulum elegans*

Table 1. Collection localities of *Strubellia wawrai* n. sp. on Guadalcanal, Solomon Islands (1–4) and Espiritu Santo, Vanuatu (5–8).

Number	Locality	Coordinates
1	Mataniko River, near Tavaruhu (3 km upstream)	S 9°27.377′, E 159°57.447′
2	Mataniko River, near Tavaruhu (3.5 km upstream)	S 9°27.517′, E 159°57.490′
3	Kohove River, Tanasawa bridge (at sea level)	S 9°25.333′, E 159°54.164′
4	Lungga River, near Mbetikama (6 km upstream)	S 9°26.916′, E 160°02.448′
5	Wounaouss River, Tapuntari Cascades (800 m upstream)	S 15°34.320′, E 167°00.159′
6	Puelapa River (Rowa River, 200 m upstream)	S 15°34.664′, E 167°01.902′
7	Wenoui River (350 m upstream)	S 15°34.826′, E 167°02.879′
8	Adson River (5 km upstream)	S 15°33.397′, E 166°58.112′

Rankin, 1979 (from St Vincent; see Neusser & Schrödl, 2007) and the radiation of comparatively giant Indo-Pacific Acochlidiidae (*sensu* Arnaud *et al*., 1986). The latter family comprises the genera *Acochlidium* and *Strubellia*, the first acochlidians discovered by the Austrian naturalist A. Strubell (1892); the type species for both genera were described from a stream on the island of Ambon (Amboina) in the Molucca archipelago of Indonesia (Bücking, 1933; Küthe, 1935). Together with the enigmatic *Palliohedyle* Rankin, 1979, several acochlidiid species have been described from island streams of Indonesia, Palau, the Solomon Islands and Fiji (Bergh, 1895; Bayer & Fehlmann, 1960; Wawra, 1979, 1980; Haynes & Kenchington, 1991; own unpublished data).

Since the discovery of *Strubellia paradoxa* (Strubell, 1892) on Ambon (Küthe, 1935; original material redescribed by Brenzinger *et al*., 2011), populations of *Strubellia* have been discovered some 3,500 km away on Guadalcanal, Solomon Islands (Starmühlner, 1976). This geographically separate population was described as the "rediscovery of *Strubellia paradoxa*" by Wawra (1974, 1988). Further examinations of island stream malacofauna showed the genus to occur even further south on Efate and Espiritu Santo Islands, both Vanuatu (Haynes, 2000; present study). In all locations, *Strubellia* is known to share its habitat with numerous limnic Neritidae and can be found hiding under calcareous rocks in brackish water from close to the river's mouth to as far as 5 km upstream. A fifth population is presently known only from a single juvenile collected on Sulawesi, Indonesia (present study).

The Indo-Pacific limnic species are generally large-bodied (crawling individuals are up to at least 4 cm long, compared to the millimetre-scale marine mesopsammic acochlidians); they should thus be ideal candidates in the search for shared morphological characters uniting Acochlidia and their panpulmonate relatives. They are also relatively easy to keep in an aquarium; observations on their biology are nevertheless scarce and mostly limited to descriptions of habitat. Life history is unknown except for the observation that *Acochlidium* veligers do not survive in fresh water (Haynes & Kenchington, 1991; own observations). Assuming an amphidromous lifestyle as in many other invertebrates found in similar habitats (see McDowall, 2007; Kano, 2009), the questions how metamorphosed individuals manage to return and maintain reproductive populations, or how they have colonized widely separated islands, remain unanswered.

We observed and examined numerous specimens from Guadalcanal and Vanuatu, using three-dimensional (3D) microanatomical reconstruction from serial semithin sections and scanning electron microscopy (SEM). Molecular data from *Strubellia* specimens from all five known localities and from closely related hedylopsacean taxa were compared in order to reveal their origin and relationships. Based on morphological and molecular evidence, the eastern Melanesian *Strubellia* is described as a new species and the evolution of the genus is discussed in the light of these new data.

MATERIAL AND METHODS

Collection and cultivation

About 90 specimens of *Strubellia wawrai* n. sp. were collected on northwestern Guadalcanal, Solomon Islands, in October 2007; further specimens from Espiritu Santo Island, Vanuatu, were collected during the Santo Expedition in September 2006 (see Table 1 for collection localities). All specimens were collected by hand in shallow water of freshwater streams flowing into the sea. The slugs were most commonly found aggregating in small groups on the underside of loose limestone rocks at the river's edge, up to 5 km upstream. In most places the rocks showed covering of algae; freshwater neritids were abundant in most places.

Living specimens were observed in petri dishes. Four specimens from Kohove River, Guadalcanal, were kept alive for several months in a small and shallow glass aquarium with a few flat rocks. Water was regularly replenished with tap water that had been allowed to stand for several days beforehand; the aquarium was ventilated by an aerating pump. Specimens were fed different types of fish feed, egg masses of *Physa* snails and egg capsules of freshwater neritids (*Neritina* cf. *natalensis*). The neritids were acquired from a zoo store and kept in a separate aquarium with added pieces of wood; chips of wood with freshly laid egg capsules were placed with the *Strubellia* specimens. Photographs of feeding specimens were made through a stereo microscope using a handheld digital camera.

For further studies, specimens were anaesthetized using menthol crystals sprinkled onto the water surface, fixed in 1.5% glutardialdehyde buffered with 0.2 M sodium cacodylate (pH 7.2) and stored in 75% ethanol for histological study or 96% ethanol for molecular analysis.

Serial sectioning and 3D reconstruction

Glutardialdehyde-fixed specimens were postfixed in 0.01 M cacodylate buffer/0.35 M sucrose (pH 7.2) and 1% osmium tetroxide. After decalcifying in 1% ascorbic acid, specimens were dehydrated in a graded acetone series and infiltrated overnight with Spurr's low-viscosity epoxy resin (Spurr, 1969) diluted with one part 100% acetone. Infiltrated specimens were placed on embedding grids, covered with pure epoxy resin and left to polymerize for 24 h at 60°C.

Serial sections of 1.5 μm were cut with Ralph glass knives (first half of series ZSM Mol-20071895) or a Histo Jumbo diamond knife (Diatome, Biel, Switzerland—all other series) with a Microm HM 360-rotation microtome (Zeiss, Germany) (Table 2). Serial sections were collected on cleaned microscopy slides, stained with methylene blue/azure II (Richardson, Jarett & Finke, 1960) and sealed with araldite. Slides were then mapped from 600-dpi greyscale scans; single sections were photographed through a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany) with mounted Spot CCD camera (Spot Insight, Diagnostic Instruments, Sterling Heights,

Table 2. Material used for morphological and phylogenetic analyses.

Species	Locality	Museum number of voucher and use of specimens			
Strubellia wawrai n. sp.	Solomons, loc. 1	ZSM Mol-*20071895* (used for 3D); *20071881*, *20071883*, *20071886*, 20071887, 20071890 (further serial sections)			
	Solomons, loc. 2	ZSM Mol-20071796 (entire lot used for SEM)			
	Solomons, loc. 3	ZSM Mol-*20071894* (used for 3D); 20071877, 20071880, *20071892* (further serial sections)			
	Vanuatu, loc. 5	ZSM Mol-20071105 (used for 3D)			
	Vanuatu, loc. 6	ZSM Mol-20071106 (used for 3D)			
		Museum number of voucher	DNA voucher DNA Bank	GenBank accession number	
				16S rRNA	COI
	Solomons, loc. 3	ZSM Mol-20080014	AB34404271	JF819728*	JF819756*
	Solomons, loc. 3	ZSM Mol-20080015	AB34404208	JF819729*	JF819757*
	Solomons, loc. 3	ZSM Mol-20080016	AB34404250	JF819730*	JF819758*
	Solomons, loc. 1	ZSM Mol-20080017	AB34404264	JF819731*	JF819759*
	Solomons, loc. 1	ZSM Mol-20080018	AB34404255	JF819732*	JF819760*
	Solomons, loc. 1	ZSM Mol-20080019	AB34404256	JF819733*	JF819761*
	Solomons, loc. 4	ZSM Mol-20071810	AB34404212	JF819734*	JF819762*
	Vanuatu, loc. 7	ZSM Mol-20071117	AB34404234	JF819735*	JF819763*
	Vanuatu, loc. 7	ZSM Mol-20080150	AB34404205	JF819736*	JF819764*
	Vanuatu, loc. 5	ZSM Mol-20080072	AB34404207	JF819737*	—
	Vanuatu, loc. 5	ZSM Mol-20080148	AB34404251	JF819738*	—
Strubellia paradoxa	Kemeri, Ambon, Indonesia	Berlin Moll 193943	AB35081823	JF819739*	—
	Watatiri, Ambon, Indonesia	Berlin Moll 193944	AB34858174	HQ168419	HQ168457
Strubellia sp.	Tambala River, Manado, Sulawesi, Indonesia	ZSM-Mol 20100339	AB35081762	JF819740*	JF819765*
Palliohedyle sp.	Tambala River, Manado, Sulawesi, Indonesia	ZSM-Mol 20100356	AB35081794	JF828040	JF828032
Acochlidium fijiense	Lami River, Viti Levu, Fiji	ZSM-Mol 20080063	AB34404244	HQ168420	HQ168458
Pseudunela espiritusanta	SE Espiritu Santo, Vanuatu	ZSM-Mol 20080117	AB34404289	JF819750	JF819775
Pseudunela marteli	Oyster Island, Vanuatu	ZSM-Mol 20080393	AB35081809	HQ168418	HQ168456
Hedylopsis ballantinei	'INMO' Reef, Dahab, Egypt, Red Sea	ZSM-Mol 20090244	AB34858170	HQ168416	HQ168454

The table lists the species names, collecting localities (number refers to Table 1), reference numbers of museum vouchers (ZSM, Bavarian State Collection of Zoology; Berlin, Museum of Natural History, Berlin), DNA vouchers deposited in the DNA Bank of the ZSM and GenBank accession numbers. Numbers in italics indicate designated paratypes; asterisks mark the sequences generated for the present study.

MI, USA). Series of photographs were downsized to *c.* 400 megabytes by conversion to 8-bit greyscale and a resolution of 800 × 600 pixels and then imported to AMIRA 4.1 software (TGS Europe, Mercury Computer Systems, Mérignac, France) for 3D reconstruction. Labeling of organ systems was done manually, with interpolation and surface-smoothing features applied to create 3D surfaces, in general following the method described by Ruthensteiner (2008). Reconstructions of four specimens are used herein: one 'male' from Vanuatu (every eighth section was photographed for the model, resulting in a virtual stack of 871 photos; Figs 4A; 9C–E), one 'female' from the Solomon Islands (693 photos, every 4th; Figs 4E; 9A, B, F) and two further specimens for the CNS (Solomon Islands: 439 photos, every section photographed, Fig. 4B, D, F; Vanuatu: 479 photos, every 2nd; Fig. 4C). All sections are deposited in the Mollusca Department, Bavarian State Collection of Zoology, Munich, Germany (see Table 2 for museum numbers).

Interactive 3D model

The interactive 3D models in the online PDF version were prepared according to Ruthensteiner & Heß (2008), although using the 3D tools of Deep Exploration v. 5.5 (Right Hemisphere EMEA, Germany) and Adobe Acrobat v. 9.0 Professional Extended (Adobe Systems GmbH, Germany) to create interactive models of the original Amira surface files. Separate surface files of each organ were exported into the former program, then grouped into a complex model and rendered. An interactive figure was then created by importing these rendered models as backdrops of Figure 4; different views of the organ systems were prefabricated to allow the reader rapidly to get a general idea of the models. Click on the interactive Figure 4A–D for models of the general anatomy and on Figure 4E, F for a more detailed model of the CNS.

Scanning electron microscopy

Several specimens were dissected and spicules, radulae and copulatory stylets were removed and cleared from tissue in diluted KOH or Proteinase K (20 µl in 180 µl ATL Tissue lysis buffer; Qiagen, Hilden, Germany; after Holznagel, 1998). The undissolved sheath of radulae was removed using tungsten minutien needles before flattening the radula. After rinsing with distilled water, samples were mounted on aluminum stubs with sticky carbon tabs, sputter coated with gold (120 s at 2.4 kV) and examined in a LEO 1430 VP scanning electron microscope (15 kV; 2×10^{-5} mbar).

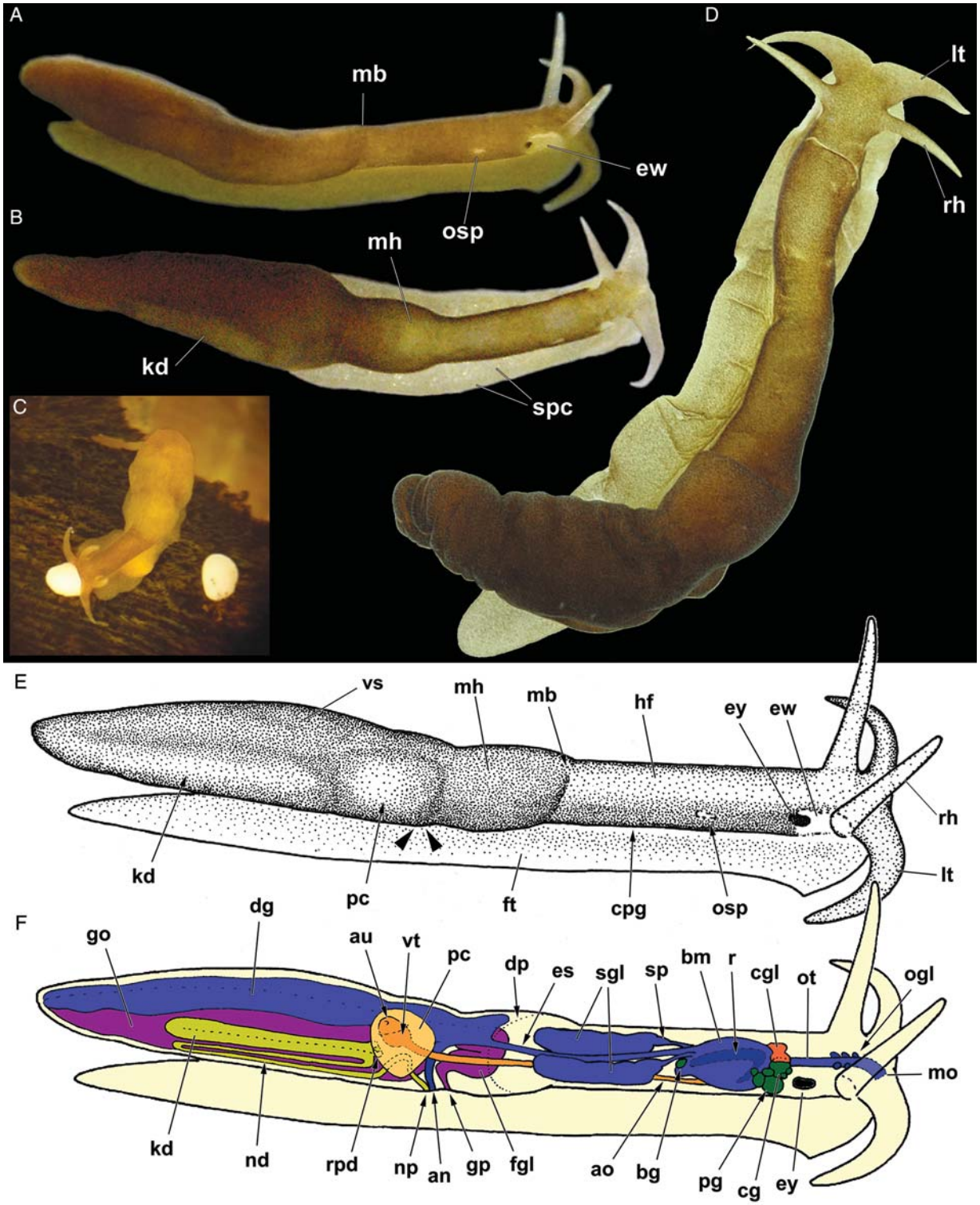

Figure 1. Live specimens and general schematic overview of the anatomy of *Strubellia wawrai* n. sp. **A–D.** External morphology of living specimens from Kohove River, Guadalcanal, Solomon Islands (**A–C**) and Tapuntari Cascades, Wounaouss River, Espiritu Santo, Vanuatu (**D**). **A.** Young specimen, *c.* 8 mm, right view. **B.** 20 mm specimen, dorsal view. **C.** Juvenile feeding on egg capsule of *Neritina* cf. *natalensis* attached to wood (experimental setting). **D.** Adult, at least 30 mm, dorsal view. **E.** Overview of external morphology, based on young specimen A, right view. **F.** Composite of internal anatomy, female phase. Abbreviations: an, anus; ao, aorta; au, auricle; bg, buccal ganglion; bm, buccal mass; cg, cerebral ganglion; cgl, "cephalic gland"; cpg, cephalopedal groove; dg, digestive gland; dp, diaphragm separating body cavities of head–foot complex and visceral sac; es, esophagus; ey, eye; ew, translucent patch over eye ('eye-window'); fgl, female gland mass; ft, foot; go, gonad; gp, genital pore; hf, head–foot complex; kd, kidney; lt, labial tentacle; mb, anterior border of mantle; mh, mantle 'hood'; mo, mouth opening; nd, nephroduct; np, nephropore; ogl, oral glands; osp, osphradium; ot, oral tube; pc, pericardium; pg, pedal ganglion; r, radula; rh, rhinophore; rpd, renopericardioduct funnel; sgl, salivary glands; sp, salivary pump; spc, spicule; vs, visceral sac; vt, ventricle. Arrowheads: position of nephropore/anus (left) and genital pore (right).

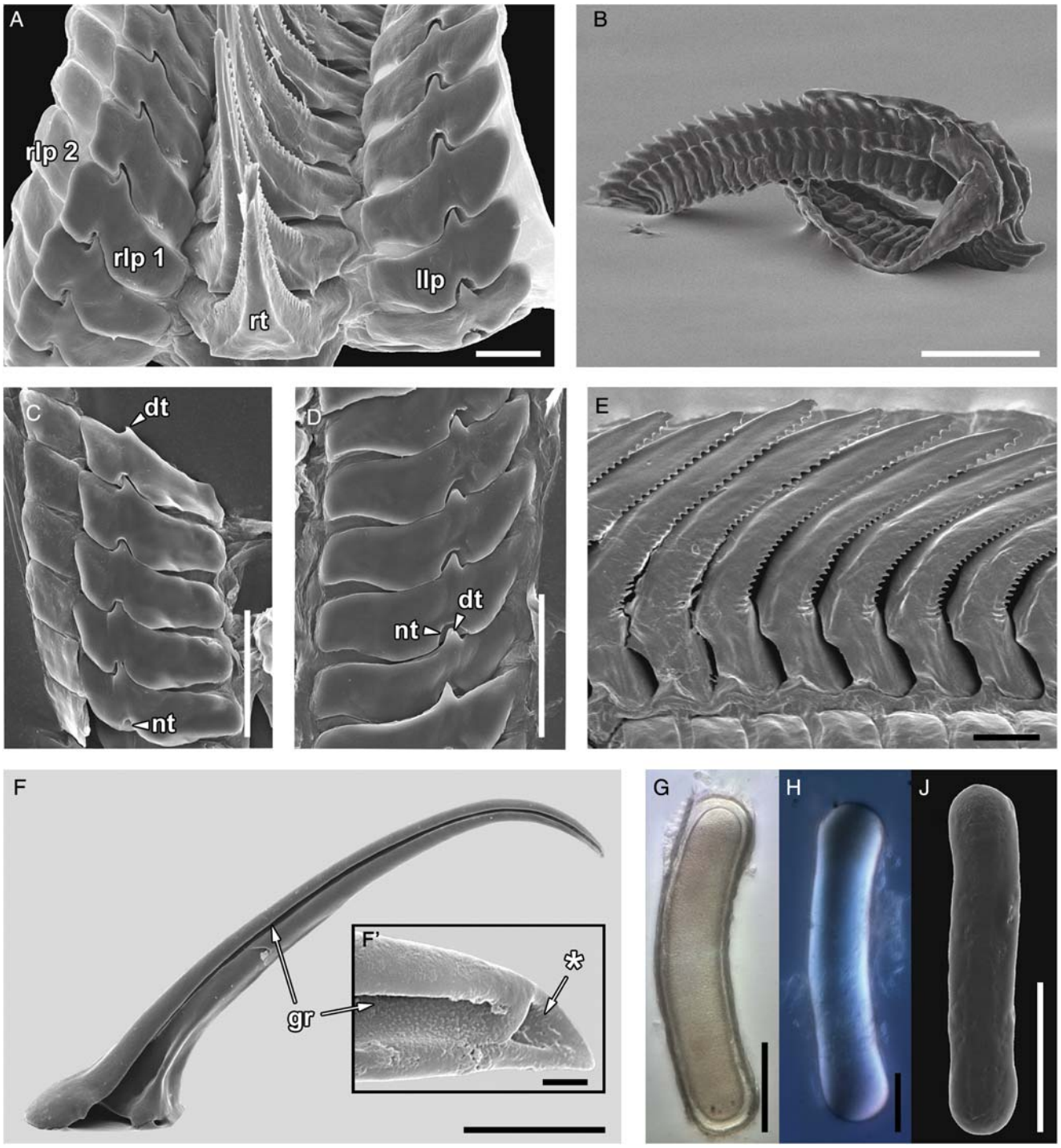

Figure 2. Microscopic views of radula (SEM), stylet of basal finger (SEM) and spicules surrounding the buccal mass (SEM, light microscopy) of *Strubellia wawrai* n. sp. **A, F, F′.** Vanuatu specimen; **others**: Solomon Islands. **A.** Functional part of radula. **B.** Complete hook-shaped radula. **C.** Right lateral teeth. **D.** Left lateral teeth. **E.** Rhachidian teeth, left view. **F.** Stylet of basal finger. **F′.** Detail of stylet tip. **G.** Spicule, phase contrast. **H.** Spicule, lateral illumination. **J.** Spicule, SEM. Abbreviations: dt, denticle; gr, groove; llp, left lateral plate; nt, notch; rlp 1 and 2, first and second right lateral plates; rt, rhachidian tooth; *, opening of hollow stylet. Scale bars: **A, C–E** = 20 μm; **B** = 100 μm; **F** = 150 μm; **F′** = 3 μm; **G, H, J** = 50 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Molecular analysis

Genomic DNA was extracted from tissue samples of the foot or entire specimens using the DNeasy Blood and Tissue Kit (Qiagen), according to the manufacturer's instructions. Two mitochondrial markers, partial 16S rRNA (400 bp) and cytochrome *c* oxidase subunit I (COI; 650 bp), respectively, were amplified using PCR (for PCR protocols and primers, see Table 3). PCR products were purified using ExoSapIT (USB, Affymetrix, Inc.); cycle sequencing and the sequencing reaction were performed by the sequencing service of the Department of Biology Genomic Service Unit (GSU) of the Ludwig-

Table 3. PCR protocols and primers used for the sequences generated within this study.

Gene	Primer	Sequence 5′–3′	Reference	PCR program
16S	16S-H	CGC CTG TTT ATC AAA AAC AT	Simon *et al.* (1994)	98°C 30 s (98°C 5 s, 48–55°C 5 s, 72°C 25 s) × 35–40,
	16S-R	CCG GTC TGA ACT CAG ATC ACG T	Simon *et al.* (1994)	72°C 60 s (Phire polymerase, New England Biolabs)
COI	LCO1490	GGT CAA CAA ATC ATA AAG ATA TTG G	Folmer *et al.* (1994)	94°C 3 min (94°C 60 s, 45–48°C 60 s, 72°C 90 s) × 35–
	HCO2198	TAA ACT TCA GGG TGA CCA AAA AAT CA	Folmer *et al.* (1994)	40, 72°C 3 min (Taq polymerase, Sigma)
	COI long r	TAA AGA AAG AAC ATA ATG AAA ATG	Stothard & Rollinson (1997)	

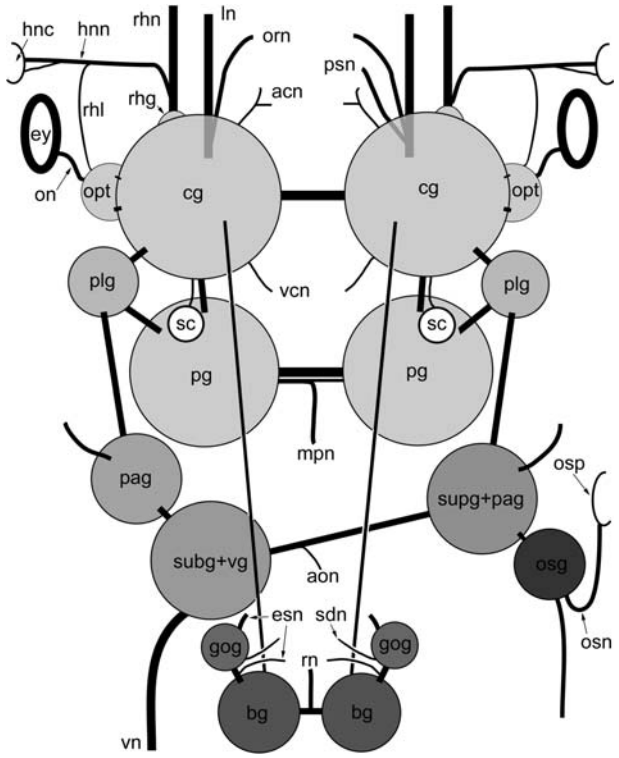

Figure 3. Schematic overview of the CNS (pedal nerves omitted for clarity) of *Strubellia wawrai* n. sp., dorsal view. Abbreviations: acn, anterior cerebral nerve; aon, aortic nerve; bg, buccal ganglion; cg, cerebral ganglion; esn, esophageal nerves; ey, eye; gog, gastroesophageal ganglion; hnc, Hancock's organ; hnn, Hancock's organ nerve; ln, labial tentacle nerve; mpn, median pedal nerve; on, optic nerve; opt, optical ganglion; orn, oral nerve; osg, osphradial ganglion; osn, osphradial nerve; osp, osphradium; pag, parietal ganglion; pg, pedal ganglion; plg, pleural ganglion; psn, penial sheath nerve; rhg, rhinophoral ganglion; rhl, rhinoporal looping nerve; rhn, rhinophoral nerve; rn, radular nerve; sc, statocyst; sdn, salivary duct nerve; subg, subintestinal ganglion; supg, supraintestinal ganglion; vcn, ventral cerebral nerve; vg, visceral ganglion; vn, visceral nerve. Not to scale.

Maximilians-University Munich, using Big Dye 3.1 kit and an ABI 3730 capillary sequencer. All fragments were sequenced on forward and reverse strand. DNA vouchers are stored at the DNAbank of the Bavarian State Collection of Zoology; sequences are deposited at GenBank (see Table 2 for accession numbers). Sequences were edited using Sequencer (Gene Codes Corporation). We applied a Blast search (Altschul *et al.*, 1990) on each sequence to check for potential contamination (http://blast.ncbi.nlm.nih.gov/Blast.cgi). MUSCLE v. 3.8.31 (Edgar, 2004) was used to create the alignments of each marker, subsequently the COI alignment was checked manually according to the translation into amino acids. Maximum-likelihood analyses of the concatenated dataset (in two partitions) were performed using RAxML v. 7.0.3 (Stamatakis, 2006) under the GTR + G model (selected for the concatenated dataset under the Akaike information criterion with jModeltest; Posada, 2008) and 1,000 bootstrap replicates were generated. Outgroups were chosen according to previous morphological and molecular hypotheses on acochlidian phylogeny (Jörger *et al.*, 2010a; Schrödl & Neusser, 2010) and retrieved from GenBank (Table 2). *Hedylopsis ballantinei* Sommerfeldt & Schrödl, 2005 was defined as outgroup.

For both markers, intra- and inter-specific variation was evaluated using Species Identifier, available from TaxonDNA (http://taxondna.sourceforge.net; Meier *et al.*, 2006) and used to cluster sequences based on pairwise distances (testing thresholds from 1 to 10%). Additionally, we calculated haplotype networks for both markers using TCS 1.21 (Clement, Posada & Crandall, 2000); the COI alignment was shortened, until all sequences had the same length; default settings (95% probability of parsimony) were used.

SYSTEMATIC DESCRIPTION

Heterobranchia *sensu* Haszprunar, 1985a
Panpulmonata Jörger *et al.*, 2010a
Acochlidia sensu Wawra, 1987
Hedylopsacea *sensu* Wawra, 1987
ACOCHLIDIIDAE *sensu* Arnaud *et al.*, 1986

Strubellia Odhner, 1937

Strubellia wawrai n. sp.

Strubellia paradoxa—Wawra, 1974: 8–10. Starmühlner, 1976: 473–656. Wawra, 1988: 163–172 (not *Acochlidium paradoxum* Strubell, 1892 = *Strubellia paradoxa*).
Strubellia sp. Haynes, 2000: 101–111.

Type material: Holotype: ZSM Mol-20100718; complete specimen stored in 75% ethanol; 7 mm preserved body length; collected in Mataniko River, Guadalcanal, Solomon Islands (locality 1, Table 1), 8/9 October 2007 by K. Jörger & Y. Kano. Paratypes: nine complete specimens stored in 75% ethanol (lot: ZSM Mol-20071797), same lot as the holotype; six serially sectioned specimens mounted on microscope slides [Mataniko River ZSM Mol-20071881, 20071883 (partial series), 20071886, 20071895; Kohove River: 20071892 (partial series), 20071894]; all paratypes collected 8/9 October 2007, together with holotype (Table 2).

Etymology: Named in honour of Erhard Wawra (1945–1994) for his pioneering work on the biology and systematics of Acochlidia and particularly the *Strubellia* of the Solomon Islands.

Interactive model: In addition to the 3D images (Figs 4, 9), see also the interactive 3D models of *Strubellia wawrai* n. sp. that can be accessed by clicking onto Figure 4A–D (general anatomy) and E, F (CNS) in the online PDF version of this article.

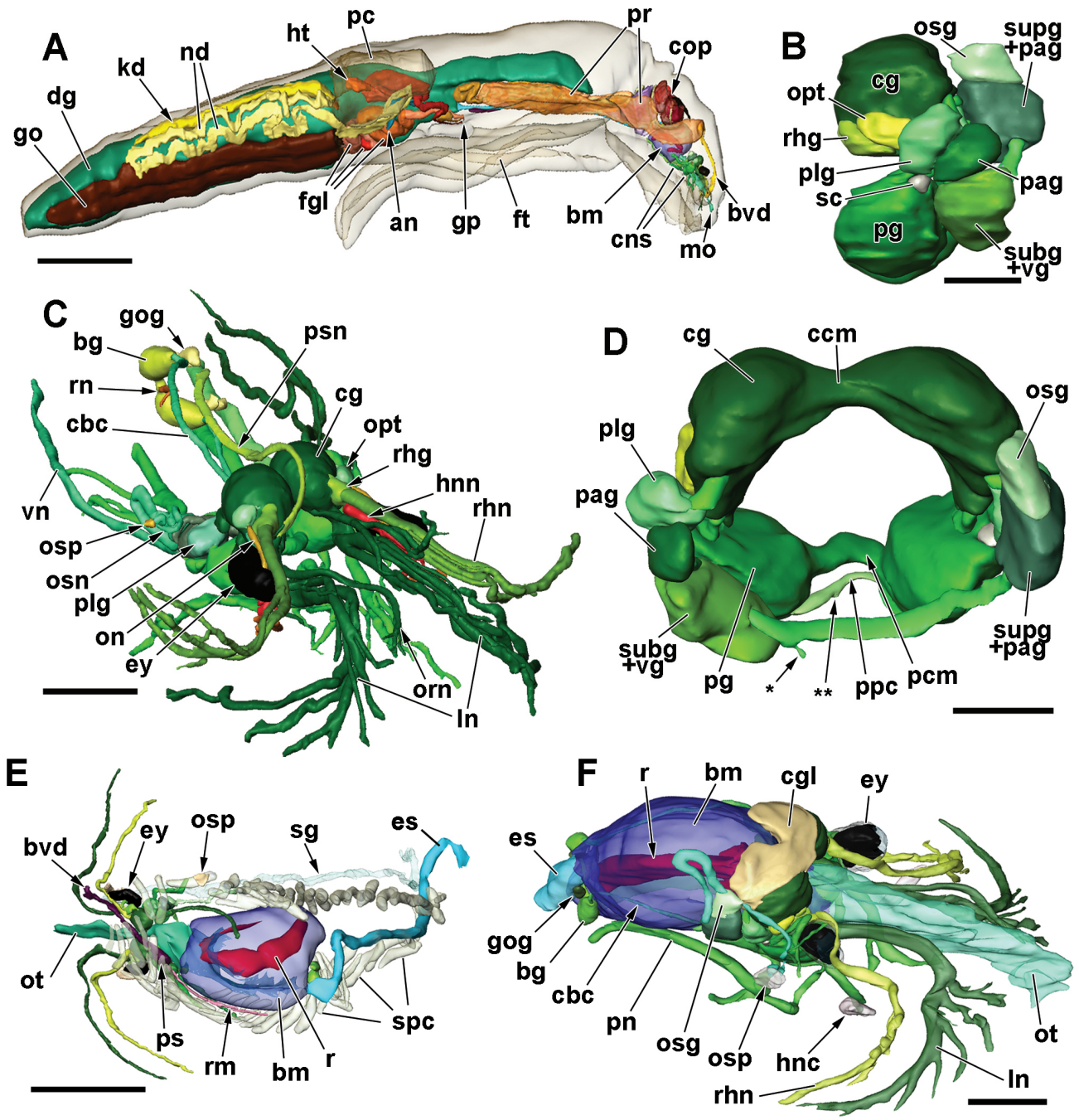

Figure 4. Three-dimensional reconstruction of general anatomy and CNS of *Strubellia wawrai* n. sp. from Vanuatu (**A, C**) and Solomon Islands (**B, D–F**). **A.** General anatomy, right view. **B.** Main ganglia, left view. **C.** CNS, anterior right view. **D.** Main ganglia, posterodorsal view. **E.** CNS with spicule grid and rudimentary penial sheath, dorsal view. **F.** CNS and buccal mass, anterior right view. Abbreviations: an, anus; bg, buccal ganglion; bm, buccal mass; bvd, posterior-leading vas deferens; cbc, cerebrobuccal connective; ccm, cerebral commissure; cg, cerebral ganglion; cgl, 'cephalic gland'; cns, central nervous system; cop, copulatory apparatus; dg, digestive gland; es, esophagus; ey, eye; fgl, nidamental glands; ft, foot; go, gonad; gog, gastroesophageal ganglion; gp, genital pore; hnc, Hancock's organ; hnn, Hancock's nerve; ht, heart; kd, kidney; ln, labial tentacle nerve; mo, mouth opening; nd, nephroduct; on, optic nerve; opt, optical ganglion; orn, oral nerve; osg, osphradial ganglion; osn, osphradial nerve; osp, osphradium; ot, oral tube; pag, parietal ganglion; pc, pericardium; pcm, pedal commissure; pg, pedal ganglion; plg, pleural ganglion; pn, pedal nerve; ppc, parapedal commissure; pr, prostate; ps, penial sheath; psn, penial sheath nerve; r, radula; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; rm, retractor muscle of penial sheath; rn, radular nerve; sc, statocyst; sg, sperm groove; spc, spicules; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve; asterisks: branching points of nerves. Scale bars: **A** = 2 mm; **B, D** = 100 μm; **C, F** = 200 μm; **E** = 500 μm. The interactive 3D models of *S. wawrai* n. sp. can be accessed by clicking onto **A**–**D** (general anatomy) and **E, F** (CNS) in the online PDF version of this article. Rotate model by dragging with left mouse button pressed; shift model: same action + ctrl (or dragging with left and right mouse buttons pressed); zoom: use mouse wheel. Select or deselect (or change transparency of) components in the model tree, switch between prefab views or change surface visualization (e.g. lighting, render mode, crop etc.). Interactive manipulation requires Adobe Reader 7 or higher.

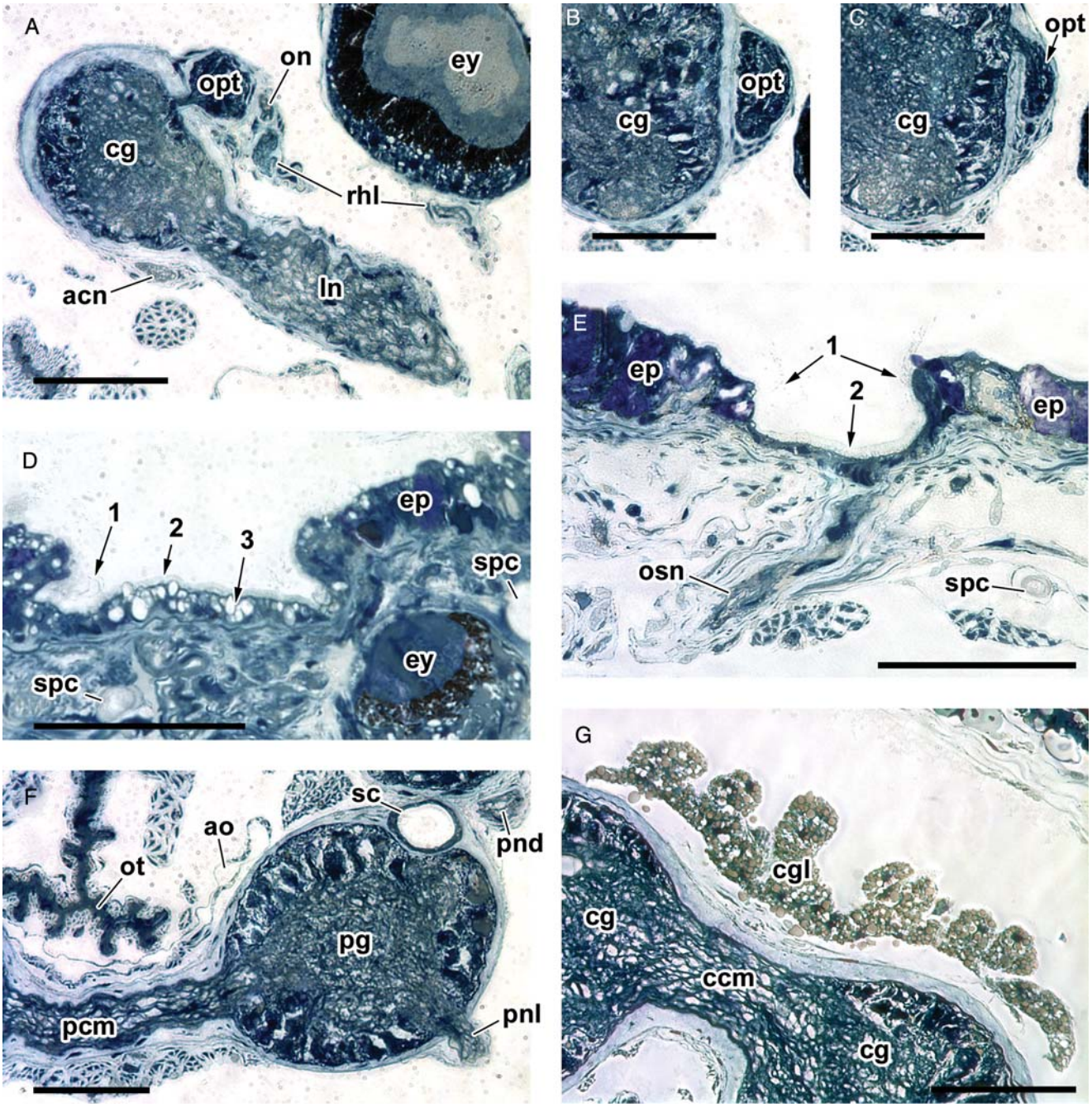

Figure 5. Semithin sections of the CNS and sensory organs (Solomon Islands specimens) of *Strubellia wawrai* n. sp. **A–C.** Cerebral ganglion and double cerebro-optic connectives. **D.** Hancock's organ. **E.** Osphradium. **F.** Pedal ganglion and statocyst. **G.** Cephalic gland dorsally to cerebral ganglia. Abbreviations: acn, anterior cerebral nerve; ao, aorta; ccm, cerebral commissure; cg, cerebral ganglion; cgl, cephalic gland; ep, epidermis; ey, eye; ln, labial tentacle nerve; on, optic nerve; opt, optic ganglion; osn, osphradial nerve; ot, otal tube; pcm, pedal commissure; pg, pedal ganglion; pnd, dorsal pedal nerve; pnl, lateral pedal nerve; rhl, rhinophoral looping nerve; sc, statocyst; spc, spicules; 1, multiciliated cells; 2, microvillous border; 3, vacuolate cells. All scale bars = 50 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

External morphology: External appearance is of a typical hedylopsacean acochlidian: elongate head–foot complex with two pairs of pointed head appendages; foot separated from body by longitudinal cephalopedal groove; uncoiled, shell-less visceral sac projecting freely behind foot, especially in fully grown specimens (Fig. 1). Epidermis appearing velvety smooth under stereo microscope; visceral sac slightly grainier. Body coloration orange to rusty brown in living specimens; foot, head appendages and translucent patch above the eye (Fig. 1A, E: ew) brighter, pale yellow; large specimens appear darker. Eyes visible externally as black dots, digestive gland as orange tube. Spicules in foot and head appendages visible as refracting bodies. Osphradium a keyhole-shaped brighter spot on right side of head–foot (Fig. 1A). Alcohol-fixed material light yellow-brown.

Crawling specimens usually between 6 and 12 mm, up to 20 mm (Solomon Islands specimens; Fig. 1B) or 35 mm long (Vanuatu; Fig. 1D). In younger specimens, visceral sac straight

and slightly shorter than foot with foot tip visible in dorsal view; larger specimens with visceral sac longer and appearing somewhat ragged and bent, with tip often pointing to right side. Pericardial space and beating of heart sometimes visible ('heart-bulb') at anterior right of visceral sac. Spacious haemocoel cavity into which head–foot can be retracted located between 'heart-bulb' and anterior mantle border (mantle 'hood' just anterior to position of diaphragm separating head–foot from visceral sac; Fig. 1). When disturbed, animals retract head–foot into this cavity and contract, visceral sac then curved, foot folded and tucked into concave side of visceral sac, head appendages project partially from underneath mantle 'hood'.

Front end of foot semicircular, edges slightly flaring; posterior end with pointed tip; foot sole wider than dorsal head–foot. Head appendages of about equal length; each appendage showing rod-like spicules sorted longitudinally. Labial tentacles slightly flattened in cross-section, held parallel to ground in crawling specimens, medially forming upper lip. Rhinophores round in cross-section, held erect.

General histology: Musculature consisting of blue staining fibres either spanning body cavity independently, or associated closely with organs. Body wall musculature a mesh of outer circular and inner longitudinal fibres. All parts of digestive system surrounded by longitudinal muscle fibres; circular fibres apparent only around salivary ducts. Transversal muscular diaphragm (Fig. 1F: dp) is punctured by aorta, oesophagus and visceral nerve, and is located at base of visceral sac, separating body cavities of head–foot and visceral sac (see mantle 'hood' above).

Connective tissue fills most spaces in foot (dense aggregates of cells), and flanks of head–foot and anterior visceral sac (less dense aggregates). Aggregates separated from central body cavity by thin longitudinal sheath of connective tissue; aggregates consisting of rather large, irregularly shaped cells staining homogeneously light blue, filled with darker grains and few yellow-stained vesicles.

Calcareous spicules embedded in most of connective tissue. In serial sections of decalcified animals only spicule cavity remaining, apparently enclosing spicule in living animals; chamber usually containing remnants of dissolved spicules visible as smaller, translucent body consisting of concentric layers of undissolved matter. Spicules themselves cylindrical, straight or slightly bent with slightly thickened, rounded tips, giving a dumb-bell-like shape. Spicules glassy transparent but strongly refracting (Fig. 2H) under light microscope. Spicule surface smooth (Fig. 2J), interior slightly yellowish to brown in phase-contrast due to organic material (Fig. 2G). Concentric lamination evident in broken spicules viewed with SEM. Spicules size differing greatly: very small and short spicules around oral opening and oesophagus; long and thin ones arranged longitudinally inside cephalic appendages, forming continuous row from labial tentacles into upper lip. Highest number of spicules (80–120 μm long) embedded in dense connective tissue of foot. Largest spicules (up to 300 μm) sorted in at least two parallel strips dorsolaterally of central nervous system (CNS) and buccal mass, forming a grid of interdigitating pieces ('cephalic spicule grid'; Fig. 4E).

Large anterior pedal gland located in anterior body cavity, ventrally to pharynx and CNS; distal part consists of paired lobes of thick glandular epithelium surrounding central lumen; cells filled with very small granules staining dark or light blue. Lobes of this gland merge anteriorly, connecting to short and wide epidermal duct leading into strongly ciliated, V-shaped longitudinal groove on dorsal side of anterior foot margin, ventrally to mouth opening. Further clusters of round foot glands located in entire foot ventrally to connective tissue, between dorsoventral muscle fibres; glands most numerous in anterior foot. Glandular cells containing many small dark blue grains, some yellow vesicles; cells open onto foot sole through very thin ducts.

Digestive system: Digestive system closely resembling that of other acochlidians: oral tube elongate, followed by bulbous pharynx containing hook-shaped radula, followed by paired salivary glands and oesophagus; direct connection into large digestive gland filling large part of visceral sac; intestine short with anal opening on right anterior side of visceral sac (Fig. 1E). No histologically detectable differentiated stomach. Ciliation of digestive tract detectable only in two places: at short strip in proximal part of oesophagus (where it projects from pharynx) and inside intestine.

Mouth opening a vertical slit located underneath upper lip; the following rather long oral tube surrounded by lateral clusters of oral glands opening into oral tube through thin ducts; oral gland cells staining dark blue (peripheral) or pale pink (closer to oral tube). Strong pair of pharynx protractors running from posterior end of oral tube to rhinophores; another pair running posteroventrally. Posterior end of oral tube is lined with thin cuticle. Pharynx egg-shaped, complex mass of muscle surrounding pharyngeal cavity; muscle surrounds posterior tip of radula (Fig. 4E, F). Pharynx protrusible anteriorly in slightly sucker-like fashion, surrounded by circular margin of epidermal tissue. Haemocoel lacunae present within pharynx, between fibres of pharyngeal muscles, supporting radula laterally and ventrally. Pharyngeal cavity lined with thin epithelium covered by equally thick, clear blue-staining cuticle (up to 15 μm thick); cavity with three longitudinal furrows, appearing as three-pointed star in cross-section (vertical furrow extending dorsally of radula). Radula originates in posterior tip of pharynx; ribbon originally still folded, embedded between large cells. Folded, upper branch runs anteriorly, emerging into pharyngeal cavity and spreading open. Radula then curves down, open part with old and worn teeth leading posteriorly again for about half length of upper branch (Fig. 2B). Radula asymmetric: single left lateral plate, prominent rhachidian tooth, two right lateral plates per row. Radular formula 40–60 × 1.1.2 (number of tooth rows in small Solomon Islands to large Vanuatu specimens, respectively). Rhachidian teeth with rectangular base and very slender, blade-like and pointed median cusp, its margins serrated (*c.* 30 or more small denticles per side) (Fig. 2A, E). Under light microscope, youngest rhachidian teeth appearing more translucent and with slimmer base than following teeth; median cusps of oldest rhachidian teeth generally worn down to stumps. First lateral plates of both sides flat and rectangular; each plate equipped with strong denticle on border to next younger plate, this border with notch into which denticle of other plate fits (Fig. 2A). Small and diamond-shaped second lateral plate on right side of radula; inner border straight, right first lateral plate appearing equally cut-off (Fig. 2A, C). Left lateral plates slightly wider than right ones (65 *vs* 50 μm in same row), outer border more rounded (Fig. 2A, D).

Salivary glands paired, connecting to posterior end of pharynx via thin salivary ducts. Each gland with two longitudinal lobes (resembling figure-of-eight in cross-section) formed by columnar cells densely filled with dark blue-stained granules. Central collecting duct strongly ciliated, showing bulbous salivary pump distal to glandular tissue (Fig. 1F: sp); spindle-shaped pumps and following salivary ducts surrounded by circular muscle fibres (contrasting with all other muscular linings of digestive system); salivary ducts opening anteriorly into lateral folds of pharyngeal cavity.

Oesophagus a simple tube projecting from posterodorsal side of pharynx; distal oesophagus widens gradually before

connecting to lumen of digestive gland. Digestive gland a long sac usually filling most of visceral sac (in mature specimens gonad more voluminous). Outer surface of gland with irregular transverse folds; inner surface highly enlarged by glandular epithelium with high columnar cells forming bundles projecting into lumen. Epithelial cells filled with numerous small blue-stained vesicles; large, spherical, yellow-stained vacuoles in an apical position make up large part of glandular mass (Fig. 7A, C). Intestine rather short and thick, emerging from digestive gland dextrolaterally to distal oesophagus. Inner surface of intestine folded longitudinally, strongly ciliated. Intestine gradually thinning towards anal opening; opening hard to detect in most specimens but very close to renal pore, both openings sometimes forming an invaginated and ciliated common cavity (possibly an artifact due to fixation).

Central nervous system—cerebral nerve ring: CNS euthyneurous, slightly epiathroid (i.e. pleural ganglia closer to cerebral than to pedal ganglia), following general acochlidian bauplan (Fig. 3). Prepharyngeal nerve ring consisting of paired cerebral, pedal and pleural ganglia; three ganglia on visceral nerve cord plus osphradial ganglion; paired buccal ganglia posterior to pharynx. Further elements: paired optic and rhinophoral ganglia (on anteroventral sides of cerebral ganglia), paired gastro-oesophageal ganglia dorsally on each buccal ganglion. Serial sections reveal numerous nerves (Figs 3, 4).

Cerebral ganglia largest ganglia, largely spherical; cerebral commissure strong (Figs 4D, 5G). Cerebropleural connective slightly shorter than cerebropedal one; static nerve very thin, emerging close to base of cerebropleural connective and running parallel to it to paired statocysts. Statocysts embedded in top of each pedal ganglion. Cerebrobuccal connectives thin, very long, running posteriorly within pharyngeal musculature laterally to dorsal branch of radula (Fig. 4F).

Labiotentacular nerves very thick (diameter *c.* 50 μm), emerging medioventrally from each cerebral ganglion; nerve splits early into thinner oral branch (running to upper lip) and thick part (to tip of labial tentacles, with thinner branches repeatedly running to anterior side of tentacles; Fig. 4C, F). Right labial nerve of some specimens with further branch extending posterodorsally, innervating penial sheath (Fig. 4C: psn).

Rhinophoral ganglion located at anteroventral part of cerebral ganglion between labiotentacular nerve and optic ganglion (Fig. 4B). Rhinophoral ganglion elongate and pear-shaped; thicker portion containing few peripheral cell bodies and connecting to cerebral ganglion by short connective, thinner part running smoothly into rhinophoral nerve. Rhinophoral nerve splitting into three branches close to its origin: thickest part continues into rhinophores (without much further branching); second, thinner part innervates Hancock's organs posterior to rhinophoral bases; third (thinnest) branch looping backwards and apparently connecting to anteroventral side of optic ganglion (Fig. 3: rhl).

Optic ganglion hemispherical, attached to cerebral ganglion laterally but separated by independent layer of connective tissue (Fig. 5B). Double, very short cerebro-optic connectives, posterior one stronger (Fig. 5A, C); third connective detected in single specimen. Optic nerve thin, rather long, joining to posteroventral portion of eye; thin and looping second nerve connecting to Hancock's organ's branch of rhinophoral nerve (see above).

Two further cerebral nerves detectable: (1) thin nerve leaving cerebral ganglion medially (Figs 3, 5A: acn), running anteroventrally along paired cephalic blood vessels before splitting into branches running towards rhinophores and to the mouth opening; (2) thin nerve emerging from posteroventral side of cerebral ganglion (Fig. 3: vcn), running into muscular lining of cephalic blood vessels.

Mass of loosely aggregated and apparently glandular cells in body cavity above cerebral ganglia and cerebral commissure ('cephalic gland'); containing numerous vacuoles staining light yellow. Gland mass without detectable connection to ganglia except for some thin fibers (connective tissue?); symmetric lobes extending slightly down sides of cerebral ganglia (Figs 4F, 5G).

Pedal ganglia spherical, only slightly smaller than cerebral ganglia; joined by thick pedal commissure (Fig. 5F) and thinner, longer parapedal commissure; very thin nerve splitting off parapedal commissure just left of midline (Fig. 4D), running to median part of foot sole and anterior pedal gland.

Six further pairs of pedal nerves detected, all running to body flanks: anteroventral, ventrolateral, posteroventral and posterodorsal nerves rather thick and running along body sides in posterior direction (except for first one); additional thin antero- and posterodorsal nerves running to sides, the former one apparently joining to anteroventral pedal nerve close to eye.

Central nervous system—visceral loop and buccal ganglia: Visceral cord with three medium-sized to large ganglia, connecting beneath anterior part of pharynx (Fig. 4B, D; nomenclature after Haszprunar, 1985a; Sommerfeldt & Schrödl, 2005): (1) left parietal ganglion (small, thin nerve running to left body side); (2) fused subintestinal/visceral ganglion (large, left of midline; giant nerve cells and very thick visceral nerve running posteriorly); (3) fused supraintestinal/right parietal ganglion (medium sized, thin nerve running to right body side). Latter ganglion with osphradial ganglion (small, cap-shaped) on posterodorsal side (Fig. 5D), both ganglia enclosed by common sheath of connective tissue. Osphradial ganglion with two nerves, one looping upwards first before running posteriorly; second: osphradial nerve innervating osphradium on anterior right body side (Fig. 4F). Ganglia on visceral nerve cord joined by short to very short connectives, only ganglia (2) and (3) with long connective passing obliquely between pharynx and aorta; thin nerve emerging from left third of long connective running downward into musculature of aorta (Fig. 4D: asterisk).

Visceral nerve strongest nerve posterior to CNS (diameter *c.* 25 μm) and running posteriorly into visceral sac, slightly left of midline (Fig. 4C: vn); nerve identifiable by surrounding longitudinal muscle fibres throughout entire length; nerve passes through diaphragm close to aorta and oesophagus.

Buccal ganglia paired, medium-sized, situated on posterodorsal side of pharynx at emerging point of oesophagus. Buccal commissure short, running ventrally to oesophagus; thin, apparent radular nerve emerging from middle of commissure, leading forward into muscular mass of pharynx (Fig. 4C, F).

Gastro-oesophageal ganglia (small, bean-shaped) on top of each buccal ganglion, connected by short vertical connective; thin oesophageal nerve from upper part of connective leading medially into muscular sheath of oesophagus; another thin nerve running from base of each gastro-oesophageal ganglion into sheath surrounding salivary ducts (Fig. 3: esn, sdn).

Sensory organs: Eyes located dorsolaterally to slightly anteriorly to cerebral ganglia, underneath translucent patch of epidermis visible in living animals (Fig. 1A, B); eyes bean-shaped, *c.* 130 μm long, facing anterolaterally (Fig. 4C, F), surrounded by thin layer of connective tissue; innervation by thin optic nerves. Prismatic (sensory?) cells with distinct nuclei form cup-shaped outer layer of eye, followed by layer of grainy black pigment; grey-blue staining irregular band (possibly sensory microvilli) between pigment layer and otherwise acellular and

light blue-staining lens (Fig. 5A). Lens covered distally by cornea consisting of single layer of flat cells.

Statocysts paired, hollow spheres (diameter 25 μm) with flat, slightly ciliated cells forming outer wall (Fig. 5F); remnants of layered single statolith inside fluid-filled cavity visible in some sections. Statocysts embedded in dorsal part of each pedal ganglion (Fig. 4B); static nerve originating in cerebral ganglia.

Hancock's organs posterior to base of each rhinophore, located inside zone of brighter epidermis over eyes; exact dimensions of organs detectable only in serial sections, there appearing as shallow patches of thin epidermis, resembling osphradium in histology (dense microvillous border, several multiciliated cells), differing in presence of rounded, apparently glandular cells with clear lumen (Fig. 5D); innervation by lateral branches of rhinophoral nerves.

Osphradium a small pit on right body side, visible in living animals as keyhole-shaped spot paler than surrounding epidermis (Fig. 1A); in serial sections a pit about 40 μm deep and 60 μm long, lined with very thin epidermis showing strong microvillous border (Figs 4F, 5E); several cells with bundles of cilia *c*. 25 μm long found inside pit but mainly close to rim; osphradial nerve emerging from osphradial ganglion, splitting up distally.

Multiciliated cells similar to putative sensory cells in Hancock's organs and osphradium found interspersed within normal epidermal cells on labial tentacles and rhinophores.

Circulatory and excretory systems: Pericardial complex located in anterior right of visceral sac, with externally visible 'heart bulb' indicated by beating of heart in living animals (Fig. 1B). Pericardial complex formed by spacious pericardium enveloping two-chambered heart; elongate kidney and looping nephroduct extending posteriorly along right side of visceral sac (Figs 4A, 6). Renal pore situated on anteroventral right, close to anal opening. Aorta extending into head–foot, passing between pharynx and pedal commissure, distally dividing into paired vessels (Figs 5F, 7); vessels terminating laterally of oral tube. In large Vanuatu specimens, second branch of aorta detectable, running posteriorly into visceral sac.

Pericardium formed by very thin wall breached in three places: (1) dorsally at venous connection between haemocoel and atrial lumen; (2) anteroventrally, where aorta extends from ventricle into body; (3) posterolaterally to heart where ciliated renopericardioduct drains off into kidney (Fig. 6). Pericardial lumen free of cells, except for few vacuolated cells at anteroventral wall which appears to wrap around distal part of nephroduct.

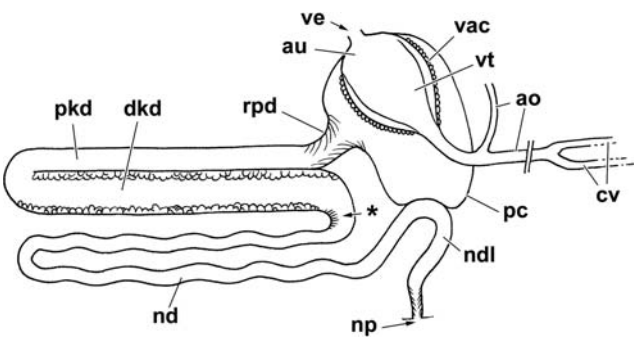

Figure 6. Schematic overview of the circulatory and excretory systems of *Strubellia wawrai* n. sp., right view. Abbreviations: ao, aorta; au, auricle; cv, paired cephalic vessels; dkd, distal kidney lumen; nd, nephroduct; ndl, nephroduct loop; np, nephropore; pc, pericardium; pkd, proximal kidney lumen; rpd, renopericardioduct; vac, vacuolated epicardium on ventricular wall; ve, venous opening; vt, ventricle; *, ciliated intersection between kidney and nephroduct. Not to scale.

Heart consisting of thin-walled auricle and muscular, ovoid ventricle. Haemocoel on right side of visceral sac connected to auricle by small hole (diameter 10 μm); opening visible only in single series where auricle clearly distinguishable from ventricle (Fig. 7A); auricle collapsed in most other cases. Ventricle continuous with auricle in its wall, ovoid form appearing more constant; ventricular wall much thicker, formed by mesh of striated muscle fibres staining blue-grey, some fibres appearing to cross ventricular lumen, forming muscular bridges (Fig. 7B).

Inside of ventricular wall covered with irregular cells, some staining darker blue or with yellow-stained vacuole; conspicuous large cells embedded in former layer and interspersed freely in the ventricular lumen: cells elongate and ovoid, showing a central body stained light grey, with concentric layers somewhat resembling a spicule.

Outer wall of ventricle covered with irregularly bordered, conspicuous lining at least as thick as muscular layer of wall. Epicardial lining consisting of vacuolate cells staining light blue to grey, with flat nuclei sorted apically staining slightly darker (Fig. 7E).

Tip of ventricle continuing into thick aorta, wall consisting of longitudinal muscle fibres, internal surface smooth. Aorta leaving pericardium on medioventral side, running anteriorly and passing through diaphragm close to oesophagus and visceral nerve, splitting into paired vessels formed only by strips of muscle fibres and membranous wall ventrally to cerebral nerve ring; cephalic vessels spacious, running parallel to oral tube (Figs 5F, 6), terminating close to mouth.

Excretory system consisting of short but well-developed renopericardioduct, elongate kidney and long and looping nephroduct. Renopericardioduct longitudinally folded, connecting to pericardium via funnel-shaped opening containing conspicuous ciliary flame; cuboidal lining with bundles of strong cilia projecting into pericardium and renopericardial duct (Fig. 7C, E), leading into kidney.

Kidney elongate, extending along two thirds of visceral sac; longitudinal interior wall separating lumen into hairpin-like loop connected only at kidney's posterior end (Figs 6, 9A, B): proximal part of lumen (running front to back) lying more ventrally, lined with regular epithelium with dense microvillous border (Fig. 7C, F); distal part of kidney lumen (running back to front) lying dorsally, more voluminous and lined with epithelium with shorter microvillous border, conspicuous unstained vacuoles giving wall spongy appearance (Fig. 7D) and accounting for much of kidney's volume. Connection to nephroduct through constriction of only about 3 μm diameter (in direct proximity to the renopericardioduct funnel), followed by short patch of dense ciliation (Fig. 7C: triple asterisk). Undulating nephroduct running posterior to tip of kidney and looping forward again; nephroduct interconnected by single muscle fibres in at least one place; lined with smooth epithelium staining light blue, with interspersed yellow-stained vesicles and a slight microvillous border (Fig. 7G). Distal loop of nephroduct differing slightly in histology (epithelium staining darker, showing fewer yellow vesicles but possibly colorless, irregular vacuoles), arching upward before running downward again towards nephropore (Fig. 9A, B); appearing to be closely associated with fold of pericardium.

Nephropore formed by ciliated and invaginated part of epidermis, situated next to anal opening or inside invaginated cloaca (artifact?), on dextroventral anterior visceral sac.

Genital system: Presence of allosperm receptacles in males, and females with rudimentary 'male' features indicate protandric hermaphroditism (as in *S. paradoxa* from Ambon). Examined specimens from Solomon Islands only juveniles and two functional 'females' (one with vestigial bursa copulatrix and penial sheath;

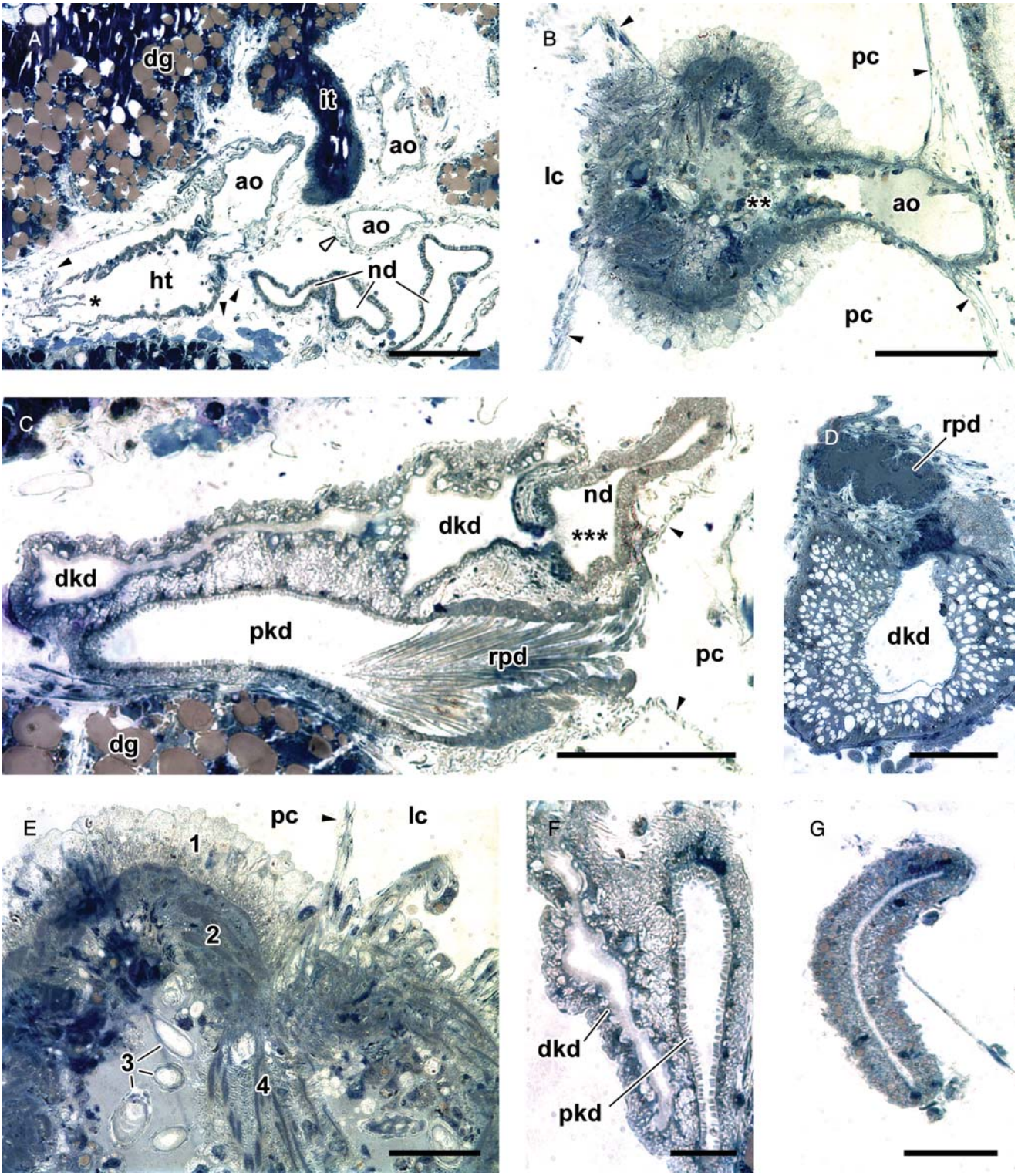

Figure 7. Semithin sections of the circulatory and excretory systems of *Strubellia wawrai* n. sp. (Solomon Islands specimens). **A.** Heart, longitudinal section. **B.** Pericardium and heart, cross-section. **C.** Anterior portion of excretory system, longitudinal section. **D.** Anterior portion of excretory system, cross-section. **E.** Wall of ventricle, cross-section. **F.** Proximal and distal kidney lumina, cross-section. **G.** Nephroduct, suspended by muscle fiber, cross-section. Abbreviations: ao, aorta; dg, digestive gland; dkd, distal kidney lumen; ht, heart; it, intestine; lc, hemocoel lacunae dorsally to pericardium; nd, nephroduct; pc, lumen of pericardium; pkd, proximal kidney lumen; rpd, renopericardioduct; black arrowheads: wall of pericardium; white arrowhead: peritoneal membrane; *, venous opening of heart to hemocoel lacunae; **, loose cells inside heart; ***, ciliated intersection between kidney and nephroduct; 1, vacuolate epicardium; 2, muscular wall of ventricle; 3, cells containing spicule-like body; 4, muscle fibers spanning ventricle. Scale bars: **A–C** = 100 μm; **D, E** = 50 μm; **F, G** = 25 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Figs 4E, 9F); Vanuatu specimens containing one juvenile and one female (gonad filled with oocytes, nidamental glands developed) but with apparently functional cephalic copulatory apparatus and two allosperm receptacles (Figs 4A, 9C, D, E).

Posterior genital system consisting of acinar gonad, proximal receptaculum seminis filled with sorted spermatozoa and glandular gonoduct leading to genital opening on anterior right of visceral sac. Ampulla thin-walled, wide; detected only in single specimen. Gonad consisting of numerous almost spherical acini, filling much of visceral sac in functionally female specimens. Each acinus formed by thin epithelial wall, filled with large spherical oocytes containing high numbers of vesicles staining brilliantly blue, with colorless vesicles filling gaps in between; acini connected to gonoduct by thin ducts (Fig. 8A). Collecting gonoduct surrounded by muscle fibres but collapsed in both specimens; strong ciliation apparent; following last acinus a very short piece of gonoduct from which receptaculum seminis (thick-walled and blind-ending sac) emerges laterally. Receptacle lined with simple blue-staining epithelium forming an undulated inner wall; numerous spermatozoa are embedded with their heads into wall. Heads of spermatozoa visible only at high magnifications as stronger refracting bodies; head short, not screw-shaped, diameter about 1 μm; flagella forming pink-stained, dense and streaked mass inside receptacle (Fig. 10B: arrowheads and asterisk).

Following receptaculum seminis another short piece of gonoduct, leading into female gland mass. Glandular mass tubular throughout, forming several stout loops in anterior visceral sac; strongly stained, columnar glandular cells surround lumen only from one side (Fig. 10A); lumen a longitudinal fold projecting in between glandular cells. Glandular cells up to almost 100 μm high, filled with granular secretions. Three differently staining zones along glandular gonoduct: (1) proximal part staining dark blue; (2) distal part blue with strong pinkish tone; (3) part in between appearing blue with slightly greenish hue (Fig. 9D, F). Distal part of glandular epithelium becomes thinner with diameter of strongly ciliated gonoduct lumen appearing to increase before opening to outside through genital pore.

Single female Solomon Island specimen with vestigial bursa copulatrix consisting of very thin duct (10 μm diameter; emerging from gonoduct close to genital opening) and almost spherical terminal bulb close to upper intestine (Fig. 9F); bulb stained very dark blue inside. Same individual with distal gonoduct containing several oval bodies with pink-stained and grainy vesicle and fully developed ciliated sperm groove running from genital opening to base of right rhinophore. Thin tube entering body and running posteriorly from anterior end of sperm groove: posterior-leading vas deferens passing cerebral commissure dorsally and terminating in elongate blind sac (an empty and reduced penial sheath); reduced, thread-like penial retractor muscle extending posteriorly from sac, ending freely in body cavity (Fig. 4E).

Cephalic male copulatory organs: One Vanuatu specimen with elaborate male and female features: external sperm groove between female genital opening and base of right rhinophore, connecting to fully developed male copulatory organs surrounded by penial sheath at left of pharynx. Copulatory organs consisting of muscular basal finger, considerably smaller penis and their associated paraprostatic and prostatic glandular systems, respectively (Figs 4A, 8B).

Posterior-leading vas deferens connected to voluminous, tubular prostate gland; prostate continuing into long and curled ejaculatory duct, entering muscular penis at its base; ejaculatory duct opening to exterior through penial papilla at tip of penis. Solid spine of *c.* 150 μm width situated next to penial papilla (Fig. 9E). Blind ending glandular paraprostate a longer and thinner tube than prostate, strongly coiled (Fig. 9C: ppr). Paraprostatic duct emerging from paraprostate and connecting to muscular basal finger, entering basal finger approximately in middle of curved muscle; duct opening apically via curved hollow stylet of about 750 μm length. Stylet with cuticular groove running along its side (Figs 2F, 10D–H). Penis and basal finger muscles interconnected at their base; both structures surrounded by thin-walled penial sheath meeting posterior-leading vas deferens before opening to exterior at base of right rhinophore.

Behaviour and feeding: Living specimens collected by hand under rocks in shallow water at sides of streams. Aggregations of up to 25 individuals found under single calcareous rocks, hidden in grooves and pits of undersurfaces. Exposure to light causes animals to move; specimens kept in a Petri dish moved around without pause until hiding place was presented. On smooth surfaces, movement was fast, about 7 mm/s, with head moving from left to right, labial tentacles held parallel to ground. Movement appeared to be caused by ciliary motion (visible in animals crawling upside down at water surface: fine particles on water surface were quickly drawn away from front margin of foot) and supported by clear mucus as observable in specimens suspended by thread of mucus from water surface.

Three small individuals (probably juveniles) were cultivated in a small aquarium for about 5 months. When supplied with calcareous egg capsules of freshwater neritids *Strubellia* individuals were observed to aggregate on the egg capsules after a few minutes. Other types of food (fish feed, algae tabs, gelatinous egg masses of *Physa* sp.) did not lead to any reaction. Individuals remained on egg capsule with anterior border of foot and mouth pressed onto capsule's surface, head appearing slightly contracted (head appendages bent backwards, eyes not visible; Fig. 1C). Slow peristaltic dilatations of entire visceral sac observed during this apparent feeding posture, accompanied by slow but strong pumping motions of heart. Each feeding period up to 15 min; between two and three egg capsules fed on per individual. Some egg capsules fed on by more than one individual, others were ignored. Continuous supply of neritid eggs over longer period of time proved difficult; specimens shrank during time in aquarium.

Molecular phylogeny: The RAxMC-tree based on 16S rRNA and COI genes recovers the monophyletic genus *Strubellia* (bootstrap support, BS = 100) as sister taxon to the genera *Acochlidium* and *Palliohedyle* (Fig. 11), all three genera forming the large-bodied and limnic family Acochlidiidae (*sensu* Arnaud *et al.*, 1986). Sampling of 13 *Strubellia* individuals reveals three clades: a basal and yet undescribed branch from Sulawesi (known only from single individual) as sister taxon to a clade formed of *S. paradoxa* from Ambon (BS = 100) and a clade consisting of all sampled individuals from Solomon Islands and Vanuatu (BS = 96). Specimens from Vanuatu are nested within populations from the Solomons.

Statistical parsimony analyses generate two independent haplotype networks (not shown) for partial 16S rRNA: *S. paradoxa* and a network uniting *S. wawrai* n. sp. populations from Solomons and Vanuatu (no 16S rRNA sequence was available for *Strubellia* from Sulawesi). Four independent networks were generated based on partial COI (reduced to 571 bp, to analyse sequences of same length): *S. paradoxa*, *Strubellia* sp. (Sulawesi), *S. wawrai* n. sp. from Solomons, and from Vanuatu.

Intraspecific variation is generally very low: in 16S rRNA (438 bp) 0.0% in *S. paradoxa* ($n = 2$), 0.68–0.91% in *S. wawrai* n. sp. from Solomons ($n = 7$) and 0.45–0.68% in *S. wawrai* n. sp. from Vanuatu ($n = 4$). Uniting both populations of *S. wawrai* n. sp. ($n = 11$), intraspecific variation ranges from 0.45 to 1.14% in 16S rRNA. Lowest interspecific variation in 16S rRNA between *S. paradoxa* and *S. wawrai* n. sp. is 4.1%; a higher selected

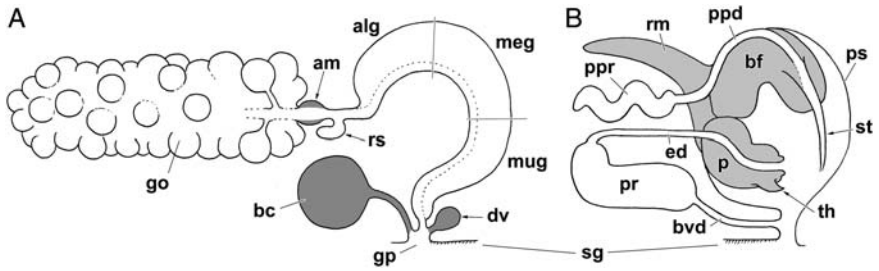

Figure 8. Schematic overview of the genital system and copulatory apparatus of *Strubellia wawrai* n. sp. **A.** Genital system, dark grey areas indicate organs that become reduced in the female phase. **B.** Copulatory apparatus. Abbreviations: alg, albumen gland; am, ampulla; bc, bursa copulatrix; bf, basal finger; bvd, posterior-leading vas deferens; dv, diverticle; ed, ejaculatory duct; go, gonad; gp, genital pore; meg, membrane gland; mug, mucus gland; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, penial sheath; rm, retractor muscle; rs, receptaculum seminis; sg, sperm groove; st, stylet of basal finger; th, spine. Not to scale.

threshold clusters both species together. In COI (571 bp) intraspecific variation ranges between 1.57 and 1.92% for *S. wawrai* n. sp. from the Solomons ($n = 7$) and 0.7% for *S. wawrai* n. sp. from Vanuatu ($n = 2$); uniting both populations ($n = 9$) the variation ranges between 2.1 and 2.8%. Interspecific variation is comparably high ranging between 12.25 and 13.31% in *S. paradoxa* and *S. wawrai* n. sp. and between 14.36 and 15.06% in *Strubellia* sp. from Sulawesi and *S. wawrai* n. sp.

DISCUSSION

Comparative morphology of the cerebral nerve ring

The CNS of *Strubellia wawrai* n. sp. has been described briefly from dissected material by Wawra (1974, as *S. paradoxa*). The general organization of ganglia resembles that of *S. paradoxa* and other hedylopsacean acochlidian species, e.g. *Pseudunela espiritusanta* (Neusser & Schrödl, 2009; Brenzinger *et al.*, 2011). Examination of serially sectioned specimens revealed several additional features, such as the previously undetected parapedal commissure and several thin cerebral nerves that complement the set of nerves beyond what is generally detectable in small mesopsammic acochlidians. Among the usually present nerves are three comparatively large anterior cerebral nerves also shown in Wawra's drawing (1974: fig. 7); we regard the nerves numbered 1.1–1.3 therein to be the labial tentacle nerve, the Hancock's and the rhinophoral nerve, respectively. *Strubellia* shows two small ganglia attached to the cerebral ganglia, as do all other hedylopsaceans: The "procerebral lobe" described by Wawra (but not depicted) can be suspected at the base of the rhinophoral and Hancock's nerve and likely refers to the rhinophoral ganglion herein. The optic ganglion appears to have been overlooked by Wawra; his "optic" nerve is shown to arise directly from the cerebral ganglion and thus might alternatively be the oral nerve which extends close to the labial tentacle nerve.

The homology of opisthobranch rhinophoral or optic ganglia and the pulmonate procerebrum (with double connectives to the cerebral ganglion) has been suggested previously (e.g. Haszprunar, 1988; Haszprunar & Huber, 1990; Huber, 1993) and a general homology of the sensory innervation among Euthyneura appears more and more likely (Jörger *et al.*, 2010a, b). Comparison of these ganglia among Acochlidia might however hint at a common anlage of both the optic and rhinophoral ganglion: the presence of a looping nerve interconnecting both (present in *S. wawrai* n. sp. and *Tantulum elegans*; Neusser & Schrödl, 2007), the variable origin of the optic nerve (usually from the optic ganglion, in *P. cornuta* it splits off from the rhinophoral nerve; Neusser, Heß & Schrödl, 2009a) and finally the presence of double connectives in one ganglion or the other. A double cerebro-rhinophoral connective is present in *Tantulum*, the microhedylacean *Pontohedyle milaschewitchii* (Kowalevsky, 1901) and *Microhedyle glandulifera* (Kowalevsky, 1901) (Jörger *et al.*, 2008; Neusser & Schrödl, 2009; own unpublished data); *S. wawrai* n. sp. is the only known species with a double cerebro-optic connective.

Differences from the CNS of *S. paradoxa* involve the apparent lack of the small cerebral nerves, the Hancock's nerve and Hancock's organs, but are likely to be artefacts (see Brenzinger *et al.*, 2011). The only evident difference between the CNS of *S. wawrai* n. sp. from the Solomon Islands and from Vanuatu is the 'penial' nerve in the examined specimen from Vanuatu, which might be present only in mature male specimens and could therefore not be detected in the female specimens from the Solomon Islands.

Visceral loop, osphradial ganglion and arrangement of buccal ganglia

Wawra (1974) described the typical acochlidian visceral nerve cord with three separate ganglia; we identify the ganglia herein as a left parietal, a fused subintestinal/visceral and a fused right parietal/supraintestinal ganglion, respectively. Nerves splitting from the connective joining the latter two ganglia and from the parietal ganglia have not been reported for any other acochlidian so far.

The additional ganglion attached to the fused right parietal/supraintestinal ganglion was mentioned by Wawra (1974); it is known for all hedylopsacean species examined in detail and also for the microhedylacean *Parhedyle cryptophthalma* (Westheide & Wawra, 1974; Jörger *et al.*, 2010b; Schrödl & Neusser, 2010). Judging from its position on the right side of the visceral loop, the ganglion was hypothesized to be homologous with the osphradial ganglion of other euthyneurans (Wawra, 1989; Huber, 1993; Sommerfeldt & Schrödl, 2005); this interpretation can be confirmed with the detection of an osphradium in *S. wawrai* n. sp. The presence of two nerves in *S. wawrai* n. sp. and a bifurcating nerve in *Pseudunela espiritusanta* suggests more than one function of the osphradial ganglion. In *Tantulum elegans*, the single nerve leaving the osphradial ganglion was mentioned to terminate close to the copulatory apparatus and hence assumed to be a "genital" or "penial" nerve (Neusser & Schrödl, 2007); innervation of the copulatory apparatus in *S. wawrai* n. sp., however, appears to be mainly by the nerve of cerebral origin mentioned above.

Buccal ganglia posterior to the pharynx are present in all Acochlidia, and associated gastro-oesophageal ganglia are known from several hedylopsacean species but not (yet) *Hedylopsis ballantinei* (Sommerfeldt & Schrödl, 2005; Wawra, 1988, 1989; Schrödl & Neusser, 2010) and also the microhedylaceans *Asperspina murmanica* (Kudinskaya & Minichev, 1978) and *Microhedyle glandulifera* (Neusser, Martynov & Schrödl, 2009b; Eder, Schrödl & Jörger, 2011). In *S. wawrai* n. sp., this arrangement of ganglia innervates the salivary ducts, musculature of the oesophagus and the radula as can be shown from

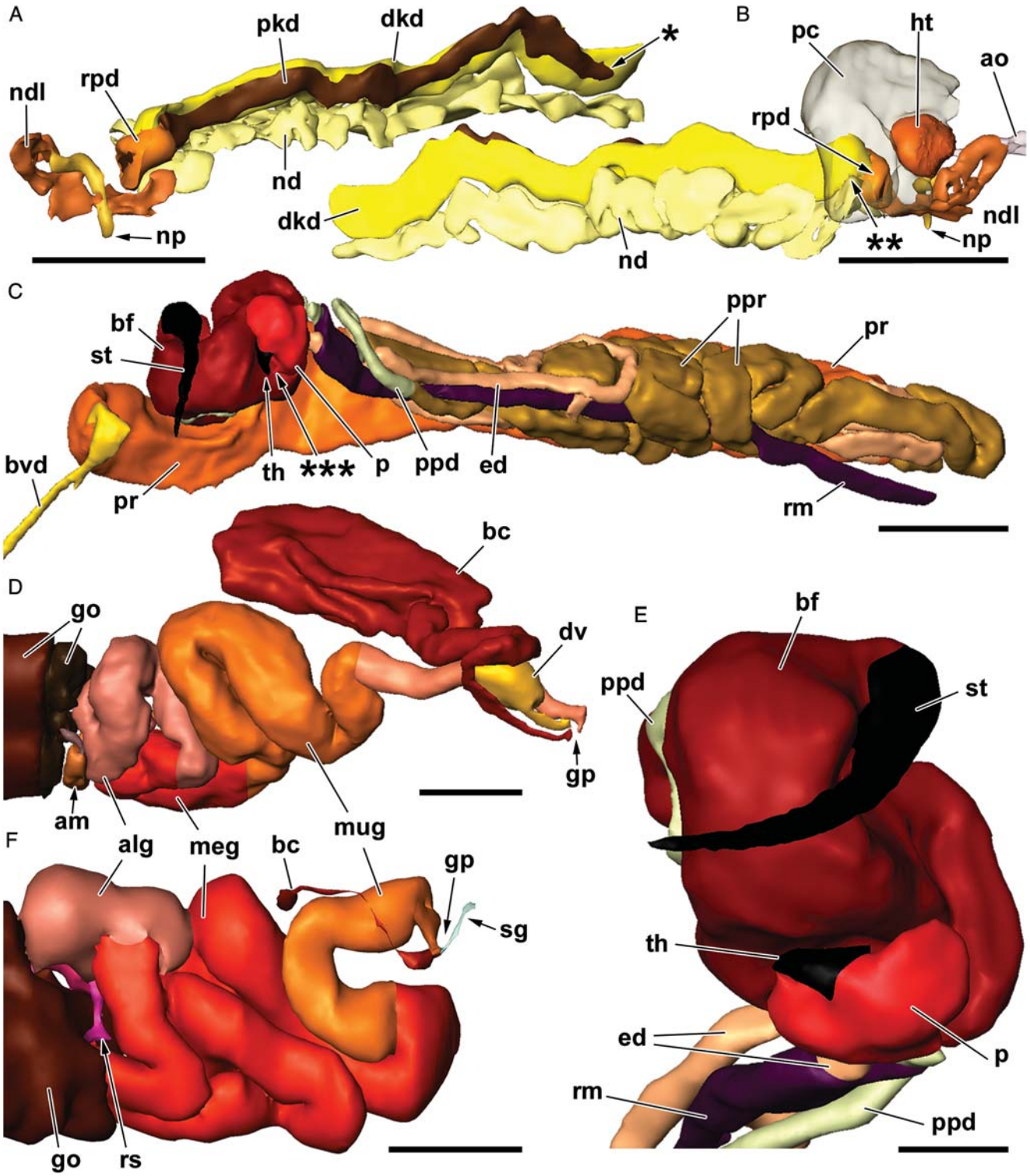

Figure 9. Three-dimensional reconstructions of the excretory, circulatory and reproductive systems of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B, F**) and Vanuatu (**C–E**). **A.** Excretory system, left view. **B.** Excretory and circulatory systems, right view. **C.** Anterior male copulatory organs and (para-)prostatic glandular systems, left view. **D.** Nidamental glands and bursa copulatrix, right view. **E.** Penis and basal finger, left view. **F.** Nidamental glands and rudimental bursa copulatrix, ventral view. Abbreviations: am, ampulla; alg, albumen gland; ao, aorta; bc, bursa copulatrix; bf, basal finger; bvd, posterior-leading vas deferens; dkd, distal kidney lumen; dv, diverticle; ed, ejaculatory duct; go, gonad; gp, genital pore; ht, heart; meg, membrane gland; mug, mucus gland; nd, nephroduct, ndl, nephroduct loop; np, nephropore; p, penis; pc, pericardium; pkd, proximal kidney lumen; pr, prostate; ppd, paraprostatic duct; ppr, paraprostate; rm, retractor muscle; rpd, renopericardioduct; rs, receptaculum seminis; sg, sperm groove; st, stylet; th, spine; *, connection between proximal and distal kidney lumina; **, connection between distal kidney lumen and nephroduct; ***, position of ejaculatory duct opening. Scale bars: **A, B** = 500 μm; **C** = 600 μm; **D, F** = 400 μm; **E** = 200 μm.

three pairs of nerves plus the unpaired radular nerve, again most of which have not been detected in other acochlidians.

The detection of a high number of previously unknown cerebral features, all possibly bearing useful phylogenetic information, again highlights the usefulness of serial sectioning and accompanying 3D reconstruction.

Osphradium

The observation of a pit-shaped osphradium is the first mention of this sensory organ in Acochlidia. In living *S. wawrai* n. sp. from Guadalcanal, the osphradium is clearly visible as a paler spot on the right body side. A similar spot is also visible in living *Acochlidium* sp. from the same locality, in this case rather inconspicuously on the anterior border of an otherwise darkly pigmented bar (own unpublished data). Interestingly, two previous accounts on the aforementioned genera mention body openings in the position of the osphradium: *S. paradoxa* was erroneously displayed to have the anus in the position of the osphradium (Rankin, 1979: 72) and the original account of *A. amboinense* Strubell, 1892 described the copulatory apparatus to open in this place (Bücking, 1933: fig. 2), contradicting observations from other sources or species (e.g. Küthe, 1935; Haase & Wawra, 1996; Brenzinger *et al*., 2011).

The position of the osphradium—far anterior to what can be considered the mantle border (see Fig. 1A)—appears strange, since the chemosensory organ is usually part of the mantle cavity organs including the gill, anus, genital opening and nephropore (e.g. Thompson, 1976). Apparently the osphradium has moved to a more anterior position after the loss of the mantle cavity in acochlidians. While it appears possible that the osphradium as a discrete organ is expressed only in the large-bodied species, it is also likely to have simply been overlooked so far in the minute interstitial species. These taxa should be critically (re-)investigated regarding the presence of a possible osphradium by searching for the osphradial nerve and its targeted epithelium as part of the epidermis.

Judging from light-microscopical observations, the osphradium of *S. wawrai* n. sp. resembles the organ of the cephalaspidean *Philine* (a pit-like structure with a flat bottom; Edlinger, 1980) and can accordingly be divided into two zones: a microvillous inner zone and a ciliated border forming the rim (Fig. 5E), similar to the condition described for the cephalaspidean *Scaphander lignarius* (Linnaeus, 1758) by Haszprunar (1985b). Since ultrastructural research on the osphradial sensory epithelia has been used to test phylogenetic hypotheses, examination of the organ in *Strubellia* might reveal features shared with other Panpulmonata that have retained the osphradium.

Hancock's organs

Hancock's organs are cerebrally innervated chemosensory organs situated on the sides of the head; they are present in most shelled opisthobranch gastropods (see Göbbeler & Klussmann-Kolb, 2007). Previously assumed to be missing in Acochlidia (see e.g. Wawra, 1987; Sommerfeldt & Schrödl, 2005), the organs were detected in four mesopsammic species including one *Pseudunela* species (Edlinger, 1980; see Neusser, Jörger & Schrödl, 2007; Neusser *et al*., 2011b; own unpublished data). As in the latter species, the Hancock's organs of *S. wawrai* n. sp. are ciliated epidermal depressions located posterior to the labial tentacles; each organ is innervated by a lateral branch of the rhinophoral nerve. The organs can only be reliably detected in specimens where the head is not strongly retracted into the visceral sac and are thus likely to be overlooked, as was probably the case in *S. paradoxa*.

Oophagy and radular characters

An asymmetric radula with a formula of $n \times 1.1.2$ is present in most hedylopsaceans and has been regarded as a possible synapomorphy of all Acochlidia (Schrödl & Neusser, 2010). Wawra (1974) described the radula of Solomon Island *S. wawrai* n. sp. (as *S. paradoxa*) with a formula of $n \times 2.1.2$, but later corrected this to $n \times 1.1.2$ (Wawra, 1979); the latter can be confirmed by our study. *Strubellia paradoxa* was also originally described with a formula of $n \times 2.1.2$ (Küthe, 1935). Reexamination of *S. paradoxa* showed that on the left side there is just a single tooth (Brenzinger *et al*., 2011). The genus shares with *Acochlidium* (and *Aiteng ater* Swennen & Buatip, 2009) the finely serrated rhachidian teeth (e.g. Haynes & Kenchington, 1991; Swennen & Buatip, 2009; Neusser *et al*., 2011a), however the very elongate rhachidians appear to be a synapomorphy for *Strubellia*. There are no clear differences in tooth morphology separating *S. paradoxa* and the Solomon Islands/Vanuatu populations. Counts of radular rows do not show consistent differences among populations and the only connection appears to be with size or ontogenetic stage: very large individuals of *S. wawrai* n. sp. from Vanuatu had *c*. 55–60 rows of teeth, medium-sized specimens from the Solomon Islands showed between 48–51 rows (Wawra, 1974, 1979) and 40–46 rows (this study).

The observation of cultured *S. wawrai* n. sp. feeding on egg capsules of *Neritina* cf. *natalensis* is the first direct observation of feeding in Acochlidia. Only *Acochlidium amboinense* has been mentioned to have "animal remains in the stomach" (Bergh, 1895), while the meiofaunal *Pontohedyle milaschewitchii* was suggested to be an unspecialized detritus grazer due to its preference of substrates with microbial mats (Hadl *et al*., 1969; Edlinger, 1980; see Schrödl & Neusser, 2010).

Clusters of neritid egg capsules were seen on rocks at most sampling localities in the Solomon Islands and are an energy-rich potential food source. However, these capsules are strongly reinforced by conchiolin and diatoms or sand grains derived from the food (Andrews, 1935), a fact that appears to deter predation effectively. Only recently have other neritids been shown to feed facultatively on egg capules of other species (Kano & Fukumori, 2010). *Strubellia wawrai* n. sp. appears to be equipped with a radula specialized for piercing the hard-shelled capsules: the rhachidian teeth are more elongate than in any other acochlidian genus and show considerable wear in the older part of all examined radulae. The finely serrated rhachidians are most likely used to create a slit in the egg capsules through which the contents of the capsules are sucked out, as is suggested by the peristaltic movement of the visceral sac during feeding and the duration of each feeding interval. The sucker-like aspect of the lips surrounding the protruding pharynx is probably related to this mode of feeding. An oophagous habit can also be assumed for *S. paradoxa*, which shows no major differences in microhabitat or radular morphology (Brenzinger *et al*., 2011). The closely related *Acochlidium* species all share the same habitat (as far as can be deduced from the literature) and exhibit highly similar radular morphology (the rhachidian teeth are wider and less dagger-shaped). One might suggest a similar mode of feeding in this genus, perhaps involving niche differentiation with regard to the durability of egg capsules that are fed on; not all egg capsules are equally reinforced and most harden further after their deposition on the rock (Kano & Fukumori, 2010). During the feeding experiment, a specimen of *Acochlidium* from Guadalcanal was attracted to the presented egg capsules but did not feed (own observations).

Spicules

Subepidermal spicules are found in a number of shell-less heterobranchs and are there considered to be an adaptation to life

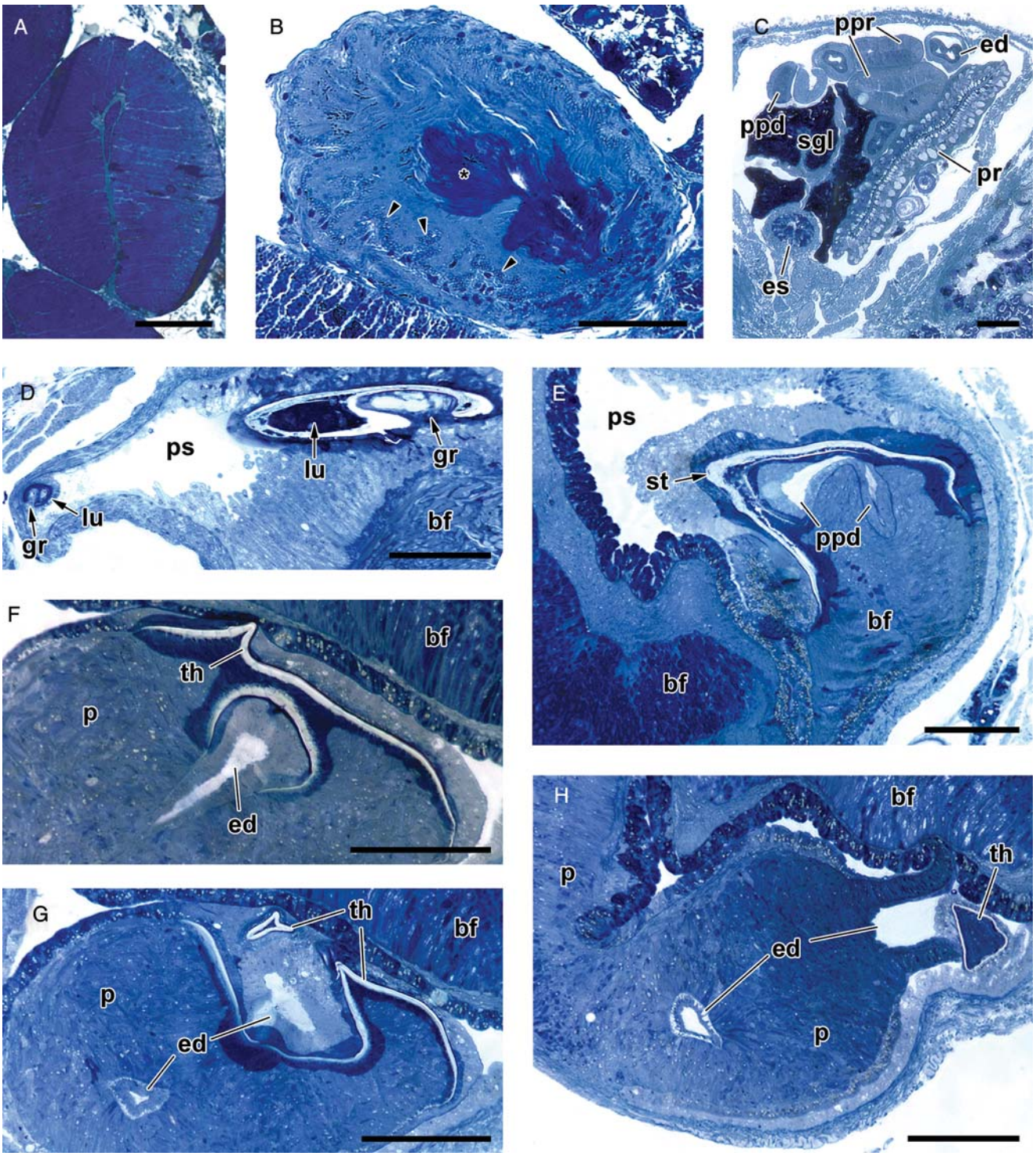

Figure 10. Semithin sections of the genital system of *Strubellia wawrai* n. sp. from Solomon Islands (**A, B**; posterior genital system in female phase) and Vanuatu (**C–H**; parts of copulatory apparatus). **A.** Membrane gland showing acentral lumen. **B.** Receptaculum seminis filled with spermatozoa, heads along the wall. **C.** (Para-)prostatic glandular system. **D.** Hollow stylet of basal finger (tip on the left, close to the base on the right). **E.** Basal finger at base of stylet. **F, G.** Penis with ejaculatory duct and thorn embedded in epithelium. **H.** Trumpet-shaped penial papilla and tip of thorn. Abbreviations: bf, basal finger; ed, ejaculatory duct; es, esophagus; gr, groove of basal finger stylet; lu, lumen of basal finger stylet; p, penis; ppd, paraprostatic duct; ppr, paraprostate; pr, prostate; ps, lumen of penial sheath; sgl, salivary gland; st, stylet of basal finger; th, spine of penis; arrowheads: sperm heads; asterisk: mass of flagella. Scale bars: **A, B** = 50 μm; **C–H** = 100 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

in the marine interstitial environment (see Rieger & Sterrer, 1975 for a review), functioning as either protective or stabilizing skeletal elements. In some doridoidean nudibranchs, defensive calcareous spicules have also been suggested to be calcium reservoirs (Cattaneo-Vietti *et al.*, 1995). Spicules are present in most acochlidians (Jörger *et al.*, 2008; Schrödl & Neusser, 2010); members of the mesopsammic *Asperspina* and *Hedylopsis* have evolved a secondary spicule 'shell' that surrounds the

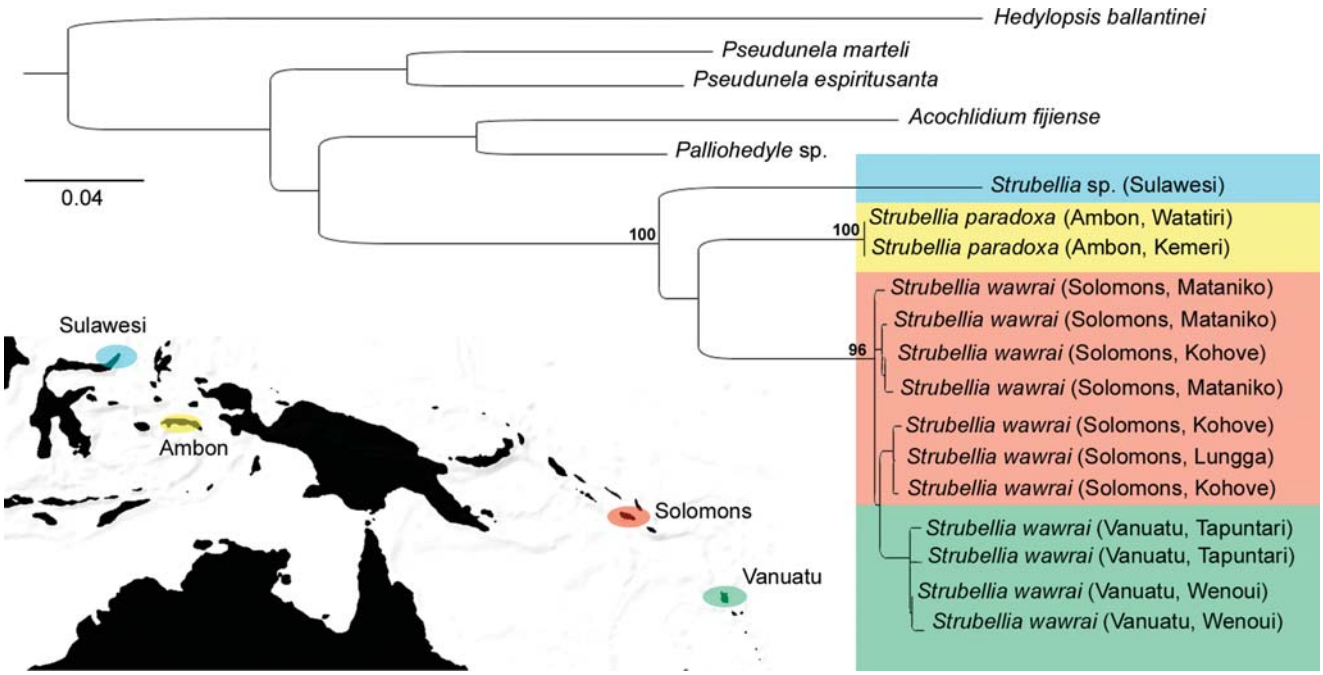

Figure 11. RAxML tree of the genus *Strubellia*, based on a concatenated dataset of mitochondrial COI and 16S rRNA (1113 bp) and colour coded distribution map of the different *Strubellia* species. Bootstrap values given above nodes.

visceral sac (e.g. Swedmark, 1968; Schrödl & Neusser, 2010). Wherever present, acochlidian spicules are calcareous, more or less elongate or forming concrements of irregularly formed grainy material.

In form, relative size and distribution, *Strubellia* spicules resemble those of *Pseudunela* or *Acochlidium* (see Bayer & Fehlmann, 1960; Neusser & Schrödl, 2009) but, judging from their location within the body, they do not function as protective elements (the lowest density of spicules is found in the dorsal surface of the visceral sac, the part of the body which remains most prominent in contracted animals). Rod-shaped spicules with blunt ends are found most numerously in the foot, in the head appendages, at the base of the visceral sac and in parallel strips dorsolaterally of the pharynx ('cephalic spicule grid'). A skeletal function appears likely for the former three examples, in a position where the spicules might well function, in bulk, as stabilizing agents. A protective function (for the CNS) seems reasonable only for the cephalic spicules, as has already been suggested for *S. paradoxa* by Küthe (1935). We hypothesize an additional function of this spicular arrangement, namely acting as a supporting structure during feeding: the spicules might interlock and thus stabilize the pharyngeal region, while the head is pressed hard onto the neritid egg capsules in order to puncture their walls with the radula. Similar aggregations of spicules close to the pharynx have been reported in other acochlidian genera: in the microhedylacean *Asperspina* and *Pontohedyle* (Jörger *et al*., 2008) and as a "post-pharyngeal spicule collar" in the hedylopsacean *Tantulum elegans* (Neusser & Schrödl, 2007; Schrödl & Neusser, 2010); in *Acochlidium bayerfehlmanni* Wawra, 1980 (Bayer & Fehlmann, 1960; as *A. amboinense*) spicules are stated to form "a ring around the esophagus" similar to the situation found in *Strubellia*.

Cephalic gland

The loose aggregation of cells covering the cerebral ganglia was present in all individuals examined in this study, but has not been reported for any acochlidian species, including *S. paradoxa*. Neusser *et al*. (2007, 2009b) mention both "cells above the cerebral commissure" and "lateral bodies" attached to the cerebral ganglia in the interstitial acochlidians *Asperspina murmanica* and *Hedylopsis ballantinei*; these cells were, however, embedded within the connective sheath of the cerebral commissure. Supposedly endocrine "dorsal bodies"—surrounded by a connective sheath and associated with the cerebral ganglia—are common among pulmonates, where there is considerable diversity regarding structure and innervation (e.g. Boer, Slot & van Andel, 1968); they have been shown to be more active during female reproduction (Saleuddin, Ashton & Khan, 1997). In *S. wawrai* n. sp. there appears to be no connective sheath and there are no histologically detectable differences between juveniles and mature specimens.

In histology (loose tissue with yellow-stained vesicles visible in serial sections) and position the structure also resembles the 'blood' gland found in some anthobranch nudibranchs, e.g. the doridoidean *Corambe lucea* Marcus, 1959 (Schrödl & Wägele, 2001) and the dexiarchian *Doridoxa* (Schrödl, Wägele & Willan, 2001). However, the presence of apparently osmiophilic, yellow-staining vesicles indicates fatty substances, as are present in the digestive gland, possibly implying a function as an additional fat-storing structure. Ultrastructural research on cell anatomy and affiliation to the CNS is needed for conclusive identification of this organ, which might represent an apomorphy for either *Strubellia* or Acochlidiidae.

Heart and kidney

Only few shell-less heterobranchs venture into habitats that are regularly influenced by freshwater, e.g. some nudibranchs and sacoglossans (Barnes, 1994). The excretory system of the sacoglossan *Alderia modesta* (Lovén, 1844), found on partially brackish intertidal mudflats (Evans, 1951), has been examined in detail but lacks a heart and shows no apparent elaboration of its sac-like kidney (Fahrner & Haszprunar, 2001). Members of the recently described Aitengidae (also Acochlidia) live

amphibiously among mangroves or coastal rocks, and show an elaborate system of branched dorsal vessels (resembling the condition found in many plakobranchioid sacoglossans) which might originate from a histologically similar and sac-like kidney (Swennen & Buatip, 2009; Neusser *et al*., 2011a). Neither condition appears very similar to that found in *Strubellia*.

The circulatory and excretory systems of *S. wawrai* n. sp. show several apparent morphological adaptations to permanent life in fresh water, namely specialized cell types in the heart, elongated lumina of the kidney and nephroduct, and possibly the loop in the distal nephroduct. A strongly vacuolated epicardium and discrete thick-walled cells inside the lumen of the heart have been described only from *S. paradoxa* and the brackish-water *Pseudunela espiritusanta* (Neusser & Schrödl, 2009; Brenzinger *et al*., 2011). These cells possibly involve a novel site of ultrafiltration (on the ventricle) and aggregations of rhogocytes, however in both cases ultrastructural investigation is needed to identify those cell types. Muscular bridges spanning the lumen of the heart, presumably an aspect of an enhanced circulation, have been mentioned for *Acochlidium amboinense* (Bücking, 1933) and *S. paradoxa*.

In *Strubellia* there appears to be a functional division of otherwise elongated excretory lumina, judging from the separation of at least three histologically different zones (proximal and distal kidney lumina and nephroduct). The presence of the conspicuous upward loop of the distal nephroduct, which is closely associated with the pericardial wall, hints at the presence of a fourth zone involved in the modification of the primary urine. Again, ultrastructural studies are needed to test these observations derived from light microscopy.

Elongation of excretory lumina has been shown to be a feature of hedylopsaceans and is conspicuously present in the coastal mesopsammic *Pseudunela cornuta* (Challis, 1970) and *P. espiritusanta* (Neusser & Schrödl, 2009; Neusser *et al*., 2009a, Neusser, Jörger & Schrödl, 2011b) and the more basal but limnic *Tantulum elegans* (Neusser & Schrödl, 2007). All of these species display an elongate kidney with divided lumina and U-shaped nephroduct with distal loop. Members of the marine mesopsammic genus *Hedylopsis* also show the elongate, complex kidney, but have a short nephroduct (Fahrner & Haszprunar, 2002; Sommerfeldt & Schrödl, 2005). This means that the elaborate excretory system found in *Strubellia* is already more or less present in marine or brackish-water *Pseudunela* species (Neusser *et al*., 2011b) and thus further adaptations to life in freshwater are likely to have happened on an ultrastructural level.

There is only scarce information on the circulatory and excretory systems of *Acochlidium* species, although it appears to be more sophisticated. Bücking (1933) mentioned a branching vessel on the dorsal side of the visceral sac (superficially similar to that found in sacoglossans or Aitengidae) and the presence of multiple renopericardial funnels. It should be critically compared with the condition found in *Strubellia* to trace the evolution of characters in these organ systems that are crucially important in the colonization of limnic habitats.

Genital ontogeny

As was confirmed for *S. paradoxa* by Brenzinger *et al*. (2011), *S. wawrai* n. sp. appears to be a sequential, protandric hermaphrodite, as is otherwise known only for *Tantulum elegans* and *Hedylopsis* species among Acochlidia (Wawra, 1989; Neusser & Schrödl, 2007; Kohnert *et al*., 2011). The change of sex during ontogeny can be deduced (1) from the presence of two allosperm receptacles in otherwise male specimens and (2) the presence of intermediate stages (females with bursa copulatrix, seminal groove and copulatory apparatus still present but in various stages of reduction) (Wawra, 1988; present study). Sperm transfer appears to be via copulation and mainly in the male phase, after which the sex changes to a female state (gonad producing oocytes; female gland mass developed) while the strictly male genital features become reduced. This change is likely to be rapid since intermediate stages have rarely been found in previous studies of *Strubellia* species (Küthe, 1935; Wawra, 1988).

Genital system (posterior part)

The genital system of *S. wawrai* n. sp. was largely described by Wawra (1974, 1988; as *S. paradoxa*), assuming first gonochorism and then sequential hermaphroditism. We can confirm the description of the posterior genital system with a full set of sperm storing organs, i.e. the ampulla for autosperm and two allosperm receptacles (receptaculum seminis, bursa copulatrix), which is a condition known from the marine *Pseudunela cornuta* and the brackish-water *P. espiritusanta*, among Acochlidia. However, in both the latter species the receptaculum seminis is situated more proximally to the gonad than the sac-like ampulla (Neusser & Schrödl, 2009; Neusser *et al*., 2009a); this is in contrast to *S. paradoxa* and *S. wawrai* n. sp. where the receptaculum seminis is distal to the tubular ampulla. Except for its functional change during ontogeny, the gonad of *Strubellia* varies from the aforementioned genus by the separation into distinct follicles and the high number of eggs, both features shared with *Acochlidium fijiense* (Haynes & Kenchington, 1991; Haase & Wawra, 1996), probably reflecting a higher reproductive potential per individual. The female gland mass, developed from the very long gonoduct in 'males', is tubular all along and shows three histologically separable parts. This organ system is highly variable among *Pseudunela* and other acochlidians (but see Neusser *et al*., 2011b), where usually at least some of the glands are sac-like extensions and sometimes there appear to be only two different glands; the situation in *Acochlidium* species is unclear (see Schrödl & Neusser, 2010; Brenzinger *et al*., 2011).

The bursa copulatrix, reduced in the female phase, is similar to that of the marine hedylopsaceans in its morphology (bulbous, with thinner stalk) and its location next to the genital opening. *Acochlidium* on the other hand has been described to lack any allosperm receptacles due to its supposedly hypodermal mode of insemination (Haase & Wawra, 1996). The genital diverticulum next to the genital opening is a feature known also from *S. paradoxa* (Brenzinger *et al*., 2011); its variability in size (largest in one specimen from Vanuatu) and reduction in females hint at a function in copulation.

Strubellia shares the supposedly 'primitive' open seminal groove connecting to the genital opening distal to the bursa with *Hedylopsis spiculifera* Kowalevsky, 1901 (see Wawra, 1989). Other hedylopsaceans have been described to have a closed vas deferens that splits off the distal gonoduct proximal to the bursa and runs below the epidermis of the right body side (e.g. Neusser *et al*., 2009a; see Schrödl & Neusser, 2010). We suggest that the open seminal groove is not a plesiomorphic character *per se*, but is likely connected with ontogenetic sex change; as a transient feature, the duct remains only as a groove and is not sunk below the epidermis.

Cephalic copulatory apparatus

We disagree with Wawra's (1974) description of the cephalic copulatory apparatus which was based on dissected material missing the penis and associated glands; as in the description of *S. paradoxa* by Küthe (1935), the basal finger was erroneously interpreted as the penis. The copulatory organs of *S. wawrai* n. sp. consist of two distinct muscles with connected (para-)

prostatic glandular systems as in *S. paradoxa*, resembling the *Pseudunela* species known in detail (Neusser & Schrödl 2009; Neusser *et al.*, 2009a, 2011b). *Strubellia*, however, lacks the hollow penial stylet and instead features a solid spine near the penial opening, precluding sperm transfer by hypodermal injection which is believed to occur in *Pseudunela*, *Acochlidium* and a number of heterobranchs that possess one or several hollow penial stylets as an extension of the distalmost vas deferens (see Gascoigne, 1974; Haase & Wawra, 1996; Neusser *et al.*, 2009a).

The long and hollow stylet of the basal finger, however, appears to be used for (hypodermal) injection of the paraprostatic secretion; only in *Strubellia* does the stylet have the longitudinal groove. Both muscle and chitinous elements are more pronounced in *Strubellia* than in other genera, which imply a relatively higher importance of the paraprostatic system in this genus. Stylet morphology (and perhaps that of the penial spine) may also present a possibility to distinguish at least male specimens from the two *Strubellia* species by SEM: the basal finger stylet of *S. wawrai* n. sp. appears to be more elongate than that of *S. paradoxa* and shows a bent or slightly hooked tip (Table 4). This distinction is however only based on few male specimens and disregards the possibility of the stylet being flexible, as is mentioned for the chitinous penial stylets of some sacoglossan species (Gascoigne, 1974).

The paraprostatic duct has been mentioned to split at the base of the stylet in *S. paradoxa* and *S. wawrai* n. sp. from Guadalcanal (Küthe, 1935; Wawra, 1974; Brenzinger *et al.*, 2011), whereas it is undivided in the specimen from Vanuatu. This feature is of unclear function and may again be related to the individual stage of ontogeny, but is hard to detect and deserves reexamination.

Species-level relationships

Molecular data indicate that there are three separate lineages in the genus *Strubellia*, the first offshoot known only from the single juvenile specimen from Sulawesi examined herein. More material is needed to establish this population as a new species.

The eastern Melanesian specimens of *S. wawrai* n. sp. form a clade that is sister group to *S. paradoxa* from Ambon, Indonesia. Both clades receive strong bootstrap support and sequence divergence in COI (*c.* 12–13%) is relatively high. Both Species Identifier and parsimony network analyses indicate specific differences between *S. paradoxa* and *S. wawrai* n. sp. Given the 3,500-km distance between Ambon and the Solomon islands, this divergence is not surprising. Separation of *S. wawrai* n. sp. by only morphological means is not straightforward, since most organ systems previously used to separate acochlidian species are highly similar. However, there are some differences in parts of the copulatory apparatus, including length and curvature of the basal finger stylet (elongate and apically curved vs. rather stout and short in *S. paradoxa*; Brenzinger *et al.*, 2011) and form of the penial spine that might be useful features discernible by SEM. In both cases these differences refer to few mature individuals only, so ranges of intraspecific or ontogenetic variations remain poorly known. Variations in radular row counts, as already mentioned, are likely to be attributable to the size of the individuals examined. The presence of a second lateral plate in *S. paradoxa* has to be formally confirmed (Brenzinger *et al.*, 2011).

Summing up, potential differences in relevant parts of the copulatory organs, together with genetic evidence, leave little doubt that the populations from Ambon and Melanesia represent distinct species.

On a population level, the observed size disparity between mature specimens of *S. wawrai* n. sp. from the Solomon Island and Vanuatu is an obvious morphological difference, especially since female individuals from Vanuatu with remaining male genitalia were larger than already fully female specimens from Guadalcanal (Table 4). This observed delay in ontogeny is hard to explain given knowledge of the genetic similarity between the populations, but is perhaps attributable to ecological factors. Observed differences in the size of the genital diverticulum and the distal division of the paraprostatic duct (present/absent) are also likely to be variable during ontogeny. Analysis of molecular divergence shows that the Guadalcanal and Espiritu Santo populations of *S. wawrai* n. sp. are very similar, with the clade comprising the latter population nested inside the former, indicating that the split is too recent to be obvious from COI divergence. We therefore regard the two populations as a single species that might be close to separating into two species, with geographic separation preventing regular gene flow.

Habitats and dispersal

The localities discovered in this study fit well with the described habitat regarding physical and chemical properties, i.e. limestone slabs at the edge of shallow streams carrying mineral-rich and slightly alkaline water. *Strubellia* species co-occur with neritid gastropods (Starmühlner, 1976; Haynes, 2000). This is significant, since we observed *S. wawrai* n. sp. feeding on neritid eggs, resolving a longstanding mystery. In addition we know that different species and populations occur in limnic systems of more or less distant islands and archipelagos surrounded by sea.

Table 4. Comparison of morphological data of *Strubellia wawrai* n. sp. and *S. paradoxa*.

	S. wawrai n. sp.			*S. paradoxa* (Strubell, 1892)	
Reference	Wawra (1974, 1979, 1988)	Present study	Present study	Küthe (1935)	Brenzinger *et al.* (2011)
Collecting site	Guadalcanal, Solomon Is	Guadalcanal, Solomon Is	Espiritu Santo, Vanuatu	Ambon, Indonesia	Ambon, Indonesia
Max. recorded body size	~2.5 cm	~2.0 cm	~3.5 cm	~2 cm	~1 cm
Radula formula	48–51 × 1.1.2	43–46 × 1.1.2	59 × 1.1.2	48–56 × 2.1.2	38 × 1.1.2
1st lateral tooth denticle	Present	Present	Present	Absent	Present
Length of basal finger stylet	1 mm	?	0.75–1 mm	0.5 mm	0.6 mm
Stylet form	Elongate, tip hooked	?	Elongate, tip bent	Rather stout	Rather stout
Distal paraprostatic duct	Divided (Wawra, 1974: fig. 4)	?	Undivided	Divided	Divided
Genital diverticle	Small	?	Large	?	Small
Penial thorn	?, curved	?	Concave, curved	Flat (?), curved	Flat, curved

So, how do limnic slugs, generally hiding away under rocks during the day, disperse to or maintain gene flow between different localities, as is implied by the molecular analysis? Other stream gastropods with similar lifestyles, such as the numerous neritid species occurring in the rivers of Indo-West Pacific islands, reach distant islands by means of planktonic larvae (Haynes, 1988; Myers, Meyer & Resh, 2000) and regularly recolonize them; juveniles of at least one species even migrate by sometimes 'hitchhiking' upstream on the shell of larger individuals (Kano, 2009). Assuming a similarly amphidromic life with larvae hatching in freshwater and returning to it after a period of time and metamorphosis in the sea (see McDowall, 2007) would explain the observed distribution in *Strubellia*—but there are yet no observations of eggs or larvae of *Strubellia*. However, *Acochlidium fijiense* is known to produce gelatinous egg masses from which veligers hatch that are apparently not able to survive in fresh water (Haynes & Kenchington, 1991). In seawater, these veligers quickly metamorphosize into 'adhesive'-type larvae which remain alive for at least 2 months and glue themselves e.g. to the wall of the Petri dish they are kept in (own observations on *Acochlidium* sp.). This shows that limnic *Acochlidium*, and possibly already the common ancestor with *Strubellia*, have evolved a specialized larval type that might be able to disperse between islands of archipelagos leading to the colonization of rivers, involving a neritid-like amphidromic lifestyle. On one hand, these adhesive larvae, if quickly glued to a substratum outside the river, could avoid being drifted away too far into the ocean. Following juvenile neritids on their necessary movement upstream (possibly while glued to a shell during metamorphosis) and then feeding on their eggs would be a novel and efficient kind of symbiosis. On the other hand, it seems possible that this type of larva is able to use more mobile and far-ranging organisms as vectors between islands (planktonic organisms, fish, birds, boats). While acochlidiid larvae can survive in the laboratory for months without any movement or food uptake, metamorphosized juveniles would have to feed. Such juveniles would still be in the size range of most marine acochlidians (1–2 mm) and are not likely to prey on adult food, i.e. strongly mineralized neritid egg capsules. A juvenile stage feeding on microbial mats, mucus, algae or detritus is thus hypothesized. Field observations and laboratory experiments are needed to confirm the hypothesized life-history traits of *Strubellia*.

Larvae sticking to floating or swimming objects may therefore be the 'missing' dispersive stages explaining interisland dispersal, such as from the Solomon Islands to Vanuatu in the case of *S. wawrai* n. sp., or the colonization of Palau or Fiji in the case of *Acochlidium bayerfehlmanni* and *A. fijiense* (Bayer & Fehlmann, 1960; Haynes & Kenchington, 1991). Since limnic Acochlidiidae are estimated to have originated in the Palaeogene (Jörger *et al*., 2010a), this long period would present a timeframe to have enabled dispersal via island-hopping, facilitated by lower sea levels and shorter distances between islands in Indonesia during much of the period. Dispersal to the west might have been limited by deeper-water currents being deflected at the border of the Southeast Asian continental shelf, as is indicated by Wallace's-line distributional patterns of marine organisms with pelagic larval stages (Barber *et al*., 2000). The lack of records of acochlidiids west of the Wallace line hints at a similar limitation. On the other hand, it appears likely that numerous populations of acochlidiids are yet to be discovered and also that many have become extinct.

Phylogeny of Strubellia *and evolution of characters*

The molecular phylogeny of the acochlidiids shows *Strubellia* to have originated in Indonesia. The genus is sister group to the morphologically more derived *Acochlidium* and *Palliohedyle*, these in turn being sister group to the marine interstitial Pseudunelidae. This configuration is congruent with the previously proposed phylogenies of Acochlidia, based on morphology (Schrödl & Neusser, 2010) or molecular markers (Jörger *et al*., 2010a).

According to the new results, the apomorphies for Acochlidiidae are the limnic habitat, benthic and probably amphidromic lifestyle, accompanied by large body size and distinct epidermal pigmentation, and the finely serrated rhachidian teeth. The visible distinction of the anterior mantle border and heart 'bulb', complex kidneys and the bipartite copulatory organs with spines and associated glands are already present in the mesopsammic *Pseudunela* species (Neusser & Schrödl, 2009; Neusser *et al*., 2009a, 2011b).

Presence of an osphradium and oophagy might represent further apomorphies; however, we suggest that the presence of epidermal sensory cells is likely at least in the hedylopsacean species with an osphradial ganglion. Furthermore, we suggest that a piercing-and-sucking mode of feeding is typical for Acochlidia, since all share the muscular pharynx, a slender radula that appears ill-equipped for grazing, and sometimes arrays of spicules surrounding the pharynx. For the meiofaunal species, instead of grazing, sucking liquid contents from soft, encapsulated food such as large-bodied protists or eggs of sand-dwelling organisms might explain the coloration of some species' digestive glands (e.g. brown or green in *Pontohedyle milaschewitchii*; Jörger *et al*., 2008), the lack of both abraded teeth and mineral residues in the digestive system. The sacoglossan-like monostich radula of the microhedylacean Ganitidae (Challis, 1968) would thus be specialized for a specific type of food, but not a unique mode of feeding within the group. Given the similarity of the pharynx and radula (slender ribbon, triangular median tooth with serrated margins, flat or reduced laterals) in Sacoglossa (especially the basal *Cylindrobulla*; Mikkelsen, 1998), Aitengidae (Swennen & Buatip, 2009; Neusser *et al*., 2011a), Amphibolidae (Golding, Ponder & Byrne, 2007) and Glacidorbidae (Ponder, 1986; Ponder & Avern, 2000), the suggested mode of feeding by piercing and sucking might represent a basal panpulmonate feature. Somewhat similar to *Strubellia*, both Sacoglossa and *Aiteng ater* are known to feed by puncturing internally soft food (siphonal algae and insect pupae, respectively) and sucking out the contents (Jensen, 1980, 1981; Swennen & Buatip, 2009); some Sacoglossa are also known to feed on the more or less gelatinous egg masses of opisthobranch gastropods (see Jensen, 1980; Coelho, Malaquias & Calado, 2006). However, some Euopisthobranchia *sensu* Jörger *et al*. (2010a) show similar, narrow radulae with serrated rhachidian and flat lateral teeth, e.g. species of the cephalaspidean *Toledonia* (Marcus, 1976; Golding, 2010), and also several nudibranchs (such as the oophagous aeolidioidean *Favorinus*; Schmekel & Portmann, 1982), making it difficult to detect phylogenetic patterns. An example is the proposed relationship of *Toledonia* and Acochlidia on the basis of radular morphology (Gosliner, 1994), which according to more recent hypotheses clearly represents a case of convergent evolution (Jensen, 1996; Sommerfeldt & Schrödl, 2005; Jörger *et al*., 2010a; Schrödl *et al*., 2011). Furthermore, a slender piercing radula is also present in *Omalogyra atomus* (Philippi, 1841) ('lower Heterobranchia'; Bäumler *et al*., 2008).

Synapomorphies of *Strubellia* appear to be the reddish-brown pigmentation, very slender rhachidian teeth, three receptacles in the male phase, the genital diverticulum, the enhancement of the basal finger with the stylet having a lateral groove, and the possession of a single flat hook on the penis instead of a hollow penial stylet.

The organization of the posterior genital system of *Strubellia* essentially conforms to the 'classic' idea of plesiomorphic monauly that was suggested to be the condition found in the hermaphroditic "opisthobranch common ancestor" (Ghiselin, 1966; Gosliner & Ghiselin, 1984), however the condition of *Strubellia* is fundamentally different. All hedylopsaceans are (special) androdiaulic hermaphrodites (Schrödl & Neusser, 2010; Schrödl *et al*., 2011) except for *Strubellia* (and *Hedylopsis* species; see Wawra, 1989; Sommerfeldt & Schrödl, 2005; Kohnert *et al*., 2011). The derived phylogenetic position of *Strubellia* (Jörger *et al*., 2010a; Schrödl & Neusser, 2010) suggests either a reversal to a monaulic system (with sperm and oocyte pathways not separated anatomically but in time, with a secondarily open seminal groove) or multiple developments of diauly among Acochlidia. The presence of allosperm receptacles already in the male phase might have led to the evolution of defined breeding seasons in *Strubellia*, hinted at by the strong skew among sexes revealed from sampling in all known localities: specimens were either predominantly juvenile, or only either male or female (Küthe, 1935; Wawra, 1988; present study). This might also be related to the observation that *Strubellia* generally aggregates in groups: If *Strubellia* has defined breeding seasons (possibly the rainy seasons accompanied by changes in riverine water levels) then aggregations of numerous specimens might mate after which the specimens change sex synchronously, spawn and then either die or fully reduce their genital organs, as was suggested for *A. fijiense* (Haynes & Kenchington, 1991). This appears at least possible, since complete reduction of the large copulatory apparatus during ontogeny can be deduced from the observations presented here, and a strong reduction of body size likely connected with a reduction of organs has been observed after periods of starvation in the specimens maintained in aquaria for this study.

Strubellia differs externally from *Acochlidium* and *Palliohedyle* by its more slender body, elongate visceral sac (versus leaf-shaped and flattened) and uniform reddish coloration (*vs* greenish-brown and black pigmentation), making it externally more similar to the aforementioned *Pseudunela* species (e.g. Haynes & Kenchington, 1991; own observations). According to the literature, internal differences from the better-known *Acochlidium* species include shape of the rhachidian teeth (very elongate in *Strubellia vs* triangular), morphology of the penis (relatively small with single apical thorn in *Strubellia vs* large and multi-spined; e.g. Wawra, 1979, 1980; Haase & Wawra, 1996) and basal finger (larger than the penis and with long stylet in *Strubellia*), the mode of genital ontogeny (protandric hermaphroditism in *Strubellia vs* hermaphroditism; Haynes & Kenchington, 1991) and the elaboration of visceral organs (multiple renopericardial funnels, digestive gland lobes, praeampullary gonoducts and branched, dorsally situated vessels connected to the heart in *Acochlidium*; Bücking, 1933; Haase & Wawra, 1996). Since the only comprehensive anatomical description of an *Acochlidium* species is very old (Bücking, 1933) and the only detailed study of the genital system includes characters that are still unclear (e.g. a connection between the ampulla and the digestive gland; Haase & Wawra, 1996), revision of the aforementioned anatomical features is urgently needed to trace the evolution of these unique limnic slugs.

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Chapter 3. Kohnert P, **Brenzinger B**, Jensen KR & Schrödl M (2013a): **3D- microanatomy of the semiterrestrial slug *Gascoignella aprica* Jensen, 1985--a basal plakobranchacean sacoglossan (Gastropoda, Panpulmonata).** *Organisms, Diversity and Evolution,* **13**:583-603.

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ORIGINAL ARTICLE

ORGANISMS DIVERSITY & EVOLUTION

3D- microanatomy of the semiterrestrial slug *Gascoignella aprica* Jensen, 1985—a basal plakobranchacean sacoglossan (Gastropoda, Panpulmonata)

Peter Kohnert · Bastian Brenzinger · Kathe R. Jensen · Michael Schrödl

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Abstract The monophyly of the panpulmonate, usually marine benthic Sacoglossa—and its basal division into shelled Oxynoacea and shell-less Plakobranchacea—is undisputed, but family relationships are in doubt. Of particular interest is the potentially basal plakobranchacean family Platyhedylidae, comprising morphologically aberrant members lacking head tentacles or body appendages. Herein we re-describe the type species of the genus *Gascoignella*, *G. aprica* Jensen, 1985, from Hong Kong. Morphological data was generated by three-dimensional reconstruction from serial semi-thin sections using Amira software. Our microanatomical results largely confirm the original description. The anterior digestive system is sacoglossan-like but modified, e.g. the ascus is not demarcated externally and pharyngeal pouches are lacking. The digestive gland is bipartite, with two rami separated by a longitudinal, muscular, median septum, but fused in the rear end. The postpharyngeally situated nerve ring contains fused cerebropleural ganglia; the short visceral loop has three ganglia. Two major cerebral nerves were identified as rhinophoral and labiotentacular nerves, innervating sensory areas on the head velum. *Gascoignella aprica* is a hermaphrodite with a truly androdiaulic genital system of which some originally ambiguous characters are clarified. Bursa and prostate insert into a fertilization chamber proximal to a sac-like albumen gland and a tubular mucus gland. The cephalic copulatory apparatus contains a penis armed with a short and straight stylet and an accessory gland of unclear function; the presumed mode of sperm transfer is discussed. A well-developed heart and a large H-shaped kidney are present; the nephroduct opens into the intestine. Epidermal glands and body tissues are described for the first time. The presence of a unique longitudinal, median septum is considered diagnostic for Platyhedylidae, multiple further apomorphies are given. Morphological evidence supports the molecular phylogenetic hypothesis that the Platyhedylidae could be a basal non-shelled sacoglossan lineage.

