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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

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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).

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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

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	Three families
+ Euthyneura Spengel, 1881	

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).

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.

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 & Schrödl, 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 MgCl₂ 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 μ m; section thickness 1.5 μ m) and one juvenile (250 μ m; 1 μ m). 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 μ m, in juvenile 250 μ m. 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 greyishwhite. 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 $5-8 \,\mu\text{m}$ thick and ciliated on headfoot and in mantle cavity (Fig. 5B), $2-3 \,\mu\text{m}$ thick and unciliated on visceral sac and in caecum of mantle cavity. Band of particularly strong cilia (15 μ m 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 μ m 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 $30-35 \ \mu\text{m}$) in posterior foot and below posterior tip of mantle cavity (Fig. 6B, white cell in 7M); cluster of smaller cells (diameter $10-15 \ \mu\text{m}$) 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: mul 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 $10-25 \,\mu$ m), 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 μ m long groove with voluminous, light pink-staining cells (30 μ m 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 μ m 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 μ m 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-bluestaining 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 $\mu m \times 20 \ \mu m$), 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 μ m) and spherical, blue-stained nucleolus (7 μ m). 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; ccl, calcium cells below caecum of mantle cavity; cc2, calcium cells on neck; cc3, calcium cells in foot; cae, caecum of mantle cavity; cm, cerebral commissure; cpc, cerebropleural connective; cpg, (left) cerebropleural ganglion; cr, columellar ridge; ct, cephalic 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); mul, 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, operculuar groove; osp, putative osphradium; pcm, pedal commissure; g, (left) pedal ganglion; pi, pigment granules on neck and head; pp, propodium; ppg, 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; ph, 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; fg0, female gland 2; mg0, male gland; g2, ring-shaped mantle gland; gd, hermaphroditic part of gonoduct; mg1, male gland 1; mg2, male gland 2; mg0, 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** = $250 \ \mu m$; **C**, **D** = $100 \ \mu m$; **E**, **F** = $50 \ \mu m$.

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 $(5-6 \ \mu\text{m})$ (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 $(1-2 \ \mu\text{m})$ 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 $(30 \ \mu\text{m})$ with large nucleus $(14 \ \mu\text{m})$ 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 arrowhead indicate clear rods inside odontophore. **D.** Head at level of female genital opening (arrowhead). White arrowhead marks duplicate mantle border (shell gland). **F.** Section at level of female genital opening (arrowhead). White arrowhead marks duplicate mantle tentacle. Asterisk mark position of mantle cavity. **G.** Section at level of glandular pocket in mantle cavity. White asterisks mark position of mantle cavity. Abreviations: 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 μ m except **A** = 250 μ m; **F** = 10 μ m.



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; ocl, 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; fgl, first female gland (putative albumen gland); fg2, second female gland (putative membrane gland); fg3, zones of third female gland (putative mucus gland); fg0, female genital opening; g1, tubular mantle gland; g2, ring-shaped mantle gland; g3, gland in mantle roof; q4, 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 l, 'male' gland 1 (putative seminal receptacle); mg2, 'male' gland 2; mg0, male genital opening; ml, mantle lobe; 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 ¹Warén (1995), ²Warén (2013), ³Rasmussen (1944), ⁴Wise (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 largerbodied 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-vellow 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 wide-spread 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 eves 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 reproduct-ive 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 spermatophoreforming 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 shortterm 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 brack-ish 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 rhodopemorphmurchisonellid 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 murchisonellidrhodopemorph 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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