Keywords Mollusca · Opisthobranch · Sea slug · Phylogeny · Morphology · Evolution

Introduction

Molecular phylogenetic studies (e.g. Klussmann-Kolb et al. 2008; Dinapoli and Klussmann-Kolb 2010; Jörger et al. 2010) challenged conventional ideas on euthyneuran gastropod phylogeny. Rather than being divided into monophyletic Opisthobranchia and Pulmonata (e.g. Gosliner 1994; Wägele et al. 2008), previous "opisthobranch orders" are distributed over the "new euthyneuran tree" (Schrödl et al. 2011a). The Panpulmonata (Jörger et al. 2010) comprise traditional pulmonates, but also previously lower heterobranch Pyramidellidae and Glacidorbidae, and two "opisthobranch orders", i.e. Acochlidia and Sacoglossa. The Acochlidia are a modestly diverse taxon of mainly tiny mesopsammic marine slugs (Schrödl and Neusser 2010), but also brackish-water, limnic, and even amphibious species exist (Neusser and Schrödl 2007, 2009; Neusser et al. 2011b; Brenzinger et al. 2011a). The

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P. Kohnert (✉) · B. Brenzinger · M. Schrödl
SNSB Bavarian State Collection of Zoology, Sektion Mollusca, Münchhausenstr. 21,
81247 Munich, Germany
e-mail: PeteKohnert@gmail.com

P. Kohnert · B. Brenzinger · M. Schrödl
Department Biologie II, Biozentrum, Ludwig Maximilians-Universität, Großhaderner Str. 2,
82152 Planegg-Martinsried, Germany

K. R. Jensen
Zoological Museum, Natural History Museum of Denmark, Universitetsparken 15,
2100 Copenhagen, Denmark

second group, Sacoglossa, are marine or brackish water species, suctorially feeding on algae (Jensen 1981, 1993a,b,c, 1997). The greenish body colouration of most members derives from the chloroplasts of their prey (Clark et al. 1990). The globally known roughly 300 species of Sacoglossa are usually characterised by having an ascus, i.e. a muscular sac storing worn radula teeth, a uniseriate radula used for piercing algae, and an esophageal pouch (Jensen 1991, 1996; Mikkelsen 1996; Wägele et al. 2008).

Because some members share a shell-less body, a uniseriate radula with dagger-like teeth and an androdiaulic genital system, Acochlidia were thought to be closely related with Sacoglossa (e.g. Gosliner 1994; Sommerfeld and Schrödl 2005). The acochlidian Ganitidae with a sacoglossan-type radula, however, were shown to be derived rather than basal acochlidians in both morphology-based and molecular phylogenetic analyses (Jörger et al. 2010; Schrödl and Neusser 2010), and acochlidian and sacoglossan androdiaulic systems differ regarding the relative insertion of the vas deferens, which is proximal in sacoglossans and distal in acochlidians (Schrödl et al. 2011a). The single described mesopsammic sacoglossan species *Platyhedyle denudata* Salvini-Plawen, 1972, was originally thought to be acochlidian due to features such as a worm-like body with reduced head tentacles, and having separate cerebral and pleural ganglia in a postpharyngeal central nervous system. However, later studies by Jensen (1985), Wawra (1988) and Rückert et al. (2008) showed distinct morphological differences, proved its sacoglossan nature and explained similarities by habitat-induced convergence. In fact, Sacoglossa clustered as sister of various clades with interstitial members (Rhodopemorpha, philinoglossid cephalaspideans and Acochlidia) in a morphocladistic analysis by Wägele and Klussmann-Kolb (2005). This association was shown to be an artefact by all available molecular data (Jörger et al. 2010; Wilson et al. 2010; Schrödl et al. 2011a). Recently, the new family Aitengidae was established for some amphibious "bug-eating slugs" that show mixed sacoglossan and acochlidian features (Swennen and Buatip 2009). However, computer-aided microanatomical three-dimensional (3D) reconstructions and multi-locus sequence marker analysis place the Aitengidae within Acochlidia (Neusser et al. 2011b).

Alternative hypotheses on the origin of Sacoglossa include descendance from cephalaspidean euopisthobranchs. The infaunal sacoglossan *Ascobulla*, having large radula teeth with long triangular cusps for piercing algae and an ascus for storing worn teeth, apart from pharynx features, closely resembles *Cylindrobulla*—an equally infaunal genus with small teeth with short triangular cusps and a poorly developed ascus (Jensen 1995, 1996). The latter genus was either considered member of cephalaspideans (Jensen 1996), or the most basal sacoglossan offshoot (i.e. sister of all other sacoglossans) (Mikkelsen 1996, 1998). Molecular data, however, show that *Cylindrobulla* is an ingroup member of panpulmonate oxynoacean sacoglossans (Mikkelsen 1998; Maeda et al. 2010; Jörger et al. 2010; Göbbeler and Klussmann-Kolb 2011; Neusser et al. 2011b), implying that similarities with euopisthobranch cephalaspideans, such as the head shield used for digging in sand, are secondarily derived (see Malaquias et al. 2009; Brenzinger et al. 2012). "Rampant parallelism" as stated already by Gosliner (1981) to be typical for opisthobranchs thus expands to all euthyneurans and molecular phylogeny shows that even more features are concerned (Schrödl et al. 2011a).

Recent multi-locus studies all place Sacoglossa within Panpulmonata, usually in a rather basal position. Sacoglossa were either recovered as sister to intertidal limpet-shelled Siphonarioidea (Klussmann-Kolb et al. 2008; Dinapoli and Klussmann-Kolb 2010; Jörger et al. 2010), as sister to all non-siphonarioidean panpulmonates (Göbbeler and Klussmann-Kolb 2011), or as sister to those panpulmonates that still bear an operculum (estuarine Amphiboloidea plus parasitic marine Pyramidellidae plus limnic Glacidorbidae) in some analyses by Jörger et al. (2010). The latter relationship, but under a traditional Opisthobranchia concept (for a rebuttal see Schrödl et al. 2011b), was recovered also by mitochondrial genomic sequence data and the group called Siphoglossa (Medina et al. 2011). Siphoglossa, however, were not recovered monophyletic in a comprehensive mitogenomic approach by Stöger and Schrödl (2012).

In addition to the unique ascus there may be more features apomorphic for sacoglossans, but this depends on the assumed origin and inclusiveness of the group. Kleptoplasty, with non-functional chloroplasts stored in body tissue was reconstructed as sacoglossan synapomorphy by Maeda et al. (2010), and retained (or lost) among shelled Oxynoacea. Functional kleptoplasty, retaining chloroplasts and using photosynthetic carbohydrates, according to Händeler et al. (2009), evolved within another major sacoglossan subclade called Plakobranchoidea, comprising sea slugs with dorsolateral prolongations of the foot edges (parapodia). Sacoglossans show a head-foot with usually one pair of longitudinally enrolled tentacles (rhinophores), except for some derived plakobranchaceans that have digitiform or bifid tentacles, or none at all, like intertidal *Gascoignella* and interstitial *Platyhedyle*; the latter genera combined into Platyhedylidae (Jensen 1985, 1996; Rückert et al. 2008).

The aberrant family Platyhedylidae comprise very few members, i.e. the mesopsammic Mediterranean *Platyhedyle denudata* Salvini-Plawen, 1973, the intertidal *Gascoignella aprica* Jensen, 1985, and two further species from intertidal mudflats of Thailand. Swennen (2001) described *G. nukuli* and *G. jabae* based mainly on external and radular rather than microanatomical details. While *G. nukuli* externally resembles other Platyhedylidae regarding the absence of tentacles and body processes, and in having a pair of digestive gland rami fusing in the rear part, *G. jabae* differs

externally, showing a pair of posterior, elongated cerata. A fourth record of a putative yet unidentified "*Gascoignella* sp." depicted in Gosliner et al. (2008: p. 65), rather appears to be an *Aiteng*. In having the head-foot separated from the visceral sac, shell-less Platyhedylidae (except *G. jabae*) resemble the shelled Oxynoacea. In morphocladistic analyses, aberrant Platyhedylidae were recovered as rather basal plakobranchacean offshoot (Jensen 1996), with exact systematic position uncertain, or forming an internal Plakobranchoidean branch (Mikkelsen 1998). Molecular multi-locus analyses showed *G. nukuli* in a basal plakobranchacean position (Jörger et al. 2010). Recent analyses additionally including *P. denudata* recovered monophyletic Platyhedylidae as a basal plakobranchacean branch (Neusser et al. 2011b). However, taxon sampling is not yet dense enough to reveal the exact origin and inner phylogeny of platyhedylids. Thus, morphology is currently the only available source of information on all known members of this fascinating sacoglossan family, and should be studied and interpreted in the light of modern euthyneuran tree hypotheses.

Gascoignella aprica Jensen, 1985, was described as the type species for the genus *Gascoignella* in the newly established family Gascoignellidae by Jensen (1985). Going into considerable morphological detail, the more or less conventional nature of the digestive system was recognised, while the complex reproductive system was apparently distinct from any other sacoglossan. The combination of reduced external and complicated reproductive characters did not fit into traditional sacoglossan family level classification. Jensen (1985) also recognised external similarities between *Gascoignella aprica* and the former acochlidian *Platyhedyle denudata*, such as the shared presence of a longitudinal septum separating the left and right halves of the visceral cavity. Main distinctive features were the fused versus separate cerebral and pleural ganglia, the absence versus presence of spicules and hermaphroditism versus dioecy. Jensen (1985) doubted *P. denudata* to be the single sacoglossan with separate cerebropleural ganglia, and predicted that, if ganglia were shown to be fused, the families Platyhedylidae and Gascoignellidae could be merged. Ultimately, Jensen's (1985) assumption was confirmed by a series of increasingly more detailed morphological re-examinations. The original description of *Platyhedyle denudata* by Salvini-Plawen (1973) was based on preserved material and squeezing techniques. Wawra (1988, 1991) corrected and supplemented it based on observations of living specimens and paraffin/paraplast based histological serial sections (7–8 μm). Huber (1993) re-examined its central nervous system and showed *P. denudata* to have the usual sacoglossan type cerebropleural ganglia. Rückert et al. (2008) used resin based histology with computer-aided reconstruction of all major organ systems from serial semithin histological sections (1.5 μm). Central nervous and other features of *P. denudata* were checked, confirmed or corrected and supplemented by details undetectable from thicker paraffin/paraplast slides. In general, recent 3D microanatomical approaches have shown earlier original anatomical descriptions to be erroneous to a surprising extent, especially referring to small euthyneuran species (e.g. Neusser et al. 2006, 2009b; Neusser and Schrödl 2007; Brenzinger et al. 2012). Careful histological analyses and reconstructing 3D models using AMIRA are well-suited to reveal detailed, accurate and reproducible data (DaCosta et al. 2007), and were applied successfully to lower heterobranchs (e.g. Brenzinger et al. 2011b; Haszprunar et al. 2011), Nudibranchia (DaCosta et al. 2007; Martynov et al. 2011), and Cephalaspidea (Golding 2010; Brenzinger et al. 2012).

This study thus uses modern semithin histological analyses and computer-aided 3D modelling with AMIRA software to explore the microanatomy of *Gascoignella aprica* in depth. Material collected at the type locality in Hong Kong were embedded in resin and cut into semithin sections. Our aims were to (1) check and supplement the original description of *G. aprica*, especially focussing on details of the taxonomically and phylogenetically important central nervous and reproductive systems, (2) compare this information with literature data on the microanatomy of *Platyhedyle denudata*, (3) find potential synapomorphies and evaluate the origin of the supposedly basal sacoglossan family Platyhedylidae on the basis of our novel morphoanatomical data.

Material and methods

The two specimens of *G. aprica* used in this study were collected by K.R.J. in a tidal mudflat in Tsim Bei Tsui, Deep Bay, Hong Kong in April 1998. They were found crawling on an exposed algal mat at low tide during the day. They were relaxed in an isotonic solution of magnesium chloride, fixed in 4 % neutral formaldehyde and stored in 75 % ethanol. The fixed specimens were stored at the Zoological Museum in Copenhagen (ZMUC, unnumbered; section series 4F3 and 4F4). Specimens were postfixed by transferring them into a 0.01 M cacodylate buffer solution with 1 % osmium tetroxide for 1.5 h, and eluted in cacodylate buffer. Both specimens of *G. aprica* were decalcified in 1 % ascorbic acid, dehydrated in a graded acetone series (30, 50, 70, 90 and 100 %) and transferred into Spurr's low viscosity epoxy resin (Spurr 1969). Resin blocks were transformed into ribboned serial sections (1.5 μm) using a diamond knife (Diatome Histo Jumbo, http://www.emgrid.com.au) installed on a rotation microtome (Microm HM 360, Zeiss, Jena, Germany), according to standard procedures (Henry 1977; Ruthensteiner 2008). Slices were stained using Richardson's stain (Richardson

et al. 1960), a 1:1 mixture of methylene blue-azure II stain and distilled water and sealed with Araldit resin (Romeis 1989) under coverslips.

Sections of specimen 4F4 were photographed using a Leica DMRD microscope, and a Spot Insight Color digital camera, (Diagnostic Instruments, http://www.spotimaging.com) and Spot 3.1 software (Diagnostic Instruments). Pictures were saved as .tif at a resolution of 1600×1200 pixels and 24-bit colour depth.

The reconstruction of all major organ systems of specimen 4 F4 was done using Amira 5.2.0 (Visage Imaging, Berlin, Germany) following basically the procedures described by Neusser et al. (2006) and Ruthensteiner (2008). Sections of specimen 4 F3 were used for comparison.

An interactive 3D model (accessible through the supplementary material) was compiled according to the procedure described by Ruthensteiner and Heß (2008).

Results

General morphology

The body (length 2.54 mm) of *Gascoignella aprica* appears dorsoventrally flattened. It consists of an anterior head-foot complex and a posterior visceral mass, separated from each other by a muscular, transversal diaphragm that forms an externally visible groove (mdg, Fig. 1a). There are no rhinophores, cerata or parapodia. The head-foot complex features a cephalopedal groove, in which the mouth is situated. The body cavity of the head-foot comprises digestive organs (oral tube, oral glands, pharynx, salivary gland and esophagus) as well as the postpharyngeal CNS, a prostate gland and the male copulatory complex (Fig. 1b). The foot is short, wide, ciliated and does not reach far under the visceral sac.

The visceral sac is in large part divided longitudinally by a vertical muscular septum. This so-called median septum (Rückert et al. 2008) is less distinct in the anterior part of the visceral sac, where it is penetrated by the intestine, the anterior anastomosis of the digestive gland and the gonoduct. Posteriorly, the septum is penetrated by a second anastomosis of the digestive gland. The septum creates an externally visible, longitudinal furrow on the dorsal side of the visceral sac. The intestine is connected to the digestive gland. The latter is a massive, split organ, which fills in large part both ventral sides of the visceral sac. The dorsal part of the visceral sac is filled with the gonad and a bursa on the left, and an arrangement of male and female glands on the right, i.e. prostate and two nidamental glands. Externally, apart from the mouth, three more openings are visible: the male

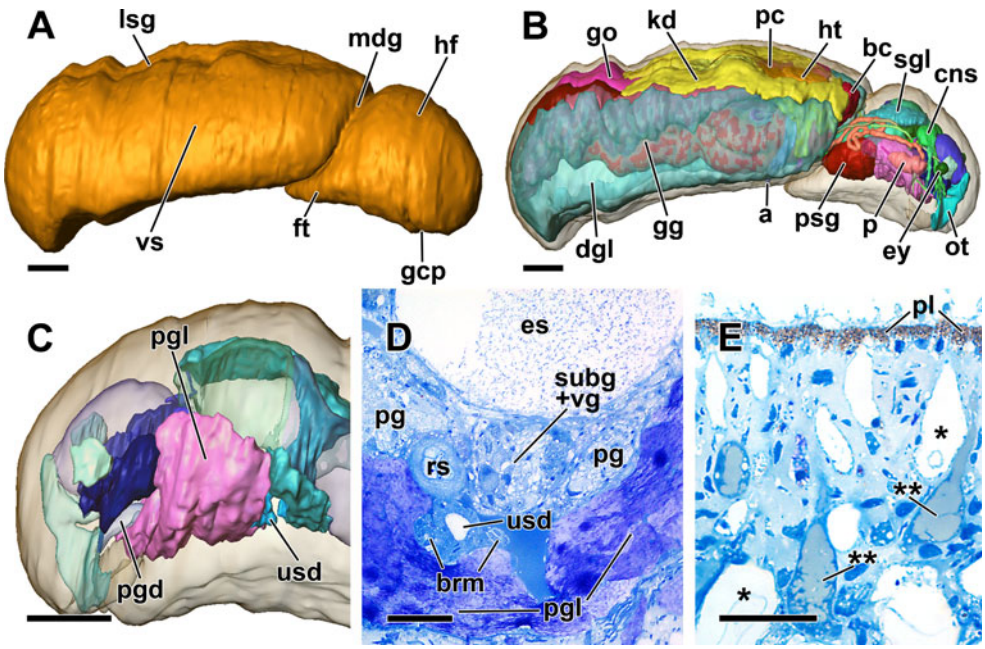

Fig. 1 **a**–**e**. Anatomical overview, three dimensional (3D) reconstruction of pedal gland and semithin cross-sections of pedal gland and epidermis of *Gascoignella aprica* Jensen, 1985. **a**–**c** 3D reconstructions: **a** external morphology of specimen 4F4, right view. **b** Overview of microanatomy showing internal organ systems, right view. **c** Pedal gland, ventrally encompassing other organs of the head-foot complex. **d** Cross section of the pedal gland ventral of the central nervous system. **e** Dorsal epidermis of the visceral sac, * Voluminous "type1" cells, ** greenish "type2" cells. *a* Anus, *bc* bursa copulatrix, *brm* buccal retractor muscle, *cns* central nervous system, *dgl* digestive gland, *es* esophagus, *ey* eye, *ft* foot, *gcp* cephalopedal groove, *gg* complex of genital glands, *go* gonad, *hf* head-foot complex, *ht* heart, *kd* kidney, *lsg* groove caused by longitudinal septum, *mdg* groove caused by muscular diaphragm, *ot* oral tube, *p* penis, *pc* pericardium, *pg* pedal ganglion, *pgd* pedal gland duct, *pgl* pedal gland, *pl* pigment layer, *psg* penial sheath gland, *rs* radula sheath, *sgl* salivary glands, *subg* subintestinal ganglion, *usd* unconfirmed salivary duct, *vg* visceral ganglion, *vs* visceral sac. *Bars* **a**, **b**, **c** 200 μm; **d** 50 μm; **e** 25 μm

genital opening under the right eye, the female genital opening laterally in the groove caused by the diaphragm, and the anus lateroventrally on the visceral sac.

Epidermis

The epidermis is rather smooth, consisting mainly of glandular structures and interspersed tegmental cells (Fig. 1e). It is mostly 35–40 μm thick, except for the groove caused by the muscular diaphragm, where the epidermis is thinner (ca. 10 μm). The epidermis of the dorsal and lateral sides of the head-foot and the visceral mass is pigmented. The yellow-to-brownish stained pigment granules are situated apically in the epidermal cells, forming a smooth surface of ca. 8 μm thickness.

There are at least three types of glandular cells in the epidermis:

"Type 1"- glands constitute the largest part of the epidermis. These glands appear to a great extent optically empty (i.e. white), but almost all of them contain spherical to longish, light gray stained and amorphous structures (Figs. 1e and 11e). The cells are bottle-shaped (diameter up to 35 μm) and open to the exterior via an apical pore. This type of gland is prevalent in the dorsal and lateral sides of the visceral-sac and the head-foot, and less frequent in the foot sole and ventral side of the visceral sac.

"Type 2"-glands are medium-sized (diameter up to 20 μm), monocellular and spherical. The light-blue-stained cytoplasm of their cells encloses a greenish stained, amorphous content. The nucleus is stained dark blue. These cells occur in the dorsal and lateral epidermis exclusively. Most of them open to the exterior via a digitiform duct and an apical pore.

"Type 3"-glands (not shown) are formed by accumulations of cells with a diameter of up to 25 μm. These cells contain a blue-stained nucleus and are filled with spherical, violet-stained vesicles. Thin ducts connect these glands to the outside.

In contrast to the dorsal and lateral epidermal cells, the ones of the foot show a dense apical ciliation. The "type 3"-glands prevail in the foot-area.

Anterior pedal gland

In the head-foot complex, a massive, sac like, light violet stained pedal gland is present (Figs. 1c, d, 2b, 7c and 11e). It opens to the outside via a broad, tubular connection (Ø up to 80 μm) to the mouth area, ventrally of the oral tube (Fig. 1c). The gland extends ventrally of the pharynx, the pedal ganglia and the esophagus. The latter two structures are encompassed laterally by the gland. This anterior pedal gland is 320 μm long and has a maximum width of 425 μm. The glandular cells are filled with small spherical granules and stained in different nuances of purple. In the posterior part of the gland, parallel structures with a filamentous appearance can be found. They are stained dark purple (Figs. 7c and 11e).

Musculature

Body musculature consists of blue staining muscle fibers that either extend through the body or are associated closely to specific organs. A thin sheath of muscle fibers (about 3 μm thick) is situated just below the epidermal basal lamina. Numerous muscle fibers pervade the head-foot area in dorsoventral orientation and cross on the ventral side, forming a basket-like web, in which the inner organs are located. The most complex muscular structures in the head-foot are the buccal mass and the penis (see digestive and genital system, respectively).

There is a paired buccal retractor (about 15 μm high and 25 μm wide) which splits up into two strands anteriorly; one part inserts close to the connection of oral tube and pharynx, the other is attached to the posterior portion of the buccal mass, ventral of the ascus. Further posteriorly the buccal retractor (Figs. 1d and 2b) connects to the diaphragm. This is a transversal, muscular structure, dividing the visceral sac from the head-foot. The diaphragm is up to 20 μm thick and penetrated by the esophagus, the aorta, the vas deferens and some nerves (see central nervous system). Additionally, a paired muscle runs from the anterior part of the buccal mass to the base of the bulge-like structures in the oral tube (not shown).

The median septum, which divides the visceral sac into a right and a left part, is not as distinct as, and thinner (up to 5 μm) than the diaphragm. However, the separation of the digestive gland in a left and a right ramus clearly shows its presence (see Figs. 3 and 4b). Two muscular layers arise from the left and right ventral side of the visceral sac and form the median septum, creating a ventral, hemolymph-filled space between their bases.

Digestive system

The mouth is situated medioventrally in the groove between the head and the foot (Fig. 4a, b). The short oral tube possesses thick columnar epithelial cells with a basal lying nucleus. Close to the pharynx, the oral tube widens, and the wall of the tube forms two lateral notches. These notches comprise bulge-like structures, which enclose the entrance to the pharynx.

Two pairs of adjacent but separate oral glands (Figs. 2a, 3 and 4d) seem to secrete into the posterior lumen of the oral tube, close to the entrance of the pharynx. Both glands (Fig. 2a) contain small, granular vesicles, but they differ in staining, location and size. The bigger glands are stained

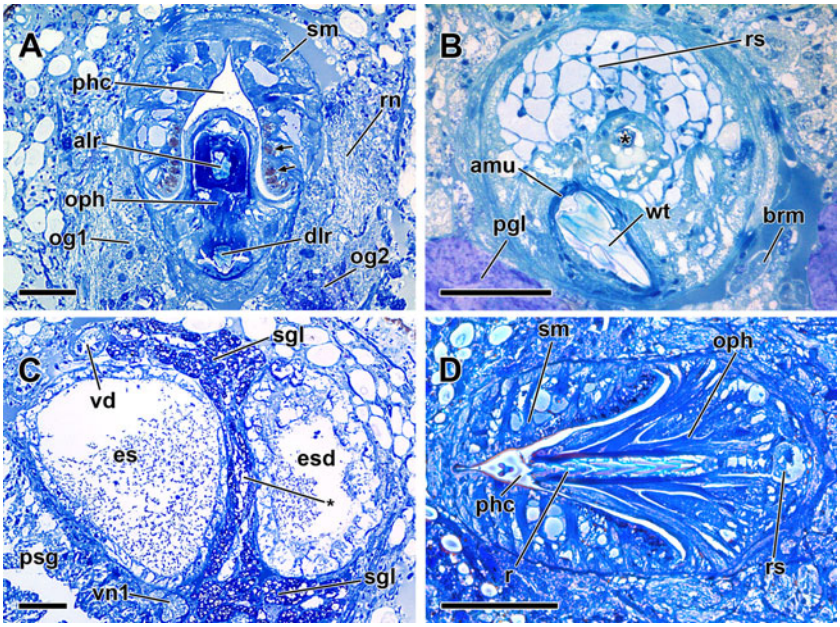

Fig. 2 Semithin cross- (**a**–**c**) and longitudinal-sections (**d**) of the digestive system of *G. aprica*. **a** Cross section at the middle of the buccal mass; *arrows* pigment granules. **b** Posterior end of the buccal mass; note origin of the radula (*) and sourrounding odontoblasts. **c** Esophagus and esophageal diverticulum, interconnection of the salivary glands (*). **d** Longitudinal section of the buccal mass of specimen 4 F3 showing the ascending limb of the radula. *alr* Ascending limb of the radula, *amu* ascus muscle, *brm* buccal retractor muscle, *dlr* descending limb of the radula, *es* esophagus, *esd* esophageal diverticulum, *og1* oral gland 1, *og2* oral gland 2, *oph* odontophore, *pgl* pedal gland, *phc* pharyngeal cavity, *psg* penial sheath gland, *r* radula, *rn* rhinophoral nerve, *rs* radula sheath, *sgl* salivary glands, *sm* septate muscle, *vd* vas deferens, *vn1* visceral nerve 1, *wt* worn teeth. *Bars* **a**–**c** 50 μm, **d** 100 μm

light grayish, and posses big, darker gray stained nuclei. Due to their unknown function and homology, we name them "oral gland 1". The second pair of glands ("oral gland 2" herein) contains vesicles of different staining, from grayish to dark blue. Nuclei are visible, but they are much smaller than the ones of "oral gland 1".

The large and bulbous buccal mass (Fig. 4c) is a prominent structure of the head-foot complex (350 μm long, 190 μm wide and 245 μm high). The pharyngeal cavity (Figs. 2a, d and 4c) is connected anteroventrally to the oral tube and lined with a thin, homogeneously blue stained cuticle. In cross section, the cavity appears nearly triangular in its anterior part, and flattens progressively towards the connection with the esophagus on the posterodorsal side of the pharynx (Fig. 2a). The cavity is encompassed dorsally by a thick and massive muscular structure of alternating bands of different orientation. This septate muscle (Gascoigne 1979) is horseshoe-shaped. On its median sides, facing the odontophore, cells filled with pigment granules are situated. On the ventral side of the cavity, the wedge-shaped, muscular odontophore carries the monostichous radula, and separates the latter in an ascending and a descending limb. The teeth of the radula are formed within the radula sheath by odontoblasts, surrounding the origin of the ascending limb (see Fig. 4c). The odontoblasts appear as dark blue, distinctly bordered cells, with an amorphous, light gray content and dark blue nuclei (Fig. 2b, d). There are 11 teeth in the ascending limb; the descending limb of the radula is surrounded by a muscular layer and carries 9–10 teeth. The descending limb leads to the ascus which is situated between the ascending and the descending limb. The ascus (Fig. 4c) is an epithelium-lined, sac-like structure on both sides of the descending limb. In this structure (110 μm long, 125 μm wide and 70 μm high), used teeth are stored in a heap without orientation (Fig. 2b). The ascus is surrounded by odontophoral musculature in the posterior part of the pharynx and therefore might be hardly discernable in a macroscopic dissection.

The paired, flat and elongated salivary glands are situated on both sides of the esophagus. They are interconnected in the space between esophagus and esophageal blind sac (Figs. 2c and 3). The right salivary gland is markedly longer (390 μm) than the left one (260 μm). The former encompasses the whole anterior half of the esophagus while the latter does not reach that far anterior. The salivary glands have a central, not ciliated lumen surrounded by dark stained, glandular mass. The cells are orientated radially around the lumen and contain colourless vesicles as well as purple or dark blue stained ones (Figs. 2c and 7b). Both salivary glands are connected to the pharynx via the thin salivary ducts (Ø 15 μm) that emerge on the anterior tip of the glands and enter the pharyngeal cavity right and left lateral of the esophagus (Figs. 4c and 7b). According to the different size of the glands, the left salivary duct is much longer than the right one. Before they enter the pharynx, the

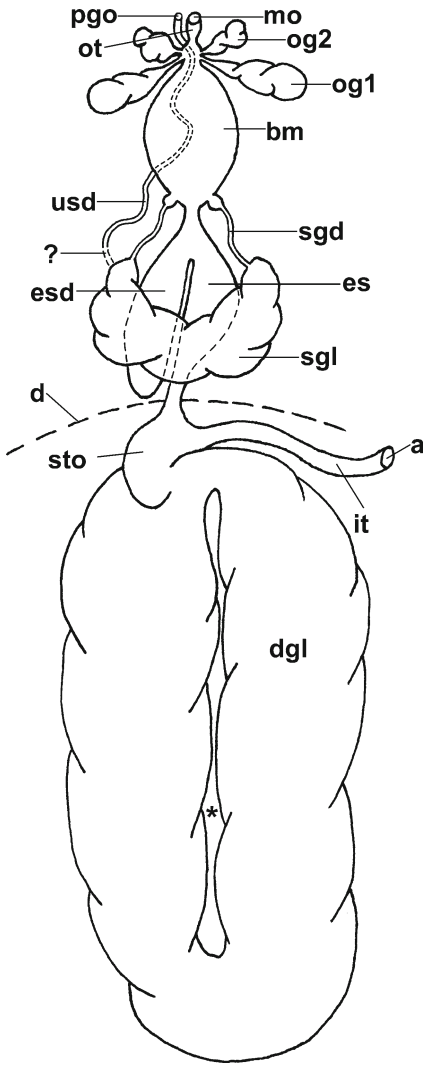

Fig. 3 Schematic overview over the digestive system of *G. aprica*. *Asterisk* indicates position of the longitudinal, median septum. *a* Anus, *bm* buccal mass, *d* diaphragm, *dgl* digestive gland, *es* esophagus, *esd* esophageal diverticulum, *it* intestine, *mo* mouth opening, *og1* oral gland 1, *og2* oral gland 2, *ot* oral tube, *pgo* opening of the unknown salivary duct to the pedal gland duct, *sgd* salivary gland duct, *sgl* salivary gland, *sto* stomach, *usd* unconfirmed salivary duct

ducts widen to a bulbous structure (Ø 25 μm). An additional duct was found to emerge from the left salivary gland. This duct (Ø 20–25 μm) is strongly coiled and runs anteriorly alongside the pharynx (Fig. 4d), until it connects with the opening of the pedal gland (see above). Due to its unclear affiliation and function, the duct is called "unconfirmed salivary duct" herein.

The esophagus (Figs. 2c, 3 and 4d) emerges from the posterodorsal area of the pharynx. It consists of three specifiable parts. The most anterior portion is a short, narrow, ciliated duct that abruptly widens into a voluminous, bulbous, tubular structure, lined with an epithelium consisting of large, elongated cells. The interior is slightly filled with granules of different size, shape and staining properties. Shortly afterwards, the esophagus is constricted vertically and divided into the left lateral esophageal diverticulum (Figs. 2c and 4d), and the right lateral part, that is tubular and narrows progressively on its way to the diaphragm. The epithelium of the blind sac differs from the epithelium of the tubular part of the esophagus by being thicker. The content of the blind sac does not seem to differ from the rest of the esophagus. Immediately after passing through the transversal diaphragm, the esophagus enters the small stomach (Fig. 4d), the epithelium of which is folded and covered densely with cilia. The digestive gland (Figs. 3 and 4a, b, d) is the most voluminous organ situated in the visceral sac. The gland stretches from the diaphragm to the posterior end of the animal, and is divided by the median septum, so that two longitudinal main branches, which only anastomose anteriorly and posteriorly, are formed (Fig. 4b). The left branch joins the stomach on its posterior end ventrolaterally on the left, the right branch on the same level in a right, dorsolateral position. Both branches possess short side branches at regular intervals, leading to distinct lobes that extend alongside the integument to the dorsal side. In this way, the other major organs of the visceral sac (i.e. posterior genital glands on the right, hermaphrodite gonad on the left side) appear enclosed by the two branches of the digestive gland. Shortly anterior of the connections to the digestive gland, set on the right side of the stomach, the intestine (Fig. 4d) arises and forms a curve over the posterior genital glands. Shortly distal of the top point of the arch, the intestine unites with the nephroduct, and alongside a side branch of the kidney, it runs to the anus.

Central nervous system

The CNS (Figs. 5, 6 and 7) is euthyneurous and consists of a circumesophageal, postpharyngeal ring of paired cerebropleural and pedal ganglia, twice connected to each other, and a visceral loop with three ganglia of different size (Fig. 6). After the nomenclature of Haszprunar (1985) and Sommerfeld and Schrödl (2005), ganglia are named as left parietal, subintestinal/visceral and right parietal/supraintestinal ganglion, respectively. Alternatively, under a triganglionate hypothesis (see Dayrat and Tillier 2000), ganglia would refer to subintestinal, visceral/genital and supraintestinal ganglion, respectively. Additionally there are paired buccal ganglia within the circumesophageal ring, slightly anterodorsally of the pedal commissure.

All ganglia are surrounded by a thick layer of connective tissue (Fig. 7c). They can be subdivided into an outer cortex that contains the cell bodies with dark blue stained nuclei, and an inner medulla that contains only nerve fibers. Therefore the medulla has the same histological appearance as nerves and connectives (i.e. light blue stained). Giant neurons are present in all major ganglia (Fig. 7c).

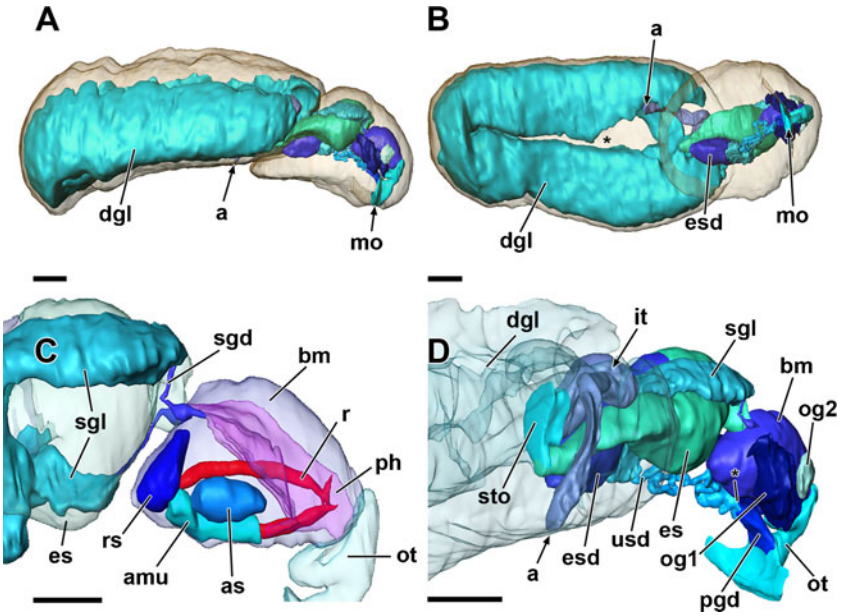

Fig. 4 **a–d** 3D reconstruction of the digestive system of *G. aprica*. **a** Localization of the digestive system in the specimen, right view. **b** Ventral view of the digestive system, note branches of the digestive gland, separated by the longitudinal median septum (*). **c** Buccal apparatus and associated organs, right view. **d** Digestive system, digestive gland transparent, right posterolateral view, note connection of unconfirmed salivary duct and pedal gland duct (*). *a* Anus, *amu* ascus muscle, *as* ascus, *bm* buccal mass, *dgl* digestive gland, *es* esophagus, *esd* esophageal diverticulum, *it* intestine, *mo* mouth opening, *og1* oral gland 1, *og2* oral gland 2, *ot* oral tube, *pgd* pedal gland duct, *ph* pharyngeal cavity, *r* radula, *rs* radula sheath, *sgd* salivary gland duct *sgl* salivary glands, *sto* stomach, *usd* unconfirmed salivary duct. *Bars* **a**, **b**, **d** 200 μm; **c** 100 μm

The paired, oval-shaped and totally fused cerebropleural ganglia (ca. 120 μm high, 130 μm wide and 110 μm long) are placed side by side, dorsally of the posterior end of the buccal mass. They are connected via a short and strong commissure (Fig. 5b). From each cerebropleural ganglion, there are two connectives to the pedal ganglion; the cerebropedal connective is placed more anterior than the pleuropedal connective (Fig. 5c). The short, double-rooted cerebrorhinophoral connectives emerge from the anterior side of each cerebropleural ganglion and lead to the small rhinophoral ganglion. The rhinophoral nerve ramifies into several branches. The labiotentacular nerve emerges from the anteroventral side of the cerebropleural ganglion, and also ramifies. Rhinophoral as well as labiotentacular nerve run anteroventrally.

Posteriorly, the cerebropleural ganglia connect to the visceral loop (Fig. 5e); beginning on the left, there is a short connective to the left parietal ganglion which is the smallest ganglion on the visceral loop. It is 45 μm high, 70 μm wide and 50 μm long, and situated adjacent to the posteroventral tip of the left cerebropleural ganglion. The left parietal nerve emerges from its posterior end and runs posteriorly along the left side of the esophagus and its diverticulum. A 40 μm long connective leads to a large, fused subintestinal/visceral ganglion, which is the biggest of the visceral loop (85×130×80 μm). It is located posterior of the left pedal ganglion, ventral of the esophagus. Two thick nerves emerge from the subintestinal/visceral ganglion and run posteriorly very close to each other, first on the ventral side of the esophagus, and then surrounded by esophagus, its diverticulum and the penial sheath gland (see Genital system). They penetrate the diaphragm dextral of the esophagus, and cling on the distal part of the oviduct. Shortly afterwards, they separate into a ventral and a dorsal branch, which run alongside the albumen gland, and the mucus gland, respectively (for gland nomenclature see Discussion).

The longest connective of the visceral loop (125 μm) connects the subintestinal/visceral ganglion and the right parietal/supraintestinal ganglion, running diagonally from ventral to lateral of the esophagus. The right parietal/supraintestinal ganglion is medium-sized (65×80×75 μm), and situated directly behind the right cerebropleural ganglion, to which it is connected via a very short connective. No other ganglia (i.e. genital or osphradial ganglia) connected to the right parietal/supraintestinal ganglion were detected, although there is a strong nerve pointing posterior and penetrating the diaphragm. Unfortunately, this nerve could not be followed further.

The pedal ganglia (105×130×105 μm) (Figs. 5b–f and 7b) are almost as large as the cerebropleural ganglia, but their commissure (Figs. 5e and 7c) is slightly longer and bridges over the posterior tip of the ascus that is located between them. Three nerves emerge from each ganglion. One (pn1) emerges on the anterior, ventral side and runs anteriorly into the foot. The second (pn2) emerges on the anterodorsal side,

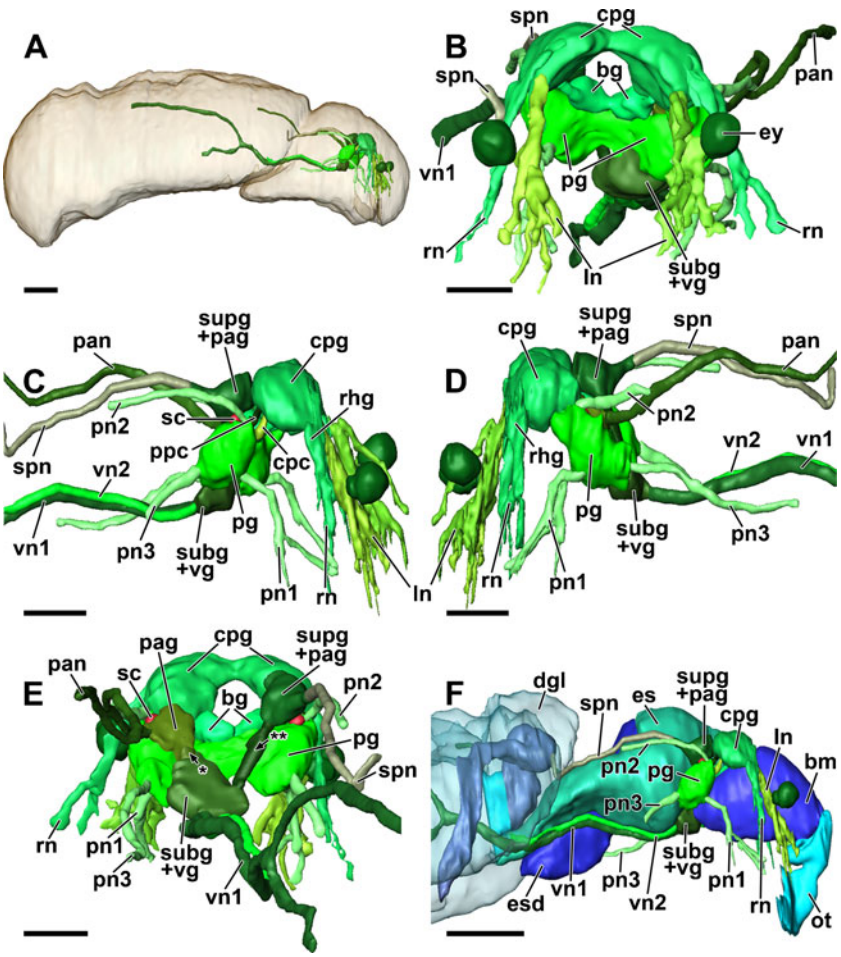

Fig. 5 **a–f** 3D reconstruction of the central nervous system (CNS) and main nerves of *G. aprica*. Optic and static nerves could not be detected. **a** Localization of the CNS in the specimen, right view. **b** Anterior view of the CNS, legend of pedal nerves omitted. **c** Left lateral view of complete CNS. **d** Right lateral view of complete CNS. **e** Posterior view of the complete CNS, connective of left parietal ganglion and fused subintestinal and visceral ganglion (*), connective of fused right parietal and supraintestinal ganglion and fused subintestinal and visceral ganglion (**). **f** CNS and encompassed digestive system right lateral view. *bg* buccal ganglion, *bm* buccal mass, *cpc* cerebropedal connective, *cpg* cerebropleural ganglion, *dgl* digestive gland, *es* esophagus, *esd* esophageal diverticulum, *ey* eye, *ln* labiotentacular nerve, *ot* oral tube, *pan* parietal nerve, *pag* parietal ganglion, *pg* pedal ganglion, *pn1* pedal nerve 1, *pn2* pedal nerve 2, *pn3* pedal nerve 3, *ppc* pleuropedal connective *rhg* rhinophoral ganglion, *rn* rhinophoral nerve, *sc* statocyst, *spn* supraintestinal nerve, *subg* subintestinal ganglion, *supg* supraintestinal ganglion, *vg* visceral ganglion, *vn1* visceral nerve 1, *vn2* visceral nerve 2. *Bars* **a**, **f** 200 μm; **b–e** 100 μm

shortly posterior of the pleuropedal connective and lateral to the statocyst. It runs to the dorsal posterior area of the foot. The third nerve (pn3) emerges from the posteroventral end of the ganglion, passes partially through the pedal gland and runs posteriorly in the foot. On top of each pedal ganglion there is a spherical statocyst (Ø 20 μm) located mediodorsally. A single, spherical but dissolved structure may refer to remainders of a statolith. A static nerve could not be detected.

The paired buccal ganglia (52×65×50 μm) (Figs. 5b, e and 7b) are located at the posterodorsal end of the pharynx, flanking the emerging esophagus. They are connected to each other via a short commissure. The connection to the cerebropleural ganglia is short and emerges from the anterior tip of the buccal ganglia.

The eyes (Figs. 5b and 7a) are nearly spherical (Ø 65 μm), and are situated lateral to the anterior part of the pharynx (i.e. prepharyngeal) in the front of the head. They are structured in several layers. The outermost layer is a brownish colored, grainy pigment layer, which forms a retinal cup. It clasps around a convex, hyaline and acellular lens with a dark blue-stained border and a brownish interior that has grainy areas. Between the lens and the pigmented layer there is a light blue coloured layer that seems to be detached from the pigmented layer. Neither optical nerves nor accessory ganglia could be detected.

Circulatory system

Gascoignella aprica possesses a monotocardian heart consisting of a thin-walled posterior auricle and a thicker-

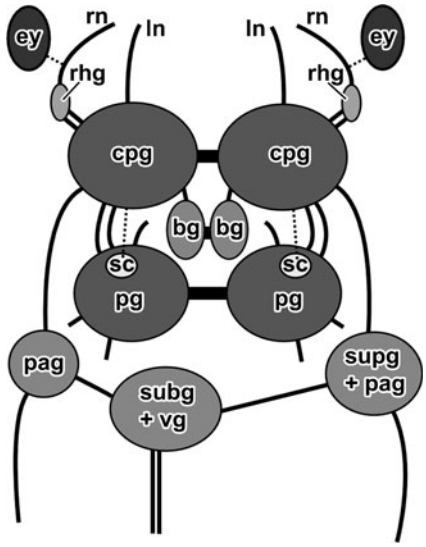

Fig. 6 Schematic overview of the CNS of *G. aprica*, dorsal view. Connectives of the cerebropleural ganglia to the rest of the visceral chord not to scale. Optic and static nerve not found. *bg* Buccal ganglion, *cpg* cerebropleural ganglion, *ey* eye, *ln* labiotentacular nerve, *pag* parietal ganglion, *pg* pedal ganglion, *rhg* rhinophoral ganglion, *rn* rhinophoral nerve, *sc* statocyst, *subg* subintestinal ganglion, *supg* supraintestinal ganglion, *vg* visceral ganglion

walled, anterior ventricle (Fig. 8b). The heart, situated in the dorsoanterior, median part of the visceral sac, is surrounded by a pericardium (Fig. 8c). The auricle is fed by a single sinus that runs anteriorly, starting at the end of the visceral sac. It penetrates the pericardium on its posterior, dextral side and leads to the auricle via a short and narrow, venous vessel. This vessel appears closely attached to the right branch of the kidney, so does the auricle in its posterior area. The auricle (125 μm long and 200 μm wide) is very thin-walled, its light grey-stained epithelium is flimsy and highly folded.

The auricle enters the bigger ventricle (260 μm long and 240 μm wide). Its content appears extremely homogenous, hyaline and of grey color. Dark blue stained, roundish cells are interspersed in the lumen of auricle and ventricle and attached to their epithelia. In phase contrast they look red.

The anteroventral tip of the ventricle leads to the aorta (Fig. 8b). It passes the arch of the intestine on the ventral side, penetrates the diaphragm, and runs further anterior on the dorsal side of the esophagus. At the anterior end of the esophagus it branches several times, the hemolymph seems to flow freely over the central nervous system.

Excretory system

The H-shaped kidney (Fig. 8c) is characterized by its spongy, highly vacuolated and light-stained tissue. It is located dorsally in the anterior two-thirds of the visceral sac, mostly directly under the integument. Both posterior branches are equally long (ca. 600 μm). The left and the right branches are connected posterior of the pericardium, their connection is 75 μm wide. The anterior branches encompass the pericardium laterally. The right anterior branch (length 540 μm) extends much further anterior than the left one (340 μm), sends a vertical branch alongside the intestine (Fig. 8a, d) and additionally encloses the pericardium anteriorly. A potential connection between the pericardium and the kidney (i.e. a renopericardial duct) shows no ciliation and therefore could not be definitively confirmed as such. The nephroduct emerges shortly posterior of the interconnection of the kidney branches. In its most proximal part, a small portion of the nephroduct is situated ventral of the left branch of the kidney, but does not connect to the latter (see Fig. 8d). The nephroduct runs alongside the ventrolateral right side of the kidney and unites with the intestine (Fig. 8f). Consequently, there is no discrete nephroporus visible from the outside. The cells of the nephroduct wall contain small granules of pigment (Fig. 8e, f).

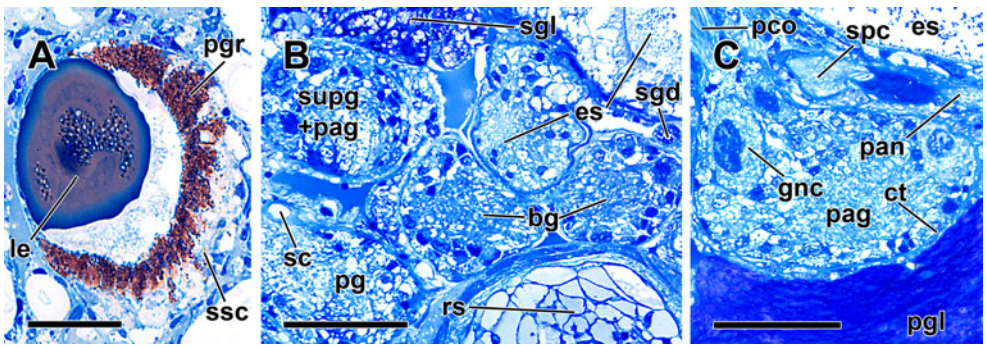

Fig. 7 **a–c** Semithin cross-sections showing aspect of the CNS and the eyes of *G. aprica*. **a** Cross-section through right eye showing layers. **b** Arrangement of several ganglia, buccal ganglia showing cortex and medulla, pedal ganglion with statocyst. **c** Left parietal ganglion with giant nerve cells, fibers of the pedal commissure. *bg* Buccal ganglion, *ct* connective tissue *es* esophagus, *gnc* giant nerve cell, *le* lens, *pag* parietal ganglion, *pan* parietal nerve, *pco* pedal commissure, *pg* pedal ganglion, *pgl* pedal gland, *pgr* layer of granular pigment, *rs* radula sheath, *sc* statocyst, *sgd* salivary gland duct, *sgl* salivary gland, *spc* connective of left parietal ganglion and fused subintestinal and visceral ganglion, *ssc* sensory cells, *supg* supraintestinal ganglion. *Bars* **a** 25 μm; **b**, **c** 50 μm

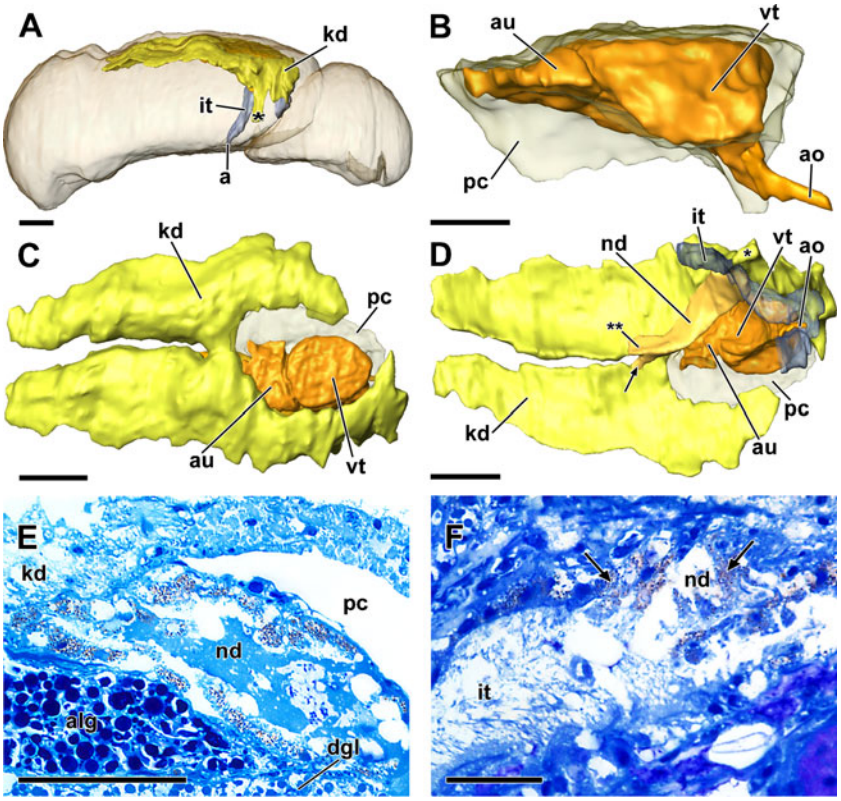

Fig. 8 **a–f** 3D reconstruction of the circulatory and excretory system, semithin cross sections of the excretory system of *G. aprica*. **a** Localization of the circulatory and excretory systems in the specimen, right view, note vertical branch of the kidney along the intestine (*). **b** Right lateral view of the circulatory system. **c** Circulatory and excretory systems, dorsal view, note H-shape of kidney. **d** Ventral view of circulatory and excretory systems, note vertical branch of the kidney (*), blind end of the nephroduct (**) and connection of nephroduct and intestine (arrow). **e** Nephroduct and adjacent structures. **f** Connection of nephroduct and intestine, arrows indicate the most distal portion of the nephroduct, characterized by the granular pigment. *a* Anus, *alg* albumen gland, *ao* aorta, *au* auricle, *dgl* digestive gland, *it* intestine, *kd* kidney, *nd* nephroduct, *pc* pericardium, *vt* ventricle. *Bars* **a**, **c**, **d**, 200 μm, **b** 100 μm, **e** 50 μm, **f** 25 μm

Genital system

The androdiaulic genital system of *G. aprica* consists of a posterior arrangement of a hermaphroditic gonad, a bursa and three voluminous glandular structures of which one, the prostate, is connected by the vas deferens to an anterior, cephalic copulatory apparatus (Figs. 9, 10 and 11). The glandular structures are situated on the right side of the median septum. They are vicinal to each other, and twist slightly around their common axis (Fig. 10d)

The gonad fills nearly the whole upper half of the left side of the visceral sac (Figs. 10d and 11a). Its total length is 1.64 mm. It is externally sac-like, but consists of several consecutive gonad lobes, which connect by their branching lumina. The gonad contains mature sperm and some yolky oocytes, as well as early stages of gametes. The oocytes contain granules of different size and color. There are small, blue or greenish colored yolk granules as well as big, optically empty (i.e. whitish) vesicles. The nucleus appears grayish, a nucleolus was not detected. Autosperm appear tightly packed in a parallel position, sometimes the heads are arranged around cells that could be nurse cells.

About one-third of its length from the anterior end, the gonad gives off a ciliated duct (Ø up to 55 μm), i.e. the proximal gonoduct that widens to the ampulla (ca. Ø125 μm), which is filled with autosperm (Fig. 10b). The ampulla runs anteriorly dorsal of the digestive gland, before it descends beside the longitudinal septum and narrows into a postampullary gonoduct. Ventrally along the anterior anastomosis of the digestive gland, the ciliated gonoduct ascends again and forms a cavity (Fig. 9) on the right side of the longitudinal septum. This cavity (herein called fertilisation chamber) separates the female and male genital systems. Four other ducts originate from the densely ciliated walls of the fertilisation chamber; the bursa stalk, the prostate, the proximal oviduct, and the vas deferens.

The bursa stalk (Ø approx 25 μm) (Fig. 10b–d) is a narrow, coiled and ciliated duct that connects to a roundish vesicle (260×190×170 μm; hereafter named bursa). It is located on the left side of the visceral sac, anterior to the gonad, and is enclosed by the left branch of the digestive gland, the right branch of the kidney and the intestine. It extends anterior to the diaphragm. Its voluminous, spherical lumen contains a grayish, hyaline mass with several blue-

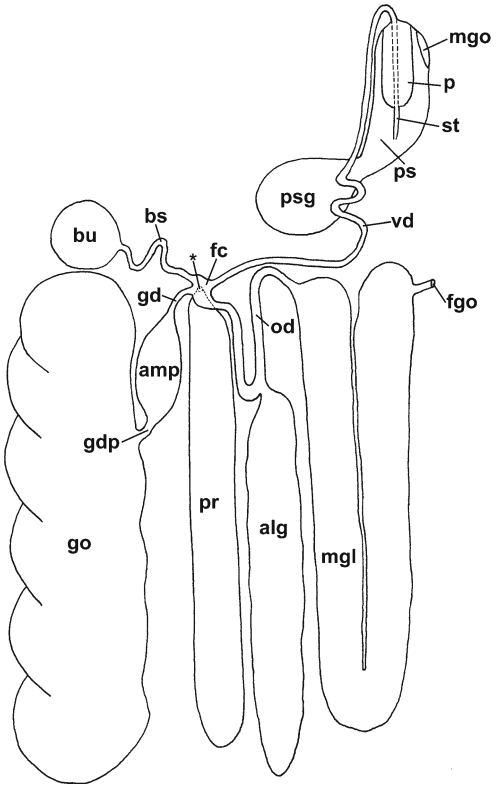

Fig. 9 Schematic overview of the genital system of *G.aprica*. Asterisk indicates opening of prostate into fertilization chamber. *alg* Albumen gland, *amp* ampulla, *bs* bursa stalk, *bu* bursa, *fc* fertilization chamber *fgo* female genital opening, *gd* postampullar gonoduct, *gdp* praeampullar gonoduct, *go* gonad, *mgl* mucus gland, *mgo* male genital opening, *od* oviduct, *p* penis, *pr* prostate, *ps* penial sheath, *psg* penial sheath gland, *st* hollow stylet, *vd* vas deferens

stained dots. The lumen itself is colored light pink. The wall is about 15 μm thick and partly vacuolated in a spongy way. No cilia are visible on the inside of this structure.

The prostate (Figs. 9, 10d and 11c) is 1.45 mm long, generally blue stained, sac like, with a central, circular and ciliated lumen that reaches to the very proximal end of the gland. The cells contain small grainy, dark blue stained vesicles as well as bigger aggregations of blue stained material. The nuclei tend to be found basally.

The ciliated oviduct (Ø ca. 45 μm) (Figs. 10a, b, d and 11a) runs posterior for approximately 200 μm, then extends into a conspicuous loop, and runs anteriorly again; 75 μm after the loop, the lumen of gland two enters the oviduct dorsomedially.

The albumen gland (Figs. 9 and 10a, d) is a multiple-lobed and flat gland. In its distal area there is only one, medium-sized, ciliated lumen that connects to the proximal oviduct. More proximally, the lumen branches several times. The cells surrounding the lumina contain numerous large, round, and very dark stained vesicles. The total length of the gland is approximately 1.3 mm. Following the oviduct for 135 μm further ahead, there is the connection to gland three (the distal oviduct, see below).

The mucus gland (Figs. 9, 10a, c and 11a) is a tubular folded gland, enclosing a broad, flat and ciliated lumen. Its most proximal part is connected to the oviduct and its distal end leads to the female genital opening, therefore it functionally constitutes the distal oviduct. The lumen is surrounded by elevated, columnar cells with a basal lying, blue stained nucleus. The cells are filled with small granular vesicles of a darker violet color. The gland per se is stained in different nuances from violet to pink. The dorsal part runs posteriorly and makes a U-turn. The ventral part runs a little further anterior almost to the diaphragm. At the distal end of the gland, the lumen opens to the ciliated female genital opening which is situated lateroventrally in the groove between foot and visceral sac.

The narrow coiled and densely ciliated vas deferens (Ø ca. 25 μm) connects the posterior genital system to the cephalic copulatory apparatus (Figs. 9 and 10b, d). Emerging most anteriorly on the ventral side of the fertilisation chamber, the vas deferens is muscular along its entire length. It penetrates the diaphragm, runs anteriorly alongside the dextral side of the esophagus and enters the penis sheath at its dorsal, anterior end. No glandular part of the vas deferens was detected. The copulatory apparatus (Figs. 10b and 11d, e) is located within the penis sheath and consists of a muscular penis (ca. 210 μm in length), armed with a straight and thin, hollow stylet (ca. 65 μm in length). The circular muscle layer within the penis is about 25 μm thick. The penis sheath opens on the right side of the animal into an extension of the cephalopedal groove (Fig. 10a). On its posterior end, the lumen of the penis sheath is connected to a conspicuous, nearly spherical structure (330 μm in length, 300 μm in diameter, "penial sheath gland" herein) situated on the ventral side of the esophagus. It is lined with thick and fringed epithelium of glandular cells (Fig. 11d). The elongate cells contain cytoplasm of blue color and grayish-white vesicles. There are no cilia and not a trace of sperm. The lumen of the penial sheath gland is connected to the exterior via the penis sheath. No other connected glandular structures or ducts could be detected.

Discussion

General morphology

In her description of *Gascoignella aprica*, Jensen (1985) examined numerous specimens that were found crawling on exposed algal mats in a tidal mudflat in Deep Bay, Hong Kong in 1983. Anatomical results came from dissection and serial sections.

We can confirm the general morphology information given by Jensen (1985), comprising a very flat body with no traces of rhinophores, cerata or parapodia, and the head-foot

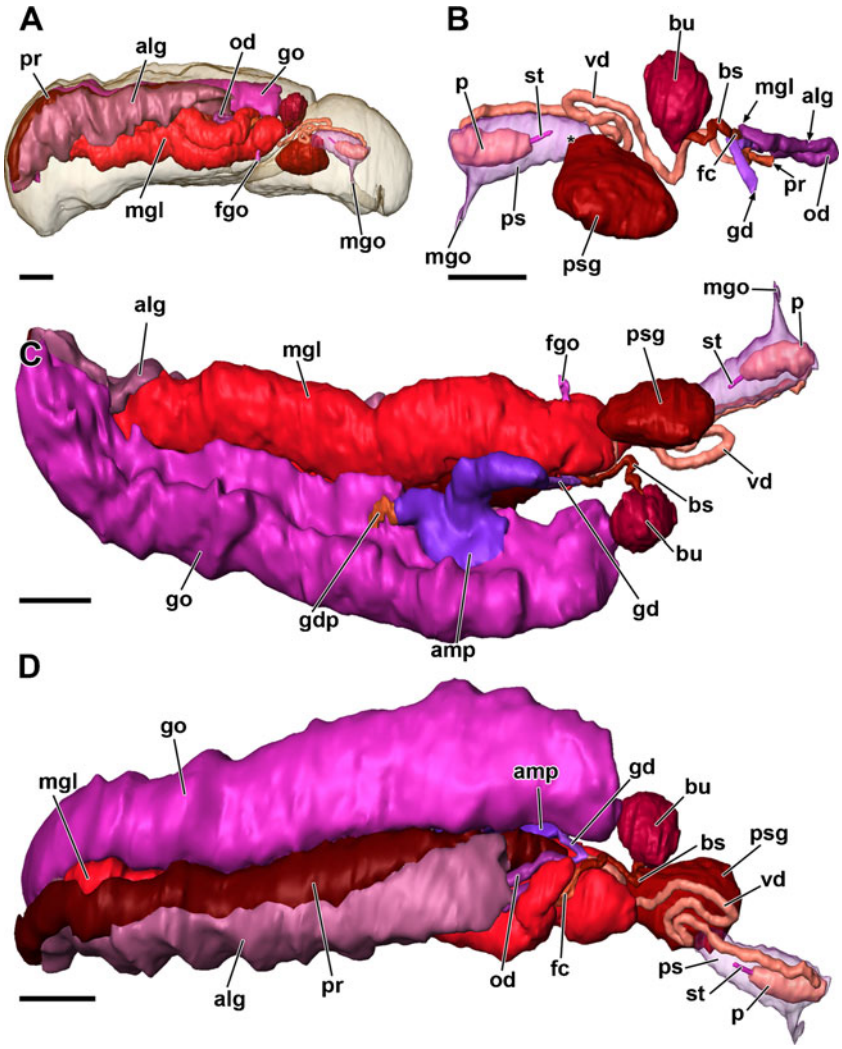

Fig. 10 **a–d** 3D reconstruction of the genital system of *G. aprica*. **a** Localization of the genital system in the specimen, right view. **b** Left view of copulatory apparatus and adjacent ducts; *arrows* connections to the respective glands, *asterisk* connection of penial sheath and unknown penial sheath gland. **c** Ventral view of the genital system. **d** Dorsal view of the genital system. *alg* Albumen gland *amp* ampulla, *bu* bursa, *bs* bursa stalk, *fc* fertilization chamber, *fgo* female genital opening, *gd* postampullary gonoduct, *gdp* preampullary gonoduct, *go* gonad, *mgl* mucus gland *mgo* male genital opening, *od* oviduct, *p* penis, *pr* prostate, *ps* penial sheath, *psg* penial sheath gland, *st* hollow stylet, *vd* vas deferens. *Bars* **a**–**d** 200 μm

very distinctly set off from the visceral mass. A head-foot separated from the more or less freely projecting visceral sac occurs among plakobranchacean sacoglossans only in *G. aprica*, *G. nukuli*, and *P. denudata*, but is common state in shelled Oxynoacea. The special body shape of shell-less *G. aprica* and *Platyhedyle* otherwise resembles acochlidian panpulmonates, a fact that has lead to confusion regarding the systematic placement of Platyhedylidae (Salvini-Plawen 1973 versus Wawra 1988, 1991). Several differences between Platyhedylidae and acochlidians were summarised by Rückert et al. (2008). An additional difference not found in any known acochlidian is the very short but broad foot of *Gascoignella*, not reaching under the visceral sac. Jensen (1985) also stated the median position of the mouth in a cephalopedal groove, which forms two distinct notches lateroventrally on the anterior end of the head. The "penial opening" was reported to be situated in the notch shortly posteroventral of the right eye (Jensen 1985). This position can be confirmed herein. A female genital opening could not be detected in the relaxed specimens, only the dextral, lateroventrally located anus was mentioned to open to the groove between the foot and the visceral mass (see also Jensen 1991). No separate renal pore was observed. In this study, however, the female genital opening (oviductal opening) was found in the groove caused by the muscular, transverse diaphragm, and the anus was found to open posteriorly to this groove on the lateroventral surface of the visceral sac. Due to the fact that the nephroduct unites with the intestine inside the body the absence of an externally visible renal pore can be confirmed.

Body wall

Jensen (1985) described *G. aprica* to be entirely black on the dorsal surface, with a yellowish-white foot sole and a green-appearing ventral surface of the visceral sac, due to the green content of the digestive gland. The eyes were stated to be surrounded by a yellowish transparent area. In this study, we also explored the composition of the epidermis. A layer of 8 μm on average of pigment granules was found to cover the dorsal and lateral sides of the animal. Numerous glandular structures were found, opening to the exterior via apical

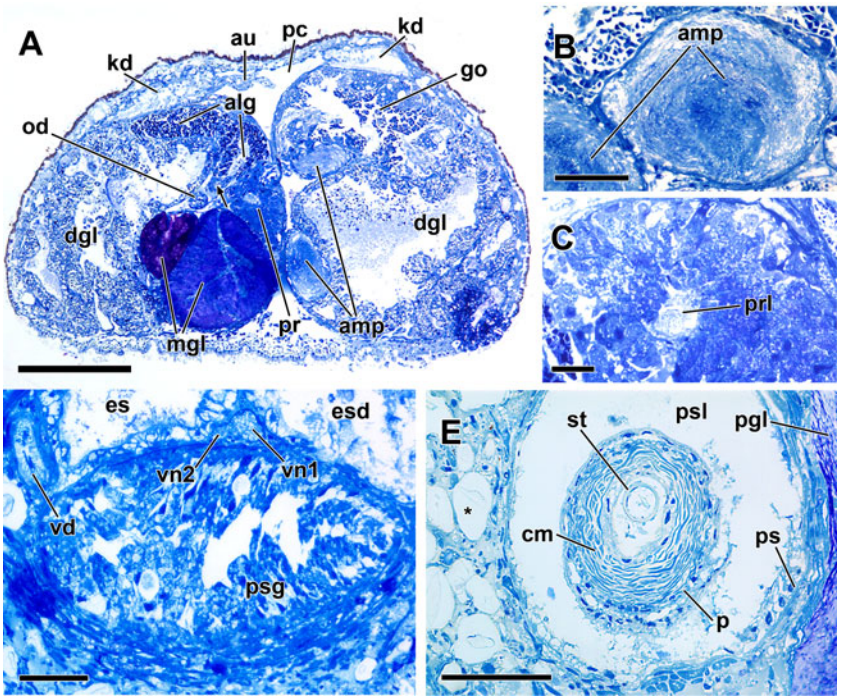

Fig. 11 Semithin sections through the genital system of *G. aprica*. **a**, cross-section through the visceral sac and overview of the genital glands, arrow indicates connection of genital gland 2 and the oviduct; **b**, ampulla with tightly stored autosperm; **c**, putative prostate **d**, penial sheath gland; **e**, cross-section through the penial sheath and the penis, asterisk indicates a very common "type 1"-gland. *alg* Albumen gland, *amp* ampulla, *au* auricle, *cm* circular muscle layer, *dgl* digestive gland, *es* esophagus, *esd* esophageal diverticulum, *go* gonad, *kd* kidney, *mgl* mucus gland, *od* oviduct, *p* penis, *pc* pericardium, *pgl* pedal gland, *pr* prostate, *prl* lumen of prostate, *ps* penial sheath, *psg* penial sheath gland *psl* lumen of penial sheath, *st* hollow stylet, *vd* vas deferens, *vn1* visceral nerve 1, *vn2* visceral nerve 2. *Bars* **a** 250 μm; **c** 25 μm; **b**, **d**, **e** 50 μm

pores. As the animals were described to crawl most often on algal mats in the open sunlight, we suggest that the thick dorsolateral epidermis with a pigmented layer and a large number of glands forms an adaptation to semiterrestrial life. Like all Plakobranchacea, *G. aprica* does not possess a shell, which means the epidermis constitutes a protective barrier against environmental stress. The semiterrestrial habitat of a tidal mudflat is characterized by continuously changing intensities concerning moisture, salinity, temperature and UV-radiation. Adaptations to this environment thus may have lead to the special external morphology (i.e. compact shape, lack of tentacles) in *G. aprica*, which is shared by some other amphibiously living plakobranchaceans as well [e.g. *Alderia modesta* (Loven, 1844)] or the amphibious, "bug-eating" acochlidian slug *Aiteng ater* Swennen and Buatip, 2009 (Neusser et al. 2011b: 332). *Aiteng ater* differs from other acochlidians in habitat and shape; in particular a thickened epidermis with underlying spongy tissue with large vacuoles obviously gives the soft notum some stability. We show that *G. aprica* also has a thick layer of notal tissue with spacious vacuolated appearance. From a histological point of view, the voluminous "type 1"-glands described in the results resemble the leftover cavities of spicules after decalcification (see, e.g. Brenzinger et al. 2011b). Spicules are characteristic for interstitial heterobranch gastropods like Acochlidia and Rhodopemorpha (Rieger and Sterrer 1975; Arnaud et al. 1986; Salvini-Plawen 1991), but have also been described for *P. denudata* (Rückert et al. 2008). However, leftover cavities found in *G. aprica* are probably not from spicules. Spicules usually leave cavities that are rather small and do not form aggregations that could be responsible for optically empty cavities found in *G. aprica*. More likely, holes are leftovers of big vacuoles in cells that build a resistant notal integument under the epidermis. Similar spongy layers of connective tissue are also known from the marine intertidal nudibranch *Corambe lucea* Marcus, 1959, where large vacuoles were confirmed by SEM examination (Schrödl and Wägele 2001). In the latter species, pairs of dorsoventral muscle bundles were suggested to enhance the sucking ability of the foot, but also keep the notum in shape and help the body wall musculature generating hemolymph pressure. We suggest that in *Gascoignella aprica* the dorsoventral longitudinal median septum in the visceral hump has a similar stabilising function.

Digestive system

The digestive system of *G. aprica* comprises a short oral tube, a well developed buccal apparatus with monostichous radula and the ascus, and an esophagus that widens to form two parallel swellings, only the right one penetrates the

diaphragm to the stomach. The latter has a connection to the left and right ramus of the digestive gland, respectively, and a connection to the intestine that runs to the lateroventrally situated anus, shortly posterior of the muscular diaphragm on the right side of the animal. These findings largely agree with Jensen's (1985) description. However, it is the right rather than the left part of the esophagus that unites with the stomach, and the anus opens on the lateroventral surface of the visceral sac rather than in the furrow between head-foot and the visceral sac built by the diaphragm. In this study, the "buccal glands" described by Jensen (1985) were found to be actually two paired and histologically different oral glands. Homology of these glands is unclear, and histological differences could occur due to different functional phases.

The bulge-like structures enclosing the entrance to the pharynx could have a function during feeding, for example as a kind of sphincter. As they are very small, they do not seem to be evertable. They might comprise a structure also referred to as "inner lips" in a schematic drawing in Jensen (1993a: fig. 1).

The digestive system redescribed for *G. aprica* herein is compared with the usual sacoglossan type as it has been examined in detail in several studies (Jensen 1980, 1981, 1991, 1993a, b, c, 1996, 1997). Generally, the suctorial pharynx of Sacoglossa is composed of four muscular units: (1) the dorsal septate muscle, (2) the odontophore, (3) the ventral, longitudinal ascus muscle, and (4) the pharyngeal pouch. The existence of muscles mentioned in point 1, 2 and 3 could be confirmed (see Fig. 4c), but a pharyngeal pouch is absent in *G. aprica*; the lack of a pharyngeal pouch corresponds to fig. 4F in Jensen (1991).

All known Sacoglossa have a uniseriate radula with an ascending and descending limb and an ascus where discarded teeth are stored. In this study, 11 teeth in the ascending limb, and 9–10 teeth in the descending limb were counted. This fits well with originally described 8–9 teeth in the ascending limb and 12 teeth in the descending limb. Jensen (1985) found at least 20 teeth stored as a heap in the ascus. This and other radula features that Jensen described (i.e. shape of teeth) cannot be efficiently studied from semithin serial histological sections and 3D-models.

The epithelium-lined ascus is a sacoglossan innovation. The ascus retains all radular teeth formed throughout the life of the animal (Jensen 1996). In *G. aprica*, the ascus and the descending limb are attached to the buccal mass (and not demarcated externally). In the reconstructed specimen, the ascus is positioned dorsal of the descending limb, connected to the latter at the posterior end, which is an unusual arrangement within the Sacoglossa. In most species the ascus is located outside the pharyngeal musculature (Jensen 1993a, b, c).

The buccal mass of *G. nukuli* differs markedly from that of *G. aprica*; Swennen (2001, fig. 1f) indicated large muscular swellings in the ventral part of the pharynx. The buccal mass of *P. denudata* possesses an identical, ventral muscular swelling (own observation) and thus seems to be more similar to *G. nukuli* than to *G. aprica*. There is thus a considerable variation in pharynx morphology among Platyhedylidae that might correspond to different food types. *Gascoignella aprica* is assumed to feed upon macroscopic intertidal filamentous chaetomorphal algae (Jensen 2003), while the only macroscopic algae available in the mesopsammic habitat of *Platyhedyle denudata* are stolons of *Caulerpa* spp. (M. Schrödl, own observation). In contrast, *G. nukuli* is assumed to prey upon subterranean branches of *Derbesia marina* or green micro-algae (Swennen 2001). Therefore, microalgae sucked in by the putative ventral sucking pump should be explored as a potential food source for *Platyhedyle denudata*.

The existence of salivary glands, corresponding paired ducts and reservoirs can be added to the description of *G. aprica*, as well as an enigmatic third, unpaired, duct that appears to emerge from the left salivary gland and connects to the pedal gland tube. A similar structure is missing in *G. nukuli* and *P. denudata*. The exact connectivity, homology and function of this duct are unclear but merits further research.

An esophageal pouch occurs in many sacoglossans [e.g. *Elysia timida* (Risso, 1818)] and differs in size as well as in muscular and glandular components (Jensen 1996). The esophageal diverticulum (= pouch) found in *G. aprica* has a thicker, more glandular epithelium than the esophagus, but is not lined by a remarkable muscular layer. A similar esophageal pouch is present in *G. nukuli* (own observations), but was not mentioned for *P. denudata* (see Rückert et al. 2008).

As in most sacoglossans, the stomach of *G. aprica* constitutes a rather small and simple part of the digestive system. While in many non-shelled Sacoglossa there appears to be a trend of enlargement of the surface area of the digestive gland (Jensen 1991), *G. aprica* only has short lobules on the long, wide, main ducts of the digestive gland. This character is shared with *Limapontia* and *Platyhedyle*, both of which do not have any cerata (Jensen 1996). The lobules of the digestive gland of *G. nukuli* and *P. denudata* seem more elaborated (own observations).

Since the intestine unites with the nephroduct at about two-thirds of its length, the distal part of the intestine functions as a joint rectum and nephroduct. The position of the anus was stated to be located in a groove on the right side of the head as in *Bosellia* and some *Elysia* spp. (Jensen 1996). As mentioned above, it is actually situated shortly posterior on the right ventrolateral side of the visceral sac.

The general arrangement of the digestive system of *G. aprica* is typical for Heterobranchia in its simplicity of e.g. the esophagus/intestine (Ponder and Lindberg 1997), but shows sacoglossan innovations that were modified in the different platyhedylid species to a variable extent, thus showing taxonomic and potential phylogenetic significance.

Central nervous system

The CNS of *G.aprica* consists of a postpharyngeal nerve ring of paired cerebropleural and pedal ganglia. The fused nature of the cerebral and pleural ganglia is shown by the fact that there are two connectives to the pedal ganglia per side. Paired rhinophoral and buccal ganglia are connected to the cerebropleural ganglia. This condition already stated by Jensen (1985) was also found in *Platyhedyle denudata* by Rückert et al. (2008) and is usual among sacoglossans. Precerebral accessory ganglia are present in mesopsammic *P. denudata*, many acochlidians (Schrödl and Neusser 2010), cephalaspidean *Pluscula cuica* Marcus, 1953 (Brenzinger et al. 2012) and rhodopemorphs (Brenzinger et al. 2011b), but were not found in intertidal *G. aprica*. This supports assumptions that accessory ganglia are an adaptation to mesopsammic habitats that independently evolved in many lineages. Giant neurons as found in *G. aprica*, however, are absent in *P. denudata*; they might have been lost due to body size reduction in mesopsammic species.

Rhinophores and other head tentacles are missing in *G. aprica*, but rhinophoral and labiotentacular nerves are present. Both major head nerves show multiple peripheral ramifications, thus indicating their sensory function in the head epithelium without forming distinct tentacles. Rhinophoral ganglia are connected with the cerebral ganglia by double nerves as found in many other panpulmonates (e.g. traditional pulmonates with procerebrum, but also several acochlidians; Neusser et al. 2007; Schrödl and Neusser 2010), possibly the mesopsammic cephalaspidean *Pluscula cuica* (Brenzinger et al. 2012), and rhodopemorphs (Brenzinger et al. 2011b).

Eyes in the examined specimens are well-developed and in an anterolateral position rather than situated in a more central position as in *P. denudata*. Similar laterally situated eyes were found in the amphibious acochlidians of the genus *Aiteng* (Swennen and Buatip 2009, Neusser et al. 2011b). *Aiteng ater* as well as *G. aprica* show clear areas above the eyes devoid of the pigment granules otherwise found in the dorsal integumental cells. Pigmentation can be explained as adaptation to semiterrestrial life and potential exposure to the sun.

The visceral loop of *G. aprica* is short and ganglionate as was observed by Jensen (1985); histology shows that there are three distinct ganglia of different sizes. The first ganglion on the left is smaller than the others, and bears a nerve leading to the body wall; it is thus identified as the left parietal ganglion. The second, most posteriorly situated ganglion is the largest; it can be identified as containing the subintestinal and visceral ganglia, since it bears two strong nerves running to the visceral mass. A genital ganglion as found on the visceral nerve of *P. denudata* by Rückert et al. (2008) was not detected in *G. aprica*. The right ganglion corresponds to the supraesophageal ganglion that connects to the putative osphradial ganglion in *P. denudata* (see Rückert et al. 2008). In *G. aprica*, the emerging nerve was detected, but no ganglion was found. There is no direct evidence on the existence and position of a right parietal ganglion in *G. aprica*. According to the Pentaganglionata hypothesis (Haszprunar 1985), euthyneurans show five separate visceral loop ganglia that may fuse during later ontogeny. No ontogenetic data is available on *G. aprica*, but interpreting the three ganglia according to their relative sizes as left parietal ganglion, fused subintestinal and visceral ganglion, and fused supraintestinal and right parietal ganglion fits with the Pentaganglionata idea. Nevertheless, this concept was criticised by Dayrat and Tillier (2000), and reliable pentaganglionate species, i.e. rhodopemorphs and acteonids, were shown to occur also outside of Euthyneura recently (Brenzinger et al. 2011b; Schrödl et al. 2011a). Thus, if there is a pentaganglionate condition involved, the concept may apply to a more inclusive group of heterobranchs rather than euthyneurans.

Circulatory and excretory system

Gascoignella aprica possesses a heart consisting of an auricle, a ventricle and a partly muscular aorta. This is in line with Jensen (1985), who stated the presence of a heart shaped as usual in sacoglossans. Some additional features can be added: the auricle is posteriorly connected to a single, dorsal hemolymph sinus. Dorsal vessels, as found in most Plakobranchacea are absent in *Platyhedyle*, *Gascoignella* and *Plakobranchus* (Jensen 1996). A similar heart is present in *G. nukuli* (see Swennen 2001), while absent in *P. denudata* (Rückert et al. 2008). In general, the molluscan heart is surrounded by the pericardial epithelium (epicardium) with an underlying extracellular matrix, representing the location of ultrafiltration (Fahrner and Haszprunar 2001). Though this cannot be confirmed by light-microscopy, it is likely that the pericardial complex of *G. aprica* is built the same way. The pericardium contains the primary urine, which is modified subsequently by the kidney. A general character in the Mollusca is the close ontogenetic and functional interrelation of the pericardial complex and the nephridia in excretion (e.g. Andrews 1988; Morse and Reynolds 1996; Baeumler et al. 2011, 2012). A nephrostome or a renopericardioduct (i.e. a ciliated connection of the pericard and the kidney) has been described for other sacoglossans with a similar renopericardial complex, e.g. *Bosellia mimetica* Trinchese, 1891 by Fahrner and Haszprunar (2001), but we failed to reliably detect such connection in *G. aprica*. The H-shaped kidney in *G. aprica* takes up a large part of the body (see Fig. 8); the large size could be due to an advanced osmoregulatory effort necessary in semiterrestrial habitats, and therefore constitute an adaptation to the latter. A similar way of adaptation is described by Neusser et al. (2011b) for the semiterrestrial acochlidian family Aitengidae. Their members possess a highly ramified system of dorsal vessels, which

constitutes a modified part of the kidney and is connected to the pericardium at least in *Aiteng mysticus*. This system is assumed to enhance respiratory, secretory and excretory processes in this secondarily amphibious lineage.

The kidney of *P. denudata* was already recognised as such by Salvini-Plawen (1973). It is located in the right anterior section of the visceral hump, and is small, compared to the other members of the Platyhedylidae. The kidney secretes via a nephroduct that unites with the intestine quite distantly from the anus (Rückert et al. 2008) as it is the case in *G. aprica* as well.

A heart has apparently been lost secondarily in a few Sacoglossa [e.g. *Alderia modesta*, *Placida viridis* (Trinchese, 1873)]. Jensen (1996) stated the loss to be most likely as an adaptation to intertidal and/or estuarine living. Gosliner (1994) also presumed the reduction in *Alderia modesta* to be due to its semiterrestrial lifestyle rather than to its small body size. This trend cannot be confirmed for the Platyhedylidae, as the mesopsammic *P. denudata* is the only member lacking a heart. However, the reduction of the heart in *P. denudata* could be due to its small body size. Decreasing body sizes were long observed as an adaptation to mesopsammic life (Swedmark 1964), and microhedylacean acochlidians were generally assumed to lack a heart (Rankin 1979). This was, however, shown to be erroneous (e.g. Neusser et al. 2009b). The heart is present in all microhedylaceans reexamined by 3D reconstruction techniques, though hearts may be simplified and contain a detectable ventricle only. In absence of auricles the site of ultrafiltration is still unknown. Similarly, future ultrastructural investigations on *P. denudata* have to show whether or not there are at least some remainders of the heart, and whether or not there is an alternative site of ultrafiltration.

Genital system

G. aprica is a hermaphrodite with an androdiaulic genital system first described by Jensen (1985), but the identities and special arrangements of complex reproductive organs in small sacoglossans are very difficult to reveal even by sophisticated gross-morphological dissecting techniques (Jensen 2001). Therefore, the 3D model approach provides substantial additional and reliable data.

The hermaphroditic gonad is a rather compact structure located entirely on the left side of the longitudinal septum. It is not divided into follicles as in most other sacoglossans. *Alderia modesta* has a similar, compact gonad (Gascoigne 1976), which may be a convergent adaptation to semiterrestrial life. The ampulla is a widened section within the proximal hermaphroditic duct, where autosperm is stored prior to copulation.

The histology of the proximally situated receptacle named "bursa" in this study matches the definition (Wägele and Willan 2000) of an at least temporarily gametolytic gland in the Sacoglossa. Accordingly the grayish, hyaline content is presumably a bolus of partly dissolved surplus reproductive products (Jensen 1996). While in most shelled Sacoglossa the genital receptacle opens by a separate duct to the female genital papilla, in the non-shelled sacoglossans the connection of the genital receptacle with the reproductive system has moved from the genital opening to a more interior position.

The elongate, sac-like prostate was, despite of its size, not mentioned in the first description of *G. aprica* by Jensen (1985). The prostate is connected to the fertilisation chamber in *G. aprica* and situated close to the exit of the vas deferens. It is histologically almost identical to the proximal prostate described for *P. denudata* by Rückert et al. (2008), which, however, is an unbranched tubule that is connected directly to the vas deferens, and which does not reach as far posteriorly as in *G. aprica*.

The most proximal, sac-like gland of the female genital system constitutes the albumen gland. Jensen (1985) already described it as a flat, much-lobed gland that winds along the dorsal side of the "large oviduct" or mucus gland, but she did not give any information about its connection to the oviduct. In fact, the histology of the albumen gland in *G. aprica* remarkably resembles the albumen gland of *Oxynoe viridis* (Pease, 1861) illustrated by Klussmann-Kolb (2001). Jensen (1996) stated that the eggs do not traverse the albumen gland in sacoglossans. This is in line with the sac-like slender appearance of the albumen gland in *G. aprica* and suggests secretion into the proximal part of the oviduct.

The more distal portion of the female glands is tubular and can be referred to the mucus gland and distal oviduct (Klussmann-Kolb 2001). There is no distinct membrane gland in *G. aprica,* which is also the case in *P. denudata* (see Rückert et al. 2008), or the membrane gland at least is not clearly recognisable as such.

The vas deferens connects the fertilisation chamber with the copulatory organ (i.e. the penis); it is non-glandular, and therefore not prostatic. In the Oxynoacea, the prostate is a glandular part of the vas deferens, while in the non-shelled Plakobranchacea it is in general a separate gland that opens into the vas deferens (Sanders-Esser 1984). The latter is the case in *G. aprica*. An additional thickened and glandular region at the distal end of the vas deferens as described for *P. denudata* was not found in *G. aprica*.

The penial stylet of *G. aprica* has the shape of an injection needle. Hypodermal injection of sperm is known for several groups of slugs (e.g. the nudibranch *Palio*, Rivest 1984) and is achieved in a precise or an imprecise way. In the precise way the injection is made directly into a seminal receptacle with oriented sperm, as observed for *Limapontia capitata* (Müller, 1774) and *Limapontia senestra* (Quatrefages, 1844) by Gascoigne (1956). A precise hypodermic injection might

be conceivable for *G. aprica*, as the stylet is about 70 µm long and therefore would reach the bursa which is lying directly under the integument. On the other hand the bursa described in this study seems to be a gametolytic sac (see above) and not a seminal receptacle. In addition, the bursa of *G. aprica* is very small compared to other species where a precise impregnation occurs. In the two specimens of *G. aprica* examined herein there is no evidence for imprecise injection, such as free sperm in the body cavity, nor would functional allosperm storage organs be expected under such sperm transfer scenario. On the other hand, imprecise sperm transfer appears common among plakobranchacean sacoglossans (Schmitt et al. 2007), despite the presence of allosperm receptacles (e.g. *Elysia filicauda*, see Jensen 1999). We cannot yet exclude the possibility that *G. aprica* copulates, transferring sperm and sperm liquid surplus that is stored and digested in the bursa. Copulation through the oviductal opening has not been observed in any non-shelled sacoglossan, though it has often been inferred, e.g. by Gascoigne (1976, 1979).

The penial sheath gland remains enigmatic. Jensen (1985) detected it and suspected that it would be a large seminal vesicle or prostate gland. Since the gland is not connected to the vas deferens or the female part of the genital system, it is unlikely that this gland could be used for the storage of sperm. However, due to its position it can be assumed that the gland is also functionally associated to the copulatory complex and plays a role during copulation, as it was suggested for the "paraprostatic" system of some hedylopsacean Acochlidia (Neusser et al. 2009a; Brenzinger et al. 2011a; Neusser et al. 2011a) or some gasteropterid cephalaspideans (e.g. *Siphopteron*, see Anthes and Michiels 2007).

Jensen (1985) assumed a pronounced protandry for *G. aprica*, since, in a serially sectioned, 1-mm-sized specimen she found the oviduct to be very small and not to connect to the oviductal opening. The hermaphrodite ampulla contained only spermatozoa. However, oocytes in different stages of maturity were found within the gonad of the two specimens examined herein.

Systematic remarks

The genus *Gascoignella* currently comprises three species: the herein redescribed type species *G. aprica*, the anatomically poorly known *G. nukuli*, and *G. jabae*, a somewhat *Olea*-like species tentatively placed into *Gascoignella* by Swennen (2001). In absence of detailed information on the others, we concentrate here on *G. aprica* and compare it with the similarly well-explored *P. denudata*, the sole described member of the genus *Platyhedyle* (Fig. 12). The first has a massive H-shaped, dorsal kidney, which is neither shared by *Platyhedyle* nor by any other sacoglossans known to us. Also, *G. aprica* has an esophageal diverticulum and a heart, which are sacoglossan symplesiomorphies. In contrast, in *P. denudata* an esophageal diverticulum and a heart are uniquely absent. While clearly separable, e.g. by the features given above, Jensen (1996) convincingly showed that the monotypic genus *Platyhedyle* is the sister group of *Gascoignella*; therefore the Gascoignellidae was stated to be a junior synonym of the Platyhedylidae. Morphological similarities between *Platyhedyle* and *Gascoignella* were also confirmed by Rückert et al. (2008), who regarded the presence of the "median septum", a unique longitudinal muscular septum dividing large parts of the visceral sac into left and right hemispheres, as diagnostic for Platyhedylidae. Our results on *G. aprica* confirm this conclusion. Further unique features found in *Gascoignella* and *Platyhedyle*, and thus potential apomorphies for Platyhedylidae include: (1) a bipartite, posteriorly fused digestive gland, with (2) short peripheral tubules, (3) a spongy subepidermal tissue, concentrated in the dorsal head-foot, (4) reduction of pharyngeal pouches, (5) reduction of a receptaculum seminis, and (6) a proximal, single, sausage-like prostate. Further apomorphies of Platyhedylidae may exist, such as the reduction of rhinophores, the distinction of headfoot and visceral sac and the very short foot. However, these characters are homoplastic among Sacoglossa, and thus their polarity depends on sacoglossan topology and the exact origin of the family within the Sacoglossa.

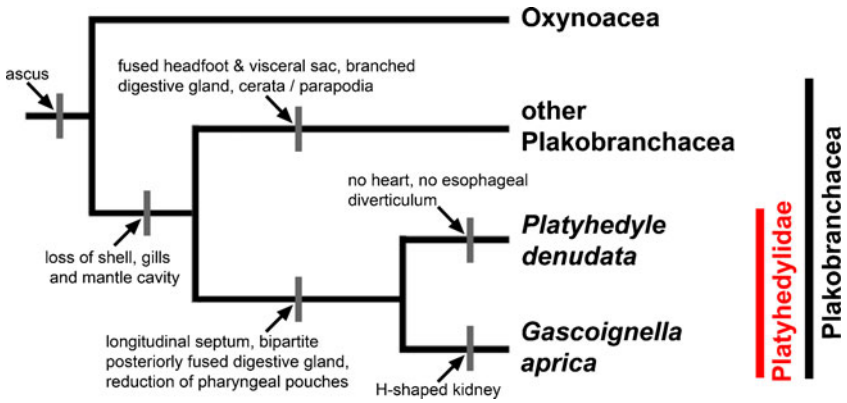

Fig. 12 Phylogenetic tree showing some potential apomorphies for sacoglossan taxa

Older morphology-based cladistic approaches (Jensen 1996; Mikkelsen 1996, 1998) recovered a basal sacoglossan dichotomy into non-shelled Plakobranchacea and shelled Oxynoacea; this was confirmed by molecular analyses that also show that *Cylindrobulla* is a member of Oxynoacea (Maeda et al. 2010; Göbbeler and Klussmann-Kolb 2011; Neusser et al 2011b). As a member of Plakobranchacea, the Platyhedylidae share the apomorphic reduction of the shell, mantle cavity, gill and glandular strips that are still present in Oxynoacea. Jensen (1996) recovered the Platyhedylidae as basal offshoot of the plakobranchacean superfamily Plakobranchoidea, and Mikkelsen's (1998) reanalysis revealed Platyhedylidae as an inner branch of Plakobranchoidea. In contrast, a recent molecular analysis showed the Platyhedylidae as most basal branch of the Plakobranchacea, i.e. sister to a common clade of limapontioidean and plakobranchoidean members (Neusser et al. 2011b). Assuming this topology is stable against adding more taxa into molecular analyses, the absence of a fused headfoot and visceral sac, the still short foot and the absence of cerata and parapodia, in Platyhedylidae could be symplesiomorphies with Oxynoacea. The loss of rhinophores would be apomorphic (and convergent to losses among derived limapontioidean lineages).

Platyhedylidae thus could be a basal non-shelled sacoglossan offshoot that is specialised to extreme habitats, i.e. intertidal, semi-terrestrial benthic or infaunal (*Gascoignella*), and a subtidal mesopsammic life (*Platyhedyle*). Several ancestral features were retained in the family, such as a rather compact body shape, but modified to resist mechanical and other environmental forces. While many apomorphies support the common origin of Platyhedylidae, the internal topology and evolution of the three currently recognised members (*Platyhedyle denudata*, *Gascoignella aprica*, *G. nukuli)* is not yet well-resolved. Special reductions found in *Platyhedyle denudata*, such as that of a heart and a well-developed kidney may be correlated to size reduction during adaptation to mesopsammic life. If this adaptation to a mesopsammic habitat occurred within *Gascoignella*, this would render the genus paraphyletic. Microanatomical data on *G. nukuli* and molecular data on *G. aprica* are needed to reconstruct the inner phylogeny and evolution of the Platyhedylidae.

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Chapter 4. **Brenzinger B**, Padula V & Schrödl M (2013b): **Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Euopisthobranchia: Cephalaspidea: Philinoglossidae).** *Organisms, Diversity and Evolution*, **13:** 33-54.

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ORIGINAL ARTICLE

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Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Gastropoda: Euopisthobranchia: Philinoglossidae)

Bastian Brenzinger · Vinicius Padula · Michael Schrödl

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Abstract Increasing molecular evidence suggests that the phylogeny of euthyneuran gastropods differs greatly from centenary textbook concepts. The presence, homology and evolution of characters in major subgroups thus need to be reinvestigated. Traditionally basal opisthobranch Cephalaspidea ("head-shield snails and slugs") were pruned to a new taxon concept, with benthic euopisthobranch and tentacle-bearing cephalaspidean lineages basal to burrowing, head-shield bearing philinoidean species. Among the latter, mesopsammic "microslug" lineages evolved at least twice. Herein we explore in 3D microanatomical detail the putatively basal philinoglossan *Pluscula cuica* (Marcus, Boletim da Faculdade de Filosofia, Ciências e Letras. Universidade de São Paulo 164:165–203, 1953a) from its type locality in Brazil. The species possesses several "accessory" ganglia and a reduced posterior mantle cavity that retains some putative shell-building tissue and an osphradium. The hermaphroditic, monaulic genital system opens in a posterior position; it retains a bursa copulatrix but lacks a distinct receptaculum seminis. Autosperm is transferred to the cephalic copulatory organ via an external sperm groove, not through the hemocoel, as suggested in the original description. The penis opens through the oral tube, sperm is transferred by a "kiss". A conspicuous yellow gland is discussed as a modified Blochmann's gland. Retaining several putative symplesiomorphies with philinoids, *Pluscula* is discussed as the most basal offshoot in meiofaunal Philinoglossidae. However, the supposed "primitiveness" of the fused rather than separate cerebropleural ganglia and the triganglionate rather than pentaganglionate visceral nerve cord was based on misobservations. Higher categories such as Philinoglossacea for Philinoglossidae, and a separate family Plusculidae for *P. cuica* are no longer warranted. Inner cephalaspidean relationships and a scenario of more or less successive philinoglossid adaptation to meiofaunal environments should be investigated by molecular studies with more comprehensive taxon sampling.

Keywords Mollusca · Opisthobranch · Meiofauna · Interstitial · Adaptation · Phylogeny

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B. Brenzinger (✉) · V. Padula · M. Schrödl
Bavarian State Collection of Zoology, Section Mollusca,
Münchhausenstr. 21,
81247 Munich, Germany
e-mail: bastian.brenzinger@arcor.de

V. Padula
e-mail: viniciuspadula@yahoo.com

M. Schrödl
e-mail: michael.schroedl@zsm.mwn.de

B. Brenzinger · V. Padula · M. Schrödl
Department Biology II and GeoBio-Center,
Ludwig-Maximilians-Universität München,
Großhaderner Str. 2,
82152 Planegg-Martinsried, Germany

Introduction

Gastropoda are renowned for their morphological, and therefore ecological, diversity (e.g., Beesley et al. 1998). In recent decades, phylogenetic studies have rapidly increased our understanding of their evolution. However, accumulating molecular evidence suggests that the topology of Heterobranchia — covering roughly half of gastropod diversity — differs greatly from traditional textbook concepts. The long held split of Euthyneura into monophyletic Opisthobranchia and Pulmonata has been challenged (e.g., Haszprunar 1985; Dayrat and Tillier 2002; Klussmann-Kolb et al. 2008; Dinapoli and Klussmann-Kolb 2010) and a "new euthyneuran tree" has emerged (Jörger

et al. 2010a; Schrödl et al. 2011a, b; Göbbeler and Klussmann-Kolb 2011), the backbone topology of which has been confirmed in phylogenomic approaches (Kocot et al. 2011; Smith et al. 2011). In the light of radically changing concepts and classifications, morphological characters, taxa and traits need to be reinvestigated (Schrödl et al. 2011a).

Among the most aberrant and problematic heterobranchs are several lineages of minute slugs that are specialized members of the meiofauna. Living in the marine interstitial or mesopsammon, i.e., the interstices between sand grains in well oxygenated sands (Swedmark 1964, 1968), all these taxa — most acochlidians, rhodopemorphs, some Cephalaspidea, Sacoglossa and Nudibranchia (Arnaud et al. 1986)—exhibit characteristic morphologies. Convergently evolved characters are small sizes, vermiform bodies, losses of body appendages, eyes and pigmentation, development of adhesive abilities, spicules and additional ganglia, and unusual reproductive traits such as the production of spermatophores, hypodermal insemination, production of only few eggs, and loss of a free-floating larval stage (Swedmark 1968, 1971; Salvini-Plawen 1973; Schrödl and Neusser 2010; Neusser et al. 2011a; Schrödl et al. 2011a). Similar features and tendencies are also found in other groups of metazoans that inhabit the same habitat (Swedmark 1964; Higgins and Thiel 1988; Rundell and Leander 2010). In addition to showing reductions and convergent innovations, the reduced adult size common to these taxa is suggestive for progenetic processes (e.g., Hanken and Wake 1993). Retaining simple juvenile features means losing diagnostic apomorphies of higher clades and gaining pseudoarchaic ones; this may lead to entirely wrong classificatory conclusions (Martynov et al. 2011; Martynov and Schrödl 2011). Furthermore, minute specimen sizes have historically hampered both collecting efforts and structural analyses. Incongruities from previous descriptions were detected and corrected during 3D microanatomical reanalyses of meiofaunal sacoglossans (Rückert et al. 2008) and acochlidians (e.g., Neusser et al. 2006, 2009a; Jörger et al. 2008, 2010b; Eder et al. 2011) that were originally examined using paraffin-based histology. Interstitial cephalaspideans have not yet been analyzed in such depth.

The Cephalaspidea or "bubble-shells" were long thought to be the most basal and conservative major opisthobranch clade, including several distinct taxa characterized by the name-giving head-shield, an organ used for infaunal digging (Gosliner 1994; Mikkelsen 1996; Burn and Thompson 1998). However, the inclusiveness of the taxon concept has decreased over time. Acteonoidea and Ringiculoidea were already excluded from Cephalaspidea on morphological grounds (Haszprunar 1985; Mikkelsen 1996, 2002); the former were placed at the base or outside Euthyneura by multilocus analyses (Göbbeler and Klussmann-Kolb 2010, 2011; Dinapoli and Klussmann-Kolb 2010; Jörger et al. 2010a; Schrödl et al. 2011a, b). The previously disputed cephalaspidean *Cylindrobulla* (Jensen 1996; Mikkelsen 1996, 1998) was confirmed as a "bubble-shelled" sacoglossan panpulmonate by molecular analyses (Händeler and Wägele 2007; Maeda et al. 2010; Neusser et al. 2011b). Finally, Malaquias et al. (2009) removed the small-sized benthic Runcinacea from Cephalaspidea; this has been confirmed by molecular studies using larger outgroup sets (Jörger et al. 2010b; Göbbeler and Klussmann-Kolb 2011). With the remaining Cephalaspidea now appearing as a non-basal taxon within so-called Euopisthobranchia (Jörger et al. 2010a), head-shield bearing lineages are scattered over the euthyneuran tree. This reclassification has important implications for the understanding of euthyneuran evolution. For example, euthyneuran head tentacles and head shields show essentially similar cerebral innervation patterns (Huber 1993; Faller et al. 2008; Staubach et al. 2008; Jörger et al. 2010b) and thus may simply transform according to habitats and life styles.

Within Cephalaspidea, morphology-based classifications are heterogeneous and authors claimed at least four 'superfamilial' ranks. The most basal Cephalaspidea in all available multi-locus studies were the little-known Diaphanoidea (e.g., Malaquias et al. 2009; Jörger et al. 2010a; Göbbeler and Klussmann-Kolb 2011). Intriguingly, this paraphyletic group (Göbbeler and Klussmann-Kolb 2011) contains tentacle-bearing members such as benthic *Colpodaspis* and infaunal *Toledonia* (Brown 1979; Golding 2010) suggesting that there is no simple ecological rule. Therefore, one might suggest that diaphanoidean tentacles may be phylogenetic remainders of a benthic euopisthobranch ancestor, while higher cephalaspideans have evolved their eponymous head-shields de novo. Stable inner cephalaspidean topologies and detailed microanatomical data to test these hypotheses are not yet available. Albeit with varying topologies, members of at least four families of the carnivorous Philinoidea commonly cluster close together: *Scaphander* (Scaphandridae), *Philine* (Philinidae), Aglajidae and Gastropteridae (Malaquias et al. 2009; Göbbeler and Klussmann-Kolb 2011). These philinoid families contain slender carnivores with a reduced or internalized shell (save *Scaphander*) and a rearward displaced mantle cavity (Burn and Thompson 1998). Mesopsammic, at least externally shell-less philinoideans have evolved independently at least twice (Arnaud et al. 1986; Malaquias et al. 2009; Jörger et al. 2010a): within the burrowing Philinidae (*Philine exigua* Challis, 1969a and juveniles of other species), and with the entirely mesopsammic 'Philinoglossacea' Thiele, 1931 of still unknown affinities.

The philinoglossans are a small group containing four genera and seven described species (four of which belong to *Philinoglossa* Hertling, 1932). These miniaturized slugs (body length rarely exceeds 4 mm) show a ribbon-shaped body with posteriorly overhanging dorsum, lack a distinguishable head-shield (except for the Mediterranean *Abavopsis latosoleata* Salvini-Plawen, 1973), a gill, and have at best a vestigial shell.

These multiple reductions have significantly hampered phylogenetic studies based on morphological data: Wägele and Klussmann-Kolb (2005) recovered philinoglossans within a group containing the meiofaunal members from several traditional heterobranch 'orders'. Molecular studies have shown to be better suited to solve similar tasks (e.g., Malaquias et al. 2009; Jörger et al. 2010a) but so far only a few have included philinoglossans in their sampling. Accordingly, their phylogenetic position within philinoid Cephalaspidea is not known: Vonnemann et al. (2005) recovered *Philinoglossa praelongata* Salvini-Plawen, 1973 basal but inside a polytomy. Both Malaquias et al. (2009) and Göbbeler and Klussmann-Kolb (2011) identify a clade of *Philinoglossa* and Gastropteridae as sister to Aglajidae plus Philinidae, with Scaphandridae basal. Jörger et al. (2010a) recovered *Scaphander* as sister to *Philinoglossa*, but without covering the aforementioned families. So far, monophyly of 'Philinoglossacea' has never been tested by including more than single representatives into molecular analyses. Not much is known about the biology of the group.

The monotypic genus *Pluscula* is represented by the Brazilian *Pluscula cuica* Marcus, 1953a, the only philinoglossan species described from the Americas. It is potentially the most basal of philinoglossans, since it is described with characters that appear to be plesiomorphic and are not found in the other genera (Marcus 1953a). These characters are a thin internalized shell, the genital opening in a posterior position, still separate cerebral and pleural ganglia, and five distinguishable ganglia on the visceral nerve cord. On the other hand, the mode of autosperm transfer is suggested to be unique and peculiar: Marcus (1953a) observed numerous spermatozoa in the body cavity and concluded that autosperm move from the gonad directly to the copulatory organ—through the hemocoel, instead of being transported along the external ciliated groove running along the right body side, as in most other cephalaspideans. Due to these peculiarities, some authors place *Pluscula cuica* in a family of its own (Plusculidae: Marcus 1959; Franc 1968; Bouchet and Rocroi 2005) or subfamily (Plusculinae: Salvini-Plawen 1973). Therefore, *Pluscula cuica* might be a key organism for the understanding of philinoglossan evolution and the internal phylogeny of philinoid groups, and interesting for its peculiar reproductive mode.

Within a framework of comparative morphological and evolutionary studies on mesopsammic heterobranchs, we analyzed the entire microanatomy of *Pluscula cuica* using computer-based 3D reconstruction from semi-thin histological sections. Our aims were to (1) check, correct, and supplement the original description; (2) elucidate the structure and function of the reproductive system, in particular with regard to the potentially highly peculiar modes of autosperm transport and transfer; and (3) evaluate potentially ancestral features in a phylogenetic context, reconsidering the familial status of the species, and the relationships of philinoglossans to other cephalaspideans.

Materials and methods

Specimens of *Pluscula cuica* were extracted from bulk samples of coarse sand taken from the uppermost subtidal at low tide at Ilhabela, São Paulo, Brazil (type locality) in 2005 following the method described by Schrödl (2006). Specimens were relaxed in isotonic magnesium chloride solution, fixed in ethanol (75 % or 96 %) or, for histology, in 4 % glutaraldehyde (in 0.2 M cacodylate buffer, 0.1 M sodium chloride, 0.35 M sucrose buffered at pH 7.2). The latter specimens were further postfixed with 1 % osmium tetroxide in 0.2 M cacodylate buffer/0.3 M sodium chloride, then dehydrated over a graded acetone series and embedded in Spurr's epoxy resin (Spurr 1969). Specimens are stored at the Bavarian State Collection of Zoology (ZSM), Department Mollusca, Munich, Germany, and in the malacological collection of Museu de Zoologia da Universidade de São Paulo (MZSP, vouchers 104098–104100), Brazil (C. M. Cunha, personal communication).

For 3D reconstruction, three specimens in epoxy blocks were trimmed and serially sectioned at 1.5 μm using either Ralph glass knives (specimens ZSM Mol-20070316, 20070323) or a HistoJumbo diamond knife (specimen ZSM Mol-20070317) (Diatome, Biel, Switzerland) with contact cement at the lower cutting edge, following the method described by Ruthensteiner (2008). Ribbons of sections were collected on microscope slides, stained with methylene blue/azure-II (Richardson et al. 1960) and sealed with araldite resin. Sections of the complete diamond-sectioned specimen—a moderately contracted adult specimen of approximately 1.7 mm length—and, separately, its central nervous system were photographed with a ProgRes C3 ccd camera (Jenoptik, Jena, Germany) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Photographs were stack processed (resized, changed to greyscale, unsharp masked) in Adobe Photoshop (Adobe Systems, Mountain View, CA) and imported into Amira 5.2 software (Visage Imaging, Berlin, Germany) with a resolution of 1,024×768 or 2,080×1542 pixels, respectively. After alignment of the photographs, organ systems were labeled manually onto the sections. Rendered 3D models of the organ systems were created for the complete specimen (based on 575 photographs, every second section was used). Details of the specimen's nervous system were analyzed in a separate aligned stack (256 photos, every section used), but labeled in the complete body's model. Anatomical features were compared among all three specimens (one juvenile, one functionally male, the other adult).

Further two specimens fixed in 75 % ethanol (lot: ZSM Mol-20070835) were photographed through a Leica dissection microscope and macerated in KOH solution for analysis of shell remnants and the radula. Radulae were viewed through above mentioned light microscope for counting of tooth rows and detection of denticulate tooth margins.

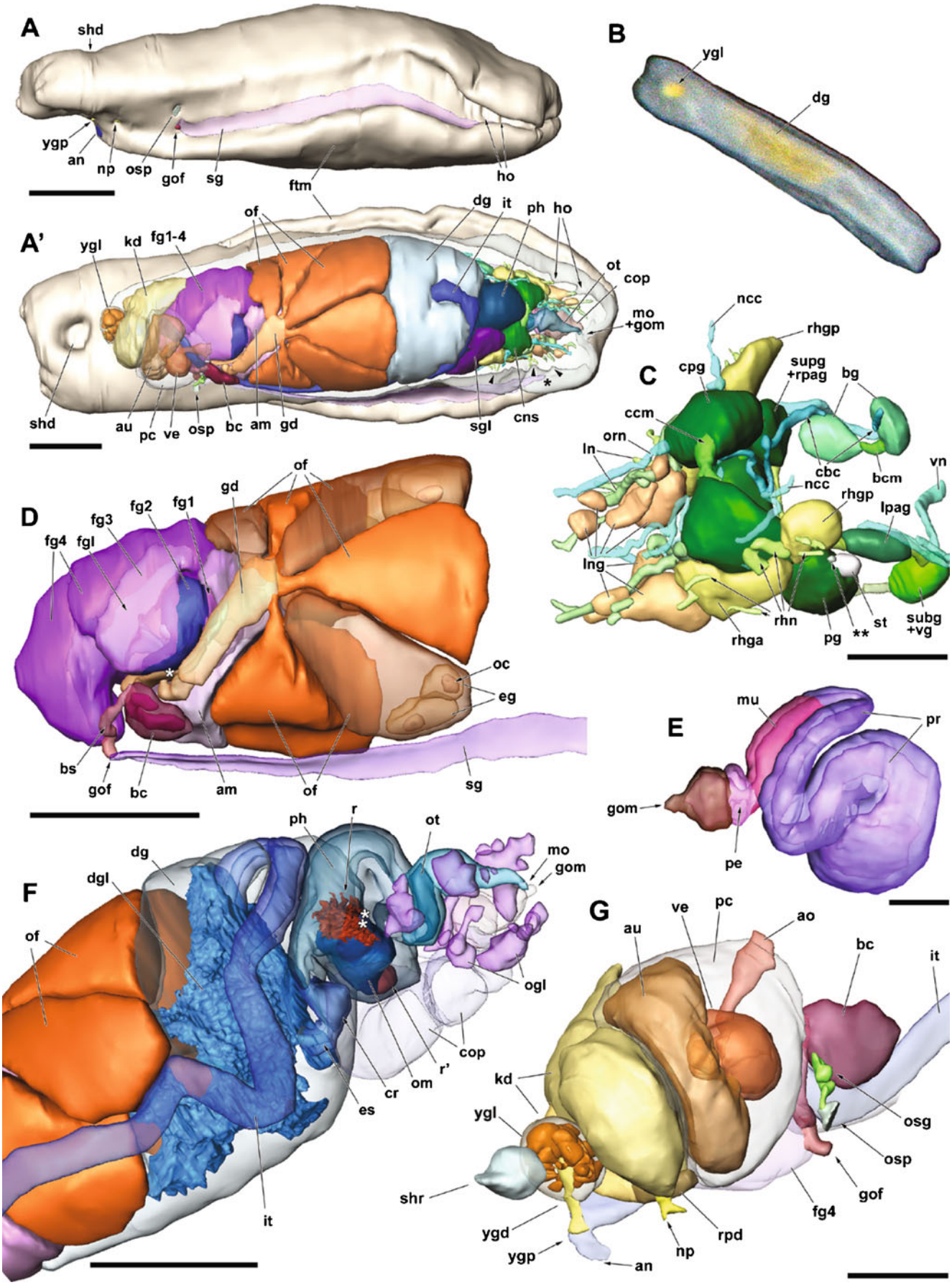
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Fig. 1 **a–g** Three-dimensional reconstructions of *Pluscula cuica* microanatomy. **a** External aspect of body showing body openings, right view. **a'** Dorsal view of body with the dorsum above body cavity and head shown transparent, showing inner organ systems, *arrowheads* short nerves innervating Hancock's organs, *asterisk* anterior end of seminal groove. **b** Live specimen, ca. 2 mm total length, dorsal view. **c** Anterior left view of the central nervous system, pedal nerves omitted, *double asterisk*: large cell next to statocyst, **d** Posterior part of reproductive system, dorsolateral right view, *white asterisk* branching point of gonoduct to female glands and ampulla. **e** Copulatory apparatus, ventral view, anterior towards left. **f** Oblique right view of digestive system, salivary glands omitted, *double white asterisks* positions of salivary duct openings and small glandular field inside pharyngeal lumen. **g** Oblique dorsolateral right view of pericardial complex and surrounding organs. *am* ampulla, *an* anus, *ao* aorta, *au* auricle, *bc* bursa copulatrix, *bcm* buccal commissure, *bg* buccal ganglion, *bs* bursa stalk, *cbc* cerebro-buccal commissure, *ccm* cerebral commissure, *cns* central nervous system, *cpg* cerebropleural ganglion, *cop* copulatory apparatus, *cr* putative crop, *dg* digestive gland, *dgl* lumen of digestive gland, *eg* egg, *es* esophagus, *fg1–fg4* nidamental glands (proximal to distal), *fgl* lumen of nidamental glands, gd gonoduct, *gof* female genital opening, *gom* male genital opening, *ho* Hancock's organs, *it* intestine, *kd* kidney, *ln* labiotentacular nerve, *lng* accessory labiotentacular ganglia, *lpag* left parietal ganglion, *mo* mouth opening, *mu* muscular tube, *ncc* nervus clypei-capitis, *np* nephropore, *oc* oocyte, *of* ovarial follicles, *ogl* oral glands, *om* odontophore musculature, *orn* oral nerve, *osg* osphradial ganglion, *osp* osphradium, *ot* oral tube, *pc* pericardium, *pe* penis, *pg* pedal ganglion, *ph* pharynx, *pr* prostate, *r* distal part of radula, *r'* origin of radula, *rhga/rhgp* anterior/posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *rpd* renopericardial duct, *sg* seminal groove, *sgl* salivary gland, *shd* shell dimple, *shr* shell remnant, *st* statocyst, *subg+vg* combined subintestinal and visceral ganglion, *supg +rpag* combined supraintestinal and right parietal ganglion, *ve* ventricle, *vn* visceral nerve, *ygd* duct of yellow gland, *ygl* yellow gland, *ygp* opening of yellow gland. *Bars* **a**, **a'**, **d**, **f** 250 μm; **c**, **e**, **g** 100 μm. Interactive version of this figure is available in the supplementary online material.

The interactive model was prepared following the protocol of Ruthensteiner and Heß (2008), using Adobe Acrobat 9.0 Professional Extended software. The model can be accessed in the supplementary online interactive version of Fig. 1.

Results

Remarks on taxonomy

Euthyneura Spengel, 1881: Tectipleura Schrödl et al., 2011a: Euopisthobranchia Jörger et al., 2010a

Cephalaspidea P. Fischer, 1883: Philinoidea Gray, 1850: Philinoglossidae Hertling, 1932 (or Plusculidae Marcus, 1959)

Pluscula cuica Marcus, 1953a (type by monotypy)

Marcus (1959) separated monotypic Plusculidae from the Philinoglossidae Hertling, 1932 (type species *P. praelongata* Hertling, 1932) based on *P. cuica* retaining a reduced circular shell, the separation of cerebral and pleural ganglia, and the posterior position of the genital opening. Other described distinguishing features include lack of eyes, presence of five distinguishable ganglia on the visceral loop and the derived mode of autosperm transport from gonad to copulatory organ (via the hemocoel), among others. Bouchet and Rocroi (2005) used Plusculidae Franc, 1968. In contrast, Salvini-Plawen (1973) used a philinoglossid subfamily Plusculinae. Other authors included *Pluscula* and all other genera among Philinoglossidae (e.g., Arnaud et al. 1986).

While generally considered as part of the Philinoidea (e.g., Burn and Thompson 1998; Bouchet and Rocroi 2005), earlier authors commonly used the now obsolete 'order' Philinoglossacea sensu Thiele, 1931 of equal rank to Cephalaspidea (e.g., Marcus and Marcus 1954; Salvini-Plawen 1973). For practical reasons, we use the term 'philinoglossan' to address *Pluscula cuica* and the three other philinoglossid genera.

General anatomy and histology

Living specimens of *Pluscula cuica* are white, with externally visible yellowish digestive gland and the conspicuous 'yellow' gland in the caudal part (Fig. 1b). The body is approximately rectangular in dorsal aspect, and about 3.5 to 4.5 times longer than wide (ca. 1.7 mm × 500 μm in the reconstructed specimen), with a smooth epidermis. The dorsal side is slightly convex; head shield and notum are fused without a detectable groove. The head end is concave with rounded corners. The overhanging posterior end of the notum has a dimple on top under which where remnants of the shell-forming tissue are located; the depression appears to be more pronounced in fixed specimens. Slightly more anterior, the conspicuous spherical yellow gland may be visible, if filled (Fig. 1a',b). Four body openings that are usually found inside the mantle cavity are located underneath the right side of the posteriorly overhanging notum (Fig. 1a). Notum and foot are separated by wide longitudinal grooves along the circumference of the body; the grooves are widest on the sides of the head, thinnest along the anterior face of the body, left and right to where the mouth is situated. The foot is only slightly indented anteriorly, it is wider than the notum in the anterior half of the body; posteriorly, the foot is shorter than the notum with a slightly pointed, but not projecting end.

Notum and foot sole show a distinct margin of short motile cilia. Small intraepidermal, light pink glands can be found, especially close to the head; numerous larger pink-staining and fewer dark blue glands are located subepithelially and open to the outside via thin ducts (Fig. 4a). Within the lateral grooves, the epidermis is thinner and lacks glands and contingent ciliation except for interspersed multiciliated cells and the motile cilia of the seminal groove. Left and right of the head, the Hancock's organs are three shallow depressions with dense microvillous border (Fig. 1a,a'; 4d).

Below the epidermis there is loose connective tissue (formed by round cells that contain an unstained vacuole) that is intersected by muscle fibers, especially in the foot.

Instead of the previously described shell, the decalcified examined specimens show only a dense batch of blue-staining, irregularly sorted fibrous material located within the connective tissue of the overhanging notum end, just below the dorsal depression (Fig. 5g). This circular shell organ/vestige (80 μm diameter, 55 μm thick; Fig. 1g) lacks any trace of a dissolved shell.

The main body cavity is round in cross-section along most of the body's length and separated from the outer connective tissue by a strong layer of mostly longitudinal muscle fibers. All major organ systems reconstructed herein are situated within this body cavity (Fig. 1a). A diaphragm is not detectable.

In the most posterior end of the body cavity lies a conspicuous gland which is visible in living specimens as a bright orange-yellow spot (Fig. 1a',b). The gland is roughly spherical (diameter 100 μm) and surrounded by a thin sheath of muscle fibers. It comprises large, columnar cells with a vacuole that in most cells contains remnants of a grey-staining liquid. The cells are of apparently holocrinous nature and discharge into a central epithelial duct (Fig. 5f); the duct opens to the outside just dorsal of the anus (Fig. 1g).

Digestive system

The mouth opening is located medially within the transversal groove separating notum and foot (Fig. 1a'). The oral tube is thin-walled, surrounded by irregular arrangements of pink-staining, single-celled glands (Fig. 4a). Approximately 50 μm from the outside, the copulatory organ branches from the ventral side of the tube. Following this split, the oral tube becomes wider, its inner wall with numerous longitudinal folds, indicating strong extendibility of this part (Fig. 4b,c). There are approximately ten elongate to egg-shaped, light pink-staining oral glands or various sizes situated around the oral tube (Fig. 1f, 4a); a connection to the tube's lumen is, however, detectable only in some.

The pharynx is elongate and curved (Fig. 1f). Its anterior part curves upward, is spacious and comparably thin-walled; in KOH-macerated specimens the pharynx reveals a thin cuticular covering. The posterior part of the pharynx curves downward, is more muscular and contains the odontophore in its ventral portion (Fig. 1f). There are small patches of violet-staining glandular cells to the left and right of the open radula (Fig. 4e). Inside the odontophore, thick longitudinal muscle fibers run parallel to the posterior two thirds of the still folded radula; only the anterodorsal part of the radula is spread open, underlain by paired fluid-filled lacunae. The radula itself has no distinct descending limb and lacks rhachidian teeth; there are approximately 16–20 rows of curved, pointed lateral teeth (six per row). The inner laterals are the largest and are widest at one-quarter of their height (masticatory border); the second and third laterals are smaller and grow continuously thinner towards the tip (Fig. 4e). Neither serial sections nor light microscopic observation of the radula showed serration of the first laterals (not shown).

The salivary glands are voluminous tubes, their cells filled with comparatively few droplets of dark-blue staining secretion. In the reconstructed individual, the right salivary gland is situated ventrally and appears considerably larger; its ciliated salivary duct can be traced to the right intersection of the thin-walled and muscular walls of the pharynx (white asterisks in Fig. 1f). The left salivary gland is situated dextrodorsally and appears much smaller (Fig. 1a'). The ciliated esophagus exits the pharynx posteriorly and curves downward where it forms a spherical chamber (a vestigial crop?; Fig. 1f); esophagus and putative crop show the longitudinal folds also found in the oral tube. From there a thinner part connects to the stomach dextroventrally. A histologically distinct stomach is not detectable; the presumed stomach lumen appears to extend dorsally, towards the intestine.The digestive gland—pale yellow in living specimens, Fig. 1b—is an externally smooth sac, its outer wall is covered by a mesh of criss-crossing muscle fibers. The digestive gland's rounded anterior face fills much of the body cavity, its posterior face slopes downward (also visible in living specimens) and ends in an elongate tip at about two thirds of the body's length (Fig. 1f). The digestive lumen is outlined irregularly by an epithelium formed mainly by high columnar cells that are rounded apically (surface shown in Fig. 1f) and filled with blue-staining droplets (Fig. 4g, 5c).

The origin of the ciliated intestine is pushed into the digestive lumen in an about 70 μm long trunk-like extension at the anterodorsal side (Fig. 4g); its connection to the stomach is unclear. From there, the intestine curves to the right and runs backwards along the body side to the end of the body, where the anus is situated medially, just dorsal of the foot sole's posterior tip (Fig. 5f).

Central nervous system

The cerebral nerve ring is situated prepharyngeally and most of its ganglia adhere closely to the dorsal and lateral sides of the pharynx (Fig. 1a'). In all ganglia, neurons are situated peripherally just underneath a blue-staining fibrous layer, with central fibrous neuropil extending to the outside as nerves. Accessory ganglia can be distinguished histologically by their distinctly smaller neurons and less obvious separation into cortex and neuropil (Fig. 4b–d).

The paired cerebropleural ganglia are the largest ganglia and are connected by the thick cerebral commissure; each ganglion is hemispherical anteriorly and oblong posteriorly. The cerebropedal and pleuropedal connectives connect each cerebropleural ganglion to the pedal ganglia. The connectives to the ganglia on the visceral loop (pleuroparietal c.) are short (left side) and very short (right side). The

cerebrobuccal connectives are long and slightly undulated; they emerge from the medioventral side of each cerebropleural ganglion and run along the sides of the pharynx. Only the right cerebro-buccal connective could be traced along its entire length.

From each cerebropleural ganglion, four nerves emerge and run laterally and frontally. The anterior and median oral nerve is of medium thickness and appears to innervate the oral tube and mouth opening; on the left side, this nerve shows a distal bifurcation. Slightly more laterally, the very thick labiotentacular nerve emerges; this nerve shows two branches that are equipped with several accessory ganglia: the lateral branch innervates a large ganglion (70×50 μm), the median branch shows along its length four smaller ganglia (25–40 μm) that are closer to the digestive tract. On the left side, the first of the small ganglia and the large ganglion are partially fused. The large ganglion emits several short nerves innervating the most anterior epidermal pit in position of the Hancock's organ, while the smaller ganglia show nerves running medially, towards the oral tube and mouth opening.

Two further nerves emerge from the sides of each cerebropleural ganglion. One is thin and extends dorsolaterally (headshield nerve; Fig. 1c). The rhinophoral nerve is very thick (diameter 20 μm) and emerges laterally; it shows a rather wide connection to the cerebropleural ganglion with possibly two separate roots in the cerebro-pleural ganglion. The rhinophoral nerve splits close to its base, each part supplying two large accessory ganglia: the anterior one is elongate and about 100 μm long, the posterior one is situated more posterodorsal and oval (70×50 μm). Again, each ganglion innervates sensory cells in pits of the Hancock's organs via at least two to three short nerves (Fig. 4d). A fifth cerebral nerve, thin and running to the oral tube, was detected only on the left side, emerging anterior of the left cerebrobuccal connective. *Pluscula cuica* lacks eyes.

The paired buccal ganglia are of medium diameter and situated at the posterior side of the pharynx just below the origin of the esophagus, under which the buccal commissure passes. Buccal nerves could not be detected.

The paired pedal ganglia are almost spherical and connected by the long pedal commissure. Several nerves of different diameter originate from each ganglion, in general running to the body sides and into the foot. One anterior-running nerve emerges just next to the cerebropedal connective, two nerves emerge close by on the anteroventral face of the pedal ganglion and run anteriorly, and a very thick posterior nerve exits from the posteroventral side. A further posterior-running nerve was found only on the left side, while a dorsolateral nerve emerging just anterior to the statocyst was detected only on the right.

The spherical statocysts are located on the posterodorsal side of each pedal ganglion; each statocyst is of approximately 30 μm diameter and contains a single statolith (Fig. 4f). The static nerve could not be detected. Just anterodorsally to the statocysts of both sides there is a conspicuous 'blister'-like cell containing a large unstained vesicle or vacuole (Figs. 1c, 4f).

There are three medium-sized ganglia on the euthyneurous visceral loop; two are close together on the left side (1, the left parietal and 2, the combined subintestinal and visceral ganglion; terminology after Haszprunar 1985), the third (combined supraintestinal and right parietal ganglion) being situated just behind the right cerebropleural ganglion. Ganglia two and three are connected by a very long connective passing below the pharynx close to the pedal commissure. The left parietal ganglion is elongate and shows a single nerve curving to the left body side. Ganglion number two (medium-sized, rounded) shows two nerves: the left one thin, the right one (visceral nerve) very thick. Both nerves run posterior inside the body cavity. Ganglion number three (medium-sized) shows another very thick nerve running posterior along the right side of the body cavity.

An additional ganglion, consisting of two to three small lobes, can be found between the female genital opening and the sac of the bursa copulatrix (Fig. 1g). The connection to the central nervous system (CNS) could not be clarified, but there is a short nerve running to a small ciliated pit located inside the right lateral groove just dorsal to the genital opening. This pit consists of higher cells than the surrounding epidermis and might represent a small osphradium (Fig. 5e); we therefore regard the associated ganglion to be an osphradial ganglion.

Pericardial complex

The pericardial complex comprises the main parts of the circulatory and the excretory systems and fills the posterior end of the body cavity.

The circulatory system consists of the thin-walled pericardium, broad auricle and oval ventricle and is located at the posterior right of the body cavity (Fig. 1a'). The auricle is almost as wide as the posterior end of the pericardium and curves around the more anterior ventricle (Fig. 1g). The proximal end of the ventricle is equally thin-walled but shows a transversal, valve-like septum separating left and right (Fig. 5d); the ventricle's distal tip points marginally to the left and has a slightly thicker, muscular wall from which the aorta emerges. The aorta exits the pericardium at its anterior tip; it runs along the upper right of the body wall, parallel to the intestine. Right of the pharynx it splits into two thin-walled hemolymph vessels (Fig. 4c,f); one turns left, runs below the pedal commissure and then anteriorly, the other passes the CNS on the right and terminates close to the oral tube (not shown).

The horseshoe-shaped kidney broadly touches the posterior wall of the pericardium and expands to the left; it is characterized by the typical vacuolate, unstained epithelium. The ciliated renopericardial duct exits from the posterior right end of the pericardium and curves to the left, leading into the thinner limb of the kidney. This runs into the larger part of the kidney at the left body side, which then curves to the front and right again. A very short and thin nephroduct connects to the renal pore located inside the longitudinal groove just right of the foot's tip (Fig. 1g).

Reproductive system

Pluscula cuica is a monaulic hermaphrodite with follicular gonad, posterior right genital opening, ciliated sperm groove on the right body side and copulatory organ opening through the mouth. The posterior part of the reproductive system fills about half of the body cavity.

In the reconstructed specimen, the gonad (ovotestis) consists of six thin-walled, cone-shaped follicles that radiate from a common mid-dorsal position in the gonoduct (Fig. 1a',d). The follicles are widest at the base where they touch the lateral and ventral body wall or the sloping posterior part of the digestive gland (Fig. 1f). Spermatozoa with screw-shaped head fill most of the follicles' volume (Fig. 5c) and are arranged around large nursing cells. Except for the most dorsal, each follicle also contains two to three oocytes in various stages of development (bright nucleus and blue-staining nucleolus without surrounding yolk, or with various amounts of blue-staining yolk droplets) in its ventral portion. Other cell types—gamete precursors or types of nursing cells—are loosely arranged around the periphery of the follicles.

All follicles discharge via short stalks into the dorsally situated gonoduct, a ciliated tube that runs posteriorly, then curves downward. A short stalk (white asterisk in Fig. 1d) leads downward and connects via a small pore to the very large ampulla—a thin-walled sac filled densely with spermatozoa and extending anterior between the gonad's follicles. Unusually for this organ, the walls of the ampulla are irregularly covered with large cells filled with up to ten very large blue-staining droplets (lipids?) (Fig. 5a,b). The postampullary gonoduct curves to the left, forming the nidamental gland mass with a thick and strongly glandular wall and irregularly shaped ciliated lumen. The entire gland mass consists of three, possibly four histologically different parts, three of which form the more convoluted but thinner part running to the left. The first gland (albumen gland) is a short tube characterized by rounded, light blue/pinkish staining cells with gaps between them (Fig. 5b); the second gland (membrane gland) is equally short and has more columnar cells filled with dark blue-staining small droplets (Fig. 5a); the third gland (mucus gland, proximal limb) is an elongate tube and shows columnar, pink-staining cells. Between glands one and two, the gonoduct wall forms a thin-walled pouch expanding dorsally (another connection could not be found); this pouch is filled densely with spermatozoa (Fig. 5b). It is not clear whether these are auto- or allosperm. The third nidamental gland turns downward. From the turning point on, a uniform part of the gland mass (mucus gland, distal limb) crosses the entire body cavity in a wide curve; its wall resembles that of gland three in histology but is much thicker (cells are at least twice as high and stain slightly darker pink) (Fig. 5a,b). Close to the right body wall, the distal gonoduct becomes non-glandular again for a short distance before opening to the outside; in this part a thin duct splits off and runs straight dorsally (Fig. 5e). Near the end of this duct a spherical pouch (bursa copulatrix) is located at the right body wall (Fig. 1d,g); the bulb is smooth on the outside and shows a more irregular inner surface, its

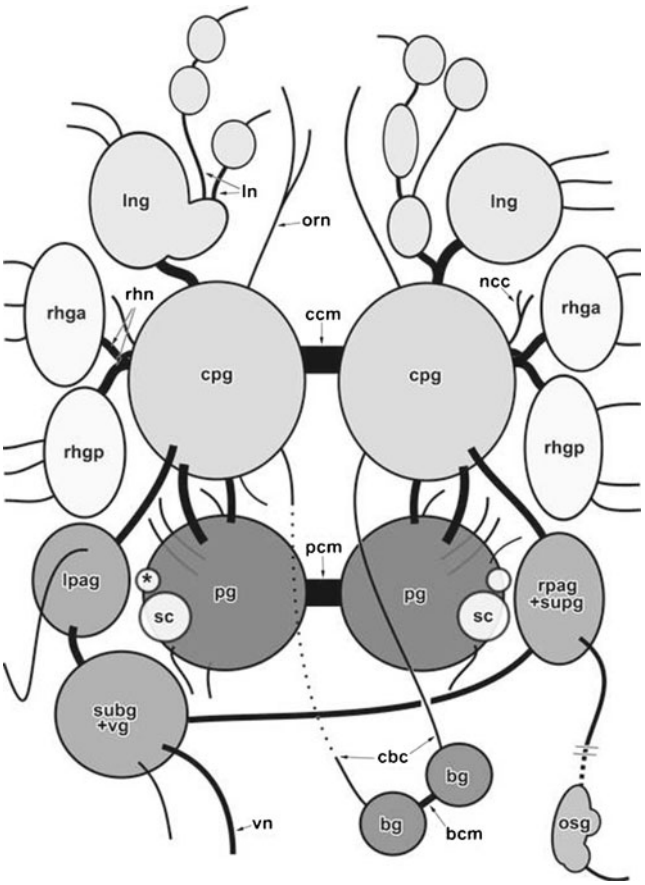

Fig. 2 Schematic dorsal view of the central nervous system (CNS) and nerves, anterior at top. Roughly to scale except for length of pleuroparietal connectives. *bg* buccal ganglion, *bcm* buccal commissure, *cbc* cerebro-buccal connective, *ccm* cerebral commissure, *cpg* cerebropleural ganglion, *ln* labiotentacular nerve, *lng* accessory labial nerve ganglion, *lpag* left parietal ganglion, *ncc* nervus clypei-capitis, *osg* osphradial ganglion, *orn* oral nerve, *pcm* pedal commissure, *pg* pedal ganglion, *rhga* anterior accessory rhinophoral ganglion, *rhgp* posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *rpag+supg* combined supraintestinal and right parietal ganglion, *sc* statocyst, *subg+vg* combined subintestinal and visceral ganglion, *vn* visceral nerve, *asterisk* large 'blister' cell next to statocyst

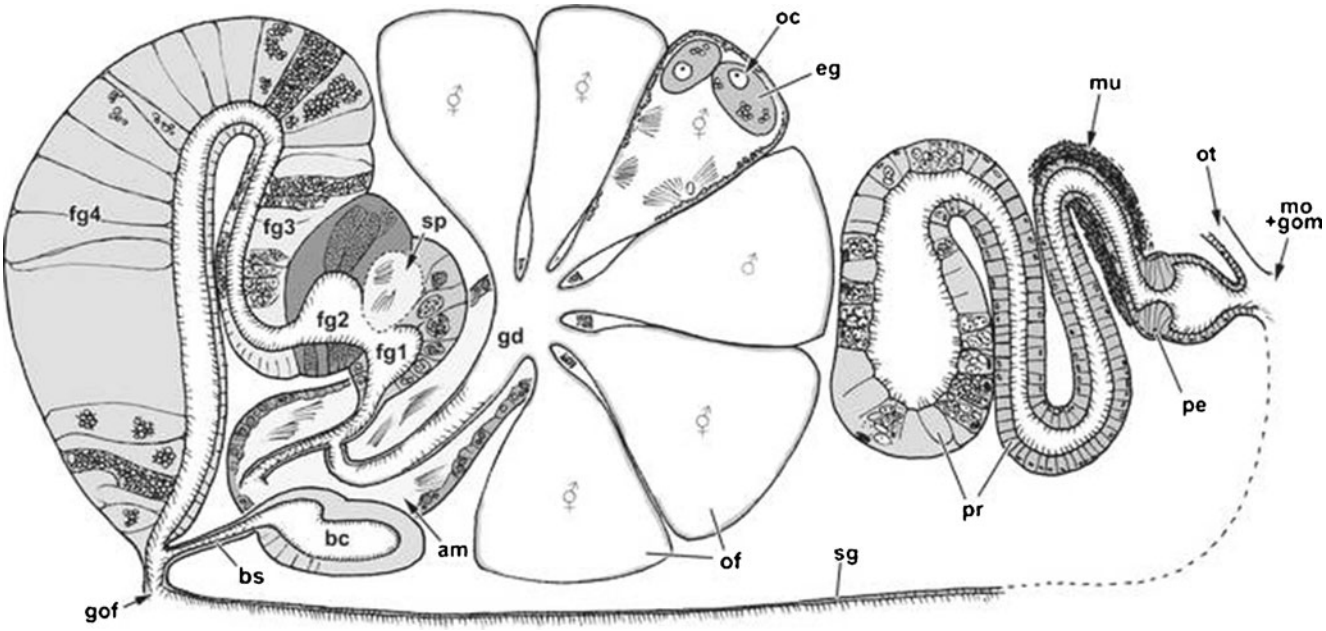

Fig. 3 Schematic dorsal view of the reproductive system, anterior at right. *am* Ampulla, *bc* bursa copulatrix, *bs* bursa stalk, *eg* egg, *fg1* albumen gland, *fg2* membrane gland, *fg3* thin portion of mucus gland, *fg4* large portion of mucus gland, *gd* gonoduct, *gof* female genital opening, *gom* male genital opening, *mo* mouth opening, *mu* muscular tube, *oc* oocyte, *of* ovarial follicles, *ot* oral tube, *pe* penis, *pr* prostate, *sp* sperm package, *sg* seminal groove

lumen is filled with a homogeneous pink-stained fluid (Fig. 5a). The genital opening is a small pore located ventrally in the right lateral groove (Fig. 1a). From the genital opening, a wide ciliated ribbon runs along the ventral portion of the right lateral groove (Figs. 1a,d,g, 5a). The ciliated strip (or sperm "groove") disappears approximately at the level of the pharynx (asterisk in Fig. 1a'), so that there appears to be no further specialized structure for sperm transport to the opening of the copulatory organ within the oral tube. In the foot margin below the end of the sperm groove, there is a group of additional glandular cells that open below the sperm groove.

The copulatory organ opens together with the mouth (Fig. 4a). It is a convoluted, blind-ending tube and extends ventrally in the body cavity as far back as the pharynx (Fig. 1a',f). It connects to the outside via a ciliated duct lined with a regular epithelium of light blue-staining cells with basal nuclei. At first the duct expands slowly before forming an almost spherical pouch, its lumen containing few spermatozoa (Figs. 1f, 2 and 3). The posterior wall of this hollow structure is considerably thicker and forms a circular rim projecting into the lumen, likely forming a penial papilla when everted to the outside (Figs. 1e, 4b). Pouch and papilla are followed by an elongate tube curving to the left; this tube shows only thin epithelial lining but is surrounded by a conspicuous mantle of thick, circular muscle fibers (Fig. 4c). The following prostate is the largest part of the copulatory organ and forms three loops before ending blindly. Its walls are thick, ciliated and glandular; the cells are filled with unstained vacuoles and mostly apically distributed blue-staining droplets (Fig. 4c,f).

The smaller examined specimen proved to be functionally male. Its gonad consists of six follicles (two large, four smaller) that contain only spermatogenesis. The gonoduct is long, sinuous and non-glandular. There is a comparatively small ampulla with characterstic histology (blue vacuoles in epithelium). The bursa is small and empty. The copulatory organ is small but shows all elements found in the larger specimen.

Discussion

As expected, histological examination of semithin sections and 3D models generated a detailed dataset of microanatomical information with the potential to correct and/or supplement the original description of *Pluscula cuica* Marcus, 1953a. We compare these data to those available in other philinoglossans, with focus on their relationship to other cephalaspideans and in the light of new euthyneuran systematics that were established by recent molecular approaches.

External morphology and habit

Pluscula cuica can be identified by its typical philinoglossan streamlined habit without an external shell or distinct head-shield. The body is ribbon-shaped and elongate, although less than in *Philinoglossa praelongata* (see Arnaud et al. 1986). The cephalaspidean head-shield is either absent

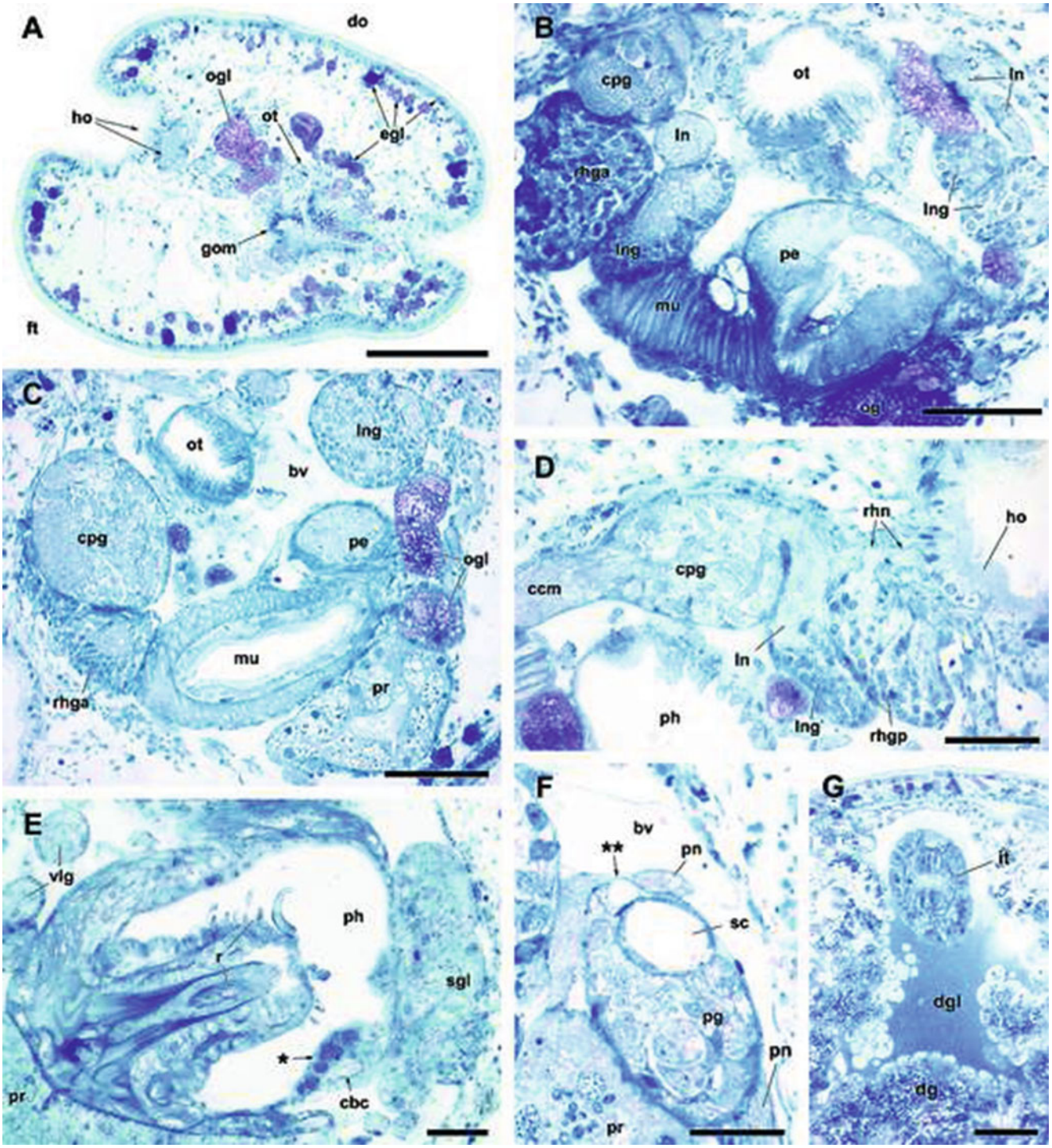

Fig. 4 **a**–**g** Semithin histological cross-sections of anterior body half. Dorsal side at *top*, in **e**: at right. **a** Level of mouth opening, showing lateral grooves. **b** Anterior part of CNS and copulatory organ. **c** Section of CNS and copulatory organ posterior to **b**. **d** Detail of right Hancock's organ and its innervation. **e** Pharynx with muscular odontophore and spread radula; *asterisk* patch of glandular cells. **f** Detail of pedal ganglion with statocyst and 'blister' cell (*double asterisk*). **g** Trunk-like anterior end of intestine inside digestive gland lumen. *bv* blood vessel, *cbc* cerebro-buccal connective, *ccm* cerebral commissure, *cpg* cerebro-pleural ganglion, *dg* digestive gland, *dgl* lumen of digestive gland, *do* dorsum, *egl* different types of epidermal glands, *ft* foot, *gom* male genital opening, *ho* Hancock's organ, *it* intestine, *ln* labiotentacular nerve, *lng* accessory labiotentacular ganglion, *mu* strong muscular lining / muscular tube of copulatory organ, *ogl* oral gland, *ot* oral tube, *pe* penis, *ph* pharynx, *pn* pedal nerves, *pr* prostate, *rhga* anterior accessory rhinophoral ganglion, *rhgp* posterior accessory rhinophoral ganglion, *rhn* rhinophoral nerve, *sc* statocyst, *sgl* salivary gland, *vlg* visceral loop ganglia (sectioned at margins). *Bars* **a** 100 μm; **b**–**e**, **g** 50 μm; **f** 25 μm

or modified into a shield confluent with the rest of the notum. We prefer the second interpretation, since the anterior part of the *Pluscula* shield is cerebrally innervated. Also, a vestigial separation of the head and body shields by a transversal groove in the first quarter of the body is present in another philinoglossid, *Abavopsis latosoleata* (Salvini-Plawen 1973, own observations). As in most other philinoglossans, the broad dorsum and foot are separated by lateral grooves that create a more or less x-shaped aspect in cross-section (an exception is *Sapha* Marcus, 1959, which is more or less round). Histological similarity of notum and foot surfaces (ciliated epithelium, epidermal glands) might be associated with the ability to crawl on either body side (observed by Hughes 1991), since all-around ciliation is present in many small-sized interstitial heterobranchs and facilitates movement between sand grains (Swedmark 1968). The foot of *Pluscula* is slightly wider than the notum and might reflect vestigial cephalaspidean parapodia. These lateral foot extensions are more pronounced in *Abavopsis,* which shows foot margins that curve upward (Salvini-Plawen 1973). This is slightly less the case in *Philinoglossa*, and *Sapha* shows only indistinct foot margins (Marcus 1959). Parapodia are a feature found in most philinoids (Burn and Thompson 1998), so the presence of a widened foot in *Abavopsis* and *Pluscula* might reflect the ancestral condition.

Pluscula shows the typical caudal overhang of the notum, underneath which the body openings are located in the body wall (see below). The caudal overhang of *Pluscula* is broad and fin-like as in *Abavopsis* and the *Philinoglossa* species; where it was observed to form a bilateral symmetric cavity if the overhang is bent downwards (Salvini-Plawen 1973). In *Sapha*, the overhang is pictured as short and pointed (Marcus 1959); an undescribed '*Philinoglossa*' from Fiji (Morse 1987) resembles this species in that aspect.

In their elongate habit and reduced shell, philinoglossans resemble most the aglajid genera *Philinopsis* or *Nakamigawaia* of which some are infaunal burrowers (Gosliner 1980). These taxa however have a fairly long head-shield (half of body length or longer) in contrast to the vestigial head-shield found in *Abavopsis*, which is rather short as in Gastropteridae (Salvini-Plawen 1973; Gosliner 1989).

Shell remnants and mantle cavity associated organs

Pluscula cuica and all other philinoglossans are externally shell-less and show a reduced mantle cavity that is roofed by the caudal overhang of the mantle. In *Pluscula*, within a short stretch of epidermis on the right body side there is the anus, the yellow gland opening, the nephropore, the genital opening and the osphradium. *Pluscula cuica* was described to possess a small internalized circular shell below a dorsal depression in the caudal end (Marcus 1953a), neither of which is present in any other philinoglossan. While not easily visible in live *Pluscula*, the dimple is quite distinct in the preserved ones examined in this study. However, no remainders of a decalcified shell in macerated specimens, or remnants of an organic matrix or empty spaces in histological sections were observed. Still, the presence of putative vestigial shell-forming tissue just underneath the dimple was confirmed herein, and this is interpreted as an ancestral feature that apparently was lost in (all?) other philinoglossans. Other features present in the putatively ancestral philinoidean mantle cavity and known only for *Pluscula* but no other philinoglossan are the osphradium (detected herein for the first time) and the genital opening associated with the mantle cavity (see the respective chapters).

The spherical yellow gland found in the caudal overhang of philinoglossans is a conspicuous histological feature and visible in many live specimens. In *Abavopsis* and *Philinoglossa* (except *P. marcusi* Challis, 1969b) it is described as an externally visible bright orange spot (Salvini-Plawen 1973, 1984), implying a strongly yellow secretion. In *Sapha*, the gland is located in the pointed tail end (Marcus 1959). Since filled glands apparently turn black by certain preserving agents, Salvini-Plawen (1984) considered them to be homologous with the 'black larval kidney' found in some other heterobranchs, implying paedomorphism (see Haszprunar 1985). These organs are in fact described to be present in larval *Philinoglossa* (Swedmark 1968), but are reduced during metamorphosis—otherwise they would be visible as conspicuous black bodies, as observable, e.g., in some post-metamorphic *Philine* (Horikoshi 1967). Alternatively, Salvini-Plawen (1973) suggested the gland to be part of an adhesive mechanism that he observed in *P. praelongata*: this species supposedly attaches to sand grains by its tail end, aided by 'glands of the epidermis and the pallial gland'. This was not observed for other philinoglossans yet but might well represent an adaptation similar to other members of the meiofauna. Some of these show localized adhesive mechanisms, e.g., rhodopemorphs that possess a caudal adhesive gland that are likely derived from glands of the foot sole (Brenzinger et al. 2011b), and thus not homologous to the gland in philinoglossans.

The nomenclature of glands located in the floor or roof of the mantle cavity in traditional opisthobranch taxa is confusing (see e.g., Wägele and Klussmann-Kolb 2005; Wägele et al. 2006 for review), therefore homologies are difficult to establish. With respect to the yellow gland of *Pluscula*, position and histology—large unstaining vacuoles in the spherical gland surrounded by muscle fibers, with epithelial duct opening ventrally, yellow secretion—were already described by Marcus (1953a). He noted similarities to the 'Blochmann's' gland in *Aplysia* but followed Guiart (1901) in simply naming it a 'pallial' gland. Salvini-Plawen (1973) highlighted the similarities to the Runcinacean 'pallial' or 'suprabranchial' gland; we confirmed this observation. In histological aspects, the gland of *Pluscula* resembles most the 'yellow' gland of

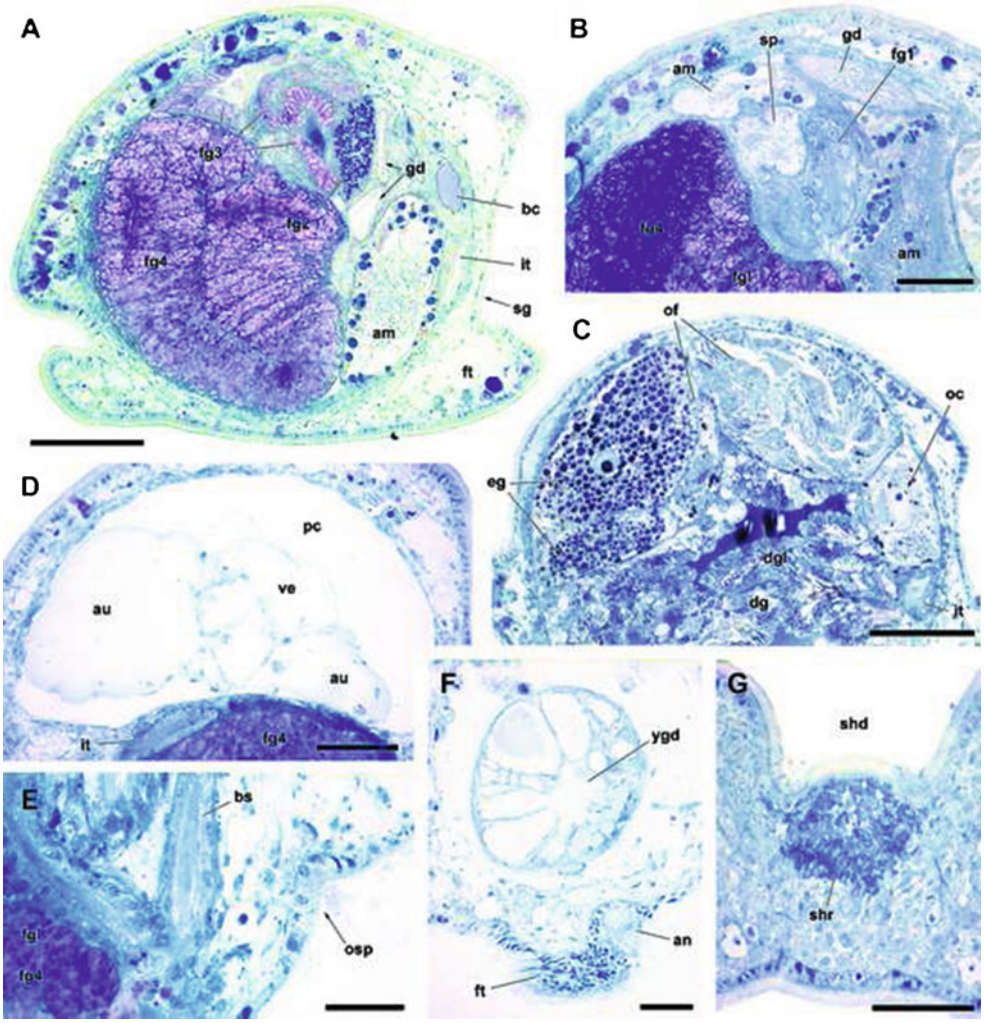

Fig. 5 **a**–**f** Semithin histological cross-sections of posterior body half. Dorsal side at top. **a** Overview at level of nidamental glands. **b** Detail of nidamental glands with interjected sperm package. **c** Ovarial follicles. **e** Most distal gonoduct and osphradium. **f** Yellow gland. **g** Caudal dorsal depression with shell 'remnant', insert: complete cross-section. *am* Ampulla, *an* anus, *au* auricle, *bc* bursa copulatrix, *bs* bursa stalk, *dg* digestive gland, *dgl* digestive gland lumen, *eg* egg, *fg1* albumen gland, *fg2* membrane gland, *fg3* short limb of mucus gland, *fg4* large limb of mucus gland, *fgl* female gland lumen, *ft* foot, *gd* gonoduct, *it* intestine, *oc* oocyte, *of* ovarial follicle, *osp* osphradium, *pc* pericardium, *shd* shell 'dimple', *shr* shell 'remnant', *sg* seminal groove, *sp* interjected sperm package, *ve* ventricle, *ygd* duct of yellow gland. *Bars* **a**, **c** 100 μm; **b**, **d**, **g** 50 μm; **e**, **f** 25 μm

aglajids which Rudman (1972a, 1978) considered unique for that family. Dayrat and Tillier (2002) rejected the homology of yellow and purple/Blochmann's glands, and Blochmann's glands were coded as absent for aglajids by Wägele et al. (2006). Most other philinoids also show glands in the mantle cavity, but these are often groups of single subepithelial cells that do not open though a common epithelial duct and are therefore difficult to homologize. For example, the 'pallial' glands of some *Philine* species consist of a patch of cells that open separately into the mantle cavity (e.g., Challis 1969a; Rudman 1972b: 'posterior' gland; Guiart 1901: 'fossette glandulaire'). Nevertheless, a conspicuous yellow secretion was reported for *P. trapezia* Hedley, 1902 (Rudman 1998) and *P. caledonica* Risbec, 1951 (Risbec 1951; which might be the same species according to Rudman, 1998). Members of the Gastropteridae sometimes show a patch of dark-staining glandular cells surrounding the anus (Brodie et al. 2001; Klussmann-Kolb and Klussmann 2003); their additional large 'posterior pedal gland' is different in structure or in position (e.g., Gosliner 1994). Lemche (1956) reported the unicellular or multicellular 'Blochmann's' glands of *Cylichna*, positioned dorsally in the mantle cavity roof and with an epithelial duct, to contain a secretion that is yellow in life but does not stain with methylene blue. *Scaphander lignarius* L., 1758, a species that produces a thick yellow fluid when disturbed (Guiart 1901), possesses single-celled Blochmann's glands that open through an epithelial duct (Perrier and Fischer 1911). Therefore, it seems that glands situated dorsally in the mantle cavity (or what is left of it) are present plesiomorphically in most philinoids, and persist in many or most other cephalaspideans and Euopisthobranchia. The aforementioned histological staining properties and position have also been reported for the Blochmann's gland of *Haminoea* by Wägele and Klussmann-Kolb (2005: Fig. 5d). Therefore, we regard the yellow gland of philinoglossids and aglajids to be a derived multicelled Blochmann's gland. The specific configuration may represent a synapomorphy of these two families. However, since the most recent molecular phylogenies never found a sistergroup relationship between the two families, the yellow gland might have been lost or modified in other philinoidean lineages, or may be a product of convergent evolution in philinoglossids and aglajids. Regarding the function of the aglajid gland,

Rudman (2001) assumed either an excretory or defensive function and observed the secreted substance to be toxic for annelids. Sleeper et al. (1980) identified the gland's secretions in *Navanax* as 'alarm pheromones', Cruz-Rivera (2011) observed an 'amber-coloured' secretion to repel potential fish predators.

Pluscula cuica thus matches other philinoglossans in the reduction of a distinct shell, although associated tissues are still present. The mantle cavity is also lost, but most organs and body openings found within the ancestral cephalaspidean mantle cavity are still present underneath the caudal overhang. Only the gill and current-inducing ciliated strips—typical for philinoidean mantle cavities (e.g., Rudman 1972b)—are lost completely, as is the case in all other meiofaunal slug lineages (Swedmark 1968; Arnaud et al. 1986). This loss of course indicates that respiration has to take place entirely through the body wall, as is supported by Bartolomaeus' (1997) observation of numerous subepidermal blood sinuses in *Philinoglossa helgolandica* Hertling, 1932.

Circulatory and excretory systems

Our findings on the circulatory and excretory systems of *Pluscula cuica* correspond well to the original description (Marcus 1953a). The heart is located slightly right of the midline, and consists of a wide auricle posterior and slightly left of the ventricle, indicating that *Pluscula* is almost completely detorted. This organization is in general agreement with Bartolomaeus' (1997) ultrastructural study on the heart and kidney of *P. helgolandica* which showed that the valve is described to consist of only a single, flattened cell. Judging from our histological sections, there appear to be more nuclei in the valve of *Pluscula*. As described by Marcus (1953a), the kidney is largely horseshoe- or 'u'-shaped and consists of a slim part running from the pericardium to the left, and a more voluminous part curving back to the nephropore at the right body side. The parts of the kidney appear very similar in histology; we were not able to detect ciliation in the proximal part described for *P. helgolandica* by Bartolomaeus (1997).

Digestive system

The digestive system of *Pluscula* conforms well to the original description and the general philinoglossan organization. Described differences among the genera can be found in the presence of denticles on the first lateral teeth, possibly the presence of a vestigial crop in *Pluscula* and in the form and dimensions of the digestive gland.

Nearly all philinoglossids are described with a long and curved pharynx similar to that of *Pluscula*, with the radula situated far posterior (Hertling 1932; Marcus and Marcus 1954; Marcus 1959). Our material suggests that the anterior part of the pharynx and especially the posterior oral tube are rather expandable due to the presence of longitudinal folds. Chitinous jaw plates present in Euopisthobranchia are secondarily lost in many Philinoideans (Burn and Thompson 1998), including philinoglossids. Jaws are present only in some philinids and allgastropterids (Rudman 1972b; Gosliner 1980, 1989), therefore jaws were lost multiple times convergently. All philinoglossans possess a radula (formula given as $n \times 3.0.3$ or 2.1.0.1.2) that especially resemble philinids and gastropterids in tooth form (see Gosliner 1994). Since reduction of the rhachidian tooth row has occurred separately in all other philinoid families, it is therefore hardly useful for phylogenetic comparison with philinoglossans (see Gosliner 1980; Rudman 1972b). The first lateral teeth of *Pluscula*, *Abavopsis*, *Philinoglossa praelongata* and *P. marcusi* are described without smaller denticles along the masticatory border; however, denticles of this size might be hard to detect without SEM studies and their number also depends on the size of specimens (see Salvini-Plawen 1973; Challis 1969b). Therefore, 'absence of denticles' in the literature might not always be a useful taxonomic character in philinoglossans, as is exemplified by *Pluscula*: neither Marcus (1953a) nor our light microscopical examination of sectioned material and separated radulae revealed denticles, but Marcus and Marcus (1954) mention about 20 denticles per tooth in later collected material. Comparative re-examination using scanning electron microscopy might be needed to reveal if denticulate teeth occur consistently in any philinoglossan, or if intraspecific plasticity reduces the taxonomic value of this character, as is known for some other marine gastropods (e.g., Padilla 1998; Reid and Mak 1999). Following the pharynx, *Pluscula cuica* shows a slightly dilated esophagus where most other philinoideans have an unarmed crop (Aglajidae, e.g., Rudman 1974, Gastropteridae: Gosliner 1989) or a gizzard armed with cuticular plates to grind up hard-shelled food (many Philinidae: Rudman 1972b). Neither crop or gizzard are described for other philinoglossans, but the structure found in *Pluscula* may represent vestiges of the ancestral condition, if not an artifact. A gizzard armed by cuticle was regarded as a synapomorphy of Euopisthobranchia (Jörger et al. 2010a), but spines or calcareous plates are reduced secondarily in many philinoideans (Burn and Thompson 1998).

Pluscula (and *Sapha*) do not possess a histologically distinct stomach between esophagus and digestive gland, in contrast to *Abavopsis* and *Philinoglossa*, which are described with a small and smooth-walled stomach (Salvini-Plawen 1973). In *Pluscula*, the pale yellow digestive gland is a single sac and located anterior to the gonad in mature specimens. The sloping rear face of the digestive gland—visible in living specimens—might be a useful diagnostic character for *Pluscula*, and was also observed in an undescribed species from Belize (K.M. Jörger, Munich, personal observation). In all other species the digestive gland extends almost to the end of the body cavity. *Sapha* and *Abavopsis* possess a single digestive gland (Marcus 1959; Salvini-

Plawen 1973); in *Philinoglossa* there are two tubular branches, one of which is long, coiling, and ventral to the gonad (Hertling 1932; Marcus and Marcus 1954; Salvini-Plawen 1973). The latter case resembles other philinoids that possess more than one digestive gland, e.g., *Philine exigua* (Challis 1969a; Martínez et al. 1993). In all philinoglossans, the intestine emerges from the stomach/ digestive gland anterodorsally and curves along the right body side; the anus is posteriomedian. Only in *Abavopsis* the intestine is described to emerge more on the left, running underneath (!) the digestive gland for much of its course (Salvini-Plawen 1973). The funnel-like extension of the proximal intestine into the digestive gland lumen was found only in the reconstructed specimen and may be an artifact, since it is not reported for other philinoglossans species.

There are no reports of philinoglossan food sources, although Marcus and Marcus (1954) mention 'a large diatom' in the intestine of *P. remanei* Marcus and Marcus, 1958. The lack of distinct cuticular armament in the gut implies that food is not hard-shelled. Radular morphology, coupled with the thin pharyngeal cuticle and infolding of the (?dilatable) preradular digestive tract, may hint at a carnivorous habit of philinoglossans on soft-bodied prey. Although predation was not observed directly, co-occuring acochlidians extracted from sand samples disappeared from Petri dishes when kept with philinoglossans over night and thus may be a possible food source, at least under lab conditions (own observations). Carnivory would be consistent with the general condition in Philinoidea.

Central nervous system

One reason to argue for a basal phylogenetic position of *Pluscula cuica* within Philinoglossidae, or for separation from the latter in its own family, was the supposed "primitiveness" of the cerebral nerve ring and the visceral nerve cord. This was based on the supposed separation of cerebral and pleural ganglia (Marcus 1953a, 1959; Salvini-Plawen 1973) and also the presence of five distinguishable ganglia on the visceral nerve cord (albeit four of them closely allied, forming two pairs; Marcus 1953a). Reexamination of the nervous system, however, shows that neither is the case. Free pleural ganglia in *Pluscula* were identified originally by Marcus (1953a) lateral to the cerebral ganglia, with connectives to the latter and the pedal ganglia. This is a misobservation, since cerebral and pleural ganglia form fused cerebropleural ganglia as is evident from semithin histological sections and visible on the 3D model. As other philinoglossans, *Pluscula* has cerebropleural ganglia showing characteristic double connectives to the pedal ganglia. Marcus' laterally situated 'pleural' ganglion therefore is most likely the (posterior) rhinophoral accessory ganglion; however, the reported connective of these laterally situated ganglia to the pedal ganglion does not exist. This unusual lateral-pedal connective was also described for the 'lateral' ganglia of *Philinoglossa remanei* and *P. praelongata* (Marcus and Marcus 1954; Salvini-Plawen 1973). It should be critically reinvestigated whether this connective presents a genuine structure.

The presence of five ganglia on the visceral cord has been proposed as a synapomorphy of Euthyneura (=Pentaganglionata, Haszprunar 1985, 1988), although most taxa possess a lower number of separate ganglia that have been interpreted as the result of various stages of ontogenetic fusion. Dayrat and Tillier (2000) challenged such a scenario claiming that there are very few reliable examples of euthyneurans showing a pentaganglionate condition, i.e., just six genera, of which two belong to basal heterobranchs according to molecular data (see Schrödl et al. 2011a). *Pluscula* was overlooked as a pentaganglionate candidate; if confirmed, it would be the only cephalaspid reliably showing five ganglia on the visceral loop. Our results, however, demonstrate that mature *Pluscula* possess only three ganglia on the visceral nerve cord. These three ganglia correspond well to the single ganglion and two closely aligned pairs mentioned by Marcus (1953a), although our material shows more than superficial fusion. The visceral nerve cord of *Pluscula* is not fundamentally different from that of other philinoglossans, since all other species are described with three ganglia, except for *P. praelongata* which Huber (1993) reinvestigated and reported four (although his Fig. 10 shows only three).

Cerebral nerves and sensory organs

Pluscula cuica possesses a set of four paired cerebral nerves (plus a single nerve on the left side) that correspond well to the nerves found in previous investigations of other cephalaspidean species (Faller et al. 2008; Staubach et al. 2008). Following the nomenclature of nerves identified by the previous authors and Huber (1993) in other heterobranchs, we identified an oral nerve (anteromedian), the labiotentacular nerve (basally branched, with one large and several small extra ganglia), the rhinophoral nerve (possibly with a double root, basally branched with two large extra ganglia), and a small nervus clypei-capitis (head-shield nerve). The single median nerve extending from the left cerebral ganglion could not be identified, and a corresponding nerve on the right side was not detected either. The finding of a vestigial head-shield nerve (n. clypei-capitis) in *Pluscula* is important since it suggests an ancestral presence and secondary reduction of a functional cephalaspidean headshield in philinoglossans. Most cephalaspideans possess an elaborate nervus clypei-capitis that innervates the posterior part of the head-shield (e.g., Staubach et al. 2008); this nerve is less branched in other heterobranchs, if identified at all (Huber 1993). Reduction of an externally discernible head-shield is thus confirmed as one of the synapomorphies of philinoglossans (Arnaud et al.

1986). Only *Abavopsis latosoleata* shows a slight transversal groove indicating remainders of a separate head-shield (Salvini-Plawen 1973), and previously only this genus was shown to possess a thin nervus clypei-capitis branching from the base of the rhinophoral nerve (Huber 1993). If confirmed, a loss of the headshield nerve in *Philinoglossa* (shown by Huber 1993) and *Sapha* might represent a synapomorphy uniting these genera.

Pluscula cuica is unusual among philinoglossans in that it lacks eyes, which appears to be an apomorphy of the species. In *Abavopsis* and *P. praelongata,* the eyes are innervated through a branch of the large labiotentacular accessory ganglia (Salvini-Plawen 1973). Among meiofaunal slugs, loss of eyes is found convergently among several taxa (Swedmark 1971), e.g., among rhodopemorphs (own observation), pseudovermids (see Urgorri et al. 1991) and some acochlidians (Marcus 1953a).

We are not aware of literature mentioning the paired 'blisters' embedded in the pedal ganglia next to the statocysts. They are not present in *Philinoglossa praelongata* (own observation). The structures might represent single specialized cells. If not for their position next to the statocysts, one might confuse the structures with the vestigial, unpigmented eyes found, e.g., in some acochlidians (see Challis 1968; Neusser et al. 2011a).

Accessory ganglia

Accessory ganglia anterior and lateral to the cerebropleural ganglia are described for all philinoglossans examined in detail, but nomenclature and proposed innervation patterns differ considerably in the descriptions (e.g., Marcus 1953a, 1959; Salvini-Plawen 1973; Huber 1993). In all cases there appear to be large ganglia (lateral and anterolateral to the cerebropleural ganglia) and distinctly smaller ones (mostly anterior and more median). In *Pluscula*, one large rhinophoral ganglion was identified originally as the pleural ganglion (see above); five further 'precerebral' ganglia were described on both branches of the labial nerve (Marcus 1953a). In *Sapha*, there are paired large 'Hancock's' and 'olfactory' ganglia, and pairs of small 'labial' and 'prepedal' ganglia (Marcus 1959); innervation of these ganglia was not described. *Abavopsis* was originally described without accessory ganglia (Salvini-Plawen 1973), but Huber (1993) showed that there are two large ganglia on each rhinophoral nerve and one large and one small on each labiotentacular nerve, similar to the condition found in *Pluscula*. *Philinoglossa praelongata* was described originally with small anterior 'accessory' ganglia and two large ganglia innervating the Hancock's organs: one 'olfactory' ganglion (with the two connectives to the cerebropleural and pedal ganglia as originally and falsely described for *Pluscula*; = accessory rhinophoral ganglion?) and one 'labial' ganglion (also innervating the eye; = large labiotentacular ganglion?) (Salvini-Plawen 1973). Except for the double connective, this configuration largely agrees with Huber's (1993) examination of the same species. A connective between the pedal and a large 'precerebral' ganglion was again described for *Philinoglossa remanei* by Marcus and Marcus (1954); this ganglion also innervates the Hancock's organ together with two 'olfactory' ganglia, besides smaller 'labial' ganglia. The number of large ganglia in *P. remanei* (two or three) is not entirely clear. Summarizing the literature and homologizing with the ganglia found in *Pluscula*, the following general pattern of innervation of the accessory ganglia appears to be present in all philinoglossans: there is one accessory rhinophoral ganglion in *Sapha* and *Philinoglossa praelongata*, and two in *Pluscula* and *Abavopsis*. These and the large accessory labiotentacular ganglion innervate the posterior and anterior parts of the Hancock's organ, as is postulated or observed for numerous cephalaspideans (e.g., Huber 1993; Mikkelsen 1996; Staubach et al. 2008). A variable number of smaller accessory labiotentacular ganglia innervate the lip and/or oral tube.

Additional, accessory ganglia innervated by cerebral nerves are characteristic features of meiofaunal slugs. These structures are described for rhodopemorphs (Salvini-Plawen 1991), pseudovermid nudibranchs (Ev. Marcus 1953a; Huber 1993), the sacoglossan *Platyhedyle* (Rückert et al. 2008), microhedylacean acochlidians (e.g., Neusser et al. 2006) and the limnic hedylopsacean *Tantulum* (Neusser and Schrödl 2007). Among Cephalaspidea, only philinoglossans and *Philine exigua* (Challis 1969a) show accessory ganglia. Wherever examined, these accessory ganglia are innervated by the rhinophoral and labiotentacular nerves (as in *Pluscula*). Accessory ganglia are often histologically distinct in lacking a separation into cortex and medulla (Neusser et al. 2006). Marcus (1953a) specifically states that this is not the case in *Pluscula cuica* (in contrast to the acochlidian *Ganitus evelinae* described in the same paper). Our material shows that the neurons in the accessory ganglia of *Pluscula* are considerably smaller than those in the other ganglia, making identification on histological sections possible at a glance. This is in contrast to the accessory ganglia of acochlidians that differ in overall organization but not in neuron size (as mentioned above). The function of the conspicuous accessory ganglia of meiofaunal heterobranchs has so far been a matter of speculation. Haszprunar and Huber (1990) argued that additional neurons were needed in small-sized ganglia to help mediating 'essential activities'. However, they also noted that miniaturized slugs that are not meiofaunal, e.g., runcinids or the nudibranch *Vayssierea*, do not show these accessory ganglia (e.g., Huber 1993; Baba 1937) and that the evolution of accessory ganglia is therefore linked to the mesopsammic habitat. Since the accessory ganglia are invariably found associated with sensory nerves, they might rather reflect the need of additional nervous capacity in this three-dimensional interstitial living space, as was argued by Jörger et al. (2008). The development

of large accessory ganglia innervating the Hancock's organs may imply comparatively enhanced chemosensory or tactile capabilities, involved in trailing chemical cues or for simply finding the easiest way to push through the complex three dimensional pore-spaces of the interstitial habitat.

Osphradium

Pluscula cuica is the so far only meiofaunal slug demonstrated to possess an osphradium with an associated ganglion. Originally, a posterior 'genital' ganglion close to the female genital opening was described for *Pluscula* and *Sapha* (Marcus 1953a, 1959), but innervation patterns were not observed. In *P. remanei*, Marcus and Marcus (1954) assumed innervation by the visceral nerve. In *Abavopsis*, a possibly similar ganglion is located at the posterior end of the copulatory organ (Salvini-Plawen 1973). Our material of *Pluscula* confirms the presence of the ganglion next to the genital opening and also shows innervation of a small pit resembling a small osphradium in histology (ciliated pit with higher, unstained, columnar cells; see Edlinger 1980) and position (right body side, close to organs and body openings plesiomorphically situated in a mantle cavity). We therefore regard this posterior ganglion to be homologous to the osphradial ganglion of other heterobranchs. In this case the ganglion should be innervated by the nerve extending from the combined right parietal and supraintestinal ganglion (e.g., Haszprunar 1988) and not the visceral nerve which leads into the same general direction. A chemosensory osphradium has not been reported for any other meiofaunal slug. Many acochlidians possess an osphradial ganglion, but an osphradium was detected only in the secondarily large-bodied *Strubellia* and *Acochlidium* (Brenzinger et al. 2011a). Osphradia are likely present in many meiofaunal slugs with an associated ganglion, but in these cases the sensory epithelium has been reduced to only few sensory cells. Presence of sensory areas in other species bearing osphradial ganglia needs reinvestigation.

Reproductive system

The reproductive system of *Pluscula cuica* unites usual and thus plesiomorphic philinoid cephalaspidean features with those that appear highly derived but typical for meiofaunal slugs. The hermaphroditic gonad of adult *Pluscula* is not divided into distinct female and male follicles save for the medial and strictly male follicle. The latter was also described by Marcus (1953a), but interpreted as an autosperm ampulla rather than part of the gonad. Contrary to *Pluscula*, *Sapha* and *Philinoglossa remanei* have strictly female acini located either at the left side or ventral of the strictly male ones, respectively (Marcus and Marcus 1954; Marcus 1959). Data on *Abavopsis* are not conclusive. Spatial separation of gamete production is a feature commonly found in meiofaunal slugs (Swedmark 1968): *Rhodope* shows a consecutive separation of male and female ovotestis follicles (Brenzinger et al. 2011b), some meiofaunal acochlidians have separate ovaries and testes (Morse 1976) or are completely gonochoric (Challis 1968; Schrödl and Neusser 2010). The meiofaunal *Philine exigua* has some follicles that produce either only one type of gamete besides follicles that produce both (Challis 1969a).

Philinoideans generally possess three different sperm storing structures (besides one associated with the copulatory organ): a proximal ampulla for autosperm, a receptaculum seminis for long term storage of allosperm, and a distal bursa copulatrix for allosperm storage and/or lysis (e.g., Gosliner 1994; Mikkelsen 1996). Identification of these structures according to their relative positions rather than histology or a combined approach is advocated (Gosliner 1994; Valdés et al. 2010), but may be a preconception that misses actual structure, homology and function (e.g., Mikkelsen 1996; Wägele and Willan 2000). Our histological data suggests that *Pluscula* possesses a stalked, sac-like ampulla that is unusual in several aspects: first, it is extremely large and splits off an unusually long part of gonoduct that is located between gonad and nidamental glands (instead of being a widening close to the gonad). The ampulla reaches far anterior, but it opens to the gonoduct at its posterior end. Second, the ampulla shows an unusual but distinct histology with large (?lipid) droplets covering the wall, instead of being a thin-walled sac conforming to the gonoduct in histology (see Gosliner 1994). A proximal ampulla is described for all philinoglossan genera; it is also sac-like but smaller in *P. remanei* (Marcus 1953a; Marcus and Marcus 1954), but tubular in *Sapha* (Marcus 1959).

Pluscula does not show a distinct receptaculum seminis: this organ usually follows the ampulla closely and would be identifiable by spermatozoa embedded into the muscularly lined wall with their heads (e.g., Beeman 1977). No such structure is found in the material examined herein, and no receptaculum is described for any other philinoglossans. Loss of a distinct proximal receptaculum seminis may represent a synapomorphy of philinoglossans, since it is present in other philinoidean groups (e.g., Rudman 1972a, b; Gosliner 1980, 1989).

We interpret the distal stalked sac, filled with pink secretion and branching from the gonoduct close to the genital opening, to be a bursa copulatrix. Marcus originally described this structure in *Pluscula* as a 'spermatheca or receptaculum seminis that contains spermatozoa' (1953: p 180); he also describes a 'red and blue' staining secretion. This histological character is typical for the allosperm-digesting bursae, but not for a receptaculum according to newer terminology (Beeman 1977; Valdés et al. 2010). No other philinoglossan is described with a similar structure, but a bursa with at least temporary gametolytic function is present in most other philinoids and may represent a plesiomorphic character in *Pluscula*.

The pocket containing spermatozoa between the membrane and mucous glands in one examined specimen is most likely not a permanent feature. It may be a received package of allosperm or a spermatophore, a temporary fertilization chamber, or a package of autosperm on its way out.

The three parts of the female gland mass of *Pluscula* correspond well to the albumen, membrane and large mucous glands of most other 'opisthobranchs' (Gosliner 1994; Klussmann-Kolb 2001), but comparison to other philinoglossans is not straightforward due to ambiguous literature. *Philinoglossa remanei* has a 'protein' gland and sac-like mucous glands (Marcus and Marcus 1954); the nidamental glands of other species are not described in further detail. In *Abavopsis*, the nidamental glands are situated posteriorly as in *Pluscula,* but are apparently followed by a long distal gonoduct part leading to the anteriorly shifted genital opening (Salvini-Plawen 1973). In *Philinoglossa* and *Sapha,* the distal gonoduct is short since the female glands are also shifted towards the genital opening (Marcus and Marcus 1954; Marcus 1959). This situation differs from that of *Pluscula* and other philinoideans and may be a synapomorphy of a *Philinoglossa*/*Sapha* clade.

The female genital opening in *Pluscula* is close to the posterior end of the body—as in other philinoids—showing its affiliation with the ancestral mantle cavity (Burn and Thompson 1998). In the remaining philinoglossans the opening is in the anterior right third, e.g., at the posterior border of the head-shield in *Abavopsis* (Salvini-Plawen 1973); therefore, the seminal groove that is present in philinoglossans is generally short compared to that of *Pluscula*. At least in *Pluscula*, there is a gap between the seminal groove and the male genital opening. Marcus (1953a) identified acidophilous glands along the rim of the anterior sperm groove in mature individuals, and assumed a role in guiding spermatozoa. We were able to identify additional glands in the foot at this position, although they seem to open through the foot sole and not the sperm groove.

Pluscula cuica possesses a sac-like cephalic copulatory organ that contains several histologically separable parts. Marcus (1953a) originally identified the following elements (from anterior to posterior): an epidermal pouch, a narrow and tubular penis, followed by a short tubular prostate, and a bulbous 'seminal vesicle'. Our material shows that the penis consists of a rather short ring-like structure at the base of the epidermal pouch which is followed by a tube with strong subepidermal circular muscles. The posterior part is histologically uniform becausethe prostate and its autosperm-storing end are confluent, instead of forming a distinct 'seminal vesicle'. In *Sapha*, the copulatory organ was also described to consist of four parts (Marcus 1959), but with a different order: following a distinct penial papilla, there is a long prostate and then a sphincter-like muscle (and not vice versa), the muscle closing the large spherical seminal vesicle. Marcus and Marcus (1958) show a similar configuration in *P. helgolandica*, but mention the short part anterior to the 'seminal vesicle' to be of glandular nature, not muscular. In *P. remanei* and *Abavopsis*, the copulatory organ is described as a simple, bag-like structure with variable orientation, even looping around the oral tube (Marcus and Marcus 1954; Salvini-Plawen 1973). It remains unclear whether the copulatory organ is truly less elaborate in the latter taxa compared to the condition found in *Pluscula*. Nevertheless, the sac-like copulatory organ of philinoglossans in general appears to differ from that of other philinoideans in being less elaborate, probably due to size constraints. Judging from histological examinations, there is no true eversible papilla (perhaps excepting *Sapha*) but only a slightly prominent ring, and there never is the cuticular armament found at least in some groups, e.g., Gastropteridae (Anthes and Michiels 2007a, b). Functionally more important, in philinoglossans there is no separate posterior-leading vas deferens ("ejaculatory duct" according to Mikkelsen 1996) leading directly to the prostate as e.g., in *Philine* species (Rudman 1972b); therefore, autosperm have to enter and exit the copulatory organ via the same opening. This two-way configuration is more similar to what is found, e.g., in the spermatophore-producing *Runcina* species (Kress 1985).

Sperm transfer by a "kiss"?

Rather than anterodextrally as in most cephalaspideans, the male genital opening of *Pluscula* is situated frontally at the head. It is joined to the anterior oral tube, as was also observed by Marcus (1953a). The same condition is reported for *P. helgolandica* (Marcus and Marcus 1958), *Sapha* (Marcus 1959) and *Abavopsis* (Salvini-Plawen 1973). This means that the copulatory organ of philinoglossans has to be everted through the mouth during copulation. It seems that philinoglossans have taken to the extreme a trend that is found in Aglajidae and Gastropteridae (see Anthes and Michiels 2007a, b), where the male genital opening is shifted to underneath the anterior side of the headshield. This is in contrast to other philinoideans that have it located more on the right side of the head (e.g., Rudman 1972b), as is the plesiomorphic condition for cephalaspideans. More specifically, the male genital opening inside the mouth is also found in the meiofaunal acochlidian *Pontohedyle milaschewitchii* Kowalevsky, 1901; this aphallic species glues spermatophores indiscriminately onto a partner's epidermis (Jörger et al. 2008, 2009). In the meiofaunal *Philine exigua*, the opening appears also to be more anterior than in other, burrowing or benthic members of the genus (Challis 1969a). The extreme anterior shift may therefore be another adaptation particular of meiofaunal groups, facilitating sperm transfer within the limited space and dynamics of sand interstices (Swedmark 1964): in an animal

moving between sand grains, it is the anterior face that touches a partner most readily. Sperm transfer would be possible by a simple "kiss" on a quickly passing partner's epidermis (in the case of hypodermal injection or dermal insemination), or on the genital opening in species that copulate. This latter head-to-tail mode of copulation can be suggested for *Pluscula* because of the opposite positions of the male and female genital openings, and because sperm transfer by a trailing 'male' is known to take place in a number of other philinoideans (e.g., Rudman 1972a). However, since the other philinoglossan genera have also shifted the female genital opening anteriorly, copulation in these genera could more be bilateral or sequential, but also more head-to-head and thus again less space-consuming.

Autosperm transport through the hemocoel?

Marcus' (1953a) original description of *Pluscula cuica* suggests a highly peculiar mode of autosperm transport, probably unique among gastropods: on their way between the gonad's follicles and the sperm-storing part of the copulatory organ, sperm were hypothesized to move directly through the hemocoel, and not along the gonoduct and external ciliated groove. This was concluded because (1) apparently all 'mature' specimens examined by Marcus showed numerous spermatozoa free in the body cavity, with the highest density between gonad and copulatory organ, and (2), the external ciliated groove was found to disappear before connecting to the copulatory organ, implying that its original function as a conveyor of autosperm was lost.

We can confirm the peculiar lack of a continuous sperm groove in *Pluscula*, although the gap could be explained by the presence of sensory epithelium (Hancock's organs) in this place (Fig. 1a). Since the lateral furrow itself is quite narrow, it might still have sufficient capability in guiding sperm towards the mouth. Furthermore, there are additional glands below the end of the sperm groove which Marcus (1953a) hypothesized to facilitate a further passage of sperm by producing 'protective secretions' (1953: p 181). The lack of a continuous sperm groove might be a consequence of an overall beneficial apomorphic anterior shift of the copulatory organ. A gap in ciliation may not be much of a hindrance to sperm transport: spermatozoa are known to be capable of moving along the epidermis of species without such a groove [Karlsson and Haase 2002 in the nudibranch gastropod *Aeolidiella*; Brown (1979) on *Colpodaspis thompsoni*]. Since our specimens examined were mature hermaphrodites and none of them contained free spermatozoa in the hemocoel (as would be expected assuming internal autosperm transport) we conclude that sperm is conveyed externally via the sperm groove, as usual.

How then to explain Marcus' observation of hemocoelic spermatozoa in *Pluscula*? If autosperm, it could be squeezing or fixation artifact, or it could have been allosperm. In other meiofaunal slugs, a proportionally common mode of sperm transfer is by hypodermal injection or dermal insemination: it was suggested for species of *Rhodope* (Brenzinger et al. 2011b) and was observed in the microhedylacean acochlidians *Pontohedyle* and *Ganitus evelinae* Marcus, 1953a (Jörger et al. 2009; Marcus and Marcus 1954). In these generally aphallic species, sperm are transferred through the epidermis; at least in *Pontohedyle* this happens by lysis of epidermal cells induced by the dermally applied spermatophore (Jörger et al. 2009). After dermal insemination, the spermatozoa move through the body cavity and fertilization supposedly takes place somewhere inside the gonoduct or directly in the gonad. Explaining Marcus' (1953a) observation of hemocoelic sperm in *Pluscula cuica* in a similar way is, however, inconsistent with the presence of a distal bursa copulatrix in the species. Such an allosperm storage organ is usually present only in copulating species, or in non-copulating species that may inject spermatozoa directly into the (large) bursa using a copulatory stylet (e.g., the acochlidian *Pseudunela*; Neusser et al. 2009b). Since hemocoelic spermatozoa have never been reported in other philinoglossans, their occurrence should be critically reinvestigated in other species.

Origin of the Philinoglossidae

The advent of molecular systematics cast doubt on long-held beliefs in euthyneuran topologies, and studies using multi-locus markers started to change our concepts of their evolution (e.g., Göbbeler and Klussmann-Kolb 2011; Jörger et al. 2010a). The backbone topology of a "new euthyneuran tree", with Nudipleura basal to the common clade of Euopisthobranchia and panpulmonates—as summarized by Schrödl et al. (2011a)—was supported by recent phylogenomic data (Kocot et al. 2011; Smith et al. 2011), and is also compatible with a recent molluscan phylogenetic study based on housekeeping genes (Vinther et al. 2011). In contrast, the traditional concept of monophyletic Opisthobranchia and Pulmonata is contradicted by all phylogenomic and other approaches that include nuclear rather than mitochondrial genes.

Rather than being basal opisthobranchs, the Cephalaspidea in a modern sense (sensu Malaquias et al. 2009) form one of several clades of the so called Euopisthobranchia (Jörger et al. 2010a) among tectipleuran Euthyneura (Schrödl et al. 2011a). Philinoglossans lack the major euopisthobranch synapomorphy, a cuticularized gizzard. Having a large body-shield rather than a head-shield, a posterior mantle cavity, and a simple, frontal copulatory organ they somewhat resemble similarly small-sized runcinids. However, molecular data clearly indicate that philinoglossans are cephalaspideans in the strict sense (Jörger et al. 2010a; Göbbeler and Klussmann-Kolb 2011). The prepharyngeal nerve ring combined with monaulic genital system qualifies Philinoglossidae as Cephalaspidea

sensu Malaquias et al. (2009), and the presence of a secondarily modified head-shield innervated by the nervus clypei-capitis fits with the placement into a higher, non-diaphanoidean clade. Having a narrow radula, a carnivorous gut type without cuticle and gizzard plates and a slender, at least externally shell-less body points towards a placement among philinoidean lineages. In fact, both multi-locus analyses with broader taxon sampling (Malaquias et al. 2009; Göbbeler and Klussmann-Kolb 2011) identify a philinoidean clade of *Philinoglossa* and Gastropteridae as sister to Aglajidae plus Philinidae, with Scaphandridae as outgroup. At the current state of knowledge, possible shared characters of a gastropterid/philinoglossid clade may be a comparatively short headshield and the anterior shift of the copulatory organ. A philinoglossid/aglajid clade on the other hand would be supported by the presence of a spherical yellow gland and the loss of jaws. Molecular hypotheses on the origin of Philinoglossidae within Philinoidea thus are consistent with morphological evidence discussed herein and by Salvini-Plawen (1973), although the exact position remains unclear. Nevertheless, a previously proposed higher category, i.e., ordinal Philinoglossacea Thiele, 1931, is no longer required.

We show that previously discussed "primitive", potentially progenetic or at least aberrant features such as separate pleural and cerebral ganglia, a pentaganglionate visceral loop, and hemocoelic autosperm transfer in *Pluscula* were due to misobservations or artifacts. A gizzard with three plates that is characteristic of ancestral, non-carnivorous cephalaspideans including philinoidean Scaphandridae and Philinidae is absent in most Aglajidae, Gastropteridae (Rudman 1978; Gosliner 1989), and likely carnivorous philinoglossans. This supports their independent origin from mesopsammic *Philine exigua* as indicated by molecular analysis (Jörger et al. 2010a). We propose that philinoglossans are small-sized, though not obviously pedomorphic invaders of mesopsammic spaces, evolving a detorted streamlined body, precerebral accessory ganglia, a frontal, potentially unilateral mode of sperm transfer, losing and modifying allosperm receptacles, reducing the ancestral shell, and reducing and modifying the mantle cavity and associated organs. All these traits are adaptive and synapomorphic for Philinoglossidae, but have evolved convergently in interstitial members of other heterobranch lineages. The conspicuous yellow gland found in *Pluscula* and other philinoglossans can be roughly homologized with similar glands in other philinoidean lineages (especially Aglajidae), but limited comparative histological knowledge inhibits definite conclusions.

Within Philinoglossidae, the case of *Pluscula cuica* showing a number of morphological plesiomorphies that support its basal position among philinoglossans is weakened. We could not find any shell, but putative vestiges of shell-forming tissue at most. An osphradium, the vestigial crop and the comparatively elaborate copulatory organ described herein might be further plesiomorphies but need comparative reinvestigation in the other genera. Stronger evidence supporting a basal position are the retained posterior position of the female genital opening and the presence of a putative bursa copulatrix. None of these features was described from other philinoglossans. If confirmed, their apparent absence might be a synapomorphic loss, indicating that *Pluscula* is the most basal branch of Philinoglossidae, as had been assumed by previous authors (Marcus 1953a; Salvini-Plawen 1973). In conflict with this scenario are the putative retention of parapodia and an at least temporarily detectable separation of the head-shield from the rest of the notum in *Abavopsis*, and presence of two digestive gland branches in *Philinoglossa* species. Both parapodia and nervus clypei-capitis are more developed in *Abavopsis*, but remainders are still detectable in *Pluscula*. We suggest that a separate family Plusculidae for *Pluscula cuica* as established in the literature (e.g., Bouchet and Rocroi 2005) is no longer warranted.

Pluscula cuica can be distinguished externally from all other known philinoglossans by the lack of eyes, the dimple in the dorsal side of the caudal overhang, the presence of only a single digestive gland with a sloping posterior face. So far identified internal features include aforementioned plesiomorphies, and possibly the presence of the paired 'blister' cells next to the statocysts.

The remaining Philinoglossidae are united by further reductions (shell-associated tissue, bursa copulatrix) and shared characters (anterior shift of the female genital opening). *Philinoglossa* appears to be most derived (vermiform, tail-end glueing, simple copulatory organ, lateral separation of ovotestis follicles; Salvini-Plawen 1973) but shows two digestive gland lobes (cephalaspidean plesiomorphy). This highlights the continuing lack of comparable microanatomical data on the philinoglossans. Some current datasets, such as the denticulation of the lateral radula teeth as a criterion for species delimitation, should be reviewed (Salvini-Plawen 1973). The origin of monophyletic Philinoglossidae from a presumed gasteropterid—or aglajid-like ancestor—and the evolutionary scenario proposed herein with more or less successive adaptation to meiofaunal environment, should be further investigated. An integrative approach combining more comprehensive molecular datasets with additional morphological data seems most promising to evaluate proposed homologies and traits of evolution.

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Chapter 5. Kubilius RA, Kohnert P, **Brenzinger B** & Schrödl M (2014): **3D-microanatomy of the straight-shelled pteropod *Creseis clava* (Gastropoda: Heterobranchia: Euthecosomata).** *Journal of Molluscan Studies*, **80**:585-603.

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3D-microanatomy of the straight-shelled pteropod *Creseis clava* (Gastropoda: Heterobranchia: Euthecosomata)

R. A. Kubilius[1,2], P. Kohnert[1,2], B. Brenzinger[1,2] and M. Schrödl[1,2]

[1]*SNSB Bavarian State Collection of Zoology, Section Mollusca, Münchhausenstr. 21, 81247 Munich, Germany; and*
[2]*Department Biologie II, Biozentrum, Ludwig Maximilians–Universität, Großhaderner Str. 2, 82152 Planegg–Martinsried, Germany*

Correspondence: P. Kohnert; e-mail: petekohnert@gmail.com

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ABSTRACT

The Thecosomata are pelagic euopisthobranch pteropods that are important for marine food chains, but threatened by ocean acidification. Members of the suborder Euthecosomata are either torted snails with a coiled, sinistral shell (Limacinidae) or are straight-shelled with a symmetrical body and an unusual ventral mantle cavity (Orthoconcha). The classification and taxonomy of euthecosomes still depends on shells, but is being challenged by initial molecular data. There is a large body of morpho-anatomical information dating from the beginning of the last century, and only biological (rather than soft-part anatomical) detail has been added since. For our initial study on pteropod morphoanatomy we have selected the potentially basal orthoconch genus *Creseis* Rang, 1828. Supplementing Meisenheimer's (1905) monographic work, we redescribe the microanatomy of the Mediterranean *C. clava* (Rang, 1828) from serial semithin sections and present 3D-models of all major organ systems. In the absence of histological differences we interpret the head to be fused with the foot, forming the wings with reduced labial tentacles and rhinophores. The postpharyngeal nerve ring is strongly condensed, showing fused buccal ganglia and a short visceral loop with two discernable ganglia. The genital system is monaulic and hermaphroditic, with female glands and a potential allosperm receptacle of unclear homology. An open seminal groove connects with the frontal copulatory organ, which shows complex penial infoldings. We confirm the 180° longitudinal rotation of the visceral organs relative to the condition in limacinids. As an alternative to ontogenetic detorsion, progenesis may have skipped torsion. Other obvious paedomorphoses such as single, basally forked tentacular nerves, suggest that heterochrony has been a driving force in thecosome evolution. Retaining the mantle cavity in a ventral position, *Creseis* has its large mantle gland—necessary for creating a mucus web for feeding—close to the mouth. Further comparative microanatomical data on orthoconchs and limacinids are needed to corroborate our assumptions of homology, and for reconstructing pteropod evolution once reliable molecular phylogenetic hypotheses are available.

INTRODUCTION

The Pteropoda are the largest taxon of holopelagic gastropods and constitute a significant part of the marine zooplankton (Klussmann-Kolb & Dinapoli, 2006). As part of the euopisthobranch heterobranchs (Jörger *et al.*, 2010; Wägele *et al.*, 2014), pteropods are closely related to cephalaspidean snails and slugs, and are usually recovered as sister to sea hares (Anaspidea) in molecular phylogenetic analyses (Klussmann-Kolb & Dinapoli, 2006; Malaquias *et al.*, 2009; Göbbeler & Klussmann-Kolb, 2011). The Pteropoda contain two traditional opisthobranch orders, the shell-less Gymnosomata (estimated 40 species) and the Thecosomata. Since Meisenheimer's (1905) fundamental monographic work, Thecosomata have been divided into two suborders: the Pseudothecosomata with 23 valid species and a reduced or absent shell, and the Euthecosomata with at least 60 shelled species (current classification according to WoRMS, 2014). Euthecosomes represent an important food source for

other zooplankton including Gymnosomata, and for higher predators, such as fishes, birds and whales (Hunt *et al.*, 2008; Comeau *et al.*, 2010), and are an essential component in the marine carbon cycle (Feely *et al.*, 2004). As a consequence of their aragonitic shells ($CaCO_3$), euthecosomes are sensitive to the rising acidity of global seas (Feely *et al.*, 2004; Orr *et al.*, 2005; Comeau *et al.*, 2010). Euthecosomes are thus important and threatened members of marine ecosystems, as well as suitable study organisms for monitoring and predicting oceanic changes (Feely *et al.*, 2004; Bednaršek *et al.*, 2014).

In sharp contrast to their ecological relevance and the increasing amount of experimental work done on their physiology and resilience to environmental stress (Comeau *et al.*, 2012; Lischka & Riebesell, 2012), there has been little modern advance regarding pteropod morphology. Most of what is known of the external and internal anatomy and organ functions originated from early monographic works, such as those of Gegenbaur (1855) and Meisenheimer (1905). While several works (van der Spoel, 1967; Rampal, 1973, 2002, 2011; Lalli & Gilmer, 1989) added information on external features and functions (Lalli, 1970a, b; Lalli & Wells, 1978), soft-part anatomy basically remained unstudied. With sea slugs and other molluscs, modern 3D-micronatomical methodology has been shown to provide detailed and accurate data (e.g. Brenzinger *et al.*, 2011a; Brenzinger, Wilson & Schrödl, 2011b; Kohnert *et al.*, 2013; Brenzinger, Padula & Schrödl, 2013a; Brenzinger, Haszprunar & Schrödl, 2013b). Software-aided reconstruction of serial semithin histological sections is a highly suitable tool to assess, correct and supplement outdated, gross-morphological or paraffin-histology based knowledge (DaCosta *et al.*, 2007; Neusser & Schrödl, 2007; Neusser, Martynov & Schrödl, 2009).

For the first time in pteropods, we here aim to present 3D-anatomical models of a representative of the shelled thecosomes, the needle-shelled *Creseis clava* (Rang, 1828). According to the recent molecular phylogenetic hypotheses of Corse *et al.* (2013), the genus *Creseis* Rang, 1828 represents one of the earliest offshoots of all the straight-shelled euthecosomes, the Orthoconcha. The taxonomy of the family Creseidae has recently been reviewed by Gasca & Janssen (2014). The family is characterized by a bilaterally symmetrical, straight and pointed shell (Lalli & Gilmer, 1989), with a round aperture. The Creseidae currently comprise three genera and six species (Gasca & Janssen, 2014). *Creseis clava* is considered to be a senior synonym of the frequently used name *C. acicula* (Rang, 1828). Comparison of *C. clava* microanatomy with that of supposedly plesiomorphically coil-shelled euthecosomes such as *Limacina* may help to assess old hypotheses on the evolutionary emergence of the Orthoconcha associated with the decoiling of their shell, as assumed by Boas (1886).

MATERIAL AND METHODS

Specimens of *Creseis clava* used in this study were collected using a plankton net towed vertically in open water near Fetovaia Bay (Elba, Italy) in June 1998. Specimens were preserved in 96% ethanol. For microanatomical examination and histology, specimens were decalcified using Bouin's fluid, dehydrated in a graded acetone series and submerged overnight in a 1 : 1 solution of Epon epoxy resin and 100% acetone. Fully infiltrated specimens were then embedded in pure Epon and set to polymerize for 1 d at 70 °C. One specimen block (ZSM Mol 20000023/1; Fig. 1) was trimmed manually with a razorblade, and serially sectioned (1.5 μm) using a diamond knife (Diatome Histo Jumbo) installed on a rotation microtome (Microm HM 360, Zeiss). Ribbons of serial sections were obtained by applying contact cement (Pattex Gel compact, Henkel) to the side of the specimen block facing the knife (Ruthensteiner, 2008). The ribbons were transferred to cleaned microscopy slides and stained with methylene blue/azure II stain following Richardson, Jarett & Finke (1960). Basophilic and osmiophilic structures stain blue, and metachromatic structures stain violet. Slides were then sealed with araldite resin (Romeis, 1989) and coverslips.

Figure 1. Examined specimen of *Creseis clava* embedded in Epon resin. Scale bar = 600 μm.

Sections were photographed using a Leica DMRD microscope with a Jenoptik ProgRes C3 digital camera and ProgRes CapturePro v. 2.8.0 software installed (Jenoptik, Jena, Germany). Every fourth image was used for 3D-reconstruction (of a total of 2,464 sections). After editing in Adobe Photoshop CS2 (Adobe Systems Inc.) software (resolution −50%, conversion to 8-bit grayscale, adjustments of contrast, brightness and sharpness) and XnView software (Kolor) (stack renaming), this resulted in a final image stack of 616 images with a resolution of 1,040 × 771 pixels (complete animal). An additional photo series of the same sections, but focusing on the central nervous system (CNS) and the penis, was created separately (477 sections with no gaps along the *z*-axis, imaged at higher magnification) using the same protocol. For 3D-reconstruction, image stacks were imported into Amira v. 5.4.3 software (Visualization Science Group, Mérignac, France) and processed according to the procedures described by Neusser *et al.* (2006) and Ruthensteiner (2008). Automatic alignment of images was carefully checked by hand; voxel size was calculated after measuring a selected area on a physical section in the microscope and applying the rule of three. In the aligned image stacks, anatomical structures were labelled manually (using 'brush' and 'lasso' tools); interpolation between sections was used where applicable. From these labels, rendered surface models were created; these are shown in Figures 2, 3, 6, 9 and 12. For histological figures, photographs were taken from the same sections.

RESULTS

General morphology

Body length of preserved *Creseis clava* specimen 3.6 mm. Body with short yet wide anterior headfoot complex and elongated, straight, conical and narrowly pointed posterior visceral mass (length 2.7 mm, maximal width 400 μm) (Fig. 2). These two body regions separated internally from each other by a muscular diaphragm. Headfoot complex partly retracted into visceral sac. Smooth transition between frontal, median cephalic lobe and lateral wings, i.e. no externally demarcated head. No rhinophores, labial tentacles or eyes detectable. Two flat and widespread wings attached to head anterolaterally, ventrally uniting in a small, median footlobe. Wingspan *c.*1.1 mm. Conspicuously ciliated field between mouth opening and median lobe, extending to half length of the wings on their ventral side (Fig. 3B). Body cavity (haemocoel) of head contains anterior digestive tract

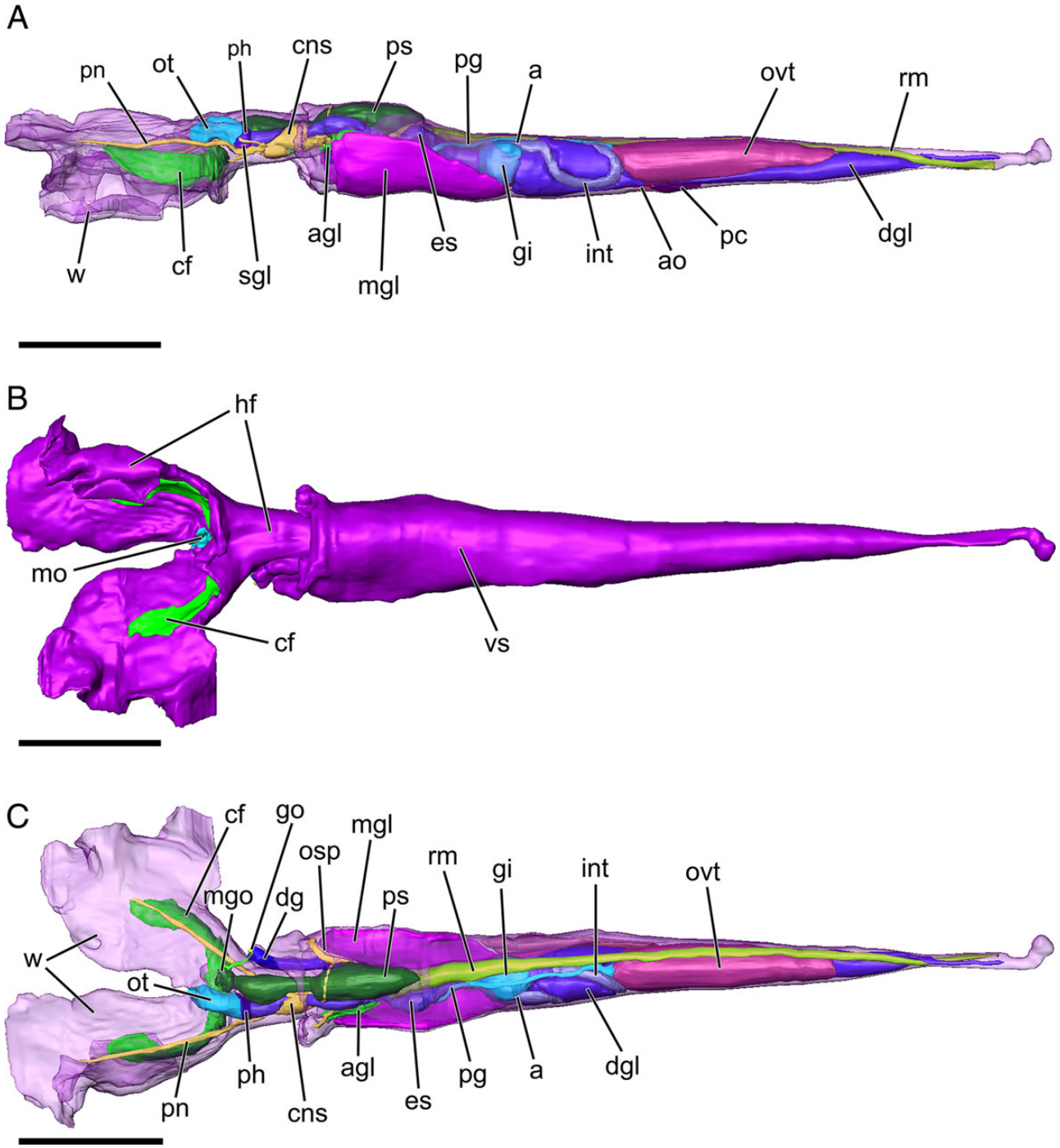

Figure 2. 3D-reconstructions showing the general morphology of *Creseis clava*. **A.** General organization of main organ systems, left lateral view. **B**. External morphology of examined specimen. **C.** General organization of the main organ systems, dorsal view. Abbreviations: a, anus; agl, anal gland; ao, aorta; cf, ciliary fields; cns, central nervous system; dg, distal gonoduct; dgl, digestive gland; es, oesophagus; go, genital opening; gi, gizzard; hf, headfoot complex; int, intestine; mgl, mantle gland; mgo, male genital opening; osp, osphradium; ot, oral tube; ovt, ovotestis; pc, pericard; pg, proximal gonoduct; ph, pharynx; pn, pedal nerve; rm, retractor muscle; ps, penial sheath; sgl, salivary gland; vs, visceral sac; w, wings. Scale bars = 500 μm.

(oral tube, pharynx, radula, salivary glands and oesophagus), CNS (postpharyngeal) and penis (Fig. 2A, C). Oesophagus penetrates diaphragm into visceral mass. Viscera enveloped by mantle. Mantle cavity situated ventrally, extending posteriorly below visceral sac for one-third of its length. Ventrally situated mantle 'roof' with large field of thick, glandular epithelium (mantle gland). Osphradium flat, elongated; situated anteroventrally, on right side of bottom of mantle cavity (Fig. 8F). Visceral sac contains posterior part of oesophagus, gizzard, stomach, caecum and intestine (Figs 4, 5). Reproductive system composed of posteriorly situated, large ovotestis (Fig. 2A, C), connected via gonoduct with complex of genital glands situated medially in anterior part of visceral sac. Genital glands connected to genital opening on right side of mantle cavity, in foremost part of visceral sac (Fig. 9A). Kidney located on right side of visceral sac, forming excretory and circulatory systems together with heart and

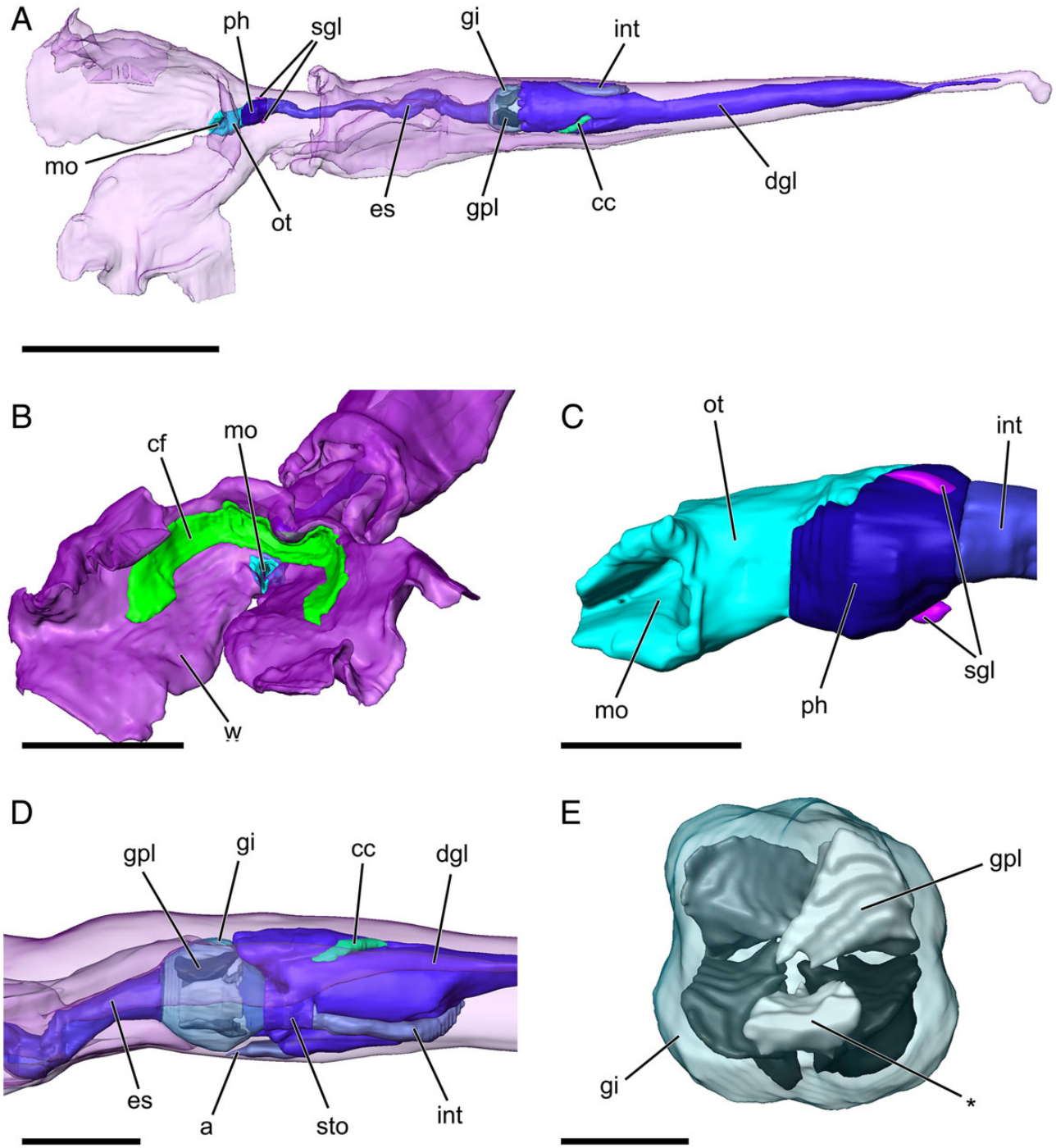

Figure 3. 3D-reconstruction of the digestive system of *Creseis clava*. **A.** Overview of digestive system within the specimen, ventrolateral view. **B.** Ciliary fields and mouth opening, anteroventral view. **C.** Ventral view of pharynx and associated organs. **D.** Arrangement of posterior digestive organs, dorsolateral view. **E.** Gizzard and gizzard plates, posterior view. Note smallest, 5th gizzard plate (*). Abbreviations: a, anus; cc, caecum; cf, ciliary field; dgl, digestive gland; es, oesophagus; gi, gizzard; gpl, gizzard plates; int, intestine; mo, mouth opening; ot, oral tube; ph, pharynx; sgl, salivary glands; sto, stomach; w, wings. Scale bars: **A** = 700 μm; **B** = 400 μm; **C** = 100 μm; **D** = 200 μm; **E** = 70 μm.

pericardium (Fig. 12). Kidney opens into anterior part of mantle cavity through nephropore on its left side (Fig. 13D).

Mantle cavity organs

Anterior mantle edge with mantle edge gland (= shell gland) forming a complete ring, consisting of glandular epidermal cells. Mantle cavity with two epidermal glands: large, outer mantle gland and smaller, inner anal gland. Mantle gland a flat, convex, very extensive organ (length 600 μm, width 350 μm), situated in anterior section of visceral sac (Fig. 2A, B), occupying entire inner epithelium of ventrally situated roof of mantle cavity. Mantle gland (= pallial gland) extending ventrally to envelop most of the anterior organs (Fig. 2A, B), such as penis sheath, female genital glands, oesophagus, osphradium gland. Mantle gland cells very large and

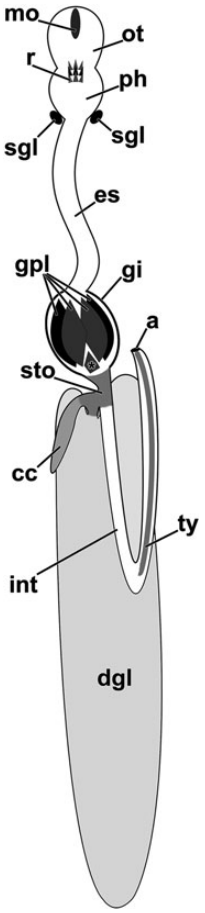

Figure 4. Schematic overview of the digestive system of *Creseis clava*, ventral view. Abbreviations: a, anus; cc, caecum; dgl, digestive gland; es, oesophagus; gi, gizzard; gpl, gizzard plates; int, intestine; mo, mouth opening; ot, oral tube; ph, pharynx; r, radula; sgl, salivary glands; sto, stomach; ty, typhlosolis. Note 5th gizzard plate (*). Connection of salivary glands to pharynx not detectable.

columnar, arranged around longitudinal axis of body, having granular cytoplasm with distinctive staining, showing transition from dark blue base to colourless apical areas. Anal gland of unclear homology; flat, 220 μm in length and 50 μm in width, situated on left side of mantle cavity, opposite to osphradium (Fig. 2A, C). Anal gland part of inner wall of mantle cavity, built up by single layer of cells, containing very large nuclei and many clear and unstained vesicles, orientated towards mantle cavity (Fig. 13E).

Retractor muscle

A 2.13 mm long, roughly cylindrical muscle extends mediodorsally throughout length of visceral sac, from posteriormost part of visceral sac to posterior part of penis sheath (Figs. 9A and 13B). Structure straight, increasing in width anteriorly (posteriorly 15 μm, anteriorly 80 μm). 3D-model incomplete because of muscle being damaged in its most anterior part, showing indistinct ramification into two branches.

Digestive system

Mouth opening located medioventrally between bases of wings. Oral tube short, thin-walled (Fig. 3A, B). Lumen of anterior part of oral tube round in cross section, roughly V-shaped approaching pharynx. Pharynx about 220 μm long, up to 120 μm wide, with thick muscular layer surrounding buccal lumen (Fig. 3C). Radula with nine teeth in three rows (radula formula 3 × 1.1.1) (Fig. 5A). Each row of one triangular median tooth and two hook-shaped lateral teeth. Two very short and small salivary glands lateral to pharynx; salivary ducts not detected. Tubular oesophagus 780 μm long, with ciliated epithelium multiply folded, lumen star-shaped in cross section (Fig. 5B.). Folding of oesophagus successively reduced towards its posterior end, with volume of lumen increasing. Gizzard short yet broad, voluminous, resembling a spherical bag. Gizzard with thick outer muscular layer; containing five almost unstained, chitinous, pyramidal gizzard plates. Gizzard plates attached basally to epithelium, with their peaks pointing towards centre of gizzard lumen. Four plates (length *c*.110 μm) form anterior ring of gizzard plates; 5th plate only 55 μm long, medioventral, slightly posterior to rest of plates (Figs 3D, E, 4, 5C). Posterior end of gizzard connected to stomach. Three different structures branch from stomach: digestive gland, caecum and intestine. Digestive gland large, lobed, 1.7 mm in length, 190 μm in maximal width, extending to most posterior part of visceral sac. Digestive gland of many peripheral lobes arranged densely around (central) main duct (Figs 3A, 5D–F). Lower density of branching lobes towards posterior end of digestive gland. Cells of digestive gland with granular cytoplasm. Caecum a blindly ending, small, elongate sac-like organ, ciliated, 120 μm long, 20 μm wide (Figs 4, 5D, E). Intestine *c*.800 μm long, densely ciliated tube leaving stomach posteriorly, looping around left anterior part of digestive gland. Loop turning at first ventrally to left side, then flipping backwards. Intestinal cells cylindrical, short. Distal third of intestine with distinct typhlosole (Figs. 4, 5F). Anus small, directed anteriorly, mediodorsal in visceral sac, directly above gizzard.

Central nervous system

CNS in head cavity, consisting of postpharyngeal, circumesophageal nerve ring and posterior visceral loop. Visceral loop ganglia densely arranged, partly fused. Each ganglion with outer thick cortex of dark blue stained perikarya, and inner light blue stained medulla containing exclusively nerve fibres. Nerve ring of paired pedal and cerebropleural ganglia. Oval pedal ganglia (length 110 μm, width 60 μm) anterior to cerebropleural ganglia, lateroventral to oesophagus. Single pedal commissure short, thick (Fig. 8A). Two statocysts embedded on top of posterior end of pedal ganglia. Pedal ganglia connected with cerebropleural ganglia by two separate connectives each. Completely fused cerebropleural ganglia large (length 130 μm, width 100 μm), encompassing oesophagus laterally, with distinct dorsal commissure 50 μm long. Buccal ganglia elongated (length 78 μm, width 30 μm), fused medially, wedged between oesophagus, cerebropleural and pedal ganglia (Fig. 8B). Buccal-cerebropleural connectives short, thin.

Direct transition of cerebropleural ganglia into fused visceral loop ganglia, without any recognizable connectives. Visceral loop short (length 90 μm, width 130 μm), comprised of two almost completely fused ganglia. Ganglia nevertheless distinguishable by rudimentary inner separations through perikarya: one larger (fused suboesophageal and visceral ganglia) ganglion (length 90 μm, width 80 μm), situated on left side of visceral loop; one smaller (supraoesophageal) ganglion (length 75 μm, width 50 μm), situated on its right side.

Each pedal ganglion with strong pedal nerve (width 20 μm) emerging anterolaterally, progressing towards anterior part of head-foot, ramifying into at least three branches (Figs 6, 7). Pedal nerves innervating both wings with at least one of their branches; exact numbers of branches, trajectories and target areas not detectable.

A paired cerebral nerve (10 μm diameter) emerges anteriorly from each cerebropleural ganglion and progresses lateroventrally along each pedal ganglion towards buccal mass, ramifying

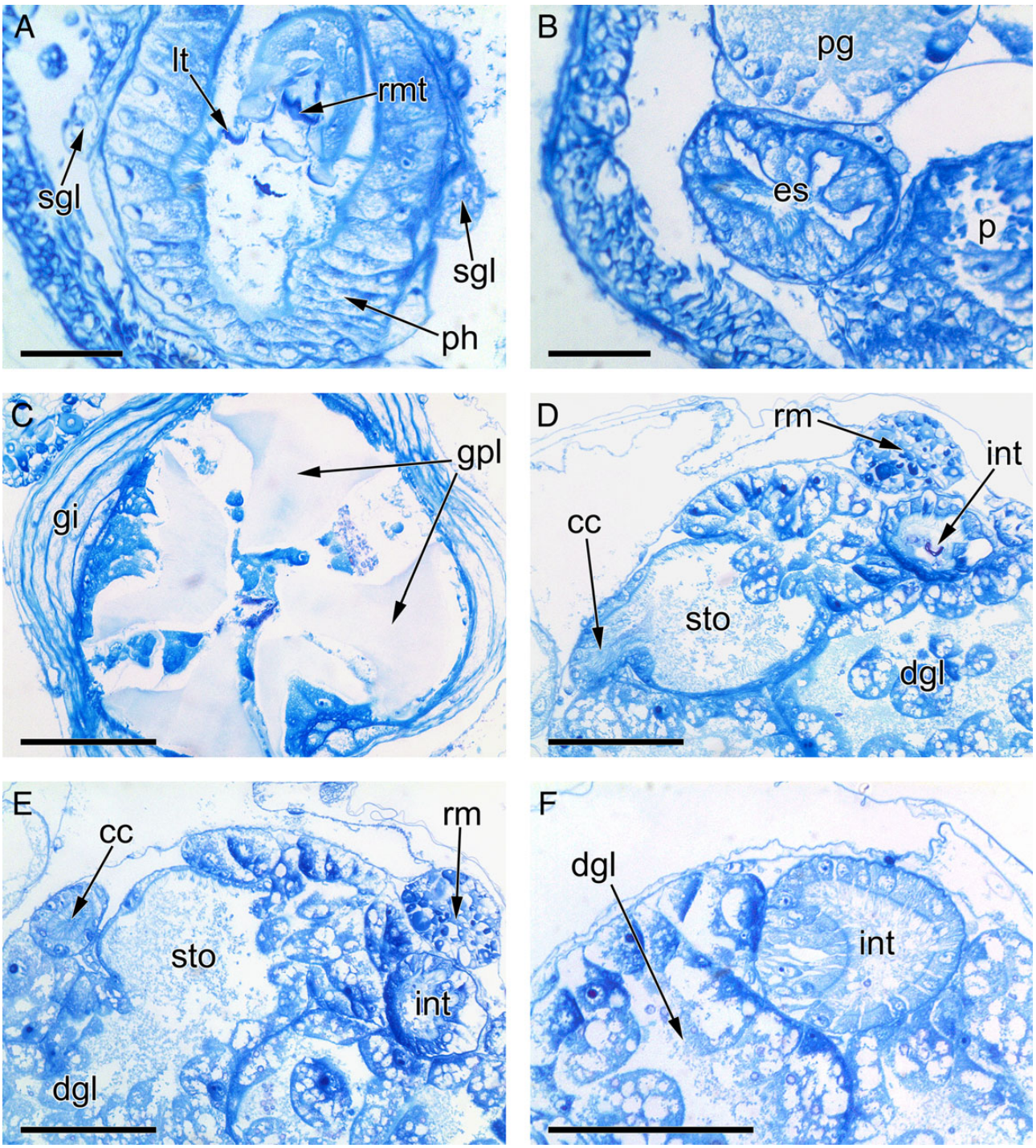

Figure 5. Semithin cross sections of the digestive system of *Creseis clava*. **A.** Pharynx. **B.** Oesophagus. **C.** Gizzard. **D.** Connection of stomach and caecum. **E.** Connection of stomach and digestive gland. **F.** Distal part of intestine. Abbreviations: cc, caecum; dgl, digestive gland; es, oesophagus; gi, gizzard; gpl, gizzard plates; int, intestine; lt, lateral tooth; pg, pedal ganglion; ph, pharynx; p, penis; rmt, radular median tooth; sgl, salivary gland; sto, stomach; rm, retractor muscle. Scale bars: **A**, **B** = 25 μm; **C**, **D**, **E**, **F** = 50 μm.

into two branches after about 100 μm. Branches of left nerve apparently innervating left side of pharynx; branches of right nerve enter right side of pharynx and anterior end of penis/penis sheath, respectively.

Fused buccal ganglia gives rise to two nerves, progressing laterally, alongside oesophagus towards buccal mass. Buccal nerves narrow (5–7 μm), without ramifications; innervating ipsilateral sides of pharynx (Figs 6C–F, 7).

Visceral loop ganglia with three distinctive nerves. Two nerves emerge from larger, left ganglion, one from smaller right ganglion. Left ganglion with delicate posterior nerve (5 μm diameter) proceeding alongside oesophagus into visceral sac; nerve convoluted, running multiple times back and forth. Second left ganglion nerve thick (15–20 μm), running for 60 μm towards posterior left part of head with U-turn shortly after penetration of diaphragm, thus progressing along

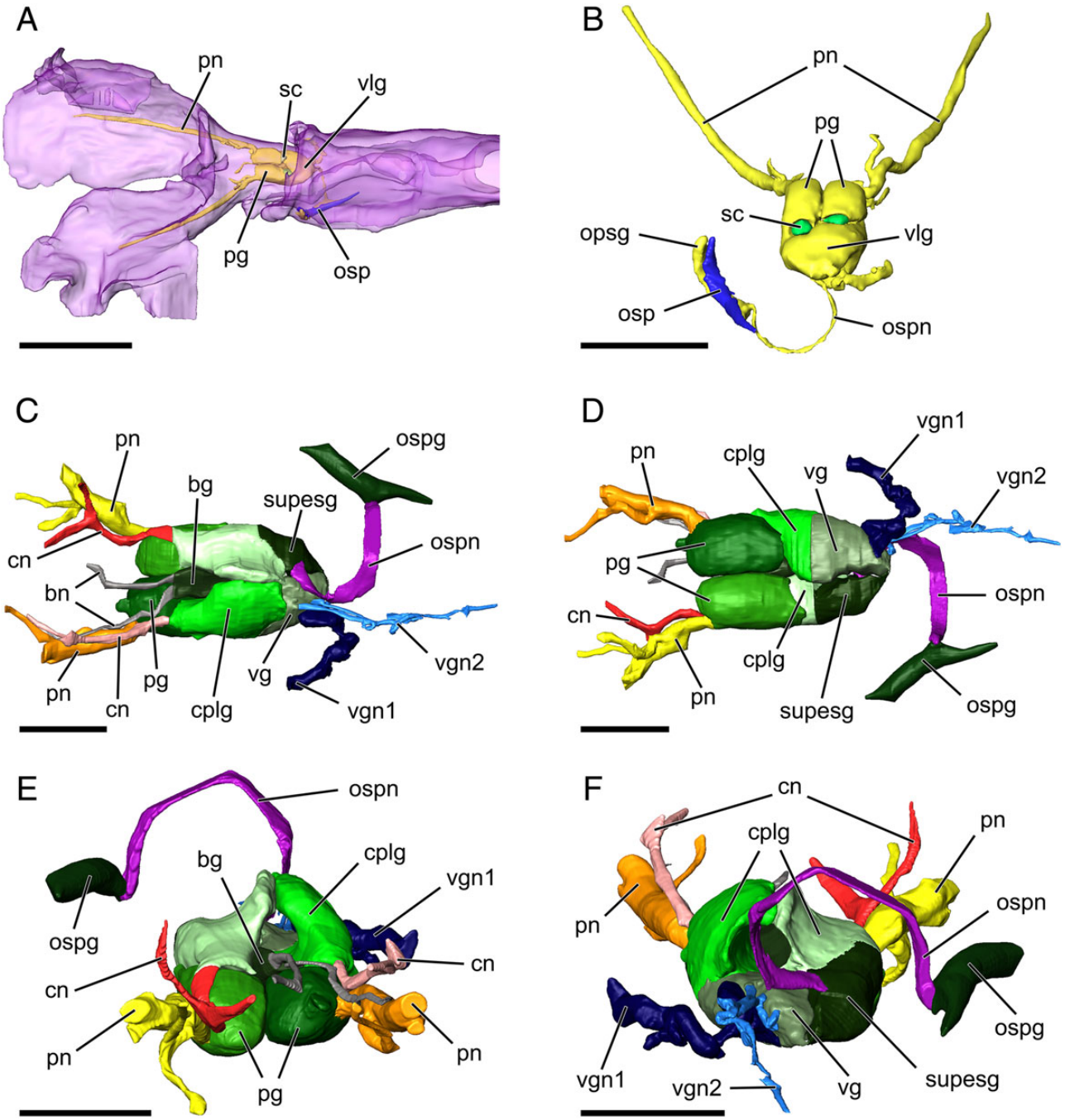

Figure 6. 3D-reconstruction of nervous system of *Creseis clava*. **A.** Localization of central nervous system (CNS) within specimen, ventral view. **B.** Posteroventral view of complete CNS. **C.** Complete CNS, dorsal view. **D.** Complete CNS, ventral view. **E.** Anterior view of complete CNS. **F.** Posterior view of complete CNS. Abbreviations: bg, completely fused buccal ganglion; bn, buccal nerve; cn, cerebral nerve; cplg, cerebropleural ganglion; opsg, osphradial ganglion; osp, osphradium; ospn, osphradial nerve; pg, pedal ganglion; pn, pedal nerve; sc, statocyst; supesg, supraoesophageal ganglion; vlg, visceral loop ganglia; vg, visceral ganglion; vgn1, visceral ganglion nerve 1; vgn2, visceral ganglion nerve 2. Scale bars: **A** = 400 μm; **B** = 200 μm; **C–F** = 100 μm.

left side of anterior part of mantle cavity wall (Figs 6C, D, F, 7); there apparently innervating mantle cavity gland. Right visceral loop ganglion with anterior, flattened nerve (width 20 μm, thickness 3–5 μm), running dorsally along and then around penis sheath, finally connecting to osphradial ganglion (length 230 μm, width 25 μm). Osphradial ganglion on right side of anterior part of visceral sac, directly below osphradium.

Sensory organs

Paired statocysts and unpaired osphradium. No traces of eyes. Two ellipsoid statocysts (50 μm long, 35 μm wide) ventral in head, between pedal and cerebropleural ganglia (Fig. 8C, E). Each statocyst with its anterior half embedded in respective pedal ganglion. Innervation of statocysts not recognized. Statocysts with thin outer wall of flattened cells with large nucleus; inner lumen, appearing empty in histological sections. Osphradium flat,

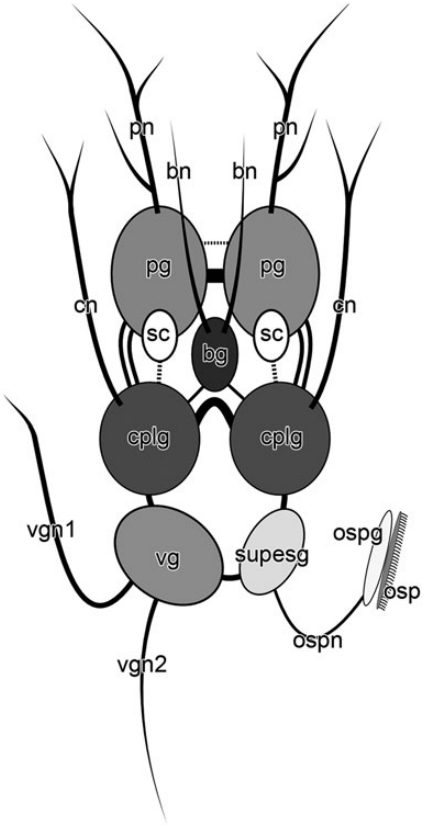

Figure 7. Schematic overview of CNS of *Creseis clava*, dorsal view. Dashed lines represent nerves that could not be identified in reconstructed specimen but were mentioned in the literature. Abbreviations: bg, buccal ganglion; bn, buccal nerve; cn, cerebral nerve; cplg, cerebropleural ganglion; osp, osphradium; ospg, osphradial ganglion; ospn, osphradial nerve; pg, pedal ganglion; pn, pedal nerve; sc, statocyst; vg, visceral ganglion; supesg, supraoesophageal ganglion; vgn1, visceral ganglion nerve 1; vgn2, visceral ganglion nerve 2.

elongated, on right of anterior part of visceral sac, of inconspicuously ciliated epithelium in outer wall of mantle cavity. Osphradial epithelium of single layer of cubic cells, with granular intracellular medium (Fig. 8F).

Genital system

Reproductive system of *C. clava* (Figs 9–11) monaulic, with posterior organs located in visceral sac (ovotestis, three female genital glands) and anterior copulatory apparatus situated in head cavity (Fig. 9A, B).

Palliovisceral genital components: Massive elongated hermaphroditic gonad (length 780 μm; width 160 μm; Figs 9A, 11A) with peripheral layer of blue stained cells; fewer large cells (potential oocytes) with blue-staining cytoplasm and dark blue stained nucleus (Fig. 11A). Proximal gonoduct with small lumen; cilia not detectable. Gonoduct straight, connecting with three densely packed, but distinct, glandular organs (Figs 9, 10). This glandular complex (Figs 9C, 10, 11B) situated medially in anterior part of visceral sac beneath oesophagus, extending towards right lateral part of neck region (Fig. 9A). First genital gland smallest (gland 1; length 80 μm, width 50 μm). Gland 2 largest (length 360 μm, width 60 μm). Short sac-like gland 3 (length 140 μm, width 25 μm) connecting to both other glands. Histology of glands 1 and 2 (potential female glands) similar, both of round and cylindrical cells, with granular content stained dark to light blue (Fig. 11B, C). Cells of gland 2 with light pink stained granules in apical parts. Cells of gland 3 (potential allosperm receptacle) stout, containing granules stained light blue. Gland 2 narrowing distally into distal gonoduct, latter part with columnar epithelium (Fig. 11B). Distal gonoduct connecting to genital opening, on right side of most anterior part of mantle cavity (Figs 9A, D, 11D).

Copulatory organ: Epidermal sperm groove (130 μm long), without clearly detectable cilia, linking genital opening to male genital opening (Fig. 11D). Male copulatory organ (i.e. cephalic penis; length 720 μm, width 130 μm) completely enveloped by a sheath, extending into posterior part of head cavity. Epithelium of sheath invaginated with complex infoldings, with multiple irregular ramifications into large and voluminous caeca along its length (Figs 10, 11F); of large, very elongated, densely packed cells with mostly dark blue stained granular content. Caeca interconnected by narrow lumina (Fig. 11E); no spines or copulatory stylets detected.

Circulatory and excretory systems

Monotocardian heart (Figs 12B, 13A) of thick-walled ventricle and thin-walled auricle. Heart medioventral, halfway through visceral sac, close to posterior end of kidney (Fig. 12A–C). Heart enveloped by flattened, thin pericardium (length 160 μm, width 100 μm). Thin-walled, flat, sac-like venous sinus penetrating pericardium on its right side (Fig. 12B), merging into auricle. Auricle (length 50 μm, width 30 μm) with many convolutions in its thin, light blue stained wall, extending through dark blue stained, thick wall of oval ventricle (length 60 μm, width 40 μm) on its right side (Fig. 13A). Aorta emerging from anterior end of ventricle, penetrating pericardium medially (Fig. 12B, C), progressing towards anterior part of visceral sac (Fig. 12A). Aorta (length 1.2 mm, width 8 μm) thin-walled, narrow, tubular (Fig. 13B), running through almost entire visceral sac, apparently opening into visceral cavity above left anterior-most edge of mantle gland, posterior to CNS.

Kidney (length 630 μm, width 90 μm) tubular (Fig. 12A), with slight dorsoventral depression, situated in middle third of visceral sac on its right side. Kidney with large lumen surrounded by single layer of large, flattened cells. These light blue stained cells with large nucleus and granules in cytoplasm (Fig. 13D). Posterior portion of kidney without visible lumen, with cells increasing in volume. Kidney connected with anteriorly situated pericardium via 10 μm long renopericardioduct (Figs 12B, 13C). Nephroduct emerging from anterior left part of kidney. Small nephropore opening into mantle cavity (Fig. 13D).

DISCUSSION

This study is the first comprehensive anatomical account of an entire pteropod for over a century. Other previous studies were mostly based on gross dissections, or on only a few histological sections, or focused solely on particular organs or the shell (e.g. Fahrner & Haszprunar, 2000; Janssen, 2012). We here attempt to establish a dataset as a basis for future studies on *Creseis* and related taxa. The homology of pteropod features needs to be re-evaluated in the light of modern hypotheses on heterobranch phylogeny and evolution (Schrödl *et al.*, 2011; Wägele *et al.*, 2014). We compare our description of the microanatomy of *C. clava* with available descriptions, particularly those of Meisenheimer (1905). However, Meisenheimer presented a comparative account of thecosome anatomy rather than providing coherent descriptions of particular species.

External morphology

The 3-D reconstruction of *C. clava* coincides in most respects with the general morphology of the Euthecosomata described

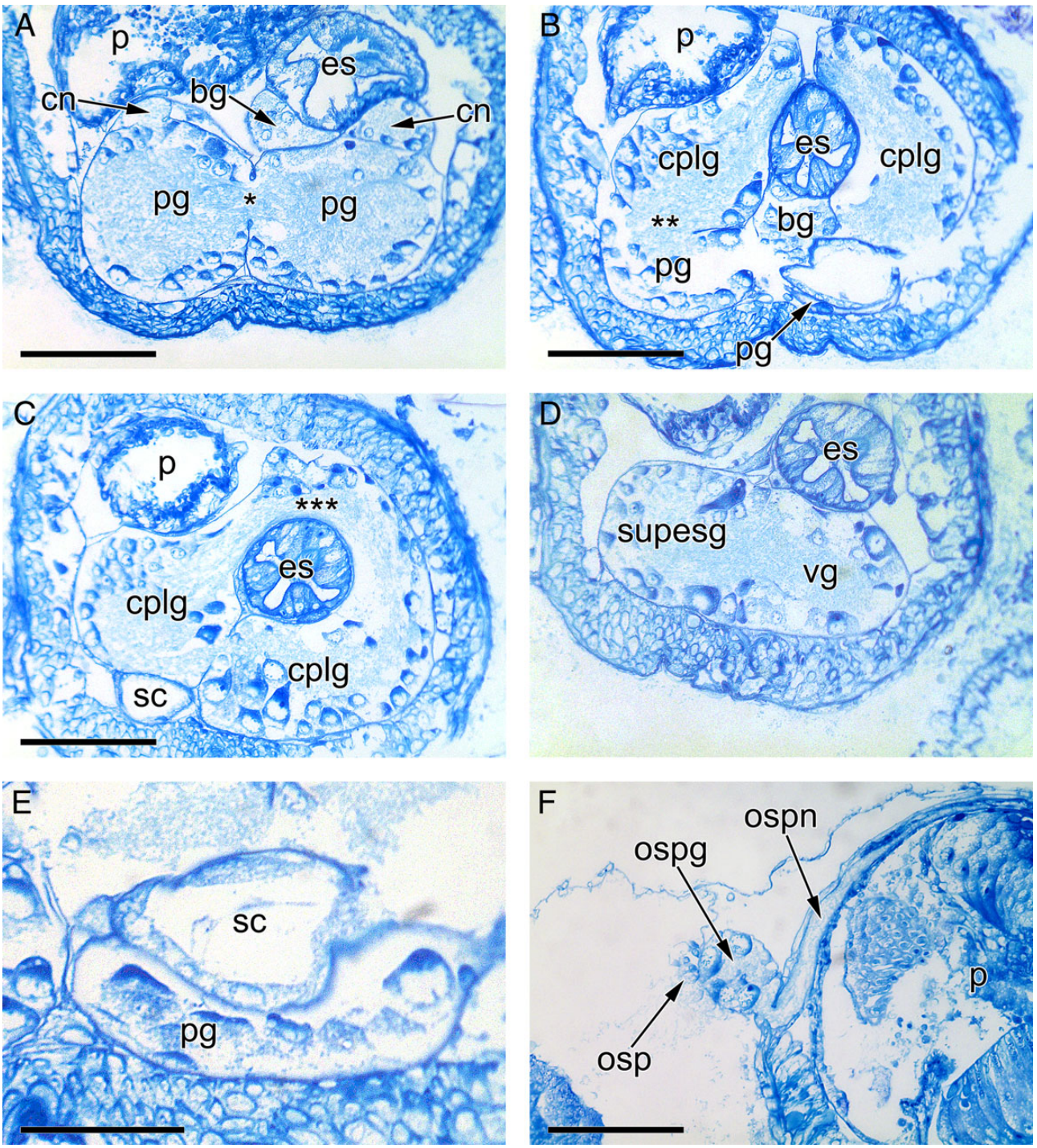

Figure 8. Semithin cross sections of nervous system of *Creseis clava*. **A.–C.** Arrangement of several ganglia of nerve ring. **A.** Cross section of pedal ganglia and surrounding organs. Note strong pedal commissure (*). **B.** Cross section of nerve ring showing cerebropedal commissure (**). **C.** Cross section of cerebropleural ganglia. Note extensive cerebral commissure (***). **D.** Cross section showing strong fusion of ganglia on visceral loop. **E.** Statocyst embedded in pedal ganglion tissue. **F.** Cross section of osphradial ganglion and osphradial nerve. Abbreviations: bg, buccal ganglion; cn, cerebral nerve; cplg, cerebropleural ganglion; es, oesophagus; osp, osphradium; ospg, osphradial ganglion; ospn, osphradial nerve; p, penis; pg, pedal ganglion; sc, statocyst; supesg, supraoesophageal ganglion; vg, visceral ganglion. Scale bars: **A–D, F** = 50 μm; **E** = 25 μm.

by Meisenheimer (1905). All thecosomes show the typical gastropod subdivision into an anterior headfoot complex and a posterior visceral sac.

The headfoot of pelagic Pteropoda, in particular euthecosomes, differs radically in external morphology from that of related euopisthobranchs. The latter plesiomorphically have a demarcated head with two pairs of head tentacles (Wägele & Klussmann-Kolb, 2005; Brenzinger *et al.*, 2013a), i.e. anterior labial tentacles and more posterior rhinophores, which are also present in sea hares, the putative sister group of pteropods. As is evident from our data, *C. clava* lacks a demarcated head and does not possess well-elaborated head appendages; there also are no unambiguously detectable eyes. Reports on other thecosomes have mentioned one pair of posterior tentacles, which could

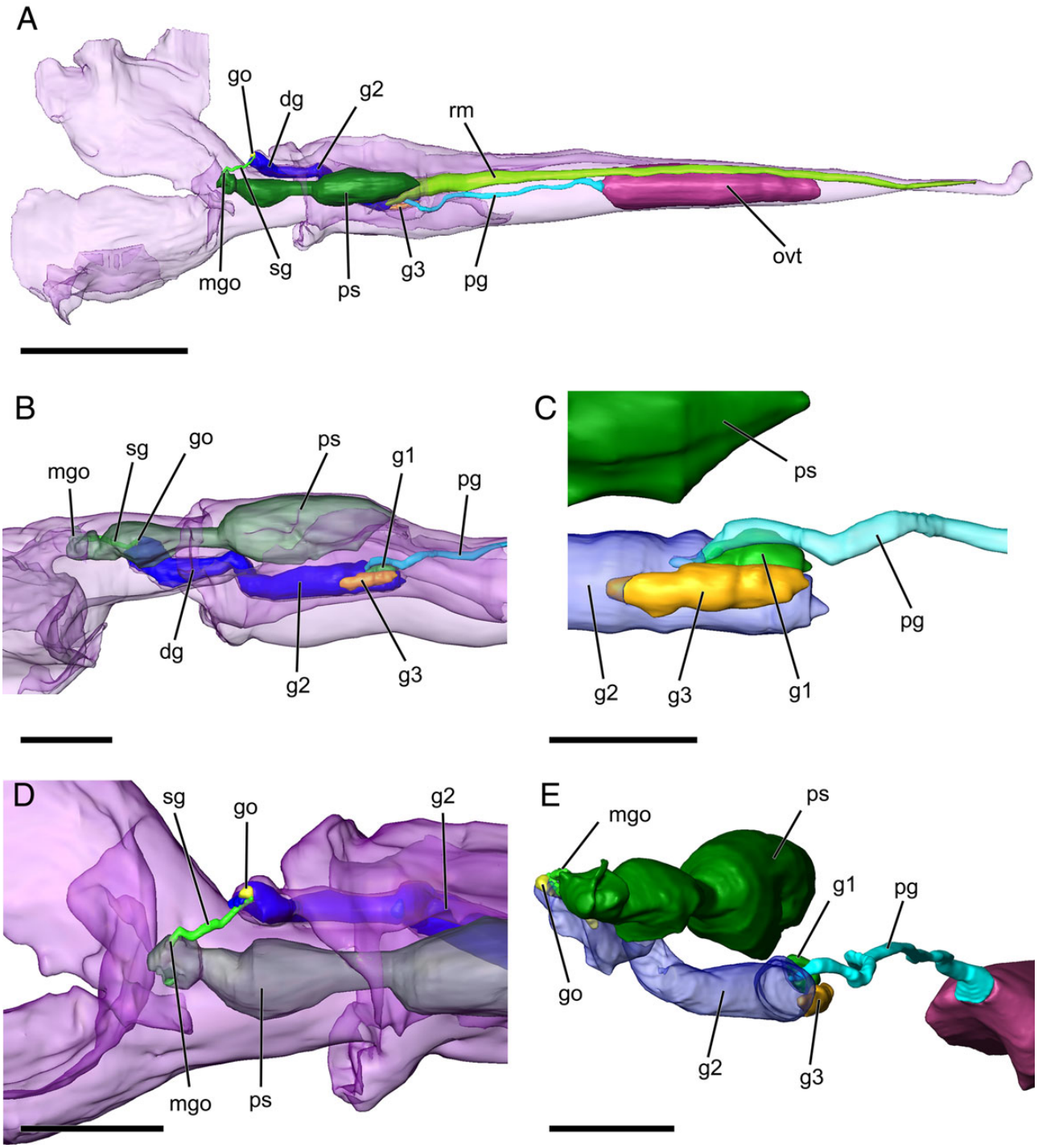

Figure 9. 3D-reconstruction of genital system of *Creseis clava*. **A.** Localization of genital system within examined specimen, dorsal view. **B.** Left lateral view of complex of genital glands and penial sheath. **C.** Complex of genital glands, left lateral view. **D.** Epidermal groove between genital openings (sperm groove), dorsal view. **E.** Anterolateral view of general genital arrangement. Abbreviations: dg, distal gonoduct; g1, genital accessory gland 1; g2, genital accessory gland 2; g3, genital accessory gland 3; go, genital opening; mgo, male genital opening; ovt, ovotestis; pg, proximal gonoduct; ps, penial sheath; rm, retractor muscle; sg, sperm groove. Scale bars: **A** = 600 μm; **B, D** = 200 μm; **C** = 100 μm; **E** = 150 μm.

represent homologues of rhinophores according to Corse *et al.* (2013). In thecosomes, the left rhinophore may be more or less reduced and the right one associated with a basal, more or less rudimentary eye. In contrast to our material, rudimentary tentacles (i.e. putative rhinophores) were indeed observed in some *Creseis* species (Gegenbaur, 1855; van der Spoel, 1967); Meisenheimer (1905) also mentioned a rudimentary, unpigmented eye for the genus.

Some thecosomes show small stubby tentacles along the anterior edge of their wings (Meisenheimer, 1905), often referred to as 'tentacle-like' structures (Corse *et al.*, 2013) or 'wing protrusions' (van der Spoel, 1967); these are visible in living specimens, but not evident from our preserved material. We interpret these tentacles as rudimentary labial tentacles, which are also present in the sister group Gymnosomata and most other, noninfaunal Euopisthobranchia.

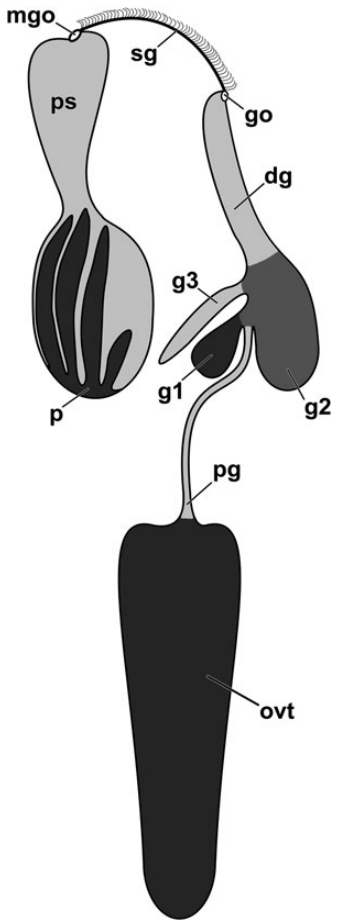

Figure 10. Schematic overview of genital system of *Creseis clava*. Abbreviations: dg, distal gonoduct; g1, genital accessory gland 1; g2, genital accessory gland 2; g3, genital accessory gland 3; go, genital opening; mgo, male genital opening; ovt, ovotestis; pg, proximal gonoduct; p, penis; ps, penial sheath; sg, sperm groove.

The foot of many euopisthobranchs carries lateral extensions (parapodia), which are sometimes used in swimming (Dayrat *et al*., 2001; Donovan Pennings & Carefoot, 2006), but it is still unclear if these are directly or completely homologous with the 'wings' of pelagic Pteropoda. Euthecosomes show two anterolaterally extending wings, which attach to the anterior part of the head, in close proximity to the mouth (Figs 2, 3B). These flat and extended wings constitute the primary locomotive organs, which characterize all euthecosomes (Lalli & Gilmer, 1989). They are also involved in the feeding process; euthecosomes feed by catching food with a spherical mucus web produced by the mantle gland; at intervals, this web is hauled in towards the mouth, using the ventral ciliated fields on the wings (Fig. 3B) (Lalli & Gilmer, 1989). According to Gilmer & Harbison (1968), some thecosomes, including *Creseis*, hang from this web upside-down, i.e. with the anatomically ventral side facing upwards. This coincides with the observation in the specimen examined here that the ciliated fields on the wings extend medioventrally along the surface of the wings, uniting directly under the mouth opening in an epidermal groove. This observation, however, differs from Meisenheimer's (1905) descriptions of the ciliated fields extending along the outer edge of the wings, without being associated with the mouth.

Meisenheimer (1905) described the foot of *Creseis* as clearly distinguishable from the wings; apparently this so-called 'foot' refers to the median footlobe mentioned herein. Judging from our material, the head of *Creseis* is not discernable as such, but appears completely fused with the foot, with unclear boundaries.

Mantle cavity

The mantle cavity of *C. clava* is anatomically ventral, elongate and with an anterior opening. There is an anal opening and nephropore, but no gill or ciliary strips were detected. The walls of the mantle cavity possess two major glands, the mantle gland (or pallial gland) and anal gland. The former, located in the mantle roof (i.e. ventral) corresponds with the hypobranchial gland (e.g. van der Spoel, 1967) and exhibits some variation among thecosome species (Meisenheimer, 1905). The latter gland is small and located at the left anterior corner of the mantle cavity and is referred to as the 'anal gland' (e.g. Tesch, 1913) herein. van der Spoel (1967) noted its position distant from the anus and instead considered it homologous with the hypobranchial gland. The pigmented nature of the euthecosome anal gland indicates a potential homology with the pigmented mantle organ (see Dayrat & Tillier, 2002) of heterobranchs. In our material, this gland seems to be innervated by the thickest nerve of the left, larger ganglion of the visceral loop. The function of this organ is still unclear; based on its histology, Meisenheimer (1905) concluded that it most probably has either a glandular or a sensory function.

Digestive system

Our 3D-model of *C. clava* visualizes and largely confirms the components of the digestive system that Meisenheimer (1905) found in Euthecosomata species. The digestive system of the Euthecosomata is generally subdivided into an anterior part located in the head cavity (consisting of the mouth opening, the pharynx, the radula, two salivary glands and the oesophagus) and a posterior part extending through the entire visceral sac, which is comprised of the gizzard with gizzard plates, the digestive gland, a caecum and the intestine (Meisenheimer, 1905). Among the Euthecosomata the radula usually shows *c.*10 rows, each carrying two thin hook-shaped lateral teeth and a pointed, serrated and triangular middle tooth (van der Spoel, 1967). The radula formula was detectable even in our 3D-reconstructed specimen, which, however, shows only three rows of radula teeth (Fig. 5A).

The very small and short salivary glands found here in *C. clava* are attached laterally to the pharynx; salivary ducts could not be discerned. This coincides with Meisenheimer's (1905) descriptions of *Creseis* and *Hyalocylis*, in which he identified two very small glands comprised of only 2–3 cells that connect laterally to the buccal apparatus; in contrast, other Euthecosomata, such as *Cuvierina* species, feature large and extensive salivary glands (Meisenheimer, 1905).

The oesophagus of *Creseis* is longer than that of other euthecosomes (Meisenheimer, 1905; this study). The epithelium of the oesophagus is characterized by ciliated cells along most of its length and features multiple longitudinal folds (Fig. 5B). This distinctive arrangement was also observed in *C. clava* and all Euthecosomata by Meisenheimer (1905), who also stated that the epithelium is more convoluted towards the end of the oesophagus; in our material, it is the other way around.

Creseis and other thecosomes have a gizzard, a character that was considered a synapomorphy of Euopisthobranchia by Jörger *et al*. (2010). In *C. clava*, the gizzard contains four large and one small gizzard plate, the smaller plate being situated ventrally and slightly shifted to the posterior end of the other plates. Meisenheimer (1905) observed the same arrangement of the plates in most euthecosome species except for *Limacina*, where the single plate is situated dorsally. This finding may be explained by the concept of de-coiling introduced by Boas (1886), hypothesizing that straight-shelled Euthecosomata (such as *Creseis*) feature a visceral sac inverted relatively to the head, in

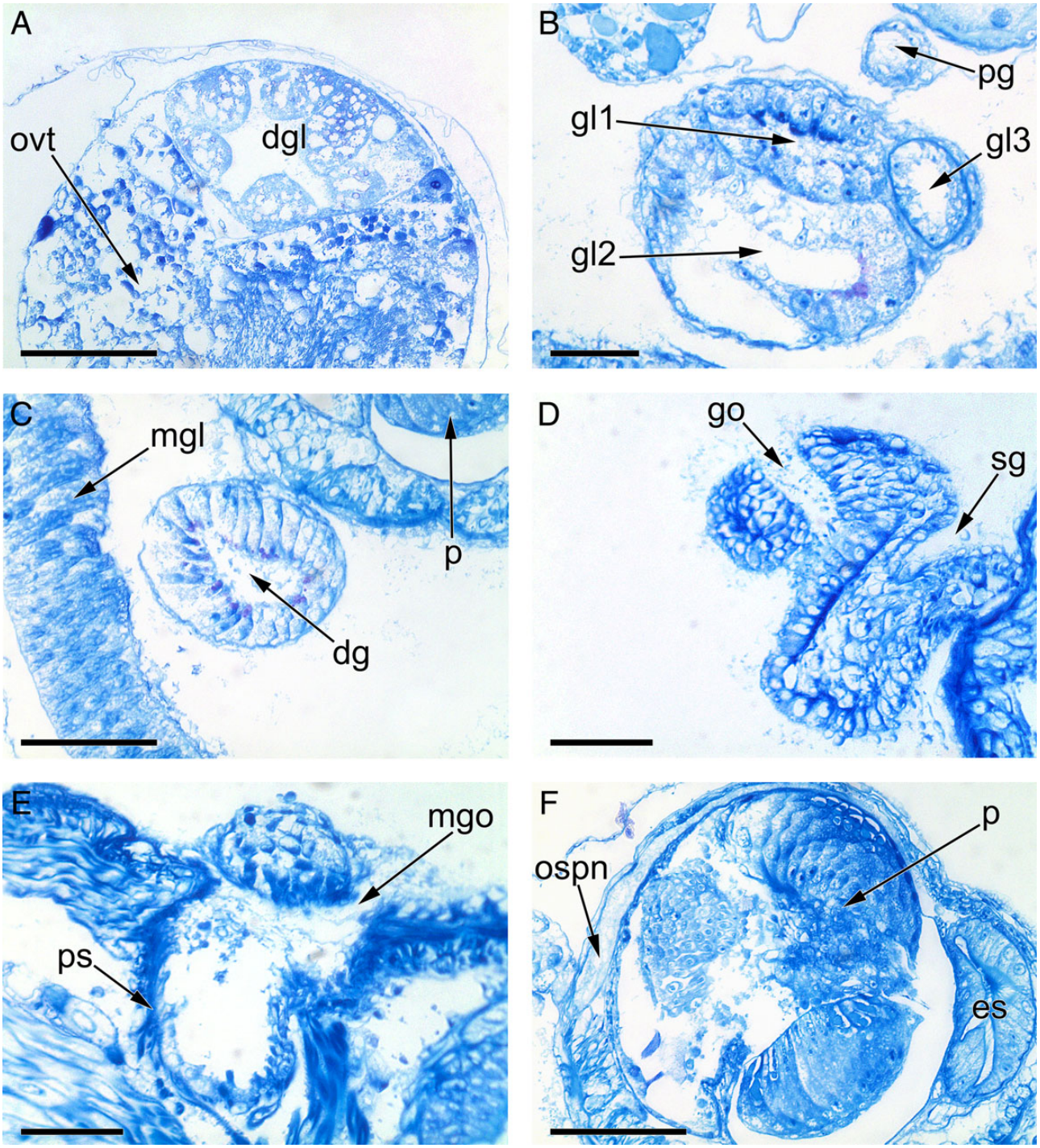

Figure 11. Semithin cross sections of the genital system of *Creseis clava*. **A.** Posterior visceral sac filled in large part by ovotestis. **B.** Cross section of the genital accessory glands. **C.** Distal gonoduct. **D.** Genital opening and adjacent epidermal sperm groove. **E.** Penial sheath connected to male genital opening. **F.** Male copulatory organ. Abbreviations: dg, distal gonoduct; dgl, digestive gland; es, oesophagus; go, genital opening; gl1, genital accessory gland 1; gl2, genital accessory gland 2; gl3, genital accessory gland 3; mgl, mantle gland; mgo, male genital opening; ospn, osphradial nerve; ovt, ovotestis; p, penis; pg, proximal gonoduct; ps, penial sheath; sg, sperm groove. Scale bars: **A, C, F** = 50 μm; **B, D, E** = 25 μm.

contrast to coiled-shelled Euthecosomata such as *Limacina*. This was explained by a rotation of the visceral sac by 180° along its longitudinal axis, thereby relocating the mantle cavity to the ventral side and rotating some, but not all, internal organs. For example, as can be seen from the 3-D model, the digestive gland of *C. clava* is situated on the right side of the body (as in all straight-shelled euthecosomes), whereas in coiled-shelled species (such as in *Limacina*) it is situated on the left side (Meisenheimer, 1905).

The digestive gland of *C. clava* extends into the tip of the strongly elongated visceral sac. Meisenheimer (1905) observed that the gland features almost no (internal) lobes in *C. clava* (in contrast to other related species) and assumed that this was due to its strongly elongated body and digestive gland. Our results

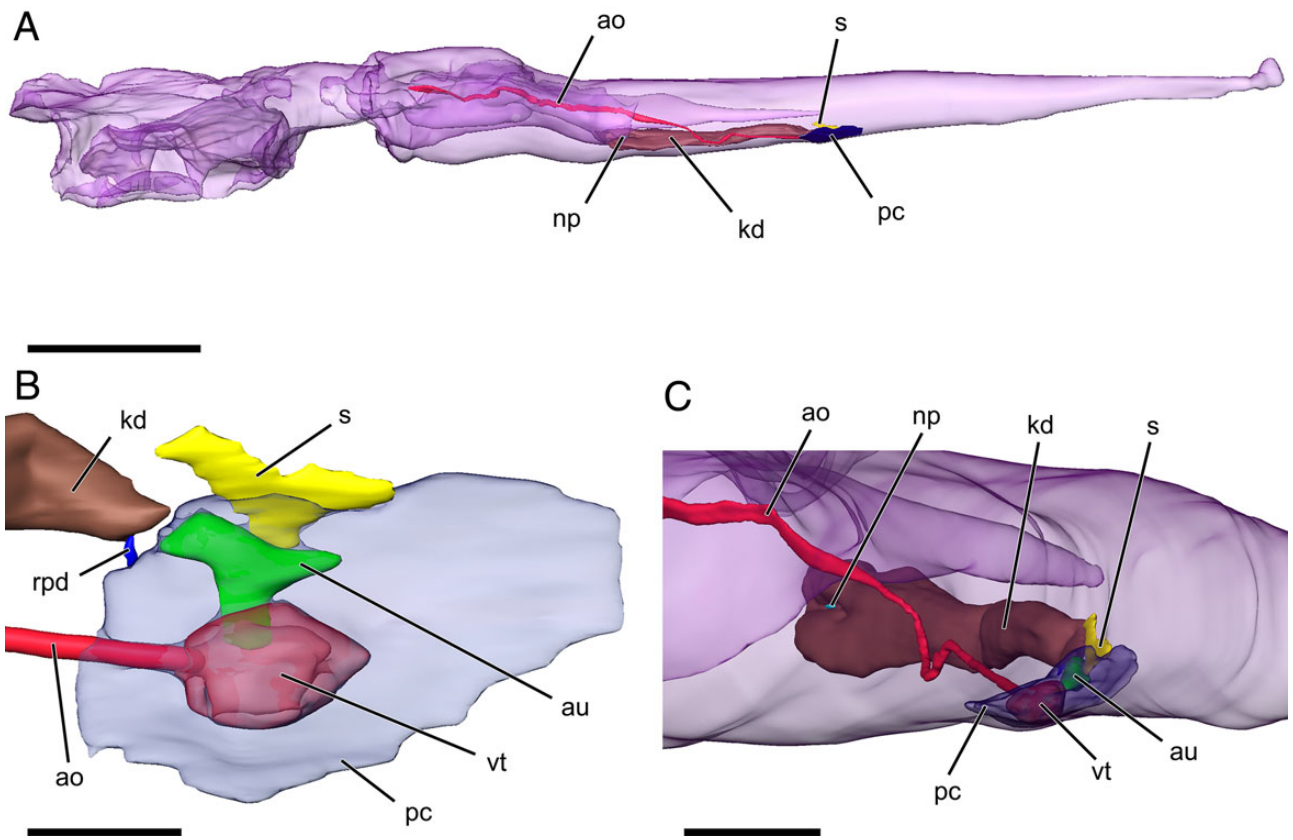

Figure 12. 3D-reconstruction of circulatory and excretory systems of *Creseis clava*. **A.** Localization of circulatory and excretory system within the specimen, left lateral view. **B.** Heart and renopericardial system, dorsal view. **C.** Heart and renopericardial complex, left posterolateral view. Abbreviations: ao, aorta; au, auricle; kd, kidney; np, nephroporus; pc, pericard; rpd, renopericardioduct; s, haemolymph sinus; vt, ventricle. Scale bars: **A** = 500 μm; **B** = 50 μm; **C** = 100 μm.

contradict previous data in that the reconstructed specimen possesses a large quantity of digestive lobes, densely packed around the gland's central lumen; most cells have a granular cytoplasm in accordance to their secretional and resorptive function in digestion (Meisenheimer, 1905).

Creseis clava possesses a tubular, highly ciliated caecum-like structure, which is closely attached to the digestive gland (Meisenheimer, 1905). This caecum is situated on the left side of the digestive gland in coiled-shelled Euthecosomata and on the right side in straight-shelled Euthecosomata, as in *C. clava* (Meisenheimer, 1905; this study). The function or homology of thecosome caeca is still unclear. Whether or not this caecum is the remainder of a style sac as in some caenogastropods (e.g. Fretter & Graham, 1962), or a reduced and specialized right branch of a paired digestive gland, as hypothesized for nudibranchs by Schmekel & Portmann (1982), remains to be studied.

The intestine, which branches from the stomach, features a loop in close proximity to the digestive gland and opens through the anus into the mantle cavity (Fig. 3D). In coiled-shelled species such as *Limacina*, this loop turns slightly towards the right side of the body, whereas in uncoiled orthoconchs it loops towards the left side (Meisenheimer, 1905; this study, Fig. 3D). The histology of the intestine found in our material coincides in large parts with Meisenheimer's descriptions. In all Euthecosomata the intestine has a longitudinal internal fold (Fig. 5F), differing in size depending on the species (Meisenheimer, 1905). Specifically in *C. clava*, Meisenheimer (1905) also identified a similar, unilateral ridge of the intestine's epithelium. He assumed that this cell enlargement increases the resorptive ability of the intestine by enlarging the epithelial surface, in analogous fashion to a typhlosole in other invertebrates; however, we assume that it is rather related to effective transport of faeces, because the intestine does not possess a particular resorptive function in molluscs.

Nervous system

The 3D-reconstruction of the CNS of *C. clava* demonstrates its highly concentrated nature, comprising an anterior nerve ring and a short visceral loop, with all ganglia closely attached to each other. This configuration corresponds in most respects to the CNS of other Euthecosomata species described by Meisenheimer (1905) and as shown by Franc (1968: fig. 398c). The presence of two separate, putative cerebropedal and pleuropedal connectives confirms the fused nature of the paired, anterodorsal cerebropleural ganglia in *Creseis*; there is not a merely superficial separation between the cerebral and the pleural ganglia, as in *Cuvierina* (Meisenheimer, 1905; this study). Furthermore, the buccal ganglia are fused as are those of the visceral loop; only the pedal ganglia appear not to be fused in any way.

Nerve ring

In the elongate cerebropleural ganglia, we were able to detect a single, anterior pair of cerebral nerves, dividing into two branches and innervating areas of the pharynx, with a branch that is tentatively interpreted as the oral (labial) nerve. The other branch leads anteriorly, apparently also innervating the anterior part of the penis. Meisenheimer (1905) roughly described these nerves in *Creseis*, but as innervating exclusively the pharynx and areas around the mouth opening, without

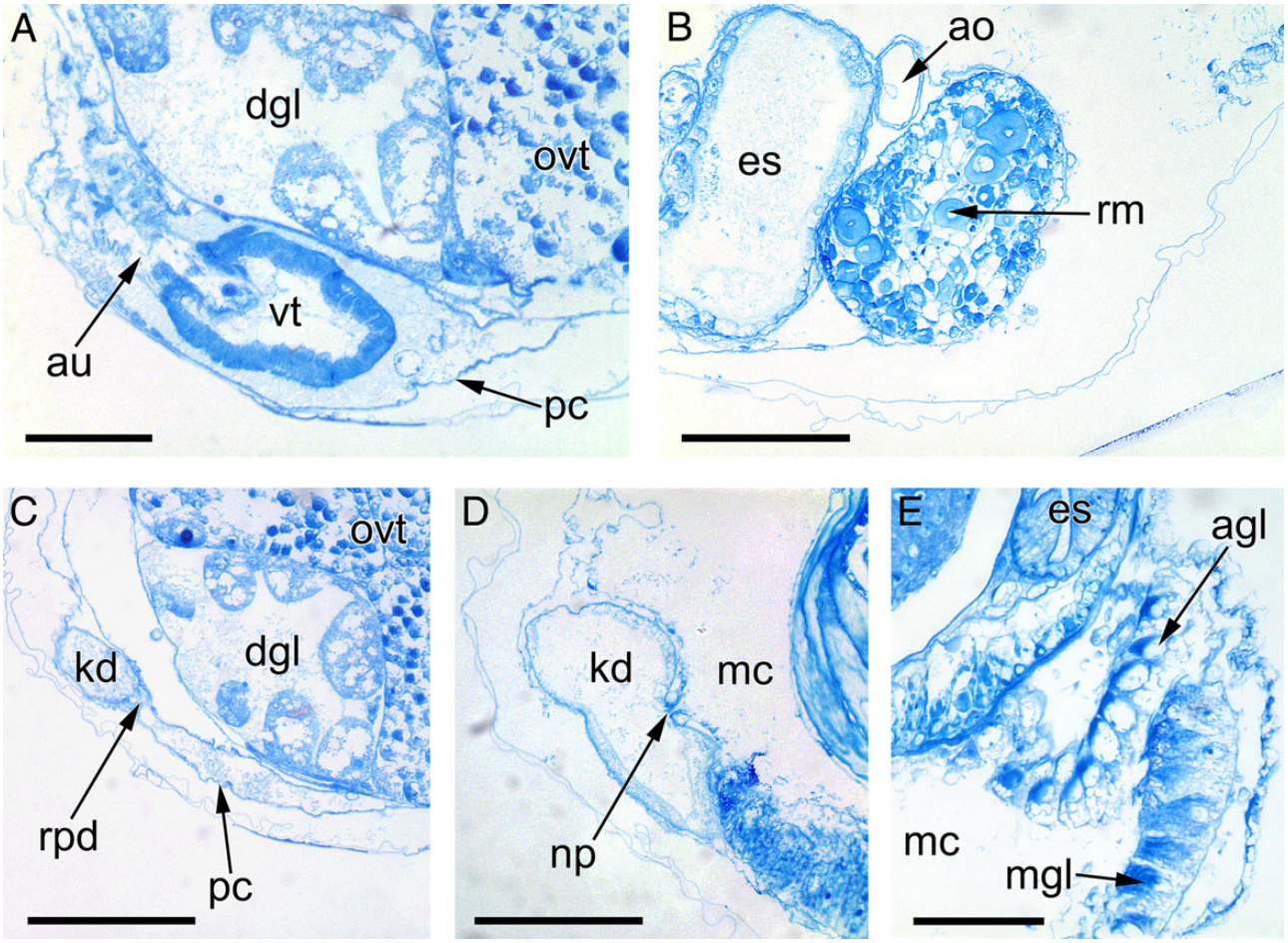

Figure 13. Semithin cross sections showing aspects of circulatory and excretory systems and mantle cavity gland of *Creseis clava*. **A.** Cross section of the heart at junction of auricle and ventricle. **B.** Aorta in anterior part of visceral sac. **C.** Connection of pericardium and kidney (renopericardial duct). **D.** Nephropore. **E.** Cross section of anal gland. Abbreviations: agl, anal gland; au, auricle; ao, aorta; dgl, digestive gland; es, oesophagus; kd, kidney; mc, mantle cavity; mgl, mantle gland; np, nephropore; ovt, ovotestis; pc, pericardium; rm, retractor muscle; rpd, renopericardioduct; vt, ventricle. Scale bars: **A** = 25 μm; **B–D** = 50 μm; **E** = 40 μm.

mentioning innervation of the penis. In *Clio pyramidata*, cerebral nerves apparently do not innervate the penis; instead they lead only into parts of the exterior head region and into the wing protrusions (Meisenheimer, 1905). Static or optic nerves were not detected in our material, but are usually very thin and difficult to discern. In general, euthecosomes have been reported to possess three pairs of cerebral nerves, namely the static nerves, labial nerves and tentacular nerves (Huber, 1993).

Huber (1993) stated that the tentacles of caenogastropods, 'archaeopulmonates' and, among others, thecosomes are innervated by the nervus tentacularis, which he homologized with the nervus clypeus capitis of the Cephalaspidea. In contrast, Staubach (2008) showed the nervus tentacularis of the caenogastropod *Littorina* to be composed of the labiotentacular and rhinophoral nerves; the nervus clypei capitis does not innervate any tentacles in the studied opisthobranchs and (pan)pulmonates (Klussmann-Kolb, Croll & Staubach 2013; Koller, Brenzinger & Schrödl, 2014). Meisenheimer (1905) reported that the tentacular nerve innervates the (posterior) 'tentacles' of thecosomes, but did not give details about the innervation of the 'wing protrusions' (anterior tentacles). We tentatively interpret the cerebral nerves in thecosomes to be combined rhinophoral and labiotentacular nerves innervating the rhinophores and, potentially, the anterior tentacles; the latter we regard as homologues with the labial (or oral) tentacles present in gymnosomes, anaspideans and some other euopisthobranchs, as well as in nudipleurans and many panpulmonates. More detailed studies are needed to confirm the exact innervation of the putative labial tentacles of thecosomes by a branch of the tentacular nerve. The 'labial' nerve of thecosomes may be homologous with the oral nerve ('N1'; e.g. Staubach *et al.*, 2008; Klussmann-Kolb *et al.*, 2013), but its target area needs to be investigated. Interestingly, Huber (1993) considered that the presence of a tentacular nerve in adult Thecosomata reflects a retained larval condition, i.e. an apomorphic paedomorphosis, rather than a plesiomorphic feature in common with caenogastropods. Recent hypotheses on heterobranch phylogeny recover Thecosomata as a derived euopisthobranch clade (Klussmann-Kolb & Dinapoli, 2006; Jörger *et al.*, 2010; Wägele *et al.*, 2014) and thus support this view.

The pedal ganglia are interconnected in the reconstructed specimen by only one short and thick pedal commissure; we could not discern an additional, much narrower parapedal commissure as reported in *Creseis* and other euthecosomes by Meisenheimer (1905) and Tesch (1913). *Creseis clava* studied herein shows that each pedal ganglion gives rise to three large nerves. The thickest of these nerves runs to the base of the wings and innervates them through all of their length; the two smaller nerves could not be followed in our specimen. Meisenheimer (1905), in *C. pyramidata*, ascribed four nerves to each pedal ganglion; Franc (1968) counted up to five pairs. The thickest of

Table 1. Comparison of the positions of particular organs in *Creseis* and *Limacina*.

3D-model *Creseis clava*	*Creseis*	*Limacina*
Mantle cavity → ventral	Ventral	Dorsal
5th gizzard plate → ventral	Ventral	Dorsal
Digestive gland → right	Right	Left
Caecum → right	Right	Left
Intestine loop → left	Left	Right
Anus → medial	Left	Right
Osphradium → right	Right	Left
Visceral ganglion → left	Left	Right
Genital opening → right	Right	Right
Heart → medial	Right	Left
Kidney → right	Right	Left
Mantle gland → ventral	Ventral	Dorsal
Mantle cavity gland → left	Left	Right

Left column: traits identified in 3D-reconstruction of *C. clava*.
Middle column: traits observed by Meisenheimer (1905) in *Creseis*.
Right column: traits observed by Meisenheimer (1905) in *Limacina*.

these nerves was reported to run into the wings, almost to their anterior edge (Meisenheimer, 1905); this corresponds to our observations in *C. clava*. In *Clio*, the remaining three pedal nerves run into different target areas, such as the base of the wings and the epidermal groove around the mouth opening. The lobes surrounding this structure were therefore defined as foot-lobes by Meisenheimer (1905). The three smaller pedal nerves described by Meisenheimer could not be correlated individually with the two smaller pedal nerves found in this study.

In *C. clava*, we found the buccal ganglia to be connected with both cerebropleural ganglia via short connectives; no visible separation of the cortices of the (ancestrally paired) buccal ganglia could be recognized. This observation coincides in large part with Meisenheimer's (1905) findings. Only in *H. striata* did he recognize a rudimentary inner separation of the buccal ganglia through a visible division of the perikarya. Most species of Euthecosomata have completely fused buccal ganglia, which is a potential apomorphy for the Euthecosomata.

Of the three nerves that emerge anteriorly from the buccal ganglia according to Meisenheimer (1905) and Franc (1968), only two could be found in the reconstructed specimen. These two nerves run exactly as described for the three nerves Meisenheimer identified, i.e. anteriorly towards the mouth region, clearly innervating the pharynx from both sides.

Visceral loop

The highly concentrated and obviously fused composition of the ganglia situated on the visceral loop in *C. clava* coincides with the condition reported in other species of Euthecosomata (Meisenheimer, 1905). According to hypotheses on the development of the CNS of Heterobranchia (Haszprunar, 1985), the visceral loop can be assumed to originate from either three or five separate anlagen during ontogeny. In adult euthecosomes, only two ganglia can be distinguished throughout all species, one on the right side of the visceral loop and one on its left side (Meisenheimer, 1905; Franc, 1968). In *Creseis*, the right ganglion is smaller than the left, whereas in the helicoid *Limacina*, it is the other way round (Meisenheimer, 1905). This may imply opposing patterns of fusion in these two taxa, as reported by Franc (1968). Tesch (1913) identified the right ganglion as the supraoesophageal ganglion, and the left one as the product of fusion of the abdominal (= visceral) and suboesophageal ganglia. This interpretation is supported by our observation of a rudimentary yet discernible internal separation of the left ganglion through the arrangement of the perikarya. Meisenheimer (1905) observed the same intrusion of the cortical perikarya into the inner medulla in *Diacria*; in *Clio* and *Cavolinia* this internal separation was less distinctive. This similarity may indicate that the latter two genera are more closely related to each other than to *Diacria* and *Creseis*, which show a presumably ancestral (i.e. less fused) pattern. Once the polarity of characters has been clarified among thecosomes and related euopisthobranchs, such morphological information could, in addition to genetic analyses, be useful to investigate ambiguous phylogenetic relationships (see Corse *et al.*, 2013), specifically between *Diacria*, *Cavolinia* and *Clio*.

In the reconstructed specimen the right visceral loop ganglion dispatches one nerve towards the right side of the mantle, connecting to the elongated osphradial ganglion, which is situated directly below the osphradium (Figs 6B, 7, 8F). Therefore, the right visceral loop ganglion corresponds to, or at least contains parts of, the supraoesophageal ganglion (see Brenzinger *et al.*, 2013b for discussion of euthyneuran nervous systems). The double innervation of the osphradium and right mantle side by this right ganglion is in agreement with Tesch's (1913) observation on *Creseis* (see also Franc, 1968: fig. 398) and coincides in most respects with Meisenheimer's (1905) descriptions. However, the latter author did not mention any looping osphradial nerve in any of the euthecosome species investigated, which could be due to the fact that eversion of the penis and penial sheath could change the conformation at least of the loop of the osphradial nerve.

The osphradium is an unpaired chemosensory organ typically situated on the inner wall of the apogastropod mantle cavity in the anterior part of the visceral sac (e.g. Haszprunar, 1985, 1988). In all coiled euthecosomes, it is located on the left side of the body, whereas in straight-shelled species it is on its right side (Meisenheimer, 1905) (Figs 6A, 7). This was confirmed by the 3D-model of *C. clava*. In all Euthecosomata the osphradium is characterized by an elongated, flat and ciliated stretch of epithelium, under which an osphradial ganglion of equal dimensions is located (Meisenheimer, 1905).

Two nerves emerge from the left, larger visceral loop ganglion of *Creseis*. As reported for *C. pyramidata* (Meisenheimer, 1905), one of the two nerves is rather narrow and proceeds towards the visceral sac, running along the digestive tract towards the posterior part of the visceral sac, where the genital organs are situated, while presumably innervating both organ systems. This is largely in agreement with Tesch's (1913) observation that there are two nerves emerging from the inner part of the visceral loop (a visceral and a genital nerve); these separate nerves may be the same as the aforementioned single nerve found in our material. The second, thicker nerve runs laterally along the left side of the mantle towards the anterior part of the visceral sac (Meisenheimer, 1905; Tesch, 1913). In contrast to the observation of Meisenheimer, who stated without any further explanation that the thick nerve's target organ is the right side of the mantle, we identified the mantle cavity gland as its target organ.

The left, putatively fused visceral loop ganglion is larger than the right ganglion, and it gives rise to two nerves. Innervating visceral organs, the euthyneuran visceral ganglion may contribute to this fused ganglion; its other portion is tentatively referred to as the suboesophageal ganglion, pointing to an at least triganglionate condition. There is not yet any indication of a pentaganglionate condition (with additional, separate or fused parietal ganglia) (see Haszprunar, 1985) in thecosomes.

Concluding, the CNS of *Creseis* is characterized by strong condensation and various assumed fusions among ganglia, such as between the cerebral and the pleural ganglia, the two buccal

ganglia and between those of the visceral loop (Meisenheimer, 1905). Further examination of nervous systems of pteropods and related euopisthobranchs is needed to infer their homologies, functions and evolution, e.g. with relation to the reduction of the head and tentacles and their potential integration into predominately pedally innervated wings. The wings thus appear to be specialized parts of the foot transformed to function in swimming.

Reproductive system

Creseis clava possesses a monaulic genital system and is a protandric hermaphrodite, as was shown by the presence of male sexual organs in early stages of life and both male and female sexual organs in older individuals (Meisenheimer, 1905); the male gonad portion is in large part replaced by the female one. The reconstructed specimen of *C. clava* was fixed in a hermaphroditic stage, possessing both female genital glands and a penis. We could distinguish only a few eggs or egg-like cells with a yolky cytoplasm, situated mostly in the periphery of the ovotestis. Meisenheimer depicted the sperm-producing tissue in one of his sketches, as a tissue constituted of closely packed dark dots. A similar observation was made in the reconstructed specimen, identifying in the centre of the ovotestis very small areas of dark blue stained dots (Fig. 11A); these are, however, not unequivocally spermatozoa.

The gonoduct is a long, narrow, tubular structure that transports the gametes to the complex of genital glands. The gonoduct features a ciliated epithelium (Meisenheimer, 1905), which, however, could not be clearly identified as such in our specimen. Meisenheimer observed throughout all the Euthecosomata that the proximal gonoduct is differentiated from the rest as an ampulla that stores mature autosperm until copulation. Specifically in *Creseis*, Meisenheimer (1905) described it as a caecum-like evagination of the gonoduct, located immediately next to the connection of the latter to the ovotestis. No such structure could be recognized in our specimen.

The complex of genital glands, through which the more distal gonoduct leads dorsally, consists of three glandular, epithelial structures (here called glands 1–3). The complex of genital glands is found in all Euthecosomata on the right side in the anterior part of the visceral sac (Meisenheimer, 1905). This position was also confirmed in the 3D-model of *C. clava*. The interconnections of the single genital glands and the gonoduct differ among species of the Euthecosomata (Meisenheimer, 1905). Meisenheimer described these interconnections thoroughly for *C. clava*; his observations coincide in most but not all respects with our own. Meisenheimer attributed to glands 1 and 2 a function in the preparation and coating of fertilized eggs, directly after copulation. Gland 1 thus could be homologous with the capsule gland identified by Klussmann-Kolb (2001) in nudibranchs. Gland 2 has a ciliated epithelium according to Meisenheimer (1905), which could not be seen in the reconstructed specimen, and could be a mucus gland. As shown in the 3D-model, gland 2 is long and tubular, proceeding into a narrower duct-like structure, which represents the distal gonoduct (Meisenheimer, 1905). This transports the gametes to the genital opening, which is located on the right side of the mantle cavity floor. The bag-like gland 3 was characterized by a slight muscular layer and a ciliated epithelium, and was identified as a receptaculum seminis (Meisenheimer, 1905) or vesicula seminalis (Tesch, 1913) by earlier workers. While these histological observations were confirmed in our specimen, we do not agree with previous interpretations: gland 3 does not contain any sperm and does not resemble a typical receptaculum or bursa as described by Wägele & Willan (2000). Its identity and function as a sperm-receiving receptacle needs further investigation.

Herein we confirm the presence of a seminal groove in *C. clava*. A monaulic genital system with open sperm groove is typical for euopisthobranchs and was long regarded as plesiomorphic for opisthobranchs in general (but see Schrödl *et al.*, 2011; Brenzinger *et al.*, 2013a). Monauly is clearly plesiomorphic for pteropods and thecosomes, but the androdiaulic condition has evolved at least once. *Cavolinia* is the only genus among the Euthecosomata that does not feature an open seminal groove, but instead a closed vas deferens, as described by Meisenheimer (1905). The closed vas deferens is an apomorphy for *Cavolinia*, which forms the most derived clade within Orthoconcha together with *Diacria* and *Clio* (Corse *et al.*, 2013). The arrangement of the vas deferens in *Cavolinia* is similar to the 'special androdiaulic' condition in panpulmonate acochlidian hedylopsaceans (Schrödl *et al.*, 2011) and obviously evolved convergently.

In *Creseis*, the penis is a complex infolded organ, enveloped by a sheath that connects to the seminal groove apically. The entire copulatory organ fills a considerable part of the head cavity in all euthecosome species except for *Cavolinia* (Meisenheimer, 1905). As described by Meisenheimer for *C. clava*, the penis sheath is attached to the long retractor muscle at its posterior end; this muscle extends to the most posterior end of the visceral sac (Fig. 9A). In both Meisenheimer's (1905) study of *C. clava* and our own, a long, narrow, putatively muscular structure was found which could not be allocated to any other organ systems. We suspect this is a homologue of the columellar muscle retracting the headfoot and buccal mass, but connections could not be seen as a result of local damage of tissues.

As described by Meisenheimer (1905), all Thecosomata except for the genus *Cavolinia* possess complex copulatory organs with stylets. The penis of *C. clava* features several side branches (caeca) that are filled with extensive glandular organs. We did not find stylets in the single specimen investigated, and the number of caeca and their ramifications could not be definitely determined. The functions of these structures (caeca, stylets and side branch) remain unclear. *Creseis* was reported to copulate reciprocally, with wings of partner intertwined and their penises connecting externally (Lalli & Gilmer, 1989: p. 109), which is similar to the condition in terrestrial stylommatophoran slugs such as *Limax*, although here the 'penises' are in fact composed of male and female gonoducts. In *Creseis* and other euthecosomes, the male copulatory organ stands alone and usually in a frontal position; this implies that there is no contact with the more posterior 'female' genital opening during copulation, so we suggest that the lateral caeca of the penis could act as temporary allosperm-receiving structures. Copulatory organs, however, show some structural variation among euthecosomes, which could be of taxonomic relevance; for example, the copulatory organ of *Diacria* species has been reported to be very large compared with the animal itself (Lalli & Gilmer, 1989). Similar copulatory organ systems that are particularly large, complex and bear stylets and accessory glands, are known from several euthyneuran groups (Sanders-Esser, 1984; Kohnert *et al.*, 2010; Brenzinger *et al.*, 2011a) and could indicate traumatic mating and sexual conflict (e.g. Lange, Werminghausen & Anthes, 2013), or possibly divergence within widespread pelagic populations as a result of sexual selection (Churchill *et al.*, 2013).

Circulatory and excretory systems

Meisenheimer's (1905) findings regarding the renopericardial complex in Euthecosomata were reexamined and in large part confirmed in *C. virgula* by Fahrner & Haszprunar (2000). The main components of the circulatory system are the aorta and a monotocardian heart, consisting of a ventricle and a single auricle. The heart is enveloped by a thin-walled pericardium, which is connected to the kidney via a renopericardioduct. As the 3D-reconstruction indicates (Fig. 12B, C) the heart of *C. clava* is

situated medioventrally halfway through the visceral sac, although described as situated on the right side by Franc (1968), which may indicate some artificial twisting of retracted specimens. This is also suggested by the fact that in our 3D-model the ventricle and auricle are arranged side by side, with the ventricle on the left (agreeing with Fahrner & Haszprunar, 2000), while described as antero-posterior (longitudinal) by Meisenheimer (1905). Only in the case of *Cuvierina* and *Hyalocylis* did Meisenheimer describe a transverse arrangement of the ventricle and the auricle.

As shown for *Creseis* by previous authors, the main components of the excretory system are the auricle wall (where the actual ultrafiltration into the pericardium takes place) and the kidney (where processing of the primary urine occurs) and the renopericardial duct features a ciliated epithelium (Meisenheimer, 1905; Fahrner & Haszprunar, 2000). This general arrangement was confirmed here for *C. clava*.

Taxonomic remarks

We identified our specimen as *C. acicula* which, according to Gasca & Janssen (2014), is a junior synonym of *C. clava*, because of its distinctive elongate shell. *Creseis virgula*, in contrast, was reported to possess a stouter, curved shell. Using the barcoding region of the COI gene as a sequence marker, Gasca & Janssen (2014) supported the specific distinction of elongate *vs* stout shell morphs. However, their *C. acicula* is paraphyletic with regard to *C. clava* in the COI tree (Gasca & Janssen, 2014) and there appears to be geographical structure. In particular, basal *C. acicula* lineages are Indo-Pacific, while the remaining clade combines specimens of *C. clava* and *C. acicula*, all from the Caribbean. We believe that considerable variation exists in *Creseis* shell shapes, and potentially also in soft-part characters, as indicated herein, so that further analyses of intraspecific *vs* interspecific variation is required. We also doubt that single pteropod species occur across oceans and hydrogeographic boundaries (Uribe *et al*., 2013). Recent molecular studies have revealed complexes of cryptic species within traditionally shell-based thecosome taxa (Hunt *et al*., 2010; Birky, 2013; Maas, Blanco-Bercial & Lawson, 2013). Higher species diversity than expected from traditional taxonomy, and more limited ranges than predicted from high dispersion ability, have been discovered in other pelagic sea slugs also (Churchill *et al*., 2014). Externally cryptic species of the nudibranch *Glaucus marginatus* complex show distinctly different reproductive organs, with a bursa developed or not, and sexual selection was hypothesized to drive speciation within wide-ranging pelagic sea slugs (Churchill *et al*., 2013). Recent results emphasize a high level of cryptic species with narrower than expected ecological and distributional ranges in other holoplanktonic groups also, such as calanoid copepods (Cornils & Held, 2014). An integrative and global approach, as performed by Jörger *et al*. (2012) for the acochlidian sea slug genus *Pontohedyle*, could be useful to resolve species limits within *Creseis*.

Orthoconchy via detorsion?

In general, helicoid thecosomes have a dorsal mantle cavity (Meisenheimer, 1905). In contrast, *Creseis* and other straight-shelled thecosomes (Orthoconcha) have a ventral mantle cavity, opening ventrally to the mouth. This condition implies a rotation of the visceral sac relative to the (reduced and transformed) headfoot by 180° along the longitudinal body axis. A comparison of other organ systems (Table 1) is consistent with this assumption. Where present in euopisthobranch gastropods, the mantle cavity is dorsal; a rotation of 180° to the ventral side, as envisioned by Boas (1886), would thus be a synapomorphy of orthoconch thecosomes. Relative rotation can be explained by secondary detorsion of the mantle and viscera, or by more or less impeded torsion. The latter appears likely according to descriptions of some ontogenetic stages of *Creseis* by Gegenbaur (1855), in which no major torsion or detorsion events were indicated. Boas (1886) also assumed that orthoconchs are decoiled. However, the apparently uncoiled nature of *Creseis* larvae (Gegenbaur, 1855) implies that a rather symmetrical, uncoiled body is present throughout orthoconch ontogeny. The lack of evident torsion and coiling in orthoconchs may be considered paedomorphic, produced by abbreviated or skipped developmental processes (which are active in coiled limacinids) and a derived condition, because limacinid shells appear earlier in the fossil record than straight shells (Corse *et al*., 2013). The relocation of the mucus-web forming mantle gland below the foot and mouth possibly provides a more efficient way of mucus-web feeding, or facilitates faster jettisoning of the web during escape from predators.

CONCLUSION

This is an initial study of thecosome microanatomy, providing data from *Creseis clava* for future comparisons with other pteropods and euthyneurans. In most respects, our results confirmed Meisenheimer's (1905) descriptions. In addition, our approach allowed for discovery of several additional details, such as those of central nervous structures, gizzard and genital organs. We also provide some first opinions on homologies of thecosome organs, in particular of cerebral nerves and tentacles, in the light of modern structural and phylogenetic hypotheses. However, our observations and assumptions will need confirmation and supplementation through study of additional and better fixed specimens, ideally of different ontogenetic stages.

We have used our data for comparison of *Creseis* (as a supposedly basal member of Orthoconcha) with other straight-shelled euthecosomes and with limacinids such as *Limacina* having helicoid shells. Both straight shells and unusually rotated posterior organs intuitively suggest that these special features of Orthoconcha are apomorphic relative to supposedly plesiomorphic *Limacina*-like thecosomes (Corse *et al*., 2013) and are functionally and/or evolutionarily related. This remains to be confirmed in the light of reliable hypotheses of thecosome phylogeny.

We hypothesize that the symmetrical, uncoiled and untorted body of larval to adult *Creseis* is produced by skipping the developmental processes of torsion and coiling, and are therefore paedomorphic features. In addition, previous authors have proposed the retention of a bifurcate tentacular nerve (Huber, 1993) and of a permanent, continuously growing functional larval shell (Haszprunar, 1985) as paedomorphic features of thecosomes. We may add to this list an incomplete differentiation and separation of the head (with more or less reduced or transformed tentacles including parts of the cerebral larval velum) and foot, allowing for transformation of the combined headfoot into the charismatic thecosome wings. Confirming and supplementing old ontogenetic descriptions of thecosomes, gymnosomes and related outgroups will be necessary for assessing heterochronic processes that have driven thecosome evolution.

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Chapter 6. Martynov AV, **Brenzinger B**, Hooker Y & Schrödl M (2011): **3D-anatomy of a new tropical Peruvian nudibranch gastropod species, *Corambe mancorensis*, and novel hypotheses on dorid gill ontogeny and evolution.** *Journal of Molluscan Studies*, **77:** 129-141.

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3D-ANATOMY OF A NEW TROPICAL PERUVIAN NUDIBRANCH GASTROPOD SPECIES, *CORAMBE MANCORENSIS*, AND NOVEL HYPOTHESES ON DORID GILL ONTOGENY AND EVOLUTION

ALEXANDER MARTYNOV[1], BASTIAN BRENZINGER[2], YURI HOOKER[3] AND MICHAEL SCHRÖDL[2]

[1]*Zoological Museum, Moscow State University, Moscow, Russia;*
[2]*Zoologische Staatssammlung München, Münchhausenstr. 21, D-81247 München, Germany; and*
[3]*Laboratorio de Biología Marina, Universidad Peruana Cayetano Heredia, Lima, Peru*

Correspondence: M. Schrödl; e-mail: Michael.Schroedl@zsm.mwn.de

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ABSTRACT

Nudibranch molluscs of the genus *Corambe* differ from most other Doridoidea by having ventral rather than dorsal anus and gills. Because of these and other features, such as separate cerebral and pleural ganglia, corambids have been considered as an archaic or enigmatic group. The first tropical eastern Pacific *Corambe* species is described in morphological and some histological detail. Selected organs such as circulatory and central nervous features are reconstructed from serial semithin histological slides and visualized in three dimensions using Amira software. Anatomical findings include two separate ganglia on the visceral loop and an additional ganglion on the right side of the body that is connected to the pedal ganglion. *Corambe mancorensis* n. sp. is dorsoventrally depressed, has an oval, fleshy notum covered with a cuticle, and has a wide posterior medial notch that can be closed completely by unique lobules. Gills are arranged in an unusual horseshoe-like manner including both phanerobranch anal (=medial) gills and corambid lateroventral gill rows, and are connected to the atrium by a complex vessel system. The three medial gills arise from a posterodorsal gill cavity within the notal notch, similar to the case in *Corambe evelinae* Marcus, 1958. By scanning electron microscopy a vestigial gill cavity is also detectable in *C. pacifica* MacFarland & O'Donoghue, 1929, but here it is situated ventrally. Our new information on adult corambids is compared with new and published ontogenetic data on phanerobranch and cryptobranch dorids, to contribute to a novel interpretation of the ontogeny of dorid mantle and gill complexes. The progenetic evolution of corambids 'recapitulates' early juvenile dorid stages – turning Haeckel's Law upside down.

INTRODUCTION

Corambid dorid nudibranchs have long been regarded as aberrant (Bergh, 1871, 1892; Fischer, 1891; Odhner in Franc, 1968). Unusual features include a depressed body shape, a fleshy notum that is covered with a shedding cuticle in most members, separate cerebral and pleural ganglia, and a peripherally lobed digestive gland that is separated by paired dorsoventral muscle bundles (Schrödl & Wägele, 2001). Differing from lower diversity estimations by Edmunds (2007), the latest analysis of corambid phylogeny recognizes 10 valid species, including the genera *Loy* (3 species) and *Corambe* (7 species), plus 4 further, still undescribed *Corambe* species (Martynov & Schrödl, in press). According to that study, members of *Loy* are characterized by inhabiting soft bottoms and having an oral veil that is partly fused with the hyponotum, while most features thought to be characteristic for corambids actually are apomorphic for *Corambe* species, such as serial ventral gills, a cuticle covering the notum and pairs of dorsoventral muscle bundles separating the digestive gland into peripheral lobes.

All corambids show a typical suctorian buccal apparatus and radula; this led Millen & Nybakken (1991) to conclude that the corambids are a basal offshoot of suctorian phanerobranch dorids. However, the ventral position of the anus and gills of all corambids known at that time was intriguing and, as an apparently ancestral condition, led to classification of the group as an order Corambida at the base of the dorids (Baranets & Minichev, 1994). Martynov (1994a) was the first to describe two northern Pacific deep-water corambid-like species with dorsal or subventral anus and gills, thus bridging the gap to 'normal' dorids with dorsal anus and circumanal gills. An evolutionary scenario of derivation of corambids from suctorial Onchidoridiidae was proposed by Martynov (1994b, 1995) and confirmed by a recent cladistic approach (Martynov & Schrödl, in press); this explained the ventral shift of the anus and gills by paedomorphosis, which could also account for the comparatively low level of cephalization in corambids. The nervous system of corambids has, however, not yet been studied in adequate histological detail. Even the presence, position and homology of the heterogeneous corambid gills have not been clarified so far. *Loy meyeni* Martynov, 1994a, b shows three non-retractile dorsal gills (and anus) in a posteromedial notal cavity, thus closely resembling the usual phanerobranch dorid condition. These three medial gills lie in a subventral position in *Loy millenae* (Martynov, 1994a, b) (as *Proloy*) and ventrally, apparently without any cavity, in *Loy thompsoni* (Millen & Nybakken, 1991) (as *Corambe*). All other corambids have ventrolateral rows or pairs of gills. Having special gill glands and a similar gill vessel system, Schrödl & Wägele (2001) showed that ventrolateral gills in *Corambe lucea* Marcus, 1959 correspond to primary dorid gills, in contrast to the secondary, lamellate lateral gills present in Phyllidiidae. The left and right lateroventral gill rows of *C. lucea* and several other corambids are, however, neither connected with each other nor in close

association with the anus as is usual in other dorids. In contrast, a continuous row of gills around the rear part of the animal is present in *Corambe pacifica* MacFarland & O'Donoghue, 1929. In the rather poorly known Brazilian *C. evelinae* Marcus, 1958 a special condition has been described: in addition to the lateral gill rows there are 2–3 medial gills situated in a more dorsal position, obviously close to the anus and within a kind of notal cavity (Marcus, 1958: pl. 7, figs 51, 54). The significance of this observation has not so far been explored.

The present paper gives detailed anatomical information on a new *Corambe* species from northern Peru, which is the first corambid record from the tropical eastern Pacific. The histology of selected organs is described; the nervous system is reconstructed from serial semithin slides and visualized three-dimensionally. The unique gill arrangement is compared with that of other corambids; it is interpreted as showing a continuous assemblage of both phanerobranch medial gills in a vestigial cavity and multiplied corambid gills in lateroventral rows. New observations on early phanerobranch and cryptobranch juveniles lead to a novel hypothesis on the ontogeny of the dorid notum, anus and gills, and a comparison with the progenetic evolution of these organs in corambids (Martynov & Schrödl, in press).

MATERIAL AND METHODS

More than 20 specimens of *Corambe mancorensis* n. sp. were collected at the pier of the fishery harbour of Mancora, Tumbes province, northern Peru by two of us (Y.H. and M.S.). Specimens were found on macroalgae (*Padina durvillea*, *Spatoglossum* cf. *congestum*) that were covered with encrusting bryozoans (*Membranipora*), at 0–3 m depth. Specimens were observed alive and photographed. Seven specimens were fixed in 96% ethanol and further specimens were fixed in 70% ethanol; the latter were used for dissections and SEM of body surfaces (after critical-point drying) and radulae. For histological study eight specimens were relaxed in isotonic $MgCl_2$ solution and then fixed in 4% glutaraldehyde buffered in 0.2 M sodium cacodylate (0.1 M NaCl and 0.35 M sucrose, pH 7.2). These specimens were rinsed several times in the buffer solution and postfixed in buffered 1% OsO_4 for 1.5 h. The specimens were dehydrated through a graded acetone series and finally embedded in Spurr's low-viscosity epoxy resin (Spurr, 1969) for semithin sectioning. Following Henry (1977) and Ruthensteiner (2008), glass knives were used to prepare two complete ribbons of serial cross-sections (1.5 μm) with a microtome (Microm HM 360, Zeiss, Thornwood, NY, USA). Sections were stained with methylene blue-azure II (Richardson, Jarett & Finke, 1960). One individual (the holotype) was used for three-dimensional reconstruction of selected organ systems using the software AMIRA 3.0 (TGS Template Graphics Software) as described, for example, by Neusser *et al.* (2006) and discussed by DaCosta *et al.* (2007).

To study the ontogeny of the direct developer *Cadlina laevis*, specimens and egg masses were taken from the vicinity of the Marine Biological Station of Moscow State University, Kandalaksha Bay, White Sea. *In vitro*, egg and juvenile development was observed daily, until juveniles reached 1 mm length. At relevant ontogenetic stages, i.e. whenever new features of body shape, anus position or structures related to notal lobes, gills or gill cavities appeared, specimens were fixed for light microscopy and SEM (CamScan; Leo II), the latter after critical-point drying (ZMMU Op-23, ZMMU Op-26). Preserved early juvenile specimens of other dorid species were available for SEM from Corsica, Mediterranean Sea (*Paradoris indecora*, ZSM 20011861; *Doris ocelligera*, ZSM 20012397; *Onchidoris neapolitana*, ZSM 20012382). Adult corambids used for light microscopy and SEM were obtained from Sevastopol, Black Sea (*Corambe obscura*, ZMMU Op-7), British Columbia (*Corambe pacifica*, ZMMU Op-14; *C. steinbergae*, ZMMU Op-31) and Peter the Great Bay, Japan Sea (*Loy meyeni*, holotype ZMMU Lc-25699).

Abbreviations: ZMMU, Zoological Museum Moscow State University; ZSM, Zoologische Staatssammlung München.

SYSTEMATIC DESCRIPTION

Family Onchidoriddidae Gray, 1827

Genus *Corambe* Bergh, 1869

Corambe mancorensis new species

(Figs 1–6)

Material examined

Holotype: ZSM 20080543 (series of histological semithin sections; Bavarian State Collection of Zoology), pier of fishery harbour of Mancora, Tumbes Province, northern Peru (4°6′36″S, 81°4′2″W), 0–3 m depth (Hooker & Schrödl col. 02/xii/2006). *Paratypes*: Seven specimens, ZSM 20080536–42 (resin blocks); 6 specimens, ZSM 20091918–23 (entire), collected with holotype. *Additional material*: Several specimens, used for dissection and SEM; collected with holotype.

Etymology

Named after the type locality Mancora.

External morphology

Length of holotype 3 mm; in preserved condition and embedded for semithin sectioning 2.6 mm. Length of eight measured living specimens 1.3–3.2 mm, width 1–3.2 mm. Consistency of living animals soft, i.e. no spicules stiffening the body. Body dorsoventrally depressed. Notum broad, almost circular, posteriorly with two broad, slightly asymmetrical lobes forming a deep notch in the middle (Fig. 1A, B). Anterior and anterolateral edge of notch of notal lobes bears 3 broad, triangular, leaf-like, inwardly directed lobules (one specimen showed only 2 lobules) (Fig. 2A, D), each protecting one of the 3 dorso-terminal gills. Living specimens capable of opening notal cavity widely by spreading the lobes and bending their edges and lobules upwards, revealing the gills (Fig. 1A). When disturbed, edges of the cavities close rapidly. Gills arranged in horseshoe pattern comprising dorso-terminal semicircle of 5 gills, including 3 dorsalmost medial gills within a notal cavity (Fig. 2B). This gill semicircle merges gradually into the remaining gills, placed in two lateroventral rows. Usually 7–9 gills on each side of hyponotum, decreasing in size anteriorly; 2–3 anteriormost gills smallest and hardly visible. A juvenile specimen (1.3 mm) with only 3 and 4 ventrolateral gills on each side of hyponotum. Largest gills are those next to gill cavity. Gill rows do not reach middle part of foot and are restricted to its posterior part. All gills unipinnate; largest ones of 4–7 leaflets; smallest do not possess recognizable leaflets (Fig. 2B). Ventral anus located just anterior to notal cavity entrance, between two largest gills (Fig. 2B). Rhinophores short, retracted into raised sheaths with smooth, soft, not indented edges (Fig. 2A); edges capable of considerable contraction in living specimens. Rhinophore with short anterior smooth stalk, wrapped within two pairs of folds, and bearing posterior unpaired fold, which is a continuation of rhinophoral stalk (Fig. 1D). Notum sparsely covered with low hemispherical tubercles (Figs 1D, 2C), which are more densely arranged on notal notch lobules (Fig. 2D). Oral veil small, trapezoid; in

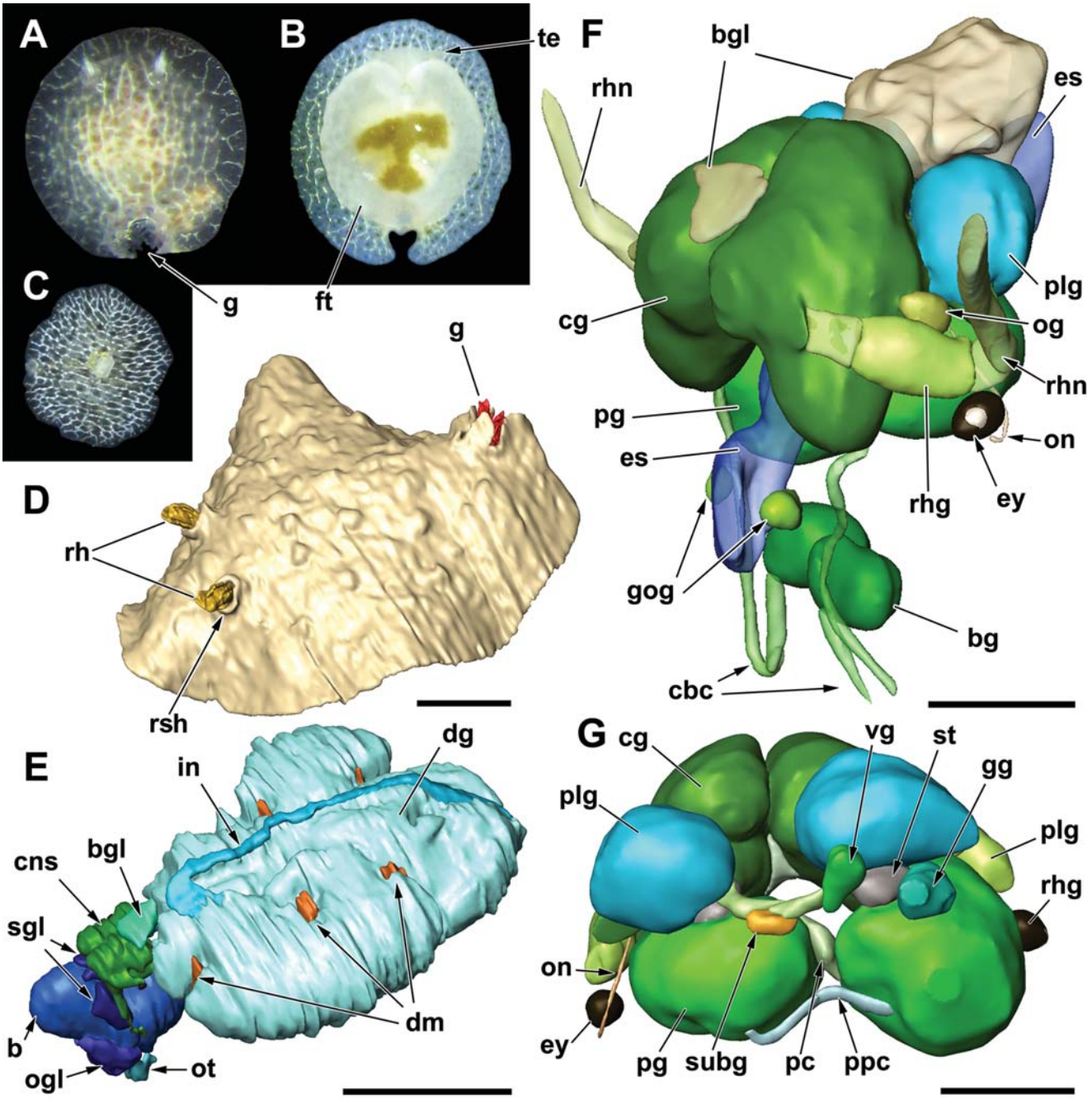


Figure 1. Living specimen of *Corambe mancorensis* n. sp. and three-dimensional reconstructions of body, digestive and central nervous systems (CNS). **A.** Dorsal view of living specimen (*c.* 3 mm). **B.** Ventral view of living specimen showing foot and lobed digestive gland. **C.** Syntopic, *Corambe* and *Membranipora* mimicking flatworm (*c.* 1 cm). **D.** Entire body; note gills partially projecting from papillate notch. **E.** Left view of digestive system and adjacent organs. **F.** Left view of CNS showing major ganglia, nerves and adjacent organ systems. **G.** Posterior view of CNS. Abbreviations: b, buccal pump; bg, buccal ganglion; bgl, blood gland; cbc, cerebrobuccal connective; cg, cerebral ganglion; cns, central nervous system; dg, digestive gland; dm, dorsoventral muscles; es, esophagus; ey, eye; ft, foot; g, gills; gg, genital ganglion; gog, gastroesophageal ganglion; in, intestine; og, optic ganglion; ogl, oral glands; on, optic nerve; ot, oral tube; pc, pedal commissure; pg, pedal ganglion; plg, pleural ganglion; ppc, parapedal commissure; rh, rhinophore; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; rsh, rhinophoral sheath; sgl, salivary glands; subg, suboesophageal ganglion; st, statocyst; te, oral tentacle; vg, visceral ganglion. Scale bars: **E, F** = 500 μm; **D, G** = 100 μm.

living specimens anterolateral corners form short narrow triangular tentacles (Fig. 1B). Foot is broad, anteriorly thickened, beneath mouth it has a deep incision forming lateral lobes; posteriorly slightly narrowed and rounded (Fig. 1B).

Colour: Living specimens semitransparent, with irregular network of opaque bright white and yellowish broken lines or spots (Fig. 1A, B); brownish and pale lilac spots scattered within network, but in a deeper integument layer. Hyponotum covered by similar network of lines (Fig. 1B). Rhinophores translucent with few small white dots. Gills translucent white; some gills, including those in notal cavity, covered dorsally with conspicuous brownish or lilac spots. Three brownish lobes of digestive gland visible through the foot.

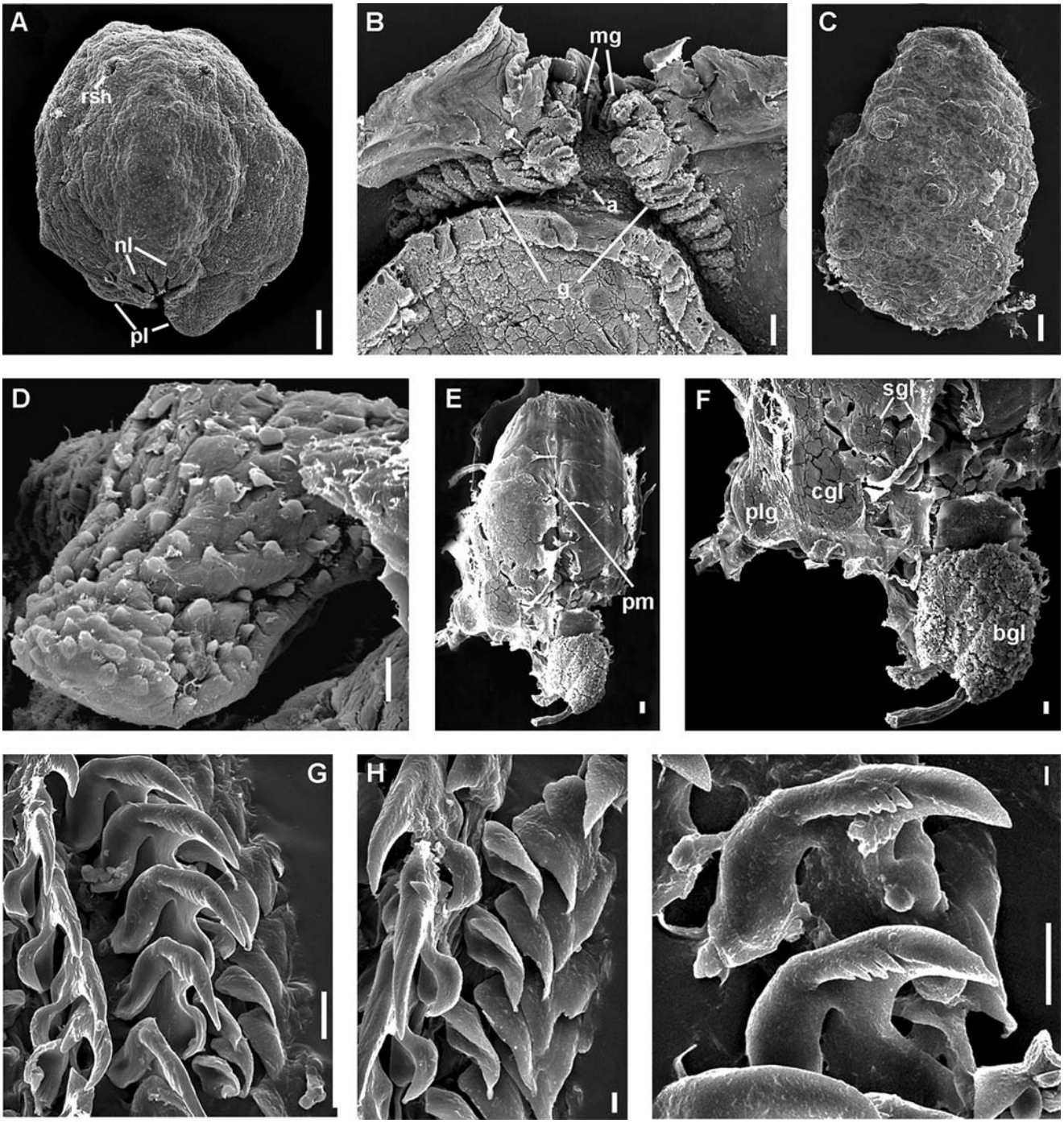


Figure 2. *Corambe mancorensis* n. sp., SEM micrographs. **A.** Dorsal view, rhinophores retracted. Note lobules of posterior mantle notch between posterior notal lobes, and small papillae on notum. **B.** Ventral view of posterior mantle margin showing three medial gills (mg) and further serial gills (g) surrounding the anus in horseshoe-like arrangement; foot and posterior notum partially removed. **C.** Detail of notal surface showing wart-like papillae. **D.** Detail of dorsal surface of one of notal notch lobules. **E.** Buccal pump, dorsal view; note peripheral muscle. **F.** CNS and blood gland, dorsal view. **G.** Radula, middle part. **H.** Outer lateral teeth. **I.** Close-up of two first lateral teeth. Abbreviations: a, anus; bgl, blood gland; cgl, cerebral ganglia; g, gills; mg, medial gills; nl, lobules of posterior mantle notch; pgl, pleural ganglia; pl, posterior notal lobes; pm, peripheral muscle; rsh, rhinophoral sheath; sgl, salivary gland. Scale bars: **A** = 0.5 mm; **B, C** = 200 μm; **D, E** = 20 μm; **F, G** = 10 μm; **H** = 3 μm; **I** = 10 μm.

Anatomy

Integument (Figs 1A, D, 3A–D): Thin (10 μm), smooth epidermis forms notal surface, with few wart-like bumps. SEM reveals evenly distributed small papillae on entire dorsal surface in one specimen. Semithin sections show cuticle (8 μm thick) covering only dorsal epidermis (Fig. 3D); slightly darker 'pegs' of cuticular material are located apically large nuclei of epidermal cells (Fig. 3C, D). Fleshy notum formed of thick layer of densely fibrous connective tissue without detectable cell borders (Fig. 3A, B, D). Spacious vacuoles form numerous spherical to slightly pear-shaped cavities (50 μm diameter)

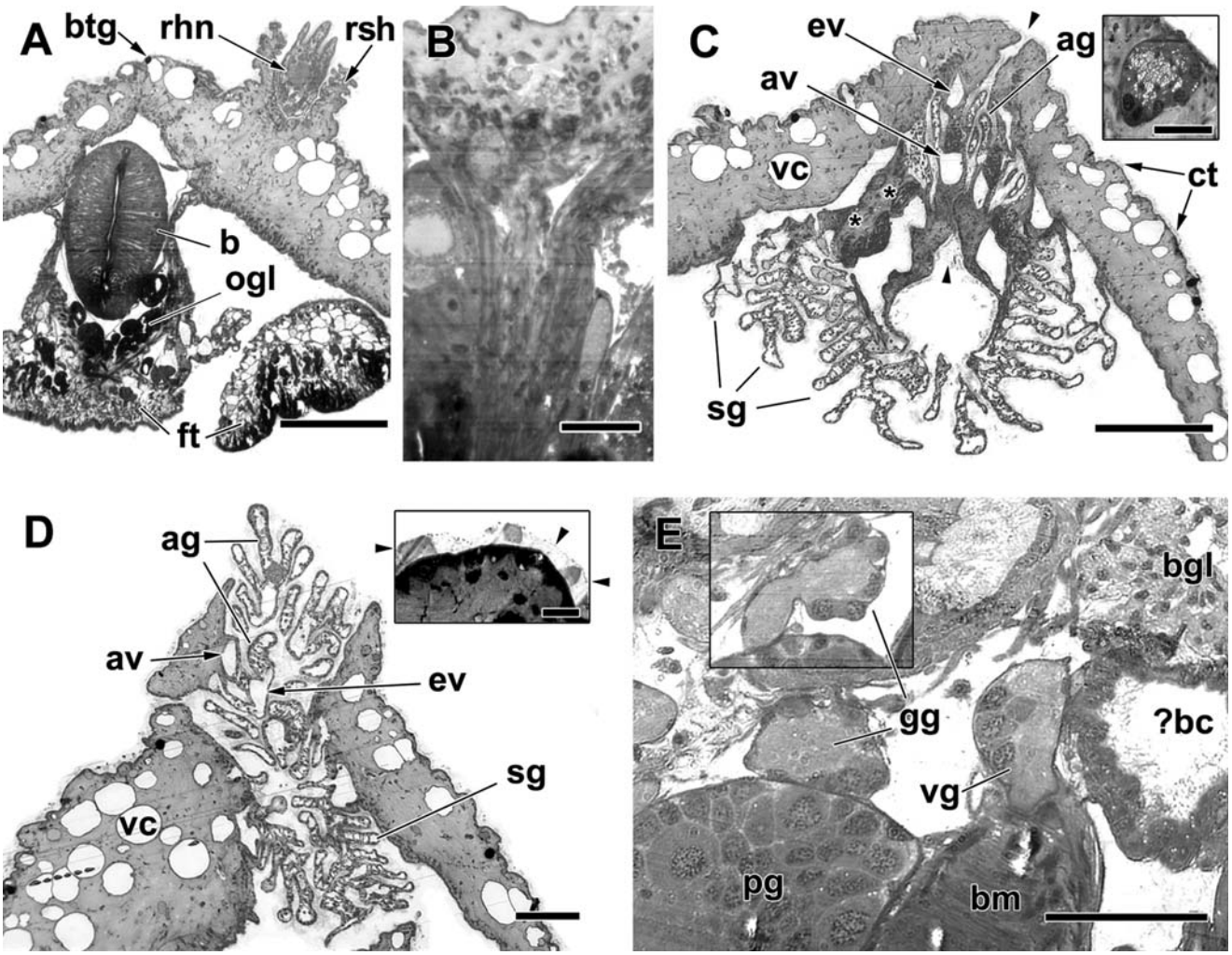


Figure 3. Semithin cross-sections of *Corambe mancorensis* n. sp. **A.** Anterior body with buccal pump and left rhinophore. **B.** Dorsoventral muscle bundle extending from notum across body cavity. **C.** Gills and lacunae; position of gill glands indicated by asterisks; arrowheads mark notal notch and vessel-like spaces that are not part of the animal. Insert shows gill gland next to haemolymph lacuna. **D.** Posterior part of mantle with projecting medial gills. Inset shows detail of cuticle with peg-like structures apically of nuclei. Arrowheads indicate the surface of cuticle. **E.** Genital and visceral ganglion. Inset shows pedally innervated 'genital' ganglion and thick nerve leading to copulatory organ. Abbreviations: ag, anal gill; av, afferent vessel; b, buccal pump; bc, bursa copulatrix; bgl, blood gland; bm, buccal muscle; btg, bottle-shaped glands; ct, cuticle of notum; ev, efferent vessel; ft, foot; gg, 'genital' ganglion; ogl, oral glands; pg, pedal ganglion; rhn, rhinophoral nerve; rsh, rhinophoral sheath; sg, serial gill; vc, large vacuoles of notum; vg, visceral ganglion. Scale bars: **A** = 200 μm; **B** = 50 μm; **C** = 200/25 μm; **D** = 100/10 μm; **E** = 50/50 μm (second number refers to scale bar of inserted frame).

within entire notum, mostly without connection to dorsal epidermis. These cavities appear empty but are lined with thin, distinct layer 2 μm thick; if apical pore is present it appears to be sealed by this lining. Dark-staining bottle-shaped glands sparsely distributed, opening through epidermis by apical pore that may be associated with aforementioned papillae (Fig. 3C). Except for dorsal surface of notum, epidermis is ciliated (patchy inside entire notal cavity, denser on gills and rhinophores, continuous on foot sole).

Digestive system (Figs 1B, E, 2E–I, 3E): Anterior part of buccal bulb modified to form prominent sessile (i.e. without stalk) buccal pump; pump encircled by narrow peripheral muscle (Fig. 2E). Rounded labial disk lined with smooth cuticle. Radula formula (in 2 studied specimens 2.5–3 mm length) 26–31 × 4.1.0.1.4. Central tooth absent. First lateral tooth large with long, wide base and long, slightly curved, attenuated beak-shaped cusp (Fig. 2G–I); that bears 5–7 sharp, long denticles. Further lateral teeth are slightly elongated small plates without cusps, all similar in size and shape. Salivary glands short. Stomach cavity large, broad, without caecum. Digestive gland of three lobes (Fig. 1B) – anterior pair and single posterior one, the latter notched terminally; anterior to and between lobes are three pairs of dorsoventral muscles (Figs 1E, 3B) connecting notum and foot.

Circulatory system (Figs 3C, D, 4): Pericardial sac with broadly triangular posterior auricle (atrium) and elongate oval ventricle. Ventricle with slightly thicker wall than auricle and no separating valve. Aorta leaves anterior tip of heart. A pair of spacious haemolymph lacunae begins at posterior tip of kidney, continuing as large paired afferent vessels leading to gills. Afferent vessels form five branches: one on each side of body connecting to all the smaller, anterior serial gills; a second paired one to largest, posteriormost serial and outer medial gills; a third single one to central medial gill (Fig. 4). Similarly, five efferent vessels emerge from gills, those from serial ventrolateral gills fuse on each side of body and three vessels enter the atrium. A lacunary space around heart also has at least one paired lateral connection to atrium. Some

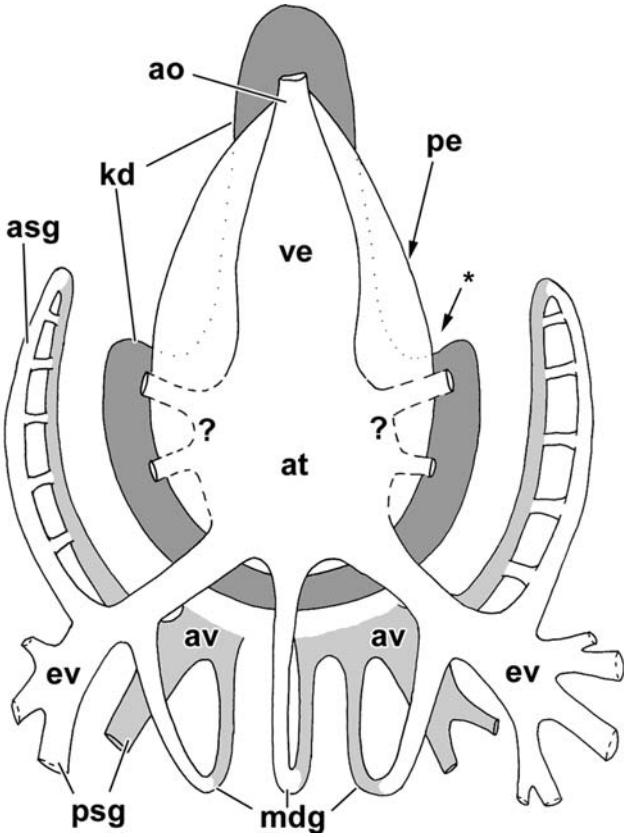


Figure 4. Schematic overview of circulatory and excretory systems of *Corambe mancorensis* n. sp.; organs situated ventrally shaded grey; ventricle in systolic phase; asterisk indicates position of syrinx (not shown). Abbreviations: at, atrium; ao, aorta; asg, anterior serial gills; av, afferent vessel; ev, efferent vessel; kd, kidney; mdg, medial gills; pe, pericardium; psg, posterior serial gills; ve, ventricle. Not to scale.

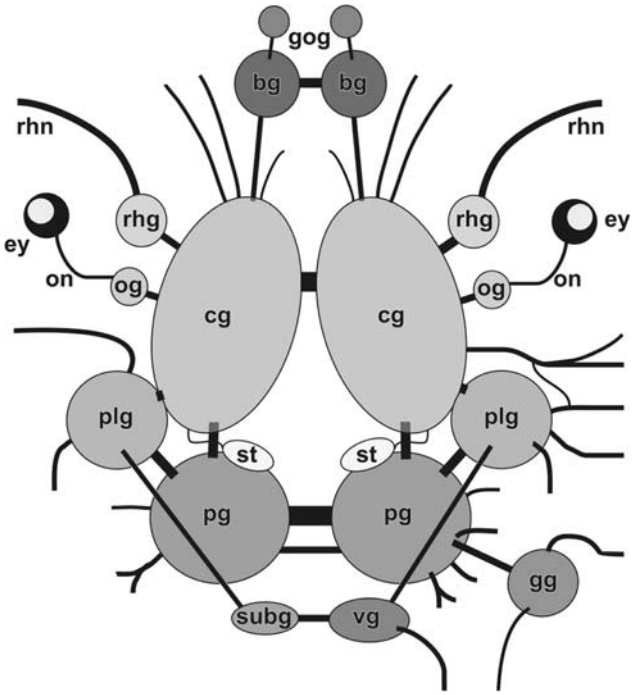


Figure 5. Schematic overview of central nervous system of *Corambe mancorensis* n. sp. Abbreviations: bg, buccal ganglion; cg, cerebral ganglion; ey, eye; gg, 'genital' ganglion; gog, gastroesophageal ganglion; og, optic ganglion; on, optic nerve; pg, pedal ganglion; plg, pleural ganglion; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; st, statocyst; subg, suboesophogal ganglion; vg, visceral ganglion.

spherical gill glands (Fig. 3C) located ventral to large afferent lacunae, but opening to outside posteriorly, close to bases of medial and large serial gills. Gill glands of several cells surrounding small central lumen filled with mucus; cells possess either large basal nuclei or smaller apical nuclei. Blood gland (Fig. 1F) posterior to central nervous system (CNS). A smaller lobe of tissue lies anterior to CNS; it is not directly connected to blood gland so its identity is unclear.

Excretory system (Fig. 4): Ciliated syrinx emerges right dorsolaterally from pericardium. Long renopericardial duct runs along ventral side of kidney within a fold, entering kidney anteriorly. Kidney large, anchor-shaped, with two posterolaterally projecting lobes ventral to heart. Wide kidney lumen surrounded by strongly vacuolated wall. Posteriorly, the short, ciliated nephroduct leads to ventroterminal nephropore, close to anal opening.

Central nervous system (Figs 1E–G, 2F, 3E, 5): Large, elongate cerebral ganglia almost fused medially; smaller pleural ganglia broadly connect posterolaterally, but are separated from cerebral ganglia by a constriction (Figs 1F, 5). At anteroventral tip of each cerebral ganglion, a bundle of three nerves emerges next to cerebrobuccal connective, presumably comprising oral and labiotentacular nerves. Spherical buccal ganglia anteroventral to cerebral ganglia, just posteroventral to most proximal oesophagus (Fig. 1F). Smaller gastroesophageal ganglia connect to each buccal ganglion dorsally. Elongated rhinophoral ganglia connected to cerebral ganglia laterally via thick connective; distally, ganglia run into thick rhinophoral nerve. Smaller spherical optical ganglia are located more posterodorsally; comparably long thin optic nerve connects to eyes (20 μm diameter) with spherical clear lens and small dark-pigmented cup. Statocysts (diameter 25 μm) on top of pedal ganglia just medially to cerebropedal connective; thin static nerve emerges from cerebral ganglia posterolaterally. An additional nerve exits right cerebral ganglion laterally, leading towards genital opening but also connecting by one branch to anteriormost nerve emerging from right pleural ganglion (Fig. 5). Cerebropedal connective short, broad. Including connectives, left cerebral ganglion bears nine nerves at least; right one bears 10.

Pedal ganglia are large, spherical, with double commissure; parapedal commissure thinner and longer than pedal one. Two nerves emerge from left pedal ganglion (one thick and branching proximally), four from right pedal ganglion. Small unpaired, additional 'genital' (or better called 'penial') ganglion (Fig. 3E) connects to right pedal ganglion posterodorsally; a thick nerve leads to penis, a thinner one runs posterodorsally.

Pleural ganglia spherical. Left one bears two nerves (one running laterally, the other posteriorly); right pleural ganglion bears one nerve running posteriorly and two laterally, of which anteriormost connects to right cerebral ganglion. Pleural ganglia interconnected by comparatively long (for nudibranchs) visceral loop, with two small, but distinct, elongated ganglia (Figs 1G, 5). Left ganglion (a tentative subintestinal ganglion) consists of only a few loosely aggregated nerve cells; larger right ganglion (visceral ganglion) is more distinct and bears a single nerve running posteriorly towards viscera.

Reproductive system (Fig. 6): Hermaphroditic gonad tissue fills anterior left body cavity and covers digestive gland. Anterior genital system triaulic. Ampulla oval, swollen, relatively short, filled by sperm in all four studied specimens. Postampullary gonoduct bifurcates into vas deferens and oviduct. Proximal part of vas deferens muscular, long, bent, attached to

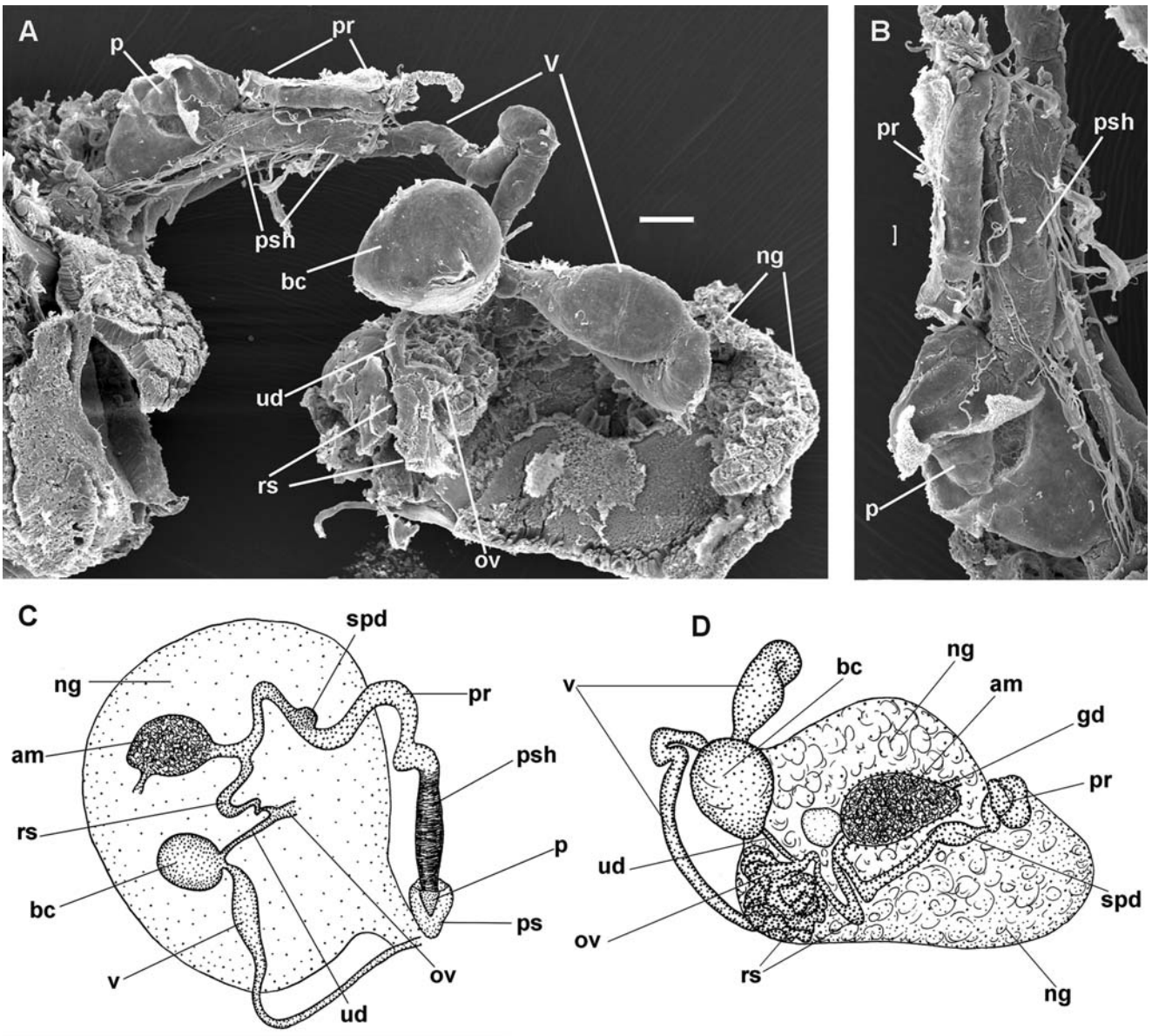


Figure 6. Reproductive system of *Corambe mancorensis* n. sp., scanning electron micrographs. **A.** Dorsal overview of reproductive system (ampulla omitted). **B.** Close up of penial sheath and penis. **C.** Schematic overview of the reproductive system. **D.** Dorsal view (drawing) of the reproductive system. Scale bars: **A**, 200 μm; **B**, 20 μ; **C, D**, no scale bars. Abbreviations: am, ampulla; bc, bursa copulatrix; gd, genital duct; ng, nidamental glands; ov, oviduct; p, penis; pr, prostata; ps, penial sac; psh, penial sheath with ejaculatory duct; rs, receptaculum seminis; spd, swollen part of the postampullar duct; ud, uterine duct; v, vagina.

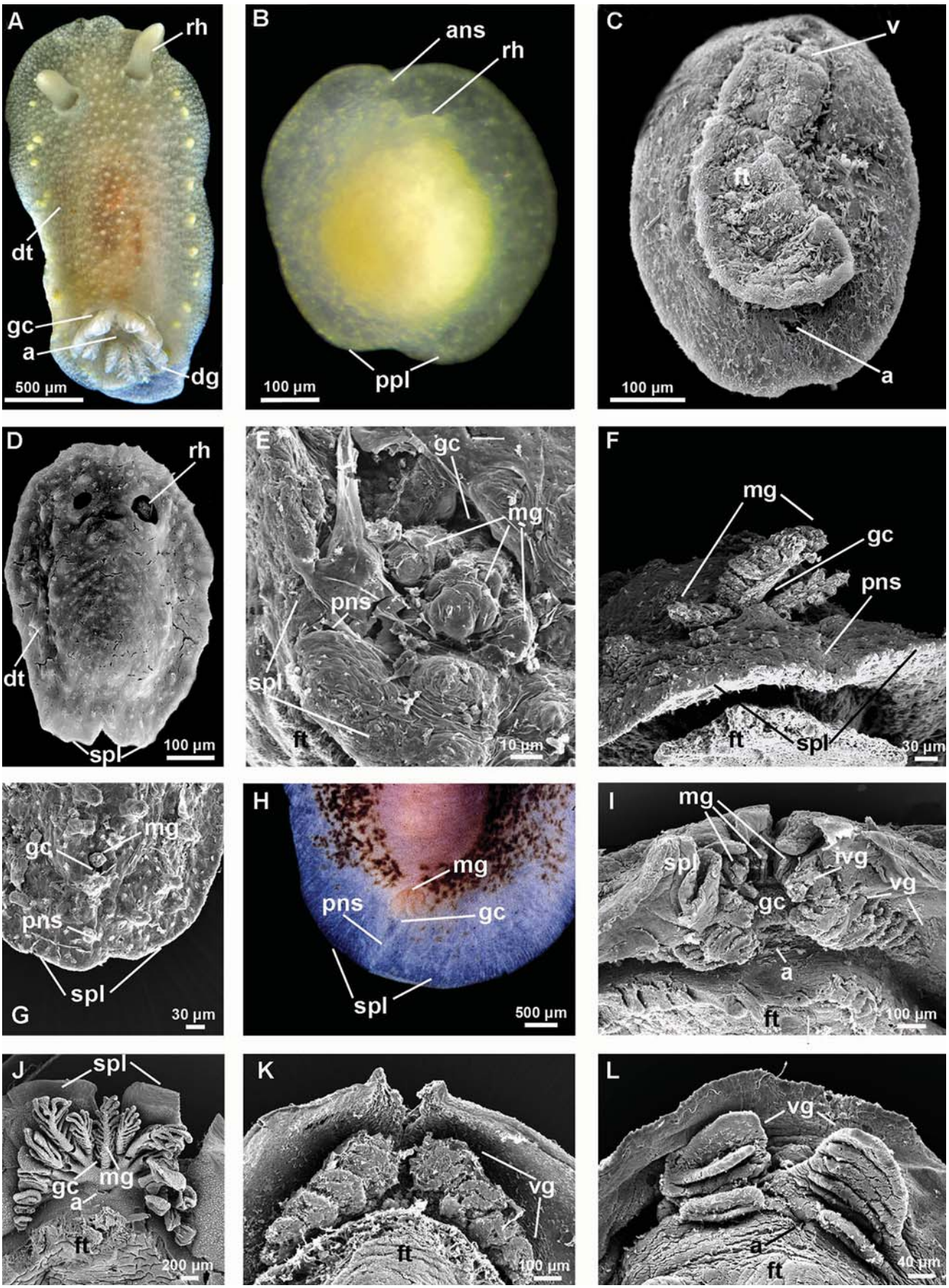
nidamental gland; forms conspicuous swollen chamber before passing into prostate (Fig. 6C, D). Tubular prostatic part of vas deferens weakly defined, short, of 2–3 slightly swollen loops, which do not encircle bursa copulatrix. Prostate transits into short, almost straight, muscular part of vas deferens, which rapidly widens and enters short penial sheath; the latter contains well-defined, short, conical penis (Fig. 6B). Vagina (Fig. 6A) very long, thin, narrow, forming swollen elongated chamber (a 'vaginal bursa') before leading into bursa copulatrix. Bursa copulatrix large, compressed, irregularly spherical. Uterine duct short, narrow, emerging at junction of bursa base and vagina, and connecting with proximal oviduct (Fig. 6C). There is no separate seminal receptacle detectable, but a bent, slightly swollen part of proximal oviduct can be regarded as a serially arranged receptacle (Fig. 6C). Oviduct enters female gland mass, the parts of which were not studied in detail.

Biology

This species inhabits shallow-water algae covered with encrusting bryozoans (*Membranipora*), on which it preys. *Corambe mancorensis* mimics *Membranipora* colonies by its flattened shape and special colour pattern. Curiously, on the same algae, two further *Membranipora* mimics were found: a rounded, depressed ascidian species and a flatworm (Fig. 1C) that can easily be mistaken for *C. mancorensis* at first glance.

Distribution

Corambe mancorensis is presently known only from the type locality Mancora, northern Peru. It is the first corambid reported from tropical eastern Pacific waters.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DISCUSSION

Taxonomy

Only four previously known species of the genus *Corambe* (*sensu* Valdés & Bouchet, 1998) possess rows of lateroventral gills and a posterior notal notch, i.e. *Corambe testudinaria* H. Fisher, 1889, *C. pacifica*, *C. lucea* and *C. evelinae*. Each of these species possesses a peculiar gill pattern that clearly differs from that of the new species. Both *C. testudinaria* and *C. lucea* have lateroventral gill rows which are not connected posteriorly (García, Urgorri & López-González, 1990; Schrödl & Wägele, 2001). They do not possess special, medially placed gills and lack any trace of a gill cavity. Due to their position, 'small central gills' found in *C. lucea* may correspond to vestiges of outer medial gills as found in *C. mancorensis*. In contrast, *C. pacifica*, *C. evelinae* and *C. mancorensis* all possess a more or less small gill cavity containing one to three medially placed gills. The gill cavity of *C. pacifica* is vestigial (Fig. 7J), and bears a single gill that is located strictly ventrally, i.e. under the notum, in between the rows of lateroventral gills. The medial gills and the gill cavity of *C. evelinae* and *C. mancorensis* are situated dorso-terminally. While the medial gills appear to be separated from the lateroventral gills in *C. evelinae*, the three medial gills of *C. mancorensis* are not arranged separately from ventral ones, but form a continuous horseshoe-shaped row. *Corambe mancorensis* is well distinguished from *C. evelinae* by a number of additional characters: the first radular teeth have longer cusps with a larger number of denticles (5–7 in *C. mancorensis* cf. 3–5 in *C. evelinae*), a short swollen ampulla (vs. a very long tube), a considerably shorter uterine duct, and a tubular and flow-through seminal receptacle (pyriform and semi-serial in *C. evelinae*). Apart from the unique gill pattern, *C. mancorensis* is well distinguished from all other known corambid species by the possession of posterior notal lobes with lobules that protect the gills. There is thus no doubt that the species described herein is distinct and undescribed. It is the first corambid species known to inhabit the tropical eastern Pacific.

Morphology and anatomy

Integument: Epidermal and notal features of *C. mancorensis* resemble those described for *C. lucea* by Schrödl & Wägele (2001). The notal cuticle of the new species is, however, thinner, and no shedding has yet been observed. Furthermore, there are small tubercles scattered over the notum and concentrated on special lobules emerging from the border of the posterior notal notch. This notch, with the aid of the lobules, is fully closable, which seems to be unique among known *Corambe* species.

Dorsoventral muscles: Three pairs of dorsoventral muscle bundles cross the body cavity of *C. mancorensis*, separating the gonad and digestive gland peripherally into lobes. A similar situation was described and discussed in *C. lucea* by Schrödl & Wägele (2001). While this appears to be the normal condition among *Corambe* species, none of the three species assigned to the genus *Loy* by Valdés & Bouchet (1998) has such muscles; at least, we could not detect them when re-examining some more or less well-preserved specimens of all of them for comparison.

Radula: The radula of *C. mancorensis* is very similar to that of congeners and *Adalaria jannae* (e.g. Marcus, 1958; Millen, 1987; Schrödl & Wägele, 2001; Martynov *et al*., 2009). In corambids the central tooth is always reduced, except for tiny and irregular central tooth vestiges in *Loy thompsoni* (see Millen & Nybakken, 1991). The first lateral teeth have long, slightly curved cusps in *C. mancorensis*, *C. lucea* and *C. pacifica*, whereas in *Loy meyeni* and *Corambe obscura* the cusp is considerably curved apically (Martynov, 1994a; A.M., unpubl.). The basal part of the cusp is provided with several distinct denticles in all corambids. Lateral tooth patterns differ between two members of the genus *Loy*, i.e. *L. meyeni* and *L. millenae*, and *Corambe*: the former have remarkable second laterals which are forked apically and long, knife-shaped further laterals (Martynov, 1994a), whereas *Corambe* species have more or less uniformly shaped elongate-triangular folded laterals with a peculiarly excavated inner side. *Loy thompsoni*, however, does not possess fork-shaped second laterals, and further lateral teeth are more similar to those of *Corambe* than the other *Loy* species. The number of outer laterals varies from 4–6 in *Corambe* species to 6–7 in species of *Loy*.

Buccal pump: There are two main types of suctorial buccal pumps within corambids: at least two species of *Loy* (*L. meyeni* and *L. millenae*) show only a poorly defined, slightly protruding elevation in the anteriormost part of the pharynx (Martynov, 1994a; A.M., unpubl.). The second type, found in *C. mancorensis* (Fig. 2E) and all other species of *Corambe*, is essentially similar to the buccal pump of noncorambid Onchidoriddidae, e.g. in the genera *Onchidoris* and *Adalaria* (Martynov, 1994b; Martynov *et al*., 2009; A.M., unpubl.). Both *Loy* and *Corambe* species have buccal pumps that are entirely encircled in the middle part by the peripheral muscle; however, there are certain differences among species: *C. pacifica* has a strongly developed, ovoid, swollen, buccal pump, *C. lucea* instead has a compressed, elongated pump, when compared at the same state of contraction. The pump of *C. mancorensis* is somewhat intermediate between *C. pacifica* and *C. lucea*, whereas *C. obscura* has a relatively short spherical pump (A.M., unpubl.).

Central nervous system: *Corambe mancorensis* shows a remarkable CNS (Fig. 5). Compared to most other dorids, it is little cephalized. Pleural ganglia are separated from cerebral ones, but not so clearly as in most other corambids, e.g. *C. lucea*. The small numbers of cerebral nerves and connectives (9 on the left, 10 on the right) reported here are provisional until more detailed study; the asymmetry is caused by an additional nerve leading to the genital opening on the right side of the body. The

Figure 7. Gills and notal lobes in juvenile and adult Doridoidea. **A.** Cryptobranch *Cadlina laevis*, living adult specimen, 25 mm length. **B.** *Cadlina laevis*, living early postmetamorphotic specimen (ca. 500 μm), dorsal view; note asymmetrical primary notal lobes. **C.** *Cadlina laevis*, SEM of early juvenile with ventral anus and reduced primary notal lobes. **D.** *Cadlina laevis*, SEM of a later stage (ca. 650 μm preserved length) showing well-defined secondary notal lobes, dorsal view. **E.** Cryptobranch *Paradoris indecora*, SEM of dorsal gill complex of juvenile (ca. 1 mm preserved length), showing fused notal lobes with suture and gills within a more or less drop-shaped gill cavity; note far posterior position of gill cavity. **F.** Cryptobranch *Doris ocelligera*, SEM of juvenile (ca. 2 mm preserved length), showing three medial gills and a vestigial suture of the notal lobes, dorsal view. **G.** Phanerobranch *Onchidoris neapolitana*, SEM of posterior part of notum of early juvenile (ca. 800 μm preserved length), showing vestige of gill cavity around anus and remnants of suture between notal lobes. **H.** Corambid *Loy meyeni*, preserved adult specimen (holotype, 6.5 mm length), showing well-defined posterior notal lobes and notal suture forming drop-shaped gill cavity. **I.** *Corambe mancorensis* sp. nov., SEM of preserved mature specimen (3 mm length), medial gills within the gill cavity. **J.** *Corambe pacifica*, adult specimen 6.5 mm length showing one medial gill within a vestigial gill cavity; ventral view. **K.** *Corambe steinbergae*, adult specimen (3.2 mm length), ventral gills, no notal lobes (artificial notch-like rupture caused by accidental damage). **L.** *Corambe obscura*, adult specimen (4 mm length), showing ventral gills without notal lobes. Abbreviations: a, anus; ans, anterior notal sutute; dg, dorsal gills; dt, dorsal tubercles; ft, foot; gc, gill cavity; ivg, innermost ventral gills; mg, medial gills; pns, posterior notal suture; ppl, primary posterior notal lobes; rh, rhinophores; spl, secondary posterior notal lobes; v, velum; vg, ventral gills.

visceral loop is relatively long for dorid nudibranchs, which usually possess just one or lack any free ganglia (Schmekel & Portmann, 1982; Wägele & Willan, 2000). At least in one corambid species, *C. testudinaria*, a distinct nonpaired visceral ganglion has been reported at the base of the visceral loop (Fischer, 1891), although subsequent study did not detect such a structure (García *et al.*, 1990). Uniquely, *C. mancorensis* possesses two clearly separated ganglia on the visceral loop (Fig. 1G). The right one of these can be supposed to be (or to contain) the visceral ganglion, because of the strong nerve emerging from it that leads backwards into the viscera. The left, very small ganglion might be parietal and/or suboesophageal. Equally unusual for nudibranchs, a small ('genital' or better 'penial') ganglion connects the right pedal ganglion with the copulatory organ; since it is neither situated on the visceral loop nor connected to it, its homology with the true genital ganglion of more basal opisthobranchs is unlikely.

Gills and circulatory system: *Corambe mancorensis* shows a very unusual arrangement of gills. As in other *Corambe* species, there are multiple gills in a posterior ventrolateral position, forming rows of feather-like gills as in e.g. *C. lucea*. These 'ventral gills' (or 'serial gills') in *C. lucea* have been shown to possess the usual dorid gill glands, and are connected to the body cavity and atrium via a paired system of vessels. Gills and gill vessels of *Corambe* have been interpreted as modified and multiplied from the normal dorid condition with 'anal gills' surrounding the usually dorsal anus in a (semi)circle (Schrödl & Wägele, 2001). According to the description and illustration of a histological slide by Marcus (1958), the Brazilian *C. evelinae* shows serial ventral gills, plus 2–3 special medial gills in a more dorsal position, obviously within the notal notch and on top of notal tissue, i.e. within a notal cavity; only the medial gills showed gill glands. Although neither the types nor any other specimens of *C. evelinae* were available for re-examination, the existence of this special condition is supported when comparing it with the gill arrangement observed in *C. mancorensis*. Here, serial ventral gills are present in two lateral rows that converge posteriorly in a horseshoe-like pattern, with the three more dorsal central gills emerging from a notal cavity. Gill glands appear to open at the bases of these medial gills and of the innermost serial gills only. Serial gill sizes increase posteriorly, with the pair of innermost serial gills being largest. Only these, and the central gills, are visible from above when the notal notch is fully opened in living specimens. A similarly continuous, though entirely ventral, placement of anal and lateral gills is found in *C. pacifica*, where just one central gill is developed, emerging from a vestigial gill cavity (Fig. 7J). Unfortunately, there is no information on gills of living *C. evelinae*, or on its circulatory system.

Based on the 3D reconstruction (not shown) of the complex circulatory system of *C. mancorensis*, a more instructive scheme was prepared (Fig. 4). All ventral gills but the innermost ones are connected to the body cavity by a common afferent vessel, and to the atrium by a common efferent vessel, as in *C. lucea* (see Schrödl & Wägele, 2001). The innermost ventral gills, however, receive their own pairs of vessels, which are basal spinoffs from the common serial gill vessels. This might be a consequence of the importance of the innermost serial gills for respiration, owing to their large size and position within the notal notch. Outer medial gills are connected to the serial gills vessel system as well, while the single inner medial gill receives haemolymph from its own, unpaired, medial vessel, and blood flows to the heart through its own efferent vessel, which enters the atrium in a terminal posterior position.

Corambe mancorensis, *C. pacifica* and most likely *C. evelinae* thus show at least two (though not strictly delineated) types of gills: (1) a multitude of serial, ventral gills arranged in rows; the innermost pairs have a special size, function, haemolymph supply, and their own gill glands at least in *C. mancorensis*; and (2) one to three central gills that are situated within a notal cavity, anterodorsally of the posteroventral anus, and provided with gill glands and their own vessel system, at least in *C. mancorensis* and *C. evelinae*. Apart from the ventral anus, this reflects the usual gill situation in dorids, and there is little doubt about the direct homology of medial gills of *Corambe* and dorid anal gills. Only those corambids with multiple ventral gills possess a fleshy notum that is covered with a cuticle, hindering oxygen diffusion; thus the transformation, multiplication and translocation of dorid anal gills into corambid serial gills correlates with, and was probably induced by, necessity for increased respiratory area on the ventral side (Martynov & Schrödl, in press).

As in the case of other genera of the Onchidoridae possessing gill pockets (Martynov *et al.*, 2009), there is little doubt about the homology of the corambid notal cavities associated with anal gills with other doridoidean gill cavities, regardless of their dorsal, subventral or even ventral position. In *C. mancorensis* the gills can contract considerably, the notal notch edges and lobules closing to seal the gap. This is a unique cryptobranch condition of a phanerobranch species, which is structurally different from – thus analogous to – the retractable gills and closable gill cavities of Cryptobranchia and the onchidoridid *Onchimira cavifera* Martynov *et al.*, 2009 (Martynov *et al.*, 2009).

The ontogeny of the dorid notum, anus and gills

The anus of adult dorids usually opens in a medial, posterior dorsal position, and is surrounded by a (semi)circle of gills, as in *Cadlina laevis* (Fig. 7A). The ontogeny of the anus and gill arrangement is, however, poorly known. The posterior part of the notum in early postlarvae (250–500 μm length, Fig. 7B, C) of both cryptobranch (*Cadlina*, *Glossodoris*) and phanerobranch (*Adalaria*) Doridoidea is not entire as in adults, but forms asymmetrical primary notal lobes that are separated by a notch; the right lobe is larger than the left in early postmetamorphic *Cadlina laevis* (Fig. 8A) and in other species with available data (Thompson, 1958, 1967; Usuki, 1967). The anus is already ventral at this stage (Fig. 7C). Apparently, the differential growth of the right lobe causes the anus to move from a (plesiomorphic) right lateral (e.g. Wägele & Willan, 2000; Martynov & Schrödl, 2008) to a posterior position. *Cadlina laevis* of *c.* 1 mm living length have been recorded to have the anus in a terminal, ventral position under the notum (Thompson, 1967), which is confirmed herein. Juveniles of 650 μm living length had a ventral anus (Fig. 7C), but had no clearly visible primary notal lobes and lacked any gills and gill pocket. Similarly, juveniles of *Glossodoris sibogae* (Bergh, 1905) of about 0.8 mm length showed no gills (Usuki, 1967). As in *C. laevis*, the postlarval (=primary) notal lobes had completely disappeared at this stage, and the posterior notum resembled the morphology of the adult, except for not showing dorsal anus and gills (Fig. 8B). Thus, during further ontogeny, which was not been directly observed for any dorid before, the presumably always functional anus must shift from the ventral to the dorsal side, and a pocket with gills must develop. It is herein proposed that by differential growth of notal tissue the anus first comes into a terminal, subventral position and then, in cryptobranchs, becomes surrounded by secondarily developed notal lobes. Evidence for the real existence of such an ontogenetic stage was obtained herein: *Cadlina laevis* of 600–1,000 μm living length developed clearly visible, secondary notal lobes (Fig. 7D), but still no signs of gills or gill cavity were detectable. Field-collected, somewhat larger early juveniles of the cryptobranch *Paradoris indecora* (Bergh, 1881)

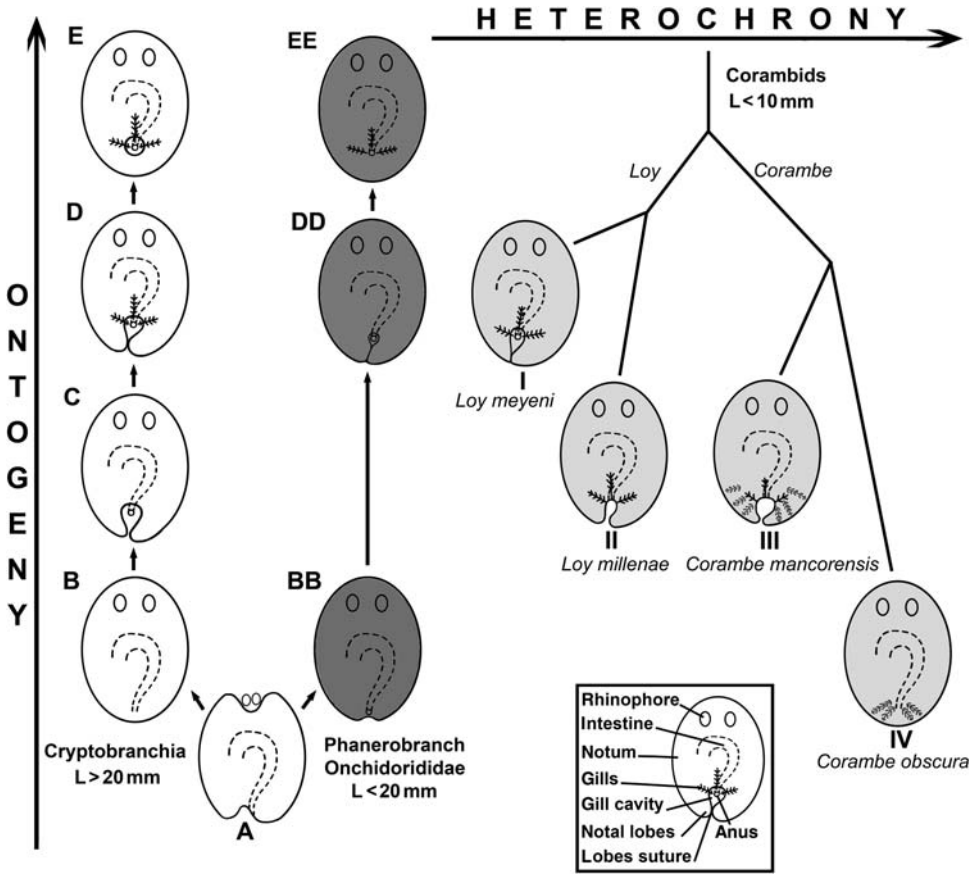

Figure 8. Dorid nudibranch ontogeny and the corresponding stages of progenetic corambid evolution. The cryptobranch and phanerobranch dorid notum and anal gill ontogeny as hypothesized herein is shown on the ordinate axis (data compiled from Thompson, 1958, 1967; Usuki, 1967; this study). Ontogenetic stages of Cryptobranchia (A–E) are indicated by white ground colour, those of 'Phanerobranchia' (BB–EE) by dark grey. Dorsal outlines of selected adult corambid species (I–IV, light grey shadows) are connected according to their relationship (Valdés & Bouchet, 1998; Martynov & Schrödl, in press) and arranged according to their resemblance to corresponding dorid ontogenetic stages (ordinate) and the relative degree of heterochronic juvenilization (abscissa). The hypothesis of successive paedomorphotic changes within corambids (Martynov, 1994b, 1995) is shown herein to be congruent with recent phylogenetic results (Valdés & Bouchet, 1998; Millen & Martynov, 2005; Martynov & Schrödl, in press). An average adult body length is given for each group. In the frame, the important structures of a juvenile doridoidean are indicated.

(1–1.2 mm preserved length) already showed three gills in a posteriormost position and that were surrounded by notal lobes, forming the drop-shaped anlage of the gill cavity (Fig. 7E). This observed stage (Fig. 8D) must obviously be preceded by a similar stage, but without gills differentiated yet (Fig. 8C). The secondary notal lobes may fuse under and posterior to the now dorsal anus, forming a dorsal cavity in which anal gills develop. In fact, a slight suture was visible as a trace of the fused posterior notal lobes in all cryptobranch dorid species with adequately sized early juvenile stages available to us, i.e. *Paradoris indecora* and *Doris ocelligera* (Bergh, 1881) (Fig. 7E, F). Finally, the suture must vanish and the drop-shaped gill cavity must transform into a normal one with rounded opening, as is characteristic for adult cryptobranch dorids (Figs 7A, 8E).

In contrast, the phanerobranch onchidoridid *Adalaria proxima*, which has a dorsal gill circle around the anus but lacks a gill cavity (Fig. 8EE), shows a shortened ontogeny (Martynov, 1994b, 1995): the anus is in a dorsal position and notal lobes have already disappeared at a postlarval stage of 250–300 μm length (Thompson, 1958; Fig. 8BB). Juveniles of the confamiliar *Onchidoris neapolitana* (Delle Chiaje, 1841) 800 μm long do not show any macroscopic cavity (Martynov *et al*., 2009); our SEM examination, however, revealed a very small but evident cavity around the anus and remnants of the suture between almost entirely fused notal lobes (Figs 7G, 8DD). This small and potentially vestigial cavity is considered homologous with the well-defined juvenile drop-shaped gill cavity of corresponding cryptobranch stages (Figs 7E, F, 8C, D). It is thus possible that Thompson (1958) just did not recognize this small cavity during light microscopic examination of his still earlier juveniles of *Adalaria proxima*, and that an ontogenetic stage with a vestigial gill cavity is present among other onchidoridids and phanerobranchs as well.

Integrating the ontogenetic data available on dorids, Figure 8 shows schematically all stages described above plus one necessary (though never directly observed) intermediary stage in Figure 8C. One lineage of ontogenetic transformation (Fig. 8A–E) refers to Cryptobranchia, of which several species of different families (i.e. Chromodorididae and Discodorididae) were examined herein and in previous studies, with congruent results. Whether or not this ontogenetic series is valid for all taxa that are currently classified as Cryptobranchia (e.g. Odhner in Franc, 1968; Schmekel & Portmann, 1982; Wägele & Willan, 2000; Valdés, 2002; Schrödl, 2003) may be tested in future studies. The assumption that the early postmetamorphic ontogeny described here for *C. laevis*, a direct developer, is the

general type for cryptobranchs needs to be confirmed by future studies of species with planktonic larvae. The other, shorter ontogenetic series (Fig. 8A–EE) refers to the few members of the phanerobranch family Onchidoriddidae for which information is available, i.e. species of the genera *Onchidoris* and *Adalaria*.

The absence of an accepted hypothesis of basal doridoidean phylogeny is problematic. Following conventional dorid classification, with monophyletic cryptobranchs that have evolved from a phanerobranch level of organization or paraphyletic phanerobranchs (e.g. Wägele & Willan, 2000; Valdés, 2002, 2004), the 'short onchidoridid ontogeny' was ancestral, and cryptobranchs not only elaborated the gill pocket but prolonged its development and even evolved an additional ontogenetic stage with secondary mantle lobes. The alternative hypothesis is that phanerobranch onchidoridids shortened an ancestral cryptobranch ontogeny, reducing the gill cavity and losing an ontogenetic stage with secondary mantle lobes. There is some evidence for the latter: some adult 'phanerobranchs' such as the basal onchidoridid genera *Onchimira* and *Calycidoris* (Martynov *et al*., 2009; Martynov & Schrödl, in press) show well-developed gill cavities (Fig. 8E) that are closely similar and thus probably homologous to cryptobranch ones rather than convergent organs. Great variation is observed in more derived onchidoridid genera. Adults of *Diaphorodoris*, *Loy* and some *Corambe* such as *C. mancorensis* (Figs 1A, B, 2B, 7I) and *C. evelinae* (see Marcus, 1958) retain a more or less well-developed gill cavity; this is very small and obviously without protective function in *C. pacifica* (Fig. 7J), but all these cavities can plausibly be regarded as remnants. Most other onchidoridids as well as other phanerobranchs lack gill cavities completely (Fig. 8EE). All these different stages can easily be explained as reductions reflecting an 'abbreviated phanerobranch type' of ontogeny which, however, remains to be tested for non-onchidoridid phanerobranchs.

Ontogenetic observations thus support a phylogenetic hypothesis in which one or several phanerobranch lineages evolved from a cryptobranch level of organisation (Martynov *et al*., 2009), implying that the possession of a gill cavity is ancestral for cryptobranchs and (at least one) phanerobranch lineages such as Onchidoriddidae. But why should such a protective gill cavity be lost during evolution? Having developed a faster way of bringing the anus into a dorsal position, or generally speeding up the entire postlarval ontogeny, the ontogenetic programme responsible for the anlage of a drop-shaped cavity could have been abbreviated or simply skipped.

The corambid time warp and the evolution of dorid notum, anus and gills

Intriguingly, adult corambids show a spectacular variety of anus positions and gill arrangements, which may reflect corresponding stages of dorid ontogeny (Fig. 8). The phylogeny of corambids is well known, for the topology by Valdés & Bouchet (1998) has essentially been confirmed by an updated and comprehensive cladistic analysis (Martynov & Schrödl, in press). The basal corambid species *Loy meyeni* has an anus that is associated with three small gills in a dorsal notal cavity (Fig. 7H). Different from a 'normal' adult dorid, but similar to an early juvenile dorid condition, is the drop-shaped rather than rounded gill cavity (compare Figs 7E, F, H, also Fig. 8C, D, I), and the presence of asymmetrical posterior notal lobes, which are nearly completely fused but still show a terminal suture (compare Fig. 7D–F). In other *Loy* species (Fig. 8II), the asymmetrical notal lobes are not fused, thus forming a notch, and the anal gills and their gill cavity are situated medially within the notch; the anus opens on the hyponotum, i.e. subventrally. The new *C. mancorensis* shows a similar terminal gill cavity with three gills and a subventral anus, but the notal lobes are more symmetrical, the notch is very well developed, equipped with protective lobules, and can be actively closed over the gills (Figs 1A, B, D, 7I). The Brazilian *C. evelinae* and the tropical Peruvian *C. mancorensis* show both gill types associated with the anus ('anal gills', 'dorid gills') and, in addition, special ventrolateral gills ('serial gills') (Fig. 8III). Most other corambid species with a notch, e.g. the Chilean *C. lucea*, have the anus in a derived ventral position between notum and foot, lack a gill cavity and lack gills clearly associated with the anus; instead, there are pairs or rows of multiple ventrolateral gills (Schrödl & Wägele, 2001). Comparing dorid ontogeny with a simplified corambid phylogeny (Fig. 8I–IV) there is evidence for a partial reversal of dorid postlarval development during corambid evolution. Within the corambid lineage, adults show an evolutionary translocation of anus and anal gills within a pocket from the dorsal to the ventral side and a successive opening of a notal notch; the underlying evolutionary process is progenesis resulting in pseudoarchaic paedomorphic features (Martynov, 1994b, 1995; Martynov & Schrödl, in press).

Accepting the presence of a gill pocket (i.e. a 'cryptobranch' condition) as the plesiomorphic state for Doridoidea, with reduction or loss in most phanerobranch lineages (Martynov *et al*., 2009), heterochronic juvenilization also explains the re-establishment of gill pockets between notal lobes in basal corambid taxa such as *Loy* (Figs 7H, 8I, II). This adult condition corresponds in shape and position to the earlier stages of gill pocket formation in cryptobranch juveniles (present study, Figs 7A–F, 8C, D). The pocket is still well developed in corambids with subventral anus and gills, i.e. *C. mancorensis* (Figs 1A, 2B, 7I, 8III) and *C. evelinae*, present but vestigial in *C. pacifica* with ventral anus and gills (Fig. 7J), and absent in other *Corambe* species. Lacking any notal notch during their entire ontogeny, *C. steinbergae* and *C. obscura* (Figs 7K, L, 8IV) may be considered as the most progenetic corambids. They may either show a very early, hypothetical ontogenetic stage before development of primary notal lobes, or a genuine modification of the latter stage. In contrast to other dorids, the posterior-ward shift of the anus is due to muscle retractor contraction rather than differential growth (Bickell, Chia & Crawford, 1981; Perron & Turner, 1977), corresponding to the fast generation times. In summary, as derived progenetic members of the Onchidoriddidae lineage, adult corambids display almost all the different stages of our novel hypothesis on notum and gill development in dorids. Interestingly, corambids such as *C. mancorensis* reflect an ontogenetic dorid stage with ventral anus and well-developed notal lobes gills and gill cavity, of which a similar type is known so far only from cryptobranchs (Figs 1A, 7D, 8C). Adult *Loy meyeni* most resembles the cryptobranch stage D (Figs 7H, 8D) and a, much smaller sized, juvenile stage of the phanerobranch *Onchidoris neapolitana* (Fig. 7G), which is in its adult condition devoid of any gill cavity. This supports our preferred hypothesis that the slow cryptobranch ontogeny is ancestral and the *Onchidoris*/*Adalaria* onchidoridid pathway represents a derived, shortened modification. Mantle and gill development may be variable among phanerobranch taxa and subject to evolutionary change according to ecological needs. Heterochrony, i.e. progenesis, is assumed to have already speeded up the ontogeny of early onchidoridids and this trend was accelerated within corambids. This 'recapitulation' of early juvenile dorid stages turns Haeckel's law upside down.

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3D MICROANATOMY OF A GASTROPOD 'WORM', *RHODOPE ROUSEI* N. SP. (HETEROBRANCHIA) FROM SOUTHERN AUSTRALIA

BASTIAN BRENZINGER[1], NERIDA G. WILSON[2] AND MICHAEL SCHRÖDL[1]

[1]*Bavarian State Collection of Zoology, Münchhausenstr. 21, D-81247 Munich, Germany; and*
[2]*Australian Museum, 6 College Street, Sydney, NSW 2010, Australia*

Correspondence: B. Brenzinger; e-mail: bastian.brenzinger@arcor.de

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ABSTRACT

The turbellarian-like, radula-lacking *Rhodope* has been a mystery to taxonomists for over 160 years and was considered a specialized off-shoot of either opisthobranch or pulmonate Euthyneura. Occasionally reported from intertidal waters and sand habitats from all continents, most species of these minute slugs are poorly known and characterized mainly by differences in pigmentation. To understand the evolution of heterobranch microslugs, we established a morphological dataset for *Rhodope* by describing a new species found in the temperate waters of southern Australia. To set a standard for rhodopids, all major organ systems of *R. rousei* n. sp. are reconstructed three-dimensionally from series of semithin sections using the software Amira. Microanatomy confirms the loss of many general gastropod features such as foot, cephalic tentacles, shell, radula, mantle cavity, gill and heart. Excretory and digestive systems are heavily modified, with free rhogocytes in the presumed position of the heart, and a secondary buccal bulb replacing the function of the vestigial pharynx. Structural details of the monaulic but hermaphroditic genital system suggest cutaneous fertilization via spermatophores formed in specialized glands. The highly concentrated central nervous system is compared to those of other species of the genus and targets of all detectable nerves are summarized. These characters are compared with adaptations shown by other interstitial gastropods.

INTRODUCTION

The tiny, worm-like Rhodopemorpha are one of the true enigmas of gastropod systematics and have puzzled taxonomists since the description of the turbellarian-like *Rhodope veranii* (Kölliker, 1847) from intertidal algae in the Mediterranean. Originally considered to be a nudibranch, its molluscan nature was questioned shortly afterwards (Schultze, 1854 described the same species as a flatworm; Bergh, 1882). The anatomy of the millimetre-sized species is characterized by the absence of many typical gastropod features (shell, head tentacles, foot, gill, heart and radula) and the reduction of the excretory system. On the other hand, anatomical features that are present include spicules, a monaulic genital system with separate male and female follicles, and a subepidermal 'vesicle' system of unknown function (e.g. Graff, 1883; Böhmig, 1893; Riedl, 1960; Haszprunar & Künz, 1996). In particular, the asymmetry of organ systems and the 'derived' architecture of the nervous system led to the conclusive placement of *Rhodope* among euthyneuran gastropods (Böhmig, 1893; Riedl, 1960; see Riedl, 1959 and Salvini-Plawen, 1970 for reviews).

Special emphasis has been placed on the highly condensed central nervous system (CNS) when developing phylogenetic hypotheses. For example, the possession of five ganglia on the visceral cord and a parapedal commissure place the genus within the Heterobranchia (*sensu* Haszprunar, 1985), and the high concentration of the ganglia was used to include *Rhodope* among 'higher' groups such as gymnomorph pulmonates (Salvini-Plawen, 1970) or nudibranch opisthobranchs (with double cerebro-rhinophoral connectives, see Haszprunar & Huber, 1990; Haszprunar & Künz, 1996). The possession of many features typical for meiofaunal opisthobranchs (e.g. worm-like shape, subepidermal spicules, adhesive gland; Swedmark, 1968) led to a grouping with the largely interstitial Acochlidia (Wägele & Klussmann-Kolb, 2005). On the other hand, Salvini-Plawen (1991) erected the taxon Rhodopemorpha—including the even more elongate worm-like and interstitial *Helminthope* Salvini-Plawen, 1991—as a "specialized off-shoot from the lower opisthobranchs" on the basis of the free visceral ganglion and its presumably primitive monauly.

All these morphology-based assumptions must be reexamined in the light of new molecular results, which have led to reorganization of traditional euthyneuran relationships (Jörger *et al.*, 2010; Schrödl *et al.*, 2011), and specific results indicating that *Rhodope* may not belong to Euthyneura (Wilson, Jörger & Schrödl, 2010), but instead form a clade with the former pyramidellids *Ebala* and *Murchisonella* (referred to herein as Murchisonellidae). The exclusion of Murchisonellidae from true (panpulmonate) pyramidellids to the 'lower heterobranchs' was indicated only by molecular analyses (Dinapoli

& Klussmann-Kolb, 2010); internal anatomy is only fragmentarily known and no synapomorphies are yet known to support a relationship with the Rhodopemorpha.

To date, rhodopemorphs are known from occasional records from intertidal to subtidal sand habitats, from temperate and subtropical waters, on all continents (Rieger & Sterrer, 1975; Salvini-Plawen, 1991; Haszprunar & Heß, 2005 for review). Besides the clearly interstitial *Helminthope* and '*Rhodope*' *crucispiculata* Salvini-Plawen, 1991 (with cross-shaped spicules), there are four nominal species of *Rhodope*, including a species from southern Australia showing three conspicuous orange bands (Burn, 1990, 1998, 2006; *Rhodope* sp. 'E' in Haszprunar & Heß, 2005). *Rhodope* species are generally distinguished by characteristic external colour patterns consisting of transverse bands; at least seven colour forms are known, including European *R. veranii* and *R. roskoi* Haszprunar & Heß, 2005, Indian Ocean *R. transtrosa* Salvini-Plawen, 1991 and undescribed species from the Caribbean and Thailand (own unpublished data). However, there also are uniformly white species (Brazilian *R. marcusi* Salvini-Plawen, 1991 and several undescribed ones).

Anatomical knowledge about species of *Rhodope* is very heterogeneous. There are detailed studies of the CNS (Haszprunar & Huber, 1990), and ultrastructure of the epidermis and excretory system (Haszprunar & Künz, 1996; Haszprunar, 1997). Another organ system of taxonomic significance, the hermaphroditic genital system, is known only from schematic representations of *R. transtrosa* and *R. marcusi* (Marcus & Marcus, 1952; Salvini-Plawen, 1991). However, the most detailed anatomical (and the only histological description including the genital system) was carried out by Böhmig (1893) on *R. veranii* from Trieste, Italy; the distal genital system has not been examined in detail since.

The use of microanatomical methods such as computer-based three-dimensional reconstruction from series of semithin sections has proved to be a useful tool for unravelling features of internal anatomy of small to microscopic gastropods (DaCosta *et al.*, 2007; Neusser & Schrödl, 2007, 2009; Brenzinger, Wilson & Schrödl, 2010; Brenzinger *et al.*, 2011; Martynov *et al.*, 2011). Herein, we use these methods to describe the above-mentioned three-banded *Rhodope* in order to establish a modern anatomical dataset as a basis for further studies of the Rhodopemorpha. This species is known from Edithburgh, South Australia and San Remo, Victoria (present study; Burn, 1990, 1998, 2006).

MATERIAL AND METHODS

Specimen sampling

Specimens of *Rhodope rousei* n. sp. were collected from subtidal sand at Edithburgh Jetty, South Australia (35°5′5.15″S, 137°44′58.73″E; 4–8 m; 2004–2007). Specimens were isolated from bulk samples using elutriation and the concentrated sample was observed under a dissecting microscope. Specimens were photographed under a stereo-microscope. One specimen of an additional undescribed *Rhodope* (principally orange) was found in the same samples.

For histological study, a 2-mm specimen (paratype, ZSM Mol-20110168) was anaesthetized using 7% $MgCl_2 \cdot 6H_2O$ in fresh water (although it remained contracted) and fixed in 4% paraformaldehyde buffered with 2 M sodium cacodylate buffer (pH 7.4) with 0.3 M sucrose. The preserved specimen was later postfixed in 1% osmium tetroxide, dehydrated in a graded ethanol series and embedded in Spurr's resin.

Serial sectioning and 3D reconstruction

Serial semithin sections of 1.5 μm thickness were obtained using a Histo Jumbo diamond knife (Diatome, Biel, Switzerland), a Microm HM 360 rotation microtome (Zeiss, Germany) and contact cement applied to the lower edge of the specimen block, following the method described by Ruthensteiner (2008). Ribbons of serial sections were collected on microscopy slides, stained with methylene blue/azure II dyes (Richardson, Jarett & Finke, 1960) and sealed with cover slips and Araldite resin.

For 3D reconstruction, photographs of sections containing all of the specimen and later only the CNS (taken at higher magnification) were taken using a SPOT CCD camera (Spot Insight, Diagnostic Instruments, Inc., Sterling Heights, MI, USA) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Photographs were converted to 8-bit greyscale TIF files prior to importing into AMIRA 4.1 and 5.1 software (TGS Europe, Mercury Computer Systems, Mérignac, France; Visage Imaging GmbH, Berlin, Germany), resulting in aligned picture stacks of the body (487 photos, downsized to resolution of 1,024 × 768 pixels) and the CNS (66 photos at 1,600 × 1,200 pixels). Organ systems were labelled in the aligned series by hand, using interpolation and surface-smoothing tools to create the rendered 3D models shown. The histological series and AMIRA files are deposited at the Mollusca Department, Bavarian State Collection of Zoology, Munich, Germany.

The series was compared with identically prepared histological series of *Rhodope veranii* from Rat Kamenjak, Istria, Croatia, and an undescribed *Rhodope* from the Caribbean.

SYSTEMATIC DESCRIPTION

Heterobranchia *sensu* Haszprunar, 1985
Rhodopemorpha Salvini-Plawen, 1970
RHODOPIDAE von Ihering, 1876

***Rhodope* Kölliker, 1847**

***Rhodope rousei* new species**
(Figs 1–4)

Rhodope sp. Burn, 1990: 9–15. Burn, 1998: 960–961. Burn, 2006: 1–42.
Rhodope sp. Brenzinger *et al.*, 2010: 269.

Type material: Holotype: complete specimen, anterior retracted, fixed in 10% formalin, stored in 75% ethanol; 2 mm preserved body length, collected under Edithburgh Jetty, South Australia, 27 February 2004, by N.G.W. and G. Rouse, deposited in Australian Museum, AM C.469551.

Paratypes: (1) Complete specimen, anterior retracted, fixed in 4% paraformaldehyde, serially sectioned by B.B. and used for 3D reconstruction (five slides). Preserved body length 2 mm, collected under Edithburgh Jetty, 21 March 2007, by N.G.W. and G. Rouse. Deposited in Mollusca Department, Bavarian State Collection of Zoology, Munich, Germany (ZSM Mol-20110168). (2) Complete specimen, anterior retracted, fixed in 4% paraformaldehyde, postfixed in 1% osmium tetroxide and stored in buffer. Preserved body length 1.5 mm, collected under Edithburgh Jetty, 31 March 2006, by N.G.W. and G. Rouse, deposited in South Australian Museum, SAM D19405.

Other material: (1) Complete specimen, anterior retracted, fixed in RNAlater. Preserved body length 1 mm, collected under

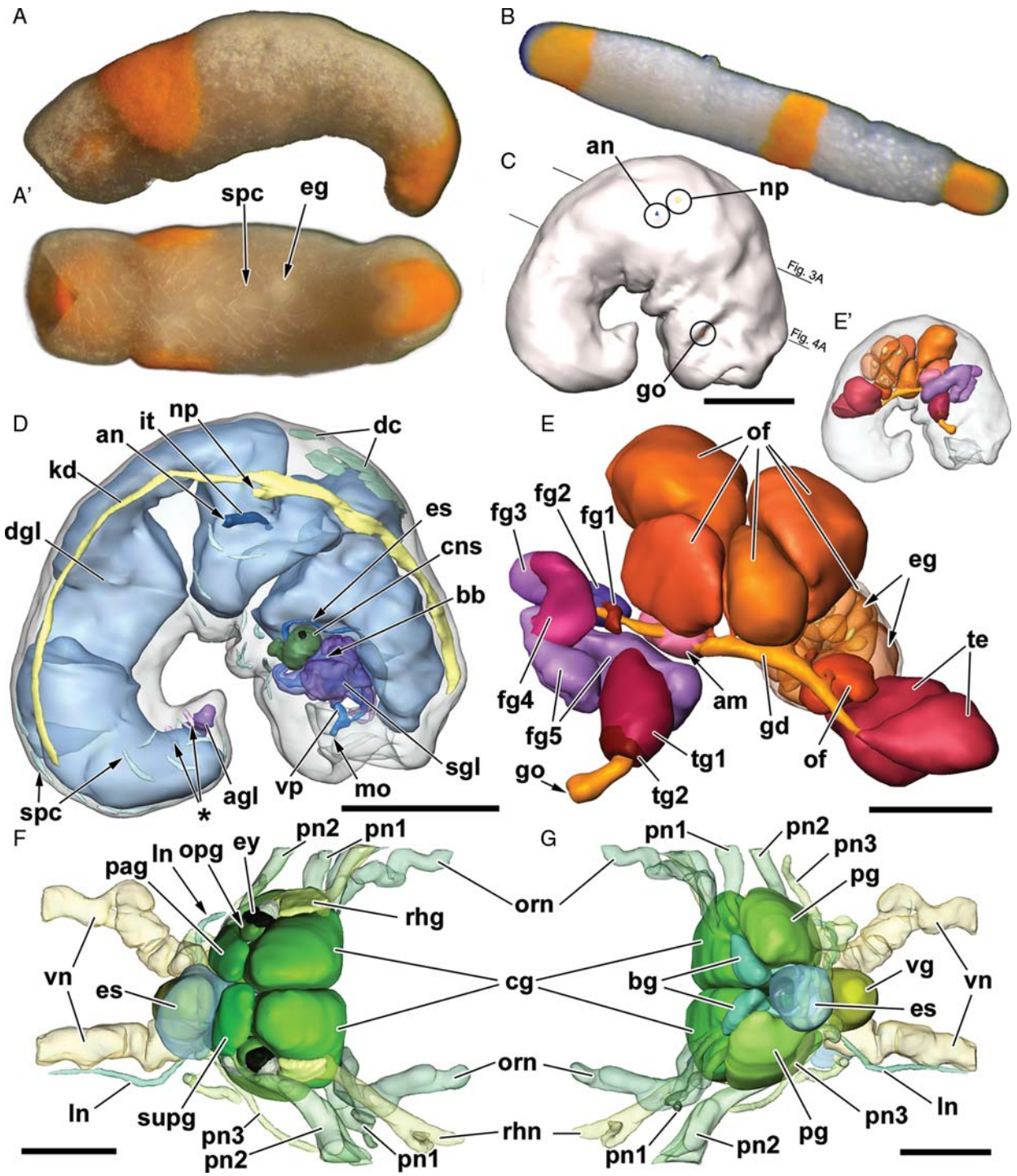

Figure 1. Live specimens of *Rhodope rousei* n. sp. (**A, B**) and 3D reconstructions of internal anatomy (**C–G**). **A.** Holotype (AM C.469551), left view, *c.* 2 mm long. Head at left, retracted. **A'.** Same as **A**, ventral view. Note subepidermal spicules and whitish eggs visible through the body wall. **B.** Dorsolateral view of crawling specimen (AM C.469553), fully extended, *c.* 6 mm long. Head at right; note whitish epidermal glands in anterior portion of body. **C.** Three-dimensional reconstruction of paratype (ZSM Mol-20110168), showing external aspect and localization of body openings, right view. Bars show section planes of Figures 3A and 4A. **D.** Internal organ systems, genital system omitted. Right view. **E.** Genital system, left view. **E'.** Dimensions of genital system in the body, right view. **F.** CNS, dorsal view, anterior side to the right. Nerves are displayed slightly transparent. **G.** CNS, ventral view, anterior side to the left. Note several nerves projecting from the intersection between two ganglia. Abbreviations **A–E**: agl, caudal adhesive gland (asterisk: ciliated openings of adhesive gland); am, ampulla; an, anus; bb, buccal bulb; cns, central nervous system; dc, patches of subepidermal, spherical 'dorsal cells'; dgl, digestive gland; eg, egg; es, oesophagus; fg1–fg5, nidamental glands (proximal to distal); gd, gonoduct; go, ciliated genital opening; it, intestine; kd, two-branched kidney; mo, mouth opening; np, nephropore; of, ovarian follicles; sgl, salivary gland; spc, subepidermal spicules; te, testes; tg1, barrel-shaped terminal gland; tg2, ring-shaped terminal gland; vp, putative vestigial pharynx. **F, G**: bg, buccal ganglion; cg, cerebropleural ganglion; ey, eye; es, oesophagus; ln, lateral nerves originating between pedal and 'visceral' ganglion; orn, oral nerve; opg, optic ganglion; pag, (left) parietal ganglion; pg, pedal ganglion; pn1–pn3, pedal nerves; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; supg, putative combined supraintestinal and (right) parietal ganglion; vg, 'visceral ganglion' = putative combined subintestinal and visceral ganglion; vn, 'visceral' nerves. Scale bars: **C**, **D** = 250 μm; **E** = 150 μm; **F**, **G** = 50 μm.

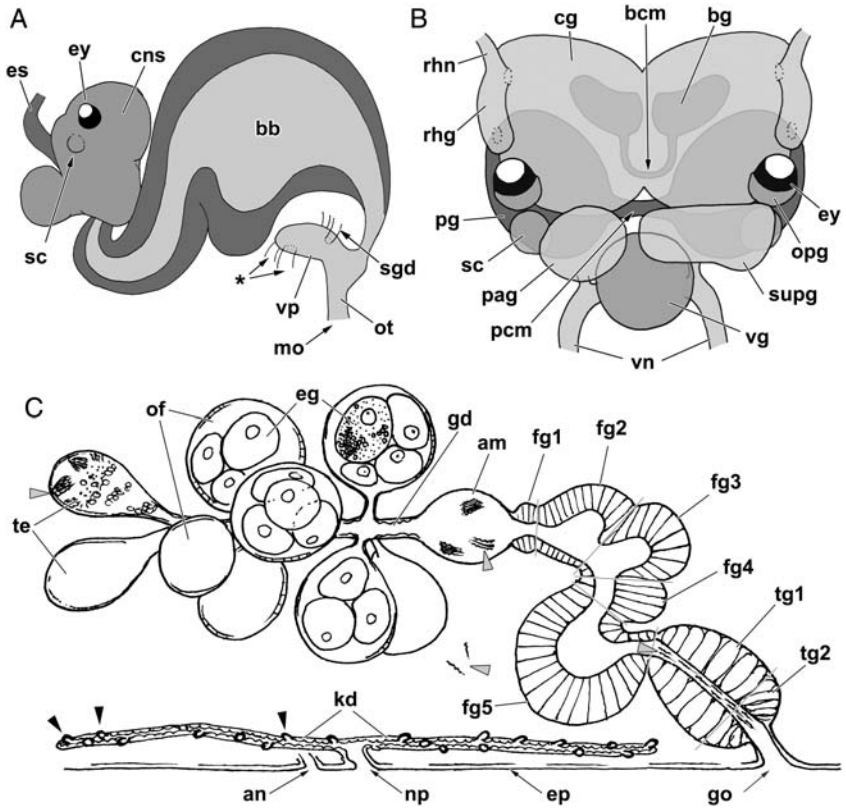

Figure 2. Schematic illustrations of anterior digestive, central nervous and genital systems of *Rhodope rousei* n. sp. paratype (ZSM Mol-20110168). **A.** Anterior digestive system and CNS, right view. Salivary glands omitted, openings of salivary ducts indicated by thin lines. **B.** CNS, showing organization of ganglia. Note that dorsal ganglia are separated only superficially. Dorsal view (see Fig. 1F). **C.** Genital system. Dorsal view, body wall below. Abbreviations: am, ampulla; an, anus; bb, buccal bulb; bcm, buccal commissure; bg, buccal ganglion; cns, central nervous system; cg, cerebropleural ganglion; fg1–fg5, nidamental glands (proximal to distal); gd, gonoduct; eg, egg; es, oesophagus; ep, epidermis; ey, eye; kd, kidney; mo, mouth opening; np, nephropore; of, ovarian follicles; opg, optic ganglion; ot, oral tube; pag, (left) parietal ganglion; pcm, pedal commissure; pg, pedal ganglion; rhg, rhinophoral ganglion, note double cerebro-rhinophoral connectives; rhn, rhinophoral nerve; sc, statocyst; sgd, insertion point of salivary duct; supg, putative combined supraintestinal and (right) parietal ganglion; tg1, barrel-shaped terminal gland; tg2, ring-shaped terminal gland; vg, 'visceral ganglion' = putative combined subintestinal and visceral ganglion; vn, visceral nerves; vp, putative vestigial pharynx; te, testes; asterisk in **A**: possible second pair of salivary ducts; arrowheads in **C**: bulbs of pseudo-protonephridia; grey arrowheads: (screw-shaped heads of) spermatozoa.

Edithburgh Jetty, 27 February 2004, by N.G.W. and G. Rouse, deposited in Australian Museum, AM C.469550. (2) Two complete specimens, both anterior retracted, fixed in 3% glutaraldehyde. Preserved body lengths 1.5–2.5 mm, collected under Edithburgh Jetty, 29 February 2004, by N.G.W. and G. Rouse, deposited in Australian Museum, AM C.469552. (3) Complete specimen, anterior retracted, fixed in 96% ethanol. Preserved body length 1 mm, collected under Edithburgh Jetty, 22 March 2007, by N.G.W. and G. Rouse, deposited in Australian Museum, AM C.469554. (4) DNA from one specimen, live crawling length 6 mm, collected under Edithburgh Jetty, 22 March 2007, by N.G.W. and G. Rouse, deposited in Australian Museum, AM C.469553.

Other records: Four individuals collected 11–15 February 2005, under Edithburgh Jetty. Photo record only, specimens lost.

Etymology: The species is named for Greg Rouse, who introduced N.G.W. to the interstitial world, and who helped collect many specimens of interstitial heterobranchs.

Distribution: Species known from two localities in southeast Australia. Known from subtidal sand at Edithburgh Jetty, South Australia (present study); previous record and illustration of a single three-banded *Rhodope* "crawling on intertidal *Zostera* on a reef flat" at San Remo, Westernport, Victoria (Burn, 1990, 1998) is believed to refer to the same species.

External morphology (Fig. 1A–C): Body elongate and cylindrical in cross-section, with no marked cephalic appendages, mantle cavity, visceral hump or foot. Snout rounded with terminal mouth opening, retractable together with anterior quarter of body. Tail end sometimes broader and slightly flattened in crawling specimens, with slightly concave underside (position of the adhesive gland).

Distinguishable from other *Rhodope* species by characteristic orange pigmentation of snout, tail end, and transversal dorsal band at anterior third of body (a constriction of body visible just anterior to this band in contracted specimens; see Fig. 1A'). Rest of dorsal side opaque with white pigment; ventral side of body colorless, translucent. Genital opening at right side, close to anterior border of median transversal band; anus dextral and close to posterior border of band (middle of animal), nephropore slightly anterodorsal to anus. Subepidermal spicules and eggs visible through ventral epidermis, whitish spherical glands through dorsal side.

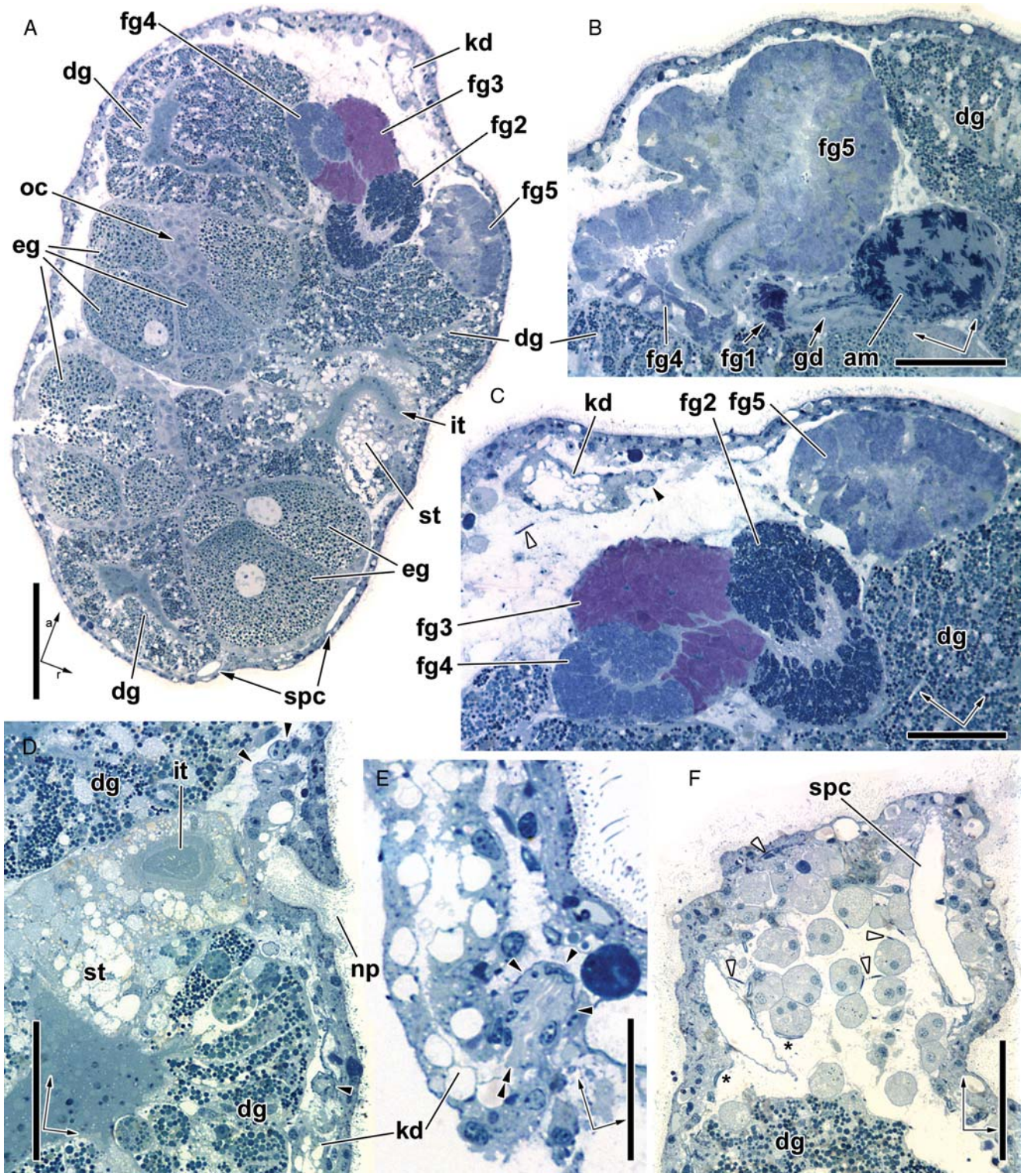

Figure 3. Semithin histological sections from *Rhodope rousei* n. sp. paratype (ZSM Mol-20110168). Anterior/right pictograms next to scale bars indicate orientation of section relative to animal. **A.** Longitudinal section through midsection of curved body (level of section indicated in Fig. 1C). **B.** More anteroventral section showing both female gland 1 and 5, and ampulla. **C.** Female glands 2 to 4, enlarged from **A. D.** Right body side showing stomach (light wall), intestine and nephropore. **E.** Longitudinal section through kidney and pseudo-protonephridium, showing ciliary flame. Epidermis at right. **F.** Putative rhogocytes (spherical 'dorsal cells'; note double nuclei in some) and parts of the 'vesicle system' below the dorsal epidermis. Abbreviations: am, ampulla filled with batches of autosperm; dg, digestive gland; eg, egg; fg1–fg5, nidamental glands (proximal to distal); gd, gonoduct; it, intestine; kd, kidney; np, nephropore; oc, oocytes; spc, spicule; st, stomach (wall); arrowheads in **C–E**: cross-section of pseudo-protonephridium, characterized by strong basal lamina; double arrowhead in **E**: ciliary flame of pseudo-protonephridium; white arrowheads in **F**: spermatozoon in body cavity; asterisks in **F**: putative tubes of 'vesicle system'. Scale bars: **A** = 100 μm; **B–D, F** = 50 μm; **E** = 20 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

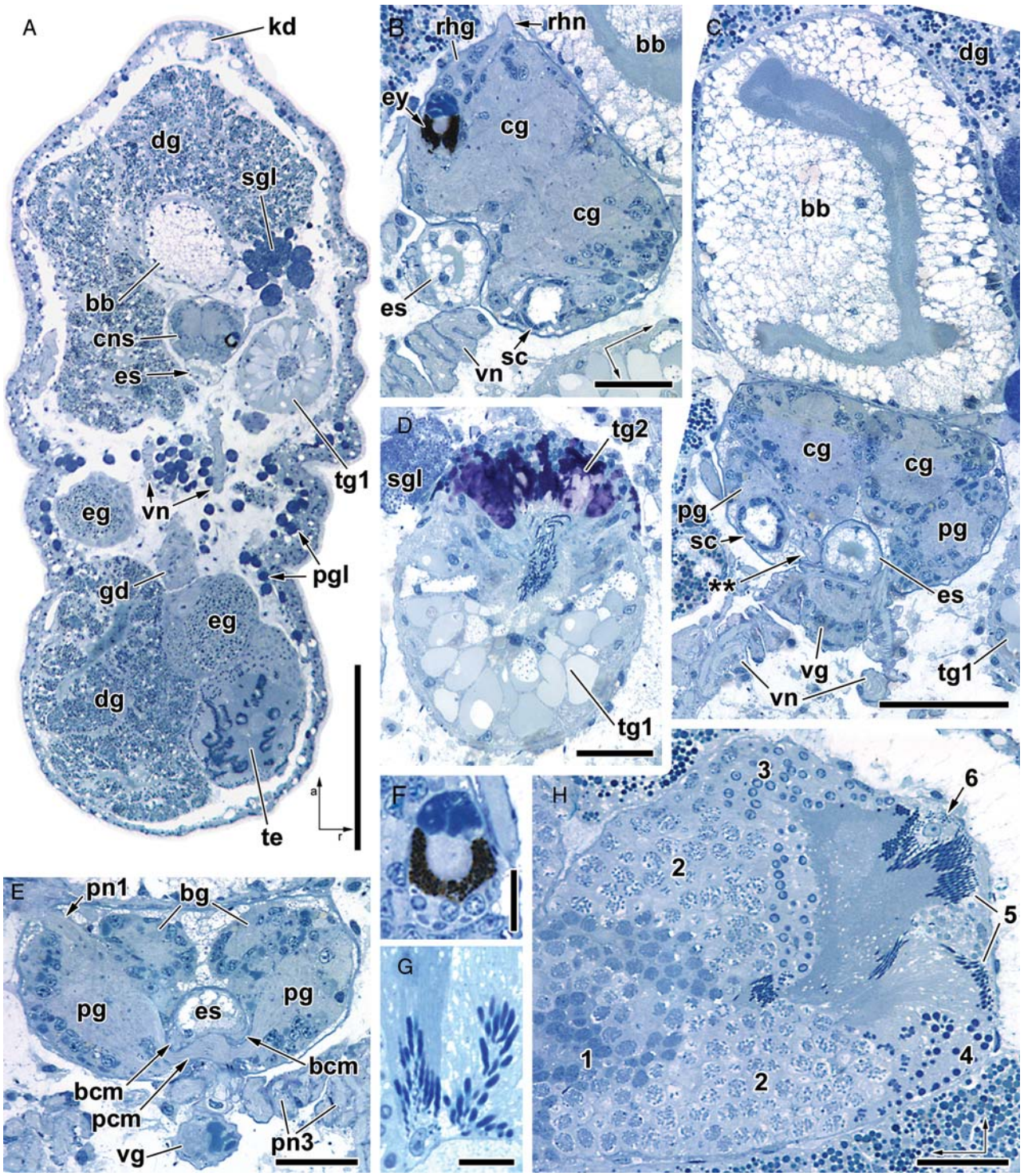

Figure 4. Further semithin histological sections from *Rhodope rousei* n. sp. paratype (ZSM Mol-20110168). See anterior/right pictogram next to scale bar in **A** for orientation (omitted in others if orientation the same). **A.** Longitudinal section through curved body (see Fig. 1C for level of section; other sections are more ventral). **B.** Longitudinal section through CNS, rather dorsal level. **C.** Buccal bulb and CNS, middle level. **D.** Terminal glands of genital system at intersection between first and second part. Note spermatozoa inside lumen. **E.** CNS, rather ventral level. **F.** Right eye. Note corpuscular lens lacking a cornea, optic ganglion below pigment cup. **G.** Enlarged area of testis wall showing almost ripe (right) next to ripe spermatozoa with nutritive cell. **H.** Section through testis showing densely packed areas of premeiotic spermatogonia/spermatids (1,2), postmeiotic spermatocytes (3,4), and ripe spermatozoa (5) crowding around nutritive cell (6). Abbreviations: bb, buccal bulb; bcm, buccal commissure; bg, buccal ganglion; cg, cerebropleural ganglion; cns, central nervous system; dg, digestive gland; eg, egg; es, (distal part of) oesophagus; ey, eye; gd, gonoduct; kd, kidney; pcm, pedal commissure; pg, pedal ganglion; pgl, pedal glands; pn1, anterior pedal nerve; pn3, posterior pedal nerve; rhg, rhinophoral ganglion; rhn, rhinophoral nerve; sc, statocyst; sgl, salivary gland; te, testis; tg1, barrel-shaped terminal gland; tg2, ring-shaped terminal gland; vg, 'visceral ganglion' = putative combined subintestinal and visceral ganglion; vn, visceral nerves; double asterisk in **C**: connective between left parietal and 'visceral ganglion'; numbers in **H**: see above. Scale bars: **A** = 200 μm; **B, D, E, H** = 25 μm; **C** = 50 μm; **F, G** = 10 μm. This figure appears in colour in the online version of *Journal of Molluscan Studies*.

Body wall (Figs 3, 4): Epidermis *c*. 8 μm thick, strongly ciliated all around. Cells with large vacuoles interspersed. Body wall musculature indistinct. Extent of orange pigmentation not detectable in histological sections. Subepidermal spicules scattered below epidermis, oriented roughly at 45° angle to body axis. Spicules *c*. 100–120 μm long, curved, narrowing towards tips (Fig. 3F). Spicule body dissolved in histological sections, surrounding layer hints at slightly rough surface. Dark-staining nucleus of spicule cell located in middle of concave side (Fig. 3F). Thin tubes of 'vesicle system' visible in sections below the dorsum, close to patches of spherical 'dorsal cells' (see Excretory system; Fig. 3F). Numerous monocellular pedal glands (diam. to 20 μm) below ventral epidermis (Fig. 4A), staining dark blue, each oval cell opening through individual apical duct. Subepidermal adhesive gland in ventral side of tail end appearing as aggregation of smaller glandular cells with grainy blue-staining interior. Adhesive gland opening through paired ciliated grooves situated lateroventrally (Fig. 1D). No aggregated muscle fibres spanning or delimiting body cavities.

Digestive system (Figs 1D, 2A): Mouth opening a transversal slit terminal on snout, followed by very short oral tube. Blind sac of about 30 μm length projecting from ventral side of oral tube; pair (possibly two) of salivary ducts opening into supposedly vestigial pharynx. Large salivary glands aggregations of oval, droplet-filled cells (staining dark blue, some with violet tinge; Fig. 4A), located left and right of oesophagus. Oesophagus lined with thick layer of irregularly sorted, vacuolated cells and mostly basal nuclei; anterior portion thin and curved. Middle part of oesophagus greatly enlarged (buccal bulb), forming an elongate oval bulb with very thick cushion-like wall and flat, ciliated lumen (Fig. 4C). Posterior part of oesophagus rather long and very thin, curving upward through cerebral nerve ring and leading into digestive gland. Tubular digestive gland with irregular inner surface of columnar, droplet-filled epithelial cells (e.g. Fig. 3B, D); short branch of gland extending anteriorly from where oesophagus enters, long and undulated posterior branch extending to tail end of animal (Fig 1D). Vacuolate, not droplet-filled, area of digestive gland wall at right body side (stomach); short and ciliated intestine exits stomach and opens at right body side (Fig. 3A, D).

Table 1. Summary of nerves in *Rhodope rousei* n. sp. paratype (ZSM Mol-20110168).

Nerve	Abbreviation in Figs	Rooted in	Targets
Rhinophoral nerve	rhn	cg/rhg	Sides of snout, branch into salivary glands, and along dorsal sides of cephalic caecum
Oral nerve	orn	cg	Sides of mouth opening, running between salivary glands and buccal bulb
Pedal nerve, anterior	pn1	pg	To anterior ventral side, flanks
Pedal nerve, lateral	pn2	pg + cg	To flanks and running posterior
Pedal nerve, posteroventral	pn3	pg	To anterior ventral side, median side
Lateral nerve	ln	pag + vg	Right side: parallel to right vn Left side: curves anteriorly
Visceral nerve	vn	vg + pag/ supg	Parallel up to tail end/ adhesive gland
Buccal nerve	—	bg	Sides of buccal bulb

CNS and sensory organs (Figs 1D, F, G, 2B, 4, Table 1): CNS a dense mass of ganglia posterior to buccal bulb, encapsulated within thin connective sheath, gaps filled with loose tissue (Fig. 4E). In large ganglia, nuclei located along periphery; central medulla a homogeneous mass, slightly fibrous, similar to nerves in histology (Fig. 4B, C). Nerves and their targets are summarized in Table 1.

Very large paired anterodorsal ganglia (cerebropleural ganglia) touching medially and separated only by slight superficial groove; cerebral commissure detectable as broad connection of medulla (Fig. 4B). Pigment-cup eyes (diam. 20 μm) located at posterior sides of cerebropleural ganglia; eyes face dorsally, lacking cellular cornea but with lens consisting of discrete cells (Fig. 4B, F). Eyes cradled by cup-shaped optic ganglia containing less than 10 nuclei; optic nerves not detectable. Elongate rhinophoral ganglia located anterior to eyes, with double cerebro-rhinophoral connectives: one connective close to the base of the ganglion, the second at tip leading into rhinophoral nerve (Figs 2B, 4B). Paired oral nerves very thick (Fig. 1G), numerous nuclei surrounding nerve fibres at nerve's base similar to rhinophoral ganglia; oral nerves extend anteroventrally from superficial gap in cerebropleural ganglia.

Paired medium-sized ganglia connecting broadly to posterior side of cerebropleural ganglia, divisible externally by shallow dorsal constrictions; left ganglion less wide (left parietal ganglion) than right (combined supraintestinal and right parietal ganglion) (Figs 1F, 2B). Medium-sized, spherical posterior 'visceral' ganglion (combined subintestinal and visceral ganglion) joined to latter ganglia posteroventrally by connectives of medium length extending around oesophagus (see Fig. 4C). Two thick, double-rooted 'visceral' nerves extend from intersection of latter ganglia and pedal ganglia (Fig. 4C); thick root inside 'visceral' ganglion, thin root in region of cerebropleural and parietal ganglia. 'Visceral' nerves very thick, undulated especially at base, containing single nuclei interspersed along their length; nerves extend parallel along ventral side of body to tail.

Large paired pedal ganglia below cerebropleural ganglia; cerebropedal connectives short and wide, pleuropedal connectives not detected, pedal commissure longer, parapedal commissure not detected. Paired spherical statocysts, slightly larger than eyes, embedded in dorsal part of each pedal ganglion (Fig. 4B); hollow capsule of few cells surrounds cavity containing remnants of single statolith. Static nerve not detectable. Three pairs of pedal nerves detectable (Fig. 1: 'pn1' to 'pn3'): First pair rather thick and extending from anterior side of each pedal ganglion (Fig. 4E), second pair very thick and extending from just anterior to statocysts, with thick second root in region of pleural ganglia, third pair extending laterally from close to base of pedal commissure. Fourth pair of thin nerves extending laterally from gap between pedal and visceral ganglia ('lateral' nerves in Fig. 1F, G) appears rooted in visceral and possibly parietal ganglia.

Paired buccal ganglia medium-sized, located in anteroventral depression between cerebropleural and pedal ganglia (Fig. 4E). Cerebrobuccal connectives short; buccal commissure rather long and thin, looping around oesophagus close to pedal commissure. Paired buccal nerves medium-sized and with very few nuclei, extending anteriorly along sides of buccal bulb (not shown).

Excretory system (Figs 1D, 2C, 3C–F): Kidney consisting of two tubular branches (collapsed diameter 50 μm) extending anterior and posterior from ciliated nephropore along right dorsolateral side. Epithelium of kidney containing rounded vacuoles; knob-shaped pseudo-protonephridia (diam. ca. 10 μm) protruding from vacuolate epithelium in irregular intervals. Each knob formed by capsule of few flat cells, outer border discernible in histological sections by conspicuously strong basal lamina (arrowheads in Fig. 3C–E); ciliary flame inside lumen of each knob, directed towards kidney lumen (Fig. 3E). Spherical light-blue-staining cells (diam. ca. 15 μm), some with two nuclei, located in loose aggregations below dorsal epidermis anterior to nephropore ('dorsal cells' = putative rhogocytes; Fig. 3F).

Genital system (Figs 1E, 2C, 3A–C, 4A, D, G, H): Monaulic genital system hermaphroditic, with spermatozoa and oocytes in separate acini. Posterior two acini (testes) drop shaped, containing spermatozoa and their precursors in distinct stages of development, sorted in batches (Fig. 4H). Following gonoduct a muscular (circular fibres) and ciliated tube, six roughly spherical ovarial acini of different sizes extending on thin stalks (Fig. 1E). Ovarian acini containing dense batches of oocytes close to the epithelial wall and 2–10 yolk-rich developing eggs (diam. to 120 μm) inside, each egg with clear nucleus and darker nucleolus (Fig. 3A). Last ovarian acinus followed by roughly spherical ampulla filled with irregularly sorted bundles of spermatozoa (Fig. 3B). Epithelium of postampullary gonoduct developed into five distinct (nidamental) glands, separable in sections by constitution of tissue and its staining properties: First nidamental gland a very small ring of small cells staining dark blue, second gland a larger sac-like extension with higher epithelium stained by dark blue granules, third gland an equally sized sac staining homogeneously pink, fourth gland a medium blue-staining tube with regular epithelium and fifth gland largest, a sac-like extension with rather loose epithelium staining light blue (Fig. 3A–C). Ciliated lumen of nidamental glands followed by compound tube of two 'terminal' glands surrounding gonoduct: first terminal gland barrel-shaped and circular in cross-section, formed by regular epithelium of apparently holocrinous glandular cells containing light blue staining vacuoles and basal nuclei; second terminal gland a short ring of columnar, irregularly dark violet staining cells (Fig. 4A, D). Gonoduct inside terminal glands filled densely with autospermatozoa (sperm heads pointing distally). Gonoduct following terminal glands short and thick, forming the strongly ciliated genital opening. Allospermatozoa with screw-shaped heads found freely distributed in entire haemocoel (highlighted in Fig. 3C, F), sometimes lodged in lining of organs, such as CNS.

DISCUSSION

Taxonomic remarks

The latest review of rhodopid species by Haszprunar & Heß (2005: table 1) recognized four described *Rhodope* (the type *R. veranii*, *R. marcusi*, *R. transtrosa* and *R. roskoi*) besides at least five undescribed species. These included *Rhodope* sp. 'E' which differs from all other known species by its possession of three orange bands. We regard our *Rhodope rousei* n. sp. from Edithburgh, Victoria to be conspecific with the aforementioned one from Westernport, Victoria (Burn, 1990, 1998, 2006), because they share the unique three-banded pattern and are both distributed in temperate southeastern Australia. Comparing it with the few *Rhodope* species known in anatomical detail, *R. rousei* n. sp. most resembles the (presumed) Indo-Pacific *R. transtrosa* (with a single orange band) in general morphology of the CNS (superficial gaps between the cerebropleural and parietal ganglia), in the length of the pedal and buccal commissures (comparatively long) and in the size of the eyes and statocysts (relatively large in comparison to the CNS) (Haszprunar & Huber, 1990). The set of nerves identified herein corresponds well to what is known for *R. veranii* and *R. transtrosa* (Haszprunar & Huber, 1990; Huber, 1993); differences are the lack of a "clearly detectable" parapedal commissure as in *R. transtrosa* and the presence of three pairs of pedal nerves instead of only one (including the double-rooted nerve termed 'pn2' herein). Two nerves leaving the 'visceral' ganglion present another shared character with *R. transtrosa*, but in *R. rousei* n. sp. the two 'visceral' nerves appear more symmetrical in their size and origin. The thin lateral nerves herein were not shown for the other species but, judging from its position, could as well refer to the right 'pallial' nerve. The genital system differs from that of *R. transtrosa* (described by Salvini-Plawen, 1991) in its possession of distinct terminal glands in the gonoduct and of more than three nidamental glands.

The nervous system presents a difficult object of study due to its strong fusion, but there appear to be morphologically 'derived' species with strongly fused ganglia (i.e. *R. veranii* in Haszprunar & Huber, 1990; *R. roskoi*: own observation) and those with superficially separated ones (*R. transtrosa*; *R. rousei* n. sp.). The genital system appears not to show much interspecific variation except in the number of ovarian follicles. The needle-like spicules were previously regarded as species-specific, e.g. by Haszprunar & Huber (1990), but have not been used to delimit species and appear not to show much interspecific variation.

We conclude that the pigmentation of *Rhodope* is still the best means to separate species but, with more data available, microanatomical information may be of taxonomic use in future. In this study we intend to set a new standard for anatomical comparison of rhodopemorph species.

Digestive system

The digestive system of *Rhodope* is highly modified due to the lack of a radula and the tubular digestive gland with a branch leading into the head (Böhmig, 1893). Especially the parts between mouth opening and digestive gland appear to be specialized for sucking soft and liquid food using the conspicuous buccal bulb; this structure appears to represent a shared feature of the Rhodopemorpha (also present in *Helminthope*; Salvini-Plawen, 1991). Judging from histology and anatomy of *Rhodope rousei* n. sp. we conclude that (1) this buccal bulb is a specialized part of the oesophagus—i.e. not homologous with the otherwise muscular pharynx of other heterobranchs as was previously assumed, and (2) that a vestige of the original pharynx is present as the blind sac close to the mouth opening into which the salivary glands open. The first is supported by essentially identical histological properties of the buccal bulb and the adjoining thinner parts of the oesophagus (the bulb is not muscular or otherwise differentiated except for its size) and that it lacks the insertion of salivary glands typical for the pharynx (see below). Regarding the second, the vestigial pharynx (mentioned by Böhmig, 1893 and Salvini-Plawen, 1991 as an "outlet of the oral glands") can be identified as such from the salivary ducts entering there, and from the observation during ontogeny of *R. veranii* that buccal ganglia develop from ectoderm just next to the mouth opening, just next to a pharyngeal anlage with a rudimentary radula (Riedl, 1960).

Pumping of the buccal bulb by dilation might be facilitated by the densely vacuolated epithelium forming an elastic wall,

although ingestion of food appears to be strongly dependent on ciliary motion (Riedl, 1959). Riedl observed *R. veranii* to be specialized for feeding on the planula-like placozoan *Trichoplax* (which is not a sponge larva, as assumed by Burn, 1998); however, bacterial assemblages, large protists or soft-shelled eggs might also fit within the food spectrum.

So far the described variation of the digestive system of *Rhodope* relates to the presence of oral glands opening next to the mouth (not obvious herein, but histologically separable from salivary glands: Böhmig, 1893; Marcus & Marcus, 1952; own observation on *Helminthope*), the form of the salivary glands (sac-like: Marcus & Marcus, 1952; or consisting of numerous acini: Graff, 1883; present study), and where the salivary ducts open (directly into the buccal bulb: Marcus & Marcus, 1952; or close to the mouth: Böhmig, 1893; present study). Judging from semithin sections, the connection to the buccal bulb is likely a mass of salivary glands that opens into the short blind sac protruding from the oral tube just behind the mouth opening. Since there appears to be more than one pair of ducts leading there, *R. rousei* n. sp. might have oral glands that are histologically similar to the salivary glands and embedded within those.

Central nervous system

The highly condensed, euthyneuran CNS of *Rhodope* has repeatedly been used to place the taxon among 'derived' heterobranchs, i.e. Euthyneura such as nudibranchs or gymnomorph pulmonates (Salvini-Plawen, 1970; Haszprunar & Huber, 1990). Judging from molecular results by Wilson *et al.* (2010), many previously assumed synapomorphies (strong fusion of ganglia, double cerebro-rhinophoral connective) are thus either analogies or simply plesiomorphic for Heterobranchia, and not synapomorphies for opisthobranchs and pulmonates, as suggested by Jörger *et al.* (2010).

Presence of giant nerve cells, a character of Euthyneura (see Haszprunar, 1985), is not evident from any of the examined material, but might be connected to the miniaturization.

The fusion of cerebral, pleural and visceral-loop ganglia in *Rhodope rousei* n. sp. is striking. While not as extreme as in *R. veranii*, it resembles closely the condition shown in *R. transtrosa* (Haszprunar & Huber, 1990). The cerebral ganglia touch broadly and the cerebral commissure is almost as thick as the contacting zone. The fusion of the pleural ganglia with the posterior part of each cerebral ganglion was observed in adult and larval *R. veranii* by Riedl (1960) and was deduced from the presence of two almost parallel connectives running from the cerebropleural ganglia into the pedal ganglia (Haszprunar & Huber, 1990); we follow this interpretation of fused cerebropleural ganglia, although a distinct pleuro-pedal connective was not detected.

Due to their fusion and close contact with the cerebropleural ganglia, the ganglia of the visceral loop can only be identified with knowledge of the ontogeny. Five separate ganglia have been observed in developmental stages of *R. veranii* (Riedl, 1960) and later fuse in a pattern which can be inferred to be present also in *R. rousei* n. sp.: three of the visceral loop ganglia are joined closely to the posterior end of the cerebropleural ganglia from which they are separated by superficial incisions. The right part is relatively larger than the left one, which can be explained—following the nomenclature used by Haszprunar (1985)—from the (also observed) fusion of both the right parietal and the supraintestinal ganglion to the cerebropleural ganglion, while on the left side only the (left) parietal ganglion is merged with the posterior side of the cerebropleural ganglion. The free ganglion below the oesophagus is ontogenetically derived from the subintestinal and visceral ganglion, which fits with the presence of two nerves leaving this ganglion, at least one of them likely to be homologous with the 'true' visceral nerve. The two nerves appear more or less symmetrical herein, but Haszprunar & Huber (1990) described two functions: a thick 'pallial' and a thinner, left, 'genitovisceral' nerve.

It should be noted that *Rhodope* is one of few heterobranchs where fusion of ganglia on the visceral loop has not been deduced solely from relative size and emerging nerves, a practice criticized by Dayrat & Tillier (2000). Together with *Helminthope*—which has been described with five free ganglia on the visceral loop (Salvini-Plawen, 1991)—the rhodopids appear to be Pentaganglionata (=Euthyneura) in the literal sense, although they formally fall outside of this taxonomic grouping judging from molecular phylogenetic data.

The pedal ganglia show three nerves, one of which shares a second root with the posterior part of the cerebropleural ganglia; this configuration is similar to that described for *R. veranii*, but not *R. transtrosa* which has been depicted with only a single pedal nerve (Haszprunar & Huber, 1990). The buccal ganglia (long connective in *R. rousei* n. sp. and *R. transtrosa*) are not reduced as suggested by Riedl (1960) and Oberzeller (1969), but are clearly developed and show conspicuously thick nerves which, judging from their position, innervate the buccal bulb and oesophagus.

Some of the very thick nerves of *R. rousei* n. sp. reflect the strong fusion of the ganglia by being rooted within two ganglia (Table 1) or branching close to or from a connective (e.g. the 'lateral' nerves herein; also the optic nerve reported by Haszprunar & Huber, 1990). Distinct neurons can be found within e.g. the oral and 'visceral' nerves, giving the nerves the appearance of medullary cords. These neurons are however never organized into 'true' ganglia (with distinct, external cortex) and also are not aggregated in thicker areas of the nerves (both being the case in *Helminthope*; own observation; Salvini-Plawen, 1991). The presence of neurons within the nerves presents an analogous character to that of other meiofaunal gastropods such as some philinoglossids (Marcus & Marcus, 1954), microhedylacean Acochlidia (Neusser *et al.*, 2006; Jörger *et al.*, 2008) or the sacoglossan *Platyhedyle* Salvini-Plawen (Rückert, Altnöder & Schrödl, 2008). The presence of accessory ganglia in these miniaturized species has been interpreted as adding extra neurons to a CNS that would otherwise be too small (Haszprunar & Huber, 1990).

Again, the CNS of *Rhodope* can be stated to show a mosaic of features that are likely to be ancestral for heterobranchs (double rhinophoral connective, see Neusser, Jörger & Schrödl, 2007; Jörger *et al.*, 2010) and those that appear highly derived (extreme fusion of ganglia) or induced by the aberrant worm-like morphology and miniature size ('outsourced' ganglia). Whether giant nerve cells (as a character of Euthyneura *sensu* Haszprunar, 1985) are present in *Rhodope* or not cannot be clarified from the present material.

Sensory organs

The eyes and statocysts are the most prominent sensory organs and are visible in live specimens, especially by transmitted light. Both organs are relatively large (compared to the rest of the CNS), differing from *R. veranii* but resembling the condition in *R. transtrosa*, as shown by Haszprunar & Huber (1990).

The peculiar pigment-cup eyes (no cellular cornea, 'corpuscular' lens with distinct cell borders) were first shown by Böhmig (1893) and their development—with ingression of primary corneal cells into the lens/vitreous body—was described by Riedl (1960). This peculiar feature appears to be derived in *Rhodope*, since the eyes of *Helminthope* do not show the corpuscular lens (own observations). Whether this

modification of the eyes affects visual performance significantly remains unclear, but it appears that the visual apparatus of *Rhodope* is not subjected to strong selection, as developmental malformations involving the eyes appear to be rather common: examples are the formation of double lenses in one eye with the other one lacking (Graff, 1883) or the formation of four eyes (Riedl, 1959, 1960).

Further sensory structures such as an osphradium or Hancock's organs are not detectable in the present material and are not reported for other species. Haszprunar & Künz (1996) mentioned sensory cells interspersed within the epidermis. According to Riedl (1960), a pit-shaped osphradium and osphradial ganglion are briefly present during early ontogeny and are innervated from the supraintestinal ganglion by what appears to be the right lateral nerve herein. Parts of the rhinophoral and oral nerve have been described to innervate the epidermis of the anterior body sides "corresponding to the anterior and posterior portions of the Hancock's organ" (Haszprunar & Huber, 1990). This follows our observation that the rhinophoral nerve ends at the sides of the snout (Table 1).

Genital system

The peculiar division into distal male and proximal female acini in the gonad (testes and ovarian follicles herein) has been reported in all previous descriptions of the rhodopid genital system. This is a rare feature in hermaphroditic heterobranchs. Exceptions include the architectonicoid *Omalogyra* and *Heliacus* (see Haszprunar, 1985) and the acochlidian *Asperspina riseri* (Morse, 1976), however ovaries and testes are described as more or less parallel in these cases. The described number of gonad acini, especially those containing sperm, varies in previous reports. While the older accounts mention up to 10 male lobes (Marcus & Marcus, 1952—only two are depicted), it appears that there really are only two in ripe specimens of any species examined more recently (Salvini-Plawen, 1991; this study). The number of developed ovarian acini, on the other hand, seems to be variable among individuals, although most described specimens contain several follicles (up to 10 in Marcus & Marcus, 1952).

The nidamental glands have been described to contain either three (Salvini-Plawen, 1991) or four lobes (Böhmig, 1893; Marcus & Marcus, 1952), the latter likely identical to the condition found in *Rhodope rousei* n. sp. In their histology the glands resemble those of nudibranchs (e.g. Klussmann-Kolb, 2001a, b), but are otherwise not very differentiated—there are no elaborate folds or similar structures. The tiny proximal gland (fg1 herein) has not been described before and appears not to be present in *R. veranii* and Caribbean specimens (own observations); its identity as a nidamental gland is not clear.

Oviposition of egg strings by a circular crawling motion was observed by Riedl (1959) in *R. veranii*; egg masses were described to contain between 6 and 30 eggs, each egg surrounded by a secondary layer and later covered by the adult with algal filaments and detritus. It is not clear if the egg masses show other heterobranch features (Haszprunar, 1985) such as inclusion of the eggs within a characteristic gelatinous capsule or if the eggs are united into strings by so-called chalazae.

The genital system of *R. veranii* following the nidamental glands was originally described as containing an eversible, spiral penis (Kölliker, 1847; Bronn & Keferstein, 1862–1866; Graff, 1883), which Böhmig (1893) interpreted to be a cone-shaped, ciliated fold inside the voluminous distal part of the genital system visible in histological sections. Marcus & Marcus (1952) describe a similar "conical, ciliated, unarmed penis" inside the "penis sheath" which is a wide and muscular bulb (the latter likely corresponding to the terminal glands herein); in *R. transtrosa*, it was explicitly mentioned to be lacking (Salvini-Plawen, 1991). While all of the specimens examined herein contained the bulbous structure consisting of the two terminal glands, there is never a cone-shaped structure inside (the wall of the terminal glands being clearly glandular and not muscular) and there obviously is no other large spiral or eversible copulatory organ. This leads to the question how sperm are transferred in *Rhodope*. Riedl (1959) assumed copulation to be taking place in specimens he observed with the anterior right side of the body touching ("typical for euthyneuran gastropods", Haszprunar & Künz, 1996) and—due to the monaulic genital system—concluded sperm transfer to be unidirectional (transfer itself was not observed), while Salvini-Plawen (1991) assumed "functional diauly". The presence of free spermatozoa in the haemocoel and the lack of allosperm receptacles, however, imply a hypodermic mode of insemination. "Fertilization by hypodermic injection" was suggested by Haszprunar & Künz (1996), but is linked with the presence of a copulatory, or at least perforating, organ. Judging from the aphallic nature of *Rhodope rousei* n. sp. and the presence of numerous autospermatozoa within the lumen of the terminal glands, we suggest instead that *Rhodope* uses dermal insemination and dermal fertilization via spermatophores, as recently described from the acochlidian *Pontohedyle milaschewitchii* (Jörger *et al.*, 2009). Spermatophores in *R. rousei* n. sp. are likely formed by the terminal glands and applied to the partner's epidermis. Sperm would have to be transferred subepidermally and into the body cavity from this spermatophore, possibly by short-term lysis of a small stretch of epidermis (as in mesopsammic acochlidians, see Swedmark, 1968; Jörger *et al.*, 2009), prior to fertilization of oocytes inside the gonad (or gonoduct). The typical heterobranch spermatozoa (cork-screw-shaped head; Healy, 1996) must hence be able to penetrate the dense basal lamina of the epidermis and the gonad epithelium, as was discussed for microhedylacean acochlidians by Jörger *et al.* (2009); it remains unclear if this is a purely mechanical process or guided by biochemical activity.

Kidney and excretory cells

Rhodope rousei n. sp. lacks a heart and shows the typical excretory system with 'protonephridium-like' knobs containing ciliary flames interspersed along the paired kidney tubes, as originally described by Graff (1883) and Böhmig (1893). As was shown from previous TEM studies, an ultrafiltration weir appears not to be present in the "pseudo-protonephridia", but only in the free haemocoelic rhogocytes (Haszprunar & Künz, 1996; Haszprunar, 1997). These were described as large, spherical cells "scattered within the body cavity" by Haszprunar & Künz (1996) for *R. transtrosa* (but not *R. veranii*). Assuming that the 'dorsal' cells in *Rhodope rousei* n. sp. are rhogocytes (see Haszprunar, 1996), then they are unusually aggregated in the place where one might expect the heart to have been (namely slightly anterodorsal to the kidney opening).

Rhodopemorpha as infaunal taxa

The rhodopids have repeatedly been treated as part of the interstitial molluscan fauna (e.g. Rieger & Sterrer, 1975; Arnaud, Poizat & Salvini-Plawen, 1986) due to their minute size, vermiform external morphology and their possession of anatomical features that are assumed to be 'typical' adaptations of interstitial molluscs. These include prominent epidermal ciliation, spicules, an adhesive gland and accessory ganglia, but also production of spermatophores (discussed above) and lack of pigmentation (Swedmark, 1968). Some

species—including pigmented ones—have indeed been found in coarse sand (Karling, 1966; Rieger & Sterrer, 1975; Haszprunar & Heß, 2005; own unpublished data), but only *Helminthope* and '*Rhodope*' *crucispiculata*—being even more vermiform and unpigmented—resemble 'full-time' infaunal animals. The anatomically described species of *Rhodope* are so far known only from algal communities on rocks (Marcus & Marcus, 1952; Riedl, 1959). The new species *R. rousei* is the first that has been sampled from both sand and algae.

Spicules

The subepidermal calcareous spicules of *R. rousei* n. sp. are typical for rhodopids in their curved form and slightly rough surface. Some other species have been shown to have a notch in the middle of the convex side of each spicule (*R. veranii* in Riedl, 1960; own unpublished data on a Caribbean species); this notch (opposite position of spicule cell's nucleus?), which is well visible in microscopic views of complete specimens, has not been mentioned for other species, but might simply have been overlooked. However, this notch is not evident in living specimens or histological sections of *R. rousei* n. sp. If variation exists among *Rhodope* species, the presence of a notch might represent a useful feature for taxonomy besides the thickness of spicules as suggested by Haszprunar & Heß (2005); the notch is clearly lacking at least in *Helminthope* (Salvini-Plawen, 1991; own observations).

Spicules are arranged at an angle of *c*. 45° to the longitudinal axis of the body, similar to what has been described for interstitial solenogasters (or gastrotrichs; Rieger & Sterrer, 1975); their uniform distribution speaks for a skeletal function in supporting the otherwise thin body wall and preventing injury by squeezing, as has been suggested for other meiofaunal gastropods that show this typical adaptation to the interstitial habitat (Swedmark, 1968; Jörger *et al*., 2008).

Adhesive gland

The caudal adhesive gland has also been described for *R. veranii* (e.g. Graff, 1883) and *R. marcusi* (Marcus & Marcus, 1952) and appears to be a general feature of Rhodopemorpha (judging from behaviour of live *Helminthope*; own observations), although it might not be easily detectable in fixed material (own observations). It is developed just after metamorphosis in *R. veranii* (Riedl, 1960). In its function as anchoring the animal to the substratum, the gland represents a character convergent with numerous infaunal worms and other organisms that quickly attach to and detach from sand grains if disturbed by quick water movement (Swedmark, 1964, 1968). In *Rhodope*, one can postulate a homology to either monocellular pedal glands, or to a posterior pedal gland as a discrete organ.

How well do we know Rhodope*?*

Our study on three-banded *R. rousei* n. sp. presents the second rhodopemorph species examined in full anatomical and histological detail after *R. veranii*, confirming several previous records and adding useful detail, e.g. to the knowledge of the genital system. Also, it represents the only temperate water species described so far from the southern hemisphere. However, collecting trips revealed it to be part of a southern Australian rhodopemorph fauna containing further undescribed morphospecies based on colour (N.G.W., unpubl.).

In general, there appears to be much diversity to be discovered among these minute and apparently quite rare slugs. The fact that at least *R. veranii* from Rovinj, Croatia, shows direct development and crawl-away larvae (Riedl, 1960) indicates low dispersal capabilities, strong tendency to localized speciation and perhaps high numbers of cryptic species, as was recently shown for meiofaunal acochlidians (Neusser, Jörger & Schrödl, 2011). On the other hand, an affinity with algae shown by some species, including *R. rousei* n. sp., might allow for rare long-range dispersal events on floating algae, as is hypothesized for the corambid nudibranchs (Martynov & Schrödl, 2011). This could help explain the presence of several undescribed *Rhodope* recorded on oceanic islands such as Madeira, Guam and the Galapagos (see Graff, 1883; Haszprunar & Heß, 2005).

The likely low dispersive capability of rhodopids, and the fact that coloration still appears to be the most practical means of separating species, hints at a possible taxonomic problem: the type species of *Rhodope*, *R. veranii*, was originally described from Messina, Sicily by Kölliker (1847), who mentioned a red transverse band only (see also Bronn & Keferstein, 1862–1866). All later studies of *R.* '*veranii*' were however done with specimens from the northern Adriatic (Trieste, Italy or Rovinj, Croatia), all showing the 'typical' crimson red transverse bar but elongated posteriorly by a longitudinal stripe (Graff, 1883; Böhmig, 1893; Riedl, 1959, 1960). The identity of these specimens as *R. veranii* has not been questioned by previous authors, but it might well be that this best-known *Rhodope* species is not conspecific with the type *R. veranii*.

This demonstrates that the rhodopemorphs still pose many questions and that further anatomical and molecular research is greatly needed.

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Chapter 8. **Brenzinger B**, Haszprunar G & Schrödl M (2013): **At the limits of a successful body plan–3D microanatomy, histology and evolution of *Helminthope* (Mollusca: Heterobranchia: Rhodopemorpha), the most worm-like gastropod.** *Frontiers in Zoology*, **10:** doi:10.1186/1742-9994-10-37.

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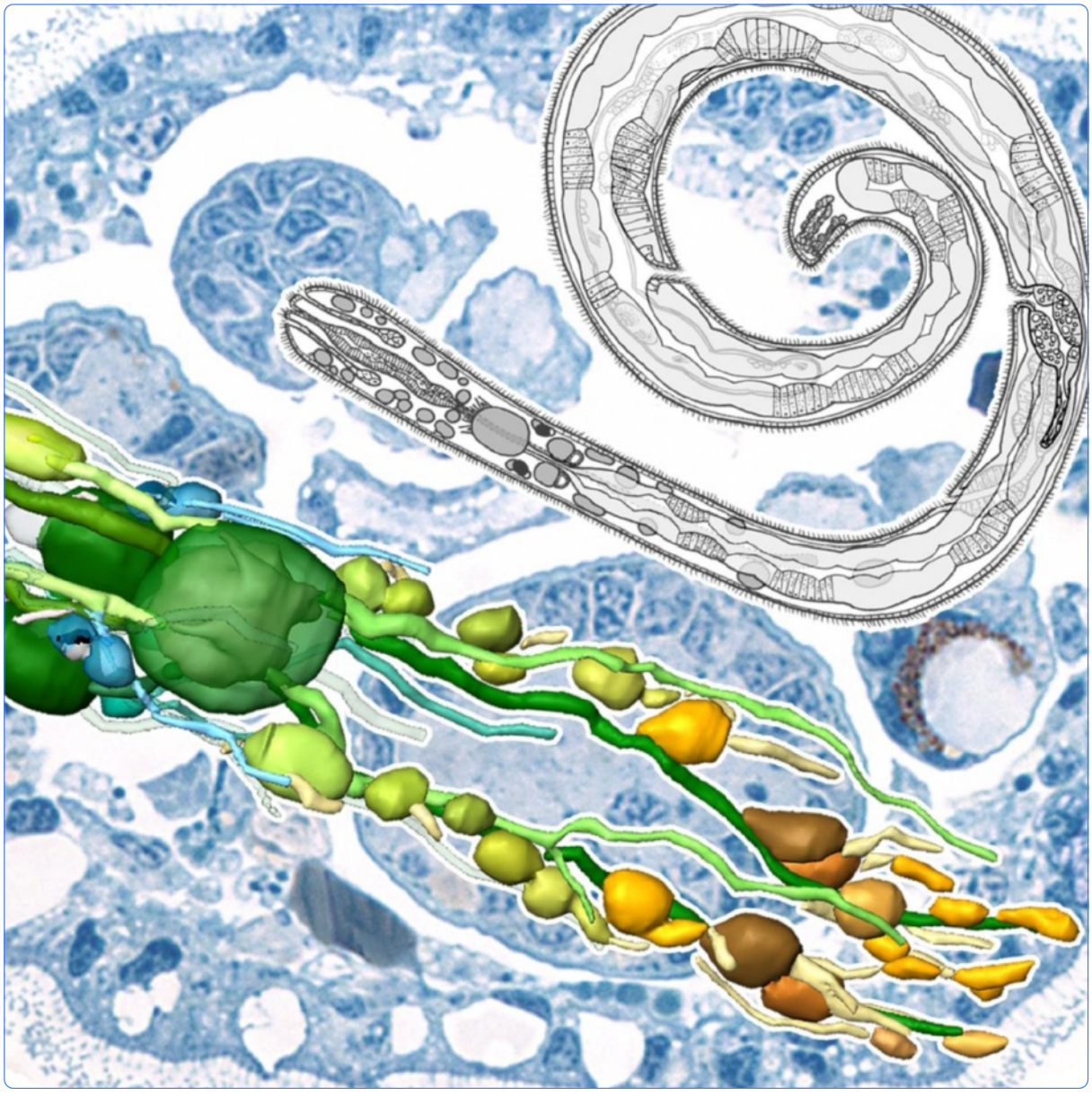

At the limits of a successful body plan – 3D microanatomy, histology and evolution of *Helminthope* (Mollusca: Heterobranchia: Rhodopemorpha), the most worm-like gastropod

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At the limits of a successful body plan – 3D microanatomy, histology and evolution of *Helminthope* (Mollusca: Heterobranchia: Rhodopemorpha), the most worm-like gastropod

Bastian Brenzinger[1,2*], Gerhard Haszprunar[1,2] and Michael Schrödl[1,2]

Abstract

Background: Gastropods are among the most diverse animal clades, and have successfully colonized special habitats such as the marine sand interstitial. Specialized meiofaunal snails and slugs are tiny and worm-shaped. They combine regressive features – argued to be due to progenetic tendencies – with convergent adaptations. Microscopic size and concerted convergences make morphological examination non-trivial and hamper phylogenetic reconstructions. The enigmatic turbellarian-like Rhodopemorpha are a small group that has puzzled systematists for over a century. A preliminary molecular framework places the group far closer to the root of Heterobranchia – one of the major gastropod groups – than previously suggested. The poorly known meiofaunal *Helminthope psammobionta* Salvini-Plawen, 1991 from Bermuda is the most worm-shaped free-living gastropod and shows apparently aberrant aspects of anatomy. Its study may give important clues to understand the evolution of rhodopemorphs among basal heterobranchs versus their previously thought origin among 'higher' euthyneuran taxa.

Results: We describe the 3D-microanatomy of *H. psammobionta* using three-dimensional digital reconstruction based on serial semithin histological sections. The new dataset expands upon the original description and corrects several aspects. *Helminthope* shows a set of typical adaptations and regressive characters present in other mesopsammic slugs (called 'meiofaunal syndrome' herein). The taxonomically important presence of five separate visceral loop ganglia is confirmed, but considerable further detail of the complex nervous system are corrected and revealed. The digestive and reproductive systems are simple and modified to the thread-like morphology of the animal; the anus is far posterior. There is no heart; the kidney resembles a protonephridium. Data on all organ systems are compiled and compared to *Rhodope*.

Conclusions: *Helminthope* is related to *Rhodope* sharing unique apomorphies. We argue that the peculiar kidney, configuration of the visceral loop and simplicity or lack of other organs in Rhodopemorpha are results of progenesis. The posterior shift of the anus in *Helminthope* is interpreted as a peramorphy, i.e. hypertrophy of body length early in ontogeny. Our review of morphological and molecular evidence is consistent with an origin of Rhodopemorpha slugs among shelled 'lower Heterobranchia'. Previously thought shared 'diagnostic' features such as five visceral ganglia are either plesiomorphic or convergent, while euthyneury and a double-rooted cerebral nerve likely evolved independently in Rhodopemorpha and Euthyneura.

Keywords: Meiofauna, Paedomorphosis, Progenesis, 3d Reconstruction, Euthyneura, Opisthobranch, Pulmonate, Morphology, Phylogeny, Histology

* Correspondence: bastian.brenzinger@arcor.de
[1]SNSB - Bavarian State Collection of Zoology, Münchhausenstr. 21, Munich 81247, Germany
[2]Department Biology II and GeoBio-Center, Ludwig-Maximilians-Universität München, Großhaderner Str. 2, Planegg-Martinsried 82152, Germany

Introduction

Gastropods are considered to be one of the most diverse major animal groups with respect to ecology and morphology and are the most species-rich taxon outside the arthropod subgroups (see [1,2]). Most gastropods are smaller than 5 millimeters (e.g. [3,4]).

Mesopsammic or meiofaunal gastropods commonly mark this lower size limit. They occupy the microscopic interstices between sand grains of marine subtidal habitats worldwide [5]. Life in these minute spaces between sand grains constrains anatomy, and these taxa commonly show convergent morphologies with other meiofaunal organisms (called 'meiofaunal syndrome' herein). This involves a modified body plan with reduction or loss of pigmentation and body appendages (tentacles, shell, gill), an elongation of the body towards a worm-like shape, development of strong epidermal ciliation, adhesive abilities, and the repeated evolution of calcareous spicules as a presumed secondary 'skeleton' [6-10]. Other characters are the production of comparatively few but large eggs besides means of direct sperm transfer such as spermatophores or stylets, and the formation of additional 'accessory' ganglia in the nervous system. The evolution of several characters and the reduction of size were assumed to be driven by paedomorphosis [11].

There are several lineages of usually shell-less meiofaunal gastropods belonging to the Heterobranchia Gray, 1840. The study of heterobranch phylogeny has recently been revitalized by molecular approaches [12-16]. This taxon covers roughly half of gastropod diversity and contains the majority of 'seaslugs', besides all lung-breathing land snails and their aquatic relatives [17,18]. Currently there are less than 100 described meiofaunal heterobranchs (e.g. [7,19]). They belong to at least six independent lineages of seaslugs including some rhodopemorphs, aeolidioidean nudibranchs, cephalaspideans, sacoglossans, and most acochlidians (e.g. [20-26]). Diversity can be expected to be much higher and undescribed species can commonly be found in sand samples from poorly studied areas – these being most of the world [27,28].

The Rhodopemorpha Salvini-Plawen, 1991 [29] or Rhodopidae von Ihering, 1876 [30] is a small group of enigmatic, minute turbellarian-like seaslugs showing characters of the 'meiofaunal syndrome', such as the possession of subepidermal spicules. The group deviates much from the general gastropod body plan in completely lacking typical external features such as a shell, mantle cavity, a demarcated foot, visceral sac or tentacles, or the typical gastropod radula [31,32]. Owing to this, the taxonomic history of the group has been much matter of debate. The best-known species, *Rhodope veranii* Kölliker, 1847 [33] lives in the littoral of the Mediterranean [32,34,35]. It was originally placed among nudibranch seaslugs, then redescribed as a flatworm [36], and later placed variously among soleoliferan pulmonate slugs, back among doridoidean nudibranchs, or outside 'higher' heterobranchs [29,37-41]. In total, there are only five described species of *Rhodope* from littoral and also mesopsammic habitats around the world (see [32,42]), and little is known about their biology. Recent sampling efforts have discovered at least as many additional morphospecies, according to pigmentation patterns (KM Jörger, NG Wilson pers. comm.).

Helminthope psammobionta Salvini-Plawen, 1991 currently is the only described member of the genus [29]. It is a meiofaunal species known only from shallow subtidal sand of Bermuda (western Atlantic). This unpigmented slug represents one of the most aberrant free-living gastropods and an extreme case of adaptation to the interstitial. Living specimens are at first glance hardly recognizable as gastropods: individuals are described as between 1 and 2.5 mm long, externally featureless thread-like worms, with a circular cross-section of 60 to 150 μm [29]. *Helminthope* can be distinguished from other interstitial 'worms' such as nemerteans by the combination of comparatively slow, sinuous movement (ciliary gliding, but never backwards), the presence of numerous curved subepidermal calcareous spicules, its conspicuous paired statocysts, and (if detectable) the asymmetric right location of body openings, owing to the original gastropod torsion. In the literature, animals resembling *Helminthope* are only recorded from the southeastern United States (as *Rhodope* sp., see [9,43]). However, recent samplings have also retrieved undescribed species from other subtropic or tropic seas (KM Jörger, NG Wilson, pers. comm.; BB, MS - own unpublished data), some of which possess unique cross-shaped spicules and may be a third, still unnamed lineage of Rhodopemorpha (see [9,42]). This indicates that the genus is much more widespread than previously thought.

Helminthope was originally placed among Rhodopemorpha [29], which was later doubted on the basis of ultrastructural characters [41]. Preliminary molecular data recover Rhodopemorpha as monophyletic and place the slug taxon as part of the still unresolved but paraphyletic 'lower Heterobranchia' or 'Allogastropoda'. More specifically, Rhodopemorpha is currently indicated as sister to the Murchisonellidae Casey, 1904 [44], a taxon of minute marine snails with high-spired shells that can be retraced from fossils back to the Triassic [45]. This phylogenetic position is far from the previously suggested origins among 'higher' heterobranchs, the Euthyneura Spengel, 1881. These comprise members with more or less detorted, i.e. 'euthyneuran' nervous systems and were also named Pentaganglionata Haszprunar, 1985 due to their possession of five ganglia on the visceral

loop [17], characters that have been given much weight in traditional taxonomy of gastropods. This leads to the observation that rhodopemorphs are, in an anatomical sense, unambiguously 'euthyneurous' and 'pentaganglionate' (according to the original description of *Helminthope* and data on *Rhodope*, [35]), but not a member of the namesake clades [15].

Due to their small size, examinations of micromolluscs are often limited to SEM study of hard parts like shells or radulae. If these features are lacking as in Rhodopemorpha, histological examination is a useful tool to characterize anatomical features. Computerized three-dimensional reconstruction facilitates understanding of complex anatomical features and can be based on histology (besides other methods), thus including information on the level of tissues and often even cells. Studies based on serial semithin sections have lately provided systematists with reliable and detailed anatomical datasets of complex organs or even entire organisms of minute taxa, improving knowledge of species that often occupy key positions in otherwise proposed phylogenies. In gastropod research, such studies have been published mainly for minute Heterobranchia (e.g. [22,24,46-53]).

In this paper, we explore at a semi-thin histological scale the 3D-visualized microanatomy of *Helminthope psammobionta*, correcting and supplementing the original description ([29], Table 1) and establishing a detailed and comprehensive dataset for comparison to *Rhodope.* This enables us to characterize presently known rhodopemorph genera. We discuss rhodopemorph evolution towards extreme body shape via putative progenetic processes. Finally, we summarize current heterobranch phylogeny and discuss placement of rhodopemorphs and compare anatomy of rhodopemorphs to other heterobranchs, in order to reconstruct and discuss their phylogenetic position and evolution as "lower" versus "higher" heterobranchs.

Results

General morphology and histology

Examined individuals of *Helminthope psammobionta* were between 1 and 3.5 mm long and roughly circular in cross-section, with a diameter of 80 to 100 μm in extended specimens to nearly 200 μm in a contracted 1.5 mm specimen. The body is completely vermiform and lacks distinction of a head, foot, mantle cavity, or visceral sac (Figures 1 and 2). The head end is rounded and slightly wider than the rest of the body; it appears not to be fully retractable. The posterior end is ventrally flattened in crawling specimens. Specimens isolated in petri dishes crawl slowly (much slower than flatworms in the same sample but similar to certain nemertines) and move their body in a sinuous fashion, with the head moving from side to side. Disturbed specimens contract slightly, but curl up at the same time (Figures 1A and 3A'). Most major internal organs are visible in live specimens, especially ganglia, statocysts and spicules, given adequate illumination.

At least in histological sections, several body openings can be discerned. The mouth opens terminally on the snout but is hard to detect due to its small size. Two small ciliated pits (discernible only in histological sections) located at the sides of the tail indicate the caudal adhesive gland. The other body openings are strongly ciliated and located along the right body side: the genital opening at approximately one quarter, the nephropore at 2/5, and the anus at 4/5 of the total length (Figures 1A and 2).

Table 1 Differences between originally described characters of *Helminthope psammobionta* and results of this study

	Salvini-Plawen, 1991 [29]	This study
Optic and buccal ganglia innervated by	branches of 'terminal cerebropleural connective'*	opg: optic nerve parallel to N4 bg: ventral sides of cpg
Buccal ganglia located	behind statocysts/pedal ganglia	anterior to pedal ganglia
Pedal ganglia	with pronounced anterior lobes	spheroid
Visceral = abdominal ganglion	with 'chiasma of fibres' indicating streptoneury	?without traces of streptoneury
Paired visceral nerves	with anterior-running branches [29:Figure 4]	not branching
Postcerebral accessory ganglia	not described	on N4, pedal nerve, ?opgn
Vesicle filled with spermatozoa is	a 'spermatheca' distal to nidamental glands	an ampulla proximal to nidamental glands
Gonad	'appears ramified'; protandric, possibly gonochoric	~ tubular; hermaphroditic (possibly protandric)
Externally visible tube below CNS is	anteriormost part of genital system (genital opening 'still appears to be absent')	single tubular salivary gland
Ciliated opening at right body side	is '(reduced) mantle cavity' (= anus and protonephridiopore)	is genital opening
Intestine located	approx. 100 μm behind visceral ganglion	in posterior fifth of body
Ventroterminal adhesive gland	not detected/ missing	present

Abbreviations as in Figure 1. (*: should be pleuropedal connective?).

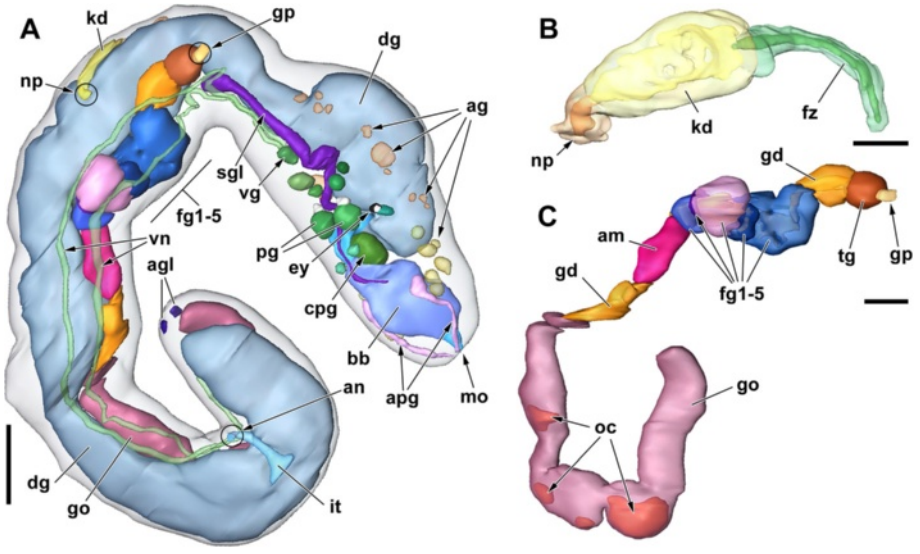

Figure 1 3D reconstruction of *H. psammobionta* (ZSM Mol-19992019/2) showing organization of major organ systems. Anterior to the right. **A**: Right view of complete, moderately contracted specimen. **B**: Kidney of same specimen, dorsal view. **C**: Reproductive system. Scale bars: **A**, 100 μm; **B**, 25 μm; **C**, 50 μm. Abbreviations: ag, accessory ganglia; agl, caudal adhesive gland; am, ampulla; an, anus; apg, anterior pedal glands; bb, buccal bulb; cpg, cerebropleural ganglion; dg, digestive gland; ey, eye; fg1-5, female glands (proximal to distal); fz, presumed filter zone; gd, (undifferentiated) gonoduct; go, gonad; gp, genital pore; it, intestine; kd, kidney; mo, mouth opening; np, nephropore; oc, oocytes; pg, pedal ganglia; sgl, salivary gland; tg, 'terminal' gland; vg, visceral ganglion; vn, visceral nerves.

The epidermis is strongly ciliated all around, with multiciliated cells, which are slender and contain a large and tall nucleus. Additionally, there are at least two distinct types of glandular cells: one type is barrel-shaped and filled with densely packed globules of pink-staining secretion, the apical opening wide and irregular ('1' in Figure 4C). The other type is very numerous and almost spherical (with a flattened, basal nucleus and large, clear or sometimes homogeneous grey vacuole opening through a terminal pore) ('2' in Figure 4C).

Below the epidermis, a variety of distinct cells surrounds the body cavity that contains the internal organs. One type of cell is largely oval and filled with numerous round blue droplets. Another type is large, amorphous and filled with a homogeneously stained, dark grey substance ('3' in Figure 4D).

In sections, spicule cells are discernible by the transparent spicule cavities enclosed by an irregular cell wall. They are located just beneath the epidermis (Figure 4C). The spicules are bent at an angle of approximately 160°; the cell's nucleus is positioned inside this bend. Judging from live photographs, the well-visible spicules have a corrugated surface, especially towards their tips. Spicules are largely sorted at an angle of 45° to the longitudinal axis of the body.

The anterior digestive tract is flanked by paired anterior 'pedal' glands (pink-staining duct and lighter posterior part with widely-spaced nuclei) that open just ventrally to the mouth within a pad-like structure (see Figures 1A, 3B and 4A,B).

The caudal adhesive gland consists of a horseshoe-shaped cluster of cells in the posteroventral part of the tail. The gland opens through paired ciliated depressions on the lateroventral sides of the tail (Figures 1A and 2). While the gland's cells themselves are difficult to detect, the ciliated pits are characterized by small strings of blue-staining secretion that project from pores through the epidermis (Figure 5G). In the reconstructed specimen (Figure 1A), the tail end is damaged so that parts of the gland are missing.

Muscle fibers are stained bright blue and are associated with the basement layers of all epithelial organs. A conspicuous pair of muscles runs along the ventral midline; both muscle bundles are fused between the pedal ganglia and the visceral ganglion (Figure 3B). The fibers attach to the anterior pedal glands anteriorly, posterior, they run along the visceral cords and the paired visceral nerve.

Digestive system

The digestive system consists of a histologically uniform anterior part with enlarged midpiece (called buccal bulb herein) and associated glands, followed by the tubular digestive gland which ends blindly close to the tail, and the ciliated intestine near the end of the body (Figures 1A and 2).

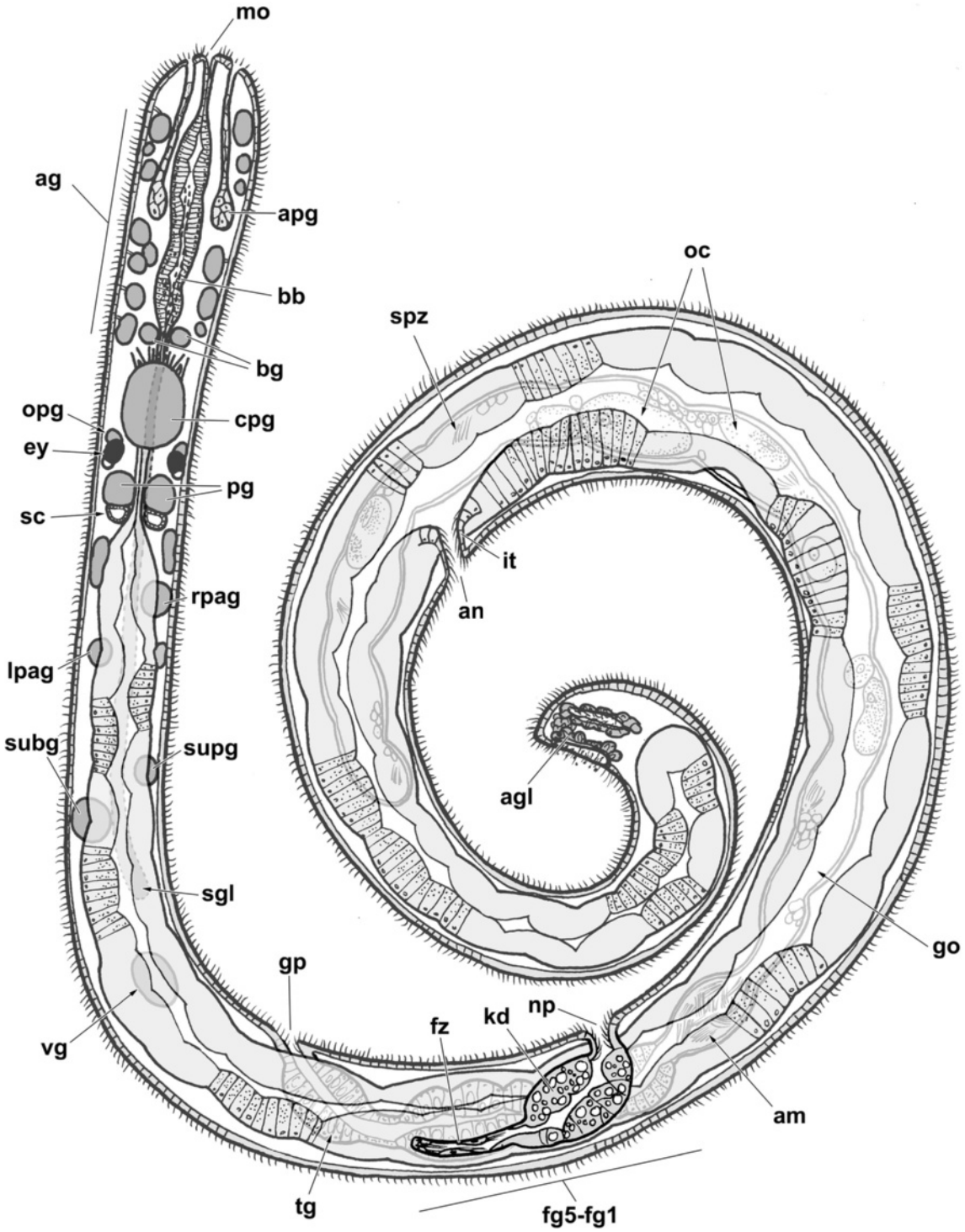

Figure 2 Schematic dorsal view of *H. psammobionta* (based on specimen shown in Figure 3A'). Abbreviations: ag, accessory ganglia; agl, caudal adhesive gland; am, ampulla; an, anus; apg, anterior pedal glands; bb, buccal bulb; bg, buccal ganglia; cpg, cerebropleural ganglion; dg, digestive gland; ey, eye; fg1-5, female glands (proximal to distal); fz, presumed filter zone; go, gonad; gp, genital pore; it, intestine; kd, kidney; lpag, left parietal ganglion; mo, mouth opening; np, nephropore; oc, oocytes; opg, optic ganglion; pg, pedal ganglia; rpag, right parietal ganglion; sc, statocyst; sgl, salivary gland; spz, spermatozoa; subg, subintestinal ganglion; supg, supraintestinal ganglion; tg, 'terminal' gland; vg, visceral ganglion.

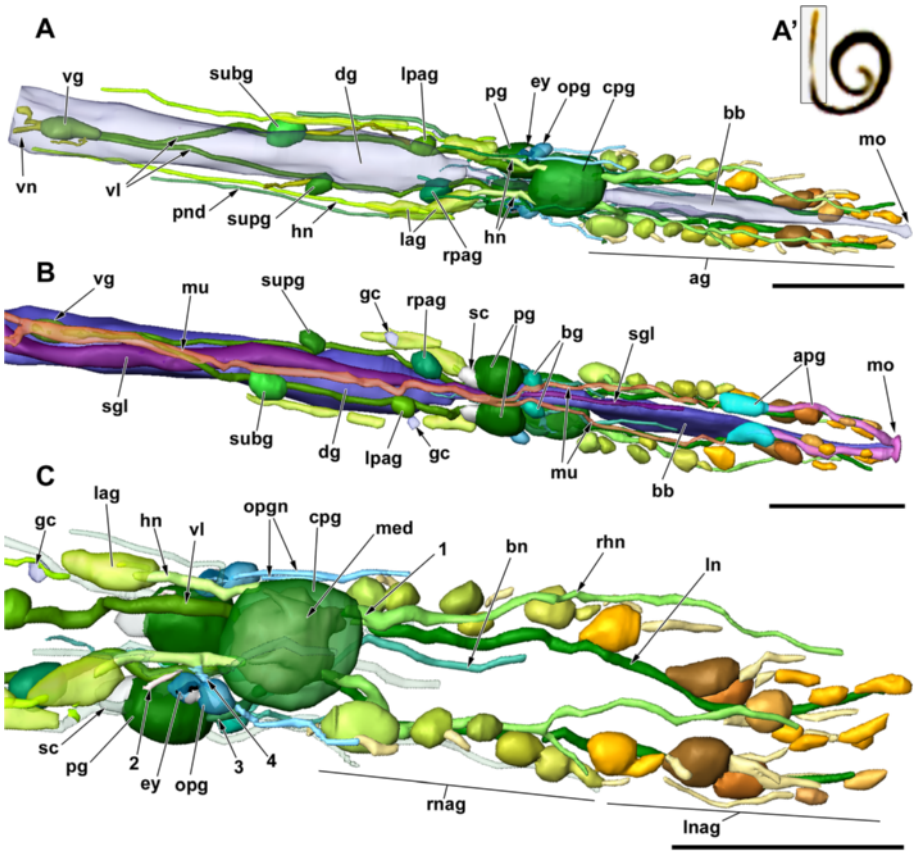

Figure 3 3D reconstruction of the anterior end of an extended *H. psammobionta* (ZSM Mol-19992020/2) showing details of the central nervous system (cns). Anterior to the right. **A**: Dorsal view of cns. Digestive system transparent, pedal nerves omitted. **A'**: The reconstructed specimen prior to sectioning, box marks region shown in this figure. **B**: Ventral view of ganglia, digestive system, and retractor muscle. Nerves largely omitted. **C**: Dorsal right view of anterior cns and details of the cerebral innervation. Pedal nerves transparent. Scale bars: all 100 μm. Abbreviations: 1, double root of rhinophoral nerve; 2, presumed pleuro-pedal connective branching from 'visceral loop'; 3, cerebropedal connective; 4, double connectives to optic ganglion; ag, accessory ganglia; apg, anterior pedal glands; bb, buccal bulb; bg, buccal ganglia; bn, buccal nerve; cpg, cerebropleural ganglion; dg, digestive gland; ey, eye; gc, bilateral 'giant cell' on headshield nerve; hn, headshield nerve; lag, accessory ganglia of headshield nerve; ln, labiotentacular nerve; lnag, accessory ganglia of labiotentacular nerve (more anterior); lpag, left parietal ganglion; med, medullary core of cerebropleural ganglion; mo, position of mouth opening; mu, ventral retractor muscle, note fused part between pedal and visceral ganglion; ogl, oral gland; opg, optic ganglion; opgn, nerves of optic ganglion; pg, pedal ganglion; pnd, dorsal pedal nerve; rhn, rhinophoral nerve; rnag, accessory ganglia of rhinophoral nerve (more posterior); rpag, right parietal ganglion; sc, statocyst; sgl, salivary gland; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve(s); vl, 'visceral loop'.

The anterior digestive tract (a derived esophagus; see [32]) is formed by a strongly ciliated epithelium of slender columnar cells filled with numerous unstained apical vacuoles, giving the epithelium a 'spongy' appearance (Figure 4D). The portion following the mouth is very thin (diameter 12 μm) before widening into the buccal bulb (laterally flattened, height approx. 60 μm) located just anterior to the cerebral nerve ring; the part following the bulb is thin again but remains histologically identical. The single, tubular salivary gland (approx. 400 μm long, 20 μm thick) is visible externally, it runs parallel to the esophagus. The posterior part of the gland consists of columnar cells containing dark violet-staining vesicles that surround a central lumen (Figure 4G). The anterior duct is so thin that is becomes undetectable along the anterior esophagus, so the exact position of its opening into the digestive tract remains unclear (Figures 1A, 2 and 3B).

The undulating digestive gland is the most voluminous organ and extends all the way to the tail end. It consists of tall columnar cells, each filled with numerous blue and fewer unstained vesicles, surrounding the unbranched central lumen. In the posterior right portion of the digestive gland there is a short sickle-shaped region of epithelium which lacks vesicles (the 'stomach' in *Rhodope*; [35]). From there, the ciliated intestine emerges and leads to the anus on the right body side, at about 4/5 of the total body length.

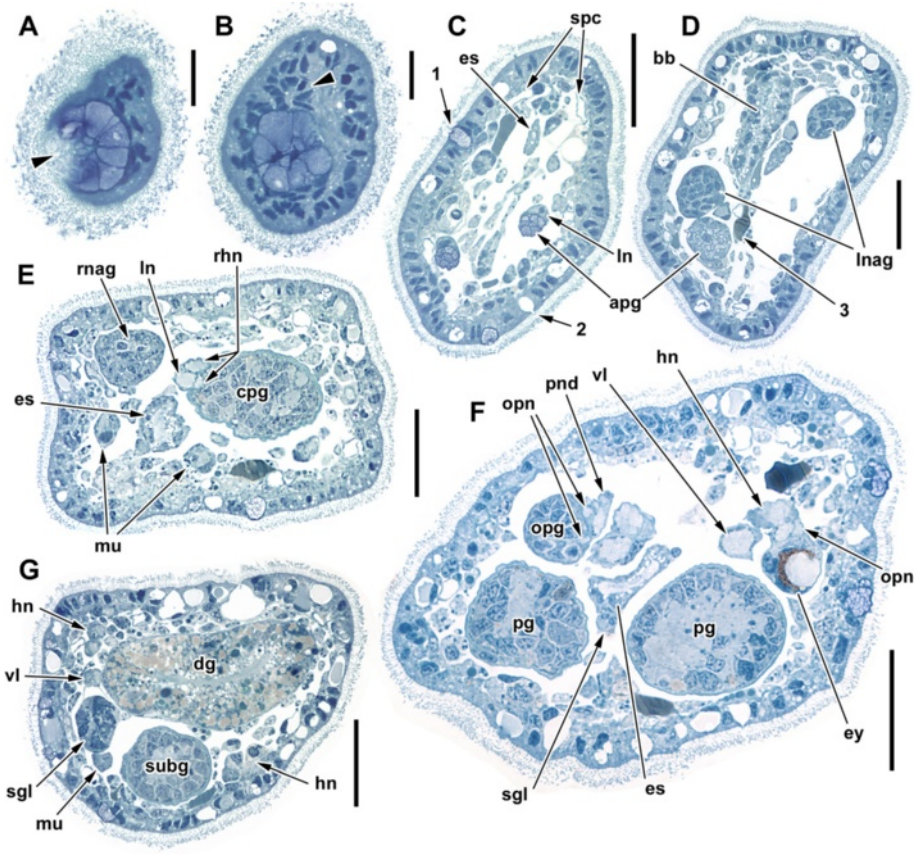

Figure 4 Semithin cross-sections showing histological aspects of the head and nervous system of *H. psammobionta*. Dorsal side to the upper right. **A**: Snout tip with opening of anterior pedal gland pad (arrowhead). **B**: Nuclei surrounding mouth opening (arrowhead) dorsal of mouth pad. **C**, **D**: Anterior head and various cell types (1-3). **E**: Front of cerebropleural ganglion (cpg). **F**: Posterior end of cpg and optic ganglion. **G**: Portion of visceral loop. Scale bars: **A**-**B**, 100 μm; **C**-**F**, 25 μm. Abbreviations: 1, pink-staining epidermal gland; 2, vacuolated epidermal gland; 3, amorphous cell; apg, anterior pedal glands; bb, buccal bulb; cpg, cerebropleural ganglion; dg, digestive gland; es, esophagus (thin portion); ey, eye; hn, headshield nerve; ln, labiotentacular nerve; lnag, accessory ganglion of labiotentacular nerve; mu, ventral muscle; opg, optic ganglion; opn, nerves to optic ganglion; pg, pedal ganglion; pnd, dorsal pedal nerve; rhn, rhinophoral nerve (double roots); rnag, accessory ganglion of rhinophoral nerve; sgl, salivary gland; spc, spicule cells; subg, subintestinal ganglion; vl, visceral loop.

Kidney

The excretory system consists of a proximal duct lying freely in the hemocoel and of the bag-like kidney (90 μm) which connects directly to the nephropore. There is no associated heart or pericardium. The anteriorly located proximal duct (about 70 μm long, 8 μm wide) consists of flat, multiciliated cells that surround a central lumen ('filter zone' in Figures 1B, 2 and 5E,F). Parts of the wall are thin but nevertheless distinct (indicating a strong basal lamina); bundles of long cilia reach down the duct towards the kidney. The kidney itself is characterized by a thickened, irregular inner wall with typical unstained, round vacuoles (Figure 5D). The kidney connects directly to the ciliated nephropore located at about 2/5 of the body length.

Reproductive system

The genital system of *Helminthope* is hermaphroditic and monaulic, i.e. a simple duct with one terminal opening. It consists of the tubular gonad followed by the ampulla, then a succession of 5 histologically separate (nidamental = eggmass-forming) glands plus a terminal (spermatophore-forming?) gland close to the ciliated genital opening (Figure 5A,B).

The gonad is an undulated tube that extends from the tail end to approximately half of the body length. It is located below the digestive gland. In the examined mature specimens it is densely filled with a variety of gamete precursors and ripe gametes, there is no remaining discernable lumen. Large oocytes can sometimes be identified by their larger nuclei and accumulation of blue-stained yolk droplets, some eventually filling most of the gonad's diameter. The examined specimens never contained more than three of these fully formed eggs. Spermatozoa and their precursors (spermatids) are conspicuous in possessing an intensely dark-staining respectively screw-shaped or teardrop-shaped nucleus. Clusters

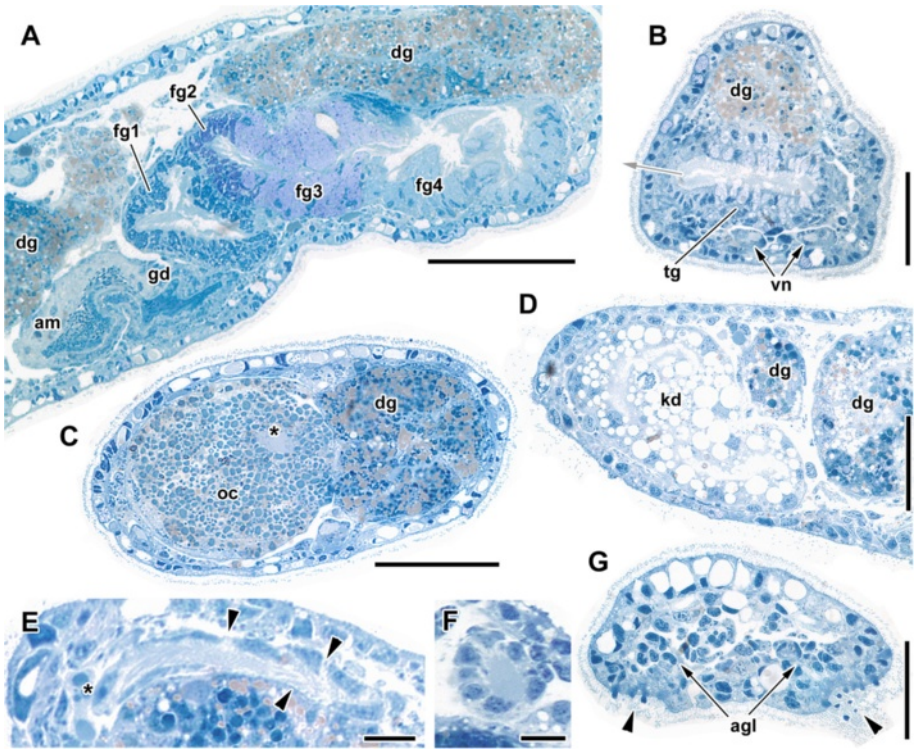

Figure 5 Semithin sections showing histological aspects of the posterior half of the body of *H. psammobionta*. **A**: Longitudinal section of reproductive system showing female glands. Anterior at right. **B**: Cross-section close to genital opening (grey arrow) and terminal gland. **C**: Yolky oocyte, nucleus indicated by asterisk. Dorsal at right. **D**: Kidney. Dorsal at left. **E**: Filter zone of kidney, sectioned longitudinally. Asterisk highlights nucleus of filter cell, arrowheads mark thin parts of wall. Dorsal at left. **F**: Cross-section through filter zone. **G**: Tail end showing ciliated openings of caudal adhesive gland (note emerging blue 'pegs', arrowheads). Scale bars: **A**-**D**, 50 μm; **E**-**F**, 10 μm; **G**, 25 μm. Abbreviations: agl, nuclei of adhesive gland cells; am, ampulla; dg, digestive gland; fg1-fg4, nidamental glands (proximal to distal); kd, kidney; oc, oocyte; tg, 'terminal' gland; vn, visceral nerve.

of spermatids were found mainly in the posterior half of the gonad; ripe spermatozoa in bundles of up to 20 are found further anterior.

Following the anterior end of the gonad and a piece of undifferentiated gonoduct (ciliated, with outer muscular layer), the ampulla is a widened part that is filled densely with ripe spermatozoa (Figure 5A).

Distal to the ampulla – at approximately half of the body length – the gonoduct wall is strongly glandular, forming five consecutive nidamental glands (Figure 5A). The first gland is a short, bag-like expansion of one side of the gonoduct, its cells show grainy vesicles staining dark blue. This is followed by a small gland 2 which shows similar grains but that stain dark violet. Gland 3 is relatively large and bulbous compared to the other glands, it stains homogeneously light pink. Gland 4 is shorter again and stains homogeneously light blue. Gland 5 is the largest; it also stains light blue but contains large interspersed cells with an unstained vacuole. Following a short piece of unmodified gonoduct, there is a final (terminal) gland which is barrel-shaped and contains columnar glandular cells with pale pink-staining vacuoles (Figure 5B). The ciliated gonopore opens at approximately 1/4 of the body length.

Central nervous system (CNS)

The CNS of *Helminthope psammobionta* consists of the spherical cerebropleural ganglion (cpg), the paired pedal, buccal and optic ganglia ventral or lateral to the cpg and five ganglia on the very long visceral loop more posterior (Figures 2, 3 and 6). Numerous large accessory ganglia are associated with the nerves emerging from the cpg, smaller ones are found on a pedal and optic ganglion nerve. The eyes are located laterally and behind the optic ganglia; the large and conspicuous statocysts sit on the posterior sides of each pedal ganglion. All of these structures are visible in living specimens with transmitted light. Histological sections show that the cpg, pedal and buccal ganglia – and, to a lesser extent the visceral loop ganglia – contain a distinct central region formed by nerve fibers (medulla) and an outer cortex containing nuclei of neurons. In the other ganglia, neurons fill the entire ganglion evenly. All ganglia are enclosed in a

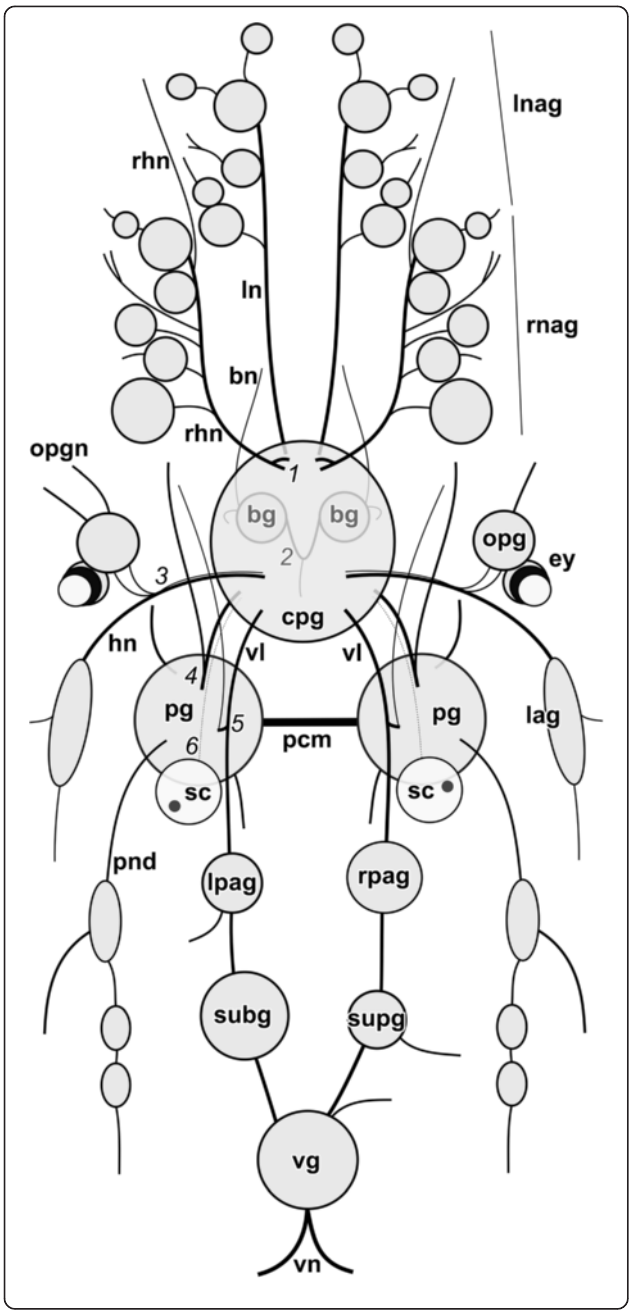

Figure 6 Schematic dorsal view of the central nervous system of *H. psammobionta*. Anterior side is up. Abbreviations: 1, double root of rhinophoral nerve; 2, buccal commissure with median nerve; 3, presumed headshield nerve with parallel nerve leading into double optic connectives; 4, cerebropedal connective with parallel static nerve and anterior pedal nerve at its base; 5, visceral loop with branch forming presumed pleuropedal connective; 6, static nerve running parallel to cerebropedal connective; bg, buccal ganglia; bn, buccal nerve; cpg, cerebropleural ganglion; ey, eye; hn, headshield nerve; lag, (lateral) accessory ganglia of headhield nerve; ln, labiotentacular nerve; lnag, accessory ganglia of labiotentacular nerve (more anterior); lpag, left parietal ganglion; opg, optic ganglion; opgn, nerves of optic ganglion; pcm, pedal commissure; pg, pedal ganglion; pnd, dorsal pedal nerve; rhn, rhinophoral nerve; rnag, accessory ganglia of rhinophoral nerve (more posterior); rpag, right parietal ganglion; sc, statocyst; subg, subintestinal ganglion; supg, supraintestinal ganglion; vg, visceral ganglion; vn, visceral nerve(s); vl, 'visceral loop'.

homogeneous blue-staining cellular capsule that contains few flattened nuclei.

The most conspicuous and central element of the CNS is the almost spherical complex of fused left and right cerebral and pleural ganglia (the cerebropleural ganglion, cpg; diameter about 55 μm). Histologically, it is distinctly divided into cortex and fibrous medulla (see Figure 3C). The cerebral commissure remains detectable only from the wide median connection of the medullar mass. Remnants of the pleural ganglia are only detectable as an aggregation of neurons at the posterodorsal side of the cpg. Two pairs of thick nerves emerge from both the anterior and posterior faces of the cpg: from the anterior side the rhinophoral and labial nerves (Figure 4E), from the posterior side the headshield/optic nerves (Figure 4F, fibers almost fused, origin in the mediodorsal part of the medulla) and the combined visceral loop/pleuropedal connective. From the ventral side of the cpg emerge the thin cerebrobuccal connectives (more anterior) and the thick cerebropedal connectives (medioventral) besides the thin static nerve. Numbers used below follow the nomenclature by Staubach and Klussmann-Kolb [54] and Staubach [55].

The thick rhinophoral nerve (N3, diameter 5 μm) emerges from the anterior face of the cpg more dorsal than the labial nerve. The nerve shows two equally thick roots, one of which originates close to the root of the labial nerve (Figure 4E). The rhinophoral nerve runs anteriorly along the sides of the head and terminates near the mouth. Up to six pairs of accessory ganglia (diameter 10 to 20 μm; only 2 to 3 in small specimens) attach laterally to the proximal half of the nerve, either by direct contact or by a short branching anastomosis (Figure 3C). The accessory ganglia are spherical and full of neurons, the neuropil being limited to the fibers of the rhinophoral nerve (Figure 4E).

The equally thick labiotentacular nerve (N2) emerges from the cpg more ventrally and features, in its distal

part, further (six) pairs of accessory ganglia. The more posterior accessory ganglia are thus innervated by the rhinophoral nerve, the anterior ones by the labial nerve (Figures 3C and 6). Thin nerves innervating the lateral epidermis of the snout are detectable in at least some of the accessory ganglia.

From the posterior face of the cerebropleural ganglion (anterior to the region with the presumed 'pleural' neurons) emerge the thick, paired headshield nerves (N4) (Figure 4F). The headshield nerves pass closely by the eyes on their way to the posterior flanks; each nerve features a single large, elongate ('lateral') accessory ganglion from which one nerve runs directly to the body wall, and another continues posteriorly (Figure 3A,C). This posterior branch is covered with neurons – some of them with a diameter of up to 10 μm – along much of its length and thus resembles a medullary cord.

The optic nerves run along the proximal part of each pleural nerve; the optic nerve then shows two connections to the optic ganglia (Figures 4F and 6). The spherical optic ganglia (Ø 15 μm) touch the anterior side of the eyes, but no direct nervous connection between the two could be detected. Each optic ganglion shows one, or possibly two additional medium-sized nerves that extend anteriorly.

The eyes face towards the sides (Figure 4F). Each eye consists of a spherical lens, followed by a cup-shaped pigment layer which is surrounded by a layer containing perhaps 5 or 6 nuclei (belonging to sensory cells?). The lens stains light grey/blue and is covered by a thin, apparently acellular but distinct blue membrane (a cornea?); the inner part of the lens shows a slight, irregular grey fringe (sensory microvilli?) (Figure 4F). The pigment layer consists of black or dark brown pigment granules. Some sections show a faint gap inside the pigment layer which might indicate that the pigment is contained within only two cells. The nuclei below the pigment cup presumably belong to the sensory cells of the eyes and the pigment cells; however, clear boundaries between the nuclei-bearing cells were not discernible in semi-thin sections.

Posterior to the headshield nerves emerge the paired visceral cords that connect to the ganglia on the visceral loop. The cords also appear to contain fibers of another origin, because after a short stretch a thick nerve branches off and connects to the posterodorsal side of the pedal ganglion ('2' in Figure 3C). Since there is no other connection between the cpg and the pedal ganglia except the more anterior cerebropedal connective, this connection should be the pleuropedal connective.

On the ventral side of the cpg, long and thin cerebrobuccal connectives emerge anteroventrally. The paired buccal ganglia usually lie more anterior and show two nerves: a paired one emerges from the base of each cerebrobuccal connective and runs along the buccal bulb ('bn' in Figure 3C); an unpaired nerve extends from the middle of the buccal commissure and extends posteriorly ('2' in Figure 6).

The paired pedal ganglia are the second largest ganglia (diameter 30 μm, 45 μm long). They are interconnected by the comparatively long pedal commissure, and together with the cpg form the cerebral nerve ring around the digestive tract. There are four connections: the paired cerebropedal connectives, and the presumed pleuropedal connectives that are present as short branches splitting off of the anterior portion of the visceral loop, approximately 50 μm behind the cerebropleural ganglion. From the bases of all connectives, thin (pedal?) nerves extend anteriorly. There are three further pairs of pedal nerves: one anterior, one posteriomedian, and one posterodorsal. The last pair extends along the flanks and features at least three small, ill-defined accessory ganglia similar to those found on the pleural nerves.

The statocysts are large, hollow spheres (∅ 15 μm) attached to the posterior face of each pedal ganglion (Figure 3B,C) and are enclosed in the same connective sheath. Each statocyst is formed by a wall of flat epithelial cells that the surround the fluid-filled lumen; there is a single spherical statolith. The presumed static nerve (a cerebral nerve) runs parallel to the cerebropedal connective, but is thin and could not be traced entirely.

The long visceral loop is untorted, i.e. euthyneurous. It features five widely-spaced ganglia – the most posterior one (the visceral ganglion) is located approximately 350 μm behind the cpg, or at one quarter of the body length. Both ganglia on the right visceral cord are located approximately 20 μm further anterior than their counterparts on the left cord (Figure 3A,B). The first pair of ganglia is separated from the back of the cpg and the front of the second pair by roughly 70 μm; the second pair is separated from the visceral ganglion by about 130 μm. The first two ganglia on the visceral loop are the left and right parietal ganglia; the right one is slightly larger (25 μm long vs. 20 μm), whereas the left one shows a thin posterior nerve (see Figures 6 and 3A). Both ganglia show at least two neurons that are larger than the others, and contain a large nucleus (but not 'giant' neurons). Second in order are the subintestinal (left) and supraintestinal ganglia (right); this time the left ganglion is larger (33 vs. 24 μm), but the right one shows a posterior nerve. The subintestinal ganglion contains two large neurons. The visceral ganglion is located medially, at the end of the visceral loop where the left and right visceral cords meet; the ganglion is about 45 μm long but elongate, it again contains two to three large neurons. A thin nerve emerges from the anterior right side, the thick visceral nerve emerges posteriorly. This conspicuous nerve splits into two equally thick branches

Table 2 Comparison of divergent characters between *Rhodope* spp. and *Helminthope psammobionta*

	Helminthope psammobionta	*Rhodope* spp.
Approx. length/width ratio (contracted - crawling)	8-25	3-9
Habitat	interstitial	littoral, interstitial (some both?)
'Vesicle' system	absent	present
Glands of the foot sole	lacking	generally present
Vestigial pharynx	not present	present
Anterior pedal = 'oral' glands	paired, tubular	paired, follicular (mixed with 'true' oral glands?)
Salivary glands	single, tubular	paired, follicular
Anterior lobe of digestive gland	lacking (or axial connection to esophagus)	extends beyond CNS
Position of intestine/anus	at 4/5 of body length, far from nephropore	at 1/3 of body length, close to nephropore
Form of kidney	sac-like, with proximal filtering duct	two thin branches with interspersed filtering knobs
Form of gonad	tubular, gametogenesis not spatially separated	2-3 posterior testicles, several anterior ovarial follicles
Number of 'terminal' glands in gonoduct	1	2
Eyes	with spherical lens, separate from cpg	with corpuscular lens, sitting
Rhinophoral nerve (double roots)	without basal ganglion, with large accessory ganglia	with basal ganglion, accessory ganglia small (or lacking?)
Labiotentacular nerve	undivided, with large accessory ganglia	bifurcated, accessory ganglia small or lacking
Postcerebral accessory ganglia	on 'pleural' nerves, also pedal nerves and possibly optic	none?
Separation of cerebral and pleural ganglia detectable	only internally	external fissures detectable in some species
Free visceral loop ganglia	5	1 (adult)
Scenario for ganglion nomenclature (parentheses indicate fusion)	(cg-plg)-1-2-3-4-5-(plg-cg)	(cg-plg-1)-(2–3)-(4-5-plg-cg) or (cg-plg-1-2)-3-(4-5-plg-cg)

Abbreviations as in Figures 2 and 6 except: cg, cerebral ganglion; plg, pleural ganglion.

just after leaving the ganglion (Figure 3A). Both branches run parallel along the ventral side of the animal (Figure 1A), and are accompanied by muscle fibers throughout their entire length. Judging from histological sections, the visceral nerves do not branch before terminating in the tail end, near (or in?) the adhesive gland.

Discussion

Helminthope psammobionta is an extreme case among marine meiofaunal heterobranchs. It lacks almost all external characters that could identify it as a gastropod, and is one of the most aberrant free-living gastropods. Only the location of the genital, kidney and anal openings on the right body side are obvious remnants of the original gastropod body plan with torsion and resulting asymmetry. Without hard parts such as a radula and shell, only internal characters can help in evaluating the relationships of *Helminthope* to *Rhodope* (Table 2), and to other heterobranchs, from a morphological point of view. The original description [29] was based on characters that are visible in squeezed specimens observed under the light microscope (spicules, many ganglia, salivary gland – [29], BB, pers. obs.). Other parts of the animal (crucial connections between ganglia, nerves) needed higher resolution and superior scrutiny. Therefore, the original description of *H. psammobionta* could be corrected and supplemented considerably by combining histological investigation with 3D reconstruction of all major organ systems (see Table 1).

Helminthope psammobionta revisited - general histology

Haszprunar and Künz [41] compared ultrastructural characters of both described rhodopemorph genera, concluding that *Rhodope* showed similarities to doridoidean nudibranchs (epidermal cells with typical vacuoles, vesicle 'network' system, possession of verrucose spicules), while *H. psammobionta* lacked these characters, supporting the author's notion that both genera were not closely related. Histology does not permit identification of the diagnostic epidermal vacuoles, but confirms that *Helminthope* lacks the enigmatic 'vesicle system'. Another difference between both genera was the

'parenchymatic', compact body cavity detected in *Helminthope* [41]; this is not apparent from our histological examination – spacing of cells may be closer in *Helminthope* due to its smaller body diameter, but we conclude here that there is no fundamental difference in the body cavity of *Rhodope* species. We were not able to correlate the conspicuous amorphous 'grey patch' cells (Figure 4C,D) found in our material with Haszprunar and Künz's results. Salvini-Plawen [29] mentioned subepidermal 'platelet-like' elements. No equivalent to these were evident in our sectioned material, although many epidermal glands show vacuoles that are visible as refracting bodies in live specimens.

Anterior pedal and caudal adhesive glands

Helminthope possesses paired anterior glands (staining pink) that appear to be homologous to the equally pink-staining but diffuse and follicular glands mentioned for some *Rhodope* species (e.g., [56]). These were interpreted as 'oral' glands by previous authors [29,32]. None of the examined *Helminthope* specimens showed a connection of the glands into the digestive tract. Instead, one specimen showed a conspicuous patch (shown in Figure 4A,B) below the mouth opening through which the glands appear to open. Reinvestigation of an undescribed *Rhodope* species also shows that at least some of the diffuse pink-staining glands open at the sides of the head and not into the digestive tract (BB, pers. obs.). Therefore, we here regard these paired anterior glands not as oral glands, but as anterior pedal glands instead (see below). *Helminthope* lacks the single-celled glands that usually open through the foot sole of gastropods and can be detected as blue-staining bodies in histological examination (e.g. [57]). These glands are present along the ventral side of the body at least in *Rhodope rousei* Brenzinger, Wilson & Schrödl, 2011 [32].

Salvini-Plawen [29] noted that *H. psammobionta* does not possess a caudal adhesive gland, separating it from *Rhodope* species. However, our results show that the gland is present. It is already externally visible in whole mounts stained with Safranin (BB, pers. obs.). Its cells are inconspicuous in histological sections, but the outline of the gland can still be reliably located by the presence of characteristic 'pegs' emerging from the cell's apices, as is also the case in *Rhodope* (BB, pers. obs.). The cells histologically resemble the 'normal' unicellular pedal glands, but judging from their position may also be homologous to the posterior pedal glands of many basal heterobranchs [58].

Putative anterior and posterior pedal glands are present as distinct organ systems in many basal heterobranchs [51,58,59] but also more derived clades such as runcinaceans (*Ilbia* Burn, 1963 [60]), acochlidians or sacoglossans [22,57]. They generally open on top of the anterior pedal sole, and on the ventral side of the posterior foot sole, respectively. These glands are either paired or fused but open close together or via a common duct. The function of the posterior gland as an adhesive structure was observed in living *Helminthope* sp. from Belize: if disturbed, specimens attached themselves to the glass of a petri dish by the flattened tail end (KM Jörger, pers. comm.). Since the conspicuous paired visceral nerves terminate in/at the gland without anterior branching, the nerves may play a crucial role in controlling the adhesive mechanism but requires TEM study to investigate. Adhesive glands are convergently present in various meiofaunal organisms such as gastrotrichs, rhabdocoel flatworms and some annelids (e.g. [5,61,62]). Because these mechanisms commonly work with a double function (adhesive and detaching gland components), the double innervation of the tail end might indicate that this is the case also in rhodopemorphs.

Digestive system

The digestive system of *Helminthope* is simplified compared to that of other gastropods, but is in principle identical to that of *Rhodope*. Histological characters are highly similar (BB, pers. obs.). Both genera lack an oral tube followed by the muscular pharynx with radula typical for gastropods. Instead, they possess a derived three-part esophagus that directly joins to the mouth opening and contains a novel 'buccal' bulb which functionally replaces a pharynx (see [32]). Both genera show a tubular digestive gland with a short intestine on the right body side. *Helminthope* differs from *Rhodope* in the marked elongation of the digestive tract (Table 2: buccal bulb is more elongate, there is no cephalic 'caecum' sensu [29], intestine and anus are shifted far posterior) and by having a single, non-follicular salivary gland. *Helminthope* lacks the small sac-like cavity into which the salivary glands open in *Rhodope* (argued to be a vestigial pharynx by [32]).

The peculiar single salivary gland of *Helminthope* is identifiable as such by histological characters (cells with dark blue-staining vesicles). The opening into the digestive tract could not be located in the examined material; it could never be traced further forward than the anterior part of the esophagus but should open far anterior, if interpretation of the anterior digestive tract as an esophagus is correct. The tubular form of the gland seems to be a result of less space in the body cavity due to body elongation. Judging from its slightly dextral position in histological sections, it might refer to the ancestrally right salivary gland. In *Rhodope*, the salivary glands are still paired, consist of numerous follicles, and (likely) open into the vestigial pharynx [32]. Salvini-Plawen [29] noted the gland's visibility in live specimens but

interpreted the gland to be a distal 'genital tube', thus locating the genital opening anteroventrally and misinterpreting other body openings (see below).

The three-part esophagus with vacuolate (and therefore elastic?) epithelium is a characteristic feature of rhodopemorphs. Its bulbous middle part was suggested to function as a sucking pump, aiding the ingestion of soft-bodied food [32]. Except for Riedl's [34] successful table-top experiment in rearing littoral *Rhodope veranii* on a diet of the basal metazoan *Trichoplax* Schultze, 1883, there are still no direct observations of rhodopemorph feeding, as is often the case for micro- and meiofaunal gastropods. One specimen of *H. psammobionta* contained food remnants in the digestive gland but which resembled the general histology of the gland, indicating that food is soft to liquid. Candidates for food organisms found in the mesopsammon are large protists or metazoan eggs. Organisms feeding as 'pump-suckers' [6] are common among meiofaunal groups such as nematodes and gastrotrichs.

The digestive gland of *Helminthope* lacks a pronounced anterior-leading part (called 'caecum' by [29]) and is much more elongate but otherwise similar to that of *Rhodope* (Table 2). Riedl ([35]: Figure 23) observed the development of two digestive gland lobes from the stomach in young *Rhodope*, the anterior lobe extending beyond the opening of the esophagus. Salvini-Plawen [29] correctly noted that the connection of esophagus and digestive gland in *Helminthope* is axial ('without anterior caecum'). The anterior lobe is either not developed in *Helminthope*, or the esophagus opening is simply shifted more anterior as a result of general body elongation.

In gastropods, the stomach is defined as the area into which the esophagus enters and from which the intestine exits; lobes of the digestive gland branch from in between [18]. Riedl [35] observed that in *R. veranii*, the ring-shaped larval stomach remains as a sickle-shaped zone surrounding the proximal intestine, close but not connected to the posterior end of the esophagus. This 'stomach' can be reliably distinguished from the surrounding digestive gland by the lack of blue- and yellow-staining vesicles, as in *Rhodope* [32]. In *Rhodope*, stomach, intestine and anus are located close to the nephropore early in ontogeny ([35]: figs. 13,15). In *Helminthope*, they are far from the nephropore and located back in the animal. We speculate that in the latter the anus is formed only after some body elongation takes place, thereby effectively relocating the stomach and anus (but not the otherwise associated nephropore) towards the tail.

Reductions of the digestive system make comparison to other basal heterobranchs difficult. Murchisonellidae are known to possess a unique 'jaw apparatus' and an apparently reduced pharynx [63]. *Henrya* Bartsch, 1947 also possesses a simple, long esophagus [64], *Koloonella* Laseron, 1959 species possess a peculiar glandularized esophagus (BB, pers. obs.). A three-part esophagus with 'spongy' epithelium at least in the midpart – possibly similar to that of rhodopemorphs – is mentioned e.g. for the valvatoid *Cornirostra* Ponder, 1990 [65,58: p. 25]. The presence of a 'derived' esophagus is noted for different basal heterobranch lineages [13,14]. This may imply a more widespread phenomenon that is secondarily lost e.g. in limnic *Valvata* O.F. Müller, 1774 (according to [58]) and the architectonicoid *Omalogyra* Jeffreys, 1859 [59], genera that grouped as a monophylum in the study by Dinapoli and Klussmann-Kolb [13]. In the marine valvatoidean *Hyalogyrina* Marshall, 1988 [51: fig. 12], the esophagus shows a histology similar to rhodopemorphs but also possesses folds not present in the latter.

Excretory system and lack of a heart

Salvini-Plawen originally described the kidney of *Helminthope* to be a 'protonephridium' positioned 'about 100 μm behind the visceral ganglion' [29: p. 307]. This fits with our results which indicate that the kidney contains two distinct parts: a proximal duct with multiciliated cells forming a ciliary flame and histologically distinct basal membrane, and a distal part with the diagnostic vacuolated epithelium. This implies that the proximal duct may function as a filter, with modification of the primary urine taking place in the vacuolated part. In *Rhodope*, the peculiar kidney has gained much attention due to its marked similarity to the branched protonephridium of flatworms (one of the factors thought to question its molluscan affinities; [31,36]). In contrast to *Helminthope*, this kidney consists of two ducts that extend along the right body side and converge at the nephropore; the ducts show the typical kidney epithelium and contain multiple interspersed filtering 'knobs' with a ciliary flame. According to Haszprunar's [66] ultrastructural examination of *R. transtrosa* Salvini-Plawen 1991, these 'pseudo-protonephridia' lack the diagnostic basement membrane with ultrafiltration weir (only free hemocoelic rhogocytes possess this prerequisite for ultrafiltration). Given the data on other groups, the branched kidney of *Rhodope* looks more derived from a hypothetical ancestor than that of *Helminthope*. These differences could be attributable to the form of the body and body volume to surface ratios – the thicker body of *Rhodope* species may need a larger number of filters than the thin body of *Helminthope*.

The excretory organ of *Helminthope* resembles the paired larval/juvenile nephridia described recently in the chiton *Lepidochitona* Gray, 1821 [67,68]: these possess 'larval' protonephridia (with filter zone and vacuolated part) that become fully reduced, and 'early adult' protonephridia with an originally similar morphology that later becomes modified to form a metanephridial sys-

tem after joining the pericardium. We assume that this mechanism is similar in heterobranch gastropods, including rhodopemorphs that possess only the (right) kidney as adults. Therefore the right-side asymmetry of the excretory system in *Helminthope* is consistent with a paedomorphic condition of an 'early adult', i.e. protonephridial-stage, nephridium.

Loss of the metanephridial system otherwise present in adult mollusks is related to the complete loss of the heart (and pericardium); for rhodopemorphs not any trace has been reported even for ontogenetic stages [31,32,35]. Lack of a heart was also described for some other small-bodied heterobranchs such as some acochlidians or the mesopsammic sacoglossan *Platyhedyle* Salvini-Plawen, 1973 [22,69], but a heart was later confirmed at least for the former [24]. Other presumably 'heart-less' gastropod taxa are the 'allogastropod' *Cima* Chaster, 1896 (according to [70]) and the sacoglossan *Alderia modesta* (Lovén, 1844) [71]. These species, however, possess a 'normal', i.e. sac-like kidney. Therefore, rhodopemorphs are unique even among other heart-less gastropods in possessing a special protonephridial-like excretory system which resembles a protonephridial-stage adult kidney.

Reproductive system

Characters of the reproductive systems are considered to be of major systematic value in heterobranchs [72-74], and many anatomical descriptions include detailed accounts of these organs. *Helminthope psammobionta* is a simultaneous hermaphrodite with an unbranched (= monaulic) genital system. Unusual for a hermaphrodite, there are no obvious structures for the storage of received sperm ('allosperm receptacles').

Our examination shows some differences in organization compared to the original description by Salvini-Plawen ([29]; see Table 1). In consequence, the reproductive system is not fundamentally different from that of *Rhodope* (see [32]). Differences include the organization of the gonad: in *Rhodope* it is ramified with posterior testicles and more anterior ovarial follicles [31,32,56]. There appear to be no separate regions of gametogenesis in *Helminthope*, oocytes equipped with yolk are located along much of the gonad, but appear to be relatively smaller than those of *Rhodope*. Spermatozoa show the corkscrew-shaped head typical for heterobranchs [75-77], but without TEM data comparison to heterobranch subgroups is not possible.

The nidamental gland mass consists of five separable glands in *H. psammobionta* and also *R. rousei* [32]. Other *Rhodope* species examined here show at least four nidamental glands (BB, pers. obs.). This is a higher number than in most other heterobranchs which are in most cases described with only three types of gland (see [78,79]). Therefore it is difficult to homologize the glands in rhodopemorphs.

Contrary to *Rhodope* species, *Helminthope* possesses only a single 'terminal' gland (Table 2). According to histological characters, the gland in *Helminthope* is homologous to the proximal of two terminal glands in *R. rousei* ([32]: 'barrel-shaped' gland) and other *Rhodope* species ([31], BB pers. obs.). In *Helminthope*, the gland is more elongate and less regular on a cellular level; also, it is separated from the last nidamental gland by a comparably longer piece of undifferentiated gonoduct. Some other basal heterobranchs (e.g. the orbitestellid *Microdiscula* Thiele, 1912, see [80]), possess prostate tissue distally to the nidamental glands, i.e. in the same position as the terminal gland(s). Because a copulatory organ located more anterior is lacking in rhodopemorphs, these glands were hypothesized to form spermatophores (see [32]). In contrast to *Rhodope* specimens that were repeatedly observed to contain free spermatozoa within the body cavity [31,32,66], our results and previous TEM studies [9,41] did not confirm this phenomenon, which is associated with hypodermal insemination, in *Helminthope*.

The reproductive system of the murchisonellid *Henrya* is depicted as monaulic and includes two seminal receptacles and a cephalic copulatory organ close to the head [64]. Nothing is known about the other supposed murchisonellids.

Central nervous system

The nervous system of *Helminthope psammobionta* is unique among gastropods in its scattered arrangement of ganglia (involving five distinct ganglia on the visceral loop and numerous 'accessory' ganglia). This is contrasted by the fusion of cerebral and pleural ganglia to an almost spherical structure. All these ganglia can be externally localized in living specimens via a light microscope ([29], KM Jörger, pers. comm.). Contrary to the original description, we were able to identify additional accessory ganglia posterior to the cerebropleural ganglion, and an extended set of nerves next to minor differences such as the anterior, not posterior position of the buccal ganglia (see Table 1).

Nervous system characters have traditionally and frequently been employed to define heterobranch relationships (e.g. [30,81], but see [82]). Especially higher taxa such as the Euthyneura = Pentaganglionata are by their name defined by nervous system characters, i.e. the untorted state of the visceral loop or the presence of five distinct ganglia on it during ontogeny. The recently recovered more basal position of rhodopemorphs, outside Euthyneura, leads to the question how and when 'typical' heterobranch nervous system features evolved, i.e. the

aforementioned characters, the considered diagnostic set of cerebral nerves with double cerebro-rhinophoral root, or the sensory Hancock's organs.

Cerebral nerves

The cerebral nerves have gained considerable attention in defining major taxa among Heterobranchia (e.g. [81,83,84]). Their correct identification is regarded as relevant for understanding questions about evolutionary patterns within Heterobranchia and their currently assumed sistergroup, the Caenogastropoda: which nerves are homologous between larger groups, how complex was the 'ancestral' pattern, and how did the nerves evolve? According to Huber [81], cerebral nerve complexity increases from caenogastropods to opisthobranchs, although 'derived' pulmonates have rather simple, i.e. plesiomorphic nervous systems. After Nordsieck [83], however, the ancestral euthyneuran already possessed a full set of nerves. Recent topologies with para- or polyphyletic opisthobranchs [13,14] imply evolution of a secondarily simple set of cerebral nerves in pulmonates, with still unclear homologies of the remaining nerves.

Heterobranchs possess several pairs of sensory cerebral nerves [81, terminology after 54–55]. The 'typical' set involves paired static, optic, oral (N1), labiotentacular (N2), rhinophoral (N3) and 'headshield' nerves (N4). Except for the first two pairs all nerves innervate larger areas of the epidermis, especially head appendages when present. It should be noted that in many taxa there is a lower number of nerves, which implies fusion or loss. Therefore, assumptions of homologies are not easy to evaluate, and nerves may have been confused frequently.

Our material of *Helminthope psammobionta* shows candidates for at least five of the six aforementioned cerebral nerves emerging from the cerebropleural ganglion (cpg). Static and optic nerves are present, as would be expected from a species that possesses statocysts and eyes. The oral nerve (N1) is either missing (due to reorganization of the anterior digestive tract?), or alternatively incorporated either into the thick labiotentacular or rhinophoral nerves (N2 and N3). The N2 is characterized by its anteroventral position in the cpg, and because it innervates the anterior sides of the snout. This area is, in rhodopemorphs, considered equivalent to the 'anterior portion of the Hancock's organs' [29,40], distinct epidermal sensory areas found at the sides of the head of many heterobranchs (e.g. [55]). In contrast, the rhinophoral nerve (N3) is more dorsal, possesses widely separated double roots (one emerging next to the labiotentacular nerve, but see below), and mainly innervates the posterior sides of the snout. The thick nerve based in the 'pleural' portion of the cpg and running parallel to the optic nerve might either be the headshield nerve (N4, nervus clypei-capitis) or a 'pleural' nerve, i.e. emerging from the pleural portion of the cpg. We prefer the first interpretation, since pleural nerves are generally lacking in normal-sized, i.e. small heterobranchs [85], but a N4 is found in some [81].

This set of cerebral nerves conforms well to that of *Rhodope* but shows distinct differences. The optic nerve of *R. veranii* was described to split off ventrally of the pleuropedal connective [40], and Salvini-Plawen [29] noted it to emerge from the 'terminal cerebropedal' = pleuropedal connective also in *H. psammobionta*. Neither is the case in our material of *Helminthope*, where the nerve emerges dorsolaterally, close to but distinct from the putative N4.

There are some differences to the nerves found in *Rhodope*. The N2 = labiotentacular nerve of *Rhodope* is basally forked, in contrast to *Helminthope*, but resembling the condition found in caenogastropods, some 'allogastropods', i.e. architectonicoids or valvatoideans (e.g. [17,51,58,86]) but also many euthyneurans, i.e. the cephalaspid *Haminoea* Turton & Kingston, 1830 (see [55]). The N3 = rhinophoral nerve is also double-rooted in *Rhodope*, but possesses a slender ganglion at its base [32,40]. In *Rhodope*, a possible equivalent to the N4 = headshield nerve is the strong 'lateral' nerve, although this nerve was described with double roots in the pleural and pedal ganglia [32,39,40]. In the same position, the nervous system of larval *R. veranii* shows distinct 'cerebropleural' nerves (the right one bifurcated) according to Riedl [35]. This nerve is possibly homologous to the 'lateral' nerve of adult *Rhodope* ([35]: fig. 15a) and innervates approximately the same area as the N4 in *Helminthope*.

Double cerebral connectives

Double connectives between the cerebral ganglion and one of the thick cerebral nerves (called rhinophoral nerve, N3 herein) were considered to be a feature diagnostic of some higher heterobranchs [40], namely opisthobranchs and Pyramidelloidea. A double connective in this nerve is also found in rhodopemorphs ([32,40], this study), which would thus support placement with traditional opisthobranchs and/or Pyramidellidae Gray, 1840. In pulmonates, the so-called procerebrum (a neurosecretory structure characterized by 'globineurons') also possesses double roots [87,88]. Jörger *et al.* [14] recovered a mix of the aforementioned clades among Euthyneura and therefore indicated both double rooted structures – rhinophoral ganglion and procerebrum – to be potentially homologous, although this possibility was earlier disregarded due to histological and ontogenetic differences (e.g. [40]). These differences may, however, not affect the presence of a double root. In more recent studies, double rooted 'rhinophoral' ganglia were found in rhodopemorphs (not Euthyneura according to preliminary

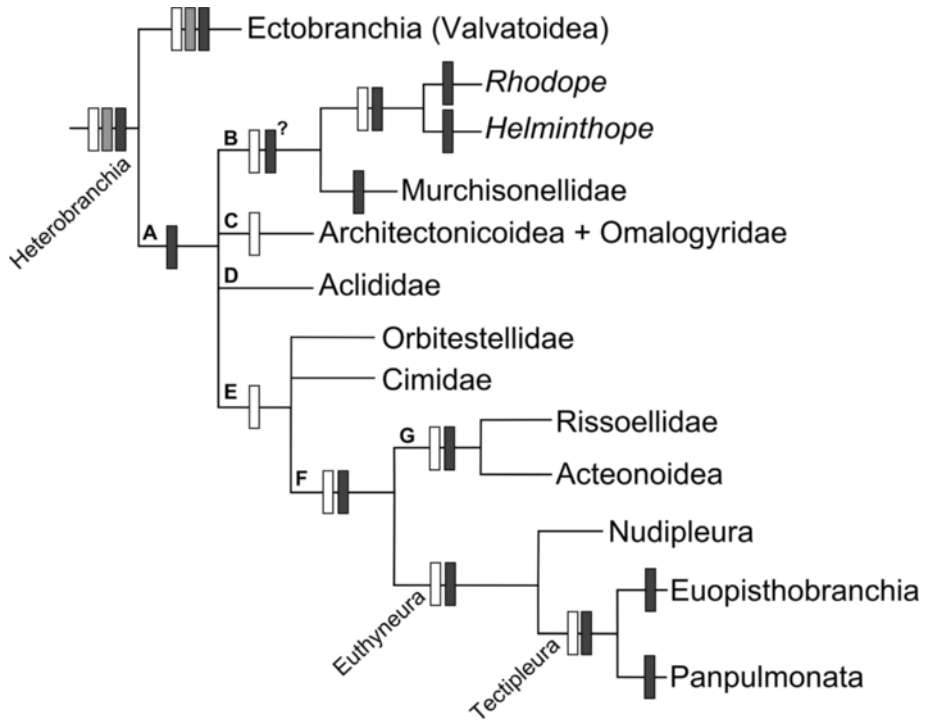

Figure 7 Simplified consensus cladogram of Heterobranchia according to [13-15,116]. White boxes: clades with strong molecular support according to the aforementioned studies. Grey boxes: possible synapomorphies regarding sperm ultrastructure [75-77,110]. Black boxes: possible morphological synapomorphies (see text for further details). Heterobranch taxon sampling and apomorphies listed here are not exhaustive, and focused on taxa and characters relevant for discussing relationships with rhodopemorphs; reversals in subgroups are not indicated. Heterobranchia: spiral sperm, hyperstrophic larval shell, original gastropod ctenidium lost, pallial kidney, simultaneous hermaphroditism with ovotestis, loss of paraspermatozoa, among others [17]. Digestive system simplified: radular cartilages and esophageal pouches lost, paired buccal retractors [51]. Special arrangement of mitochondrial genes [117]. Ectobranchia: specialized ectobranch gill, paired pallial tentacles, sperm characters [51]. Node A: ciliary tracts present in mantle cavity; gill, jaws lost (?) Early development of 4d-mesentoblast (?). Node B: pharynx reduced; esophagus vacuolated (?). Rhodopemorpha: body wormshaped, meiofaunal syndrome characters (e.g., loss of body appendages and mantle cavity; presence of caudal adhesive gland, accessory ganglia, spicules); euthyneurous, pentaganglionate nervous system with double-rooted rhinophoral nerve; esophageal pump present/pharynx vestigial or lost; protonephridial-stage kidney retained in adults, among others ([32], this study). Nodes C,D,E: unknown. Node F: Giant neurons (in macroscopic members), possibly pentaganglionate condition (at least in early ontogeny). Euthyneura: Euthyneury (several reversals in subgroups), pentaganglionate CNS at least during ontogeny (?), rhinophores (?). Euopisthobranchia: esophageal gizzard with cuticle [14]. Panpulmonata: double-rooted rhinophoral nerve (?).

molecular data, [44]) and, inside Euthyneura, so far only among panpulmonate pyramidelloids, 'opisthobranch' sacoglossans and acochlidians [47,53,81]. Several other panpulmonates possess the neurosecretory procerebrum with double roots (see [40,86]). We are not aware of further records of double connectives among the remaining Euthyneura or acteonids, and only few euopisthobranchs have been indicated to possess the double connective, i.e. *Runcina* [81] and possibly *Pluscula* [26]. It remains unclear whether these double roots *per se* are homologous, since it is so far not clear which nerve tracts originally fused (or divided) to form the double roots; ontogenetic data on this particular phenomenon are entirely lacking. However, different nerves of the aforementioned 'basic' set were suggested to play part in the double root: some examples are the putative inclusion of nerves N3+4 in the sacoglossan *Elysia* Risso, 1818, *Gascoignella* Jensen, 1985 or *Platyhedyle* ([81: p. 400], [22,53]) or the N3 + optic nerve in some acochlidians [14,25,47,52]. In *Helminthope*, one root of the N3 emerges close to the N2, therefore the double-rooted N3 may be product of partial fusion of fibers of N2+3, or one root may have originated from the otherwise missing N1. If rhodopemorphs are basal heterobranchs, as indicated by molecular data, then the double roots evolved convergently to those of panpulmonates (see Figure 7). Counter to our *a priori* homology assumption, which was based on criteria of structure and relative positions, an origin of rhodopemorphs among lower heterobranchs may also support an alternative scenario. The innermost cerebral nerve could refer to the N1, and the thicker, double-rooted cerebral nerve of *Helminthope* could be a fused N2 and N3. This possibility needs to be evaluated in the light of clarifying the identity and homology of bifid "tentacular" nerves of caenogastropods and "lower" heterobranchs versus "higher" heterobranchs often having separate cerebral N1-4.

Accessory ganglia

Salvini-Plawen [29] described *Helminthope psammobionta* to possess 'two complexes of accessory ganglia' anterior to the cerebropleural ganglion (his Figure 4 shows approximately 5 pairs of ganglia), and assumed them to be associated with the cerebral nerves. We can show that these anterior ganglia are innervated by the putative labiotentacular nerve (N2) and the more posterior ones by the rhinophoral nerve (N3). The number of accessory ganglia appears to vary between individuals; some possess less than the 12 pairs shown in Figure 6.

Accessory ganglia on the same nerves are known for at least some *Rhodope* species ([40], BB, pers. obs.) but are always rather inconspicuous in histological sections. Accessory ganglia on the N2 and N3 are known for the majority of meiofaunal slugs (e.g. [7,22,25,81,89]), and, in combination with otherwise regressive features, also are typical of the 'meiofaunal syndrome'. In short-headed taxa such as acochlidians the ganglia form a large, compact mass. More similar to the condition found in *Helminthope*, the nudibranch *Pseudovermis* Périaslavzeff, 1891 appears to possess numerous smaller ganglia along the sides of its 'acorn-shaped' snout [81,89]. Since the ganglia are supplied by sensory nerves, they were argued to be part of an enhanced sensory apparatus, facilitating food detection or path finding among three-dimensional interstitial pore spaces [26].

Helminthope is so far the only known microslug that possesses accessory ganglia also behind the cerebral nerve ring. These postcerebral accessory ganglia are innervated by at least one of the pedal nerves, possibly the additional nerve of the optic ganglia, and most prominently the headshield nerve. All these ganglia appear to innervate the flanks of the anterior body half and are elongate instead of spherical.

The formation of accessory ganglia in rhodopemorphs is correlated to the fact that many larger nerves contain nuclei/neurons along their length, giving the impression of medullary cords [29,40]. Due to the elongation of the body and nerves in *Helminthope*, the formation of additional ganglia may be necessary for fast processing of signals.

Sensory organs

The eyes of *Helminthope psammobionta* show a spherical, solid lens, as usual in gastropods [18]. *Rhodope* species characteristically possess a lens made up of discrete bodies and seem to lack a cornea [31,32]. Therefore, *Helminthope* presumably shows the ancestral eye type, whereas the corpuscular lens of *Rhodope* appears to be an autapomorphy of the genus. At least one *Helminthope*-like rhodopemorph lacks eyes (MS, pers. obs.), which is not unusual for meiofaunal taxa [7].

The optic ganglia of *Helminthope* are large (compared to the eyes) and possess an additional nerve that runs along the flanks. This nerve is presumably the reason for the presence of double connectives of the optic ganglion, indicating that the ganglion is a product of fusion. Double cerebro-optic connectives are otherwise known for the acochlidian *Strubellia* Odhner, 1937 [52]; there, an additional nerve of unknown function connects to a branch of the rhinophoral nerve. The optic ganglia of *Rhodope* were described to be cup-like structures embedding the eyes [32,40]. Given the present results, the cells in *Rhodope* may alternatively be the sensory cells of the eyes as in *Helminthope*, and not a ganglion per se.

Statocysts are conspicuous elements in the CNS of *Helminthope* and *Rhodope*. They are large (compared to the body diameter) in *Helminthope*, but middle-sized to small in *Rhodope* species [32,40]. The presumed static nerve could not be followed along all of its length in our material and was not mentioned for other rhodopemorphs.

Epidermal sense organs such as Hancock's organs on the sides of the head or an osphradium on the right side are not detectable in *Helminthope*. However, the presence of accessory ganglia on sensory nerves in the sides of the snout indicates that equivalents of the former might be present. A chemosensory osphradium, innervated by a nerve of the supraintestinal ganglion, was indicated for larvae (but not adults) of *R. veranii* [35]. *Helminthope* possesses a 'supraintestinal' nerve, but no apparent associated organ.

Visceral loop ganglia and nerves

Salvini-Plawen [29] described the expanded pentaganglionate and euthyneurous visceral loop of *Helminthope psammobionta* and named the five free ganglia (from front to back) as the left and right parietal ganglia, the sub- and supraintestinal ganglia, and the visceral (=abdominal) ganglion. We follow the same interpretation here.

Helminthope varies greatly from *Rhodope* which possesses only a single free ganglion on the comparatively short visceral loop. This ganglion was considered to be a fused subesophageal and visceral ganglion [32,35] or simply the visceral ganglion [40], the remaining ganglia of the loop being joined anteriorly to the cerebropleural ganglia (see Table 2). The visceral loop of *Helminthope* resembles that of larval *Rhodope* [35] in possessing a true pentaganglionate condition with five unfused ganglia. *Helminthope* is therefore one of the few known heterobranchs to possess five free ganglia as an adult (see below), but is not part of the current Euthyneura = Pentaganglionata according to preliminary molecular results. Salvini-Plawen [29] gave phylogenetic emphasis to the left position of the visceral ganglion in rhodopemorphs, however, lies in a median position.

Our material of *Helminthope* shows nerves only on the left parietal, supraintestinal, and visceral ganglia. Riedl [35]

identified nerves in the sub- and supraintestinal ganglia plus two strong nerves emerging from the visceral ganglion. Salvini-Plawen [29] did not show nerves of the visceral loop ganglia except for the paired visceral nerves. He found traces of streptoneury in the nerve fibers of the visceral ganglion that lead into the visceral nerves; we were not able to confirm this.

The visceral nerve of heterobranchs usually is a single strong nerve innervating the inner organs of the visceral sac. In rhodopemorphs there are two equally thick branches that run along the ventral side of the body and terminate near the caudal adhesive gland (this study, [32]). This unusual presence of two nerves instead of one might indicate that the nerves and the ganglion are a product of fusion, which is reflected in the confused nomenclature found in previous studies. In *Helminthope* the nerves split just behind the ganglion ([29], this study); originally, the right nerve was called the visceral nerve and the left one a 'genital nerve'. In *Rhodope veranii* and *R. transtrosa*, the nerves even appear to originate partly in both the more anterior ganglia and the sides of the visceral ganglion, indicating fusion of ontogenetically separate nerves. Accordingly, Haszprunar and Huber [40] identified the left branch as a 'genitovisceral' nerve, and the right one (with more obvious partial root in the supraesophageal ganglion) as a 'pallial' nerve. In *R. rousei*, both nerves show at least some fibers that originate outside of the visceral ganglion [32]. On the other hand, the paired visceral nerves originate directly in the visceral ganglion in larval *Rhodope* [35], as they do in *Helminthope*.

The presence of five visceral loop ganglia in rhodopemorphs is of considerable phylogenetic interest. As stated by Schrödl et al. [15], rhodopemorphs are Heterobranchia that are pentaganglionate and euthyneurous but fall outside the current concept of the taxon Pentaganglionata = Euthyneura (*sensu lato*, including Acteonoidea). This leads to three possible scenarios: 1), the pentaganglionate condition evolved earlier than thought, i.e. at least in the last common ancestor of rhodopemorphs and euthyneurans, but was lost independently or not yet detected in intermediate (paraphyletic) 'basal' heterobranch taxa, 2), the pentaganglionate condition evolved convergently among rhodopemorphs and euthyneurans, or 3), the phylogenetic position of rhodopemorphs (outside of Euthyneura) recovered in molecular studies is wrong.

The taxonomic importance of the visceral loop configuration lies in the considerable attention it gained as a means to delineate major taxa. Inspired by Schmekel [90], Haszprunar [17] created the taxon Pentaganglionata to include all heterobranchs with five ganglia on the visceral loop at least during some point in ontogeny, as opposed to triganglionate heterobranch 'allogastropods' and all other gastropods. The additional (= left and right parietal) ganglia were presumed to be 'derived from the pleural ganglia through elongation of the cephalopedal mass' at an early point of ontogeny [17]. One can easily imagine this scenario of elongation to be the case in *Helminthope*.

However, only few Pentaganglionata have been observed to possess the namesake five ganglia at some point of their ontogeny (most possess fewer, but some even more than five, e.g. the 'hexaganglionate' *Chilina* Gray, 1828; see [82]), and is not clear if these ganglia represent homologous structures: a pentaganglionate visceral loop was reported for few members of all four major euthyneuran s.l. clades: in some *Acteon* species, ontogenetic stages of the nudipleuran *Aeolidiella* Bergh, 1867, in the euopisthobranch *Akera* O.F. Müller, 1776, and in the panpulmonates *Lymnaea* Lamarck, 1799 ([91-93], see [18,82]). Other taxa have been reported to lack five separate ganglia during their ontogeny (e.g. the panpulmonate *Ovatella* Bivona-Bernardi, 1832, [94]). In general, few species have been studied in sufficient histological detail and in sufficiently early larval stages to exclude the existence of a pentaganglionate stage. The presence and identity of potentially fused visceral loop ganglia in triganglionate systems remains to be tested by more sensible, e.g. immunocytochemical, techniques. It therefore remains unclear whether the pentaganglionate condition is homologous or even shared among Euthyneura (s.l.) and if yes, at which phylogenetic level (Ur-Euthyneura or elsewhere) it occurred for the first time. While the Pentaganglionata sensu Euthyneura hypothesis is rejected, we would not dismiss the possibility that the two additional, parietal ganglia on the visceral loop are an innovation of the last common ancestor of Rhodopemorpha and euthyneurans.

Meiofaunal syndrome at an extreme

Meiofaunal slugs resemble small, unpigmented 'worms' that can be extracted from subtidal, well oxygenated sands (see [95]). Many species possess a set of typical characters (herein summarized as 'meiofaunal syndrome', [5-8]), aspects that are in this combination not found in small slugs that are not mesopsammic, e.g. the littoral runcinids or some progenetic nudibranchs (*Vayssierea* Risbec, 1928) and sacoglossans (*Limapontia* Johnston, 1836) [81,96,97].

Helminthope psammobionta is an exemplary meiofaunal slug that takes adaptations to the extreme: it shares with *Rhodope* the wormlike habit without body appendages, the strong ciliation, curved spicules, caudal adhesive gland, and accessory ganglia (see [32]). *Helminthope*, however, differs in its extreme elongation of the body (with parallel elongation, narrowing and simplification of internal organs) and complete loss of pigmentation (described *Rhodope* species are opaque white

and may possess one or more colored bands; [42]). A still unexamined group of apparently mesopsammic rhodopemorphs with peculiar cross-shaped spicules (see [9,29]) is externally similar to *Helminthope* in habit (thread-like, unpigmented, with spheroid cerebropleural ganglia; BB, pers. obs.) and was indicated to represent a separate lineage [42]. Not all rhodopemorph species are meiofaunal, but they all show the morphological adaptations typical for interstitial sand-dwellers and appear well-adapted to interstitial life. Some coloured members of *Rhodope* may have recolonized (epi)benthic habitats, or may alternatively represent phylogenetically basal forms retaining plesiomorphic features.

Compared to other meiofaunal slugs, *Helminthope* externally resembles most closely the aeolid nudibranch *Pseudovermis*: both share the very elongate body and the slightly widened ('acorn-shaped') head presumably used as a wedge for digging [6]. *Pseudovermis* species, however, differ in the possession of more or less rudimentary dorsal body appendages (cerata, typical for aeolids), and internal organ systems of the genus are not as simple and paedomorphic/aberrant as in *Helminthope* but otherwise resemble other aeolids (e.g. [89,98]). No other free-living gastropods are similarly wormlike (judging from length/width ratios); only some endoparasitic eulimoid caenogastropods have similarly elongate, externally featureless bodies [99,100]. Among other meiofaunal metazoans, the almost threadlike habit is convergently found in particular 'subsurface intertidal' turbellarians [101], several nemertines, and lobatocerebrid worms that share their habitat with *Helminthope* [43, GH, pers. obs.].

The role of paedomorphosis

Both the morphology of meiofaunal organisms and that of early Heterobranchia has frequently been associated with paedomorphosis, i.e. the retainment of larval or juvenile characters in the adult (see [102] for terminology). Alternatively, selection for small body sizes may simply lead to miniaturization [103], but not modification of adult morphologies. The idea that meiofaunal metazoans have largely evolved through such progenetic processes has been examined in particular for annelids (e.g. [103-105]). For Heterobranchia it has been assumed that the smallness and reduction of anatomical features found in many basal taxa were partly due to progenesis in the common ancestor [18,58]. Rhodopemorphs lack many typical heterobranch and general gastropod characters (e.g. those associated with the shell, mantle cavity, and pharynx). We hypothesize these reductions and the 'larval' organization of e.g. the visceral loop and the kidney to be indicators of progenesis.

Riedl's [35] investigation of the ontogeny of *Rhodope veranii* is of particular importance for this: he showed that development (at least in the examined species) is unique but lacks a long-lived planktonic larval stage, which is quite typical for many microgastropods [58]. The hatching stage is a derived crawl-away larva of elongate drop-shaped appearance (called 'Reisinger' larva by Riedl [35]); it does not develop a shell (although a putative shell gland is present for a short time), operculum, or the cephalic velum otherwise typical for larval gastropods. Rhodopemorphs largely retain this 'drop-shaped' outer appearance after metamorphosis. Adult organ systems do not increase much in complexity during ontogeny and therefore appear paedomorphic. For example, the simple digestive system without a muscular pharynx and radula (which are usually developed late in ontogeny; [106]) and with only a short intestine (considered paedomorphic at least for patellogastropods; [107]) is similar to early ontogenetic stages. The tubular gonad and the unbranched gonoduct appear similar to the anlagen of these organs, i.e. paedomorphic: the former originates from a simple band of mesoderm (e.g. [48]), the latter is formed from a tubular invagination of ectoderm [106]. As discussed above, the configuration of ganglia in *Helminthope* (except for the accessory ganglia) is highly similar to what Riedl [35] observed in 13 days old *Rhodope*, with still unfused visceral loop ganglia spread along the longitudinal body axis. Also, the lack of a heart (in mollusks developed shortly before metamorphosis, [108]) and therefore presence of only a protonephridial-type kidney (present before the heart; [68]) are early ontogenetic characters persisting in the adult. While heterobranchs are hypothesized to have evolved from an apogastropod ancestor in the centimeter size range by progenetic miniaturization and simplification especially of digestive and mantle cavity organs [18], rhodopemorphs have reduced body complexity even further parallel to their invasion of meiofaunal habitats accompanied by progenesis. *Helminthope* is at the current meiofaunal syndrome and progenetic extreme.

What mechanisms cause *Helminthope* to be so extraordinarily elongate? There are currently no developmental data on early ontogeny of *Helminthope*, but comparison to developmental stages of *Rhodope veranii* described by Riedl [35] suggests that a large part of longitudinal extension in *Helminthope* takes place in an early stage of development, i.e. before the equivalent of larval stages found at day 10 to 12: at this point, larval *Rhodope* possess still unfused visceral ganglia on a long visceral loop, and the anus is not yet formed [[35]: figs. 13–16]. In *Helminthope*, a scenario with an early elongation (i.e. accelerated somatic growth or peramorphosis, [102]) would explain why ganglia on the visceral loop remain unfused and paedomorphic (the loop becomes stretched) and why the position of the anus is far posterior, separate from the nephropore (because it is only formed after considerable

elongation of the body). We thus hypothesize that *Helminthope* originated from a stouter-bodied, more *Rhodope*-like ancestor by progressive progenesis coupled with peramorphosis (body hypertrophy) at an early ontogenetic stage, thus resulting in a habit partially resembling an over-elongate larva of already paedomorphic *Rhodope*. To test this hypothesis, ontogenetic data on *Helminthope* are required.

Origin of Rhodopemorpha

The historical confusion surrounding the phylogenetic position of *Rhodope* – gastropod or not? Opisthobranch, or pulmonate, euthyneuran? – was most recently summarized by [42] and [32]. Rhodopemorphs are fascinating and highly unusual – they look like worms but are gastropods since they retain some aspects of the original gastropod torsion, i.e. the position of some body openings asymmetrically on the right. They are specifically heterobranch gastropods due to the spiral sperm heads, the epiathroid, euthyneurous and pentaganglionate nervous system [17], and other characters such as the 'typical heterobranch' mode of copulation and the form of the spawn [34].

Helminthope was originally described as part of Rhodopemorpha by Salvini-Plawen [29]. Later, its affiliation to *Rhodope* and rhodopemorph affinities to some spicule-bearing doridoidean nudibranchs were doubted due to the wide nervous system and lack of the enigmatic 'vesicle system' in *Helminthope* [41]. However, close relationship between both genera is supported by numerous shared morphological characters and has recently been affirmed by preliminary multi-locus sequence analyses [15,44]. Morphological characters uniting Rhodopemorpha are the wormlike, round body with no division of the body into visceral sac and headfoot, the complete loss of shell, mantle cavity (and gill) or head appendages. Internal anatomical features are 1) boomerang- or cross-shaped, verrucose spicules, 2) the reduction or loss of pharynx and radula with parallel modification of an esophageal pump, 3) pentaganglionate and euthyneurous nervous system with fused cerebral and pleural ganglia, double rhinophoral nerve roots, accessory ganglia, and paired visceral nerves, 4) monaulic genital system without allosperm receptacles or cephalic copulatory organ but with spermatophore-forming gland(s), 5) lack of heart, with protonephridial-stage kidney retained as adults, and 6) development of a caudal adhesive gland ([32], this study). However, characters 2 to 6 cannot be evaluated satisfyingly due to the lack of comparable data on the potential sister group of rhodopemorphs. Furthermore, phylogenetic analysis is hindered by meiofaunal/paedomorphic modifications found in Rhodopemorpha that involve characters commonly used to delineate Heterobranchia (Figure 7; see [17,51]), i.e. the complete loss of the shell (hyperstrophic larval shell?), mantle cavity (formation of a pallial kidney, ciliated strips, ctenidium/gill?), or due to the modification of the digestive tract (lack of a pharynx with jaws). Thus, within Heterobranchia, hypotheses on the origin of *Rhodope* and *Helminthope* from morphological and molecular data were incompatible.

Herein we reconsider newly available morphological evidence and discuss the fact that according to molecular data, rhodopemorphs are not closely related to any of the euthyneuran slugs but should instead be placed among paraphyletic 'lower' heterobranchs, close to the equally minute but shell-bearing, high-spired Murchisonellidae [44]. This phylogenetic position is currently counterintuitive from a morphological point of view, and similar placement was never suggested by previous authors. Not much is known about the internal anatomy of Murchisonellidae. An exception is the unusual cuticular 'jaw' apparatus described for murchisonellids [63,64] which implies that the radula (and pharynx?) may also be modified and largely reduced. Given these data, the reduction of pharynx and radula with parallel modification of the esophagus (elongation, vacuolization) could be a synapomorphic trait for equally minute murchisonellids and rhodopemorphs. Both also share a similar habitat, namely subtidal reef flats or rubble among seagrass [45,109]. The Caribbean *Henrya morrisoni* Bartsch, 1947 was even described as 'infaunal' [64].

Heterobranch relationships revisited

Figure 7 attempts to provide an overview of current heterobranch phylogeny – which is in a state of reassembly – addressing the origin of Rhodopemorpha and mapping possible morphological characters onto a summarized version of recent molecular topologies. It includes taxa that were covered by recent molecular studies [13,14]. Some further potential 'basal' heterobranch taxa – e.g. the family Ringiculidae Philippi, 1853, Tjaernoeiidae Warén, 1991, 'caenogastropod' Cingulopsidae Fretter & Patil, 1958 (see [110]) and potentially misidentified "Pyramidellidae" – are not included due to the current lack of molecular coverage.

The origin of a possible Rhodopemorpha + Murchisonellidae clade (B in Figure 7) among Heterobranchia is still unresolved. Molecular studies [13,14] currently suggest at least four other likely monophyletic lineages at a similar phylogenetic level that are candidates for a sistergroup to the putative rhodopemorph- murchisonellid clade (see Figure 7). Those lineages are the Ectobranchia Fischer, 1884 (=Valvatoidea Gray, 1840), C) Architectonicoidea (Architectonicidae Gray, 1850 plus Mathildidae Dall, 1889) and Omalogyridae Sars, 1878, D) Aclididae Sars, 1878, and E) a monophylum of Orbitestellidae Iredale, 1917, Cimidae Warén, 1993, and the remaining

Heterobranchia. The latter (F) include a monophylum of Acteonoidea + Rissoellidae Gray, 1850 (G) as sister to the Euthyneura (sensu [14]). Many of the aforementioned taxa consist mainly of small-bodied members, and detailed microanatomical studies are lacking. Therefore, published data are mostly not sufficient to evaluate homologies. For example, some ectobranch as well as other lower heterobranch species do possess an esophagus that is at least histologically similar to that of Rhodopemorpha and Murchisonellidae [51,58].

The Ectobranchia (= Valvatoidea) include planispiral, minute snails with deep-sea and limnic lineages among more conventional subtidal groups (e.g. [56,65,86]). Haszprunar et al. [51] regarded them as the most basal heterobranch offshoot retaining plesiomorphies (e.g. broad, rhipidoglossate radula in Hyalogyrinidae) and showing some unique autapomorphies such as a typical 'ectobranch' gill (in contrast to the general gastropod ctenidium). This topology is neither unambiguously supported nor rejected by (not yet representative) molecular results which do, however, tend to place the Ectobranchia closer to clade C. Sperm ultrastructure (see [110]) suggests that Architectonicoidea are even more basal than Ectobranchia. Also, the rhipidoglossate radula of Hyalogyrinidae is unique also among Apogastropoda and thus could alternatively be considered autapomorphic for the family rather than assuming multiple independent origins of a narrow (taenioglossate) condition in at least the ancestral caenogastropod, in non-ectobranch heterobranchs and in non-hyalogyrinid ectobranchs. Ontogenetic transitions between rhipidoglossate, grazing radulae and more narrow ones are known in vetigastropods [111], so this character may be variable also among basal heterobranchs with unknown ontogeny. We still prefer hypothesizing Ectobranchia as sister to the remaining heterobranchs, because clade A) is supported by the unique presence of ciliated strips in the mantle cavity [17]. Further but still ambiguous apomorphies of clade A) are the lack of jaws, a taenioglossate radula, and the loss of a gill. Some derived and larger-bodied taxa among A) do possess a gill (then considered to be a novel structure, [17]), broad radulae, or jaws, so alternatively these features may be convergently reduced in all/most small-bodied basal taxa. Rhodopemorphs do not share any of the aforementioned ectobranch apomorphies, and do not possess ciliary strips; the latter may be explained by the absence of a shell and mantle cavity. Exploring Murchisonellidae in microanatomical depth may also reveal their 'jaw apparatus' to be a reduced and narrow radula [63; BB, pers. obs.], which would fit with apomorphies of clade A). An earlier development of the mesentoblasts during ontogeny (cell 4d differentiated at the 24-cell stage, and not later) was suggested to be a shared character of "opisthobranchs and pulmonates" [51], but was also observed for *Rhodope* [35]. If not evolved convergently, we suggest this is another potential synapomorphy of clade A).

Clade C) of large-bodied Architectonicoidea (globular to planispiral Architectonicidae plus medium to high-spired Mathildidae) and minute, planispiral Omalogyridae is supported by molecular results and some possible apomorphies such as an specialized eversible proboscis besides loss of a copulatory organ (see [51,59,112]), a character that is, however, also found in clades B and D. The high-spired and minute Aclididae (D) are known to possess a 'narrow' radula [113], but there are no anatomical descriptions.

The monophylum E) of Orbitestellidae (small, planispiral; [80]), Cimidae (small, high-spired; [114]) and the remaining Heterobranchia is indicated by molecular results [13,14,16,115], but not yet supported (or rejected) by morphological evidence. The remaining heterobranchs (F) include a monophylum of Acteonoidea + Rissoellidae (G) as sister to Euthyneura (e.g. [13,115]). Clade F) is possibly united by the presence of giant neurons, which are, however, present in larger-bodied taxa only (see [53]). Potential apomorphies for clade G) are the bilobed head appendages (developed into a head-shield in acteonoids – sometimes still with pointy corners); the shared androdiaulic condition of genital ducts of Acteonoidea and Rissoelloidea instead appears to be plesiomorphic (see [15]).

The Euthyneura (sensu [14]) comprise most of known heterobranch species diversity, and the node is robustly supported in recent multi-locus studies (for discussion see [15,116,117]). Morphological evidence for Euthyneura is less straightforward; a potential apomorphy refers to the presence of rhinophores (innervated by N3), if this is not already another synapomorphy of clade F). Rhodopemorphs do not possess any head tentacles, and the identity of the N3 (separate, or fused with N2) is ambiguous, so this feature is little informative for tracing their origin. Standard multilocus sequence marker based studies retrieve three major euthyneuran subgroups that are different from traditional morphological hypotheses, namely Nudipleura (including the speciose nudibranchs) as sister to a clade of Euopisthobranchia and Panpulmonata (e.g. [13,14]). The latter two tectipleuran clades contain rearranged lineages of traditional opisthobranchs, pulmonates, and the 'basal heterobranch' Glacidorbidae Ponder, 1986 and Pyramidellidae (see [13-16]). Although now contradicted by preliminary molecular results [44], older morphological studies placed Rhodopemorpha within Euthyneura based on the common possession of a euthyneurous, pentaganglionate nervous system [35,40,91]. These characters are neither unique for nor ubiquitous within Euthyneura, as

is indicated by the present study. More specifically, Haszprunar and Künz [41] followed Boettger's [118] and Odhner's [38] proposals of including *Rhodope* within doridoidean nudibranchs (Nudipleura) due to the presence of spicules, a 'modified' pharynx without radula, shared reductions, and presumed ultrastructural characters. Monauly in *Rhodope* (among otherwise diaulic or triaulic nudibranchs) was explained as a consequence of paedomorphosis [41] and the occurrence of specialized mode of sperm transfer, namely hypodermal injection (see also [32]). All these characters are homoplastic in a topological framework based on molecular data (Figure 7); e.g. calcareous spicules occur convergently in rhodopemorphs, several nudipleuran subgroups [119], but also in (some) sacoglossan and acochlidian panpulmonates [22,120], pharynx reductions are common not only among nudibranchs, and paedomorphic reductions or unilateral sperm transfer are herein discussed as 'meiofaunal syndrome' causing similar morphology and biology in independent lineages via habitat-specific selection pressure. Therefore, the latest morphological hypothesis of rhodopemorph origin is currently neither supported by morphology nor molecular data.

Other hypotheses based on morphology placed *Rhodope* among tectipleuran Euthyneura, a clade consistently retrieved in molecular studies (e.g. [13,16]). According to recent topologies these appear to be characterized by their primarily monaulic genital ducts (see [15]), which would be consistent with a relationship to Rhodopemorpha. Diagnostic features missing in the latter such as giant neurons [17] may be reduced due to the small body size. Euopisthobranchia possess, among morphological synapomorphies, an esophageal gizzard [14,15,121]. This structure is lacking in rhodopemorphs but loss can be explained by a secondary reduction coming with small body size, as a gizzard is also missing e.g. in the meiofaunal philinoglossid cephalaspideans [21,26]. In fact, morphology-based cladistic studies [121, see also 122] recovered *Rhodope* clustering with meiofaunal Cephalaspidea (Euopisthobranchia) and panpulmonate Acochlidia. This particular grouping is polyphyletic according to molecular results (see [14]), suggesting that it is a result of homoplasies ('meiofaunal syndrome') overriding other morphological characters [15,120]. Other authors assumed rhodopemorph affiliations to panpulmonate Gymnomorpha, i.e. Onchidiidae, based on *Rhodope* possessing a putative mantle cavity – herein shown to be erroneous – and a highly concentrated nervous system [19,35,39,123]. This placement was later doubted due to the lack of the diagnostic pulmonate neurosecretory procerebrum in rhodopemorphs [29,40]. However, as outlined above, the double-rooted rhinophoral ganglion of *Rhodope* could still prove to be homologous to the double-rooted procerebrum, and thus the double roots could be interpreted as a synapomorphy of (many) panpulmonates and rhodopemorphs. This interpretation is, however, in conflict with general morphology and structural differences weakening homology probability; in rhodopemorphs there are no 'globineurons' as typical for the pulmonate procerebrum [40,87,88]. Molecular results (Figure 7) indicate that a double-rooted rhinophoral nerve has evolved independently in rhodopemorphs and panpulmonates and thus constitute potential apomorphies of the respective groups.

Conclusions

Microanatomical exploration of rhodopemorphs provides strong evidence that the aberrant morphology of members refers to features (complex nervous system, presence of spicules, special reproductive strategies, adhesive glands) and regressive processes we account to a taxonomically widespread 'meiofaunal syndrome'. We interpret *Helminthope*, the most worm-like free-living gastropod, to be a progenetic sister of *Rhodope*, i.e. referring to an over-elongate and premature larval stage. We explore the diverse and largely incompatible previous morphology-based hypotheses on the origin of rhodopemorphs among heterobranch gastropods. Any earlier proposed relationships to euthyneuran opisthobranchs are not supported in the light of currently available microanatomical data, and are contradicted by (still preliminary) molecular evidence. Should future molecular studies corroborate placement of Rhodopemorpha among 'lower heterobranch' taxa, then more knowledge is needed on the minute, shelled basal heterobranch groups for better resolution and support for future phylogenies. 3D reconstruction has been demonstrated to be suited for anatomical examination of small-bodied taxa, and should be equally useful for studies on still barely known heterobranch groups such as Murchisonellidae, Aclididae, Cimidae, or the legions of snails that are currently pooled into vetigastropod or caenogastropod taxa just for their small size and shell features. Especially murchisonellids need anatomical study to test for possible anatomical synapomorphies with rhodopemorphs.

Because murchisonellid genera have been shown to exist as 'living fossils' since the Triassic [45], the putative murchisonellid-rhodopid split is potentially almost as old. The basal phylogenetic position of rhodopemorphs therefore makes them a candidate for the oldest lineage of meiofaunal slugs, and also for one of the oldest living slug lineages at all. Rhodopemorphs represent a fascinating, highly modified gastropod taxon among the otherwise typical snail-like lower heterobranchs, and give valuable insight into the enormous evolutionary potential of that much larger group.

Material and methods

About 20 specimens of *Helminthope psammobionta* Salvini-Plawen, 1991 were extracted from bulk samples of coarse subtidal sand taken from 2–4 meters depth at Police bay, Bermuda (close to the type locality), during October 1999. Specimens were anesthetized using isotonic magnesium chloride solution mixed with seawater, then fixed in 4% glutaraldehyde. All vouchers are stored at the Bavarian State Collection of Zoology, Munich (ZSM).

Several glutaraldehyde-fixed specimens were postfixed with 1% osmium tetroxide buffered with 0.2 M cacodylate / 0.3 M sodium chloride, then dehydrated in a graded acetone series and embedded in Spurr's epoxy resin.

3D reconstruction was done following largely the protocol described by Ruthensteiner [124]. Series of semithin histological sections (1 μm) were obtained using a diamond knife (Diatome HistoJumbo, Biel, Switzerland) and stained with methylene blue/azure II stain [125]. Photographs were taken of each section using a ProgRes C3 digital camera (Jenoptik, Jena, Germany) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). Digital images were imported into Amira 5.2 software (Visage Imaging, Berlin, Germany) as greyscale *.tif*-files with a resolution of 1600 × 1200 dpi. Images were aligned semi-automatically and organ systems labeled manually on the screen. From these labels, rendered 3D models were created of an entire, moderately contracted 1.5 mm specimen (ZSM Mol-19992019/2; 613 photos used; see Figure 1), the kidney of this specimen (61 photos; Figure 1B) and of the anterior body containing the central nervous system (CNS) of another 3 mm specimen (ZSM Mol-19992020/2; 358 photos; see Figure 3). Additional aligned image stacks of approximately 100 images with higher resolution and color were used to analyze very small features present in the aforementioned specimens. Histological features were furthermore compared with section series of two further specimens (ZSM Mol 20120177 and 20120178).

Interactive models of the 3D reconstructions were prepared following the protocol of Ruthensteiner and Heß [126], and are accessible as two clickable Additional files 1 and 2.

Additional files

Additional file 1: Figure S1. 3D reconstruction of *H. psammobionta* (ZSM Mol-19992019/2) showing organization of major organ systems, anterior to the right. **A**: Right view of complete, moderately contracted specimen. **B**: Kidney of same specimen, dorsal view. **C**: Reproductive system. Scale bars: A, 100 μm; B, 25 μm; C, 50 μm. Abbreviations: ag, accessory ganglia; agl, caudal adhesive gland; am, ampulla; an, anus; apg, anterior pedal glands; bb, buccal bulb; cpg, cerebropleural ganglion; dg, digestive gland; ey, eye; fg1-5, female glands (proximal to distal); fz, presumed filter zone; gd, (undifferentiated) gonoduct; go, gonad; gp, genital pore; it, intestine; kd, kidney; mo, mouth opening; np, nephropore; oc, oocytes; pg, pedal ganglia; sgl, salivary gland; tg, 'terminal' gland; vg, visceral ganglion; vn, visceral nerves. Click to activate interactive 3D model (requires Adobe Reader 7.0 or higher). Use mouse to rotate model, shift model (hold ctrl) or zoom (use mouse wheel). Switch between prefabricated views or select components in the model tree and change visualization (e.g. transparency, lighting, render modes, or crop).

Additional file 2: Figure S3. 3D reconstruction of the anterior end of an extended *H. psammobionta* (ZSM Mol-19992020/2) showing details of the central nervous system (cns), anterior to the right. **A**: Dorsal view of cns. Digestive system transparent, pedal nerves omitted. **A'**: The reconstructed specimen prior to sectioning, box marks region shown in this figure. **B**: Ventral view of ganglia, digestive system, and retractor muscle. Nerves largely omitted. **C**: Dorsal right view of anterior cns and details of the cerebral innervation. Pedal nerves transparent. Scale bars: 100 μm. Abbreviations: see main document Figure 3. Click to activate interactive 3D model (requires Adobe Reader 7.0 or higher). Use mouse to rotate model, shift model (hold ctrl) or zoom (use mouse wheel). Switch between prefabricated views or select components in the model tree and change visualization (e.g. transparency, lighting, render modes, or crop).

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

BB carried out the morphological analysis and drafted the manuscript. GH supplied materials and unpublished information and discussed results. MS conceived and supervised the study and helped writing the paper. All authors read and approved the final manuscript.

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Microanatomy of shelled *Koloonella* cf. *minutissima* (Laseron, 1951) (Gastropoda: 'lower' Heterobranchia: Murchisonellidae) does not contradict a sister-group relationship with enigmatic Rhodopemorpha slugs

Bastian Brenzinger[1,2], Nerida G. Wilson[3] and Michael Schrödl[1,2]

[1]*SNSB – Zoologische Staatssammlung München, Münchhausenstr. 21, D-81247 Munich, Germany;*
[2]*Department Biology II, BioZentrum, Ludwig-Maximilians-Universität, Großhadernerstr. 2, 82152 Planegg-Martinsried, Germany; and*
[3]*Western Australian Museum, Locked Bag 49, Welshpool DC, Perth, WA 6986, Australia*

Correspondence: B. Brenzinger; e-mail: bastian.brenzinger@arcor.de

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ABSTRACT

The Murchisonellidae are a small taxon of minute snails with a high-spired shell that occur in shallow marine habitats. Molecular phylogenetics recently revealed that they are not members of the externally similar yet phylogenetically derived Pyramidellidae, but instead potentially one of the oldest clades among the heterobranch Gastropoda. Furthermore, current data surprisingly indicate a sister-group relationship with Rhodopemorpha, highly aberrant marine slugs with previously unclear affinities. Murchisonellidae are characterized by a specialized pincer-like radula, but very little further data exist on soft-body anatomy for most species, and there are only a few observations of living animals. Investigation of the anatomy of Murchisonellidae may thus yield new data providing insights into early heterobranch evolution and that of enigmatic Rhodopemorpha. We collected live specimens of the murchisonellid *Koloonella* cf. *minutissima* (Laseron, 1951), a member of a genus known mainly from eastern Australia. We provide detailed live photographs and interactive 3D data on all major organ systems, based on serial histological sections. The mantle cavity is shown to contain several distinct glands, a pair of which is conspicuously similar to glands found in Rhodopemorpha. The anterior digestive system contains a unique four-toothed radula, a feeble pharynx and a special, vacuolated oesophageal bulb. The reproductive system is complex and diaulic, and contains unusual structures. These results highlight structural diversity among minute lower Heterobranchia. Soft-body characters do not contradict, and may even support, the counterintuitive sister-group relationship with shell-less, wormshaped Rhodopemorpha. The classification of Murchisonellidae is discussed and a revised scheme is proposed.

INTRODUCTION

In recent years, the study of gastropods in the major clade Heterobranchia Gray, 1840 and their phylogeny have been revitalized by molecular studies (Klussmann-Kolb *et al.*, 2008; Dinapoli & Klussmann-Kolb, 2010; Jörger *et al.*, 2010). Two surprising results in particular motivated the present study. One was the removal of Murchisonellidae Casey, 1904 from the Pyramidelloidea (Dinapoli & Klussmann-Kolb, 2010). Molecular phylogenetic analyses that included the Pyramidelloidea, one of the largest family-level taxa among Heterobranchia and comprised of mostly minute and high-spired marine snails that are ectoparasites, showed that the majority of species were recovered in a derived phylogenetic position among Panpulmonata (Jörger *et al.*, 2010; Dayrat *et al.*, 2011; Dinapoli, Zinssmeister & Klussmann-Kolb, 2011). Additionally, the Murchisonellidae were found to be potentially some of the oldest heterobranchs (Dinapoli & Klussmann-Kolb, 2010); they are a small group with fossil analogues dating back to the Triassic (Bandel, 2005). Warén (2013) recently reviewed the family and characterized it as a good example of 'living fossils'. The other surprising result of recent studies was the proposed sister-group relationship

of Murchisonellidae and Rhodopidae. The latter are a small group of minute, worm-like slugs that are some of the most aberrant free-living gastropods—their distinctiveness is reflected in the commonly used order-level name Rhodopemorpha—and have puzzled systematists for over 150 years (Wilson, Jörger & Schrödl, 2010; Brenzinger, Wilson & Schrödl, 2011; Brenzinger, Haszprunar & Schrödl, 2013a).

Rhodopemorphs are the only slugs among the otherwise shelled, minute marine gastropods collectively known as lower (or basal) Heterobranchia, 'Heterostropha' or 'Allogastropoda'. These are a paraphyletic assemblage of about a dozen distinct lineages that were recovered in published analyses (see Ponder, 1998; Brenzinger *et al*., 2013a; Wägele *et al*., 2014 for reviews), although not all potential families have been covered in published analyses, and further distinct lineages are to be expected (Table 1). Relationships among these lower Heterobranchia are still poorly resolved, but they remain of considerable interest. This is because they connect the two largest gastropod, and therefore mollusc, taxa, namely the species-rich crown group of Heterobranchia, the Euthyneura (including historical opisthobranch and pulmonate taxa) and the similarly speciose heterobranch sister group, the Caenogastropoda (Haszprunar, 1985a; Ponder & Lindberg, 1997). However, due to the small size and the difficulty of collecting live specimens, little is known about the anatomy and biology of most lower heterobranchs. Reconstruction of early heterobranch evolution is thus hampered by a lack of biological and anatomical characters that are meaningful in terms of evolutionary relationships.

This also holds true for the Murchisonellidae. Living murchisonellids are tiny, high-spired marine snails found in marine subtidal habitats, associated with sea-grass beds or lagoon habitats. Published records indicate an almost worldwide distribution. Several accounts have described the shells of murchisonellids (mostly classified among Pyramidelloidea), but only a few have gone beyond that: Rasmussen (1944) gave notes on veligers and adult specimens of the European *Ebala nitidissima* (Montagu, 1803), one of the most commonly recorded species (often classified as *Anisocycla* Monterosato, 1880). Warén (1995) described the peculiar 'jaw' apparatus that is now regarded as a synapomorphy of the family. The currently most comprehensive anatomical account is by Wise (1999) on the Caribbean *Henrya morrisoni* Bartsch, 1947, including descriptions of major organs systems from dissections. Most recently, Warén (2013) presented live photographs and SEM scans of further species and summarized what was known about the taxonomy of Murchisonellidae, indicating that there may possibly be two distinct lineages within the family (Ebalinae and Murchisonellinae). In total, current classification lists about 60 species in six genera (Bouchet, 2013). To date, very little data exist about *Murchisonella* Mörch, 1874, *Koloonella* Laseron, 1959 or *Pseudoaclisina* Yoo, 1994.

Table 1. Currently recognized suprageneric taxa among 'lower' Heterobranchia, including Euthyneura (crown group including Nudipleura, Euopisthobranchia and Panpulmonata).

Taxon	Notes
Murchisonellidae Casey, 1904	
Rhodopidae von Ihering, 1876	= Rhodopemorpha Salvini-Plawen, 1970
Ectobranchia Fischer, 1884	= Valvatoidea Gray, 1840; 4 families
Architectonicoidea Gray, 1850	Two families, including Mathildidae Dall, 1889
Omalogyridae G.O. Sars, 1878	
Graphididae Barros *et al.*, 2003	Elevated to family status by Warén (2013)
Cimidae Warén, 1993	
Orbitestellidae Iredale, 1917	
Ringiculidae Philippi, 1853	Morphologically distinct but not yet included in a molecular study
Tjaernoeiidae Warén, 1991	Morphologically distinct but not yet included in a molecular study
Rissoellidae Gray, 1850	
Acteonoidea d'Orbigny, 1843 + Euthyneura Spengel, 1881	Three families

Classification based on molecular results of Dinapoli & Klussmann-Kolb, 2010, as reviewed by Brenzinger *et al.*, 2013a and Wägele *et al.*, 2014; see Discussion for further references. Cingulopsidae Fretter & Patil, 1958 were suggested to be possible heterobranchs based on morphology, but molecular data confirm classification among Caenogastropoda (Dayrat & Tillier, 2002; Criscione & Ponder, 2013).

Koloonella (with 15 currently described species) is a genus originally described from the Australian east coast, with species also occurring in southern Papua New Guinea and Tasmania (Laseron, 1951, 1959). The type, *K. moniliformis* (Hedley & Musson, 1891), is from an estuary near Sydney; other species have been collected in moderately deep, fully marine or brackish habitats. A recent survey of Australian murchisonellids yielded live specimens of several millimetre-sized *Koloonella* suitable for both molecular analysis and for the present study of soft-body characters.

In the past decade, computerized 3D reconstruction based on semithin section series has been used as a tool to study and visualize (sometimes as interactive digital models) anatomical details of several taxa among minute Heterobranchia. Studies already exist for members of the three euthyneuran clades (Nudipleura: DaCosta *et al*., 2007; Martynov *et al*., 2011; Euopisthobranchia: Golding, 2010; Brenzinger, Padula & Schrödl, 2013b; Panpulmonata: Ruthensteiner, Lodde & Schopf, 2007; Ruthensteiner & Stocker, 2009; Neusser, Heß & Schrödl, 2009; Neusser *et al*., 2011; Kohnert *et al*., 2013). Most recently, other studies have used a similar approach on shelled lower heterobranchs (Haszprunar *et al*., 2011; Hawe, Heß & Haszprunar, 2013; Hawe & Haszprunar, 2014) and Rhodopemorpha slugs (Brenzinger *et al*., 2011, 2013a), thus expanding the dataset needed to unravel lower heterobranch phylogeny.

In addition to making comparisons with other shelled basal heterobranchs, we wanted to address whether Murchisonellidae snails potentially share any synapomorphic anatomical characters with aberrant Rhodopemorpha slugs, their sister group according to molecular data. To date, the divergent morphology has made it impossible to place the latter in a morphology-based phylogenetic tree. Herein, we aim to establish a comprehensive dataset on the microanatomy and histology of Murchisonellidae that can be used to test already existing or future phylogenetic hypotheses of lower heterobranch evolution. For this, we used series of semithin histological sections to reconstruct and analyse the microanatomy of *Koloonella* cf. *minutissima* (Laseron, 1951), collected in Port Stephens, New South Wales, Australia.

MATERIAL AND METHODS

Specimens were collected from a bulk sediment sample (undisturbed coarse sand covered with a fine algal or bacterial growth at 6 m) taken using SCUBA at Nelson Bay, Port Stephens lagoon (New South Wales, Australia: 32°43′3.64″S, 152°8′28.44″E). Live specimens were observed and photographed through a Leica S8 APO stereo microscope, relaxed in isotonic $MgCl_2$ and fixed either in 98% ethanol (one specimen, Australian Museum reg. no. AM C469741; Fig. 1C) or Karnovsky's paraformaldehyde (for microanatomy: one mature and one juvenile specimen, AM C469740.001 and 469740.002; Fig. 1A, B, Supplementary Material, File S2).

For microanatomical study, both specimens were washed in 0.1 M phosphate-buffered saline, decalcified in 3% ascorbic acid, dehydrated in a graded acetone series and embedded in Epon epoxy resin. Series of semithin histological sections were

obtained from both specimens, one mature (size of shell approx. 900 μm; section thickness 1.5 μm) and one juvenile (250 μm; 1 μm). Sections were made using a HistoJumbo diamond knife (Diatome, Biel, Switzerland) and stained with methylene blue/azure-II. For 3D reconstruction, sections were photographed semi-automatically using an Olympus Dotslide Virtual Slide system slide scanner mounted on an Olympus BX61V5 light microscope. Image stacks were stack-processed in Adobe Photoshop. Alignment of images, labelling of structures and rendering of surface details was done using Amira v. 5.3 (Visualization Sciences Group, Mérignac, France). Except where stated, all descriptions refer to the mature specimen; data on the juvenile specimen are summarized separately at the end.

Histological study and 3D reconstruction largely followed the protocol described by Ruthensteiner (2008). Rendered 3D Amira files were exported into the interactive format according to Ruthensteiner & Heß (2008). An interactive pdf version of the 3D reconstruction is provided in Supplementary Material, File S1.

All microanatomical work was done at the facilities of the Bavarian State Collection of Zoology, Munich (ZSM), Germany.

RESULTS

External morphology of living specimens (Figs 1, 2): Shell smooth, translucent, high spired. Aperture oval, convex posteriorly. Lip smooth. Whorls rounded, sutures distinct. Height of shell in larger adult specimen 900 μm, in juvenile 250 μm. Large adult with 4.5 teleoconch whorls, smaller one with 3.5, juvenile with 1.5, respectively. Protoconch *c.* 1.2 whorls; marked by distinct growth line (Fig. 1A″, B, C: white arrowheads). Protoconch sinistral, hyperstrophic (angled at *c.* 120°), glossy, smooth. 1st teleoconch whorl (or protoconch II?) with fine, distant speckles (Fig. 1A, C; Supplementary Material, File S2); also demarcated by growth line. Rest of teleoconch without speckles but faint spiral ornamentation and slightly opisthocline growth lines (not shown).

Head short; with wide snout, two lateral tentacles and vertical anterodorsal cleft (Fig. 1F). Snout bilobed, with rounded edges and median intersection (e.g. black arrow in Fig. 1A″). Tentacles flat, elongate, with rounded tips. Tips directed posteriorly in living specimens (Fig. 1A), but anteriorly in retracted/fixed specimens (Fig. 1E, F). Posterior side of each tentacle (dorsal in fixed specimens) with a sharply bordered, unciliated longitudinal groove (Figs 2B, 5A: arrowhead).

Median cleft between both tentacles contains mouth opening (Figs 1A″, E, 2A, 5D, Supplementary Material, File S1). Male gonopore below right tentacle (Fig. 2A: asterisk).

Foot short, narrow (Fig. 1A′, B″). Anterior end wide, thick (propodium, Fig. 1B″, E, F), distinctly ciliated. Operculum on posterior side of foot translucent, oval, paucispiral (Figs 1A″, 2B: op). Monolayered, about 8 μm thick in middle, thinning to 2 μm at outer margin (Fig. 5A). Base colour of body greyish-white. Black pigment granules in epidermis of mantle over neck, and on base and between tentacles forming 'mask-and-hood'-like pattern (Fig. 1A, D, 2C; see below). Digestive gland dark rusty brown (Fig. 1A′). Brighter red area towards anterior end of digestive gland and below intestine possibly part of reproductive system (male glands; Fig. 1A″, Supplementary Material, File S2). Ovary colourless, with large ova visible as whitish spheres. Area of mantle caecum speckled yellow (Fig. 1A′, B″, C′). Heart a translucent bag in anterior corner of mantle caecum. Finely reticulated area in mantle roof (kidney? Fig. 1A). Row of crimson red glands parallel to mantle edge (Figs 1A″, 2B′); Blochmann's glands visible as slightly opaque spherules behind red glands (Fig. 1A″). Calcium cells visible as refracting bodies in neck (Fig. 1A′).

Living observations: Snails move smoothly on glass surface; larger specimens pull shell behind in a jerking motion (Supplementary Material, File S2). Motion of cilia visible at anterior margin of snout, heartbeat on ventral side of first whorl. Shell of specimens covered in stalked diatoms (red specks in 1A; brighter red unmarked 'balloon' near apex in Fig. 1B′, B″).

Skin and subepidermal structures (Figs 2, 5): Epidermis 5–8 μm thick and ciliated on headfoot and in mantle cavity (Fig. 5B), 2–3 μm thick and unciliated on visceral sac and in caecum of mantle cavity. Band of particularly strong cilia (15 μm long) along anterior margin of snout and foot; strong ciliation in right corner of mantle cavity.

A histologically distinct strip of epidermis between 3rd whorl and anterior left corner of mantle cavity, alongside left margin of columellar retractor muscle ('cr' in Fig. 2A, B, E). Cells irregular and voluminous, with pale pink-staining vacuole (Figs 5C, G, 6A, 7N). Narrow opercular groove near anterior end and across posterodorsal side of foot, *c.* 10 μm deep, between opercular margin and glandular cells (Figs 2B, E, 5A: or 7M: arrow).

Black pigment granules found apically in many epidermal cells of headfoot (in particular dorsal side of tentacles), mantle margin and scattered in mantle cavity (esp. right corner) (Fig. 2A: pi). Further pigment in right corner of mantle cavity (Figs 5E: pl; 7B).

Calcium cells isolated spheres embedded in subepidermal tissue; with unstained interior often containing remnants of organic matrix (Figs 5A, B, C, F, 6B, 7M: cc). Two to three very large cells (diameter 30–35 μm) in posterior foot and below posterior tip of mantle cavity (Fig. 6B, white cell in 7M); cluster of smaller cells (diameter 10–15 μm) in neck (Figs 1A′, 2E).

Columellar muscle a thick, flat band spiralling along columellar part of visceral sac and posterior side of headfoot; extending from below operculum to *c.* 2nd body whorl. Main part with roughly 60 distinct fibres. Fibres fanning out in three places and directions: towards anterior right mantle skirt, into head tentacles, and to operculum (Figs 2E, 5E, 6A: mu1 to mu3; Supplementary Material, File S1). Other fibres (not shown) along sides of pharynx and into tentacles; no distinct buccal retractors found.

Aggregations of large subepidermal glandular cells (anterior pedal gland) in anterior portion of foot (Fig. 2E: apg). Cells irregular, wedged between muscle fibres; staining grey, with tiny blue vesicles (Fig. 5B). Gland opening presumed anteriomedian, in fold between upper lip and foot.

Large flask-shaped glandular cells (posterior pedal gland) found inside posterior, dorsal edge of foot below formation zone of operculum; cells stain blue (Figs 2E, 5A: ppg; dark blue cells in Fig. 7M).

Mantle cavity (Figs 2, 5 and 8): Mantle cavity opening anteriorly and slightly to the right, as wide as body whorl and extending posteriorly along approximately half of first whorl (Supplementary Material, File S1). Outline roughly triangular, posterior tip shifted slightly to the right (dorsal view of entire mantle cavity in Fig. 8B). Caecum emerging from left side of the triangle, extending along outer side of one half whorl (Fig. 2C: cae); outline marked by yellow specks in live specimens (Fig. 1A′, C′; source of colour not evident in histological sections). Caecum unciliated, inner lining smooth; situated just below epidermis, outer wall very thin (Fig. 6A). No further discrete organs/openings inside caecum.

Mantle border smooth, with two short tentacles at right corner of mantle cavity: short, solid and ciliated mantle tentacle at roof and longer, flat, second tentacle (mantle lobe herein) formed from mantle skirt at posterior right (Figs 1A″, 2A, 5F: mt and ml). Area between these appendages strongly ciliated (Fig. 8B). Anterior mantle margin duplicate (position of shell gland; Fig. 7A: arrowhead).

Anus in posterior right corner of mantle cavity; intestine along posterior edge (Figs 2A, 8B: an). Kidney in left half of

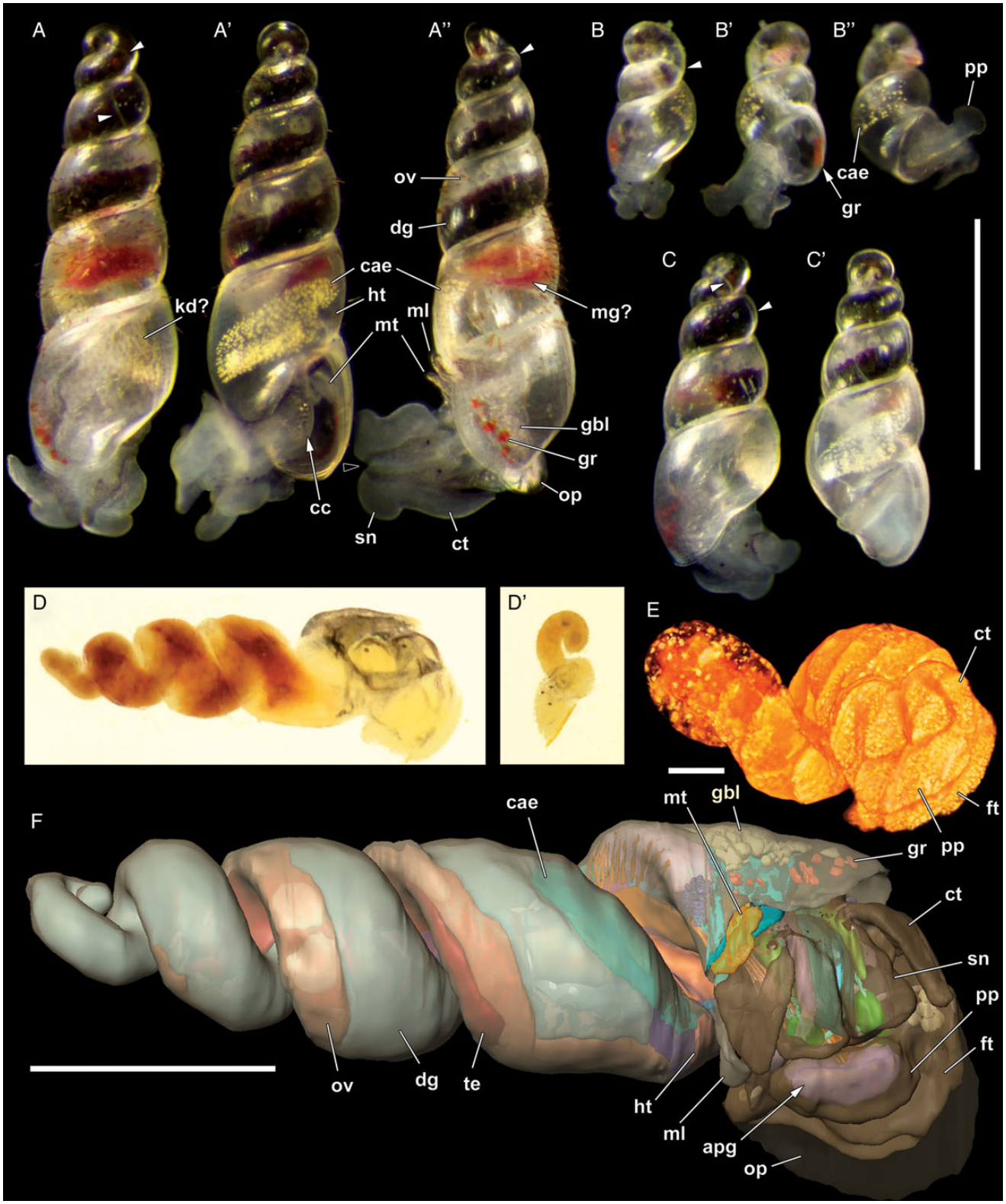

Figure 1. Live specimens of *Koloonella* cf. *minutissima* (Laseron, 1951) from Nelson Bay, Port Stephens, NSW, Australia and 3D reconstructions of complete specimens. **A, A′, A″.** Specimen AM C469740.001, mature individual used for 3D reconstruction (shown in **D**, **F**, and all other figures). Dorsal, ventral and right views. Shell size *c.* 1 mm. **B, B′, B″.** Specimen AM C469740.002, juvenile specimen used for 3D reconstruction (see also **D′, E**). Dorsal and ventral views. **C, C′.** Specimen AM C469741. Third specimen not sectioned in this study. Dorsal and ventral views. Shell size *c.* 300 μm. **D, D′.** Specimens embedded in epoxy resin prior to sectioning. Note pigment patterns on neck and head, and black cell near apex in **D′. E.** Specimen AM C469740.002. 3D reconstruction of complete specimen, anterior view. Volume rendering based on contrast values of individual images. Note morphology of the headfoot; operculum and shell not visible. **F.** Specimen AM C469740.001. 3D reconstruction of complete specimen, surface rendering of organ systems (shell not shown). Abbreviations and symbols: arrowhead, anterodorsal cleft that contains mouth opening; white

mantle roof, nephropore presumed at left of anus. Heart at left of kidney and anterior to intestine (Fig. 2D: ht). Female genital opening on floor of mantle cavity, at anterior right (Figs 2D, 8B: fgo). No gill or distinct ciliated strips; but strong ciliation in right corner of mantle cavity (Fig. 8B: cil). Folded area with voluminous cells in anteromedian part of roof (osphradium?; Figs 2D, 5E, 7B, 8B: osp).

Kidney in left part of mantle roof anterior to intestine, large, drop-shaped (Fig. 2C, D). Dense tissue with interspersed unstained vacuoles (Fig. 5F, G: kd). Lumen flattened in present material, nephropore not detected.

Heart located between origin of intestine and kidney (Fig. 2D), in 2nd body whorl. One part with strongly staining nuclei (ventricle?; Fig. 5F: ht), other part with wider lumen and smooth wall (auricle?; Fig. 5G). Pericardium or blood vessels not detected.

Epithelium in left half side of mantle cavity flat, not glandular, unciliated, as in caecum. Various epidermal and subepidermal glands in roof and along right edge of mantle cavity:

Crimson red glands at mantle rim (Fig. 1A″: gr) identifiable in histology as medium-sized to very large, rounded cells with median nucleus and vacuolated, pink-staining interior (Fig. 5C: gr; large cells in Fig. 7A).

Blochmann's gland a wide patch in roof of anterior mantle cavity (Figs 1A″, 2C, D: gbl). Spherical clusters of cells (or very large single cells?; diameter 10–25 μm), tightly spaced, with apical pore into mantle cavity (Figs 5E, 7C: gbl). Unstained interior with barely stained borders between vesicles, if visible at all.

Glands 1 and 2 apposed in right edge of mantle cavity, i.e. posterior to mantle tentacle (Figs 2C, D, 8B) and to right of female genital opening (Fig. 4F). Gland 1 posterior to gland 2, along edge of mantle cavity, approx. 150 μm long groove with voluminous, light pink-staining cells (30 μm tall; Figs 5G, 7F: g1). Gland 2 located in right corner of mantle cavity, c-shaped, with small, intensely violet-staining cells (Figs 5G, 7G: g2).

Glands 3 and 4 (presumed hypobranchial gland) near mantle tentacle (Fig. 2D). Gland 3 at base of tentacle, dorsal, with regular, blue-staining epithelium (Figs 5G, 7D: g3). Gland 4 ventral, a short strip opposing gland 3 and mantle tentacle, cells more prominent than gland 3 but otherwise similar (Figs 5F, 7E: g4).

Digestive system (Figs 3, 5, 6): Mouth opening in dorsal transversal groove on snout/upper lip (Fig. 1A″; arrow in Fig. 2A; Fig. 3A: mo). Oral tube very short (50 μm long), ciliated (Fig. 5D: asterisk).

Pharynx elongate-ovoid, with muscular layer *c.* 20 μm thick. Anterior walls of pharynx with blue-staining glandular cells (visible in Fig. 5C′); middle and posterior parts with thin, clear blue-staining cuticle, but no jaws. Odontophore slim, upright, protruding into pharyngeal cavity (Fig. 5C, C′). Paired, clear rods inside, *c.* 40 μm long (Fig. 5C′: white arrowheads), converging between root of odontophore and base of teeth (Fig. 3C–F: rr). One rod unpaired, anteromedian (Fig. 3E).

Radula on tip of odontophore, with four pointed, curved teeth (20 μm long), their tips interdigitating (Figs 3E; 5C′, 8A: black arrowheads). Radular formula 2 × 1.0.1 (derived from serial sections). Possibly one minute median tooth more anteriorly (Figs 3D, F, 8A: rtu).

Salivary gland horseshoe-shaped, on proximal oesophagus, i.e. posterodorsal to pharynx (Figs 3B, C, 8A: sg). Gland with *c.* 20 large cells with very large nuclei and minute, light-blue-staining droplets (Fig. 5E). No median boundary detected (i.e. left and right halves not separable). Salivary ducts not detected, but paired pockets in lumen of oesophagus indicate positions on each side (Fig. 3C: asterisks).

Oesophagus as wide as pharynx (Fig. 3B: es), with wide lumen and strongly ciliated cells. Epithelium glandular, a single, large blue-violet-staining vacuole per cell (Fig. 5E, F, G).

Stomach an indistinct stretch of glandular, ciliated wall between connections to oesophagus and intestine.

Single digestive gland extending to apex, in lower part of each coil (Figs 1F, 3A: dg). Cells tall and large (40 μm × 20 μm), with clear spherical vesicles; ciliation of epithelium sparsely visible (Fig. 6F, G). Lumen filled with homogeneous, uncharacterizable mass of food.

Intestine a ciliated, thin tube, emerging at right of oesophagus (Fig. 3B). Long dorsal loop to left and around posterior margin of mantle cavity (Fig. 3A); anus located in posterior right corner of mantle cavity (Figs 2C: asterisk; 3A, 6A, 8B). Epithelium ciliated (Fig. 6B, D: it); proximal and distal ends of intestine with blue-staining vacuoles (Fig. 5G).

Central nervous system and sensory organs (Figs 2F, 5 and 8): Nerve ring wide, with four ganglia located around pharynx and two others postpharyngeally (Figs 2F, 3A).

Cerebropleural ganglia elongate, drop-shaped; lateral of pharynx, interconnected by long cerebral commissure (Fig. 2F: ccm). Two connectives per cerebropleural and pedal ganglion (cerebropedal and pleuropedal connectives) (Figs 2F, 8C: cpc, ppc). Pedal ganglia roughly spherical, interconnected by long pedal commissure below pharynx. No parapedal commissure detected.

Two further ganglia (buccal/visceral loop ganglia? see Discussion; Fig. 8C) posterior to nerve ring and ventral to oesophagus. Left one elongate, curved (two annexed ganglia?) (Fig. 2F: lg); right one slightly larger and oval (Fig. 2F: rg). No nerves or connectives found in these ganglia.

Aggregations of nuclei left and right of cerebropleural ganglia may be potential further ganglia, but boundaries or interconnections impossible to delimitate in sections (Figs 5E, 8B: ag?).

Eyes laterally on cerebropleural ganglion. Lens clear and spherical; with black pigment cup and basal sensory cells (Fig. 5B, E). Optic nerve short, no connection to cerebropleural ganglion found.

Statocysts a hollow sphere dorsally on each pedal ganglion (Fig. 5B: sc). Paired static nerves to cerebropleural ganglion parallel to cerebropedal connectives; no contact of nerve to cerebropleural ganglion found.

No innervation detected of tentacles or other sensory organs (e.g. putative osphradium).

Reproductive system (Figs 4, 5, 6): Hermaphroditic, with separate ovary and testis (Figs 4B, 8D). Gonoduct diaulic: male part with internal vas deferens and cephalic penis (Fig. 4C), female part with nidamental glandular mass (Fig. 4D).

Ovary extending along adapical and outer sides of 2nd to 4th whorl (Figs 1, 4A: ov). Oocytes densely packed, large ones as wide as ovary, with large oval nucleus (25 μm) and spherical, blue-stained nucleolus (7 μm). Cytoplasm homogeneous grey, or with dense aggregates of blue-staining yolk droplets in larger cells (Fig. 6E, F: oc).

arrowheads, growth lines between protoconch I/II and teleoconch; apg, anterior pedal gland; cae, caecum of mantle cavity (spotted yellow); cc, calcium cells (refracting spherules); ct cephalic tentacle; dg, digestive gland (dark red); ft, foot; gbl, Blochmann's gland (whitish granules); gr, red gland at mantle rim; ht, heart; kd?, putative position of kidney; mg?, position of male glands (bright red); ml, mantle lobe; mt, mantle tentacle; op, operculum; ov, ovary with oocytes (white); pp, propodium; sn, snout. Scale bars: **A–C** (at right) = *c.* 500 μm; **E** = 50 μm; **F** = 250 μm. Additional files (File S1: interactive 3D model; File S2: live video) are available as Supplementary Material at *Journal of Molluscan Studies* online.

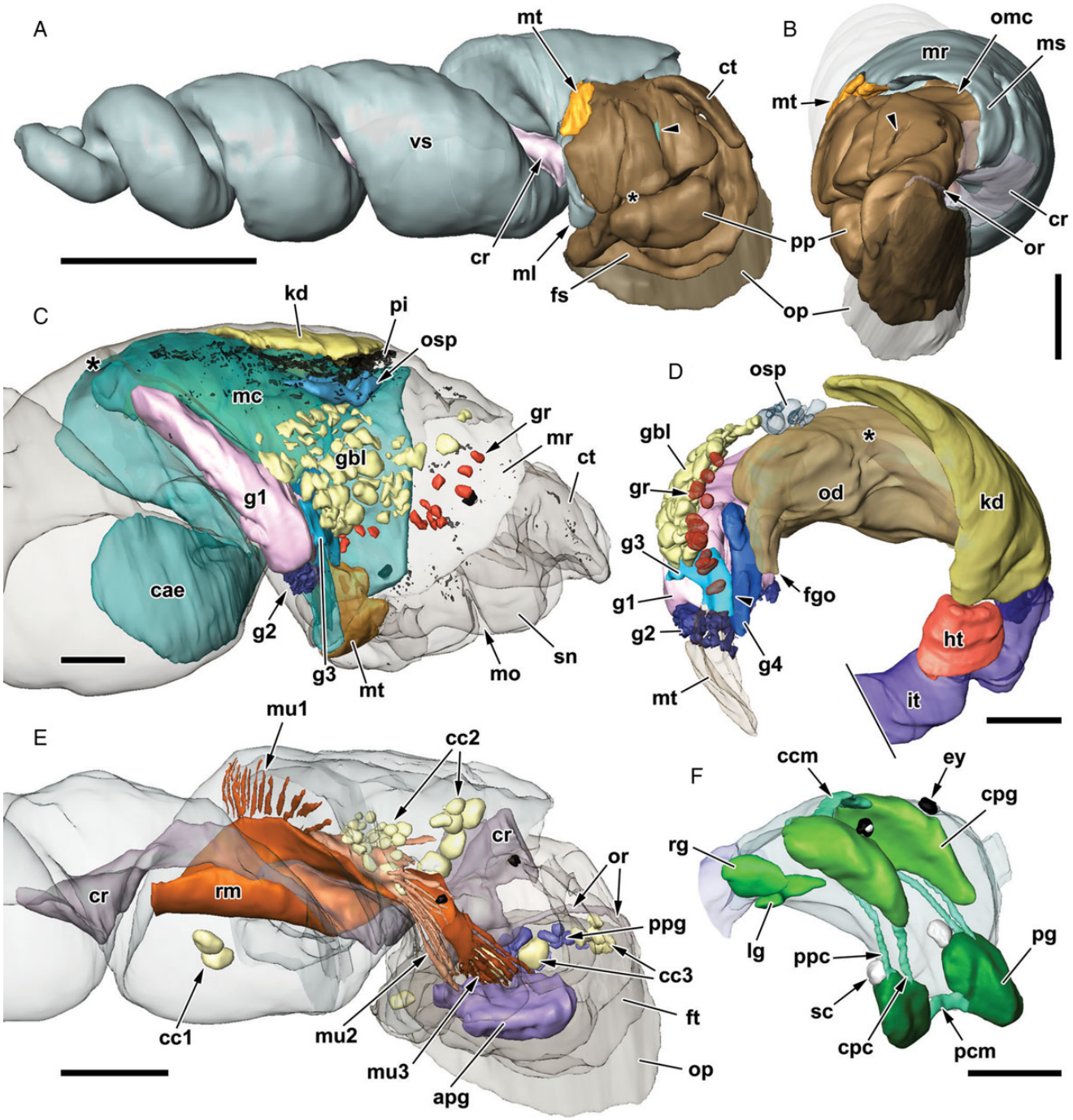

Figure 2. 3D reconstructions of microanatomy of *Koloonella* cf. *minutissima* (Laseron, 1951). Aspects of general anatomy, mantle cavity and central nervous system. **A.** External view of body, right view. Arrowhead indicates mouth, asterisk position of male genital opening inside cephalopedal groove. **B.** Left view of headfoot. Arrowhead marks groove on posterior face of head tentacle. **C.** Dorsal view of anterior body showing aspects of mantle cavity. Asterisk indicates position of anus. **D.** Mantle cavity associated organs. Anterior view. Arrowhead marks gap between floor and roof of mantle cavity, asterisk position of anus (in background). **E.** Further internal aspects of anterior body. Right view. **F.** Central nervous system, right view. Digestive tract shown transparent. Abbreviations: an, anus; apg, anterior pedal gland; cc1, calcium cells below caecum of mantle cavity; cc2, calcium cells on neck; cc3, calcium cells in foot; cae, caecum of mantle cavity; ccm, cerebral commissure; cpc, cerebropleural connective; cpg, (left) cerebropleural ganglion; cr, columellar ridge; ct, cephalic tentacle; ey, eye; fgo, female genital opening; fs, foot sole; ft, foot; g1, tubular mantle gland; g2, ring-shaped mantle gland; g3, gland at base of mantle tentacle; g4, gland opposite of mantle tentacle (hypobranchial gland); gbl, cells of Blochmann's gland; gr, red gland at mantle rim; ht, heart; it, intestine; kd, kidney; lg, left posterior ganglion; mc, mantle cavity; mr, mantle roof; ml, mantle lobe (on mantle skirt); mo, mouth; mt, mantle tentacle (on roof of mantle cavity); mu1, muscle fibres at right margin of mantle cavity; mu2, muscle fibres into head and tentacles; mu3, muscle fibres into foot; od, oviduct; omc, opening of mantle cavity; op, operculum; or, opercular groove; osp, putative osphradium; pcm, pedal commissure; pg, (left) pedal ganglion; pi, pigment granules on neck and head; pp, propodium; ppg, posterior pedal gland (at formation zone of operculum); rg, right posterior ganglion; rm, columellar retractor muscle. Scale bars: **A** = 250 μm; **B** = 100 μm; **C–E,** = 50 μm; **F** = 200 μm.

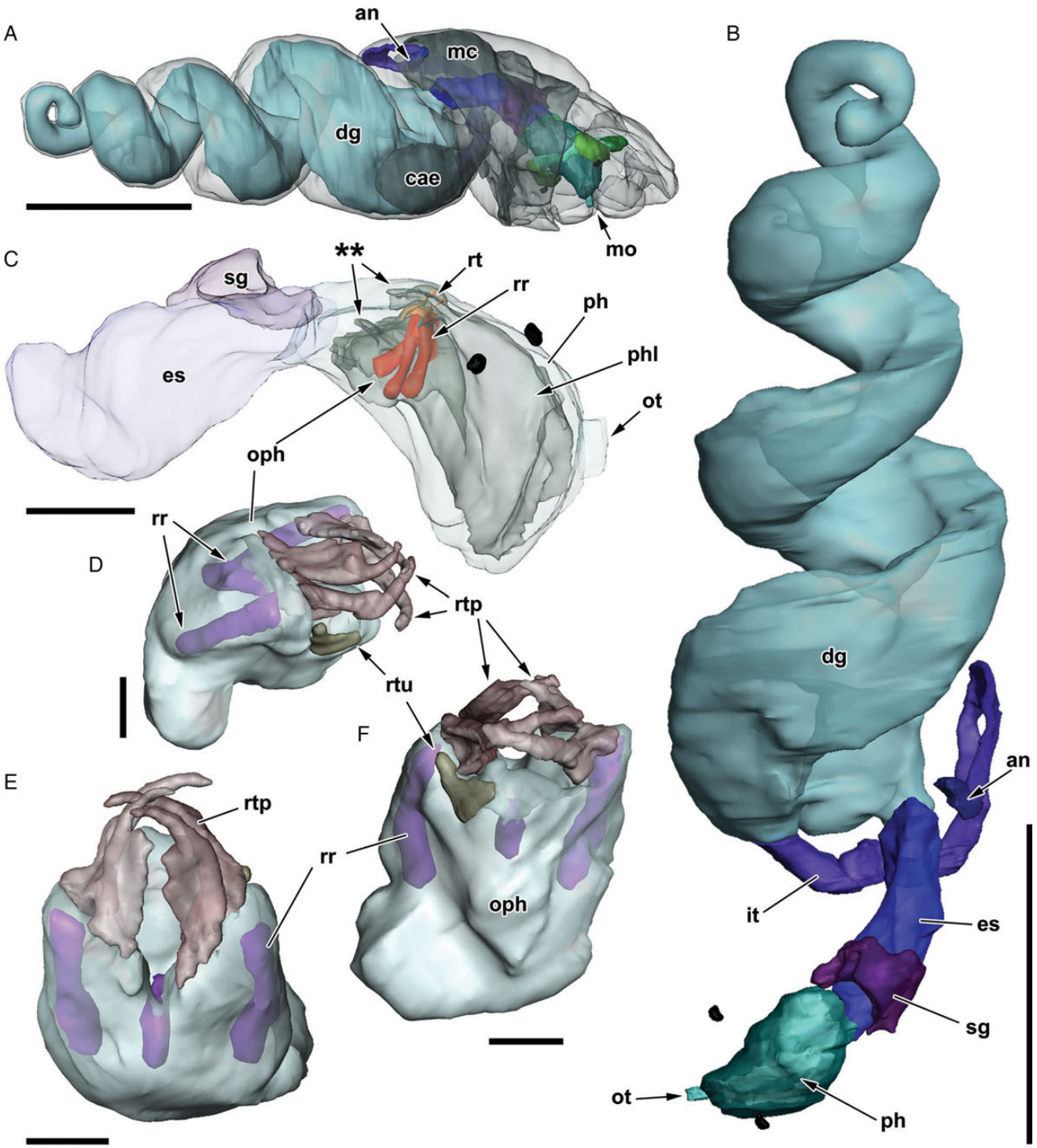

Figure 3. 3D reconstructions of alimentary organs of *Koloonella* cf. *minutissima* (Laseron, 1951) **A.** Overview, body outline shown transparent. Dorsal view. **B.** Complete alimentary system, dorsal view. **C.** Anterior part of alimentary tract. Right view. Asterisks indicate paired pockets of pharynx lumen (putative openings of salivary ducts). **D–F.** Details of odontophore and radular apparatus of juvenile specimen (AM C469740.002). **D.** Anterior right view. **E.** Dorsal view. Medial serration of teeth is an artefact. **F.** Ventral view. Abbreviations: an, anus; cae, caecum of mantle cavity; dg, digestive gland; es, esophagus; ey, eye; it, intestine; mc, mantle cavity; mo, mouth; oph, odontophore; ot, oral tube; ph, pharynx; phl, pharynx lumen; rr, radular rods; rt, radular teeth; rtp, paired radular teeth; rtu, unpaired radular element; sg, salivary gland. Scale bars: **A**, **B** = 250 μm; **C** = 50 μm; **D–F** = 10 μm.

Testis along columellar part of whorls 2.5 to 4 (Fig. 4A: te). Testis packed with irregular, blue-stained sperm precursor cells and interspersed bundles of *c.* 10–15 spermatozoa with long, smooth heads pointing towards particular (nurse?) cells (Fig. 6F). Some spermatozoa in testis with cone-shaped, externally smooth heads appearing hollow internally (Fig. 6F′: arrowheads).

Distinct lobe at base of testis a putative ampulla (or immature second testis, see Discussion; Fig. 4B, CM: am); this region filled densely with spherical cells, but no spermatozoa (Fig. 6E).

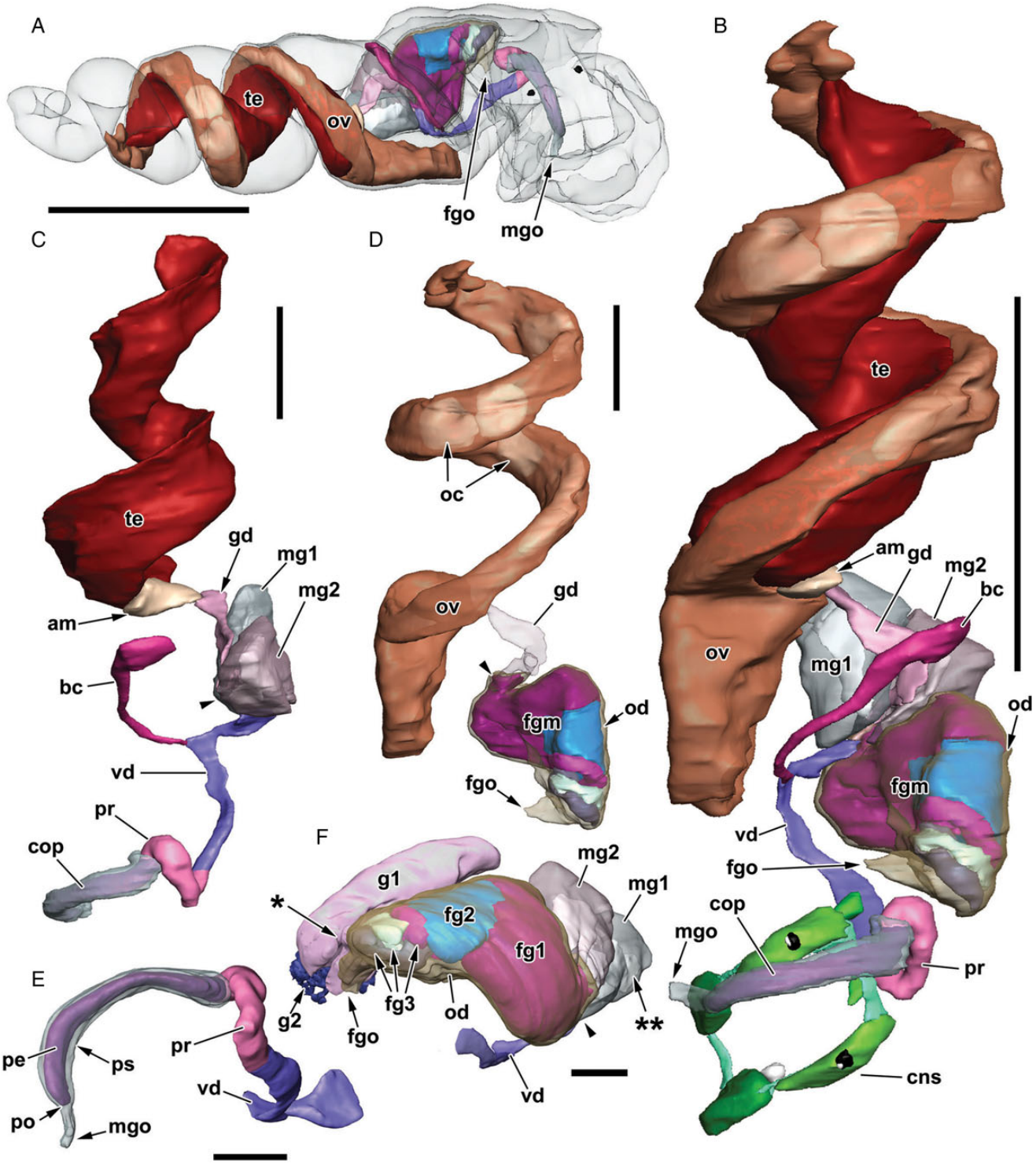

Figure 4. 3D reconstructions of reproductive organs of *Koloonella* cf. *minutissima* (Laseron, 1951) **A.** Overview, body outline shown transparent. Anterior/right view. **B.** Complete reproductive system, dorsal view. **C.** 'Male' part of reproductive system. Dorsal view. Arrowhead indicates where female system splits off from common gonoduct. **D.** Female part of reproductive system. Dorsal view. Arrowhead indicates where male system splits off. **E.** Detail of copulatory organ. Left view. **F.** Detail of reproductive glands below mantle cavity, and associated mantle glands. Left view. Asterisk indicates glandular groove opposite female genital opening. Double asterisk indicates position of presumed spermatozoa inside 'male' gland 1. Arrowhead indicates where vas deferens splits off from common gonoduct. Abbreviations: am, ampullary region; bc, putative bursa copulatrix; cns, central nervous system; cop, copulatory organ; fg1, first female gland (putative albumen gland); fg2, second female gland (putative membrane gland); fg3, zones of third female gland (putative mucus gland); fgm, female gland mass; fgo, female genital opening; g1, tubular mantle gland; g2, ring-shaped mantle gland; gd, hermaphroditic part of gonoduct; mg1, male gland 1; mg2, male gland 2; mgo, male genital opening; oc, ripe oocytes; od, (wall of) glandular oviduct; ov, ovary; pe, penis; po, penial opening; pr, prostate; ps, penial sheath; te, testis; vd, vas deferens. Scale bars: **A, B** = 250 μm; **C, D** = 100 μm; **E, F** = 50 μm.

Common gonoduct thin-walled and ciliated (Fig. 6B: gd). Splitting into separate 'male' and 'female' pathways (vas deferens and oviduct; split marked by arrowhead in Figs 4C, D, F; 8D); with distinct glands.

'Male' pathway with two proximal bag-like glands, next to female glands and opposing each other (area possibly corresponding to brighter red zone in Fig. 1A; Fig. 4B, C, F). First 'male' gland (putative receptaculum seminis, see Discussion) with thick wall, irregular cells, basal nuclei and conspicuous clear blue-stained vesicles (5–6 μm) (Fig. 6A, B: mg1); apical end of lumen with thin wall and bundle of *c*. 50 spermatozoa different from most of those in gonad (Fig. 4F: double asterisk; Fig. 6D, D′: white arrowhead). Second 'male' gland also sac-like, with wider lumen; cells more regular, with large, blue-staining basal nucleus and clear, pale pink-staining vesicles (1–2 μm) in cytoplasm (Fig. 6B, C: mg2).

Following gonoduct again thin and ciliated, running anterior along neck. Blind-ending duct (bursa copulatrix?) with long stalk and spherical head located between tip of mantle cavity caecum and retractor muscle (Fig. 4A, B, C: bc); duct thin and ciliated (Fig. 6A: bs), bulb with fluid-filled lumen staining pink (Fig. 6C: bc). Vas deferens in neck thicker (with tubular prostate; Figs 4B, C, E, 8D: pr), ciliated, slightly glandular (Fig. 5E); straight connection to lumen of penis. Penis tubular, hollow, with apical pore (Fig. 5C, E: pe). Penis retracted into penial sheath at dorsal right of pharynx and central nervous system (Fig. 4B); penial sheath thin, epithelial, unciliated. Male genital opening at anterior right side of head, between margins of foot and right side of snout (Figs 2A: asterisk; 5C: white arrowhead).

Female pathway of gonoduct a strongly glandular oviduct with columnar epithelium; in anterior floor of mantle cavity/posterior part of neck (outer wall marked od in Fig. 4B, D). Three consecutive glandular areas (or five, see Discussion; Figs 4F, 8D: fg1–fg3): first zone with tall cells and pink, irregular vesicles (Fig. 7H), second with cells staining smoothly blue (Fig. 7J), third part with shorter cells and distinct round droplets in three differently staining zones (Fig. 5F, G, H): first zone pinkish (Fig. 7K), second almost unstained (Fig. 7L: below), third ink blue (Fig. 7L: above). Female genital opening at right side of mantle cavity floor (Figs 2D, 8B: fgo; 5F: arrow).

Juvenile specimen (Figs 1, 3D–F): Morphology of head as in adult specimen, tentacles stubbier (Fig. 1B, E). Fewer pigment granules in epidermis.

Glands in mantle cavity less developed: cells of Blochmann's gland not as fused as in adult specimen. Glands 2 and 3 not present, gland 1 (pink) smaller.

Two very large single cells (30 μm) with large nucleus (14 μm) below epidermis close to recurving apical whorl (black patch near apex in Fig. 1D′ is one cell); some vacuoles with distinct black granules. Epidermis slightly frayed in this area.

Nervous, excretory, and digestive systems essentially as in adult specimen. Radula possibly with one very small cuticular element anterior to four radular teeth (Fig. 3D, E, F).

Gonad anlage a short band at outer side of first whorl, densely filled with irregular, blue-staining cells. No gonoduct detected.

DISCUSSION

Our study is the first comprehensive study of the anatomy of a member of the Murchisonellidae, and the first 3D reconstruction of a high-spired gastropod. The high degree of anatomical complexity revealed in this tiny gastropod highlights the usefulness of 3D reconstruction for the examination of taxa that lack easily accessible anatomy and those that lack character-rich hard parts. It also gives a glimpse of diversity that may otherwise be underrated from the study of shells or molecular data alone.

Taxonomy

Currently, the family Murchisonellidae is classified as consisting of five valid genera and *c*. 60 nominal species (Bouchet, 2013; see Warén, 1995, 2013 for discussion). Warén (1995) recognized the presence of a pincer-like 'jaw' apparatus in *Ebala* and *Murchisonella* as a synapomorphy and as a difference from Pyramidellidae (which have a piercing stylet). *Henrya* was similary reclassified as a murchisonellid by Wise (1999). However, except for these records and Rasmussen's (1944) observation of live *Ebala nitidissima*, all other works on Murchisonellidae have consisted only of records of shell characters (e.g. Fretter, Graham & Andrews, 1986; van Aartsen 1994, 1995; Peñas & Rolán, 2013).

The genus *Koloonella* was established by Laseron (1959) for minute, smooth and translucent pyramidellid-like shells otherwise identified as *Eulimella* Forbes & MacAndrew, 1846, all found in Australia and Papua New Guinea. Shell characters are similar to *Ebala* and *Henrya*, but *Koloonella* was only recently confirmed as a murchisonellid due to its shared possession of the jaw apparatus (Warén, 2013). This new placement is corroborated by the results of this study and preliminary molecular data (N.G.W., unpubl.).

According to shell characters, our material most closely resembles *Koloonella minutissima* (Laseron, 1951). Both are very small (about 1 mm) compared with most other *Koloonella*, some of which may reach up to 6 mm (Laseron, 1951, 1959). Accordingly, the protoconch is relatively larger with respect to the rest of the shell (see also Laseron, 1951: fig. 72). The locality of our material (Port Stephens) is *c*. 200 km north from the type locality of *K. minutissima* (Port Jackson; Laseron, 1951) and shows similar characteristics in habitat. According to shell characters, the second closest match to our material is an undescribed *Koloonella* figured by Laseron (1959: fig. 201), but which is found further north, in tropical waters.

Furthermore, Warén (2013) identified *K. minutissima* to be the species from which one of the two only hitherto published molecular sequences of Murchisonellidae was derived (Genbank COI FJ917277 from Moreton Bay, Queensland; Dinapoli *et al*., 2011). We agree with Warén's identification based on original photographs; again, shell characters and distribution fit with Laseron's (1951) description. Preliminary comparison of Dinapoli *et al*.'s sequence with that of our material (N.G.W., unpubl.) indicates a close relationship, if not conspecificity, of both samples. However, Dinapoli *et al*.'s specimen is coloured uniformly brown (observation by B.B. on original photos supplied by A. Dinapoli) in contrast with ours. Because there may be several similar species of *Koloonella* in the area, material of *K. minutissima* from the type locality needs to be compared with both sequences for a conclusive species identification of the specimens used in Dinapoli's paper and *K.* cf. *minutissima* of the present study.

Warén (2013) suggested that there were two distinct lineages among Murchisonellidae and therefore (re)established the subfamily Murchisonellinae Casey, 1904 to include all genera except for *Ebala* (the latter included in Ebalinae Warén, 1995). This was supported specifically by radular characters. We do not agree with this proposed classification (see Table 2), because we believe that *Koloonella* is more similar to *Ebala* and *Henrya* than to *Murchisonella* according to our data and to published accounts of the former two genera. Accordingly, we regard the subfamily Ebalinae to contain *Ebala*, *Koloonella* and *Henrya*; with *Murchisonella* and, according to shell features, *Pseudoaclisina* included in Murchisonellinae (Table 2).

General morphology

The shells of the material studied herein were decalcified prior to histological sectioning. Therefore, details of shell structure are

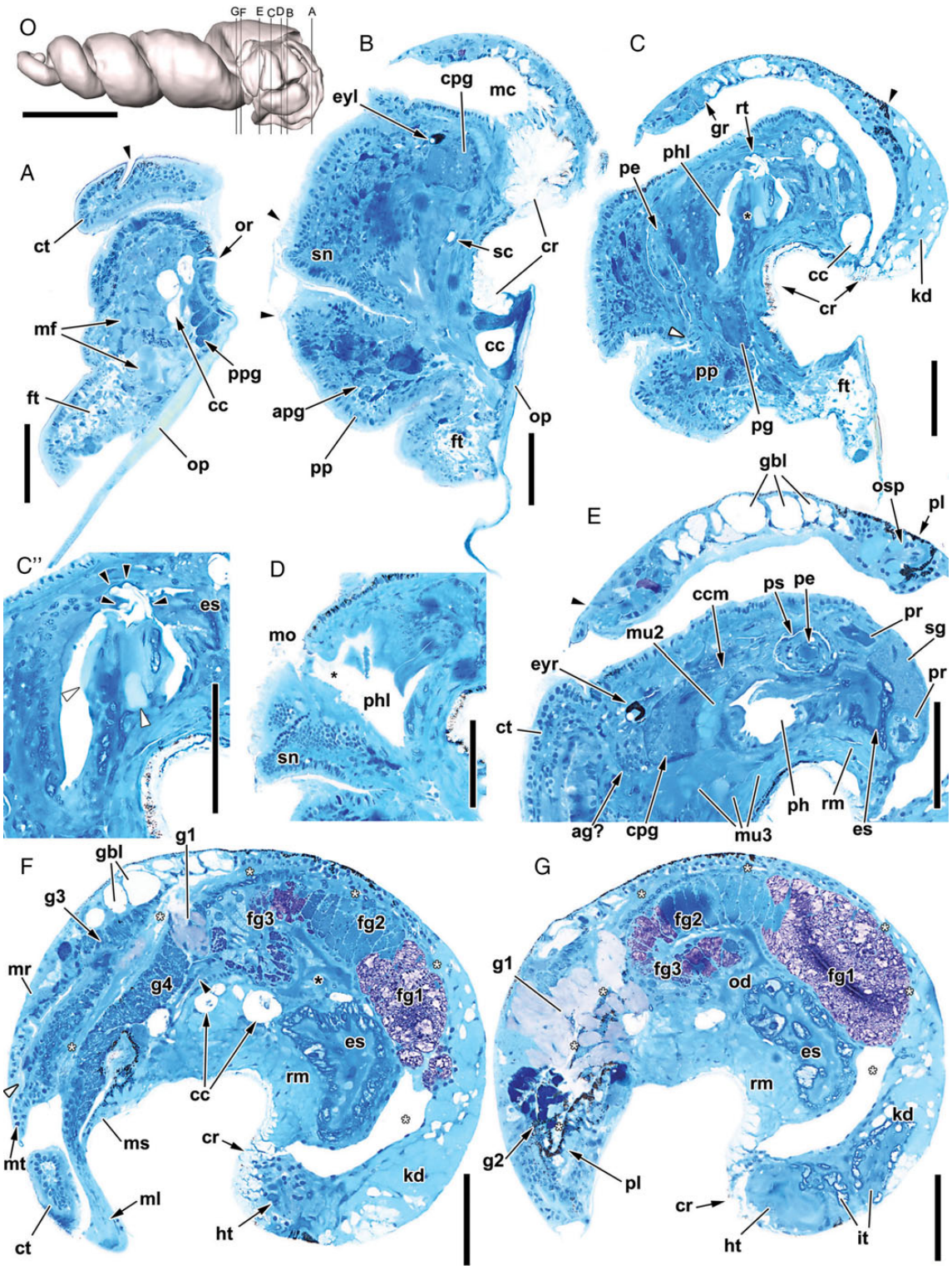

Figure 5. Histology of *Koloonella* cf. *minutissima* (Laseron, 1951) Semithin sections of anterior body, stained with methylene blue/azure-II. **O.** Overview of body, with sections shown in this figure highlighted. **A.** Head tentacle and foot. Arrowhead marks groove in tentacle. **B.** Headfoot at level of left eye. Arrowheads mark strong ciliation on anterior snout and propodium. **C.** Section at level of odontophore and male genital opening. Arrowhead marks fold in mantle roof (pigment layer below kidney). White arrowhead position of male gonopore. Asterisk indicates odontophore. **C′.** Detail of **C**, odontophore. Arrowheads mark tips of teeth. White arrowheads indicate clear rods inside odontophore. **D.** Head at level of mouth. Asterisk marks oral tube. **E.** Section at posterior end of pharynx. Arrowhead marks duplicate mantle border (shell gland). **F.** Section at level of female genital opening (arrowhead). White arrowhead marks gap between mantle border and mantle tentacle. Asterisk shows lumen of gonoduct. White asterisks mark position of mantle cavity. **G.** Section at level of glandular pocket in mantle cavity. White asterisks mark position of mantle cavity. Abbreviations: ag?, potential accessory ganglia; apg, anterior pedal gland; cc, calcium cells; ccm, cerebral commissure; cpg, cerebropleural ganglion; cr, columellar ridge; ct, head tentacle; es, esophagus; eyl, left eye; eyr, right eye; fg1, first female gland (putative albumen gland); fg2, second female gland (putative membrane gland); fg3, zones of third female gland (putative mucus gland); fgm, female gland mass; ft, foot; g1, tubular mantle gland; g2, ring-shaped

no longer visible in the reconstructed material. However, the quality of the photographs of living animals allow for some observations on the shell. Details of the shells of *Koloonella* were depicted by Laseron (1951, 1959) and Kay (1979); both characterized the shells as elongate, with rounded whorls lacking sculpture or columellar folds. The shell of the species examined herein is smooth and glossy, as reported by Laseron (1951, 1959), but also shows faint spiral striation, depending on the angle of illumination. This ornamentation was also observed in species of *Ebala* (e.g. Warén, 2013). Striation is more distinct in other *Ebala* (e.g. *E. striatula*; Öztürk & Bakir, 2013), while species of *Murchisonella* and *Pseudoaclisina* always show more or less sculptured shells.

Koloonella lacks a sinus located in the adapical edge of the lip, where the mantle tentacles protrude. This is also the case in *Henrya* and most *Ebala*. Most *Murchisonella* are characterized by a distinct sinus that creates the characteristic angular shoulder on the top quarter of each whorl; in *Pseudoaclisina*, the sinus is not prominent (Peñas & Rolán, 2013).

The protoconch of *Koloonella* is inflated and hyperstrophic, as is typical for Heterobranchia. The protoconch possesses a sinistrally coiled part that is little larger than 1 whorl; this part is inverted and angled at *c.* 120° to the teleoconch axis. All of the Australian *Koloonella* described by Laseron (1951, 1959) possess this short, oblique and 'tilted' protoconch of 'few' whorls (i.e. clearly <2 full whorls), described with an almost tubular part where the coiling direction is reversed. Our material agrees with these observations. Numerous accounts of murchisonellid shells show similarly short protoconchs, e.g. for *Ebala* (Rasmussen, 1944; Thorson & Jørgensen, 1946; Rodriguez Babio & Thiriot-Quiévreux, 1974; Fretter *et al.*, 1986; Bogi, 1987; Warén, 1995; Peñas, Templado & Martínez, 1996), *Murchisonella* (Bogi, Buzzurro & Greppi, 1995; Peñas & Rolán, 2013) and *Henrya* (Wise, 1996). In contrast to most of these records, all live specimens of *K.* cf. *minutissima* examined herein showed two distinct growth lines near the apex (Fig. 1): the first growth line marks the first whorl of the protoconch where coiling direction changes from sinistral to dextral, and the second one follows after one further complete whorl (then already dextral). This distinct second growth line observed here is also visible in some published figures of *Ebala* (Warén, 1995: fig. 1C; Peñas & Rolán, 2013: pl. 13, fig. 5). Thorson & Jørgensen (1946) described veligers and adult shells of *E. nitidissima*; they depicted the first teleoconch whorls as smooth, without the spiral sculpture found in the following parts (1946: fig. 123D-G). Whether this structurally different part (located between both growth lines in *Koloonella*) is a protoconch II, or a distinct first whorl of the teleoconch, is not known. In *K.* cf. *minutissima*, this whorl shows minute distant speckles (Fig. 1A); it is not clear from our material whether this is a character of the shell or the underlying soft body, because the shell is translucent. None of the previous studies on other murchisonellids reported similar distinct pitting different from the remaining shell or truly multispiral protoconchs. Among other basal heterobranchs, Bieler, Ball & Mikkelsen (1998) noted the presence of distinct growth lines in the (not multispiral) protoconch of cornirostrid Valvatoidea.

Two-part protoconchs are known for other gastropod taxa, and sculptural characters of the protoconch are considered to have implications about larval development of the snail (e.g. Bouchet & Warén, 1979). Our observation of *Koloonella* could imply that the phenomenon also occurs in at least some members of the genus, meaning that the protoconch includes a sinistral part (the 'embryonic' shell formed by the larval shell gland) and a single dextral whorl (the 'larval' shell formed by the mantle skirt). On the basis of larval shell characters, *E. nitidissima* was interpreted to have a long-lived, planktotrophic veliger stage (Rasmussen, 1944; Thorson & Jørgensen, 1946), which is consistent with its purported wide range throughout European waters. In contrast, data on *Koloonella* species (Laseron, 1959) currently suggest that their ranges are rather restricted, which could indicate that larval development in the genus is different (i.e. without a long-lived planktonic stage). It is not clear from our data how informative protoconch morphology is with respect to larval development in murchisonellids, and further SEM study of *Koloonella* shells is needed to test if protoconchs are different from that of other murchisonellids. Furthermore, current classification of *Koloonella* (Bouchet, 2013) also includes species that show different protoconchs with more than 1 sinistral whorl, e.g. western African *K. ignorabilis* (Peñas & Rolán, 1997: fig. 253). Robba (2013) also identified fossil *Koloonella* to be distinguished from pyramidellids by the inflated, flat-spired protoconch of three sinistral whorls or less. This configuration with several sinistral whorls is different from that observable in the *Koloonella* examined herein. Whether the aforementioned taxa with more protoconch whorls are truly *Koloonella*, or murchisonellids at all, remains to be confirmed by molecular analysis of extant species.

Overall, shells of murchisonellids can be distinguished from those of pyramidellids by the combination of the characteristic angle of the protoconch, lack of columellar lamellae or tooth, being very small and thin, and by the presence of an apertural sinus in the position of the mantle lobe (in *Murchisonella*). The shells of *Ebala*, *Koloonella* and *Henrya* are rather similar (smooth, with no sinus or shoulder), while those of *Murchisonella* appear distinct (sculptured, with adapical sinus in lip of shell) (Table 2). Other potential murchisonellids currently classified among Pyramidellidae on the basis of a similar small, translucent shells, may include, e.g. species placed in *Eulimella* Forbes & MacAndrew, 1846, *Careliopsis* Mörch, 1875, *Tathrella* Laseron, 1959 and *Instarella* Laseron, 1959.

In external morphology, murchisonellids resemble pyramidellids in the gross morphology of the foot (short) and the head (with two rather flat tentacles, and the mouth on top of a transverse shelf with a longitudinal dorsal groove). Murchisonellids possess one pair of head tentacles, and a slightly bifurcated snout. This is consistent with the pattern found in many basal heterobranchs (Ponder, 1990a, b, 1991; Bieler *et al.*, 1998). Among murchisonellids, *Murchisonella* has the most 'typical' tentacles (pointed, rather round in cross-section) compared with other basal heterobranchs (Warén, 2013). The tentacles of *E. nitidissima* are more triangular, and slightly flattened with rather wide bases (Rasmussen, 1944). In *Koloonella* and *Henrya*, the paired head tentacles are shorter and flattened, rabbit-ear shaped. In combination with the stubby sides of the snout and the middorsal cleft, the head of *Koloonella* resembles the headshield of some Acteonoidea or euopisthobranch Cephalaspidea (Burn & Thompson, 1998). This may be related to the potentially more infaunal lifestyle of these two genera as observed in this study and by Wise (1999). The sharp-bordered groove on one side of the tentacles in *K.* cf. *minutissima* has not been mentioned for

mantle gland; g3, gland at base of mantle tentacle; g4, gland opposite of mantle tentacle (hypobranchial gland); gbl, Blochmann's gland; gr, red glands of mantle rim; it, intestine; kd, kidney; mc, mantle cavity; mf, muscle fibres; mf, muscle fibres; ml, mantle lobe; mo, mouth; mr, mantle roof/rim; ms, mantle skirt; mt, mantle tentacle; mu2, muscle fibres into head and tentacles; mu3, muscle fibres into foot; od, oviduct lumen; op, operculum; or, opercular ridge; osp, putative osphradium; pe, penis; pg, pedal ganglion; ph, pharynx; phl, pharyngeal lumen; pl, pigment layer; pp, propodium; ppg, posterior pedal (opercular) gland; pr, prostate; ps, penial sheath; rm, retractor muscle; rt, radula teeth; sc, statocyst; sg, salivary gland; sn, snout. Scale bars: all 50 μm except **A** = 250 μm; **F** = 10 μm.

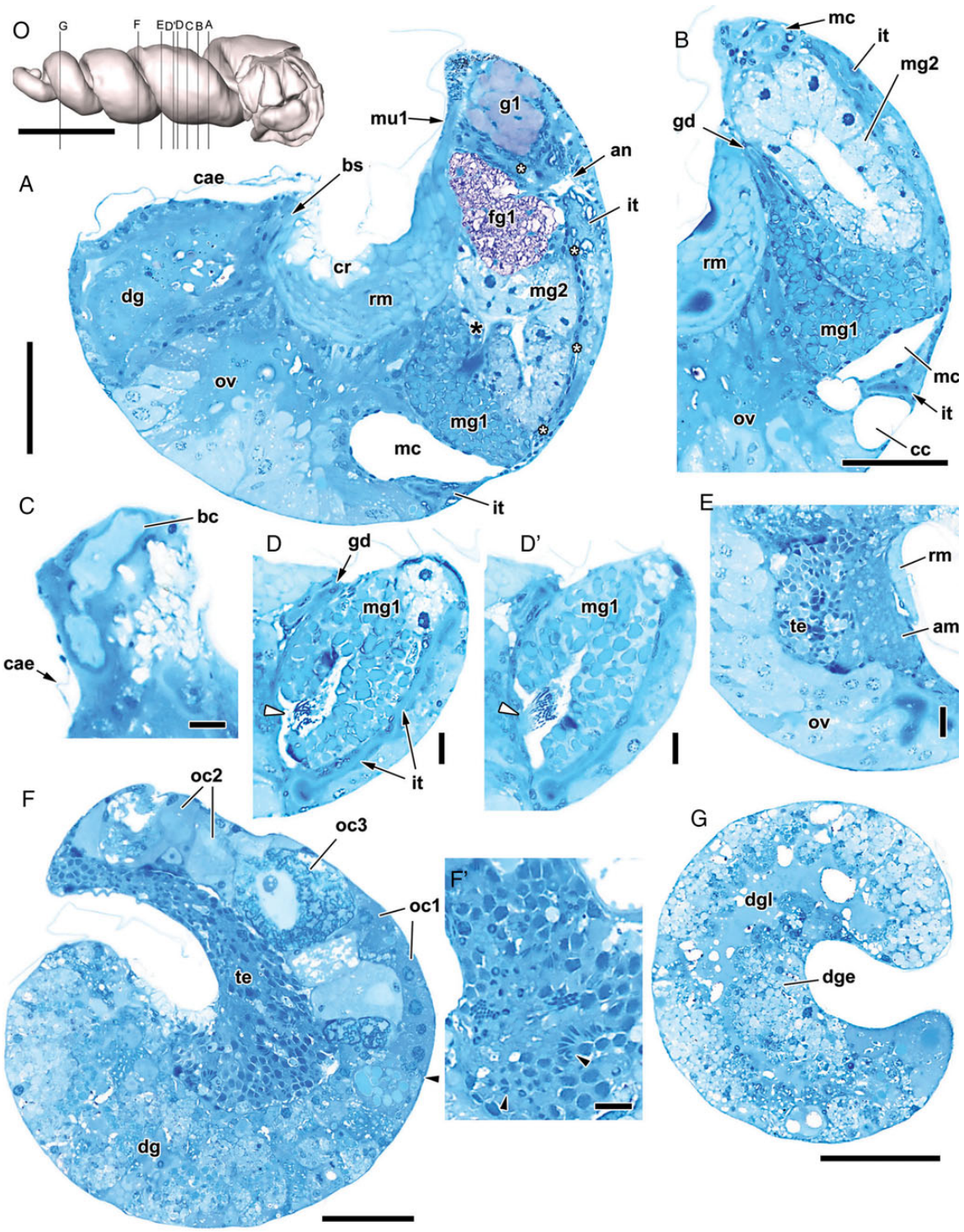

Figure 6. Histology of *Koloonella* cf. *minutissima* (Laseron, 1951) Semithin sections of posterior body, stained with methylene blue/azure-II. **O.** Overview of body, with sections shown in this figure highlighted. **A.** Glandular area at origin of vas deferens. Asterisk marks lumen of vas deferens, white asterisks mark position of mantle cavity. **B.** Detail of 'male' glands. **C.** Detail of bursa copulatrix. **D, D′.** Detail of putative receptacle, with bundle of putative spermatozoa (white arrowheads). **E.** Detail of anterior testis and second lobe (putative ampulla or second testis). **F.** Posterior body and gonads (arrowhead marks margin between ovary and digestive gland). **F′.** Detail of testis. Arrowheads indicate 'hollow' heads of spermatozoa. **G.** Posterior end of body containing only digestive gland. Abbreviations: am, putative ampulla; an, anus; bc, head of bursa copulatrix; bs, bursa stalk; cae, caecum of mantle cavity; cc, calcium cell; cr, columellar ridge; dg, digestive gland; dge, digestive epithelium; dgl, lumen of digestive gland; fg1, first female gland (putative albumen gland); g1, tubular mantle gland; g2, ring-shaped mantle gland; gd, common gonoduct; it, intestine; mc, mantle cavity; mg1, 'male' gland 1 (putative seminal receptacle); mg2, 'male' gland 2 (putative prostate); mu1, muscle fibres at right margin of mantle cavity; oc1, young oocytes; oc2, medium oocytes; oc3, yolky oocyte with reticulated appearance; ov, ovary; rm, retractor muscle; te, testis. Scale bars: all 50 μm except **A** = 250 μm, **F** = 10 μm.

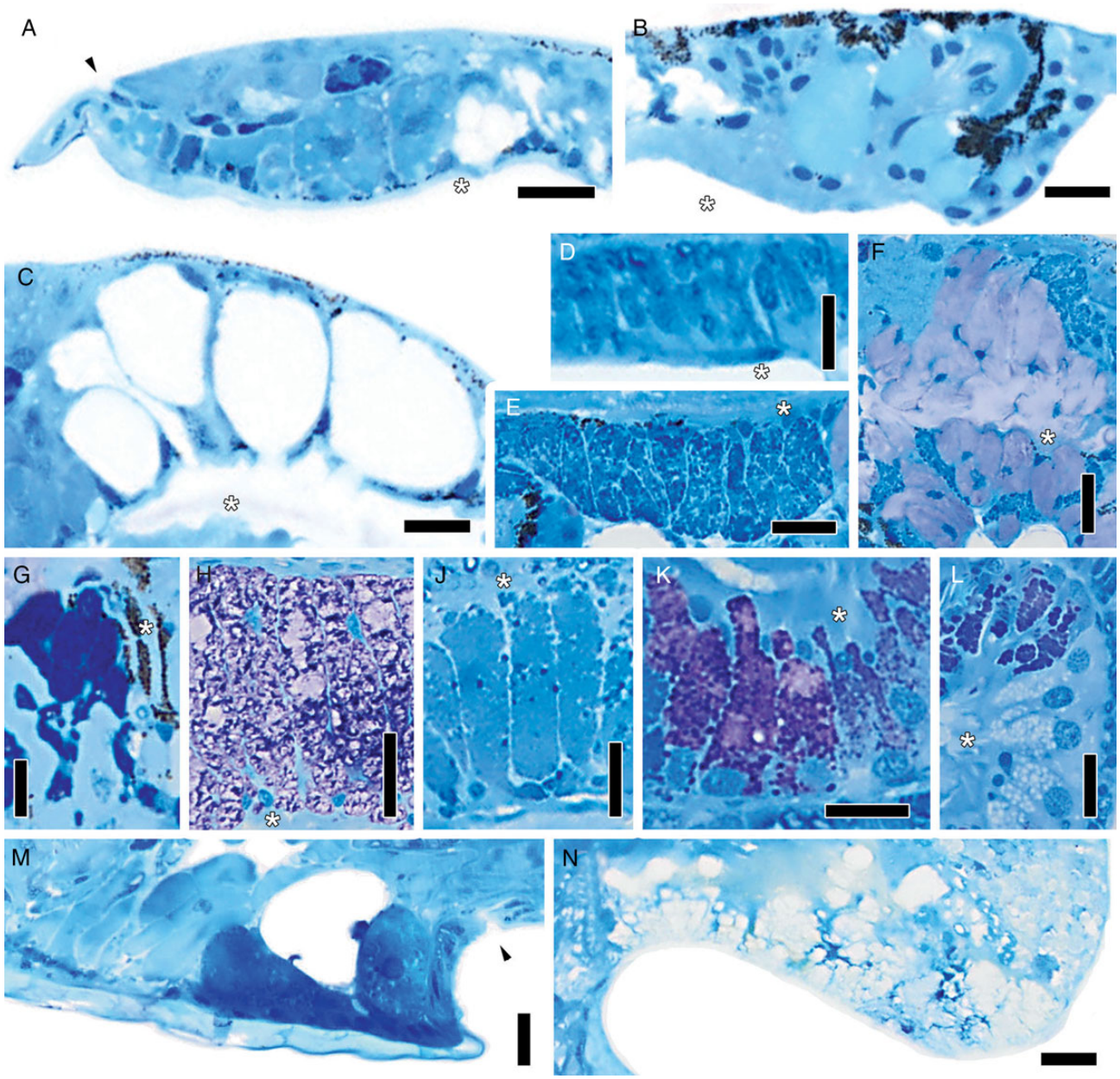

Figure 7. Details of histology of *Koloonella* cf. *minutissima* (Laseron, 1951) White asterisks mark position of mantle cavity (**A–G**) or oviduct lumen (**H–L**). **A.** Mantle rim with 'red' glands (two large, squarish cells in middle). Arrowhead marks duplicate mantle border (shell gland). **B.** Infolded area of putative osphradium and pigment granules in dorsal epidermis. **C.** Vacuoles of Blochmann's gland. **D.** Gland at base of mantle tentacle (gland 3). **E.** Gland opposite of mantle tentacle (gland 4). **F.** Pink-stained cells of tubular mantle gland (gland 1). **G.** Dark blue-stained cells of ring-shaped gland (gland 2). **H.** Columnar cells of female gland 1 (putative albumen gland), nuclei (blue) at top. **J.** Female gland 2 (putative membrane gland). **K.** Female gland 3, first region (putative mucus gland). **L.** Female gland 3 (putative mucus gland), second region (below, white vesicles, large nuclei) and third region (above, purple vesicles). **M.** Region of foot showing calcium cell (white), cells of posterior pedal gland (= opercular gland; round, dark blue cell), operculum (clear blue), and opercular groove (arrowhead). **N.** Tissue of columellar ridge on visceral sac (clear cells, apices of cells at upper right). Scale bars = 10 μm.

other murchisonellids. Because it is positioned on what is the ventral side of each tentacle in extended crawling specimens, it is probably visible only in retracted specimens or in histological sections. The configuration of the murchisonellid head with a fairly wide, flat snout has been called a 'mentum' by previous authors, in accordance with the structure found in pyramidellids (Wise, 1996, 1999), cimids and graphidids (Warén, 1993, 2013). As in *Koloonella*, the pyramidellid mentum is located below the mouth and above the male genital opening (Fretter & Graham, 1949; Wise, 1996). In pyramidellids, it acts as a specialized support for the protruding, long proboscis found in this family (Peterson, 1998), and for this reason it also carries a dorsal gutter in many cases, as in *Koloonella*. However, here we refrain from calling the structure found in basal heterobranchs a 'mentum', due to the potentially specialized morphology and the derived phylogenetic position of pyramidellids (Jörger *et al.*, 2010; Dayrat *et al.*, 2011; Dinapoli *et al.*, 2011), and instead regard the snout of *Koloonella* to be homologous with that of other basal heterobranchs.

The foot of murchisonellids is short, and shows a wide, conspicuously ciliated anterior margin (B.B., personal observation

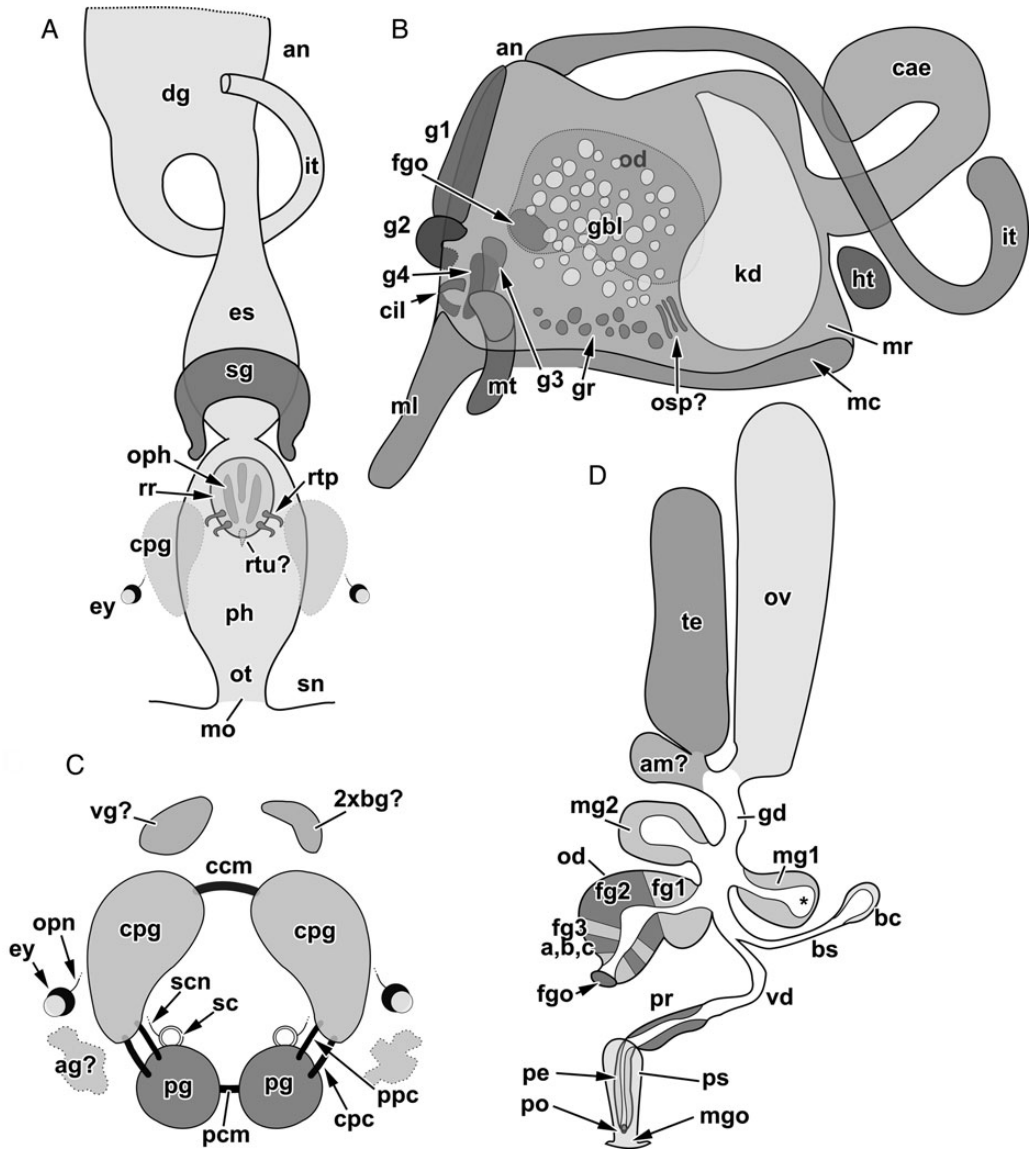

Figure 8. Schematic overviews of *Koloonella* cf. *minutissima* (Laseron, 1951) microanatomy. All dorsal view, anterior towards below. **A.** Digestive system. **B.** Arrangement of organs associated with mantle cavity as seen from above. Mantle roof lighter grey, lower-lying structures drawn with stippled lines. **C.** Central nervous system. **D.** Reproductive system. Asterisk indicates position of putative spermatozoa. Abbreviations: ag?, cell bodies of putative accessory ganglia; am?, putative ampulla; an, anus; bc, head of putative bursa copulatrix; bs, bursa stalk; cae, caecum of mantle cavity; ccm, cerebral commissure; cil, ciliated area between mantle lobe and tentacle; cpc, cerebropleural connective; cpg, cerebropleural ganglion; dg, digestive gland; es, esophagus; ey eye; fg1, first female gland (putative albumen gland); fg2, second female gland (putative membrane gland); fg3, zones of third female gland (putative mucus gland); fgo, female genital opening; g1, tubular mantle gland; g2, ring-shaped mantle gland; g3, gland in mantle roof; g4, gland in mantle floor; gd, common gonoduct; gbl, Blochmann's gland; gr, red glands at mantle rim; ht, heart; it, intestine; kd, kidney; mc, mantle cavity; mg 1, 'male' gland 1 (putative seminal receptacle); mg2, 'male' gland 2; mgo, male genital opening; ml, mantle lobe; mt, mantle tentacle; mo, mouth; mr, mantle roof; od, oviduct; oph, odontophore; opn, optic nerve; osp?, putative osphradium; ot, oral tube; ov, ovary; pcm, pedal commissure; pe, penis; ph, pharynx; po, penial opening; ppc, pleuropedal connective; pr, prostate; ps, penial sheath; rr, radular rods; rtp, paired radular teeth; rtu, unpaired radular element; sc, statocysts; scn, static nerve; sg, salivary gland; sn, snout; te, testis; vd, vas deferens; vg?, putative visceral ganglion; 2xbg?, putative annexed buccal ganglia.

of *Koloonella* and *Murchisonella*). This ciliation appears to be important in locomotion. Rasmussen (1944) depicted the anterior margin of the snout as ciliated in *Ebala*, but not the foot margin; we assume this to be an observational error. The posterior part of the foot is wider in *E. nitidissima* (Rasmussen, 1944: fig. 8A).

Black pigmented patterns on the headfoot are found in some other murchisonellids. A mask-like pattern as found in this study in *Koloonella* is also shown for *Henrya morrisoni* (with a conspicuous middorsal stripe on the head in the position of the mouth; Wise, 1999) and some *Murchisonella* (Redfern, 2001; Warén, 2013). Dark pigmented areas on the headfoot and visceral sac are also present in other *Henrya* (Warén, 2013) and *E. nitidissima* (mantle described as "black pigmented", Rasmussen, 1944: 216), but are entirely lacking in other species (N.G.W., personal observation; Warén, 2013). Whether pigment patterns are species-specific remains to be discovered.

General histology

The columellar retractor muscle is the largest muscle of the animal; it runs from the columella to the operculum and is used to retract the animal into the shell. In gross morphology, the

Table 2. Proposed classification of Murchisonellidae.

	Ebalinae Warén, 1995			Murchisonellinae Casey, 1904*
	Ebala Gray, 1847	*Henrya* Bartsch, 1947	*Koloonella* Laseron, 1959	*Murchisonella* Mörch, 1875
Shell surface	Smooth, some with fine spiral ridges[1,3]	Smooth[2,4]	Smooth	Sculptured, with distinct spiral ridges
Shell sinus	None, sometimes faint	None[4]	None	Present
Mantle tentacles	Short[3]	Short[2,4]	Short, finger-shaped	Long, club-shaped[2]
Head tentacles	Triangular, broad[3]	Short, stubby (headshield-like)[2,4]	Wide, ear-shaped (headshield-like)[2]	Elongate, pointed[2]
Radular teeth	Hook-shaped, slightly serrate[1]	Hook-shaped, slightly serrate[2,4]	Hook-shaped, smooth[2]	Wide, denticulate[2]
Rows of radular teeth	1–2[1]	1–2[2,4]	2[2]	about 10[2]

*Shell sculpture and shape place *Pseudoaclisina* Yoo, 1994 among Murchisonellinae, but there currently is no information available on soft-body anatomy. Main sources are [1]Warén (1995), [2]Warén (2013), [3]Rasmussen (1944), [4]Wise (1996), and results of the present study. See Discussion for explanation and further references. Warén (2013) placed *Koloonella* and *Henrya* among Murchisonellinae.

muscle resembles the spiraled band shown for other lower Heterobranchia by Haszprunar (1985b). The fibres extending to the right corner of the mantle cavity are consistent with the retractors of the mantle edge reported by Fretter & Graham (1962) for several 'prosobranch' taxa. A histologically distinct zone for adhesion between the columellar muscle and the shell (discussed below) was not found, but is assumed to be near the apical end of the muscle, where it is slightly upraised.

The conspicuous band of tissue herein termed 'columellar ridge' runs along the columellar side of the body; it is associated with the columellar muscle (on its left side, with respect to the longitudinal axis of the body) but extends further towards the apex and even onto the left side of the neck. Particularly towards the apex, some of the flask-shaped cells of the (epidermal?) tissue extend deep into the body (even into the digestive gland). In histology, it does not resemble any structure described in other lower heterobranchs. In its course, it is superficially similar to the scar of the columellar muscle and the 'adhesive ridge' shown for the acteonoid *Ringiculoides* (Minichev, 1967), but due to its glandular appearance it is not particularly similar to the 'adhesive pads' reported for the euopisthobranch *Philine aperta* by Brace (1997) or the 'adhesive zone' found in caenogastropods (resembling a microvillar brush border; Fretter & Graham, 1962). Strong & Glaubrecht (2008: figs 2c, 8b) depicted a band or groove along the columellar muscle in some high-spired cerithioidean caenogastropods, but did not further mention it in the text. Therefore, the identity of this quite conspicuous band of tissue in *K.* cf. *minutissima* is not clear. Judging from its position and presumably glandular character, this organ may functional not as an adhesive but as a lubricating organ, allowing for faster retraction of the soft body into the shell, along the columella (G. Haszprunar, personal communication). Alternatively, it could be a stabilizing structure. Therefore, it would be mainly necessary in high-spired gastropods whose shell is relatively longer with respect to the animal.

The opercular groove is in the position shown the caenogastropod *Littorina* by Fretter & Graham (1962: 18). Its position near the thin, wavy edge of the operculum is consistent with its function in depositing the opercular material that is secreted by the underlying flask-shaped glandular cells termed 'opercular glands' herein. Judging from the cells' position (subepidermal, along the posterior sides of the foot, next to horseshoe-shaped groove) and histology (granules staining violet instead of blue as other pedal gland cells)—but not relative size—the 'opercular' gland may homologous with the caudal adhesive gland of Rhodopemorpha (Brenzinger *et al.*, 2011, 2013a), thus indicating that the foot in Rhodopemorpha extends along the entire ventral side.

'Calcium' cells are conspicuous in histological sections and have been reported for other lower heterobranchs (e.g. Haszprunar, 1996; Haszprunar *et al.*, 2011; Hawe & Haszprunar, 2014). Similar cells are found throughout molluscan clades (e.g. pulmonate and prosobranch gastropods, bivalves; Fournié & Chétail, 1982); they are assumed to play a role in mineral storage, mainly calcium carbonate. Haszprunar (1996) hypothesized calcium cells to be homologues of excretory rhogocytes, since both are capable of accumulating metal ions and found in loose aggregates or singly inside the body cavity; in some taxa, ultrastructural characters also agree (Haszprunar, 1996: 191 and references therein). In light of the potential relationship with spicule-bearing Rhodopemorpha, we hypothesize that the calcium cells in murchisonellids might be homologues of spicule cells in rhodopemorphs (and, potentially, also in at least some other spicule-bearing taxa). This would be consistent with the similar morphology of calcium cells and spicules (layered mineral body with organic matrix) location and function in the body (both are subepidermal and calcium storing) (Rieger & Sterrer, 1975; Brenzinger *et al.*, 2011; this study). However, characteristic slits for ultrafiltration, typical of rhogocytes, have not been reported for the spicule cells of Rhodopemorpha, or those of other spicule-bearing slugs such as Acochlidia (Rieger & Sterrer, 1975).

Mantle cavity

Mantle cavity characters are important for the anatomical study of shelled gastropods. Especially in minute, thin-shelled taxa, many characters can be reliably observed even in live specimens and thus may be useful for taxonomy, such as colourful glands (hypobranchial gland/pigmented mantle organ) (e.g. Ponder, 1991; Caballer, Ortea & Narciso, 2011; Haszprunar *et al.*, 2011) or tentacles at the mantle edge.

Heterobranchia are assumed to have lost the original ctenidium of other gastropods (Haszprunar, 1985a), with distinct ciliary strips or ridges (and sometimes associated tentacles) being used for ventilation instead and a secondary gill or the kidney—located in the mantle roof—as a respiratory organ. The location of the kidney in *Koloonella* is thus typically heterobranch, but its surface facing the mantle cavity is not particularly folded. The heart is two-chambered, judging from histology, and in the position likewise shown in a drawing of live *Ebala* by Rasmussen (1944).

A distinct, folded gill is also not present in the mantle epithelium of *K.* cf. *minutissima* and has not been observed in live murchisonellids. To the right of the kidney, there is a reticulated, nonglandular area where the gill would be expected to be located; this area is folded in *E. nitidissima* (B.B., personal observation) and may therefore be a reduced gill or at least have a function in gas exchange. This folded area is not present in the examined species of *Koloonella*, but may be present in larger-bodied congeners.

Dorsal and ventral ciliary strips at the right side of the mantle cavity have been described in *Henrya* (Wise, 1996). They are not evident from our histological examination, although the right corner of the mantle cavity between the mantle tentacle and lobe is strongly ciliated. It is not clear from our material if these are the aforementioned ciliary strips. These ciliated ridges were considered a diagnostic character for early Heterobranchia (Haszprunar, 1985a) and are usually prominent in histology and distinctly ciliated (e.g. Wise, 1996; Haszprunar *et al.*, 2011).

Tentacles on the right side of the mantle cavity are present in many basal heterobranch taxa. Murchisonellids are characterized by having two such tentacles. As shown histologically by our material, at least in *K.* cf. *minutissima* the first of these two structures is round in cross-section and hangs from the roof of the mantle cavity (called mantle tentacle herein), while the second, more posterior one (mantle lobe) is rather flat and formed by the edge of the mantle. This is in accordance with Wise (1999: figs 11, 12), who showed the posterior structure (called the "siphon") to be part of the mantle edge. The tentacles of both *Koloonella* and *Henrya* are rather small and may not be conspicuous in live specimens (Fig. 1; Warén, 2013). In *Ebala*, Rasmussen (1944: fig. 8b) showed two small ciliated protrusions at the right corner of the mantle cavity. In contrast, both tentacles are rather large and club-shaped in *Murchisonella* (Warén, 2013; B.B., personal observation); this is correlated with the presence of a sinus in the lip of the shell where the tentacles protrude. Many other basal heterobranchs possess tentacles or lobes at the right corner of the mantle cavity; these structures are presumably involved in ventilation of the mantle cavity (Haszprunar, 1985a). In *Rissoella caribaea*, two finger-like tentacles of equal size were shown by Wise (1998) to be connected by a single, curving ciliary tract. According to Ponder (1990a), a single but bilobed tentacle is present in *Orbitestella*. Two tentacles of different size and form are found e.g. in the valvatoid *Xylodiscula* (Warén, 1992; Høisæter & Johannessen, 2001); some other valvatoids, *Graphis* and *Cima*, possess only one externally visible tentacle (Warén, 1993, 2013; Haszprunar *et al.*, 2011). Judging from morphology and histology, the anterior tentacle of *Koloonella* (termed mantle tentacle herein) is probably homologous with the single one of other taxa. It is less clear if the second 'tentacle' (mantle lobe herein) of *Koloonella*, the rather cylindrical second tentacle of the aforementioned taxa, or the flattened lobe covering parts of the shell e.g. in the valvatoid *Xenoskenea* (Warén, Gofas & Schander, 1993) are homologous structures.

Published data on the form or outline of the mantle cavity are difficult to compare with our results. It appears that the mantle cavity in *Koloonella* is deeper than in other basal heterobranch taxa due to the presence of the unciliated caecum on the left side. This is probably not homologous with the so-called 'pallial caecum' of some groups (e.g. *Acteon*, *Scaphander*; Haszprunar, 1985a; Rudman, 1972), because this strongly ciliated structure is located at the right side of the mantle cavity and is closely associated with the tentacles at the mantle border, both caecum and tentacle being involved in creating water currents in the mantle cavity (Haszprunar, 1988; Ponder, 1991). In the live specimens examined, granules of conspicuous sulphur-yellow colour highlight the outline of the caecum, especially in lateral or ventral view. A structure with similar dimensions (but with a more frilly outline), position and almost identical colour is visible in some *Murchisonella* species shown by Warén (2013: pl. 3; B.B. personal observation of the species shown in Warén's figs 3–5); it is not visible in *Henrya* or *Koloonella* shown in the same plate. In *Henrya morrisoni*, a structure at the left side of the body whorl was interpreted to be the hypobranchial gland by Wise (1998), characterized by a "clear matrix in which large and small cells containing yellow substance" were located; snails were reported to "release thick, pale-yellow exudates" if stressed. We do not agree with naming the structure a hypobranchial gland but, judging from the colour and location, the structure described by Wise is most likely identical to the caecum of the mantle cavity described herein, according to its position, size and dimensions. Wise's description of repugnatorial function does not contradict this observation; a defensive, glandular function would explain why the structure is not always externally visible in freshly collected, disturbed specimens. In our material of *Koloonella*, it is not clear where the yellow pigment is located histologically, because glandular structures are not evident; larger cells below the caecum (visible in Fig. 6A) appear to be cells of inner organs instead and not glands of the epidermis related to the caecum. Nevertheless, this externally visible structure might be useful in separating potential murchisonellids from other externally similar gastropods, and warrants comparison among other basal heterobranchs with regard to potential function and homology.

The so-called hypobranchial gland is a structure found in the mantle cavity of many gastropods; it generally consists of different types of mucus-producing glands that are assumed to work in cleaning the mantle cavity, or as defensive glands (Fretter & Graham, 1962). The structures are commonly called hypobranchial glands or 'pigmented mantle organs'. However, it is not clear if hypobranchial glands are homologous among Gastropoda (Ponder & Lindberg, 1997) and therefore it is currently still difficult to tell how the various pigmented patches or fields inside the mantle cavity of many heterobranchs are phylogenetically related. The identity of the 'pigmented mantle organ' is even less clear, because some authors use the term for the paired excretory organs found in many larval gastropods that sometimes persist into the adult stage (Haszprunar, 1985a). As there are several histologically distinct glandular areas in the mantle cavity of *Koloonella* and not much information on related taxa, it is difficult to interpret homologies. Data on 'opisthobranch' taxa reviewed by Wägele, Ballesteros & Avila (2006) are useful for general comparison.

In histology and position, the glandular fields associated with the mantle tentacle in *K.* cf. *minutissima* (glands 3 and 4 herein) are the most similar to a 'hypobranchial' gland reported for other taxa.

The strip of crimson red glands in the anterior mantle roof is conspicuous in our live animals. The same structure was also shown for a species of *Koloonella* by Warén (2013: "crimson pigmented mantle organ" in pl. 3, fig. 4c) and is also visible in another photograph of the *K. minutissima* specimens shown therein (pl. 6, fig. 5b; observation by B.B. on another photograph of the same individual supplied by A. Dinapoli). It has not been mentioned for other murchisonellids. A brick-red crescent of glands was also shown for a live valvatoid, *Xenoskenea* (Haszprunar *et al.*, 2011: fig. 1). It should be noted that dorsal bands with similar colours are quite typical for *Rhodope* species (see Haszprunar & Heß, 2005). However, the homology and function of the crimson glands are unclear; a function as repugnatorial gland may be suggested at least in *Koloonella*.

The field of nonstaining, large cells in the mantle roof is similar to the glandular cells found in some Valvatoidea (Haszprunar *et al.*, 2011: fig. 8: 'mg3'). At least in *Koloonella*, they are also very similar in position and histology to the

Blochmann's gland of some euopisthobranch or acteonoid taxa (see Wägele *et al.*, 2006) and may be homologous and widespread among Heterobranchia.

The pair of glands along the right margin of the mantle cavity is histologically distinct (Figs 2C, D, 5G: g1, g2). In position (facing the female genital opening), they resemble the 'glandular pocket' shown for the valvatoid *Cornirostra*, an organ that was hypothesized to be involved in oviposition (Ponder, 1990a; Bieler *et al.*, 1998). Similar glands have not been reported for the valvatoids that were examined using the same staining protocols as in the present study (Haszprunar *et al.*, 2011; Hawe *et al.*, 2013). Open glandular tracts in the mantle cavity that may be potential homologues are present in some Caenogastropoda including basal cerithioidean groups (e.g. Houbrick, 1981); they are considered either potential homologues of the hypobranchial gland or precursors or parts of the closed gonoduct of other taxa (Fretter & Graham, 1962; Haszprunar, 1988). Both in histology and position, the closest match to the two glands are the two 'terminal' glands of Rhodopemorpha that were speculated to be involved in spermatophore formation (Brenzinger *et al.*, 2011). In both *Rhodope* and *Koloonella*, one gland is a large, elongate tube or groove that contains pale pink-staining voluminous cells, while the more distal one is a short ring or c-shaped groove with smaller cells staining strongly violet. Both glands are close to the nidamental glandular mass; in *Koloonella*, the oviduct opens right next to the groove/pocket formed by the glands; in rhodopemorphs, the gonoduct discharges through these glands that form a closed tube. We therefore regard the 'terminal' glands of rhodopemorphs to be a possible homologue of the respective mantle glands in murchisonellids, and suggest that they may also play a role in reproduction in the latter.

Digestive system

The digestive system possesses all elements of the generalized gastropod digestive system, but several organs are small and reduced. Stomach (indistinct), digestive gland (single) and intestine (looping) are similar to those of other shelled basal heterobranchs in morphology and histology (rhodopemorphs differ e.g. in having a very short intestine, while valvatoids have a more complicated stomach; Brenzinger *et al.*, 2011; Hawe *et al.*, 2013).

The anterior part of the digestive system possesses some modifications. The mouth opening is situated on the upper side, between the head tentacles and not below the anterior margin of the snout. Therefore the mouth is in a more dorsal position than in other basal heterobranch taxa, but in a similar place as in at least some pyramidellids, in which the mouth is situated on top of the shelf-like mentum (Wise, 1996).

The mouth leads almost directly into the pharynx, because the oral tube is very short. The pharynx is the single largest structure inside the head, but comparatively thin-walled and weakly muscular compared with that of closely related groups that possess a pharynx. The odontophore and radula are weakly developed, yet functional.

The radula is as described for other *Koloonella*, with long, curved and smooth teeth (Warén, 2013). These are presumably the first laterals, while rachidian teeth may be missing. The unpaired anterior 'tooth' described for the genus by Warén (2013: fig. 5b) was found only in the juvenile specimen examined herein; it is not clear from our material if it really is a tooth or something else. Nevertheless, the radula resembles that of *Ebala* and *Henrya* in having only four, hook-shaped radular teeth (Wise, 1999; Warén, 2013: figs 2, 3). This pincer-like radula with flaring basal elements is shared among the former three genera, but not all Murchisonellidae: the radula of *Murchisonella* is quite different, with more numerous teeth (*c.* 10 rows) that are wide with strongly denticulate margins (Warén, 2013: figs 1, 4).

Scanning electron micrographs of the murchisonellid radula (Warén, 1995; Warén 2013: figs 3, 5) furthermore show that it is attached to much larger, wing- or rod-like elements that extend ventrally, i.e. what would be along the sides of the odontophore in living specimens. These elements withstand processing for SEM, and thus appear to be cuticular in nature, and are likely derived from the radular membrane of other gastropods. Warén (2013: pl. 5, fig. 5b) also showed three such elements, one connected to the aforementioned unpaired tooth. Our sectioned material shows three distinct, homogeneous, rod-like structures below the radula (one median, unpaired), but it is not clear from histology whether these rods are the same structure as the aforementioned 'wings' (i.e. cuticular). They may simply not be spread open in their normal position on the odontophore, or they may be intramuscular structures inside the odontophore not visible in published SEM images. In their histology, the rods do not resemble the odontophore cartilages of caenogastropods (assumed to be lost in Heterobanchia; Haszprunar, 1985a; Ponder & Lindberg, 1997).

The radula of at least Ebalinae (*Ebala*, *Henrya* and *Koloonella*) can be assumed to work in a pincer-like fashion (holding on to food) and, due to a low number of teeth, not as a typical rasping organ. To our knowledge, a radula with only four teeth or less is unique among gastropods. Morphologically, the radular apparatus conspicuously resembles that of certain caudofoveate molluscs (Chaetodermatidae, especially *Falcidens*) that also possess only four curved teeth, flaring lateral membranes and even a median cone-shaped structure (Scheltema, 1989, 1998: fig. 2.5; Cruz, Lins & Farina, 1998). The feeding mode of chaetodermatids, with the radula holding the head in place and the pharynx sucking in food (Scheltema & Jebb, 1994; Scheltema, 1998) might therefore be functionally similar to that of murchisonellids, or at least Ebalinae.

The salivary gland of *K.* cf. *minutissima* (Laseron, 1951) is unusual in being horseshoe-shaped and apparently unpaired; most gastropods possess paired glands separate from the pharynx and each other. It is not clear from histology whether there is really only a single gland, or if there are paired glands that are distally attached or joined. Paired pockets in the sides of the pharyngeal lumen indicate that there are also paired salivary ducts. In this context it may be noteworthy that Wise (1999) did not mention salivary glands in his description of *Henrya* (although small, they are usually easy to find in dissections; e.g. Wise, 1996). Furthermore, the thread-like rhodopemorph *Helminthope psammobionta* was also shown to possess only a single salivary gland (Brenzinger *et al.*, 2013a), indicating that loss of a salivary gland did occur in a closely related taxon (*Rhodope*, on the other hand, does possess the usual pair of glands; Brenzinger *et al.*, 2011).

The oesophagus of *Koloonella* is not merely a simple, thin tube connecting pharynx to stomach, but a strongly glandular and ciliated structure of similar dimensions to the pharynx. Therefore, the oesophagus and pharynx are not easily demarcated externally, which can also be seen in the depiction of *Henrya* by Wise (1999: fig. 13; the anterior alimentary tract shown there is also remarkably long). Judging from histology, the oesophageal epithelium of *Koloonella* could be rather rigid (owing to hydrostatic pressure in large vacuoles) or, alternatively, strongly secretory. Given the feeble musculature of the pharynx, the oesophagus may be mainly responsible for the uptake of food into the digestive tract by ciliary action, supported by the hydrostatically stiffened wall. In most closely related groups, the oesophagus appears to be relatively thinner, although it has been described as 'glandular' for some taxa (Ponder, 1990a; Hawe & Haszprunar, 2014). In rhodopemorphs, however, a vacuolized oesophagus with bulbous midpart is the primary organ of feeding, while the oral tube and pharynx are completely reduced or vestigial (Brenzinger *et al.*, 2011, 2013a).

Therefore, an oesophageal bulb with characteristic histology (epithelium with large and ubiquitous vacuoles) may be a synapomorphy of a murchisonellid + rhodopemorph clade. It is paralleled by the reduction (murchisonellids) or loss (rhodopemorphs) of pharynx and radula, contrasting with its presence in the other related groups.

Finally, it should be stated that the entire anterior digestive tract is fundamentally different from that of 'true' pyramidellids, among which murchisonellids were previously placed. As shown by Warén (1995, 2013) using SEM, the chitinous elements of the pharynx are radically different (no hollow stylet, but a true radula). As is now evident from our study of *Koloonella*, there is also no complicated buccal apparatus and no buccal or salivary pumps as considered synapomorphic for Pyramidellidae (see e.g. Maas, 1965; Wise, 1996).

Central nervous system and sensory organs

Six distinct ganglia were detected: four forming the cerebral nerve ring around the middle part of the pharynx (paired cerebropleural and pedal ganglia) and two below the anterior oesophagus. The cerebropleural ganglia can be confirmed as such by the presence of paired connectives to each pedal ganglion (the cerebropedal and pleuropedal connectives, respectively). This configuration (merged cerebral and pleural ganglia) is also found in other basal heterobranchs (valvatoids, *Omalogyra* and rhodopemorphs; Bieler *et al*., 1998; Bäumler, Haszprunar & Ruthensteiner, 2008; Haszprunar *et al*., 2011; Brenzinger *et al*., 2011, 2013a). The nuclei left and right of the cerebropleural ganglia may well be 'accessory' ganglia of the large cerebral nerve(s) innervating the tentacles, but our material does not permit further analysis. Accessory ganglia are typical for the larger nerves of rhodopemorphs (Haszprunar & Huber, 1990; Brenzinger *et al*., 2013a).

Due to the lack of connectives or nerves, it is not possible to conclusively identify the two uneven-sized ganglia behind the nerve ring and below the oesophagus. Judging from their position, the ganglia could be buccal ganglia (usually paired), or ganglia of the visceral loop (between one and five in basal heterobranchs; Haszprunar, 1985; Brenzinger *et al*., 2013a). Bieler *et al*. (1998) showed a superficially similar configuration of the ganglia in the valvatoid *Cornirostra* and interpreted the posterior ganglia to be buccal ganglia, with visceral loop ganglia annexed anteriorly to the pleural ganglia. The correct interpretation of the visceral loop in *Koloonella* is of some interest, because a pentaganglionate loop would be a shared character with rhodopemorphs and convergent with 'higher' heterobranchs (Euthyneura). The two ganglia in *K*. cf. *minutissima* (Laseron, 1951) could be interpreted as only visceral loop ganglia, but then buccal ganglia would be missing (and vice versa). Assuming that no ganglia were overlooked in our examination of *K*. cf. *minutissima* (Laseron, 1951), we currently interpret the ganglia to represent both structures, i.e. that the left ganglion (curved, elongate) represents closely annexed or fused buccal ganglia, while the larger, rounder ganglion on the right is part of the visceral loop.

Further examination of murchisonellids, ideally including early ontogenetic stages, is warranted to tell if this interpretation is correct. A configuration with only one visceral loop ganglion would be very similar to that of adult *Rhodope*. There, it was assumed that in adults some of the original five visceral loop ganglia are fused to the posterior ends of each cerebropleural ganglion (Riedl, 1960; Haszprunar & Huber, 1990; Brenzinger *et al*., 2011), with only the 'visceral' ganglion or a combined 'visceral/subesophageal ganglion' remaining free (see Brenzinger *et al*., 2013a). If this scenario of fusion to the cerebropleural ganglia is also the case in *Koloonella*, it could also explain the elongate form of the cerebropleural ganglia, with both connectives to the pedal ganglia located more anteriorly (which seems not to be the case at least in valvatoids). The right ganglion of *Koloonella* could then be homologous with the single ganglion of *Rhodope*. If this is correct, then murchisonellids are euthyneurous (i.e. possess an untorted visceral loop) as are Rhodopemorpha, in contrast to, e.g. valvatoids, which show a torted, streptoneurous visceral loop (Haszprunar *et al*., 2011).

The eyes and statocysts conform to those of other basal heterobranchs (e.g. Haszprunar *et al*., 2011; Hawe & Haszprunar, 2014). An osphradium could not be detected, but the infolded structure found in the anteriomedian mantle roof could be this chemosensory organ. It does, however, not look like the osphradium of valvatoids (Haszprunar *et al*., 2011), which is also located more to the left.

The only nerves detectable in our material were, curiously, the minute, short optic nerves and the static nerves that run parallel to the pleuropedal connectives. These nerves are usually rather difficult to detect; in both cases, the origin in the cerebropleural ganglia could not be detected. The only published details on the murchisonellid nervous system are by Huber (1993), who compared the cerebral nerves of *Ebala* with some pyramidellids. One major difference between the two taxa was found to be the lack of a nerve to the "lateral wall of the head" (i.e. the rhinophoral nerve) and its associated ganglion in *Ebala* (Huber, 1993: 386 ff.). Mainly because of this, Huber (1993: fig. 32) placed *Ebala* outside Pyramidelloidea and in a more basal phylogenetic position, closer to Architectonicidae. Huber (1993) noted that, contrary to pyramidellids, the eyes in *Ebala* are "attached to the cerebral ganglion" (and not the tentacular nerve); judging from the position of the eyes close to the cerebropleural ganglion, this is apparently also the case in *Koloonella*. Besides the optic and static nerves, Huber mentioned a particular pedal nerve (the "lateral" one) and three cerebral nerves (tentacle, mentum, and oral nerves), the former with a "basal accessory ganglion". It should be noted that this pattern of cerebral nerves in *Ebala* (but not its terminology) is again consistent with the pattern found in the nervous system of *Rhodope* (see Haszprunar & Huber, 1990; Huber, 1993: 404, 408; Brenzinger *et al*., 2011). The configuration of cerebral nerves is different in the meiofaunal rhodopemorph *Helminthope psammobionta*, which could be related to the extreme worm-like morphology and inferred progenetic nature of this species (Brenzinger *et al*., 2013a).

Reproductive system

Our data on *K*. cf. *minutissima* (Laseron, 1951) show yet again that small heterobranchs possess complex reproductive organs. Some of these organs are of unusual histology, and their function remains largely obscure. A number of features of the reproductive system of *Koloonella* appear unusual.

The gonad of *Koloonella* is unusual for a hermaphrodite heterobranch in possessing a separate ovary and testis. However, the gonad is also not altogether hermaphroditic in all lower heterobranchs, entirely separate male or female follicles are for example also found in large-sized Architectonicidae (Haszprunar, 1985c), Orbitestellidae (Hawe & Haszprunar, 2014), *Rhodope* (but not *Helminthope*) (see Brenzinger *et al*., 2013a), but also in the minute acochlidian panpulmonate *Asperspina riseri* (Morse, 1976).

There were only few spermatozoa to be found in the examined adult specimen. Those present in the testis apparently do not show the spiral heads and nuclei that are considered to be a distinct autapomorphy of all Heterobranchia (see Haszprunar, 1985a; exceptions are some chromodorid nudibranchs and the hedylopsacean Acochlidia; Wilson & Healy, 2002, 2006; Schrödl & Neusser, 2010). Instead, most (but not all) of the few spermatozoa found in the gonad appear to have hollow, externally smooth heads (Fig. 6F′). Healy (1993) studied the ultrastructure of spermatozoa of 'basal' heterobranchs, including Pyramidelloidea and *Ebala*; in contrast to the former, *Ebala* was shown to possess

a comparatively long, spirally keeled nucleus, with the axoneme/coarse fibre complex penetrating the nucleus completely. Whether this is also responsible for the hollow appearance of the spermatozoa found in *K.* cf. *minutissima* (Laseron, 1951) cannot be determined here; we do think that the nonkeeled, spiral spermatozoa observed in this study are not mature cells. The putative spermatozoa in the receptaculum (see below) also do not show a spiral nucleus.

Hermaphroditic gastropods such as heterobranchs commonly possess structures for the storage of endogenous sperm (ampulla and prostate) and received sperm (mostly a distal bursa copulatrix or gametolytic gland, and mostly a proximal receptaculum seminis) (Beeman, 1977; Wägele & Willan, 2000). *Koloonella* possesses such structures, but all are either unusual in histology, or position (including those termed 'male glands' herein, see below).

The ampulla or 'vesicula seminalis' is usually a widened part of the most proximal gonoduct that stores ripe autosperm in an irregular mass; histologically, it is virtually identical to the remaining proximal gonoduct, being thin-walled and ciliated. The structure termed 'ampullary region' herein is in the corresponding position, but does not contain spermatozoa and resembles the testis in histology (with closely packed, irregular cells and typical mesodermal cells). It is conceivable that this structure is, instead, a not fully developed second testis (see above), and that an ampulla *per se* is not discernible due to the lack of spermatozoa.

The structure termed receptaculum seminis is usually located in a position proximal to the nidamental glands; it stores and maintains allosperm until they are needed for the fertilization of ova, prior to coating with mucus substances by the nidamental glands (Wägele & Willan, 2000). The lining of the receptaculum is therefore capable of secreting nutrients for spermatozoa that are typically stored with their heads embedded into the organ's wall. The 'male glands' herein are both located in this particular position (near the split of the gonoduct into oviduct and vas deferens), but neither look like typical receptacula in histology due to their glandular appearance. The first 'male' gland, however, is possibly a receptaculum, because it contains a bundle of cells that are most probably spermatozoa with a small, strongly staining and rod-shaped head facing the outer, thin-walled tip of the bag-like 'gland', where there is a short spot of ciliation. The cells possess long cilia (flagella?) that are aligned and project into the gland's lumen. However, they do not resemble much the spermatozoa found in the gonad (the 'heads' are thinner and more elongate, and also not clearly spiral) and there are only very few cells (<50). The histology of the gland itself is, furthermore, very untypical for receptacles, having a thick lining with very large, clear vesicles but no clear cell boundaries (rather resembling a yolky oocyte in this respect) and a smooth inner lumen, in contrast to the usually thinner but distinctly epithelial wall which is slightly infolded (see Wägele & Willan, 2000). This raises doubts about the interpretation as a receptaculum seminis; instead, it may be a structure involved in the formation of a spermatophore.

Not much can be said about the second 'male' gland. It is clearly glandular (with large, basal nucleus and homogeneous vesicular cytoplasm). From its position, it could also be a receptacle, the first part of the nidamental glands (an albumen gland), a kind of fertilization chamber, an additional proximal prostate or a spermatophore-forming structure. In its histology, it does not fit particularly well with any of these interpretations except for the latter.

Haszprunar *et al.* (2011: fig. 21) noted two or three 'blind sacs' of unclear function in the proximal and middle parts of the gonoduct of some Hyalogyrinidae (marine valvatoids); at least in *Hyalogyrina depressa*, these structures showed somewhat similar staining and histological properties (Haszprunar *et al.*, 2011: fig. 11) and might correspond to the 'male' glands of *Koloonella*. However, these taxa possess a distal receptaculum seminis.

The structure which we presume to be a bursa copulatrix is typical in its histology: there is a long, ciliated epithelial duct and an apical bulb with a pink-staining lumen, indicating that secretions were present inside the lumen at fixation (in contrast to all other reproductive organs; see e.g. Brenzinger *et al.*, 2013b). However, as this organ branches from the distal vas deferens and is not close to the female genital opening, it is not placed in a suitable position to receive allosperm. Therefore, it might have some other function.

Finally, the prostate as a glandular part of the distal vas deferens can be assumed to store spermatozoa directly before copulation, or additionally to function as a spermatophore-forming organ. The copulatory organ is unarmed and simple; in outer form, it resembles that of *Henrya* as shown by Wise (1996: fig. 17) but lacks the bulbous basal portion. Many basal heterobranchs (including rhodopemorphs) transfer sperm via spermatophores and thus lack a 'penis', a condition that was assumed to be plesiomorphic by Haszprunar (1988) because cerithioid Caenogastropoda, basal taxa among the heterobranch sister group, are also aphallate and transfer spermatophores. This leads to the assumption that copulatory organs evolved independently among Heterobranchia. Within basal heterobranchs, patterns are not clear; there are phallate (e.g. *Borysthenia*, *Valvata*) and aphallate taxa (e.g. Hyalogyrinidae) among Valvatoidea (Haszprunar *et al.*, 2011; Hawe *et al.*, 2013); elsewhere, existing information is ambiguous (for *Cima*, see statements by Graham, 1982 and Warén, 1993). Therefore it is uncertain if the condition in *Koloonella* is derived or not.

The oviduct with its glandularized epithelium is situated in the floor of the mantle cavity, as is typical for Heterobranchia. Usually it is assumed that there are three consecutive glandular areas, which may be tubular or sac-like. Fertilized eggs thus pass successively through the albumen, membrane and mucus glands, each of which possesses different histological staining properties (Klussmann-Kolb, 2001). In *Koloonella*, there are three major areas, but the last part contains three distinct zones with vesicles that stain differently (blue, pale blue and dark violet). It is not clear if these three zones represent three functionally different glands, or simply vesicles in various stages of maturity or regeneration after egg-laying has taken place. In Rhodopemorpha, investigations using the same staining agents as in this study also found four or five different glands (Brenzinger *et al.*, 2011, 2013a; B.B., personal observation). However, these differ in staining properties and are therefore difficult to homologize.

The reproductive system of murchisonellids is so far known only for *Henrya morrisoni* (Wise, 1999: figs 16, 17). This species was described to possess an ovotestis, followed by a large stalked 'seminal vesicle' (i.e. an ampulla, according to more recent nomenclature; Beeman, 1977; Wägele & Willan, 2000), a small stalked seminal receptacle (either a receptaculum seminis for long-term allosperm storage, or a bursa copulatrix for short-term storage), the glandular oviduct, and a cephalic penis with bulbous base. This configuration appears monaulic (i.e. eggs and autosperm pass through the same duct and opening, followed by an extra path only for autosperm) and thus essentially similar to that of panpulmonate pyramidellids; however, the connection to the copulatory organ was not found. Given the minute size of the *Henrya* specimens examined by Wise (1 mm) and the method used (dissection), it seems conceivable that structures such as an internal vas deferens, with associated glands that are close to the remaining nidamental glands, may have gone unnoticed. In gross morphology and arrangement of the organs, *Henrya* as depicted by Wise very much resembles *Koloonella*. For example, the 'seminal vesicle' identified by Wise looks like the testis of *Koloonella* in form, relative size and position. Therefore, we suggest that the 'ovotestis' described for *Henrya* could be an ovary only (as in *Koloonella*), and the

'receptacle' shown by Wise the structure identified as a bursa copulatrix herein (although it is depicted slightly more upstream in *Henrya*). *Koloonella* cf. *minutissima* (Laseron, 1951) is diaulic, owing to the proximal split of the female and male gonoducts; we would expect that reexamination of the reproductive system of *Henrya* would reveal a similarly diaulic system (with internal, more proximally branching vas deferens), as this would be predicted from its phylogenetic position (see Schrödl *et al.*, 2011). On the other hand, the organization of reproductive systems is known to be of considerable variability even within family-level taxa of basal heterobranchs (as was shown for marine valvatoids of the Hyalogyrinidae; Haszprunar *et al.*, 2011), so differences in genital system patterns need to be compared on a smaller phylogenetic scale to be informative.

At first glance, the diaulic reproductive system of *Koloonella* obviously differs from that of monaulic Rhodopemorpha. The latter lack a cephalic copulatory organ and allosperm receptacles, sperm transfer is hypodermal and gonads are follicular (at least in *Rhodope*) (see Brenzinger *et al.*, 2011). The aforementioned peculiar division of the gonad into separate ovaries and testes, and the presence of two characteristic glands distal to the nidamental glands, may in fact represent shared characters in the light of molecular phylogenetic data. If the latter 'terminal' glands are truly derived mantle cavity glands—i.e. mantle glands of *Koloonella* and terminal glands of *Rhodope* are homologous structures—this would also imply that the genital opening and distal 'gonoduct' of Rhodopemorpha are in fact vestiges of a murchisonellid-like mantle cavity.

Notes on distribution

The *c.* 10 *Koloonella* species described by Laseron (1951, 1959) occur in an area spanning tropical (Port Moresby, Papua New Guinea) and temperate waters (Tasmania), but the genus may be still more widespread. Further species classified in the genus (Bouchet, 2013) are from Hawaii (Kay, 1979) and West Africa (Peñas & Rolán, 1997), but these are known only from shells. Warén (2013) identified a smooth-shelled species from the Caribbean (Guadeloupe) with short head tentacles as a *Koloonella*. Molecular analysis is needed to confirm whether non-Pacific murchisonellids belong to the genus *Koloonella*, or are something else.

While most murchisonellids are known from only few specimens and localities, the European *Ebala nitidissima* has been reported to be locally common in a wide area ranging from temperate waters (Scandinavia, Britain: Rasmussen, 1944; Fretter *et al.*, 1986; Warén, 1995; Høisæter, 2009) to subtropical parts of the Mediterranean (southern France: Rodriguez Babio & Thiriot-Quiévreux, 1974; Turkey: Öztürk & Bakır, 2013, as *Anisocycla*; van Aartsen 1994, 1995). Again, molecular analysis is needed to test whether these taxa are truly wide ranging, or members of more than one genus or species with narrower distributions.

Although it is one of the widespread taxa in current taxonomy, live *Murchisonella* have been depicted only from Hawaii (Pittman & Fiene, 2013), the Caribbean and Papua New Guinea (Redfern, 2001; Warén, 2013) and there have been no observations on biology.

Notes on ecology

The type species *Koloonella moniliformis* (Hedley & Musson, 1891) was described from brackish water among the filamentous alga *Spirogyra*, a genus known to grow in dense mats. Laseron (1951: 299) recorded it "abundantly . . . in the sand at the roots of reeds and grass at the edge of the water" and suggested the habitat of *Koloonella* to "possibly extend into estuarine or brackish water" (Laseron, 1959: 181). This habitat is unusual for lower Heterobranchia, among which only some Valvatoidea are known to live in nonmarine conditions (the exclusively freshwater Valvatidae; Hawe & Haszprunar, 2014). Even other species of *Koloonella* recorded by Laseron are described from deeper water (60–100 m), but it is not clear if these are records of empty shells only. Bandel (1991) explained the occurrence of dead murchisonellid shells in shell wash at outer reefs by resedimentation from shallower waters by currents. Peñas & Rolán (2013) assumed similar explanations for deeper-water records of empty *Murchisonella* shells, as live records indicate habitats in shallower water, similar to those of other murchisonellids (e.g. dredged from "sand and grass" in shallow water, Redfern, 2001; Peñas & Rolán, 2013). *Pseudoaclisina* is so far known only from the western Pacific and there are no records of live specimens or soft-body characters (Peñas & Rolán, 2013).

The habitats of Murchisonellidae are commonly in shallow, intertidal to subtidal waters. Most live specimens were recorded from dredgings or bulk samples of coarse sediments (Rasmussen, 1944; Warén, 1995, 2013; Peñas & Rolán, 2013; this study). Bandel recorded murchisonellids from shallow coral reefs in the Red Sea (Bandel, 2005). *Ebala* is characteristically found among the rootlets of *Zostera* in eelgrass beds (Rasmussen, 1944; Høisæter, 2009). Wise (1999) recorded *Henrya morrisoni* near mangrove swamps and considered it to be "infaunal" (it was sieved from "mostly mud"); the type localities of *Henrya* species are near coastal or superficially landlocked (and therefore hypersaline?) lagoons in Florida and the Bahamas (Bartsch, 1947). The three aforementioned genera have also been mentioned to occur in high densities, at least at certain times of the year. In general, all these habitats are potentially characterized by unusual salinities and/or low oxygen contents, and are also not dissimilar to the habitats of at least some (also infaunal, even interstitial) Rhodopemorpha.

Relationships of Murchisonellidae

Molecular phylogenetics have shown that Murchisonellidae do not belong with Pyramidelloidea, but are a distinct family among 'basal' heterobranchs (Dinapoli & Klussmann-Kolb, 2010; N.G.W., unpubl.) and are closely related to Rhodopemorpha (Wilson *et al.*, 2010). Scattered earlier anatomical data, e.g. on characters of the nervous system (Huber, 1993), spermatozoa (Healy, 1996) or the 'jaw' apparatus described by Warén (1995), had already hinted at a position isolated from other, 'true' pyramidellid taxa. Pyramidellidae, in contrast, have been convincingly shown to be part of Panpulmonata, a much more derived taxon, by recent molecular studies using molecular clock approaches (Dinapoli & Klussmann-Kolb, 2010; Jörger *et al.*, 2010; Dayrat *et al.*, 2011; Dinapoli *et al.*, 2011). This is also consistent with the much younger reported ages of pyramidellid fossils compared with those of other lower heterobranch taxa, especially Murchisonellidae (see Bandel, 2005; Wägele *et al.*, 2007; Warén, 2013).

Therefore, the following characters can be seen as convergences between Murchisonellidae and most ectoparasitic Pyramidellidae: a high-spired shell, possession of a flat snout (or, alternatively, a 'mentum'), flattened tentacles, (possible) euthyneury, a modified anterior alimentary tract indicating feeding by suction, and perhaps a similar mode of life. Whether the similar morphology of the head and shell could be convergent aspects of a parasitic mode of life is unclear, as murchisonellids have never been observed feeding. It may, however, be supported by the fact that some of the aforementioned characters are also shared with some Eulimidae and Aclididae (parasitic caenogastropods; see Ponder & Lindberg, 1997) and also lower heterobranch Graphididae (Fretter *et al.*, 1986; Warén, 2013); the latter have been shown to be parasites or at least commensal on tubeworms of the genus *Sabellaria* (Killeen & Light, 2000).

Rather unexpectedly, analysis of the murchisonellid soft body in this study revealed characters that may be shared between Murchisonellidae and aberrant Rhodopemorpha (Brenzinger *et al.*, 2011, 2013a). These characters are (1) the modified anterior digestive tract with shortened oral tube, reduced radula and pharynx (the latter two lost completely in rhodopemorphs); (2) a large, bulbous and vacuolated oesophagus that presumably is the main organ of ingestion; (3) potentially, a euthyneurous nervous system with cerebropleural ganglia fused with parts of the visceral loop; (4) two histologically similar 'mantle' glands at the right side of the body that are associated with the nidamental gland mass and may play a role in reproduction and (5) presence of flask-shaped gland cells in the posterior foot (opercular gland and caudal adhesive gland). Further similarities are the presence of subepidermal calcium concretions (calcium cells and spicules, which may be homologous structures) and the habitats (subtidal, potentially infaunal in at least some murchisonellids). Further analysis of other Murchisonellidae, especially *Murchisonella*, is needed to evaluate if these characters are found among all Murchisonellidae (or only among Ebalinae; Table 2), if they represent potential synapomorphies of a rhodopemorph-murchisonellid clade, and to compare with outgroup taxa among lower heterobranchs. More data on nervous systems are needed, as well as critical evaluation of the aforementioned characters 4 and 5 as they may be present in other lower heterobranchs as well.

Nevertheless, soft-body anatomical characters do not contradict the sister-group relationship of Murchisonellidae and Rhodopemorpha as indicated by molecular phylogenetics, and may even support it. This result may be unexpected, given the extreme reductions found in the rhodopemorph *bauplan*. So far no attempts have been made to date the murchisonellid-rhodopemorph split. It is potentially ancient, indicating that evolution of shell-less taxa (slugs) is a very old phenomenon among Heterobranchia. Rhodopemorpha may be one of the oldest, if not the oldest, extant slug taxon, while Murchisonellidae appear to have changed comparatively little over a long period of time.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Journal of Molluscan Studies* online.

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Chapter 10. Kano Y, **Brenzinger B**, Nützel A, Wilson NG & Schrödl M. **Ringiculid bubble snails recovered as the sister group to sea slugs (Nudipleura).** *Scientific Reports (Nature group),* in review (revised version).

The manuscript version of the article is included in the present dissertation.

Ringiculid bubble snails recovered as the sister group to sea slugs (Nudipleura)

Yasunori Kano[1], Bastian Brenzinger[2,3], Alexander Nützel[4], Nerida G. Wilson[5] & Michael Schrödl[2,3]

[1]Department of Marine Ecosystems Dynamics, Atmosphere and Ocean Research Institute, The University of Tokyo, 5-1-5 Kashiwanoha, Kashiwa, Chiba 277-8564, Japan. [2]SNSB-Bavarian State Collection of Zoology, Münchhausenstr. 21, 81247 München, Germany. [3]Department Biology II, BioZentrum, Ludwig-Maximilians-Universität, Großhadernerstr. 2, 82152 Planegg-Martinsried, Germany; [4]SNSB-Bavarian State Collection of Paleontology and Geology, Geobio Center LMU, Richard-Wagner-Str. 10, 80333 München, Germany; [5]Western Australian Museum, Locked Bag 49, Welshpool DC, Perth, WA 6986, Australia. Correspondence and requests for materials should be addressed to Y.K. (email: kano@aori.u-tokyo.ac.jp)

Euthyneuran gastropods represent one of the most diverse lineages in Mollusca (with over 30,000 species), play significant ecological roles in aquatic and terrestrial environments and affect many aspects of human life. However, our understanding of their evolutionary relationships remains incomplete due to missing data for key phylogenetic lineages. The present study integrates such a neglected, ancient snail family Ringiculidae into a molecular systematics of Euthyneura for the first time, and is supplemented by the first microanatomical data. Surprisingly, both molecular and morphological features present compelling evidence for the common ancestry of ringiculid snails with the highly dissimilar Nudipleura—the most species-rich and well-known taxon of sea slugs (nudibranchs and pleurobranchoids). A new taxon name Ringipleura is proposed here for these long-lost sisters, as one of three major euthyneuran clades with late Palaeozoic origins, along with Acteonacea (Acteonoidea + Rissoelloidea) and Tectipleura (Euopisthobranchia + Panpulmonata). The early Euthyneura are suggested to be at least temporary burrowers with a characteristic ‘bubble’ shell, hypertrophied foot and headshield as exemplified by many extant subtaxa with an infaunal mode of life, while the expansion of the mantle

might have triggered the explosive Mesozoic radiation of the clade into diverse ecological niches.

The traditional gastropod subclass Euthyneura is a highly diverse clade of snails and slugs with at least 30,000 living species[1,2]. They are ubiquitous in aquatic and terrestrial environments and benefit and harm human life as food, pests, hosts of parasites, and sources of natural products for medical use[3–5]. They also serve as biological models, especially in neuroscience[5,6], and as indicators for environmental conservation and climate change studies[7,8]. The traditional classification of Euthyneura, which remains in many contemporary textbooks and biodiversity databases, still recognizes Opisthobranchia (sea slugs and snails) and Pulmonata (lung snails and slugs; see[9] for review). However, molecular phylogenetic analyses have demonstrated the non-monophyly of these taxa[10–13] and induced drastic reclassification (reviewed in[2,9,14,15]).

Recent multi-locus phylogenies recovered three major clades in Euthyneura (*sensu lato*), namely Acteonacea, Nudipleura and Tectipleura (Fig. 1)[15,16]. Of these, Acteonacea consists of shelled snails in two small superfamilies, Acteonoidea and Rissoelloidea[15]. Nudipleura is a clade of sea slugs without a shell or with a highly reduced shell; the species-rich, popular and often very colourful Nudibranchia (with the subclades Anthobranchia and Cladobranchia) as well as the less known Pleurobranchoidea belong here[2,9]. The last and most diverse clade Tectipleura comprises two reciprocal sister subclades, Euopisthobranchia and Panpulmonata. Euopisthobranchia is most famously represented by sea hares and pteropod sea butterflies but also includes bubble snails in the strict sense (Cephalaspidea *s.s.*)[17]. Panpulmonata encompasses all traditional pulmonates (including common garden snails and slugs) and several, morphologically highly disparate non-pulmonate groups such as sacoglossan sea slugs and ectoparasitic pyramidellid snails[12,13,15]. Recent phylogenomic studies in principle support these relationships. Although the first, taxon-limited analysis for Euthyneura suggested a paraphyletic Nudipleura[18], the latest study with a denser sampling has confirmed its monophyletic nature with maximum support[16]. Such substantial changes in phylogenetic hypotheses inevitably entail a fundamental reconsideration of traditional assumptions on the homology of characters and traits of evolution, as well as on the systematics of fossil taxa[9,15].

From a palaeontological point of view, our understanding of euthyneuran evolution based on molecular phylogeny still wants for important elements from key taxa with supposed

late Palaeozoic or early Mesozoic origins. Particularly important and yet entirely neglected was the ancient snail family Ringiculidae in its own superfamily Ringiculoidea[19]. Ringiculidae comprises at least several dozens of extant species in such genera as *Ringicula, Ringiculopsis, Ringiculoides* and *Microglyphis*[20,21]. They inhabit sand and mud bottoms from the intertidal to abyssal depths worldwide[19,22]. The ringiculid anatomy is characterized by a hypertrophied head for burrowing, which is called headshield, and a mid-dorsal siphon (Fig. 1) for directing sand particles upwards whilst burrowing and for exchanging water in the mantle cavity for respiration[22–24]. They feed on interstitial copepod crustaceans and foraminiferans by crushing prey exoskeletons with a specialized portion of the stomach[22,25]. The small but often very solid shells of Ringiculidae are recovered in the fossil record from the Middle Jurassic of 161–165 Mya (million years ago)[26] and they flourished as one of the commonest euthyneuran groups in the Late Cretaceous[27,28]. These fossils bear surprising resemblance to living ringiculids and accordingly, most are classified in recent genera, including the oldest, Middle Jurassic *Ringicula buchholzi*[26].

Historically, ringiculids had been classified in distantly related groups of Gastropoda outside Euthyneura based on conchological characteristics[29]. Succeeding authors classified them as basal members of the 'opisthobranch' bubble snails on the grounds that they share the oval shell and headshield (Cephalaspidea *s.l.*; Fig. 1)[19]. Another, entirely different scheme of classification based largely on anatomical characters[30] recognized the Ringiculidae as closely allied to the similarly bubble-shelled Acteonoidea and Diaphanoidea, which collectively formed Architectibranchia, again outside Euthyneura. This basal position of Ringiculidae away from (Eu)opisthobranchia was confirmed by cladistic analyses of morphological data[25,31]. Diaphanoidea was later excluded from Architectibranchia based on multi-locus phylogenies (Fig. 1)[17,32], while none of previous studies had incorporated any molecular data from Ringiculidae, whose position thus remained contentious. An inclusive taxon set representing all major extant lineages is crucial for reconstructing and understanding evolutionary origins and consequences.

Here we present a molecular phylogeny of Euthyneura with the first DNA sequences of Ringiculidae. The new sequences originate from seven ringiculid species that cover all major phenotypes (and thus generic diversity) of the family and were analysed with all presently available data for major euthyneuran clades as well as related outgroup taxa. Our Bayesian and likelihood-based reconstructions clearly reject the original and modern Architectibranchia concepts[25,30–32], but instead indicate an unexpected sister group relationship of ringiculid snails to nudipleuran sea slugs. This once again highlights the

enormous evolutionary plasticity of Euthyneura. We furthermore provide microanatomical details derived from 3D reconstruction of serial histology sections to investigate homologies in this sister relationship. Combined with fossil evidence, the new molecular and anatomical data suggest that morphological innovations for burrowing and crawling in soft sediment occurred very early in the evolutionary history of Euthyneura. This represents the plesiomorphic condition, from which various body plans have arisen as a result of succeeding adaptive radiation into diverse aquatic and terrestrial ecosystems.

Results and Discussion

Molecular phylogenetic reconstruction. The seven study species of Ringiculidae (Ringiculoidea) formed a robust clade as a well-supported sister group to the Nudipleura in both Bayesian and maximum-likelihood analyses (Fig. 2). This relationship is highly remarkable and counterintuitive; thick-shelled ringiculid snails hardly resemble nudipleuran slugs and their external anatomy also shows many discrepancies (see below). With such dissimilarity, no earlier study suggested their close affinity[9,15]. There are two possibilities to explain our tree topology: the phylogenetic reconstruction is erroneous, e.g. suffering from potential long-branch attraction or under-sampling of relevant groups, or ringiculids are genuinely related to nudipleurans but these sister taxa are morphologically different to an unexpected extent.

The quality of a molecular phylogeny depends on careful selection, control and processing of sequence data. We followed best practise procedures[33]: BLAST-checking all novel sequences as well as included data from Genbank, generating and masking alignments with several different settings, and performing multiple phylogenetic analyses under different taxon and data selection regimes. All trees recovered from concatenated genes are highly compatible and most nodes receive maximum Bayesian posterior probability, while bootstrap indices are somewhat lower for basal nodes, as in comparable multi-locus studies[11,12]. Also, our sensitivity analyses reveal that the clade of Ringiculidae and Nudipleura is robust against variation of taxon sets, i.e. removing either of the two major nudipleuran subclades, contradicting the assumption of potential long branch attraction [Supplementary Figure S1; note that bootstrap support is even higher (98%) with Pleurobranchoidea alone]. The within-group branches of Ringiculidae are not noticeably longer or shorter than those of Nudipleura or the stem branches leading to the two clades (Fig. 2). Lastly, our topology is largely congruent with the latest phylogenomic phylogeny (without Ringiculidae)[16] and thus it is not

accountable for stochastic, potentially misleading histories of few genes. All these points justify the monophyletic nature of Ringiculidae + Nudipleura, for which we propose a new taxon name Ringipleura.

Microanatomy and synapomorphies of Ringipleura. Despite the highly different body plans of ringiculids and nudipleurans, we discovered similarity in the nervous system, a suite of morphological characters that are often regarded as crucial for resolving molluscan phylogenetic relationships[30,34]. Previous authors postulated that the nervous system of Ringiculidae 'primitively' retained a very long and crossing visceral loop with a ganglion on it[20,22,23]. A crossing visceral nerve loop is plesiomorphic for the entire Gastropoda and represents a configuration called 'streptoneury,' as the counter-concept to 'euthyneury' where the loop is straightened out or shortened[25,30].

However, the concept of a streptoneurous Ringiculidae and hence its phylogenetic position outside Euthyneura[20,22,23] were refuted by our 3D reconstruction of semi-thin histological sections. The two crossing nerve cords (Fig. 3b: N1 and N2) are not interconnected posteriorly to each other and therefore do not constitute the visceral loop as previously suggested[20,22,23]. Instead, the one originating from the left side of the cerebral nerve ring terminates near the anus, and the other, more dorsal one from the right side reaches a ganglion that is associated with the epithelium of a chemosensory organ called the osphradium (Fig. 3b: GO and underlying blue area). This osphradial ganglion had been interpreted as a different kind of ganglion on the visceral loop[20,22], but again the former can be histologically differentiated from the latter in having a more flat form, a deeper stain of neurons and a less distinct separation of the cortex and neuropil in Euthyneura[35]. The dorsal cord can then be regarded as the osphradial nerve, but not a part of a visceral nerve loop (see Haszprunar, 1988: fig. 3[36]). This in turn identifies its swollen anterior root as the supraintestinal ganglion (Fig. 3b,c: G1) and the other cord as the visceral nerve that originates from the true visceral ganglion (G2). The supraintestinal and visceral ganglia are in theory linked to each other, but we were not able to detect such a connective.

Visceral loop ganglia annexed or fused to the cerebropleural ganglia (so that no ganglion remains separate on the loop) have previously been found in many nudipleuran slugs but are otherwise very rare in the gastropod nervous system[34,37]. Even more interestingly, the connective between the supraintestinal and visceral ganglia is often lacking in previous descriptions of the nudipleuran nervous system as in our reconstruction for Ringiculidae, while this connective is always thick and easily traced in other euthyneuran groups[35]. Some

authors have successfully found this connective in Nudipleura as a very thin nerve running along the much thicker pedal commissure (e.g.[38]), potentially explaining the lack of observation by others, as well as in our reconstruction. The approximate course of the hitherto undetected visceral loop in Ringiculidae may be hypothesized as shown in Figure 3c (yellow line). To conclude, the unique condition of the visceral loop and its ganglia seems to represent supporting evidence of Ringipleura.

A second potential synapomorphy for this clade is the fusion of the head and mantle. Nudipleuran slugs are characterised by their continuous dorsal body wall called the notum, which is formed by the mantle fused to the head and overgrowing the visceral sac (Fig. 2i–k)[34,37]. The external anatomy of ringiculid snails superficially shows a close resemblance to acteonoid and euopisthobranch bubble snails, not only by retaining the shell but also in having the headshield for burrowing and crawling in soft sediment (Fig. 2f,g,l,m)[19]. However, we found that the headshield in ringiculids is most likely fused posteriorly to a hypertrophied, everted part of the mantle, and is not solely composed of the head as in acteonoids and euopisthobranchs. The posterior part of the fused 'headshield' bears compound defensive glands (Fig. 3a,d: DGL)[22] that are present in the mantle margin of the latter taxa (orange areas in Fig. 3j,l)[24,35]. The separate innervation of anterior and posterior parts of the shield by the cerebropleural ganglia further corroborates the fusion of the head and mantle in Ringiculidae: the anterior part receives four pairs of anteriorly projecting cerebral nerves (Fig. 3a–c: NC) while the posterior part is controlled by more dorsal, presumed pleural or parietal nerves (Fig. 3b,c: NPL; see[34,39]).

Internal relationships and divergence times of Ringiculidae. The present molecular phylogeny also provides insights into the evolutionary trends of shell shapes and hence the evaluation of the fossil record of the Ringiculidae. Many species of the family share a distinctive teleoconch morphology with pitted spiral ornaments and a complex aperture that bears multiple columellar folds and a thickened outer lip, as well as a heterostrophic coaxial larval shell[19,21,27]. Such distinctive and complex characteristics minimize the risk of misidentification of fossil specimens due to convergence. The oldest known ringiculid, *Ringicula buchholzi* from the Callovian (Middle Jurassic) of northeastern Germany, gives a reliable minimum age of the family at 161–165 Mya[26,40]. The second oldest *Ringicula blaszyki* was described from the Late Valanginian (Early Cretaceous) of Poland at 134–136 Mya[41]. These Mesozoic species are so similar to the Recent *Ringicula* that the modern representatives of the family can be regarded as 'living fossils.' Although inconspicuous in the

present era, ringiculids were one of the most flourishing euthyneuran groups in the Cretaceous period[27,28].

In the light of the present phylogeny, however, we assume that the origin of Ringiculidae is actually much older than the ages of the above fossils and that Jurassic or even Triassic ringiculids without the diagnostic apertural characters might have been erroneously placed in other euthyneuran families. The living species of Ringiculidae seem to fall into two major subclades: *Ringiculoides*, and all remaining genera (Fig. 2). *Ringiculoides* is a monotypic genus with the type species *R. kurilensis* occurring on the abyssal plain of the western North Pacific[20]. The shell of *R. kurilensis* is unique among ringiculids in having a proportionally large, oval body whorl with only one columellar fold, a sharp outer lip and a round base without a siphonal canal (Fig. 3e). Interestingly, such a condition of the shell is shared by some of other euthyneuran bubble snails, with particularly similar species in Acteonidae (Fig. 3k)[19,21,42]. *Ringiculoides* is therefore suggested to retain the plesiomorphic shell morphology of Euthyneura, while the more complex and solid shells of *Ringicula* (including the Jurassic *R. buchholzi*) and its allied genera (Fig. 2c,d) are most plausibly interpreted as an apomorphic condition in the family. Of the derived characters, the terminal thickening of the outer lip seems to have been lost independently in the putatively polyphyletic *Microglyphis* (Figs. 3f,i, 4).

Our divergence time estimates based the molecular data and four fossil-based calibration points (see Methods) suggested a late Palaeozoic euthyneuran diversification that leads to the major crown groups including Acteonacea, Tectipleura and Ringipleura (Fig. 4). The divergence between ringiculids and nudipleurans was estimated to date back to 270 Mya of the Permian period [with a 95% highest probability density (HPD) interval of 223–321 Mya]. Sensitivity analyses using only two of the three euthyneuran priors resulted in similar estimates for this split with modes at 252–285 Mya (Supplementary Figure S2). This is approximately the time when several stem groups representing what were formerly called ‘shelled opisthobranchs’ existed, with their first undoubted occurrence in the earliest Triassic of some 250 Mya[40]. These early Mesozoic bubble snails in such extinct families as Acteonellidae, Cylindrobullinidae and Tubiferidae are similar enough in general shell morphology to the living Acteonidae and to the putative plesiomorphic ringiculid *Ringiculoides*. The late Palaeozoic family Acteoninidae with a comparable teleoconch shape might belong to the same stem line of Euthyneura[40]. It can therefore be speculated that some of these early fossils represent stem groups of Ringipleura or Ringiculidae, or even stem nudipleurans retaining external shells.

Regardless of their current taxonomic position in Acteonidae, the Middle Jurassic to Late Cretaceous species of *Tornatellaea* (72–174 Mya) are much less ambiguous members of Ringiculidae with a close resemblance to the Recent *Microglyphis* (see[26,27,40,41]). The first occurrences of *Tornatellaea* and *Ringicula* agree well with the estimated date of divergence between the two ringiculid subclades at 195 Mya in the Early Jurassic (95% HPD: 141–250 Mya; Fig. 4) with modes in sensitivity analyses at 182–207 Mya (Supplementary Figure S2).

Bubble-shelled ancestry of Euthyneura and origin of nudipleuran slugs. The topology of the present molecular trees clearly rejects the Architectibranchia concepts, old[30] or new[32]. A close relationship between Ringiculidae and Acteonoidea[32] was refuted by the clustering of the latter with Rissoelloidea (Acteonacea; Fig. 2 and see also[16]). However, there remains a fundamental uncertainty regarding the position of Ringipleura. The Bayesian reconstruction using MrBayes resulted in an unresolved trichotomy at the base of Euthyneura *sensu lato* (Acteonacea, Ringipleura and Tectipleura; Supplementary Figure S3). The RAxML tree recovered Acteonacea as an unsupported sister to Ringipleura (Fig. 2), while BEAST analyses rendered Acteonacea sister to all other euthyneurans but again with low Bayesian posterior probabilities (Fig. 4). The recent phylogenomic analysis by Zapata *et al*.[16] resulted in similarly incompatible and poorly supported topologies for the early branching events in the Euthyneura. The three crown groups probably diverged within a relatively short period of time in the late Palaeozoic.

Because of the unresolved basal euthyneuran relationships, it makes little sense yet to reconstruct ancestral character states for ringipleurans in detail. As mentioned above, however, the common ancestor of Euthyneura might have had a relatively thin, oval shell with a large body whorl and a smooth surface with or without pitted spiral ornaments, as seen in the early Mesozoic ‘shelled opisthobranchs’ and Recent bubble snails including *Ringiculoides* and many acteonoideans and cephalaspideans (Fig. 3). Ecologically, such a thin and smooth shell with a large body whorl (hence a large aperture) in shallow marine environments is often associated with an infaunal lifestyle or at least temporary burrowing in the top layer of sediment. Snails with these conchological characteristics are vulnerable to crushing predation and abiotic breakage, which are however less important as a selective agency in soft sediment[43]. On the other hand, a large aperture is most often accompanied by a large foot that enables rapid and efficient burrowing[43], as does the headshield[22,31]. The infaunal mode of life has already been suggested by Brace[44] for the common ancestor of Euthyneura, from which epifaunal lineages were independently derived after varying intervals of time. We propose that

this very plausible hypothesis can be extended to the cause of the parallel shell reduction in Tectipleura. Many infaunal snails, including ringiculids and euopisthobranchs, bear an expanded mantle that partly or entirely covers the shell for further facilitating locomotion (Fig. 3)[31]. We suggest that this relaxed connection between the mantle margin and shell lip, in conjunction with the acquisition of chemical defensive devices[45], might have triggered the internalization, reduction and loss of the shell for the exploitation of new ecological niches, both within and outside soft sediment, and also on land (see[15,46]).

The fused head and mantle in Ringipleura (Fig. 3a–d) might have paved the way to the more elaborate and flexible notum of Nudipleura for crawling on a variety of three-dimensional substrates and feeding upon various sessile and mobile invertebrates[37,45]. Nudipleura is composed of two reciprocally monophyletic subclades: Nudibranchia and Pleurobranchoidea (Fig. 1). Although the postmetamorphic shell is lacking in all nudibranchs, a very thin, helicoid or plate-like teleoconch is retained under the notum of Pleurobranchidae of the latter subclade[31]. The ontogenetic extension of the mantle over the shell with the eventual inclusion of the latter, described for pleurobranchids[47], may recapitulate the evolutionary transition from bubble snails to shell-less slugs in Ringipleura. Such fragile shells of Pleurobranchidae are understandably scarce in fossil material. Pacaud *et al*.[48] mentioned a Palaeocene occurrence (*Berthella* sp.; 62–66 Mya), but this species was neither illustrated nor described in detail. The oldest reliable fossil of the family, hence the whole Nudipleura, dates back only to the late Oligocene (24–26 Mya)[49]. Based on these fossils and on the observation that several basal nudibranchs are restricted to deep or polar waters, Schrödl[50] suggested the early diversification of Nudipleura was related to the cooling of Antarctica since some 40 Mya. This view was supported and elaborated by Wägele *et al*.[2], but their hypotheses relied on an assumption that the Nudipleura were phylogenetically close to the externally shelled Umbraculoidea (= Tylodinoidea), which are actually a basal offshoot of Euopisthobranchia (Fig. 2).

More recent time-calibrated phylogenies suggest the origin of the Nudipleura, i.e. their split from Tectipleura or Tectipleura plus Acteonacea, in the Permian or Triassic period[11,12,16]. Our BEAST analyses resulted in similar dates, despite the inclusion of Ringiculidae as the sister group of Nudipleura. Ringipleura was estimated to have diverged into these subclades at 270 Mya of the Permian (95% HPD: 223–321 Mya; modes in sensitivity analyses at 252–285 Mya) and the first nudipleuran split into Nudibranchia and Pleurobranchoidea at 212 Mya of the Triassic (158–265 Mya and 197–222 Mya, respectively; Fig. 4, Supplementary Figure S2). The internalization, reduction and complete loss of the shell, which are adaptive for actively

carnivorous nudipleurans with chemical defence, should thus have occurred during the early to middle Mesozoic to give rise to one of the first slugs in the gastropod evolution—when many other predatory animals originated and diverged in the shallow sea[43].

Conclusions

New molecular and anatomical data indicate that ringiculid snails represent an ancient sister clade of nudipleuran sea slugs; a link previously missing to the remaining Euthyneura. The early Euthyneura are suggested to be at least temporary burrowers in soft sediment in the late Palaeozoic, with a characteristic bubble shell with a large body whorl as well as a hypertrophied foot and headshield for the infaunal mode of life. We hypothesize that early euthyneurans relaxed the strict connection of the shell and mantle margin for further facilitating locomotion in soft sediment, thereby releasing the mantle from morphological constraints and allowing the creation of evolutionary novelty, as conceptualized for other animal taxa[51]. This helps to explain the astonishing parallelism found across a number of lineages of euthyneuran slugs and semi-slugs[15,46]. Furthermore, the increased flexibility of the body plan might have been a key preadaptive trait behind the explosive Mesozoic radiation of Euthyneura into various ecological niches, including their multiple invasions of the freshwater and terrestrial realms.

Methods

Sampling and preparation of specimens. Ringiculid species that cover the generic and conchological diversity of the family were collected from coastal to abyssal waters as shown in Table 1. Most live snails for DNA extraction were boiled in 70–90 ºC water for 0.1–1 min and preserved in pure ethanol. The animals of *Ringicula doliaris* for serial sectioning were relaxed in 7.5% magnesium chloride, fixed for 24 hours in a solution of 10% neutral-buffered formalin in sea water, then preserved in 75% ethanol. Voucher material has been deposited at Atmosphere and Ocean Research Institute, The University of Tokyo (AORI), or Bavarian State Collection of Zoology, Germany (ZSM). All shell, radula and cephalic part of the animal were kept undamaged in most specimens for future taxonomic studies.

DNA extraction, PCR amplification and sequencing. DNA was extracted with DNeasy Blood and Tissue Kit (Qiagen) from the foot tissue of eight ringiculid specimens (Table 1), following the manufacturer's instructions. Portions of nuclear (18S and 28S rRNA) and

mitochondrial (COI and 16S rRNA) genes were amplified using primers shown in Supplementary Table S1; see[52] for amplification conditions and other details. New DNA sequences have been deposited in the DDBJ/EMBL/GenBank with accession numbers LC150577–LC150593 (Table 1 and Supplementary Table S2). Amplicons were purified by ExoSAP-IT (Affymetrix) following the described protocol. Purified PCR products were sequenced with the amplification and sequencing primers (Supplementary Table S1); sequencing reactions were prepared using a Big Dye Terminator Cycle Sequence Kit 3.1 (Applied Biosystems). The reaction mixtures were analyzed on ABI PRISM 3130xl sequencers after purification with a Big Dye XTerminator Purification Kit (ABI).

Taxonomic sampling for molecular phylogeny. For phylogenetic analyses of euthyneuran gastropods, we used 44 operational taxonomic units (OTUs) listed in Supplementary Table S2. These include two Rissoelloidea, three Acteonoidea, eight Ringiculidae (Ringiculoidea), four Nudipleura, six Euopisthobranchia and 17 Eupulmonata, as well as four species from the 'lower Heterobranchia' and Caenogastropoda for outgroup comparison (see[16]). Criteria for our selection of ingroup taxa were (1) the coverage of the phylogenetic diversity of Euthyneura, (2) consistency of evolutionary rates among OTUs, and (3) completeness and accuracy of sequences of all four gene fragments. Many of the lower heterobranch families were not included in our dataset because of the highly accelerated evolutionary rates of their nuclear rRNA and mitochondrial genes and/or the lack of available data. The accuracy of each sequence fragment was checked by BLAST searches and comparison with homologous sequences from related taxa, and species with dubious data were excluded from the succeeding analyses. The final dataset was double-checked by reconstructing single gene trees using the Maximum Likelihood method (see below; Supplementary Figures S4, S5).

Sequence alignment and phylogenetic reconstruction. The sequences of the four genes were aligned individually by MAFFT 7.182[53] with the L-INS-i strategy; the COI sequences were aligned as amino acids. Each aligned dataset was masked to remove alignment ambiguous sites by Gblocks Server 0.91b[54] with one of three options for a less stringent selection ('Allow gap positions within the final blocks').

Phylogenetic trees were reconstructed from a concatenated four-gene dataset using the Bayesian and Maximum-Likelihood (ML) methods in MrBayes 3.1.2[55] and GUI version of RAxML 7.4.2[56,57], respectively. In the Bayesian analysis, each gene and codon position was allowed to have different parameters, resulting in a total of six unlinked partitions. The model,

shape, proportion of invariant sites, state frequency and substitution rate parameters were estimated for each partition (see Supplementary Figure S3). Two parallel runs were made for 10 M generations with a sample frequency of 1,000, using the default value of four Markov chains. The first 5,000 trees for each run were discarded to make sure the four chains reached stationarity by referring to the average standard deviation of split frequencies[55]. The consensus tree and posterior probabilities (BPP) were computed from the remaining 10,000 trees (5,000 trees, two runs). The ML analyses were performed using the same partitions as the Bayesian analysis and following commands: a rapid bootstrap analysis (1,000 replicates) and search for the best-scoring ML tree in a single program run under the default GTR + G model, following the software manual[56]. Bootstrap proportions (BP) of ≥ 75% and BPP of ≥ 0.99 were considered significant support.

Divergence time estimates. The divergence dates between euthyneuran clades and between ringiculid taxa were calculated using the same data set and a relaxed molecular clock model in BEAST 1.5.4[58]. The tree was time-calibrated by setting the ages of the following four nodes: (1) the basal node of the tree, i.e. between Caenogastropoda and Heterobranchia, (2) the first split within Euopisthobranchia, (3) the split between the ellobiid genera *Carychium* and *Smeagol*, and (4) divergence between *Ringiculopsis foveolata* and three other ringiculids. The first calibration point was set at a minimum of 400 million years ago (Mya) with a 95% upper limit of 440 Mya (Gamma distribution, Shape: 1, Offset: 400, Scale: 13.34; see[12]), based on the Devonian occurrences of protoconchs characteristic to Caenogastropoda (408–417 Mya) and Heterobranchia (400 Mya)[59]. The second calibration point, the earliest split within the Euopisthobranchia, was set to have a minimum bound of 190 Mya (Scale: 6.33, 95% upper limit: 209 Mya). This interval encompasses the Early Jurassic period, when multiple extant families of euopisthobranch snails first appeared in the fossil record[2]. The third calibration point was constrained at a minimum age of 152 Ma (Scale: 5.07, 95% upper limit: 167.2 Mya) by referring to the earliest fossils of Ellobiidae and phylogenetic relationships within the family[13,60]. Lastly, the similar and characteristic shells of the Recent and Cretaceous *Ringiculopsis* (Fig. 3g)[27,42] were considered to justify the long existence of the genus since at least the Santonian age (Offset: 86, Scale: 2.87, 95% upper limit: 94.6 Mya)[28]. Meanwhile, the type genus of the family, *Ringicula*, has even older and more continuous records since the Callovian, Middle Jurassic (161–165 Mya)[26,41]. This genus as currently conceived seems to represent a non-monophyletic taxon with plesiomorphic shell features from which some other

ringiculid genera had originated, and the Jurassic record therefore could not be used to calibrate the age of a particular node (see Results and Discussion).

The GTR + G model was applied and parameters were unlinked across the six partitions; branch lengths and dates were estimated with an uncorrelated lognormal relaxed-clock model and a Yule prior on the tree. A single run consisted of 100 M generations (with a sample frequency of 1,000) produced 100,000 estimates of divergence dates. The convergence and mixing of the chain were assessed in Tracer 1.5.0 and first 50,000 estimates were discarded as burn-ins. In addition to this main reconstruction with all four calibration points, three separate BEAST analyses without one of the three euthyneuran priors and with 50 M generations were conducted to test the sensitivity of divergence time estimates to possible errors in adopting fossil records (Supplementary Figure S2).

Microanatomy. Relaxed and formalin-fixed specimens of *Ringicula doliaris* (ZSM Mol 20140460–20140464) were decalcified using Bouin's fluid, stained in a solution of Safranin in ethanol, dehydrated in an ascending acetone series, and embedded in Epon epoxy resin. Ribbons of serial semithin sections with a thickness of 1.5 to 2 µm were obtained using a Diatome HistoJumbo diamond knife and a Zeiss Microm rotation microtome. Sections were stained using Richardson's stain and photographed using a ProgRes C3 ccd camera (Jenoptik, Jena, Germany) mounted on a Leica DMB-RBE microscope (Leica Microsystems, Wetzlar, Germany). A 3D reconstruction of the entire body was made for one specimen (ZSM Mol 20140461) from the micrographs (greyscale *.tif*, 1024x759 pixels) in Amira 5.2 (Visage Imaging, Berlin, Germany). Presented images are surface renderings or drawings derived from the reconstructed central nervous system. Histology was compared among four sectioned specimens.

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Author Contributions

Y.K., B.B. and M.S. conceived the study and drafted the manuscript; Y.K. and M.S. collected the samples; Y.K. performed the molecular work; B.B. conducted histological examination and reconstructed the 3D microanatomy; A.N. and N.G.W. contributed to the interpretation of the data; all authors have read and approved the final manuscript for publication.

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Table 1. Ringiculid specimens used in this study. *Ringicula doliaris* from Ibusuki were formalin-fixed and used for microanatomy; others represent pure-ethanol preserved material for molecular phylogeny.

Species	Locality	Cruise/Station	Coordinates	Depth	Voucher
Ringicula doliaris	Nogama Is., Amakusa, Japan		32º35'N, 130º23'E	0–1 m	AORI YK#901
	Ibusuki, Kagoshima, Japan		31º15'N, 130º39'E	0–1 m	ZSM Mol 20140461–0464
Ringiculia sp. cf. *pilula*	W of Amami Is., Japan	T/V Nagasaki-maru N319, J-6(4)	28º33'N, 127º02'E	588–621 m	AORI YK#1463
Ringiculopsis foveolata	W of Amami Is., Japan	T/V Nagasaki-maru N307, J-6(2)	28º33'N, 127º02'E	606–607 m	AORI YK#1461
Microglyphis japonica	E of Kamaishi, Honshu Is., Japan	R/V Tansei-maru KT-12-18, St. 15	39º02'N, 142º24'E	1019–1041 m	AORI YK#2528
Microglyphis sp.	SE of Falkland Is., Drake Strait	R/V Polarstern ANTXIX5, PS61/150-1	54º30'S, 56º08'W	286–291 m	ZSM Mol 20140700 (B008)
'Microglyphis' sp.	W of Amami Is., Japan	T/V Nagasaki-maru N319, J-6(5)	28º33'N, 127º02'E	608–631 m	AORI YK#1460
Ringiculoides kurilensis	S of Kamchatka, Russia	R/V Hakuho-maru KH-14-2, NBD-1	47º00'N, 160º02'E	5179–5223 m	AORI YK#2531
	SE of Hokkaido Is., Japan	R/V Sonne SO223 (KuramBio), St. 09-09	40º35'N, 150º59'E	5398 m	ZSM Mol 20130355 (B319)

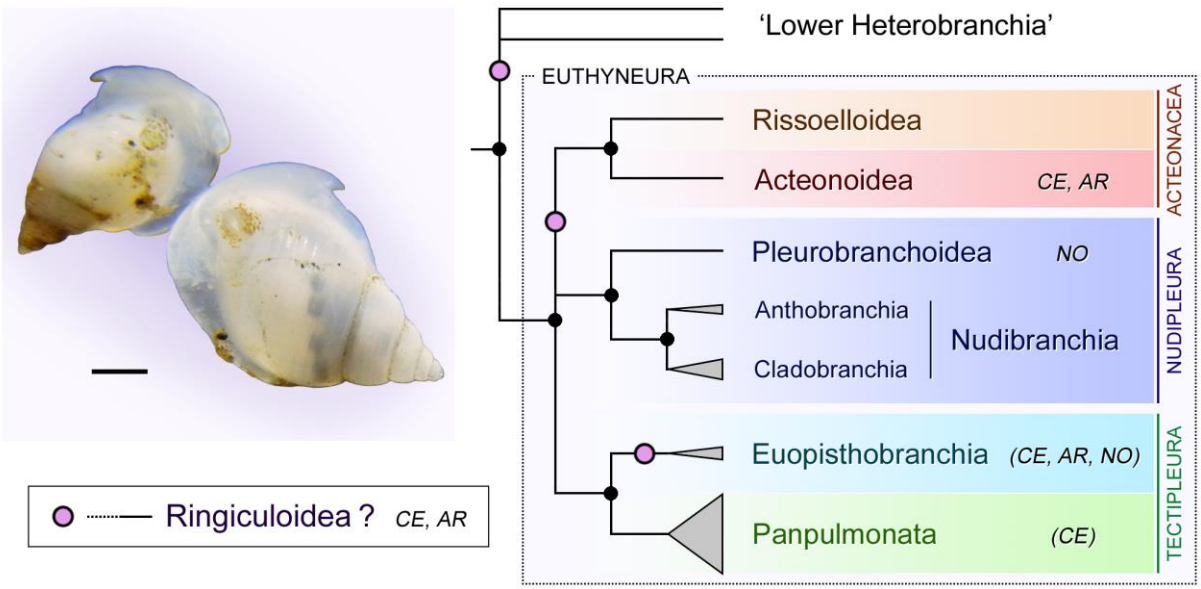

Figure 1. Current consensus phylogeny of Heterobranchia showing relationships among major clades of Euthyneura (after Wägele *et al.*[9]). Black dots indicate strongly supported clades; purple circles denote previously hypothesized positions of Ringiculidae (Ringiculoidea). Vertical height of each triangle represents approximate number of extant species. Acteonoidea, Pleurobranchoidea and Ringiculoidea as well as some subtaxa of Euopisthobranchia and Panpulmonata were traditionally classified in Cephalaspidea (CE), Architectibranchia (AR) or Notaspidea (NO). Left inset shows two live individuals of *Ringicula doliaris* from Kagoshima, Japan (Scale bar: 1 mm).

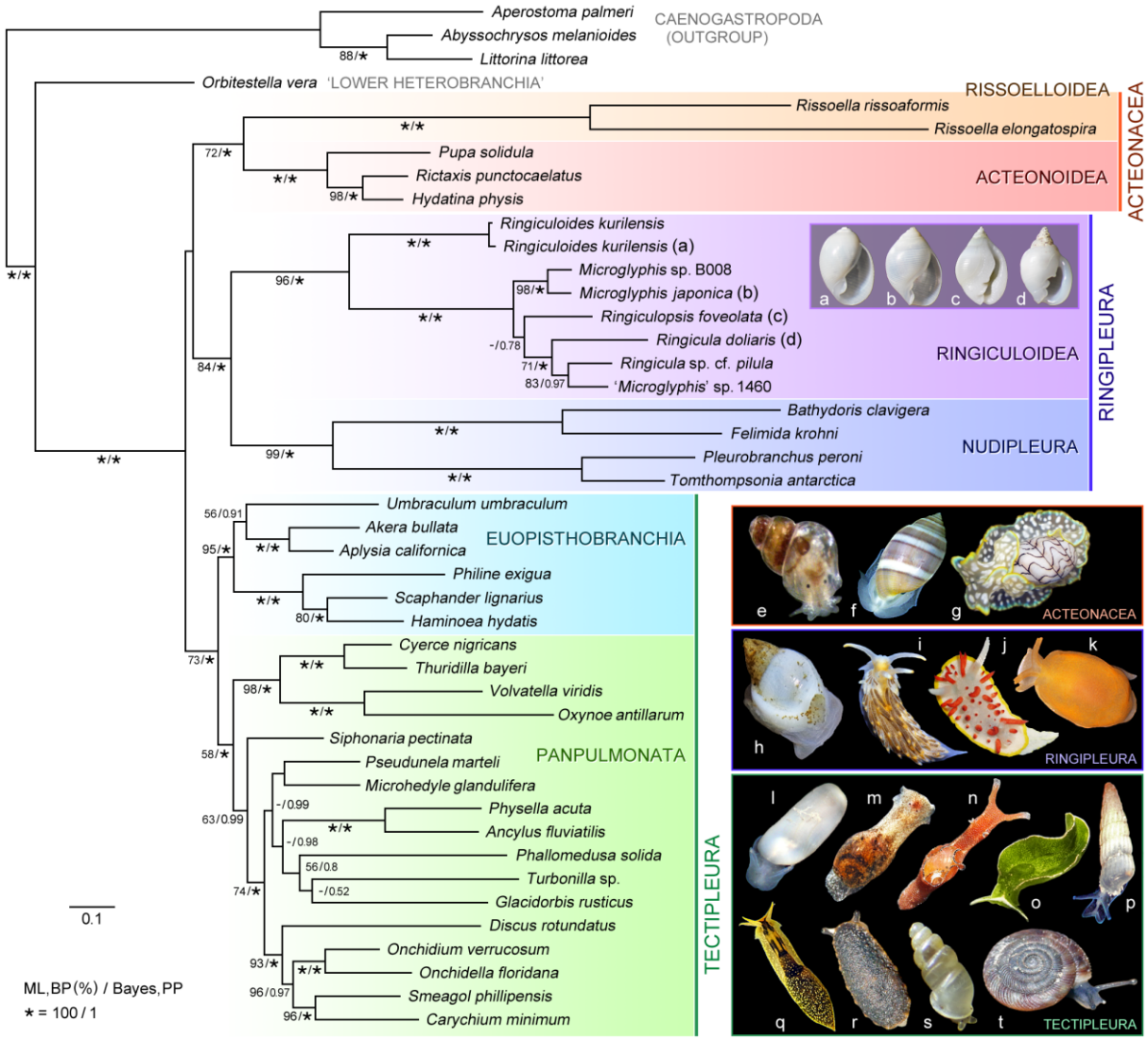

Figure 2. Maximum-likelihood phylogeny of euthyneuran gastropods. Tree reconstruction was performed in RAxML based on combined nucleotide sequences of nuclear 18S and 28S rRNA and mitochondrial 16S rRNA and COI genes (a total of 3,679 sites after exclusion of alignment ambiguous sites). Numerals on branches denote bootstrap values shown as percentages (BP, left) and Bayesian posterior probabilities computed by MrBayes (BPP, right). Significant support in bold (BP ≥ 75%, BPP ≥ 0.99); asterisks denote maximum BP and BPP values (100%, 1.00). **(a–d)** Shells of sequenced Ringiculidae (Ringiculoidea): **(a)** *Ringiculoides kurilensis*, **(b)** *Microglyphis japonica*, **(c)** *Ringiculopsis foveolata* and **(d)** *Ringicula doliaris.* **(e–t)** Live-taken images of representative species of Acteonacea, Ringipleura and Tectipleura: **(e)** *Rissoella opalina*, **(f)** *Acteon tornatilis*, **(g)** *Micromelo undata*, **(h)** *Ringicula doliaris*, **(i)** *Aeolidiella alderi*, **(j)** *Diaphorodoris papillata*, **(k)** *Berthella* sp., **(l)** *Retusa* sp., **(m)** *Haminoea* sp., **(n)** *Aplysia parvula*, **(o)** *Elysia* sp., **(p)** *Turbonilla acutissima*, **(q)** *Acochlidium bayerfehlmanni*, **(r)** *Onchidella celtica*, **(s)** *Carychium pessimum* and **(t)** *Discus rotundatus*.

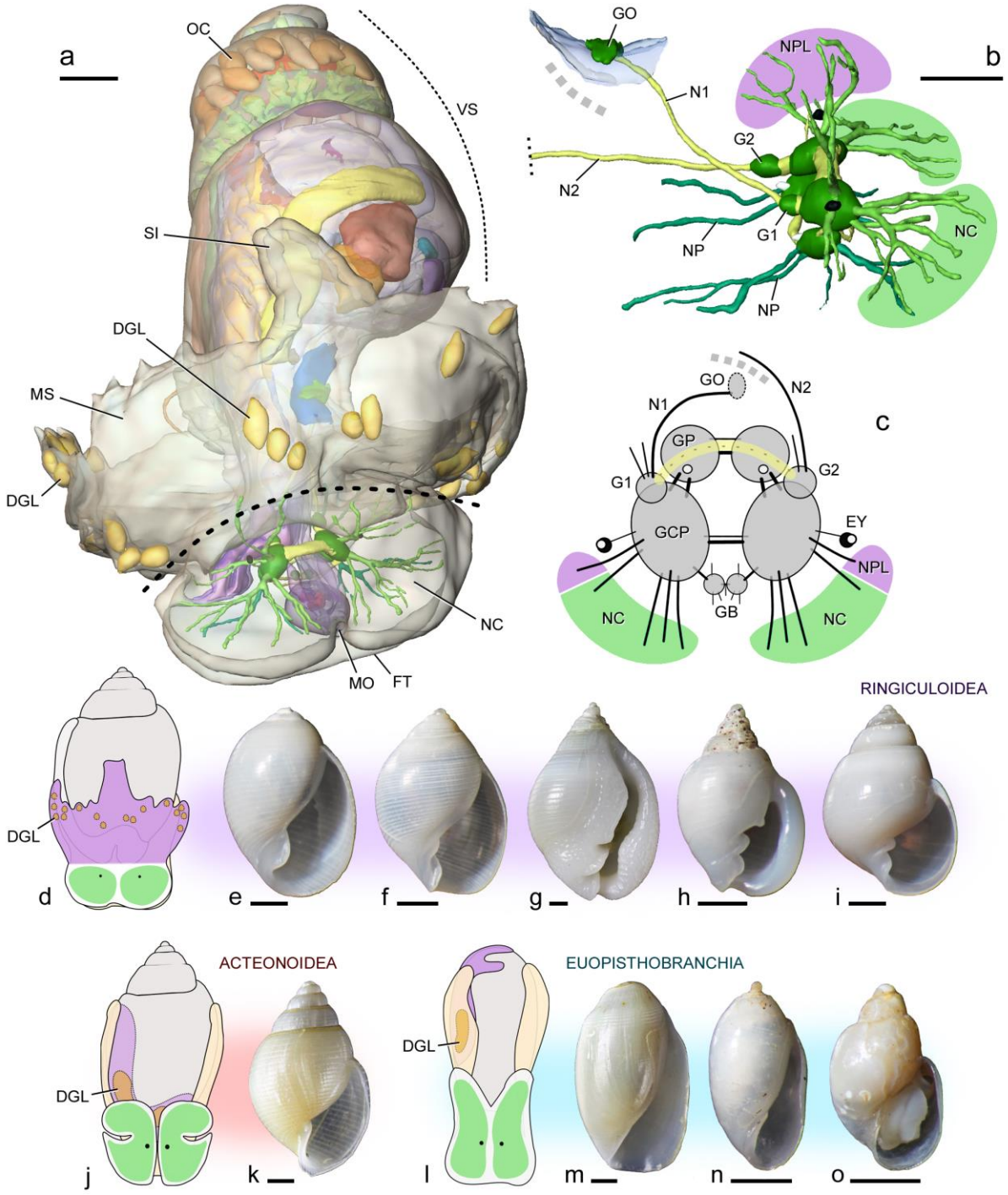

Figure 3. Morphological comparison of euthyneuran bubble snails. (a–c) Microanatomy of *Ringicula doliaris* with emphasis on head and nervous system. **(a)** 3D reconstruction of entire animal, anterodorsal view. Broken line indicates separation of headshield into head and mantle parts. **(b)** Central nervous system, oblique right view, highlighting lack of connection between nerves N1 and N2 (grey dotted line). Head (green) and mantle (purple) are innervated by cerebral and pleural (or parietal) nerves, respectively. **(c)** Schematic drawing of central nervous system, orientation as in (a). Hypothetical course of visceral loop is shown as yellow line. **(d–i)**.

Ringiculidae (Ringiculoidea). **(d)** Schematic drawing of head-hoot, mantle and shell. Green areas denote head with cerebral innervation and purple area represents mantle innervated by pleural or parietal nerves. **(e–i)** Shells of sequenced specimens: **(e)** *Ringiculoides kurilensis*, **(f)** *Microglyphis japonica*, **(g)** *Ringiculopsis foveolata*, **(h)** *Ringicula doliaris* and **(i)** '*Microglyphis*' sp. **(j,k)** Acteonoidea. **(j)** Schematic drawing. Cream area denotes expanded margins of foot or parapodia. **(k)** Shell of a representative species, *Punctacteon teramachii*. **(l–o)** Euopisthobranchia. **(l)** Schematic drawing and shells of **(m)** *Cylichnium ancillarioides*, **(n)** *Acteocina gordonis* and **(o)** *Toledonia* sp. Scale bars: 200 μm for 3D reconstruction; 1 mm for shells. Abbreviations: DGL, defensive gland, seen transparency in (i); EY, eye; FT, foot; G1, supraintestinal ganglion; G2, visceral ganglion; GB, buccal ganglia; GCP, cerebropleural ganglia; GO, osphradial ganglion; GP, pedal ganglia; MO, mouth; MS, mantle shield; N1, osphradial nerve; N2, visceral nerve; NC, cerebral nerves and innervated area; NP, pedal nerves; NPL, pleural or parietal nerves; OC, oocytes; SI, siphon; VS, visceral sac with internal organs.

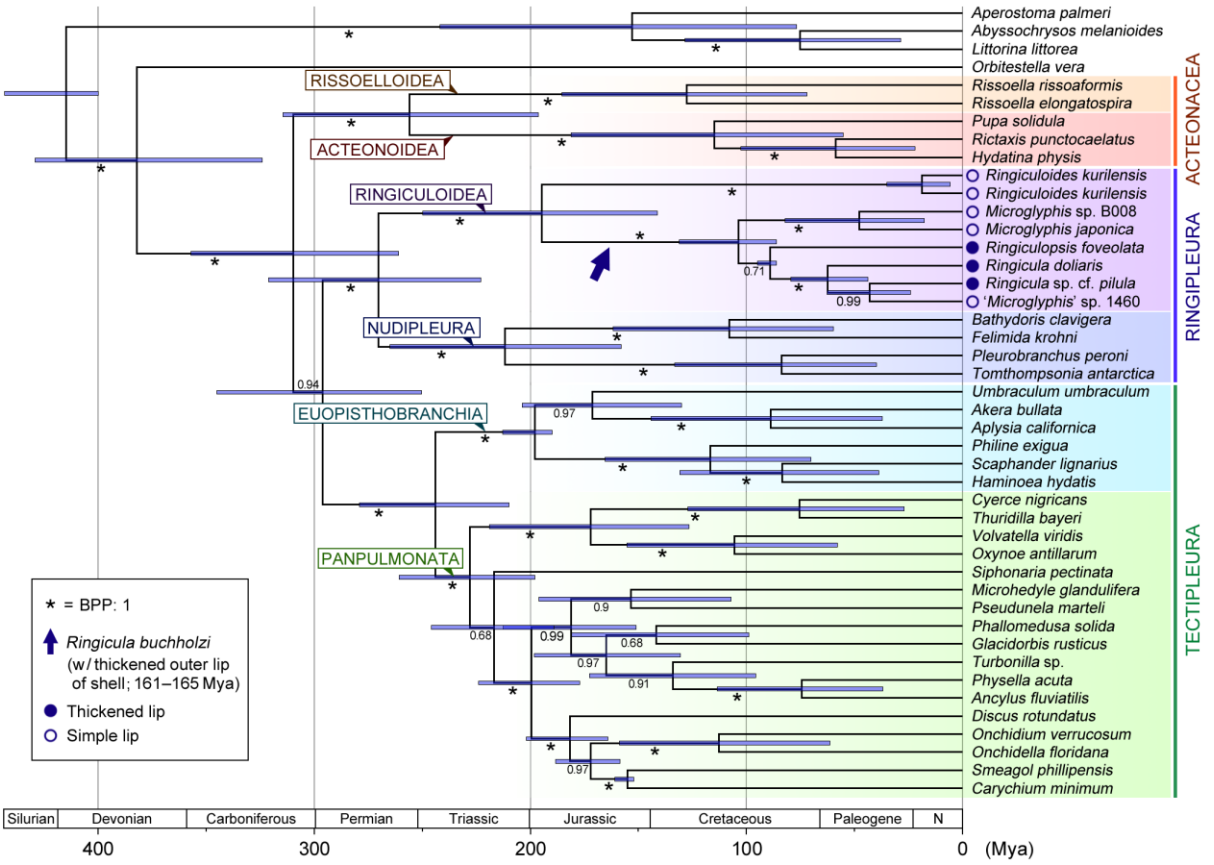

Figure 4. Time-calibrated phylogeny of euthyneuran gastropods. Reconstruction was based on concatenated four-gene sequences (3,679 sites) and four calibration priors on node ages and performed in BEAST. Numerals on branches denote Bayesian posterior probabilities (BPP); asterisks denote maximum value (1.00). Arrow points to age of earliest fossil occurrence of unambiguous Ringiculidae (*Ringicula buchholzi*), which bore a thickened outer lip of shell (plausibly a derived condition within the family). Filled and open circles indicate presence and absence of thickened lip, respectively, in modern ringiculids; note independent losses in putatively polyphyletic *Microglyphis*. Estimated nodal ages (in million years ago, Mya) and 95% credibility intervals (HPD) for first split within clades: Acteonacea, 256 (HPD: 196–315); Ringipleura, 270 (223–321); Tectipleura, 244 (210–279); Rissoelloidea, 128 (72–185); Acteonoidea, 115 (55–181); Ringiculoidea, 195 (141–250); Nudipleura, 212 (158–265); Euopisthobranchia, 198 (190–213); Panpulmonata, 228 (198–261).

4 DISCUSSION

In the last decade and during the time of this thesis, several phylogenetic or anatomical studies focused on more or less large subgroups of the Heterobranchia. The present thesis and the papers presented herein are the most up-to-date integration of this knowledge into a larger picture.

I will herein give a comparative overview of selected organ systems of Heterobranchia where data are available for comparison and that are of particular importance for phylogenetics (homologies, chapter 4.1), propose a novel consensus tree of Heterobranchia based on molecular and anatomical data, and map selected morphological features on this tree (synapomorphies, chapter 4.2). Based on this, I will propose a new classificatory scheme for Heterobranchia and subtaxa (classification, chapter 4.3) and discuss evolution of the Heterobranchia along this tree in an attempt to extract new patterns, hypotheses, and problems from morphology and anatomy (evolution, chapter 4.4.). This will be followed by an overview on larger topics and open questions (outlook, chapter 5).

4.1 Homologies: comparative morphology of selected organ systems (Figs. 2-5, 6B & 7B)

Anatomical studies prior to the "molecular age" in malacology have compiled a wealth of anatomical data, and current studies have continued to do so (see Tab. 1). However, only few studies in the last two decades have attempted to summarize the morphological knowledge on Heterobranchia as a whole (e.g. in parts: Haszprunar 1985a, 1988; Salvini-Plawen 1990, 1991; Ponder 1991; Bieler 1992). This is due to the fact that still no consensus hypothesis on the evolution of Heterobranchia exists, and "rampant parallelism" (Gosliner 1991) has rendered morphology-based cladistics a weak tool with respect to relationships of larger clades (Ponder & Lindberg 1997; Dayrat & Tillier 2002; Wägele et al. 2008).

Given the taxonomic scope of this thesis, an attempt focusing on selected organs systems will be made herein. This comparison cannot be exhaustive; I therefore comparatively describe and discuss the anatomy of organ systems where sufficient data for comparison are available. These are structures that are generally easily examined (external morphology and shell), regarded to be informative at the level of higher clades (central nervous system), and are advantageous to be examined by the histological method of sectioning and staining (e.g. for aspects of soft organs in the mantle cavity). This chapter will summarize morphological characters of taxa examined in the present thesis, and compare them with what is found in others, preferably closely related, taxa. In many cases, it will follow a separation into prosobranch-like "lower" Heterobranchia and opisthobranchs and pulmonate "higher" Euthyneura (monophyletic, and including the majority of species).

4.1.1 Body sizes and habitats (Figs. 2, 3A-D)

Heterobranchs range in body form from typical snails with a coiled external shell protecting the viscera and pronounced head tentacles (lower heterobranchs, many members of Euopisthobranchia and Panpulmonata; Fig. 2J, 3A) to those with modified heads (tentacles lost or derived as headshield;

Lower Heterobranchia in general: Fretter & Graham 1982, Haszprunar 1985a, 1988, Ponder et al. 1998, Warén 2013. Valvatoidea: Ponder 1990a, 1991, Warén et al. 1993, 1997, Ponder et al. 1998, Bieler et al. 1998, Haszprunar et al. 2011, Hawe et al. 2013, 2014. Architectonicoidea: Robertson et al. 1970, Bieler 1988, 1993, Haszprunar 1985b,c. Omalogyridae: Fretter & Graham 1982, Bäumler et al. 2008. Orbitestellidae: Ponder 1990b, Hawe & Haszprunar 2014. Cimidae: van Aartsen 1981, Fretter & Graham 1982, Graham 1982, Warén 1993, 2013. Graphididae: Fretter & Graham 1982, Warén 2013. Murchisonellidae: Warén 1994, 2013, Wise 1999, Brenzinger et al. 2014. Rhodopidae: Böhmig 1893, Riedl 1960, Haszprunar & Huber 1990, Salvini-Plawen 1991, Brenzinger et al. 2011b, 2013b.

Parvaplustra: Tjaernoeiidae: Warén & Bouchet 1988, Warén 1991, Brenzinger et al. in prep. Parvaplustridae: Powell 1951, Marcus & Marcus 1969, Chaban & Chernyshev 2013, Brenzinger et al. in prep.

Acteonacea: Rissoellidae: Fretter 1948, Ponder & Yoo 1977, Haszprunar 1988, Wise 1998. Acteonoidea: Fretter & Graham 1954, Rudman 1972c,d, Valdés 2008, Göbbeler & Klussmann-Kolb 2010a.

Ringipleura: Ringiculidae: Fretter 1960, Morton 1972, Bouchet 1975, Kano et al. in review. Nudipleura: Hoffmann 1939, Thompson 1976, Gosliner 1991, 1994, Rudman & Willan 1998, Wägele & Willan 2000.

Euopisthobranchia in general: Perrier & Fischer 1911, Hoffmann 1939, Gosliner 1994, Mikkelsen 1996, 2002, Rudman & Willan 1998. Tylodinoidea: Vayssière 1883. Cephalaspidea: Guiart 1901, Rudman 1972a,b, Mikkelsen 1996, Ohnheiser & Malaquias 2013, 2014. Runcinacea: Vayssière 1883, Burn 1963, Huber 1993. Aplysiidae: Guiart 1901, Klussmann-Kolb 2004. Akeridae: Morton & Holme 1955, Morton 1972. Pteropoda: Meisenheimer 1905, Lalli & Gilmer 1989, Kubilius et al. 2014.

Panpulmonata in general: Hubendick 1978, Smith & Stanisic 1998, Barker 2001. Sacoglossa: Jensen 1996, 2011. Siphonariidae: de Villiers & Hodgson 1986, Ruthensteiner 2006. Pyramidelloidea: Fretter & Graham 1949, Ponder 1987, Wise 1996. Amphiboloidea: Golding et al. 2007. Glacidorbidae: Ponder 1986, Haszprunar 1988, Ponder et al. 1998, Rumi et al. 2015. Hygrophila: Hubendick 1947. Acochlidia: Neusser & Schrödl 2007, Schrödl & Neusser 2010, Neusser et al. 2011a,b, Brenzinger et al. 2011a. Systellommatophora: Fretter 1943, Smith & Stanisic 1998. Ellobioidea: Morton 1955, Haszprunar & Huber 1990, Martins 1996. Stylommatophora: Smith & Stanisic 1998.

Table 1. Summary of main references used for anatomical comparison, including coding of Figs. 6 and 7. See text and classification for further details and taxon authorities.

♦

many clades) and shells (semislugs or slugs, with viscera sunk into the foot (Fig. 3C); limpets – multiple clades; see below); some taxa have become increasingly wormlike (interstitial taxa; see Fig. 2A,G-H) or are aberrant in shape (some planktonic Nudibranchia and Pteropoda, see Fig. 2E). In slugs or semislugs, mantle and foot are often enlarged and encase the visceral hump and its organs, while

the mantle cavity becomes more open to the outside and generally shifted (by clockwise "detorsion" of the visceral sac with respect to the headfoot) more to the right of the animal (compare Fig. 3A, D and C).

Heterobranchs range in body sizes from below one millimeter to over a half meter (Gofás & Warén 1998; Moroz 2010). Small to minute body sizes, with maximum shell diameters or spire heights of 4 millimeters or below are recorded for most shelled lower Heterobranchia (e.g. Ponder 1991, 1998, Brenzinger et al. 2014), for Rhodopemorpha (e.g. Salvini-Plawen 1990, Brenzinger et al. 2013b), and several lineages among Euthyneura: Rissoellidae Gray, 1850 (among Acteonacea), Tergipedidae Bergh, 1889 and Okadaiidae Baba, 1930 (among Nudibranchia), Runcinacea, some Cephalaspidea and many Thecosomata (among Euopisthobranchia), the majority of Sacoglossa, Glacidorbidae, Pyramidellidae, and Acochlidia, and many stylommatophorans (among Panpulmonata)(see Tab. 1 for references). Meiofaunal (sub)taxa are exclusively small-bodied (e.g. Jörger et al. 2014a; Fig. 2A,G-J). Taxa with sizes above 4 mm and growing beyond the centimeter-mark are found in some Architectonicidae Gray, 1850 and Mathildidae Dall, 1889 (Bieler 1993, 1995). Within the four lineages of Euthyneura *sensu lato* most taxa are relatively large, meaning they do grow beyond the 4 millimeter mark and regularly to several centimeters (e.g. most Acteonoidea, almost all Nudipleura, many Euopisthobranchia, and non-marine Panpulmonata).

The habitats of Heterobranchia are originally marine and benthic (see Fig. 6B: first box). Lower heterobranchs appear in deep to shallow-water dredgings of hard substrates such as rubble and seagrass, on sunken wood or underneath rocks (Valvatoidea and Orbitestellidae; Ponder 1990a,b, 1998, Warén et al. 1997), in dredgings of coarse sand or mud (*Tjaernoeia*, Murchisonellidae, Graphididae Barros et al., 2003 and *Cima* Chaster, 1869 – Rasmussen 1944; Warén 1994; Rodriguez Babio & Thiriot-Quievreux 1974), in rubble near or on their coral hosts (Architectonicidae and Mathildidae – Robertson et al. 1970, Climo 1975), among intertidal algae (omalogyrids, *Rhodope* – Fretter 1948; Böhmig 1893; Marcus 1953), or interstitially in coarse sand (*Rhodope*, *Helminthope* – Karling 1966; Salvini-Plawen 1990; Brenzinger et al. 2011b, 2013b). Unusual are limnic habitats of Valvatidae Gray, 1840 (e.g. on rocks and leaves – Myzyk 2002; Hauswald et al. 2008) and the hyper- to hyposaline conditions reported for some Murchisonellidae (Wise 1999; Brenzinger et al. 2014; A. Warén, Stockholm – pers. comm.; E. Strong, Washington – pers. comm.). Euthyneura are found in a large variety of habitats; ranging from deep water to coastal habitats or those in non-marine conditions. Few graze on hard subtrates such as rocks (some Siphonariidae Gray, 1827). Others are found on or among intertidal algae (Rissoellidae, Runcinacea, Anaspidea, many Sacoglossa), on soft bottoms, often burrowing (Acteonoidea, Ringiculidae, many Nudipleura, euopisthobranch Cephalaspidea, Akeroidea/Akeridae Mazzarelli, 1891, some panpulmonate Sacoglossa, Pyramidelloidea), or are pelagic (all Pteropoda, nudibranch *Phylliroë* Peron & Lesueur, 1810) or neustonic (nudibranch *Glaucus* Forster, 1777) (Lalli & Gilmer 1989), or associated and mainly located on their sessile food animals (many Nudibranchia, Tylodinoidea, Pyramidelloidea). Non-marine habitats are supratidal (panpulmonate Siphonariidae, Systellommatophora: Veronicelloidea Gray, 1840, acochlidian *Aiteng* Swennen & Buatip, 2009, sacoglossan *Gascoignella*, cephalaspidean *Smaragdinella* A. Adams, 1848), on mudflats with sometimes hyposaline conditions (*i.e.,* near river inlets; Amphibolidae Gray, 1840), in limnic systems (Glacidorbidae, Hygrophila, some Acochlidia) (see Tab. 1 for references). Airbreathing forms are found among Acochlidia (one *Aiteng* species, Kano et al. 2015), and many Systellommatophora and Ellobioidea L. Pfeiffer, 1854. Stylommatophora are fully terrestrial.

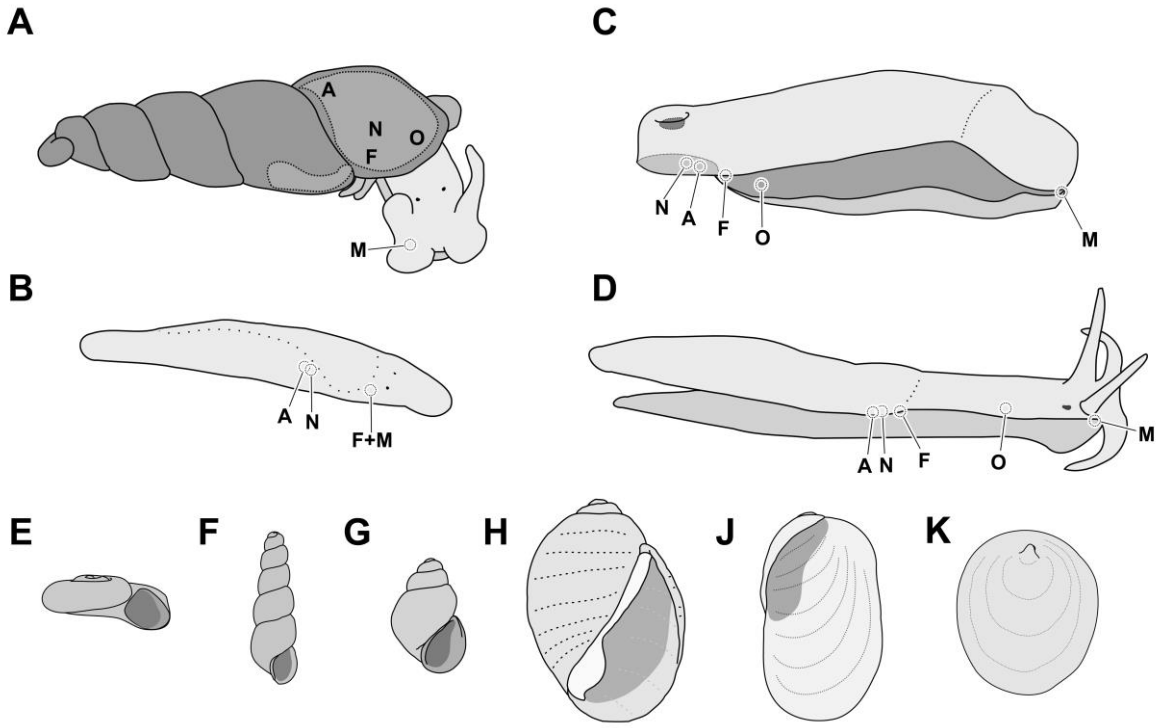

Figure 3. Examples of morphotypes described in the text: external morphology and shells.

A-D. Schematized external morphologies, right lateral views. Letters mark position of major body openings, grey stippled area in A and C extent of mantle cavity. E-F. Shell morphotypes. All apertural view; K: apical view. **A.** Shelled lower heterobranch (*Koloonella*, after chapter 9). **B.** Rhodopemorph slug (*Rhodope*). **C.** Cephalaspidean semislug (*Pluscula*, after chapter 4). **D.** Acochlidian slug (*Strubellia*, after chapter 2). **E.** Flat-spired (valvatiform) shell (*Xylodiscula* B.A. Marshall, 1988, after Warén 1991b). **F.** High-spired shell (*Cima*, after van Aartsen 1981). **G.** Globular shell. (*Rissoella*, after Ponder & Yoo 1977). **H.** Bubble shell, noted columellar fold and spiral ornament (*Ringiculoides* Minichev, 1967, original). **J.** Auriform shell (mostly internal). (*Berthella* Blainville, 1824, after photo by C. Pittman/seaslugsofhawaii.com). **K.** Limpet (external shell) (*Williamia* Monterosato, 1884, after Ruthensteiner 2006). Abbreviations: position of body openings: A, anus; N, nephropore; F, female genital opening; O, osphradium; M, male genital opening.

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Minute interstitial forms that are unpigmented and worm-shaped occur in several lineages, once among lower heterobranch Rhodopemorpha, and more than half a dozen times among Euthyneura(among Nudipleura, Cephalaspidea, panpulmonate Sacoglossa and Acochlidia – Swedmark 1968, Arnaud et al. 1986, Jörger et al. 2014a for reviews, Fig. 2A). These “microslugs” (or rather, “meioslugs”) show characteristic adaptations summarized as “meiofaunal syndrome” – multiple reductions of body appendages and internal organs, but also convergent development of calcareous subepidermal spicules and epidermal adhesive glands – by Brenzinger et al. (2013b; see chapter 5).

4.1.2 Morphology of the shell (Fig. 3E-K, 6B)

A diversity of shell forms exist in Heterobranchia, and can be grouped into several categories (Fig. 6B: second box; Tab.1 for additional references). Prosobranch-like, multispiral shells (with more than 3-4 whorls slowly growing in diameter) are found in many lower Heterobranchia, and several euthyneuran clades. These largely follow two types: Low-spired shells that are rather flat (discoidal) or even almost planispiral are found in most Valvatoidea (Fig. 3E) and Architectonicidae, all Omalogyridae and Orbitestellidae, some euopisthobranchs (among thecosome pteropods: *Limacina* Bosc, 1817), panpulmonate Glacidorbidae, Hygrophila (Planorbidae Rafinesque, 1815), and many Stylommatophora. High-spired ones that are multiwhorled, pointed and screw-shaped are found in lower heterobranch Mathildidae, Cimidae Warén, 1993, Graphididae and Murchisonellidae (Fretter et al. 1986, Bieler 1995, Warén 2013; Figs. 2J, 3A, F), and among panpulmonate Pyramidellidae and Stylommatophora. Intermediate shells that are more or less globular in outline (*i.e.,* only marginally wider or narrower than tall) are found (rarely) in some *Valvata* O. F. Müller, 1774 (see Hauswald et al. 2007), architectonicid *Heliacus* d'Orbigny, 1842 (see Bieler 1984, Stanic & Schiaparelli 2007), in *Tjaernoeia* (Warén 1991a), and occasionally in Euthyneura *s.l.* (*Rissoella*: Fig. 3G, some Cephalaspidea, and thecosome Pteropoda: *Peracle* Forbes, 1844, some *Limacina*). Characteristic shells with oval to egg-shaped outline and large body whorl and lip, and elongate to tear-shaped aperture that is longer than half of the shell length are found in many Euthyneura: in Acteonoidea, in Ringiculidae (Figs. 2K, 3H), among euopisthobranch Cephalaspidea and Akeridae (see Rudman & Willan 1998, and Tab. 1), and in several Panpulmonata. The latter include some shelled Sacoglossa, Pyramidellidae, freshwater Chilinidae Dall, 1870 and many Lymnaeoidea Rafinesque, 1815, and in many eupulmonate Ellobiidae L. Pfeiffer, 1854, stylommatophoran Succineidae Beck, 1837 and other subgroups (see Hubendick 1978Smith & Stanisic 1998; see also chapter 4.4.4). The internal shell of nudipleuran *Tomthompsonia* Wägele & Hain, 1991 (Fig. 11M) could be counted as such. Ear-shaped (auriculate) shells with a low apex, very few initial whorls and a large, flattened and flaring lip (Fig. 3J) are found in some Pleurobranchoidea (Nudipleura), several philinoid Cephalaspidea and Aplysiidae Lamarck, 1809 (Euopisthobranchia), and in terrestrial stylommatophoran (semi)slugs; these shells are often thin and internalized and do not cover the body. The external shell of the ellobioid *Otina* Gray, 1847 (Morton 1955) could also be categorized as such. Flat, limpet-like shells (roughly circular, external, and tough) into which the body cannot be retracted (but which may still cover the body and are thus protective) are found in euopisthobranch Tylodinoidea, and in panpulmonate subgroups (Siphonarioidea Gray, 1827, Amathinidae Ponder, 1987, Trimusculidae Burch, 1945, and among several taxa within Hygrophila) (Fig. 3K). Complete lack of shells in the adult stage is found in lower heterobranch Rhodopemorpha, all Nudibranchia, some Cephalaspidea, most Aplysiidae and Runcinacea, all Gymnosomata, most Sacoglossa, all Acochlidia, Systellommatophora, ellobioid *Smeagol* Climo, 1980, and in many lineages of Stylommatophora.

The spicular "skeleton" of some meiofaunal taxa (e.g. Acochlidia: *Asperspina* Rankin, 1979, *Hedylopsis* Thiele, 1931) was suggested to act as a secondary, internal shell (e.g. Rieger & Sterrer 1975). Spicules in *Rhodope* were hypothesized to be homologous to calcium cells of shelled lower heterobranchs, and subepidermal spicules of meiofaunal slugs (Acochlidia, *Platyhedyle*) may generally be homologues to calcium cells of shelled taxa (see Brenzinger et al. 2014). Subepidermal spicules are also present in many dorid nudibranchs and some Pleurobranchoidea (Cattaneo-Vietti et al. 1995, Penney 2008). Other types of secondary "shell" are the external, gelatinous pseudoconchs of pteropod Pseudothecosomata (Meisenheimer 1905, Lalli & Gilmer 1989).

Heavily calcified shells are found only in some of the larger-bodied taxa (Architectonicoidea, Ringiculidae, Tylodinoidea, some Cephalaspidea; Siphonariidae, some Pyramidellidae). Not shown here are the minute external shells of some Runcinacea and Cephalaspidea which consist of little more than a protoconch (e.g. Burn 1963) and the bilaterally symmetric, uncoiled shells of some Thecosomata (Orthoconcha; Kubilius et al. 2014; Fig. 2E).

Besides form of the shell, several ornamental elements exist in the shells (see Fig. 6B – second box): Columellar folds are one or several strongly calcified longitudinal ribs running along the columella. In contrast to Haszprunar (1985a: p. 19 and table 1 therein) who assumed these to be a character of Architectonicoidea and "most basal Heterobranchia", the only Recent Heterobranchia bearing such folds are several euthyneuran taxa bearing bubble shells: in Acteonacea (most Acteonoidea), in Ringipleura (*Ringicula*; Kano et al. in rev.), in Euopisthobranchia (some Cephalaspidea formerly grouped as "Cylichnidae" Adams & Adams, 1854), and in panpulmonates (among Pyramidellidae, Chilinidae, and Ellobiidae); especially in the latter this may be a simple tooth-like projection, as is also found in some terrestrial stylommatophorans (e.g. *Placostylus* Beck, 1837). Shells with distinct surface ornamentation consisting of ribs and/or intersecting spiral lines are found in several lower Heterobranchia (strong: most Architectonicidae and Mathildidae; thin to faint ribs: in Graphididae, some Murchisonellidae, and Orbitestellidae; knobs along the apical part of the whorls: Omalogyridae, some Orbitestellidae), while others have smooth shells. Among Euthyneura, rather prominent ribs may be found in many Pyramidelloidea (e.g. *Turbonilla* Risso, 1826 – Dall & Bartsch 1909, Laseron 1959, Fretter et al. 1986; *Amathina* Gray, 1842 – Ponder 1987) and in the thicker-shelled members of Ellobiidae (spiral ribs, e.g. *Pedipes* Férussac, 1821; see Martins 1996). A covering of minute, irregularly sorted pits on the otherwise smooth shell is found in lower heterobranch *Tjaernoeia* (see Warén & Bouchet 1988, Warén 1991a), in *Parvaplustrum* Powell, 1951 (see Chaban & Chernyshev 2013), and the valvatoidean *Tomura umbiliobsessa* Rolán & Rubio, 2008. A characteristic type of ornament (spiral grooves consisting of oval to rectangular, chain-like connected pits that may be discernible only under SEM) is found in many Acteonoidea (e.g. Sasaki 2008, Valdés 2008), *Ringicula* (Gründel 1997), some Cephalaspidea (*Philine* Ascanius, 1772, *Scaphander* Montfort, 1810: Gosliner 1991), some Amathinidae (e.g. *Leucotina* A. Adams, 1860: Sasaki 2008). The internal shells of some pleurobranchoideans show similar sculpture (*Berthella* Blainville, 1824; Gosliner 1994, Schrödl 1999). Shells of lower heterobranchs are usually translucent and colourless. Strong brown or reddish pigments in the shells are found in Mathildidae, Architectonicidae and Omalogyridae (Bieler 1993, Sartori & Bieler 2014). Pigment patterns consisting of spirally arranged blotches are found in Acteonoidea (black to red spots or lines), in euopisthobranch Tylodinidae, Haminoeidae and Bullidae Gray, 1827 (Cephalaspidea) (Rudman & Willan 1998), and patterns of zig-zag flames are present in several Panpulmonata, especially larger-bodied taxa: some Amphibolidae, Pyramidellidae (*Otopleura* P. Fischer, 1885, *Pyramidella* Lamarck, 1799), Chilinidae, Ellobiidae, and terrestrial Stylommatophora (Smith & Stanisic 1998).

4.1.3 Morphology of the mantle margin and cavity (Figs. 3A, 4)

The mantle is the characteristic molluscan organ that carries the shell and creates it by secreting new shell material along its edge. Furthermore, the mantle forms the dorsal and lateral walls of the mantle cavity which acts as an additional body compartment which opens to the outside – several organ systems are characteristically associated. In Heterobranchia, the roof of the mantle (cavity)

generally houses the heart and kidney (subepithelially), the intestine runs along the posterior margin of the cavity towards the right and the female gonoduct along its floor towards the right; all three organ systems open into the mantle cavity (in the roof, the right margin, and the right floor, respectively). Additionally there may be several epithelial structures (the gill, paired ciliary ridges, complex glands, the sensory osphradium, see Haszprunar 1988 and Fig. 4A-C). No revision of the details of the mantle configuration described by Brace (1977a,b) (musculature, attachment to shell) is attempted herein.

In shelled and aquatic taxa with multispiral shell, the mantle cavity opens anteriorly over the neck and somewhat along the right side of the foot (area marked by stippled line in Fig. 3A); it bears all aforementioned organs and a variety of glands (Lower Heterobranchia except Rhodopemorpha; Acteonoidea, Ringiculoidea, many Cephalaspidea; opercula-bearing panpulmonates, most Hygrophila). In taxa with reduced shell, the cavity is shallow and elongate, and in shell-less taxa it is mostly missing (see Mikkelsen 1996: p. 384). In these cases gill, strips and glands are generally reduced or fully lost, and body openings (anus, nephropore, female genital opening) and the sensory osphradium are distributed more or less closely together along the right side of the body. This is the case in Rhodopemorpha (Fig. 3B), Nudibranchia, some Cephalaspidea (Fig. 3C), Runcinacea, Gymnosomata, shell-less Sacoglossa, and Acochlidia (Fig. 3D). In those taxa with a partially reduced or modified yet still relatively large shell (semislugs), the mantle cavity is usually shallow and carries a lower number of epithelial structures but often retains a gill (Aplysioidea Lamarck, 1809, Akeroidea, some Cephalaspidea) or is fully lost, leaving the gill exposed on the right side between shell and foot (some Runcinacea – Burn 1963; “side-gilled” Pleurobranchoidea, Tylodinoidea: Thompson 1976, Mikkelsen 1996, 2002). In air-breathing “pulmonate” slugs, the mantle cavity may be distinct, opening to the outside by a small opening (the pneumostome) created by the mantle margin and bearing a vascular net used for gas exchange; gill and glands may be reduced (Eupulmonata). Some Acteonoidea, Cephalaspidea, and Akeridae carry a so-called “pallial caecum” on the right side (Mikkelsen 1996; Fig. 4A,C: cae); this blind sac contains extended ciliary strips (see below) and may run parallel to the coils of the visceral sac (e.g. Morton 1972). In others, there may be an unciliated blind sac on the left side; this appears to bear glandular cells (in *Koloonella* and *Ringicula*; Brenzinger et al. 2014, own unpubl. obs.); such a glandular mantle “caecum” on the left (Fig. 4B,C: gbs) may be a new structure not described in the previous literature.

The mantle margin is the rim of the mantle roof and generally the area that forms the periostracum and shell by secretion along its outer edge (e.g. Fretter & Graham 1962). Beyond this, the more inner part of the margin the gill and the rim, may be glandular as may be the anterior floor of the cavity. The mantle edge remains near the lip of the shell at all times in actively crawling animals of most multi-coiled taxa among shelled lower Heterobranchia, and in some Euthyneura. An extended mantle roof, which may extend over the lip of the shell and expand dorsally, is found in other Euthyneura (“new” mantle in Fig. 6B: third box; see also 4.4.3). This expanded mantle covers parts of the last whorl in crawling animals in *Ringicula* (Kano et al. in review, own unpubl. data; Figs. 2K, 5H), *Parvaplustrum* (see Powell 1951), and panpulmonate Lymnaeoidea (e.g. Hubendick 1978). The mantle may permanently enclose large parts of the shell in Pleurobranchoidea, some Cephalaspidea, Aplysiidae, and in many stylommatophoran semislugs. In taxa with reduced shell and mantle cavity, the mantle may 1) be confluent with the head and neck (*e.g.* Fig 3C,D) and 2) form a glandular covering of a substantial part of the dorsal body, including the visceral sac: this is exemplified by the

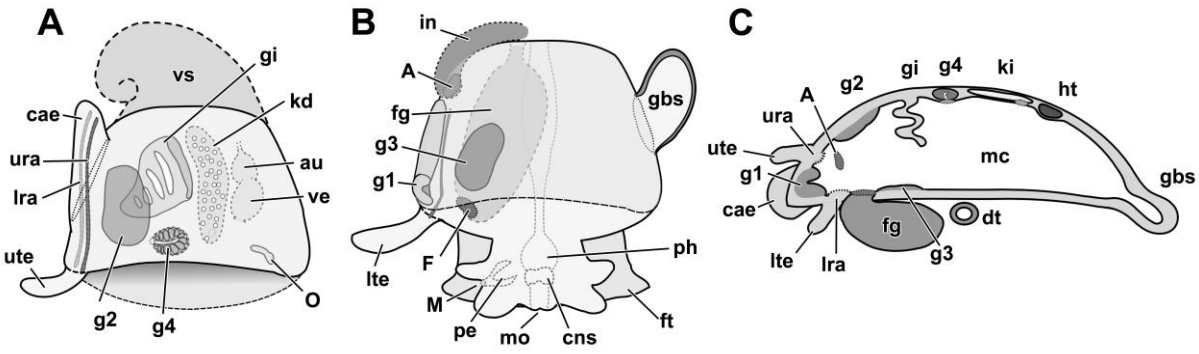

Figure 4. Exemplary organization of the heterobranch mantle cavity and associated organs.

Schematized dorsal views of mantle cavity associated organs. **A.** Organs of pallial roof. **B.** Organs of pallial floor (roof shown transparent, without organs). **C.** Schematized cross-section, anterior view. Presence, proportion and position of structures may vary in taxa. Abbreviations in capital letters, position of: A, anus; N, nephropore; F, female genital opening; O, osphradium; M, male genital opening. Further abbreviations: au, auricle; cae, "pallial caecum" of literature with ciliary strips; cns, central nervous system; dt, digestive tract (esophagus/oral tube); fg, female glandular mass (distal female genital tract); ft, foot; gbs, glandular blind sac at left side; gi, gill; g1-g3, mantle cavity glands; g4, Blochmann-like defensive glands; ht, heart; in, intestine (rectum); ki, kidney; lra, lower "raphe" or ciliary strip; lte, lower tentacle on mantle margin; mc, mantle cavity lumen; mo, mouth; pe, penis; ph, pharynx; ura, upper "raphe" or ciliary strip; ute, upper tentacle on mantle margin; vs, visceral sac.

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notum of nudibranchs (e.g. Tardy 1970, LaForge & Page 2007, Martynov et al. 2011, Figs. 2F, 5J), systellommatophorans (Solem 1978), sacoglossans (e.g. Kohnert et al. 2013) or the mantle "hood" of acochlidians (see Brenzinger et al. 2011a; Figs. 2A, B, 3D); the condition in non-euthyneuran *Rhodope* is unclear.

The gill of Heterobranchia is an evagination of the mantle roof epithelium and highly variable in form. It may show a tree-like structure (protrusible from the mantle cavity, in Ectobranchia; Yonge 1945). Another type of gill is the so-called "plicatidium" (*sensu* Morton 1972), a single sheet of mantle roof epithelium folded back upon itself (Fig. 4C: gi). This "plicate" gill may be a single, triangular sheet hanging freely into the mantle cavity (*Parvaplustrum*, Acteonoidea, *Ringicula*, Cephalaspidea, many Euopisthobranchia; see e.g. Morton 1972, Mikkelsen 1996) or quite complex, resembling a feather (Pleurobranchoidea, Tylodinoidea, some Cephalaspidea; Thompson 1976), or it may be attached largely to the roof of the mantle cavity (Sacoglossa, Siphonariidae) (de Villiers & Hodgson 1987, Jensen 2011). The so-called "foliobranch" gill consists of parallel sheets of tissue formed by the mantle roof (some Pyramidelloidea, shelled Sacoglossa; Haszprunar 1985b, Wise 1993, Jensen 2011) or the hypobranchial gland (Architectonicoidea; Haszprunar 1985b,c); in some ringiculids, the latter structure is present besides a plicate gill (own unpubl. observation). The gills of nudibranchs may be feathery structures on the back of the notum, or found underneath the mantle margin (Potts 1981, Martynov et al. 2011). A gill is lacking in Omalogyridae, Orbitestellidae, *Cima*, *Graphis*, Murchisonellidae, *Tjaernoeia* and *Rhodope* (Bäumler et al. 2008, Hawe et al. 2014, Brenzinger et al. 2011b, 2014; Ponder 1991). Many slugs and semislugs once placed among opisthobranchs possess

"secondary" (or tertiary?) gills, e.g. the serial dorsal gills of some nudibranchs (Cladobranchia – "Tritonioidea" Lamarck, 1809; Wägele & Willan 2000).

A network of hemolymph vessels located in the mantle roof forms the lung in air-breathing Panpulmonata (Eupulmonata), functionally replacing the gill. A network of superficially similar "dorsal vessels" has also been described for aquatic, gill- and shell- less Sacoglossa (*Elysia* Risso, 1818: e.g. Swennen 2011) and Acochlidia (Bücking 1933; Neusser et al. 2011b). Homologization of these vessels is still a matter of debate, as connections of the vessels to the kidney, heart, or pericardium and hence their function is still largely unexamined (see Neusser et al. 2016).

Opposed dorsal and ventral tracts of ciliary epithelium are found in numerous heterobranch clades and are regarded to be responsible for creating a ventilatory current of water through the mantle cavity, enabling oxygenation and waste emission (Ponder 1991; Fig. 4: ura, lra). Histologically, they may be distinguishable simply by their stronger ciliation, or by a more conspicuous underlying columnar epithelium (e.g. *Ringicula*, *Acteon* Montfort, 1810; Fretter & Graham 1954, Fretter 1960, own obs.). These strips (or "raphae") are reported in most lower Heterobranchia and shelled members of Euthyneura. The strips are reported to be located on the left of the mantle cavity in Architectonicidae and Mathildidae (Haszprunar 1985b,c; this was however refuted for Omalogyridae by Bäumler et al. 2008), and to the right in all other taxa that have them (Fig. 4A,C). Ciliary strips are effectively known for all aquatic taxa that have a mantle cavity, including Acteonacea, Ringiculidae (Fretter 1948, 1960, Fretter & Graham 1954), many Cephalaspidea (Rudman 1972a,b), Pyramidellidae (Wise 1993) and Amphibolidae (Pilkington et al. 1984). Ciliary strips are lacking in most slug and semislug clades with a reduced or open mantle cavity (including Nudipleura, Rhodopemorpha, some Cephalaspidea, and most Pteropoda) and in all terrestrial Panpulmonata. They are also lacking in shelled lower heterobranch Valvatoidea (Haszprunar et al. 2011), Murchisonellidae (Brenzinger et al. 2014) and apparently *Tjaernoeia* and *Parvaplustrum* (own unpubl. observation).

The gastropod mantle cavity generally houses glandular areas of epithelium. In Heterobranchia, more than one histologically distinct type of gland cells may be found (g1 to g4 in Fig. 4A-C), and several names exist for the structures often based on external appearance; homologization of these structures (called hypobranchial gland, or "pigmented mantle organ") will not be attempted here. Two structures warrant inclusion here, however: a histologically conspicuous pair of glands is found at the distal gonoduct of Rhodopemorpha (which lack a mantle cavity *per se*); this pair of glands was hypothesized to present a spermatophore-forming organ (and thus a secondary copulatory organ of the taxon; Brenzinger et al. 2011b, 2013b). A histologically similar pair of glands was found in the left corner of the mantle cavity in Murchisonellidae (Brenzinger et al. 2014; in position of g1 in Fig. 4B), and potentially in Valvatoidea (Cornirostridae Ponder, 1990a), and suggested to be homologous to the glands of Rhodopemorpha. Furthermore, another histologically and ecologically conspicuous type of gland, consisting of single holocrinous cells with a large, unstaining lumen and a surrounding muscular coat; these cells may exude a viscous, white to yellow fluid in living animals when disturbed (e.g. Fretter 1960, Pinchuck & Hodgson 2010, Cruz-Rivera 2011). Compound glands of this type with a presumed defensive function have been called "Blochmann's" glands by various authors (Guiart 1901; Perrier & Fischer 1991, Wägele et al. 2006). Single irregular layers of this cell type were observed for Murchisonellidae (Brenzinger et al. 2014), *Omalogyra* Jeffreys, 1859 (Bäumler et al. 2008), and *Tjaernoeia* (own unpubl. data). The configuration with grape-like clusters of such cells surrounding a central secretory duct (g4 in Fig. 4C) is found in *Rissoella* (own unpubl. obs.), *Ringicula*

(Fretter 1960, Kano et al. in rev.), Cephalaspidea (Rudman 1972a, see Brenzinger et al. 2013a for discussion), Runcinacea (*Ildica* Bergh, 1889: Marcus & Marcus 1963). Potentially homologous "Blochmann-like" glands are described for a diverse number of euthyneuran taxa, *e.g.* dorid nudibranchs ("gill" glands; see Martynov et al. 2011); Anaspidea (as defensive ink glands, Guiart 1901) or in Siphonariidae (Fretter & Graham 1962). Extrusion of viscous, yellow or white defensive fluid has been reported *e.g.* for Cephalaspidea (Rudman 1972a, Cruz-Rivera 2011), Sacoglossa (e.g. Lewin 1970, Marín & Ros 2004), Pyramidellidae (Wise 1996), and Amphibolidae (Golding et al. 2007).

4.1.4 Headfoot morphology and central nervous system (Figs. 4B, 5)

Gastropods possess a distinct headfoot, formed by the head (bearing mouth, paired tentacles and sensory organs, and sometimes an unpaired copulatory organ) and the foot, the primary locomotory organ. Posterior to the mouth is the buccal cavity with muscular pharynx housing the radula (Fig. 4B: ph). Radular characters are used in many taxonomical treatises, but no detailed revision or discussion will be attempted here. The central nervous system consists of one ring of ganglia that encloses the digestive tract (just anterior or posterior to the pharynx) and the more ventral buccal and visceral connectives looping ventrally; the latter carry varying combinations of further, buccal and visceral, ganglia (see schemes in e.g. Haszprunar 1988, Brenzinger et al. 2011a, 2013a). The head also bears several sensory organs and structures (paired subepidermal eyes, epidermal tentacles and sensory patches known as Hancock's organs). Posterodorsally, the neck connects to the visceral sac bearing the viscera and the mantle with its associated organs (Figs. 3A, D, 4B).

The foot is a large, muscular organ with a flat underside touching the substrate and used for locomotion (crawling, climbing, swimming, burrowing); this is aided by the presence of dense ciliation and that of glands (unicellular, and sometimes complex). The anterior margin of the foot sole may be modified by an anterior indentation (into which a large anterior pedal gland opens; Fig. 5A-C, Q), and pointed anterolateral corners of various sizes (Bieler et al. 1998, Ponder et al. 1998, Warén 2013). This is the case in all lower heterobranchs except for Omalogyridae, some Murchisonellidae and *Rhodope* which all bear a more or less modified head (Fig. 3A,B). Less prominent median indentations are also found in various Euthyneura. Among Euthyneura, pronounced laterally expanded corners (sometimes called propodial tentacles) are found in many Acteonoidea, in Nudipleura (not in dorids) and some *Ringicula*. A bifid posterior foot end is found in the valvatoid *Tomura,* the cephalaspidean *Diaphana* T. Brown, 1827, and the panpulmonate *Glacidorbis* (Ponder 1986, 1990a, Bieler et al. 1998, Ohnheiser & Malaquias 2014). In *Rhodope*, the foot is reduced.

A broadened foot is found in many Euthyneura, sometimes with fleshy structures formed by the sides of the foot (parapodia). These sometimes encase the shell and visceral hump at least partially and may function as a sediment screen; such parapodia are found in Acteonoidea, especially Hydatinidae Pilsbry, 1895 (Rudman 1972c), in some shelled Sacoglossa (Panpulmonata), and in all Euopisthobranchia except for Tylodinoidea and some "diaphanoid" Cephalaspidea (e.g. *Newnesia* E. A. Smith, 1902, *Toledonia* Dall, 1902; Eliot, 1906, Golding 2010) (see also Fig. 7: column at very right). Parapodia act as swimming organs, either during brief escape responses (some Cephalaspidea e.g. Gastropteridae Swainson, 1840, *Atys* Montfort, 1810), or during longer bouts of directional swimming (Akeridae, some Aplysiidae) and as main means of directed locomotion in holopelagic Pteropoda (Fig. 2E; see also chapter on classification).

An adult operculum, a cuticular shield attached to the posterodorsal side of the foot in crawling animals and protecting the shell aperture in retracted ones, is present in the majority of lower heterobranchs save *Rhodope,* and perhaps *Tjaernoeia* (own obs.). It is lacking in the majority of Euthyneura except for most Acteonacea (lacking only in Hydatinidae), some cephalaspideans (*Retusa* T. Brown, 1827 and *Cylichna* Lovén, 1846; e.g. Minichev 1967) and coiled-shell Pteropoda, and three families of Panpulmonata (Pyramidellidae, Amphibolidae, and Glacidorbidae – see classification below and further Discussion).

The male copulatory organ is situated inside or on the right side of the head (Fig. 4B: pe). A penis as intromitting organ is present in many Heterobranchia; it may be tubular, or with open ciliated groove transmitting sperm, and sometimes bears cuticular hooks or injectory stylets; it is usually functionally distal to a glandular duct that acts as a prostate. A simple and non-retractile penis is attached externaly to the side of the head in most Caenogastropoda (Ponder & Lindberg 1997), and also in Valvatoidea, Orbitestellidae, *Cima*, *Tjaernoeia*, *Parvaplustrum*, *Rissoella*, and Acteonoidea (Ponder 1991, Brenzinger et al. in prep and Tab. 1 for additional references), and euopisthobranch Tylodinoidea (Rudman & Willan 1998) (see also Fig. 6B: last box). A copulatory organ that is retracted into the cephalic hemocoel (and presumably becomes functional by eversion) is present in Murchisonellidae, *Ringicula*, Nudipleura, and the majority of Euopisthobranchia and Panpulmonata. In *Parvaplustrum* and many Euthyneura it also carries cuticular hooks or stylets (*Parvaplustrum*: Marcus & Marcus 1969, Brenzinger et al. in prep.; Nudibranchia: e.g. Rivest 1984, Gosliner 1994; Ringiculidae: own obs.; Euopisthobranchia: Gosliner 1994, Anthes & Michiels 2007, Lange et al. 2014; Panpulmonata: Gascoigne 1974, Jensen 1996, Brenzinger et al. 2011a). Prostatic tissue may be proximal to the penis *per se*, or parallel ("accessory") to it resulting in two-part copulatory organs. Such two-part organs are found e.g. in *Ringicula* (Fretter 1960, own. unpubl. obs.), some Cephalaspidea (Gosliner 1989, 1994), and among some Panpulmonata (some Amphiboloidea, Hygrophila: hedylopsacean Acochlidia). In many taxa however, a penis is not present at all and instead, spermatophores are formed by the prostatic tissue (Ghiselin 1966); this was observed or assumed e.g. for lower heterobranch Architectonicoidea (Robertson 1973) and *Rhodope* (Riedl 1959, Brenzinger et al. 2011b), euopisthobranch *Runcina* (Kress 1985) and at least some Cephalaspidea (Brenzinger et al. 2013b) and Pteropoda (Ghiselin 1966, Gosliner 1994, Lalli & Gilmer 1989), and among panpulmonate Pyramidellidae and Acochlidia (taxa summarized in Mikkelsen 1996, Jörger et al. 2009).

The mouth of heterobranchs may be on the tip of a dorsally visible, short snout which may possess a bifid tip (Architectonicoidea, Valvatoidea, Orbitestellidae, *Graphis* and *Cima*, *Tjaernoeia*: Ponder 1990a,b, 1991, Ponder et al. 1998, Warén 1991a,b, 1993, 2013; Figs. 5A-C, 6B); a pair of short tentacles is present on the tip of the snout in Cornirostridae and *Tjaernoeia* (Bieler et al. 1998, own unpubl. obs.). The mouth may be situated below a short and broad upper lip with a median indentation (Murchisonellidae, Euthyneura: Acteonoidea, Rissoellidae, Pyramidellidae), or may be covered by a wide and fleshy upper lip and thus not visible dorsally (other Euthyneura; Fig. 5R, 6B). The mouth is followed by a ciliated oral tube, the muscular pharynx containing the radula, and the tubular esophagus; these become partially everted during the feeding process. In some taxa oral tube or esophagus are very long, and during feeding turn into a long proboscis with the pharynx at its distant tip; a proboscis is reported for lower heterobranchs *Graphis* (assumed by Fretter & Graham 1982, yet not observed), Architectonicidae (e.g. Robertson et al. 1970), euthyneuran Hydatinidae (Acteonoidea; Rudman 1972c,d), nudipleuran *Pleurobranchus* Cuvier, 1804 (Nudipleura; Thompson

1976), some pteropod Gymnosomata (Pseudothecosomata, gymnosome *Cliopsis* Troschel, 1854; according to Hoffmann 1939, P. Kohnert pers. comm.) and panpulmonate Pyramidellidae (Fretter & Graham 1949). The oral tube and the pharynx have a reduced size in Murchisonellidae, many Nudibranchia, some Cephalaspidea; in *Rhodope*, both are reduced completely (see Brenzinger et al. 2011b, 2013b).

A taenioglossate radula (with triangular median tooth and 2 or three denticulate teeth on each side) is found in most Valvatoidea, in Orbitestellidae, and Rissoellidae (Ponder 1991, 1998) and many Caenogastropoda. Variations occur in some Valvatoidea that have a broader, *i.e.,* rhipidoglossate radula similar to the more distant prosobranch outgroup, "Archaeogastropoda" (see Warén & Bouchet 1993, Warén et al. 1993, Haszprunar et al. 2011). In other taxa, many modifications of this pattern occur, with narrowing of the radula (by loss of the median or side teeth) or broadening (by multiplication of the side teeth), and considerable change of form in particular teeth. The triangular median tooth (rachidian) is lost in a number of taxa, including lower heterobranch *Xylodiscula*, *Cima*, *Graphis* (?), Murchisonellidae, *Tjaernoeia*, many Ringiculidae, and many other euthyneuran subtaxa (e.g. Bouchet 1975, Ponder et al. 1998).

Gastropods usually possess paired sensory lobes or tentacles on the laterodorsal sides of the head. In Heterobranchia, their number varies between none and three pairs (See Figs. 5, 6B: last box). They are innervated by sensory nerves of the cerebral ganglia (see below). Form of tentacles is highly variable and group-specific. There usually is one pair of tentacles at the level of the eyes; a second pair associated with the mouth and forming an upper lip is present mainly in Euthyneura. If two pairs are present, the posterior tentacles are called rhinophores and held erect, the anterior ones are called labial or oral tentacles. Tentacles may be more or less shortened and flattened or lobe-like; if they form a largely rectangular and flattened disk this is called a headshield. Tentacles or head-lobes are completely absent in some majorly meiofaunal taxa (Rhodopemorpha, nudibranch Pseudovermidae Thiele, 1931, sacoglossan Platyhedylidae Salvini-Plawen, 1973; Brenzinger et al. 2011b, 2013b) and in some holopelagic Pteropoda (Pruvot-Fol 1954).

A sole pair of posterior tentacles (and no upper lip) is present in many lower heterobranch taxa (Fig. 5A-C); the tentacles may be finger-shaped and long (in Valvatoidea, most Architectonicoidea, Orbitestellidae, *Cima*, *Graphis*, e.g. Climo 1975, Haszprunar 1985b, Ponder 1990a,b, 1998; Fig. 5A-C), triangular and flattened or rather stout (murchisonellids *Ebala* and *Murchisonella*, e.g. Rasmussen 1944), or forming short rounded lobes or stubs (Omalogyridae; Ponder et al. 1998). Tentacles are rounded and head-shield like in the murchisonellids *Henrya* Bartsch, 1947 and *Koloonella* (Wise 1999, Brenzinger et al. 2014; see e.g. Fig. 3A); the sides of the mouth (no snout) also form small lobes which could be described as tentacles. Minute finger-shaped tentacles are present on the snout of Cornirostridae (Valvatoidea) and *Tjaernoeia* (Ponder 1990a, Warén 1991a, Bieler et al. 1998; Fig. 5C).

Two pairs of tentacular structures are present in *Tjaernoeia* and Euthyneura *sensu lato*. Distinct finger-shaped tentacles forming four sensory tips that are, however, fused at their bases to a more or less large degree are found in *Tjaernoeia,* Rissoellidae, and *Parvaplustrum* ("bifid" tentacles; own obs. on photo by G. Rouse/ A. Warén, Figs. 5C-D, 9B). Tentacle bases are further separated in the remaining Euthyneura. Tentacles may be flat and forming a headshield. Headshields are described for a number of taxa and are variable, especially with respect to their posterior margin and expression of their corners (Gosliner 1992, Rudman & Willan 1998, Mikkelsen 2002). Headshields may have rather large and flaring, lobe-like corners, sometimes forming a third pair of posterior

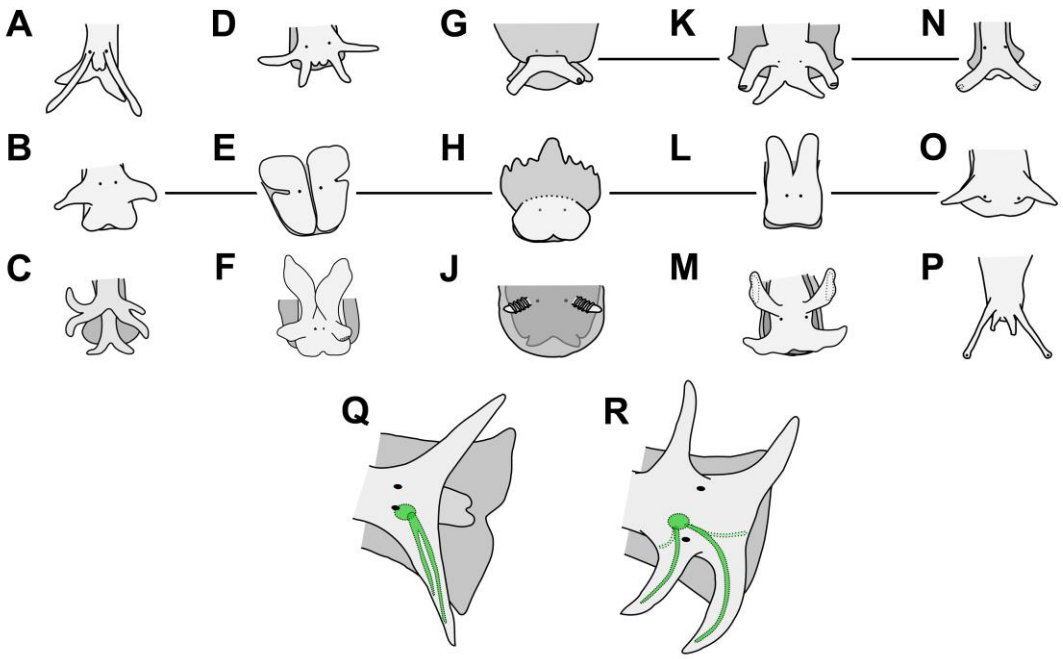

Figure 5. Examples of morphotypes described in the text: head morphologies and configuration of tentacle nerves.

A-P. Head morphotypes. All dorsal to anteriodorsal views, anterior below. Line connecting top row: types with elongate or enrolled tentacles; line connecting middle row: types with headshield-like, flattened tentacles. T-U. Schematized dorsal views of head tentacles and their innervation by cerebral nerves; schematized dorsal right views.

A-C. "Lower" heterobranchs: *Cima*, *Koloonella, Tjaernoeia* (after Warén 1991a, 1993, 2013). **D-F.** Acteonacea: *Rissoella* (after Ponder & Yoo 1977), *Acteon* (after Staubach 2008), *Micromelo* Pilsbry, 1895 (after Rudman & Willan 1998). **G-J.** Ringipleura: *Berthella* (after Wägele & Willan 2000), *Ringicula* (after Paper 10), *Corambe* (after Paper 6). **K-M.** Euopisthobranchia: *Tylodina* (after Rudman & Willan 1998), *Haminoea* Turton & Kingston, 1830, *Aplysia* (after Staubach 2008). **N-P.** Panpulmonata: *Elysia*, *Lymnaea* Lamarck, 1899, *Helix* Linnaeus, 1758. **Q.** Schematized head and tentacles of a lower heterobranch, with cerebral ganglion and bifid tentacle nerve (in green). **R.** Schematized head and tentacles of a euthyneuran (*Strubellia*, after Brenzinger et al. 2011a and Staubach 2008), with simple nerves in each tentacle and each nerve bearing a basal branch (to lip and area of Hancock's organ).

♦

processes covering the shell (in acteonoidean Hydatinidae; Fig. 5F; Rudman 1972c,d, Rudman & Willan 1998), or rounded anteriorcorners and a posterior frill covering the shell, sometimes forming a siphon (in Ringiculidae; Fretter 1960, Minichev 1967, Kano et al. in rev.; Fig. 5H). The headshields may have a rounded anterior margin and more or less pronounced, sometimes medially fused posterior corners (in many Cephalaspidea; e.g. Haminoeidae Pilsbry, 1895, Bullidae, Philinidae *s.l.*, Scaphandridae G. O. Sars, 1878, Gastropteridae; Gosliner 1994; Fig. 5L), or have short and slightly enrolled anterior corners and a posterior margin confluent with the neck (most Runcinidae Adams & Adams, 1854, Akeridae; Morton & Holme 1955, Bielecki et al. 2011; Fig. 3C), or may be an elongate wedge-shaped structure with median groove (sacoglossan *Cylindrobulla* P. Fischer, 1857 and *Ascobulla* Ev. Marcus, 1972*;* Jensen 1996*,* Laetz et al. 2014). The head of lower heterobranch

Koloonella (with posterior and anterior lobes; see Figs. 3A, 5B, and above) could be described as headshield-like as well.

In other cases the two pairs often morphologically distinct from each other, the anterior tentacles often being short and wide, the posterior ones longer and narrow.

The posterior tentacles (rhinophores) may be finger-shaped (Nudibranchia, some Gymnosomata, some shell-less Sacoglossa, Glacidorbidae, Acochlidia, some Hygrophila, and Eupulmonata – the latter partially bearing the eyes on the tips of the tentacles) or triangular, rather flattened lobes (some Sacoglossa – *Volvatella* Pease, 1860, most Pyramidelloidea, Hygrophila). In many cases the rhinophores have a sensory groove along their outer and posterior side, giving the impression of an enrolled tube in Pleurobranchoidea, Tylodinoidea (Fig. 5G, K), some Cephalaspidea (e.g. *Colpodaspis* M. Sars, 1870; see Brown 1979), and some shelled and unshelled Sacoglossa (Fig. 5N). Others have rhinophores with a depression only near their tip (resembling an ear: some Cephalaspidea and Runcinacea, Anaspidea – Rudman & Willan 1998; many Pyramidelloidea; Ponder 1987) or they may be simply triangular and flat (as in many Hygrophila – *Physa* Draparnaud, 1801, *Lymnaea*, *Chilina* Gray, 1828*;* Fig. 5O). This is similar to forked tips found in sacoglossan *Cyerce* Bergh, 1870 and gymnosome *Hydromyles* Gistel, 1848 (Rudman & Willan 1998). Additional ridges, sheaths or papillae enlarging the sensory epithelium of finger-shaped tentacles are present in many Nudibranchia (Wägele & Willan 2000; Fig. 5J). Pteropod *Limacina* possess a single, right rhinophores only (P. Kohnert, Munich - pers. comm.). Rhinophores are reduced to short stubs or minute bumps in sacoglossans *Limapontia* Johnston, 1836 and Platyhedylidae (Kohnert et al. 2013; Fig. 2C), in Siphonariidae, Amphibolidae, Trimusculidae, and acochlidian *Aiteng* (all intertidal to amphibious taxa; Morton 1955, Yonge 1957, Golding et al. 2007, Neusser et al. 2011b). Sensory structures associated with the rhinophores are the unpaired so-called “caruncle” of nudibranch *Janolus* Bergh, 1884 (medially between the rhinophores), the median “siphon” of some gastropterid cephalaspideans (*Siphopteron* Gosliner, 1989) and the paired so-called Hancock’s organs (frilly lateral protuberances described for Acteonoidea, and some Cephalaspidea; ciliated pits described for Tylodinoidea, Runcinacea, Anaspidea, and Acochlidia) (e.g. Gosliner 1994).

The anterior tentacles (oral or labial tentacles) are fused medially and form a more or less pronounced upper lip or velum above the mouth. This may result in a wide, triangular velum (Pleurobranchoidea, some cladobranch Nudibranchia, Tylodinoidea, Aplysiidae; Fig. 5G,K,M) or motile, flattened tentacles (“palps”) in some Siphonariidae, Systellommatophora and ellobioid *Trimusculus* F. C. Schmidt, 1818 and *Otina* (all air-breathing animals; Morton 1955, Yonge 1957). The tips of the labial tentacles may be long and finger-shaped (nudibranch Cladobranchia, Acochlidia especially Hedylopsacea, and the majority of Eupulmonata; Figs. 3D, 5P) or short and finger-shaped (nudibranch Anthobranchia; Fig. 5J); a relatively simple, bilobed and flat upper lip is present in some Sacoglossa (Fig. 5N), Glacidorbidae, Hygrophila (Fig. 5O), and most Stylommatophora. In Pyramidellidae, the upper lip is broad and triangular, with a dorsal median groove (called mentum, e.g. Ponder 1987, Wise 1996). Sensory structures associated with the labial tentacles are the so-called “lip” organ of Acteonoidea and Cephalaspidea (Staubach et al. 2008) and the pads bearing sensory cilia in aglajid Cephalaspidea (Gosliner 1994) (see discussion, Table 2).

Sensory structures of Heterobranchia are innervated by paired nerves of the cerebral ganglia. These innervate the mouth region, the tentacles and associated sensory areas, the sides of the head or neck, and the eyes and statocysts. Especially the configuration and number of the tentacle nerves has

been regarded as highly relevant for phylogenetics (Leyon 1947, Huber 1993, Staubach 2008), and therefore will be discussed here. Currently, data published on the cerebral nerves could be categorized as 1) studies showing the course of whole nerves *in situ*, including the target areas (e.g. by *in situ* nerve staining, 3D reconstruction of complete specimens; e.g. Wollesen et al. 2007, Klussmann-Kolb et al. 2013; papers presented herein; also van Mol 1967), 2) studies of isolated nervous systems derived from dissection (showing nerves, but not targets) (e.g. Rudman 1972b,c, Gosliner 1994), and 3) studies showing ganglia but only severed nerves (e.g. Huber 1993). In the following I will focus on studies of the first category as they make correlation of individual nerves easiest.

Tentacle nerve configuration of several lower heterobranch taxa still remains unknown (Mathildidae, *Cima*, *Graphis*, most Murchisonellidae). A single, paired and simple nerve innervating the pair of tentacles are described for Omalogyridae (Huber 1993, Bäumler et al. 2008). A single paired nerve that is bifid, splitting early into equally thick branches of which both run along the length of the tentacle is known for Caenogastropoda (Ponder 1991) and was described for several flat-spired lower heterobranchs, including Architectonicidae (Haszprunar 1985b, Huber 1993), Valvatoidea (Ponder 1990a, Bieler et al. 1998, Haszprunar et al. 2011, Hawe et al. 2013), and Orbitestellidae (Hawe & Haszprunar 2014) (see Fig. 5Q). It is unclear whether the single pairs of nerves mentioned for *Rissoella* by Huber (1993) are bifid or not; the same author described separate tentacle nerves (unclear if bifurcated, but with basal ganglion) and a nerve of the mentum (*i.e.,* the lateral corners of the snout) for the murchisonellid *Ebala*, Huber (1993: p. 338). There may be one or two pairs of simple nerves in Pteropoda (Huber 1993, Kubilius et al. 2014, P. Kohnert pers. obs. on *Limacina*).

Two pairs of simple nerves innervating each tip of the bifid tentacles (*i.e.*, basally separate and unbranched nerves) are found in incertae sedis *Tjaernoeia* and, possibly, *Parvaplustrum* (Brenzinger, Kano & Schrödl 2016, in prep.). In shell- and tentacle-less Rhodopemorpha, there are two pairs of thick anterior nerves with unclear homology; the posterior, dorsal one is simple (but possesses double roots), the anterior and ventral one is bifurcated in *Rhodope* or simple in *Helminthope* (Haszprunar & Huber 1990, Brenzinger et al. 2011b, 2013b, own unpubl. observations). Two pairs of nerves are present in Euthyneura *s.l.*, and each nerve has a thick branch leading to the tip of the tentacle and a thinner one innervating the area near the tentacle base (nerves N2 and N3 according to Faller et al. 2008, Staubach et al. 2008, Klussmann-Kolb et al. 2013; see Fig. 5R). This condition was described for Acteonoidea, Nudipleura (Faller et al. 2008, Staubach et al. 2008, Staubach & Klussmann-Kolb 2007), Ringiculidae (Kano et al. in rev.), and for tentacle-bearing Euopisthobranchia including Tylodinoidea (Vayssière 1883, Huber 1993), Cephalaspidea (e.g. Huber 1993, Staubach et al. 2008, and Brenzinger et al. 2013a, own unpubl. data on Gastropteridae), Runcinacea, Akeridae and Aplysiomorpha (e.g. Huber 1993, Faller et al. 2008, Wollesen et al. 2007). A comparable situation with split nerves N2 and N3, each with a thinner basal branch, was found in panpulmonate Acochlidia (e.g. Brenzinger et al. 2011b, Neusser et al. 2009). In contrast to Huber (1993: p. 207) who assumed (ancestrally) bifid nerves innervating the posterior tentacles in “archaeopulmonate” Ellobioidea, *Chilina* and other Hygrophila (but not *Siphonaria* G. B. Sowerby, 1823), the details depicted by van Mol (1967, 1972) are congruent with those described above for Euthyneura, as are the drawings of dissections e.g. of Hygrophila (Lever et al. 1965) and Ellobioidea (Haszprunar & Huber 1990, Martins 1996). This indicates that the pattern of N2 and N3 may be present within Panpulmonata as well. Exceptions are the Sacoglossa, with multiple nerves entering the tentacles (Russell 1929, Salvini-

Plawen 1991, Huber 1993, Jensen 1996); homologies of these nerves to those of other taxa are still unclear.

A ganglion is present at the base of the tentacle nerve in Architectonicidae (Haszprunar 1985b), *Rhodope* (Haszprunar & Huber 1990, Brenzinger et al. 2011b) and, usually, in Euthyneura *s.l.* This ganglion may have double roots, for example in *Rhodope* (see also Böhmig 1893) and in many panpulmonates (Fig. 7; see Neusser et al. 2006, Brenzinger et al. 2011a, 2013a,b; Kohnert et al. 2013). Full homology of this ganglion to the neurosecretory "procerebrum" of limnic and terrestrial panpulmonates, a glandular structure located on top of the cerebral ganglia (e.g. van Mol 1967, 1973, Ruthensteiner 1999, Chase 2000 – see Fig. 7: columns at right) was suggested but remains contentious (see below).

Additional, so-called "accessory" ganglia are found on the cerebral nerves of all meiofaunal "microslugs" (see Haszprunar & Huber 1990, Salvini-Plawen 1991, Huber 1993, Rückert et al. 2006, Brenzinger et al. 2011b, 2013a,b, Jörger et al. 2008, 2014c); these are characterized by a typical histology (see below).

Ganglia posterior to the cerebral nerve ring are found on the so-called visceral connective or "loop" below the digestive tract. The visceral loop is highly variable in configuration between taxa, bearing between two and six ganglia on either a short and somewhat twisted loop, a long and untwisted one, or a short and untwisted one (see. Haszprunar 1988: fig. 3). In the majority of lower heterobranchs this loop is twisted and oblique, more or less "torted" (="streptoneurous"), and bears two ganglia (e.g. Haszprunar 1985a, Bieler et al. 1998, Hawe et al. 2013, 2014). In rhodopids it bears either one ganglion in *Rhodope*; (Haszprunar & Huber 1990, Brenzinger et al. 2011b) or five in *Helminthope* (Salvini-Plawen 1991, Brenzinger et al. 2013b). In Euthyneura (syn. Pentaganglionata Haszprunar, 1985a), it was hypothesized to possess five ganglia, at least during ontogeny. Long loops with several ganglia are found mainly in taxa bearing "bubble" shells (see Guiart 1901). Rather distinctly, Nudipleura (e.g. Hoffmann 1939, Wägele 1989, Wägele & Willan 1994) and Ringiculidae (Kano et al. in rev.) were shown to possess untorted loops that lack ganglia in the majority of cases; the progenetic nudibranch *Corambe* is an exception (Martynov et al. 2011; Fig. 2F).

The osphradium is a sensory epithelial organ innervated by the supraintestinal ganglion on the visceral loop and is located towards the anterior margin of the mantle cavity (e.g. Mikkelsen 1996 p. 384); in taxa with a shallow mantle cavity both the ganglion and the osphradium may be shifted in their position, often to the posterior right. The osphradium has not been focus of studies since Haszprunar's work in the 1980's (e.g. 1985d). Work during this thesis discovered a small osphradium in limnic Acochlidiidae Küthe, 1935 (Brenzinger et al. 2011a) and Philinoglossidae (Brenzinger et al. 2013a), indicating that the organ may be small but not necessarily lost in small-bodied taxa.

4.2 Synapomorphies: the "New Heterobranch Tree" revisited (Figs. 6 and 7)

Paradigms of euthyneuran phylogeny have changed radically in the last ten years, and efforts were undertaken to create larger datasets of the entire clade and of subgroups (e.g. Wägele et al. 2008, Malaquias et al. 2009, Dinapoli & Klussmann-Kolb 2010, Dayrat et al. 2011, Jörger et al. 2010, 2014b). At the same time, views on the relationships of "lower" heterobranch taxa still oscillated between the extremes of Allogastropoda (originally defined as "all non-euthyneuran Heterobranchia are

monophyletic"; Haszprunar 1985a) and "step-by-step evolution" of paraphyletic "lower" taxa towards Euthyneura at the "pinnacle" (Haszprunar 1988: see Fig. 1A). Since then lower heterobranchs were not attempted to classify into a (low) number of larger monophyletic taxa, but were instead treated and displayed as a polytomy (e.g. Brenzinger et al. 2013b, Jörger et al. 2014a, Wägele et al. 2014). Also, so far no attempt was undertaken to delineate and interrelate major lower Heterobranchia taxa using modern methods. Both have been hampered by the rarity and minuteness of many taxa which made it difficult to sample well-preserved material in sufficient amount.

Except for the comparatively speciose Architectonicidae (Bieler 1988), no detailed attempt had yet been undertaken of a (morphology-based) phylogenetic analysis of lower Heterobranchia as a whole or of subgroups, because taxon sampling of many other lineages is still too low: Haszprunar et al. (2011) estimated the sampling of Valvatoidea - with regards to anatomy - at "less than 10%". Other lineages are known from the studies on single species only (e.g. Mathildidae: Climo 1975, Haszprunar 1985c) or remain to be examined in more than cursory anatomical detail at all (e.g. Graphididae, Cimidae, *Murchisonella*, Tjaernoeiidae, *Parvaplustrum*). Consequently, knowledge on comparative morphology of lower heterobranchs still presented an "impenetrable mosaic" (Ponder 1991; Ponder & Lindberg 1997; A Hawe, Munich - pers comm.), and approaches to lower heterobranch phylogeny using morphology remained even more limited in taxon sampling as was the molecular tree by Dinapoli and Klussmann-Kolb (2010).

Furthermore, molecular sequences of many lineages were entirely lacking before 2015 (Mathildidae; first sequences by Y. Kano, Tokyo and own data for Ringiculidae, Tjaernoeiidae, *Parvaplustrum*) or were represented by single specimens only. Nevertheless, new perspectives are beginning to emerge on taxa representing distinct terminal branches.

Aim of this section is twofold: first, to present a new topology (Figs. 6A, 7A and 8), expanding the tree by Dinapoli and Klussmann-Kolb (2010; see Fig. 1B) by more recent data (molecular/unpublished) and literature review; second, to provide a matrix of selected morphological and ecological features for the respective clades. The latter matrices will be used for outgroup comparison in order to discern whether presence or absence of features is plesiomorphic or apomorphic. The herein presented backbone topology (Fig. 6) is based on published and preliminary, still unpublished

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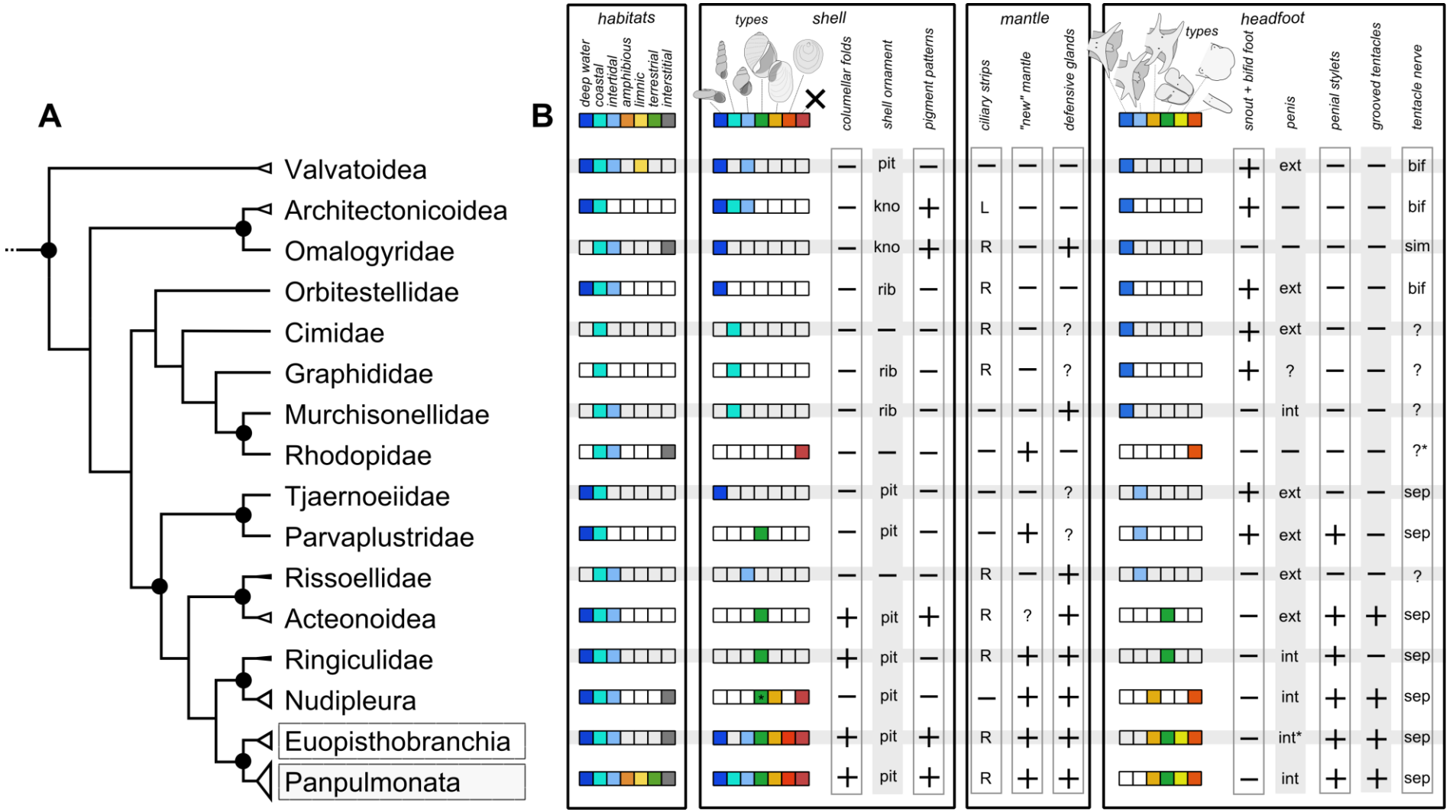
Figure 6 (following page). Backbone topology for Heterobranchia, with focus on "lower" clades, and comparative morphology.

A. Proposed new topology for Heterobranchia. Black circles: robustly retrieved nodes in molecular studies (pp > 0.98, bs > 0.95; after: Dinapoli et al. 2010, Jörger et al. 2010, Wilson et al. 2010) and unpublished studies (Kano et al. in prep.). Terminals indicate approximate species diversity (according to marinespecies.org, Wade & Mordan 2008); simple line: less than 50 described species, black triangle: 50+ species, white triangles: 100+/1000+/10000+ species, respectively. **B.** Plots of selected ecological and morphological characters discussed in this thesis. See text for details; coding after various references (see Tab. 1). Abbreviations: +, structure assumed plesiomorphically present; -, structure assumed plesiomorphically absent; ?, structure not revised. Shell ornament: pit, if present consisting of minute pits; kno, if present consisting of strong knobs and ribs; rib, consisting of fine ribs and striae. Penis internal/external: ext, external, non-retractile; int, internal, retractile. Tentacle nerve: bif, bifid; sim, simple, sep, separate (and simple). Ciliary strips: L, left; R, right. Asterisks: shell of pleurobranch *Tomthompsonia* coded as bubble-shell here; penis of Euopisthobranchia internal except for Tylodinoidea (external); tentacle nerve of *Rhodope*: examined yet homology unclear.


A
Valvatoidea
Architectonicoidea
Omalogyridae
Orbitestellidae
Cimidae
Graphididae
Murchisonellidae
Rhodopidae
Tjaernoeiidae
Parvaplustridae
Rissoellidae
Acteonoidea
Ringiculidae
Nudipleura
Euopisthobranchia
Panpulmonata
B
habitats
deep water
coastal
intertidal
amphibious
limnic
terrestrial
interstitial
types
shell
columellar folds
shell ornament
pigment patterns
mantle
ciliary strips
"new" mantle
defensive glands
types
headfoot
snout + bifid foot
penis
penial stylets
grooved tentacles
tentacle nerve
pit kno kno rib rib pit pit rib
L R R R R R R R R R R R
ext ext ext ? int ext ext ext ext int int int* int
bif bif sim bif ? ? ? ?* sep sep ? sep sep sep sep sep

Bayesian and Maximum Likelihood analyses of "standard markers" (partial sequences of the CO1, H3, 16S, 18S and 28S genes; see below for references) and phylogenomics (Zapata et al. 2014). For the lower clades (Fig. 6) this comprises preliminary 28S data that show distinctness of 1) Valvatoidea and Architectonicoidea, 2) close relationships between several other families (see below), 3) close relationship of *Tjaernoeia* + *Parvaplustrum*, and 4) and robust affinity of the latter two genera to Euthyneura *s.l*, (Y. Kano unpubl.; Brenzinger, Kano & Schrödl 2015, 2016, in prep.). The topology of Euthyneura (Fig. 7) is based essentially on published studies by Dinapoli & Klussmann-Kolb (2010) and Jörger et al. (2010, 2014b) and incorporated into the new tree; this does present the main scope of this thesis and will hence be discussed in less detail in many cases. The resulting new topology for Heterobranchia will be critically reviewed, and implications for the reconstruction of heterobranch phylogeny will be discussed in the next chapter.

The new tree hypothesis of Heterobranchia (Fig. 6) contains the following distinct lineages, sorted by order of branching: 1) Valvatoidea (Ectobranchia) as the most basal clade, followed by 2) Architectonicoidea + Omalogyridae (=Architectonicoidea *s.l.* herein), 3) a clade of the non-valvatoid small-bodied taxa, this then followed by 4) *Tjaernoeia* and *Parvaplustrum* which form a clade that is sister to 5) Euthyneura *s.l.* Therein, there are three major clades: Acteonacea, Ringipleura, and Tectipleura; topology of these three remains unresolved, but a particular version is preferred here. Tectipleura (Fig. 7) consist of Euopisthobranchia and Panpulmonata, again reflecting radical changes compared to traditional taxon concepts.

1.) Valvatoidea (= Ectobranchia) is a distinct clade on the conservative 28S gene, and contains one freshwater and three marine families of flat-spired snails with a protrusible gill and several plesiomorphies not found in the remaining heterobranchs (gill-like organ present but not supported by ciliary strips, rhipidoglossate radulae in some).
2.) Architectonicoidea *s.l.* contains two families of snails which parasitize Cnidaria and one of minuscule snails found mainly in the sub- and intertidal; both families have ornamented and pigmented shells.
3.) The next, hitherto unnamed clade contains four families of minute snails and one family of aberrant slugs, and is indicated by independent yet partially preliminary analyses of the 28S gene which independently indicated several sister group relationships: Murchisonellidae and Rhodopidae (Wilson et al. 2010), Graphididae and Murchisonellidae (Dinapoli & Klussmann-Kolb 2010), Orbitestellidae, Cimidae and Graphididae (Kano, unpubl.), and Cimidae and Orbitestellidae closer to Euthyneura than to Valvatoidea and Architectonicoidea *s.l.* (Dinapoli & Klussmann-Kolb 2010). This clade includes minute snails taxa of low-spired taxon (Orbitestellidae) at the base, and a monophylum uniting all minute lower heterobranchs with high-spired shells and Rhodopemorpha slugs (Micracicula tax. nov.; see next section).
4.) *Tjaernoeia* and *Parvaplustrum* form a distinct lineage Parvaplustra tax. nov. and are sister to Euthyneura *s.l.,* together forming Physotesta tax. nov. Parvaplustra is characterized by plesiomorphies (split foot, snout, small body size, largely valvatiform shell in *Tjaernoeia*) and derived characters appearing intermediate to Euthyneura and the other lower heterobranch snails: split tentacle nerves and bifid tentacles, inflated body whorl and punctate ornament. The sistergroup relationship to Euthyneura is supported by preliminary analysis of several markers (Kano, Brenzinger & Schrödl, 2015, 2016, in prep.) and anatomical synapomorphies, namely the shell (with pitted ornament, and rapidly expanding body whorl and large aperture: bubble shell morphotype) and the

occurrence of split cerebral nerves N2 and N3, parallel with the specialization of two pairs of tentacles.

5.) The next clade contains a tritomy of robustly supported lineages of Euthyneura *s.l.* (following the redefinition of the taxon by Zapata et al. 2014); Kano et al. (in rev.) show conflicting topologies between analyses, but the following topology shows higher support and is thus favored here: Acteonacea as the first lineage containing morphologically disparate Acteonoidea and Rissoellidae (supported by molecular data but not yet characterized as a morphological clade), and the following two clades as sister taxa.

6.) Ringipleura tax. nov. is a superordinal taxon of disparate Ringiculidae snails and Nudipleura semislugs and slugs that is recovered from genetics and characterized by several potential synapomorphies of the soft body (Kano et al. in rev.; Brenzinger, Kano & Schrödl in prep.).

7.) Tectipleura is a large taxon containing the majority of heterobranchs with two lineages: marine snails and slugs (Euopisthobranchia) and mostly coastal to freshwater and terrestrial snails and slugs (Panpulmonata) (Fig. 7). Euopisthobranchia contain 7 lineages with rather well resolved branching pattern; a new clade of parapodiate taxa is tentatively suggested here, and one of swimming taxa. Interrelationships of the 10 panpulmonate lineages are less clear; a clade of taxa still bearing an operculum is suggested here but remains unnamed.

Disregarding the high degree of homoplasy which would be expected from a taxon this old, local patterns of morphology support the signal on the 28S gene: 1), the genetic distinctness of Valvatoidea and Architectonicoidea *s.l.* is coupled with morphological distinctness as a whole, consistent with long evolutionary trajectories, 2), presence of a highspired clade together with Rhodopemorpha can be associated with characters of morphology (similarities in shell, long body, lifestyle), 3), the position of *Tjaernoeia*/*Parvaplustrum* and *Ringicula* as sisters of larger clades (Euthyneura *s.l.* and Nudipleura, respectively) can be supported by morphological synapomorphies and intermediate characters, anchoring the larger taxon more firmly with the outgroups, and 4) the same can be said for hitherto unmapped patterns of morphology with respect to higher taxa within Euopisthobranchia and Panpulmonata. Furthermore, the similarities in headfoot morphology can be explained as a symplesiomorphy of all Heterobranchia.

Problems with this proposed topology arise with regards to the rather derived position of Murchisonellidae and *Graphis* and the old age of their fossils (e.g. Bandel 1996, 2005), prompting earlier authors to hypothesize them to be among the oldest of Heterobranchia and to compare them to other, more popular “living fossils” like the shelled cephalopod *Nautilus* Linnaeus, 1758 (Warén 2013). However, the presence of old fossils may be explained by the (presumably infaunal) lifestyle of the highspired taxa and higher stability of the narrow shells, which could explain higher rates of fossilization in contrast to epibenthic, discoidal-shelled groups with fragile shells. No morphological synapomorphy could be identified that unites Orbitestellidae and the highspired clade, and molecular support for a close relationship of the highspireds to rhodopids and murchisonellids is also weak; the latter two diverge from the others in head and CNS morphology and their burrowing habit, characters which are rather more similar to Euthyneura. Furthermore, there are now indications that some of the molecular sequences analyzed by Dinapoli and Klussmann-Kolb (2010) and which were

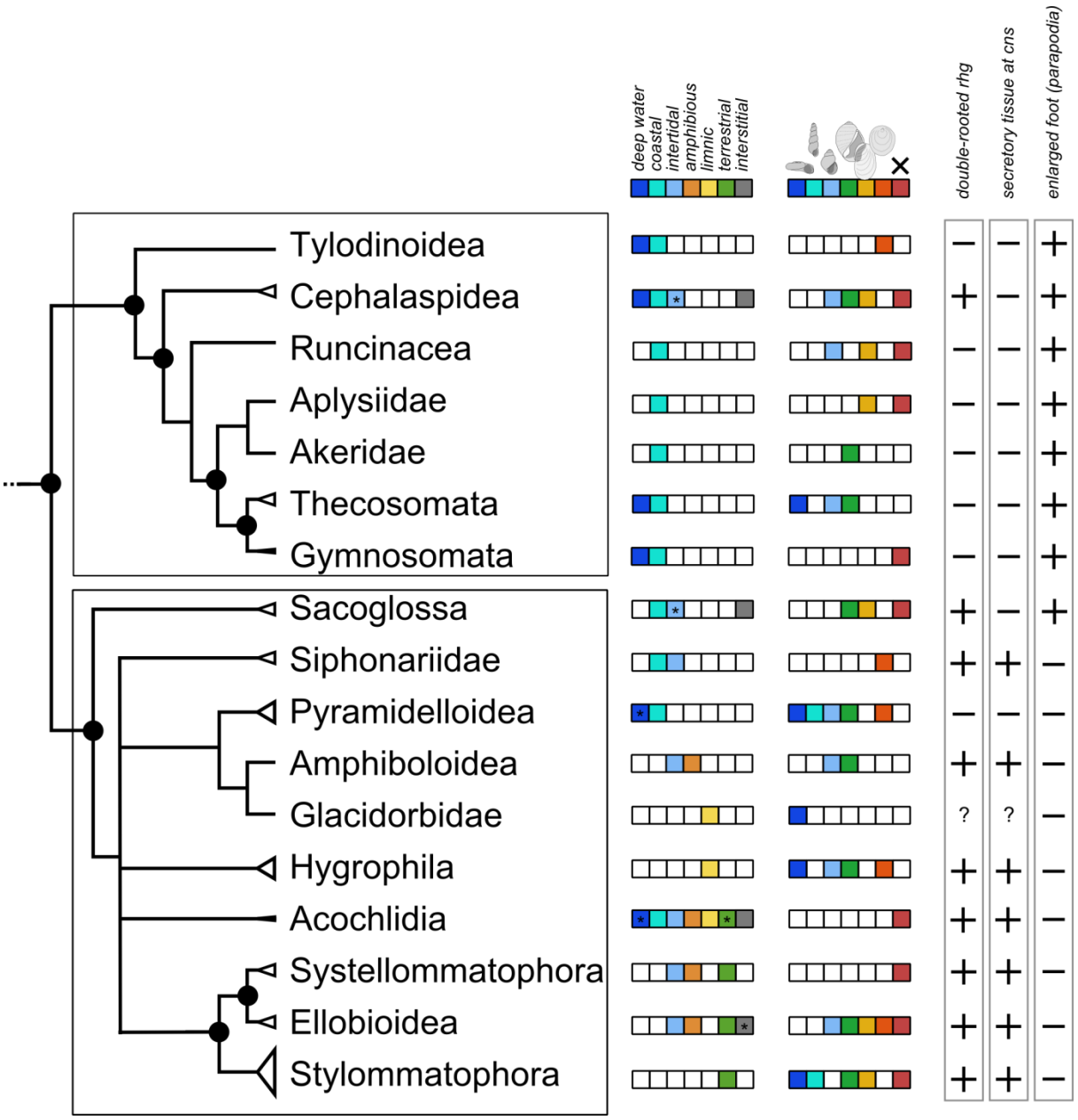


Figure 7. Backbone topology for Tectipleura, and comparison of ecology (habitat), shell morphology, and selected soft-body characters.

Topology after Schrödl et al. 2011a, coding after various references (see Tab. 1). Plots refer to presumed plesiomorphic state. Coding: +, character present; -, character not present; ?, character not revised or unknown. Asterisks: see text for details.

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reused by later authors are in fact contaminated (Heneberg 2013 on 18S sequences of Göbbeler & Klussmann-Kolb 2010b using similar methodology and, likely, protocols; Y. Kano, Tokyo – pers. comm.:18S of highspired taxa in Dinapoli & Klussmann-Kolb 2010). This highlights the need of careful reexamination of datasets incorporating sequences from Genbank. In the meantime the new tree

herein (Figs. 6, 7) can be used as a scaffold to evaluate previous hypotheses on the evolution of taxa, for example whether or not data on sperm morphology (e.g. Healy 1990, 1993, 1996, Hodgson & Healy 1998) make sense with respect to new topologies.

This new tree implies a classification fundamentally different from previous ones, especially with regard to lower heterobranchs (e.g. Haszprunar 1985a, 1988, Ponder & Lindberg 1997, Bouchet & Rocroi 2005). Therefore, an renewed preliminary classification better reflecting current advances in the definition of Euthyneura and other heterobranch subgroups will be attempted in the following.

4.3 Classification of Heterobranchia (Fig. 8)

Giving a new classification is important: it provides a testable hypothetical framework, being a guiding concept for future studies (e.g. de Queiroz & Gauthier 1992). Furthermore, it makes taxa visible: without a name, a taxon may remain invisible to the scientific community at large (e.g. Bouchet & Rocroi 2005). Zoological nomenclatory rules apply only to family level and below; herein it was attempted to find commonly used names to ordinal levels and above.

The herein revised, molecular-based topology of the "New Heterobranch Tree" is reinforced with new sets of potential synapomorphies as inferred by outgroup comparison (Figs. 4 & 5, and Tab. 1 for references). A non-comprehensive list of characters is provided, leading to the following proposed reclassification. Sources are given for molecular or morphological studies showing support for particular nodes. Numbers in () at left refer to numbered nodes in Fig. 8. Etymology in {} is given for herein proposed new taxa (in bold); these new names will be discussed below and published in a separate paper. Daughter taxa are given in [] at the end of each section.

(1) HETEROBRANCHIA Gray, 1840: Hyperstrophic protoconch, discoidal to globular teleoconch with gradual increase of whorl diameter; snout, bifurcated foot, tentacle at right pallial edge; digestive system simplified: radular cartilages and esophageal pouches lost, paired buccal retractors, taenioglossate radula?; original gastropod ctenidium lost, pallial kidney and heart; penis external, spiral sperm, simultaneous hermaphroditism with ovotestis, loss of paraspermatozoa; progenesis - annual life cycle? Molecular support by standard markers, phylogenomics, special arrangement of mitochondrial genes (e.g. Jörger et al. 2010, Kocot et al. 2014; Zapata et al. 2014; Schrödl & Stöger 2014) [Valvatoidea + node 2]

(--) VALVATOIDEA Gray, 1840 (= ECTOBRANCHIA Golikov & Starobogatov, 1975): Specialized ectobranch gill, paired pallial tentacles, some with rhipidglossate radula, sperm characters? [5 families]

(2) "CILIOTRACTA" Haszprunar, 1988 nov.: Planispiral shell with ornament consisting of knobs or intercrossing lamellae; opposed ciliary strips present in mantle cavity, branched gill lacking, Blochmann-like glands present; posterior pedal gland present?; early development of 4d-mesentoblast? (Riedl 1960, van den Biggelaar & Haszprunar 1996) [Architectonicoidea *s.l.* + node 4]

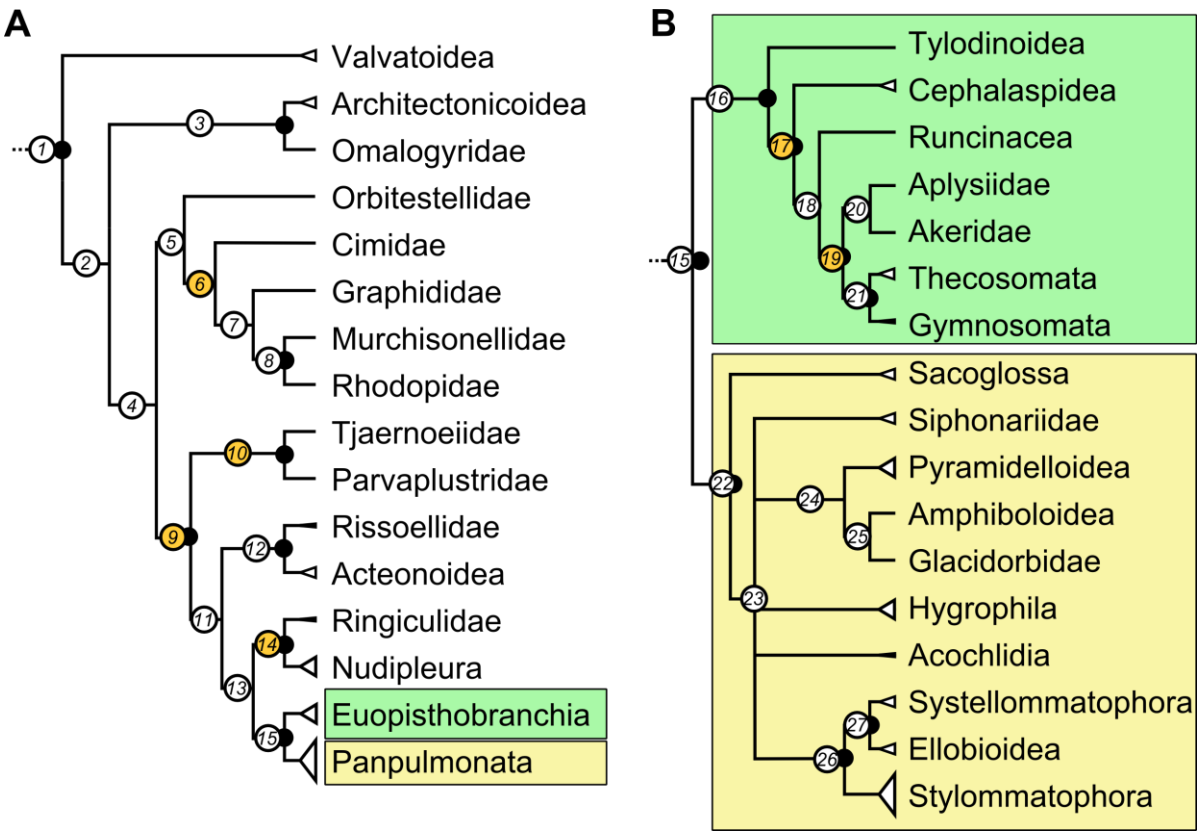

Figure 8. Classification of Heterobranchia. Topologies as in Figs. 7 and 8, numbers on nodes refer to proposed synapomorphies (see Table A). Orange circles highlight clades named in this thesis. Nodes with black dots are recovered in molecular analyses.

A. Topology of revised "New Heterobranchia tree". Topology as in 4A, after various sources (Jörger et al. 2010, Schrödl et al. 2011a, Brenzinger et al. 2013b, and unpublished results of colleagues). Node 6: Micracicula nov., node 9: Physotesta nov., node 10: Parvaplustra nov., node 14: Ringipleura nov. Node 11: Euthyneura sensu lato (Zapata et al. 2014). **B**. Subtree of Tectipleura, green box: Euopisthobranchia, yellow box: Panpulmonata. Node 17: parapodiate clade nov., node 19: swimming clade nov.

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(3) ARCHITECTONICOIDEA *s.l.*: Shell with strong brown to orange pigment; pallial tentacle lost; jaws lost?; penis lost. Position of ciliary strips only at left refuted by Bäumler et al. 2008). Sperm characters (Healy 1988). Molecular support (e.g. Dinapoli et al. 2010, Jörger et al. 2010) [Architectonicoidea s.s. (2 families) + Omalogyridae]

(4) "DEXTROTRACTA" Haszprunar, 1988 nov.: Ciliary tracts at right side of mantle cavity, mantle glands diverse [nodes 5 and 9]

(5) UNNAMED CLADE: Gill lost, monauly?, jaw specialized?; retrieved on 28S gene (Kano, unpubl.) [Orbitestellidae + node 6]

(6) **MICRACICULA** nov.: Highspired shell, ornament of fine intercrossing lines or lost, narrow radula with reduced central tooth, red glands in mantle roof?, sec. diauly?, preliminary 28S gene data (Kano unpubl.), to be confirmed {Etymology: contraction of *mikró*= small; *aciculum* = little needle. Refers to

the small body sizes and needle shaped shell, or internal spicules in Rhodopemorpha.} [Cimidae + node 7]

(7) PARASITIC CLADE nov.: Radula very small, food uptake by sucking?, ectoparasitism, stomach simple? [Graphididae + node 8]

(8) **MURCHISONELLOIDEA + RHODOPEMORPHA** nov.: Head modified: snout lost, foot not bifid, pharynx reduced, esophagus vacuolated, pump-suckers; infaunal habitat, shallow water?; penis internal or lost, distinct pair of mantle glands at right; molecular support by standard markers (Wilson et al. 2010, Jörger et al. 2016) [Rhodopidae/Rhodopemorpha + Murchisonellidae]

(9) **PHYSOTESTA** nov.: Shell aperture elongate-oval in outline (narrowing posteriorly), large, inflated body whorl (bubble shell), teleoconch ornament/ribbing lost, ornament of minute pits present; sensory head tentacles split in two, N2 + N3 basally split; plicate gill (a longitudinal sheet), mantle reflected over shell margin; ?switch to soft bottoms, cuticular spines and stylets on copulatory organ. Molecular support by 28S gene (Kano et al. in prep.; Brenzinger et al. 2016) {Etymology: *physis* = bubble, *testa* = shell. Refers to the presence of a so-called bubble shell in all major lineages} [Parvaplustra nov. + Euthyneura *s.l.*]

(10) **PARVAPLUSTRA** nov.: bifid tentacles large and flattened, foot end long and narrow, operculum lost, eyes lost, ciliary strips lost?; narrow radula with thin, leafshaped teeth (Brenzinger et al. 2015, 2016); molecular support on 28S gene (Kano et al., in prep.) {Etymology: after *Parvaplustrum* Powell, 1951 = "small *Aplustrum*", small bubble shells. Resembling small specimens of a genus of Acteonoidea.} [**Parvaplustridae fam. nov.** + Tjaernoeiidae]

(11) EUTHYNEURA Spengel, 1881 *s.l. = sensu* Zapata et al., 2014 = nov.: Bubble shells with regular pitting in spiral cords, columellar folds, periostracal zig-zag-patterns of brown; size increase (many reversals); headshield, snout lost; anterior foot margin without notch; new mantle, long neck (or node 13?), pallial tentacle reduced, compound Blochmann's glands?, gill reinstated (in large forms); radula wide (in adults of large forms); N2 and N3 with additional branches innervating lip and Hancock's organs, ?grooved posterior tentacles, giant neurons (in macroscopic members), long visceral loop in bubble-shelled taxa, possibly pentaganglionate condition, euthyneury convergent in many, but not all, subtaxa; soft bottoms in adults, temporarily infaunal. Recovered by standard markers (e.g. Dinapoli & Klussmann-Kolb 2010) and phylogenomics (Zapata et al. 2014). The latter authors (p. 7) advocated redefinition of the taxon Euthyneura as including (non-euthyneurous) Acteonoidea+Rissoelloidea to "maintain stability" [Acteonacea + Euthyneura *s.s.*]

(12) ACTEONACEA Schrödl, 2014: Upper lip with notch, tentacles somewhat bifid; change in receptacles? Molecular support (Dinapoli & Klussmann-Kolb 2010, Göbbeler & Klussmann-Kolb 2010b, Zapata et al. 2014) [Rissoellidae + Acteonoidea]

(13) EUTHYNEURA Spengel, 1881 *s.s.* = Pentaganglionata Haszprunar, 1985a: Two distinct pairs of head tentacles, presence of enrolled rhinophores, upper lip forming velum (in Doridacea also with notch); euthyneury (several reversals in subgroups), pentaganglionate CNS at least during ontogeny?; "new" mantle (fused to head); major evolutionary step proposed: mantle floor with defensive glands fused to head forming neck, grows over shell, shell smaller, slugs; penis retractile/internal; etc. Molecular support by standard markers (e.g. Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010) but in contrast to Göbbeler & Klussmann-Kolb (2010b) and unresolved by phylogenomics (Zapata et al. 2014) [Ringipleura nov. + Tectipleura]

(14) **RINGIPLEURA** Kano et al., manuscript in review: Mantle margin overgrows shell; visceral loop a naked connective ("Nudiringa"); complex renopericardioduct (syrinx); molecular support by standard

markers (Kano et al. in rev.) {Etymology: contraction name of the daughter taxa, as opposite to sister taxon Tectipleura} [Ringiculidae + Nudipleura]

(15) TECTIPLEURA Schrödl et al., 2011a: Shell reductions. Pallial tentacle lost (or node 13?). Loss of cuticular shield in stomach? Monauly. Euphotic intertidal – Algivorous!? Molecular support (Jörger et al. 2001, Zapata et al. 2014) [Euopisthobranchia + Panpulmonata]

(16) EUOPISTHOBRANCHIA Jörger et al., 2010: esophagus with cuticular teeth; foot widened and very broad; molecular support (Jörger et al. 2010, Dinapoli et al. 2010, Zapata et al. 2014) [Tylodinoidea (syn. Umbraculoidea Dall, 1889) + node 17]

(17) PARAPODIATE CLADE nov.: Foot margins enlarged laterodorsally, able to cover sections of body. Esophageal gizzard with reduced number of gizzard plates (vs. Anaspidea?). Molecular support (Zapata et al. 2014) [Cephalaspidea + node 18]

(18) UNNAMED CLADE?: Herbivory? Loss of (zig-zag) shell patterning. [Runcinacea + node 18]

(19) SWIMMING CLADE nov.: Parapodia used for swimming, as means of locomotion (not only escape). Caecum in digestive tract (secondarily lost in Aplysiidae), glands of Bohadsch? (Klussmann-Kolb 2004, Medina & Walsh 2000, Klussmann-Kolb & Dinapoli 2006). Genetics (Jörger et al. 2010, Zapata et al. 2014). [Anaspidea + Pteropoda]

(20) ANASPIDEA Fischer, 1883: Macroherbivorous? Multiple gizzard plates forming filter chamber (Mikkelsen 1996). Large body size. [Akeridae + Aplysiidae]

(21) PTEROPODA Cuvier, 1804: Head modified. Foot enlarged into paired, rounded fins; molecular support (Klussmann-Kolb & Dinapoli 2006, Zapata et al. 2014). Progenesis/paedomorphosis of head and shell. Holopelagic. [Thecosomata + Gymnosomata]

(22) PANPULMONATA Jörger et al., 2010: Double-rooted N3, double-rooted procerebrum= rhinophoral ganglion? Extended vascularization of mantle roof? Coastal intertidal to brackish habitat? Food? Molecular support by "standard" markers (Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010) and phylogenomics (Kocot et al. 2013, Zapata et al. 2014). [Sacoglossa + node 23]

(23) UNNAMED CLADE/ PULMONATA Cuvier in Blainville, 1814 *s.l.*?: Neurosecretory cerebral gland and dorsal bodies? Procerebrum with globineurons? Complex kidney? Plicatidium-type gill lost. Two-part copulatory organs? Heterochrony (shifts to progenetic and peramorphic clades). Brackish-coastal habitat/Intertidal habitat? [Siphonariidae + operculate clade + Hygrophila + Acochlidia + node 26]

(24) OPERCULATE CLADE nov.: Symplesiomorphy: operculum persistent in adults. Progenesis: return to multispiral shell. Habitat: estuaries/nutrient rich brackish/coastal areas? [Pyramidelloidea + node 26]

(25) GONDWANAN CLADE nov.: Complex copulatory organ with multiple fleshy substructures. Sperm characters (Hodgson & Healy 1998). Originally estuarine? [Amphiboloidea + Glacidorbidae]

(26) EUPULMONATA *sensu* Nordsieck 1993 (*non* Haszprunar & Huber, 1990; *non* Stanisic & Smith 1998): Airbreathing with lung. Loss of gill leaflets and ciliary strips. Eyes on stalks? Molecular data (e.g. Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010, Dayrat et al. 2011) [Stylommatophora + Amphipulmonata]

(27) AMPHIPULMONATA Schrödl, 2014: No morphological synapomorphies identified herein. Molecular support (e.g. Dinapoli & Klussmann-Kolb 2010, Jörger et al. 2010, Dayrat et al. 2011). [Systellommatophora (=Onchidioidea + Veronicelloidea) + Ellobioidea]

Major changes proposed in this classification are 1), the reorganization of “lower” Heterobranchia into only three monophyletic clades; 2), the grouping of all highspired lower heterobranchs into a larger monophylum (Micracicula), together with Rhodopemorpha; 3), the recognition of a lineage of lower heterobranchs and traditional cephalaspids (Parvaplustra) as sister to remaining Euthyneura; 4), the recognition of a bubble-shelled clade (Physotesta) as crowngroup of Heterobranchia, containing a total of 5 clades containing such shells; 5), the recognition of Ringiculidae as sistergroup to Nudipleura (Ringipleura), forming one of three major clades in Euthyneura; 6), the naming of a parapodiate and a less inclusive, swimming taxon within Euopisthobranchia; and 7), the recognition of an operculate clade within Panpulmonata.

This new scheme reflect less recent changes for Euopisthobranchia that are 1) the dissociation of “Notaspidea” with exclusion of Pleurobranchoidea and grouping with speciose Nudibranchia into a separate clade, 2) the dissociation of Cephalaspidea in the traditional sense into several lineages and relocation of Acteonoidea, Ringiculidae, and headshield-bearing Sacoglossa outside of Euopisthobranchia, 3) the removal of Sacoglossa, Acochlidia and Rhodopemorpha into other lineages. This scheme again contradicts the proposal of several major clades within Euthyneura based on mitochondrial genes (Medina et al. 2011), including Placoesophagea (which is either synonymous to Euopisthobranchia or not recovered; Schrödl et al. 2011b); Siphoglossa (a clade of Siphonarioidea and Sacoglossa) and Actopleura (a clade containing Acteonoidea and Nudipleura) by the same authors were also not recovered in subsequent analyses (see Stöger & Schrödl 2013, Jörger et al. 2014b, Zapata et al. 2014). The position of Ringiculidae within Ringipleura confirms Architectibranchia to be polyphyletic (Malquias et al. 2009, Kano et al. in rev.)

Radical changes compared to preceding hypotheses (Fig. 1A) are reflected within traditional pulmonates (Jörger et al. 2010, Schrödl et al. 2011a, Schrödl 2014), leading to more inclusive Panpulmonata and more exclusive Euopisthobranchia. Among the former, the changes are: 1) opisthobranch Sacoglossa as sister to remaining clades; 2) traditional lower heterobranch (Pyramidelloidea, Glacidorbidae) and opisthobranch clades (Acochlidia) interspersed within traditional “Basommatophora” (Siphonarioidea, Hygrophila, Amphiboloidea); and 3) Ellobioidea as a more inclusive and morphologically diverse taxon (containing formerly trimusculoid limpets and smeagoloid slugs) and in a derived position, next to taxa with eyes on stalks.

4.4 Evolution of the Heterobranchia: new patterns emerging

As summarized in chapter 4.1., a wealth of anatomical observations on Heterobranchia has accumulated during studies with or without focus on comparative evolution. However, without a robust tree hypothesis as background, existing homoplasy is likely to mask patterns, e.g. by heterochrony which is likely to create pseudoarchaic morphologies (e.g. Martynov et al. 2011, and below). With a robust topology at hand, however (Figs. 6, 7), new potential patterns emerge from combining existing morphological and ecological data with the new tree as scaffold, leading to new evolutionary hypotheses. This chapter is an attempt to infuse a selection of own and reviewed literature data into the “New Heterobranch tree of life”. Following the new topology suggested in

chapter 4.2, and the creation of new taxa in chapter 4.3, I will now discuss potential evolutionary implications.

4.4.1 The Micracicula concept – minute highspired snails as stationary parasites, and ancestors of rhodopid slugs

A monophylum of minute and highspired Murchisonellidae, Graphididae and Cimidae snails together with shell-less Rhodopemorpha as shown in Figs. 6 and 8 can be hypothesized on the basis of preliminary molecular results, and may also be supported by a set of anatomical and ecological observations.

First, existing soft-body data and descriptions of radulae (e.g. Graham 1982, Warén 1993 on *Cima*; Warén 1994, 2013, Wise 1999, Brenzinger et al. 2014 on Murchisonellidae) suggest that the radula is aberrant, narrow and much shorter in comparison to the more or less taenioglossate outgroups (see Warén et al. 1993). Bandel (2005) emphasized traditional taxonomy that had previously grouped the muchisonellid *Ebala* among "aglossan" Pyramidellidae or Aclididae, together with the graphidid *Graphis* (Thiele 1931); this may indicate a reduction of full loss of the radula in Graphididae as well (also own obs. on the graphidid *Larochella toreuma* Powell, 1927). A correlation with food specialization (consistent with parasitism) may be assumed, and may be supported by the trophic relationship with stationary filter feeders indicated for *Ebala* (filter-feeding gastropod *Turritella* Lamarck, 1799; Gofas et al. 2011) and *Graphis* (colonial tubeworm *Sabellaria* Lamarck, 1818; Killeen & Light 2001). The food of *Cima*, having a peculiar asymmetric radula (Warén 1993), is not known but may be suggested to be small and asymmetric. The rhodopemorph *Rhodope* was reared on the basal metazoan *Trichoplax* Schultze, 1883 by Riedl (1959) and shown to ingest it by suction, which is consistent by morphological observations that show full loss of a pharynx and modification of an esophageal pump in all rhodopids (Brenzinger et al. 2011b, 2013b). All these observations are congruent with trophic specialization, and suction feeding, in many micraciculan taxa and may be synapomorphic.

Second, an interstitial or burrowing habit is present at least in rhodopids and murchisonellids and may be the case in *Cima* and *Graphis* was well, but this is not sure. Murchisonellidae *Ebala* and *Murchisonella* are often found abundantly in dredgings in mud of shallow lagoons and in seagrass meadows, sometimes in brackish conditions (implying a partially infaunal lifestyle; Rasmussen 1944, Bandel 2005); *Henrya* in the mud of hypersaline lagoons (Wise 1999). *Cima* is known from dredgings of coarse sand also in deep waters (Warén 1993). Rhodopemorpha live among intertidal algae or in coarse sands (Brenzinger et al. 2011b, 2013b) and thus in a similar habitat as at least the direct sistergroup, Murchisonellidae. The body is very narrow and elongate in all these taxa (see Fig. 2G-J) and may thus be a shared character; in fact, some contracted and coiled specimens of *Helminthope*-like animals do resemble decalcified specimens of the highspired taxa (own obs.), demonstrating that anatomy may not be too dissimilar after all.

The highspired shell of Micracicula is paralleled only in few, distantly related heterobranch taxa: Pyramidellidae are also ectoparasitic on a wide variety of soft-bodied invertebrates, especially mollusks and annelids; Mathildidae are parasitic on octocorals (Climo 1975). Similar highspired shells are found in some Caenogastropoda that also are infaunal or rather sedentary (Terebridae Mörch,

1852, Turritellidae Lovén, 1847, many Cerithiidae Fleming, 1822; Allmon 2011, Strong et al. 2011) or parasitic (many Eulimidae Philippi, 1853, Cerithiopsidae Adams & Adams, 1853, Triphoridae Gray, 1847, Epitoniidae Berry, 1910; e.g. Fretter 1951; Warén 1983; Albano, Sabelli & Bouchet 2012; Gittenberger & Hoeksema 2013). Cain (1977) suggested that shell form in coiled gastropods is largely correlated with their ecology, or more precisely "with their preferred way of moving and their feeding places". The functional correlation of the highspired shell in the aforementioned taxa may thus be that these animals generally move only for short distances and drag their shell through the substrate, or climb slowly on vertical surfaces. This is the case in most ectoparasitic gastropods, which do not venture far from their host and otherwise hide in the substratum. It may also be similar to many highspired Stylommatophora which often drag their shell behind through leaf litter or climb on vertical surfaces such as tree bark or rocks. The extinct Nerineoidea (lower Heterobranchia *incertae sedis*; see Haszprunar 1985a, 1988) with highspired, yet large shells were suggested to be "shell draggers and probably deposit feeders" (Kollmann 2014) and hence also fit this pattern. Thus, highspired snails may be characterized by shared aspects of their ecology.

Further implications of the Mircacicula concept would be that the ancestor of Rhodopemorpha likely was a highspired, small animal, infaunal, with narrow and elongate body and reduced pharynx. It may be suggested that the uncoiled shell of an undescribed taxon imaged by Sasaki (2008: fig. 12F,G) may represent an intermediate between an *Ebala*-like murchisonellid (protoconch, form of aperture) and an interstitial organism resembling prosobranch *Caecum* Fleming, 1813, approaching the rhodopid condition. Discoidal-shelled Orbitestellidae as direct sister to highspired Micracicula, away from Valvatoidea, supports the idea that discoidal shell morphology was plesiomorphic for all Heterobranchia, as suggested by Ponder (1991). However, the Micracicula hypothesis needs further testing and corroboration by analysis of more comprehensive molecular datasets, and comparative anatomy of *Cima* and *Graphis* to evaluate the existing differences between murchisonellids and rhodopids (divergent head and CNS morphology, burrowing habit, weak molecular support) and the other three families.

4.4.2 Towards a new head: diversity of heads and sensory nerves and sensory specialization hypothesis

In Gastropoda, the paired cerebral ganglia are usually the main centers integrating sensory input. Several paired sensory nerves emanate from the cerebral ganglia and innervate the mouth area, the cephalic tentacles, the sides of the head, the eyes, and the statocysts. Tentacle form, ecology, and underlying nervous architecture are correlated and prime targets for research of morphological evolution. Among Heterobranchia, the easiest and therefore most intensively studied are the nerves innervating the cephalic tentacles, and these will be discussed here.

A single pair of simple head tentacles in lower Heterobranchia, innervated by a basally bifurcating nerve, is noted as a plesiomorphy of (lower) Heterobranchia shared with many Caenogastropoda (Ponder 1991, Koller et al. 2014); nerve branches are equally thick and run along the length of the tentacles (Fig. 9A). In contrast, Euthyneura have two tentacle pairs supplied by its own paired nerve that is either simple or bears a thinner basal branch, but never equally thick ones; diversity among the tentacles is large and correlated with ecology, and each tentacle pair has different chemosensory functions (Agersborg 1925, Croll 1983, Chase 1986; Fig. 9D).

By the use of axonal backfilling techniques that stain characteristic neuron somata in the ganglia, Klussmann-Kolb et al. (2013) identified four pairs of homologous nerves innervating sensory epidermis among euthyneuran Acteonoidea, Pleurobranchidae Gray, 1827, Nudibranchia, and several Euopisthobranchia (Tab. 2). N2 innervates the labial tentacles and N3 the rhinophores, N2 bears a thinner branch near its base that innervates either the velum or anterior part of the anterior tentacles ("lip organ"; see also Staubach & Klussmann-Kolb 2007, Staubach et al. 2008, Staubach 2008). A similar basal branch can be found in the N3 as well (Brenzinger et al. 2011a, 2013a) (see Fig. 9C,D). This pattern of four presumable homologous adult nerves could largely be confirmed by the herein presented studies of *Ringicula*, the cephalaspidean *Pluscula*, and the acochlidian *Strubellia* (see Brenzinger et al. 2011a, 2013a, Kano et al. in rev.). Additionally, critical comparison with preceding studies examining full nerves (Klussmann-Kolb et al. 2013: Table 1; also Leyon 1947, van

Nerve	Target	Terminology herein	possible equivalent in van Mol (1967, 1974)
N1	lip left and right of mouth	Oral nerve	nerf labial interne
N2a	median upper lip, oral veil, lip organ (= ASOa *sensu* *)	Labial nerve, inner branch	nerf labial median (thick)
N2b	labial tentacle (=ASOb *sensu* *); or corresponding sensory grooves if reduced	Labial nerve, outer branch	nerf tentaculaire (thick)
N3a	rhinophores (=ommatophore of Stylommatophora), or folded Hancock's organ in this position (=PSO *sensu* *)	Rhinophoral nerve	nerf tentaculaire (thick)
N3b (herein)	Sensory pit at base of rhinophores, siphon in Gastropteridae; often associated with optic nerve and basal ganglion/procerebrum with double roots	Rhinophoral nerve, basal branch	nerf péritentaculaire (externe)
N4 = nclc	posterior part of headshield (if present), otherwise small nerve innervating sides of head	Headshield nerve	nerf péritentaculaire interne (?)

Table 2. Characterization of cerebral nerves in Euthyneura.

Description and numbering of nerves following table 2 of Klussmann-Kolb et al. (2013; * refers to that paper).

Mol 1967, 1974– see Tab. 2) shows that the pattern may be widespread not only in opisthobranch taxa but also in the variety of pulmonates. Patterns of cerebral nerves are more complicated in Sacoglossa, probably owing to fusion of nerves early in ontogeny (Salvini-Plawen 1990, Huber 1993, Kohnert et al. 2013, Jensen et al. 2014), the ones in Pteropoda are simpler, perhaps due to paedmorphosis (e.g. Kubilius et al. 2014).

Huber's (1993) taxonomically comprehensive study is problematic, because nerves were not followed in their entirety and thus homology assumptions between taxa and naming of nerves often were fuzzy and inconsistent. This means that nerves were homologized *a priori*, without identifying them by correlation to particular targets which they innervated, and so basally split nerves (such as N2a and N2b) may have been regarded as separate nerves while others may have remained undetected. Thus, homologies remained largely unclear (see Klussmann-Kolb et al. 2013); reexamination will be necessary to evaluate Huber's results.

How did the cerebral nerves evolve in Heterobranchia? One of the most interesting results is the deeply conserved underlying pattern in neuronal architecture of the cerebral ganglia, as revealed by axonal staining methods applied by Klussmann-Kolb et al. (2013): the cerebral nerves originate from a conserved number of identifiable neuron clusters that retain a signature three-dimensional pattern; the N2 originates from five of these clusters, and the N3 from six. Staubach (2008 – unpublished thesis), using the same method, showed that the bifurcated nerve innervating the single tentacle in the caenogastropod *Littorina* Férussac, 1822 is associated with an homologous set of eleven clusters, which is the sum of both. From this it can be deduced that the bifurcated nerve of Caenogastropoda originates from the same clusters of neurons as do the terminally split rhinophoral and tentacular nerves of Euthyneura (see Koller et al. 2014). This again corroborates the idea that during the evolution of Euthyneura the originally bifid tentacle nerve became split basally (see Fig. 9A-C), correlated with specialization of the tentacles into separate structures for the detection of "smell" (*i.e.*, far-reaching chemoreception of substances in the water column: rhinophores) and "taste" (*i.e.*, touch: labial tentacles), as e.g. examined for several taxa (Chase 1986, Wertz et al. 2006). Furthermore, results on *Achatina* (Staubach 2008) show that the ommatophore of land snails is homologous to the rhinophore of aquatic Euthyneura; so, rhinophores are not diagnostic for opisthobranchs, nor is a "true rhinophoral nerve with basal, double-rooted ganglion diagnostic for Opisthobranchia", as put forth by Haszprunar and Huber (1990) and Huber (1993) The rhinophoral nerve of opisthobranchs is thus homologous to the tentacle nerve of pulmonate taxa, and partially homologous to the bifid tentacle nerve in lower heterobranchs (Brenzinger et al. 2013a,b; Koller et al. 2014).

The phylogenetic position of the still undescribed taxon of lower-heterobranch-like *Tjaernoeia* and bubble-shelled *Parvaplustrum* as sistergroup to Euthyneura was indicated by molecular genetics (Kano in prep.). Anatomically, this new taxon presents an intermediate between Euthyneura and the lower heterobranch outgroups: protruding snout and bifid foot resemble lower heterobranchs. The bifurcated tentacles that are still broadly fused at their base (already shown by Warén 1991, Powell 1951, Marcus & Marcus 1969, and Chaban & Chernyshev 2013) are unusual and also resemble a hypothetical stage of not yet completely separated tentacles, but nerves are basally split as in Euthyneura and not only bifurcated (Fig. 9B; Brenzinger, Kano & Schrödl, 2015, 2016, in prep.). It is also evident that the small tentacles on the tip of the snout in both taxa are not homologous to the labial tentacles of Euthyneura, as they are innervated by the N1.

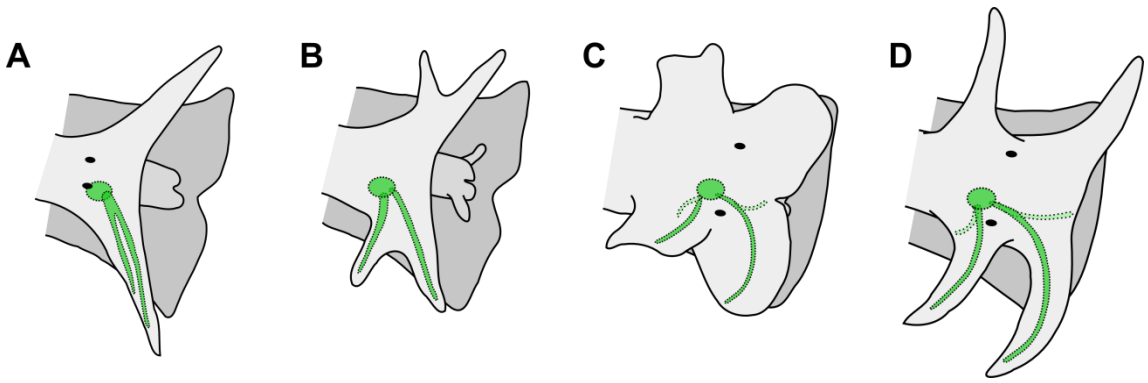

Figure 9. Hypothetical scenario of the evolution of the tentacle nerve in Euthyneura. Schematic dorsal right views of head. Green: cerebral ganglion and nerves innervating sensory tentacle (other sensory, cerebral nerves not shown).

A. Lower heterobranch condition, with single tentacle and bifurcated nerve. **B**. Condition in Parvaplustra (*Tjaernoeia* + *Parvaplustrum*), with bifurcated tentacles and separated, simple nerves. **C**. Hypothetical condition of euthyneuran ancestor, with two pairs of flattened, enrolled tentacles (a lobed headshield) and separate nerves (N2 and N3) with incipient basal branches (N2b and N3b). **D**. Euthyneuran condition as in Acochlidia, with two pairs of tentacles and separate nerves with prominent basal branch.

♦

Detailed data on Rhodopemorpha exist, but interpretation remains equivocal: they were considered opisthobranchs or pulmonates on the basis of their nervous systems (see Haszprunar & Huber 1990). However, it remains difficult to homologize their cerebral nerves due to the lack of data on the direct lower heterobranch outgroups (Murchisonellidae) and their aberrant head morphology with reduced head tentacles and pharynx. There are several possible scenarios of homology to nerves of other taxa, including potential loss (of a N1) and fusion of nerves. Huber (1993) examined *Ebala* in the context of a close relationship with Pyramidellidae; he noted distinct differences but did not mention a bifid tentacle nerve. Thus, the tentacle nerves of murchisonellids may be distinct from those of other, shell-bearing "lower" heterobranchs of the Micracicula and rather more similar to those of Euthyneura; this still needs confirmation but may change the interpretation of the rhodopemorph CNS as well.

Data presented in this thesis suggest that the presence of double roots and a basal ganglion also in the "rhinophoral" nerve of rhodopids, however, may indicate either convergence of these traits or evolution already in the last common ancestor of rhodopemorphs and Euthyneura, with secondary loss in many other taxa. These double roots were also confirmed for panpulmonate Sacoglossa (Kohnert et al. 2013, Jensen et al. 2014) and Acochlidia (Neusser et al. 2007, Brenzinger et al. 2011a). However, the potential presence in the meiofaunal euopisthobranch cephalaspidean *Pluscula* (Brenzinger et al. 2013a) needs comparative examination of other taxa, but may hint at a wider distribution of the trait.

Studies of structures associated with the cerebral nerves showed that, in principle, Acochlidia possess several "unusual" structures which may be homologues to the procerebrum and dorsal

bodies of “Pulmonata” (discussed in Neusser et al. 2007). Among them, observation of both a double-rooted ganglion and a second ganglion on the rhinophoral nerve (= double rooted optic ganglion with smaller neurons and cephalic gland *sensu* Brenzinger et al. 2011a: fig. 5) is congruent with the idea that the procerebrum and rhinophoral ganglion in fact have different origins (cited in Haszprunar & Huber 1990, van Mol 1967; Ruthensteiner 1999). This would mean that the rhinophoral ganglion and the so-called procerebrum are not fully homologous, contrary to present assumptions. Furthermore, presence of potentially secretory tissue above the brain in acochlidian *Strubellia* (cephalic gland *sensu* Brenzinger et al. 2011a) may be another panpulmonate character expressed in this taxon historically associated with opisthobranchs.

Summarizing, these steps may be proposed for the evolution of the cerebral nerves and visceral loop, with accompanying changes in external soft-body anatomy (Fig. 9):

1) Simple tentacles with bifurcated nerve (ancestral N2+3; Fig. 9A), snout area innervated by N1 only
2) Simple tentacles with bifurcated tentacle tips, basally separated nerves N2 and N3 (Fig. 9B)
3) Separate tentacles, basally broad; specialization of large basal branch in nerves N2 and N3 (Fig. 9C)
4) Specialization of rhinophores (inrolled, with basal branch N3b innervating enlarged zone at base) and wide labial tentacles, snout overgrown by base of labial tentacles (Fig. 9D); growth of N4 innervating sides of the head and neck or reduction
5) Development of accessory neural structures in Panpulmonata: double roots and specialized ganglion at base of N3, remaining ectodermal tube as glandular organ (cerebral gland), mesodermal cells above cerebral commissure (dorsal body)

The presence of accessory ganglia on nerves N2 and N3 is typical for meiofaunal slugs (Brenzinger et al. 2013b and references therein). These have evolved convergently and are morphologically variable: they may be sorted serially (e.g. *Helminthope*, Brenzinger et al. 2013b), or parallelly (two large ganglia in the N3 of Philinoglossidae: Brenzinger et al. 2013a, Huber 1993), there may be one or several externally irregular complexes (microhedylid Acochlidia: Neusser et al. 2006, Jörger et al. 2008) or an aggregation of discrete small ones (Rückert et al. 2006 on *Platyhedyle*; Jörger et al. 2014c on *Pseudovermis;* own unpubl. obs. in *Rhodope*). One hypothesis explained their presence by the small body sizes (or rather, diameters) and functionally necessary outsourcing of neurons (e.g. Niven & Farris 2012). Small adult body size, interestingly, does not appear to be correlated with the presence of accessory ganglia alone, but meiofaunal habitat is: non-meiofaunal sister taxa of meiofaunal slugs do not exhibit conspicuous accessory ganglia wherever examined in detail (Hedylopsidae Odhner, 1952/other Hedylopsacea, *Platyhedyle*/*Gascoignella*, *Helminthope*/*Rhodope* or Rhodopemorpha/ Murchisonellidae). Other small slugs such as Runcinidae or many sacoglossans (e.g. 2 mm *Ercolania* Trinchese, 1872; Jensen et al. 2014; Huber 1993) do not have them, nor are accessory ganglia known for any equally minute snails such as Murchisonellidae, Rissoellidae, or lower Heterobranchia (see also Haszprunar & Huber 1990). Ruthensteiner (1999) and Schaefer & Ruthensteiner (2001) noted the presence of accessory ganglia on several nerves during the development of *Ovatella* Bivona-Bernardi, 1832 and *Haminoea*; but not in metamorphosized adults. This may also be another example of larval structures being present in the adult, a paedomorphosis. Additionally, Brenzinger et al. (2013b) hypothesized the ganglia to be related to the complex three-dimensional habitat of meiofaunal slugs, necessitating advanced means of chemical detection to home in on prey. This would follow the general observation that gastropod “olfaction requires large

numbers of neurons" (Chase 1986), which would explain a high number of potentially olfactory neurons was well.

Evolution of specialized tentacles, supplied by separate nerves, may be correlated to different ecology of Euthyneura, e.g. moving on soft substrata and enhanced motility (see 4.2); association of olfactory organs with the head (rhinophores) may have been advantageous in homing in on more distant, or patchier, food sources. Thus the rhinophores functionally replace the gastropod osphradium as main olfactory organ. This is congruent with Morton's (1972) suggestion that the chemosensory Hancock's organs of aquatic Euthyneura (which are innervated by the rhinophoral nerve=N3) functionally replace the osphradium of prosobranchs, and also the existence of an elaborate osphradium only in lower heterobranch Architectonicidae and Mathildidae (Haszprunar 1985b,c) which also have only one, *i.e.,* non-specialized, pair of head tentacles. Directional movement towards smell sources is dependent on the presence of both rhinophores in euthyneuran taxa (see Croll 1983); this setup may be functionally equivalent to the combination of a motile siphon and the osphradium in Caenogastropoda.

4.4.3 A new type of mantle as key to the diversification of Euthyneura?

Jörger et al. (2010) placed the radiation of Euthyneura *s.l.* in the late Paleozoic to early Mesozoic at ca. 280 million years ago (middle Carboniferous to late Permian, early Triassic), Kano et al. (in rev.) estimated similar ages at 350 to 250 mya. In both scenarios, the major Recent euthyneuran lineages (Acteonacea, Ringipleura, and Tectipleura: Euopisthobranchia and Panpulmonata) emerged within a timespan of only approximately 50 million years. What triggered this rapid diversification?

Herein, no attempt will be undertaken to link this with factors of historical biogeography and ecology, but it will be noted that particular changes in morphology may have been key events for the diversification of Euthyneura: besides the aforementioned change in cerebral innervation, another one is the advent of bubble-like shells (see next chapter). Primarily, this change in shell morphology should be correlated with a change in morphology of the (shell-forming) mantle, which accordingly may have paved the way to numerous other innovations. First, there are many examples in Euthyneura where the mantle margin is enlarged: primarily, it may simply expand slightly over the lip of the shell in crawling specimens (e.g. *Parvaplustrum*, Ringiculidae, Lymnaeoidea – Powell 1951, Kano et al. in rev., Hubendick 1947) but may also be large, overgrowing much of the shell (many parapodiate Euopisthobranchia, Pleurobranchoidea, many Stylommatophora; Rudman & Willan 1998, Smith & Stanisic 1998). It would be expected that such a change of the mantle margin also influenced the specialized area that secretes the shell and periostracum, and thus the shell itself. Particular shell forms typical for Euthyneura are the bubble shell (often associated with overgrown lips), and auriculate shell types (embedded in mantle tissue). Both these shell types bear a roof of the mantle cavity that is considerably larger than in the physotestan outgroups; this creates more space, for example, for the growth of vascularized spaces (see Brace 1983) or of glandular areas. Secondly, expansion of the mantle margin increases the area of tissue exposed to the outside; in many euthyneurans, everted mantle tissue covers much of the back of the animal (e.g. the notum of nudibranchs and systellommatophorans, the mantle shield of Cephalaspidea, the cerata-bearing areas in plakobranchacean Sacoglossa, the glandular mantle of Acochlidia and Stylommatophora). This larger mantle area is often strongly glandularized and acts as a defensive organ by means of

chemicals exuded from epidermal and subepidermal glands (see 4.1.3). These glands are located inside the mantle cavity in aquatic, shelled taxa. A large diversity of glands may have already been present in lower Heterobranchia (see Brenzinger et al. 2014 on Murchisonellidae): the so-called "pigmented mantle organs", "hypobranchial or "mantle" glands *et cetera* of still unclear homology across taxa. Among those, only the Blochmann-like glands are the clearest potential homologues due to their comparatively complex and characteristic histology (with clear-staining large vacuoles, muscular coat; discussed in Brenzinger et al. 2013a). It may be suggested that formation of complex glands of this type and the enlargation of the mantle are a main prerequisite for the functioning of chemical defense which is considered typical for "opisthobranch" seaslugs (e.g. Faulkner & Ghiselin 1983, Marin et al. 1999, Wägele et al. 2006, Cimino & Ghiselin 2009). Thus, defensive glands in *Ringicula* (clearly of the Blochmann type) and so-called "mantle dermal formations" (MDF's) of certain nudibranchs (e.g. García-Gomez et al. 1999 and previous references) may be synapomorphic for Euthyneura, as are the "yellow glands" of some Cephalaspidea (Rudman 1972a, Brenzinger et al. 2013), the purple and opaline glands of Anaspidea (Klussmann-Kolb 2004), and the defensive "mantle" glands of aquatic Systellommatophora (see Pinchuck & Hodgson 2010).

Enlargation of the mantle roof and margin may also be suggested as prerequisite for the radiation of Panpulmonata: primarily, an enlarged roof with different vascularization is basis for the formation of complicated vessels systems found in some sacoglossan and acochlidian slugs (Bücking 1933, Swennen 2011, Neusser et al. 2011b), and all the air-breathing panpulmonates formerly grouped as "Pulmonata" (see study by Brace 1983 for comparative discussion in opisthobranch taxa; Haszprunar 1988). It is still unclear if the so-called "dorsal vessels" among plakobranchoidean Sacoglossa and Acochlidia are homoplasies owing to similar ecology and oxygen regimes (related to photosynthetic activity in Sacoglossa? – Jensen 1996; related to freshwater or semiterrestrial habitat in Acochlidia? – Neusser et al. 2011b, 2016) or if they are rather homologous to those vessels functionally forming the lung in "pulmonate" taxa. This would mean that lung-like organs are already present at the base of Panpulmonata (being secondarily lost in the operculate clade). Additionally, an expanded mantle margin is crucial in forming the wall around the pneumostome, which closes off parts of the mantle cavity (the "lung"; Ruthensteiner 1997); this structure is present in the majority of airbreathing Panpulmonata and is considered essential in reducing water loss. Thus, anatomical changes found in early Euthyneura are still pertinent in terrestrial taxa.

On a wider view, reorganization of the mantle with looser association of its margin to the shell and enlargation of the last whorl of the shell in the last common ancestor Physotesta may have freed the mantle from constraints and presents the morphological basis for an explosive radiation of morphologies, with the bubble shell presumably being an external indicator of such a "new mantle". Presence of the latter may be evident only by rather inconspicuous changes to the soft body morphology in some taxa. For example, the ventral part of the mantle may simply be fused anteriorly to the back of the head, forming a new "neck" area in many elongate slugs and semislugs (see mantle hood in Acochlidia, mantle frill in *Ringicula*; Brenzinger et al. 2011a, Kano et al. in rev.), or it may overgrow the head (nudibranch notum), or the visceral sac (Anaspidea, Cephalaspidea, Runcinacea). In other cases, the mantle cavity became reorganized radically: organs were lost, form and size reduced drastically and was often reduced; all these features are associated with size increase and hypertrophy of the mantle. It should be noted that the most species-rich euthyneuran subtaxa conform less to this morphotype: the nudibranchs, Pyramidelloidea, and Stylommatophora. The high

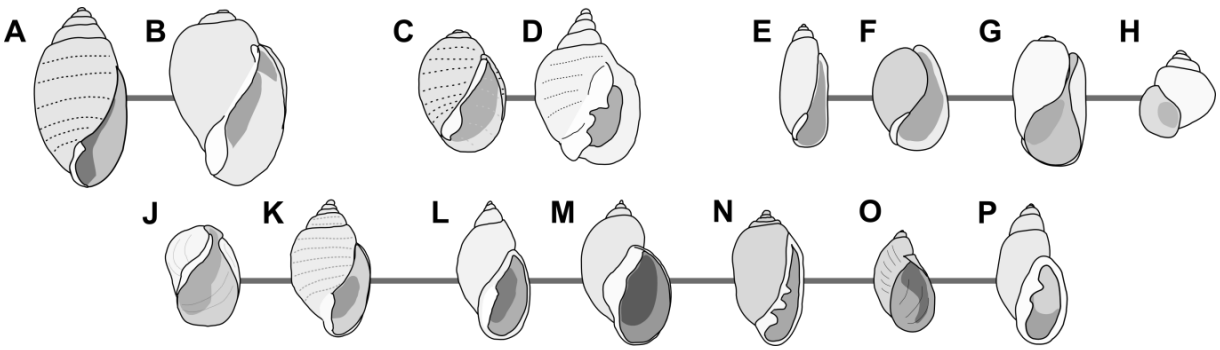

Figure 10. Examples of traditional "bubble" shells in Euthyneura *s.l.*, and of types not traditionally regared as such. All apertural views, not to scale.

A-B. Acteonoidea: **A**. *Acteon tornatilis* (Linnaeus, 1758), Acteonidae. **B**. *Aplustrum amplustre* (Linnaeus, 1758), Hydatinidae. C-D. Ringipleura, both Ringiculidae: **C**. *Ringiculoides kurilensis* Minichev, 1967 (original). **D**. *Ringicula doliaris* (after Kano et al. in rev.). E-H. Euopisthobranchia: **E**. *Acteocina exserta* (Hedley, 1903), Acteocinidae. **F**. *Bulla ampulla* Linnaeus, 1758, Bullidae. **G**. *Akera soluta* (Gmelin, 1791), Akeridae. **H**. *Limacina trochiformis* (d'Orbigny, 1834), Pteropoda: Limacinidae Gray, 1840. J-O. Panpulmonata: **J**. *Oxynoe olivacea* Rafinesque, 1814, Sacoglossa: Oxynoidae. **K**. *Leucotina* sp., Pyramidelloidea: Amathinidae. **L**. *Chilina parchappii* (d'Orbigny, 1835), Chilinidae. **M**. *Bulimnea megasoma* (Say, 1824), Lymnaeidae Rafinesque, 1815. **N**. *Pseudomelampus exiguus* (Lowe, 1832), Ellobiidae. **O**. *Succinea putris* Linnaeus, 1758, Stylommatophora: Succineidae. **P**. *Placostylus fibratus* (Martyn, 1784), Stylommatophora: Bulimulidae Tryon, 1867. Redrawn from various sources: A,B,E,F,J,K,N: gastropods.com. G: Seaslugforum.net. H: S. Grove/ molluscsoftasmania.net. P: National Museum Wales. L: C. & A. Evanno/flickr.com. M: M. Kohl/mkohl1.net.

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species diversity of these three clades can, however, be explained by the further invasion of novel ecological niches: 1) Nudibranchia (approx. 3000 species; Wägele & Klussmann-Kolb 2005) by the evolutionary novelty of a large notum and chemical defence; 2) Pyramidelloidea (estimated 6000 species; Lygre & Schander 2010) by occupation of the parasite niche; 3) Stylommatophora (estimated 30 000 species; Smith & Stanisic 1998) by the invasion of land including dry areas coupled with low dispersal abilities and endemism. The aforementioned clades may however be derived from bubble-shelled forms (see following chapters), which evolved morphological prerequisites for later diversification.

4.4.4 The bubble shell as key element in Physotesta?

Shells of Euthyneura are variable with respect to coiling pattern, including multispiral ones, egg-shaped ones, and various forms of flattened shells with very large apertures. Shells of lower heterobranchs are different in form and ornament, they are multispiral and invariably characterized by their regular increase in whorl diameter and their roughly circular aperture. Bubble shells occur only in Physotesta, but are there found in all major lineages. Thus it may be suggested in the following that the bubble shell presents a crucial apomorphy for Physotesta (this section), and that

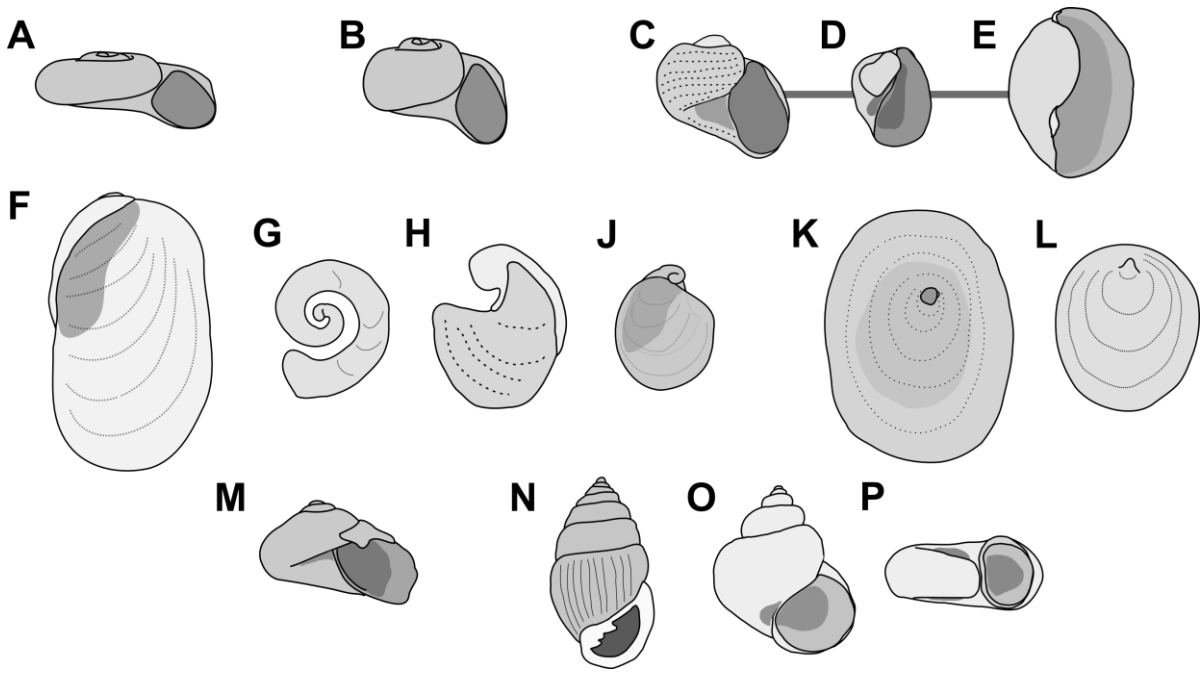

Figure 11. Scenario of the evolution of the bubble shell, and potential modifications.

A-E. Hypothetical scenario of derivation of a bubble shell from a discoidal, "lower heterobranch"-type shell. **A**. Valvatoidean *Xylodiscula boucheti* Warén et al., 1992 (after Warén 1991b). **B**. Same as first, image artificially stretched along longitudinal axis. **C**. *Tjaernoeia exquisita* (Jeffreys, 1883), resembling the previous shell in outline (after Warén 1991a). **D**. Juvenile shell of *Parvaplustrum japonicum* Chaban & Chernyshev, 2013, resembling adult *Tjaernoeia* in angle of spire and form of aperture but lacking a full second whorl. **E**. Adult shell of *P. japonicum*, a typical bubble shell with inflated body whorl, and involute spire (after Chaban & Chernyshev 2013). Roughly to scale.

F-L. Examples of shell morphologies potentially derived from a bubble shell by peramorphosis, *i.e.,* by abbreviation of a whorled stage and hypertrophy of the lip. Auriculate, semiinternal shells: **F**. *Berthella stellata* (Risso, 1826), Nudipleura Pleurobranchidae (after seaslugsofhawaii.com). **G**. *Nakamigawaia spiralis* Kuroda & Habe, 1961, Cephalaspidea Aglajidae Pilsbry, 1895 (after Sasaki 2008). **H**. *Dolabella auricularia* (Lightfoot, 1786) (after seaslugforum.net). **J**. *Aplysia parvula* Mörch, 1863 (after seaslugsofhawaii.com), both Euopisthobranchia Aplysiidae. Limpets: external, circular shells. K. *Tylodina perversa* (Gmelin, 1791), Euopisthobranchia Tylodinidae Gray, 1847 (after gastropods.com). **L**. *Williamia radiata* (Pease, 1860), Siphonariidae (after Ruthensteiner 2006). G and J: apertural views, others apical views.

M-P. Examples of shell morphologies potentially derived from a bubble shell by paedomorphosis, *i.e.,* through suppression of a large last whorl with inflated lip, and growth of multiple whorls. **M**. *Tomthompsonia antarctica* Wägele & Hain, 1991, the only pleurobranchoid with multiwhorled shell (after Wägele & Hain 1991). Panpulmonata, operculate clade: **N**. *Otopleura glans* (Reeve, 1843), Pyramidellidae (after gastropods.com). **O**. *Phallomedusa solida* (Martens, 1878), Amphibolidae (after Golding et al. 2007). **P**. *Patagonorbis nahelhuapensis* Rumi & Gutierrez Grégoric, 2015, Glacidorbidae (after Rumi et al. 2015). Apertural views.

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this presents a starting point for the formation of other shell types including the return to multispiral shells (next section).

The egg-shaped "bubble" shell has several coils followed by a rapidly expanding last whorl, bearing a large lip. The aperture is narrow posteriorly, resulting in a narrow or teardrop-shape; the columella sometimes bears additional, columellar folds (see examples in Fig. 10). Traditional Cephalaspidea were partially diagnosed by the presence of such a "bubble" shell (e.g. "Architectibranchia": first three "opisthobranch" lineages in Fig. 1A; taxa in Gosliner 1991, 1993, Rudman & Willan 1998), but are now considered to be polyphyletic (Malaquias et al. 2009, Kano et al. in rev.). Classical cephalaspids with this shell type occur in Acteonoidea (Fig. 10A-B), Euopisthobranchia (in Cephalaspidea and Akeridae; Fig. 10E-G), and Panpulmonata (in shelled Sacoglossa; Fig. 10J). Furthermore, two additional high-ranking lineages with this shell type could be identified during the work for this thesis: Kano et al. (in review) showed that Ringiculidae form a lineage independent from the aforementioned ones that links Nudipleura to the rest of Euthyneura; a bubble shell existed in the Ringiculidae stemline and potentially in the stemline of Nudipleura as well, with basal Ringiculidae (Fig. 10C) most likely lacking the complex lip and aperture of the best-known genus, *Ringicula* (Fig. 10D). Second, herein included preliminary data (Kano, Brenzinger, Schrödl, 2016, unpubl./ in prep.) show bubble-shelled *Parvaplustrum* as a further independent lineage (Parvaplustra tax. nov.) as sister to Euthyneura. Therefore, typical bubble shells occur in all five physotestan lineages.

Several of these ornamental elements appear scattered across the tree and may or may not be symplesiomorphic, but warrant further examination: superficial, spiral chords of "chain-like connected pits" also occur in all major lineages of Euthyneura: Acteonoidea (Valdés 2008, Gründel & Nützel 2012, Salvador & Cunha 2016; Fig. 10A), in Ringiculidae (Minichev 1967, Gründel 1997; Fig. 10C), some Cephalaspidea (e.g. some *Toledonia*, Cylichnidae, "Philinidae" Gray, 1850, Scaphandridae: Marcus 1957, Ohnheiser & Malaquias 2013, Eilertsen & Malaquias 2013), and also in Panpulmonata (pyramidelloid *Leucotina*, synonym*: Adelactaeon* Cossmann, 1895: Thiele 1931, van Aartsen et al. 1998, Sasaki 2008; Fig. 10K). Function and formation of this rather complex and distinctive pattern are not known; it is mainly found in infaunal taxa with a fairly large shell and may hence be connected to movement through the substrate (less adhesion of small particles to the shell?). Nevertheless, multiple convergence may be less likely than it being a plesiomorphy for Euthyneura, given that it is also found in a variety of fossil bubble-shelled taxa (Gründel & Nützel 2012: Tubiferidae Cossmann, 1895, *Tornatellaea* Conrad, 1860; see also Salvador & Cunha 2016). Also, similar ornament is found on the early whorls of the internal shells of some Pleurobranchoidea (Gosliner 1994: fig. 4D, Schrödl 1999: fig. 3). It may be suggested that presence of the pattern is a euthyneuran character, and that the irregularly sorted and roundish pits of Parvaplustra (SEM shown by Warén 1993, Chaban & Chernyshev 2013) already present parts of this pattern as an apomorphy for Physotesta (this study; Fig. 11C). Columellar folds occur only in bubble-like shells with fairly strong calcification: some Acteonoidea, Ringiculidae, in cephalaspidean Cylichnidae, but also in panpulmonate Pyramidellidae, Chilinidae and many Ellobiidae; remnants may perhaps even be present in some terrestrial Stylommatophora (e.g. *Placostylus*; Fig. 10P). This observation is in contrast to Haszprunar (1985a: p. 19 and table 1) who assumed "true" columellar folds (*i.e.*, lamellae and not only tooth-like projections) to be mainly a character of "most basal Heterobranchia" including Architectonicoidea and fossil Nerineoidea. Whether spiral patterns of brown pigment (sometimes zig-zag-shaped blotches; not shown) that are found in the periostracum of Acteonoidea

(Bullinidae, Hydatinidae), euopisthobranch Tylodinidae and Bullidae, and panpulmonate Amphibolidae, Chilinidae, Ellobiidae and Stylommatophora (see Rudman & Willan 1998, Smith & Stanisic 1998) also are an apomorphy of Euthyneura or a habitat-convergent element similar to the patterns found in many other Apogastropoda is an open question.

It may be suggested here that the smooth oval shells of several other "bulimoid" panpulmonates (with a relative high spire yet large last whorl) could be directly derived from bubble-shells by simplification: the inflated shells of basal aquatic Hygrophila (Chilinidae and Lymnaeoidea; Fig. 10L-M), the oval shells of most semiterrestrial Ellobiidae (Fig. 10N for example), and even the majority of "primitive" Stylommatophora like Succineidae and the achatinoid families (Wade et al. 2006) possess such shells (Fig. 10O-P). This pattern may not be restricted to Panpulmonata; "bulimoid" thecosomes (*Limacina bulimoides*; Fig. 10H) may display remnants of a bubble-shell morphology as well.

How did this shell type evolve? As presented earlier, the newly detected close relationship of lower heterobranch-like *Tjaernoeia* (Fig. 11C) and bubble-shelled *Parvaplustrum* (Fig. 11D,E) presents potential intermediates in a low-diversity clade. This may explain evolution from a lowspired, discoidal shell as found in the lower heterobranch outgroups. The shells of both genera have in common drop-shaped apertures and ornament consisting of minute etched pits that are, in contrast to Euthyneura, not sorted in spiral lines. This may present an original condition in the evolution of punctate spirals. Form of the aperture and last whorl may hint at changes in mantle (roof) morphology that approach the euthyneuran type. Compared to the outgroups, the globular shell of *Tjaernoeia* appears like a somewhat distorted valvatiform shell with additional surface pitting; its outline can be almost perfectly derived from a flatter valvatoid shell by simple elongation of the anterior-posterior (=apex-umbilicus) axis; digital distortion e.g. of a *Xylodiscula* shell (Warén 1991b) can be used to theoretically demonstrate this (Fig. 11A-C). Shells of adult *Parvaplustrum* do not resemble *Tjaernoeia*, but juvenile ones have a very similar general outline (Fig. 11D; after Chaban & Chernyshev 2013: fig. 1E) including presence of an elongate umbilicus and teardrop-shape of the aperture. Therefore, ontogenetic stages of Parvaplustra shells (Fig. 11C-E) may present a sequence reminiscent of the evolution of the euthyneuran bubble shell from a valvatoid-like, flat-spired lower heterobranch. The following simple steps, involving simple morphometric changes, may have led from a valvatoid-like shell to a bubble one:

1) Elongation along longitudinal axis
2) Formation of surface pits
3) Rapid enlargement of last teleoconch whorl around preceding coils, leading to bubble shell outline and loss of umbilicus
4) Formation of single columellar rib
5) Formation of spiral ornament

To my knowledge, no comparable recent hypothesis exists on how the euthyneuran bubble shell may have evolved. Parallel changes in the soft body anatomy (reconfiguration of the mantle edge, organs in the mantle roof and musculature) are still open to be examined. Assuming that the bubble shell is derived in the ancestor of Physotesta, and not secondarily lost in the lower heterobranch clades or evolved several times convergently, it can be regarded as a key innovation that is found in across all 5 major clades.

Expansion affects mainly the last whorls of the shell, and this change appears late in ontogeny; thus, occurrence of the shell-type should be sensitive to heterochronic changes. Together with evolution

of a new mantle (preceding chapter), heterochrony could explain the presence of other shell forms in the euthyneuran clades, including the subsequent lack of a shell.

4.4.5 Implications for the diversity of shell forms – the Heterochronic Pendulum hypothesis

As already noted, Euthyneura are much more variable in shell morphology than lower Heterobranchia, including forms with multipiral shells (discoidal, globular, or highspired), flattened shells (limpets, ear-shaped ones), or slugs, besides the bubble-shell ones. Assuming a bubble-shelled ancestry for all Euthyneura (or Physotesta for that matter), how can the diversity of shell expression be explained?

Heterochrony, a rebalancing of ontogenetic sequences such as development of the shell, may be the most likely candidate for this. These processes can be grouped into two principal categories, namely paedomorphosis (by abbreviation of ontogenetic trajectories) and peramorphosis (by extension; see McNamara 1986 for nomenclature and concepts).

Ontogeny of the bubble shell can be described as: I) protoconch stage, II) early teleconch with low number of short, rounded coils, III) late teleoconch with elongate and inflated last whorl, and with large lip (see Fig. 12: top row). Judging from their position on the shell, formation of spiral pitted chords and columellar folds, if present, can be assumed to start in stage 2.

Based on this sequence, limpets and auriculate shells can be derived by peramorphosis by extension of stage III (while at the same time reducing stage II; Fig. 12: middle row), thus essentially representing a protoconch with largely expanded lip. The asymmetric, auriculate shell may be seen as one that skips much of the coiling, representing essentially a protoconch and larval shell with attached flaring lip (see e.g. Horikoshi 1967 fig. 18). The often internalized shells of Pleurobranchoidea, philinoid Cephalaspidea, and Aplysiidae fit this pattern (Fig. 11F-J; Gosliner 1994), as do the external shells of some Sacoglossa (e.g. Fig. 10J - *Oxynoe* Rafinesque, 1814 and also bivalved Juliidae E. A. Smith, 1885), some Amathinidae, and possibly the ellobioid *Otina* (Morton 1955). Circular limpets are mainly found in animals that lived exposed on hard substrates; examples are present mainly in Panpulmonata (Siphonariidae, several lineages of Hygrophila, and ellobioid Trimusculidae). Euopisthobranch Tylodinoidea are unusual in that they live on sponges but are also rather motile.

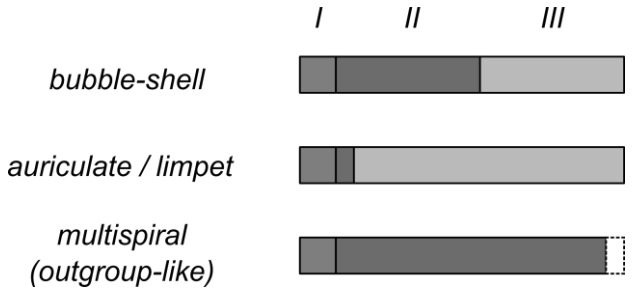

Figure 12. Ontogenetic sequences of shell types in Euthyneura.

Relative length of bar indicates duration of development of stages I-III; I), protoconch; II), coiled teleconch; III), inflated last whorl. See text for details.

On the other hand, shells developed through paedomorphosis should experience loss of later stages, skipping stage III and possibly extending stage II (Fig. 12: lower row). This would logically lead to short, multispiral shells with no significantly enlarged lip and somewhat rounded whorls. Examples for such shells could be seen in Rissoellidae, many Cephalaspidea ("Diaphanoidea" Odhner, 1914), some Thecosomata, and the operculate Panpulmonata (Figs. 3G, 10H, 11N-P). Further abbreviation of stage II would lead to a shell resembling a protoconch with similar further whorls, and examples for this that have been suggested to be progenetic forms may be the flat-spired thecosomes, *Glacidorbis*, many Hygrophila (among Planorbidae), and perhaps some derived Stylommatophora. Further succession of this pattern would lead to suppression of shell coiling, resulting in the retainment of not much more than a protoconch, as is the case in some small euopisthobranch slugs: the runcinacean *Runcina divae* (Marcus & Marcus 1969), some philinoid taxa (own unpubl. obs., some minute Aglajidae: Gosliner 1994, Ortea et al. 2014); the tube-like bilaterally symmetrical shells of orthoconch Thecosomata may be specialized cases of this scenario (Kubilius et al. 2014). No equal scenario for the evolution of highspired snails is presented here, but it is suggested that they also evolved by progenetic pathways.

It should be noted that logical extension of each scenario may in effect lead to the loss of the shell, *i.e.,* formation of slugs: either by subsequent internalization of an auriculate shell ("slow" scenario) during evolution, or by a rather abrupt change early in ontogeny leading to an abrupt loss of the protoconch in an early stage ("catastrophic" scenario).

All in all, ontogeny in Euthyneura may be characterized by heterochrony, oscillating between multispiral shells and limpet-like ones during evolution, with slugs having evolved on both extremes of this "heterochrony pendulum" (Fig. 13), and all forms being derived from a bubble-shelled ancestor. What would the implications be for the understanding of the heterobranch tree of life, and how could it be tested?

First, body size of adult slugs may perhaps indicate whether a progenetic or peramorphic pathway happened: the first scenario may be suggested to lead to slugs with large bodies that have sister taxa with auriculate shells or that are semislugs. Examples for such pairs of sister taxa may be seen within Aplysiidae (Klussmann-Kolb 2004), philinoid Cephalaspidea (e.g. Aglajidae and Philinidae; Fig. 11G), and many Stylommatophora (e.g. in Vitrinidae Fitzinger, 1833; Hausdorf 2001). The second scenario would create slugs that are physically small, aberrant and thus may be phylogenetically distinct (e.g., underwent a lot of morphological evolution which may also be visible in branch lengths on molecular phylogenetic trees); examples could be Rhodopemorpha, Gymnosomata, some Runcinacea, plakobranch Sacoglossa, and Acochlidia. Meiofaunal slugs, being small, may all fall into this category (see next chapter). However, some taxa do not fit either scenario particularly well: Systellommatophora and Nudibranchia are rather large slugs completely lacking shells but no intermediate stages to shelled forms are known; nevertheless, it may be hypothesized that either taxon fulfilled the scenarios of "large/subsequently internalized shell in ancestor" or "small/protoconch-stage shell lost" along their evolution, and their respective sister taxa are large-bodied as well. Furthermore, in some slugs either scenario is equally likely, such as philinoglossid Cephalaspidea (see Brenzinger et al. 2013a). It may be proposed here that there may essentially be two fundamental trajectories of "how to become a slug", but both may intergrade and overlap to some extent.

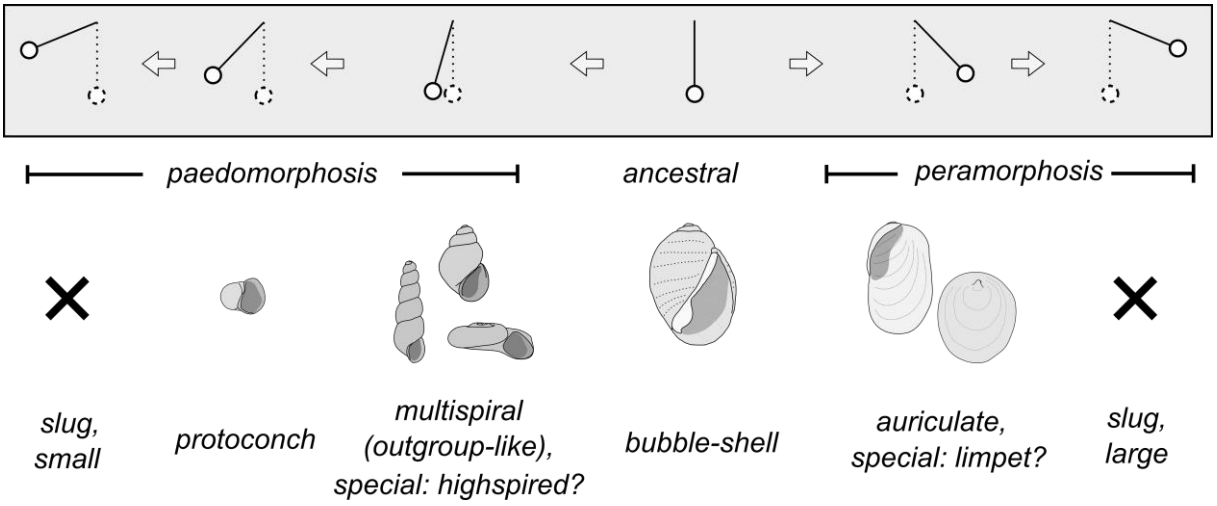

Figure 13. The heterochrony pendulum and shell morphology in Euthyneura.

The top row illustrates movement of the pendulum from its original, ancestral position. The second row describes processes referring to a swing of the pendulum. Bottom rows: resulting shell and body morphologies, see text for details.

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Second, this scenario of an evolutionary ontogenetic "pendulum" has the power to explain the occurrence of outgroup-like (in this case, multispiral and "prosobranch"-like) shell morphologies inside larger euthyneuran taxa as being morphologically "pseudoancestral", yet evolutionarily derived and progenetic forms (equaling movement of the "pendulum" to the left) (see Fig. 13). This would further challenge shell-based phylogenies (including those based on fossils), but could in principle become testable through molecular phylogenies with comprehensive taxon sampling. In concordance with already published molecular results, the following may be suggested to be examples: 1), *Rissoella* (Fig. 3G) may be regarded as derived from an acteonoidean-like shell through progenesis, 2), multispiral *Tomthompsonia* to be derived from an auriculate-shelled pleurobranchoidean, and not as primitively multispiral (Göbbeler & Klussmann-Kolb 2010b; Wägele & Hain 1991; see shells Fig. 11F,M), 3), polyphyletic Diaphanoidea (*Toledonia* and *Diaphana*) as derived and secondarily multispiral cephalaspideans (in concordance with Oskars et al. 2015), 4) the operculate panpulmonates Pyramidellidae, Amphiboloidea and *Glacidorbis* as displaying pseudoancestral morphologies due to paedomorphosis (as in Jörger et al. 2010, Dinapoli et al. 2011, Dayrat et al. 2011; shells in Fig. 11N-P), 5), bulimoid *Limacina* to be more ancestral than planorbiform ones (*L. bulimoides* vs. *L. helicina*; Fig. 10H) (not yet tested), 6), the rarity of very small bubble shells (exception: *Parvaplustrum*) but the presence of very small multispiral shells. The common lack of spiral ornamentation among many Cephalaspidea (see Ohnheiser & Malaquias 2013, 2014) could be explained by progenesis as well, as Chaban and Nekhaev (2013) demonstrated clades where spiral microsculpture occurs late in ontogeny of the teleoconch. (On the other hand, the presence of spiral ornament already in the early teleoconchs of some *Philine* species (Pruvot-Fol 1954 fig. 15a, Horikoshi 1967) may be explained by a peramorphic scenario, *i.e.,* skipping of a whorled stage). This pattern may also explain the presence of "primitive", streptoneurous visceral nerve loops in rather

derived Euopisthobranchia (see Gosliner 1991: tables 7 and 8) as secondary, anatomically correlated with bubble-shell morphotypes.

Third, planispiral taxa or minute slugs on the far left of the pendulum would present paedomorphic taxa (Fig. 13) with abbreviated life cycles, and would thus be well suited to live in dynamic, possibly ephemeral habitats; examples for this may be *Glacidorbis* and Planorbidae (ponds and streams; Ponder 1986, Smith & Stanisic 1998), Rissoellidae (Fretter & Graham 1948), shell-less Sacoglossa and Runcinidae (intertidal phytal; Rudman & Willan 1998), and perhaps Pteropoda (plankton) and interstitial slugs.

Fourth, because one evolved from the other, there should in principle be more sister pairs of limpets/auriculate-shelled taxa and bubble-shelled members than such pairs of limpets and lower heterobranch-like morphologies; the latter would be expected to be relatively more common in deeper nodes with longer evolutionary history. Albeit scant ingroup sampling in many taxa, this evolutionary pattern can indeed be found among planorboid Hygrophila: there, several independent limpet lineages are respective sisters to "bulimoid" taxa, but the typical planispiral taxa are all restricted to one clade with none being sister to limpets (see Albrecht et al. 2007: fig. 2). Other examples of limpet-bubble shell pairs may be: 1), also among Hygrophila: limpet-like *Latia* Gray, 1850 and bubble-shelled *Chilina* (Fig. 10L); 2) Tylodinoidea limpets (Fig. 11K) as sister to remaining Euopisthobranchia (Figs. 10E,F, 11G); 3) auriculate basal Aplysiidae (Fig. 11H,J) and bubble-shelled *Akera* O. F. Müller, 1776 (Fig. 10G); 4) Siphonariidae (Fig. 11L) and remaining Panpulmonata (e.g. Fig. 10J-P); 5) limpet *Trimusculus* and bubble-like *Pedipes* among Ellobioidea (Dayrat et al. 2011, shells not shown) and 6) limpet *Amathina* and bubble *Leucotina* (Fig. 10K) as the only such shells in speciose Pyramidellidae (Y. Kano, T. Takano, Tokyo - pers. comm.).

This pattern of a "heterochronic pendulum" is not obvious in lower Heterobranchia due to the lack of shell diversity, and this may be suggested to be ancestrally so. Only minute rhodopemorph slugs may have evolved by a scenario resembling the "catastrophic" loss of the shell and now fully suppress shell formation, as described by Riedl (1959); according to the new topology presented here, their ancestor likely was a highspired, small animal, infaunal, with narrow and elongate body and reduced pharynx, congruent with a scenario of progenesis.

Several historical scenarios placed *Acteon* as "most primitive opisthobranch" (e.g. Bouvier 1893, Guiart 1901, Perrier & Fischer 1911, Fretter & Graham 1954), in overlap with the herein presented idea that the bubble shell is the symplesiomorphic for Euthyneura and all other shells are derived. However, the present scenario is different in assuming symplesiomorphy and subsequent heterochronic shifts instead of "parallel evolution" of adult characters (Ghiselin & Gosliner 1984, Gosliner 1991). This resembles Martynov's (2011) proposal of viewing ontogenetic series, not only adult ones, as the to-be-observed unit in comparative morphology; however, the present approach does not assume heterochrony *a priori* to create a tree but rather, vice versa, reads this from existing trees. This follows earlier suggestions that "studies of ontogenetic processes are fundamentally dependent on hypotheses of phylogeny" (Fink 1982).

With more and more parts of the heterobranch tree of life being revealed by phylogenetics, a growing number of patterns can be revealed about the evolution of this diverse taxon. This also affects elements of one of the oldest metazoan ecological guilds, the meiofauna (next chapter).

5 CONCLUSIONS AND OUTLOOK: EUTHYNEURAN DIVERSITY AND EVOLUTION OF MEIOFAUNAL HETEROBRANCHIA

Why is it that there are few lower Heterobranchia, and so many Euthyneura? As outlined above, all the following factors may have led to higher morphological and ecological diversity, but also to morphological convergence: ecological shift (digging lifestyle, food sources), morphological novelty (mantle, central nervous system), and heterochrony ("pendulum"). Processes of heterochrony have been regarded as one of the fundamental factors in evolution of animals *per se* (e.g. Gould 1977), but there exist contradicting views on its importance: for example, Raff (1996) cautioned that heterochrony should not be used as a catch-all explanation, while McNamara (1997) effectually suggested to do so (see McKinney 1999). Progenesis, among heterochronic processes, is frequently seen as one of the main factors in the evolution of minuscule, meiofaunal taxa (Westheide 1987, Rundell & Leander 2010), and this includes interstitial "opisthobranchs" living in the interstices of marine coarse sands (Jörger et al. 2010, Brenzinger et al. 2013b).

Interstitial heterobranch slugs share a suite of morphological characters that were described as "meiofaunal syndrome" by Brenzinger et al. (2013b). These characters are not found in equally small or closely related, non-interstitial taxa: 1) tiny, worm-like bodies, 2) lack of body appendages, pigmentation and, often, eyes, 3) presence of spicular "skeletons", adhesive mechanisms, and accessory ganglia in the head area, and often 4) lack of copulatory organs and specialized ways of insemination (by spermatophores, sometimes hypodermal) (see e.g. Jörger et al. 2009, 2014a). "Rampant parallelism" was identified as a major hindrance to morphology-based phylogenetic, especially cladistic, studies on opisthobranchs (Gosliner & Ghiselin 1984, Gosliner 1991). The "meiofaunal syndrome" is a prime example for such a problem: in contrast to intuitive results and recent molecular studies that showed Rhodopemorpha, Cephalaspidea and Acochlidia on widely separated regions of the heterobranch tree (e.g. Malaquias et al. 2009, Wilson et al. 2010, Jörger et al. 2010, Oskars et al. 2015, see Figs. 6, 7), the morphocladistic study by Wägele & Klussmann-Kolb (2005) recovered all interstitial opisthobranchs as a monophyletic clade.

As mentioned in the beginning, studies focusing on the anatomy and phylogeny of interstitial and non-interstitial Acochlidia (e.g. Neusser et al. 2006, 2009, 2011a; Brenzinger et al. 2011a, Schrödl & Neusser 2010, Jörger et al. 2010, 2014b) had great impact on the recent reclassification of Heterobranchia, but prior to 2010 other taxa had remained largely unexamined in comparable detail. New perspectives were opened up by the herein presented studies that investigated morphology and relationships of previously unexamined groups of interstitial heterobranchs (rhodopids, philinoglossids) (Brenzinger et al. 2011b, 2013a,b, Jörger et al. 2014c) and non-interstitial sister taxa (Brenzinger et al. 2011a, 2014, Kohnert et al. 2013). First, the question of "what are the oldest Recent slugs?" may become answerable, and interstitial slugs may be potential candidates: Brenzinger et al. (2013b) identified Rhodopemorpha as the potentially oldest slugs because they are sister to "living fossil" Murchisonellidae (Warén 2013), however, as of yet no molecular time tree including the taxon exists. The high morphological disparity of Rhodopemorpha would fit with an early origin of the group. In contrast, the idea that Rhodopemorpha evolved by catastrophic loss of the shell would also be consistent with a recent evolutionary origin. A second candidate are the Nudipleura, with Kano et al. (in rev.; Rhodopemorpha however not included) presenting a time tree

focusing on Ringiculidae which showed Nudipleura as the only slug lineage possibly evolving prior to the Triassic-Jurassic boundary at 200 Mya (fig. 4 therein). This result of old nudipleurans is congruent with studies focusing on acochlidians as well (Jörger et al 2010, 2014b).

Second, as outlined above, there may be two fundamentally different ways of becoming a slug –does this also hold true for interstitial taxa? With the sistergroups of interstitial taxa becoming more and more known, this possibility can slowly be reconstructed.

Some interstitial taxa resemble minute versions of their respective sister-clades, and may have been derived by subsequent miniaturization, *i.e.,* a "slow" evolutionary pathway. Such are 1) interstitial sacoglossan platyhedylids that are sister to mud-digging ones; this clade is again sister to cerata-bearing *Costasiella* Pruvot-Fol, 1951 s.s. (Swennen 2001, Kohnert et al. 2013, Krug et al. 2015), 2) philinoglossids that are divergent to their stoutbodied, partially shelled sister-taxon of soft-bottom Gastropteridae and Colpodaspididae Oskars et al., 2015 that have short headshields with a posterior crest and anterior genital openings (Brown 1979, Gosliner 1989, Brenzinger et al 2013a), and 3) meiofaunal "philinids" that appear preadapted as members of burrowing, shelled cephalaspidean "Philinidae" *sensu lato* (Oskars et al. 2015). In these cases, evidence for paedomorphosis of body parts may only be found by comparative morphology of soft organs and may not be too evident.

Other interstitial taxa may have evolved following a rather "fast" scenario, with abrupt loss of the shell at some stage of evolution, thus dramatically affecting early ontogeny. This may hold true for rhodopemorphs that are sister to a shelled taxon (Wilson et al. 2010, Jörger et al. 2016) which is equally minute, slender, and has a burrowing lifestyle but otherwise highly divergent; early ontogeny of rhodopemorphs is also highly aberrant, according to Riedl (1959, 1960). It may also be valid for Acochlidia that are nested near coastal and limnic, shelled panpulmonates but whose direct Recent sister group still remains unknown (Jörger et al. 2010, 2014b). In acochlidians, original loss of the shell is also conceivable by catastrophic loss early in ontogeny as suggested by Jörger et al. (2010) who referred to the observation of an aberrant specimen of aeolid nudibranch with free visceral sac resembling that of an acochlidian (observed by Tardy 1970: fig. 20). However, pathways within Acochlidia may already be divergent: minuscule Microhedylacea may follow a "fast" route with regressive evolution and loss, equaling paedomorphosis, while only partially meiofaunal hedylopsaceans show complex organ systems and derived ecologies including peramorphosis of organ systems such as the copulatory organs (Schrödl & Neusser 2010, Jörger et al. 2014b). Furthermore, a panpulmonate relationship of Acochlidia makes them appear preadapted to their invasion of limnic habitats, unique for "opisthobranch" slugs (Brenzinger et al. 2011a, Jörger et al. 2014b).

Summarizing, it can be stated that recent advances in (molecular) phylogeny have been corroborated by detailed studies of microanatomy: genetic data is needed for a robust tree and good anatomical data to fill this tree with life. Detailed study of interstitial opisthobranchs and related taxa has provided important pieces for disentangling the puzzle of the heterobranch tree of life and, as was attempted to show in the present thesis, novel explanations can be found for the evolutionary and ecological diversity of Euthyneura. Comparative anatomy and molecular phylogenetic study can go hand in hand, filling phylogenetic trees with life.

To test these hypotheses, expanded datasets will be needed. Current taxon coverage among lower Heterobranchia remains low for any type of phylogenetic study – molecular or morphological.

Existing sets of molecular markers are still unsufficient to resolve relationships among lower heterobranch taxa because these sets of “standard” markers (*i.e.,* the five mostly used genes, see 4.2) are in many cases either incomplete or missing completely, because PCR procedures are problematic or it may be difficult to obtain specimens at all (due to the small size and remote habitats of several taxa, e.g. some Valvatoidea and Architectonicoidea). Furthermore, existing sequences of some taxa were shown to be contaminated (see 4.2) or generally show long branches, e.g. those of Architectonicoidea *sensu lato* or Rissoellidae (e.g. Jörger et al. 2010). This indicates that standard markers *per se* might be poorly suited to fully resolve phylogeny of lower heterobranchs or the crucial basal tritomy within Euthyneura.

Advances in phylogenomics, using thousands of genes sequenced from expressed sequence tags (“EST’s”), indicate that this method should be highly suitable for solving lower heterobranch relationships. In contrast to mitogenomics (Stöger & Schrödl 2013, Stöger et al. 2013), it has already yielded robust results for other heterobranchs (e.g. Kocot et al. 2013, Zapata et al. 2014). However, because it should be even more difficult to obtain adequately preserved tissue for phylogenomic study, many minute and hard-to-collect lower heterobranch taxa may still remain elusive in the future (Giribet 2016). Until then morphology, such as taken from microanatomical studies, is left as one of the more potent sources of phylogenetically valuable data.

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8 APPENDICES

Appendix 1 – Declaration of contributions to each publication

Chapter 1. Jörger KM, Neusser TP, **Brenzinger B** & Schrödl M (2014): **Exploring the diversity of mesopsammic gastropods: How to collect, identify, and delimitate small and elusive sea slugs?** *American Malacological Bulletin*, **32**(2): 290-307.

I drafted figure 1, compiled data used in table 1, partially wrote appendices 1 and 3; contributed to and discussed the final version of the article.

Chapter 2. **Brenzinger B**, Neusser TP, Jörger KM & Schrödl M (2011a) **Integrating 3D-microanatomy and molecules: natural history of the Pacific acochlidian freshwater slug *Strubellia* Odhner, 1937, with description of a new species.** *Journal of Molluscan Studies*, **77:** 351-374.

I performed morphological analysis on Solomon Islands specimens (live observation, feeding, dissection, SEM, histology, and 3D reconstruction); designed and created figures and tables; drafted and wrote the manuscript.

Chapter 3. Kohnert P, **Brenzinger B**, Jensen KR & Schrödl M (2013a): **3D-microanatomy of the semiterrestrial slug *Gascoignella aprica* Jensen, 1985--a basal plakobranchacean sacoglossan (Gastropoda, Panpulmonata).** *Organisms, Diversity and Evolution,* **13**:583-603.

I co-instructed histology and 3D reconstruction; helped draft, discuss, and write the manuscript.

Chapter 4. **Brenzinger B**, Padula V & Schrödl M (2013b): **Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Euopisthobranchia: Cephalaspidea: Philinoglossidae).** *Organisms, Diversity and Evolution*, **13:** 33-54.

I performed a large part of the 3D reconstruction (labeling, rendering); created figures, tables, and interactive 3D model; drafted and wrote the manuscript.

Chapter 5. Kubilius RA, Kohnert P, **Brenzinger B** & Schrödl M (2014): **3D-microanatomy of the straight-shelled pteropod *Creseis clava* (Gastropoda: Heterobranchia: Euthecosomata).** *Journal of Molluscan Studies*, **80**:585-603.

I co-instructed histology and 3D reconstruction; helped draft, discuss, and write the manuscript.

Chapter 6. Martynov AV, **Brenzinger B**, Hooker Y & Schrödl M (2011): **3D-anatomy of a new tropical Peruvian nudibranch gastropod species, *Corambe mancorensis*, and novel hypotheses on dorid gill ontogeny and evolution.** *Journal of Molluscan Studies*, **77:** 129-141.

I performed parts of the morphological analysis (SEM; histology, 3D reconstruction); created figures 1, 2A, 3, 4 and 5; wrote parts of the results section and discussed the manuscript.

Chapter 7. **Brenzinger B**, Wilson NG & Schrödl M (2011): **3D microanatomy of a gastropod 'worm', *Rhodope rousei* n. sp. (Heterobranchia) from Southern Australia.** *Journal of Molluscan Studies*, **77:** 375-387.

I performed all morphological analysis (histology and 3D reconstruction); designed and created figures and tables; drafted and wrote the manuscript.

Chapter 8. **Brenzinger B**, Haszprunar G & Schrödl M (2013): **At the limits of a successful body plan–3D microanatomy, histology and evolution of *Helminthope* (Mollusca: Heterobranchia: Rhodopemorpha), the most worm-like gastropod.** *Frontiers in Zoology*, **10:** doi:10.1186/1742-9994-10-37.

I performed all morphological analysis (histology and 3D reconstruction); designed and created figures, tables, and the interactive 3D model; drafted and wrote the manuscript.

Chapter 9. **Brenzinger B**, Wilson NG & Schrödl M (2014): **Microanatomy of shelled *Koloonella* cf. *minutissima* (Laseron, 1951) (Gastropoda: 'lower' Heterobranchia: Murchisonellidae) does**

not contradict a sister-group relationship with enigmatic Rhodopemorpha slugs. *Journal of Molluscan Studies*, **80**(5): 518-540.

I performed all morphological analysis (histology and 3D reconstruction); designed and created figures, tables, and the interactive 3D model; drafted and wrote the manuscript.

Chapter 10. Kano Y, **Brenzinger B**, Nützel A, Wilson NG & Schrödl M. **Ringiculid bubble snails recovered as the sister group to sea slugs (Nudipleura).** *Scientific Reports (Nature group),* in review (revised version).

I performed morphological analysis (histology and 3D reconstruction), provided rendered images, drawings and photographs for figures; partially designed and created figures 1 and 3; wrote parts of the manuscript (on morphology), discussed and commented on the final manuscript.

I hereby confirm the above statement. Munich, July 8, 2016

(Dipl.-Biol. Bastian Brenzinger) (Prof. Dr. Michael Schrödl)

Appendix 2 – List of publications

Publications in peer-reviewed journals (sorted chronologically)

1. Martynov AV, **Brenzinger B**, Hooker Y & Schrödl M (2011): **3D-anatomy of a new tropical Peruvian nudibranch gastropod species, *Corambe mancorensis*, and novel hypotheses on dorid gill ontogeny and evolution.** *Journal of Molluscan Studies*, **77:** 129-141. [Chapter 6 of this thesis]
2. **Brenzinger B**, Neusser TP, Glaubrecht M, Haszprunar G & Schrödl M (2011). **Redescription and three-dimensional reconstruction of the limnic acochlidian gastropod *Strubellia paradoxa* (Strubell, 1892) (Gastropoda, Euthyneura) from Ambon, Indonesia.** *Journal of Natural History*, **45** (3): 183-209.
3. **Brenzinger B**, Neusser TP, Jörger KM & Schrödl M (2011) **Integrating 3D-microanatomy and molecules: natural history of the Pacific acochlidian freshwater slug *Strubellia* Odhner, 1937, with description of a new species.** *Journal of Molluscan Studies*, **77:** 351-374. [Chapter 2]
4. **Brenzinger B**, Wilson NG & Schrödl M (2011): **3D microanatomy of a gastropod 'worm', *Rhodope rousei* n. sp. (Heterobranchia) from Southern Australia.** *Journal of Molluscan Studies*, **77:** 375-387. [Chapter 7]
5. **Brenzinger B**, Padula V & Schrödl M (2013): **Insemination by a kiss? Interactive 3D-microanatomy, biology and systematics of the mesopsammic cephalaspidean sea slug *Pluscula cuica* Marcus, 1953 from Brazil (Euopisthobranchia: Cephalaspidea: Philinoglossidae).** *Organisms, Diversity and Evolution*, **13:** 33-54. [Chapter 4]
6. **Brenzinger B**, Haszprunar G & Schrödl M (2013): **At the limits of a successful body plan–3D microanatomy, histology and evolution of *Helminthope* (Mollusca: Heterobranchia: Rhodopemorpha), the most worm-like gastropod.** *Frontiers in Zoology*, **10:** doi:10.1186/1742-9994-10-37. [Chapter 8]
7. Kano Y, Fukumori H, **Brenzinger B** & Warén A (2013): **Driftwood as a vector for the oceanic dispersal of estuarine gastropods (Neritidae) and an evolutionary pathway to the sunken-wood community.** *Journal of Molluscan Studies*, 79(4): 378-382.
8. Kohnert P, **Brenzinger B**, Jensen KR & Schrödl M (2013): **3D- microanatomy of the semiterrestrial slug *Gascoignella aprica* Jensen, 1985--a basal plakobranchacean sacoglossan (Gastropoda, Panpulmonata).** *Organisms, Diversity and Evolution,* **13**:583-603. [Chapter 3]
9. **Brenzinger B**, Wilson NG & Schrödl M (2014): **Microanatomy of shelled *Koloonella* cf. *minutissima* (Laseron, 1951)(Gastropoda: 'lower'Heterobranchia: Murchisonellidae) does not contradict a sister-group relationship with enigmatic Rhodopemorpha slugs.** *Journal of Molluscan Studies*, **80**(5): 518-540. [Chapter 9]
10. Kubilius RA, Kohnert P, **Brenzinger B** & Schrödl M (2014): **3D-microanatomy of the straight-shelled pteropod *Creseis clava* (Gastropoda: Heterobranchia: Euthecosomata).** *Journal of Molluscan Studies*, **80**:585-603. [Chapter 5]
11. Koller K, **Brenzinger B** & Schrödl M (2014): **A caenogastropod in 3D: microanatomy of the Munich endemic springsnail *Sadleriana bavarica* Boeters, 1989.** *Spixiana*, **37**(1): 1-19.
12. Jörger KM, Neusser TP, **Brenzinger B** & Schrödl M (2014): **Exploring the diversity of mesopsammic gastropods: How to collect, identify, and delimitate small and elusive sea slugs?** *American Malacological Bulletin*, **32**(2): 290-307. [Chapter 1]
13. Jörger KM, **Brenzinger B**, Neusser TP, Martynov AV, Wilson NG & Schrödl M (2014): **Panpulmonate habitat transitions: tracing the evolution of Acochlidia (Heterobranchia, Gastropoda).** *bioRxiv*, 010322.
14. Kano Y, **Brenzinger B**, Nützel A, Wilson NG & Schrödl M. **Ringiculid bubble snails recovered as the sister group to sea slugs (Nudipleura).** *Scientific Reports (Nature group),* in review (revised version). [Chapter 10]

Further publications

15. **Brenzinger B** (2015): **Identification guide to Northern European interstitial opisthobranchs (Gastropoda: Heterobranchia). Version 2.1, with Key appendix.** *NMBAQC 2014 taxonomic workshop, Dove Marine Laboratory, August 2015.* 26pp. Available online at http://www.nmbaqcs.org/scheme-components/invertebrates/literature-and-taxonomic-keys.aspx

Congress contributions

16. **Brenzinger B,** Neusser TP & Schrödl M (2008): **The 3D microanatomy and redescription of the acochlidian *Strubellia paradoxa* (Gastropoda, Opisthobranchia).** IN: Abstracts of the *1st International Congress on Invertebrate Morphology, Copenhagen, Denmark*. *Journal of Morphology,* **269**(12): 1488. [Poster]
17. **Brenzinger B,** Neusser TP, Jörger KM & Schrödl M (2010): **120 years after Strubell: 3D microanatomy and biology of the limnic acochlidian slug *Strubellia* (Odhner).** *World Congress of Malacology, Phuket, Thailand. Tropical Natural History*, **Suppl. 3**: 48. [Talk]
18. **Brenzinger B,** Wilson NG & Schrödl M (2010): **3D Reconstruction of a gastropod "worm": *Rhodope* sp. from Southern Australia (Heterobranchia: Euthyneura).** *World Congress of Malacology, Phuket, Thailand. Tropical Natural History*, **Suppl. 3**: 269. [Poster]
19. Schrödl M, **Brenzinger B,** Hooker YM & Martynov AV (2010): **Turning Haeckel's Law up side down? Morphology and evolution of corambid sea slugs.** *World Congress of Malacology, Phuket, Thailand. Tropical Natural History*, **Suppl. 3**: 42. [Talk]
20. **Brenzinger B,** Haszprunar G & Schrödl M (2010): **The most worm-like gastropod: Microanatomy of *Helminthope psammobionta* Salvini-Plawen, 1991 (Heterobranchia, Rhodopemorpha).** *Third International Workshop on Opisthobranchs, Vigo 2010 – Book of Abstracts. J.S. Troncoso, J. Moreira & G. Díaz-Agras, eds., UVIGO & EBMG-USC. Vigo 2010*, **Suppl**: 20. [Talk]
21. Neusser TP, **Brenzinger B,** Fukuda H, Jörger KM & Schrödl M (2010): **Sacoglossa or Acochlidia? 3D micromorphology of Aitengidae** [abstract]. *Third International Workshop on Opisthobranchs, Vigo 2010 – Book of Abstracts. J.S. Troncoso, J. Moreira & G. Díaz-Agras, eds., UVIGO & EBMG-USC. Vigo 2010*, **Suppl**: 25. [Talk]
22. Jörger KM, **Brenzinger B** & Schrödl M (2011): **Sneaking into the meiofaunal world – evolution and adaptations in microslugs (Heterobranchia, Gastropoda)**. In: *2nd International Congress on Invertebrate Morphology, Museum of Comparative Zoology, Harvard University, Program & Abstracts,* **Suppl**: 84. [Talk]
23. Neusser TP, **Brenzinger B,** Jörger KM & Schrödl M (2012): **Small molluscs – big insights: the mesopsammic contribution to malacology.** [abstract] In: *Abstracts of the 78. Annual Meeting of the American Malacological Society*. Edited by P. Callomon, G. Rosenberg & A.S. Lawless. Cherry Hill, New Jersey, USA. **Suppl**: 63 [Talk].
24. Jörger KM, Schrödl M, **Brenzinger B** & Neusser TP (2013): **Back to the sea? First ontogenetic data of limnic slugs (Acochlidia, Heterobranchia).** *Abstracts of the BioSyst.EU 2013 Global Systematics, Vienna, 2013:* 103 [Poster]
25. **Brenzinger B,** Neusser TP, Glaubrecht M, Jörger KM & Schrödl M (2013): **A new piece in the puzzle for the riverine slugs of the Acochlidiidae (Panpulmonata: Acochlidia).** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, **Suppl**. 8: 193 [Poster]
26. **Brenzinger B,** Wilson NG & Schrödl M (2013): **A glimpse into early heterobranch evolution – microanatomy of Murchisonellidae.** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, **Suppl**. 8: 180. [Talk]
27. Neusser TP, **Brenzinger B,** Strong EE & Schrödl M (2013): **The enigma from the deep – integrative study of a remarkable heterobranch sea slug from off Mozambique.** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, **Suppl**. 8: 181. [Talk]

28. Kohnert P, Hein P, Kubilius RA, **Brenzinger B** & Schrödl M (2013): **Addressing thecosome pteropods (Gastropoda: Euopisthobranchia) soft bodies in 3D microanatomical detail.** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, **Suppl**. 8: 187. [Talk]
29. Jörger KM, **Brenzinger B,** Schrödl M, Andrade SCS, Giribet G & Riesgo A (2013): **Slug spicules: adding insights from histology and transciptome analyses to the already complex picture of biomineralization in Gastropoda.** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, **Suppl**. 8: 193. [Poster]
30. Jörger KM, Neusser TP, **Brenzinger B** & Schrödl M (2013): **Out of the marine mesopsammon, into the (pan) pulmonate world: historical biogeography and habitat shifts in the evolution of acochlidian slugs.** *World Congress of Malacology, Ponta Delgada, Açores, July 22-28. Açoreana*, Suppl. **8**: 139. [Talk]
31. **Brenzinger B,** Neusser TP, Glaubrecht M, Jörger KM & Schrödl M (2013): **A new piece in the puzzle for the riverine slugs of the Acochlidiidae (Panpulmonata: Acochlidia).** Abstracts of the *106th Annual Meeting of the German Zoological Society, Munich September 2013*: 67 [Poster]
32. **Brenzinger B,** Kano Y & Schrödl M (2014): **Headshield anatomy in the 'cephalaspid' gastropod *Ringicula*, and its context in the phylogeny of Heterobranchia.** *Abstracts of ICIM3 – 3rd International Congress of Invertebrate Morphology, Berlin, 3rd to 7th August 2014,* 274 [Poster]
33. Jörger KM, **Brenzinger B,** Heß M, Schrödl M & Neusser TP (2014): **Tales of miniaturization and progenesis: a comparative histological and ultrastructural approach to investigate morphological convergence in mesopsammic slugs.** *Abstracts of ICIM3 – 3rd International Congress of Invertebrate Morphology, Berlin, 3rd to 7th August 2014,* 162 [Talk]
34. Laibl C, Kohnert P, **Brenzinger B** & Schrödl M (2014): **The "Disko sea butterfly": 3D-reconstruction and anatomy of the thecosome pteropod *Limacina helicina* (Gastropoda, Euopisthobranchia, Thecosomata) from Disko Bay, Greenland.** *Abstracts of ICIM3 – 3rd International Congress of Invertebrate Morphology, Berlin, 3rd to 7th August 2014,* 239 [Poster]
35. **Brenzinger B,** Schrödl M, Nützel A, Wilson NG & Kano Y (2015): **Shells versus sequences? Origin of the "architectibranch" Ringiculidae.** *Abstracts of the 5th International Workshop on Opisthobranchs, ICBAS-UP, Porto (Portugal) – July, 2015*, 34. [Talk]
36. Biging A, **Brenzinger B,** Neusser TP, Schrödl M & Jörger KM (2015): **Island hopping along the Indo-Pacific Archipelago – Molecular species delineation and biogeography in the freshwater slug *Acochlidium* (Acochlidia, Heterobranchia).** *Abstracts of the 5th International Workshop on Opisthobranchs, ICBAS-UP, Porto (Portugal) – July, 2015*, 52. [Poster]
37. Hübner A, Biging A, **Brenzinger B,** Jörger KM, Schrödl M & Neusser TP (2015): **An integrative approach to the diversity of the limnic slug *Acochlidium* (Gastropoda, Panpulmonata, Acochlidia).** *Abstracts of the 108th Annual Meeting of the German Zoological Society, Graz, September 9-12, 2015* - 120 [Poster]
38. **Brenzinger B,** Kano Y & Schrödl M (2015**): A small snail with a difficult name, and implications for the heterobranch tree of life.** *Abstracts of the Molluscan Forum 2015, held Natural History Museum London, 19th November 2015,* 6 [Talk]
39. **Brenzinger B,** Kano Y & Schrödl M (2016): **A new superfamilial taxon of heterobranch snails, and implications for the euthyneuran gastropod tree of life.** *Abstracts of the 17th Annual Meeting of the Gesellschaft für Biologische Systematik, 21.-24.2 February 2016, Munich.* In*: Zitteliana* **88***:* 16*.* [Talk]
40. Jörger KM, **Brenzinger B,** Wilson NG, Worsaae K & Schrödl M (2016): **The worms among the gastropods: phylogeny and evolution of Rhodopemorpha (Heterobranchia, Gastropoda).** *Abstracts of the 17th Annual Meeting of the Gesellschaft für Biologische Systematik, 21.-24.2 February 2016, Munich.* In*: Zitteliana* **88***:* 29*.* [Talk]