COMPARATIVE MICROANATOMY AND ULTRASTRUCTURE OF THE EXCRETORY SYSTEMS OF OPISTHOBRANCH GASTROPODA (MOLLUSCA)

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Alexander Fahrner
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Gutachter: Prof. Dr. Gerhard Haszprunar
PD Dr. Ralph Tollrian

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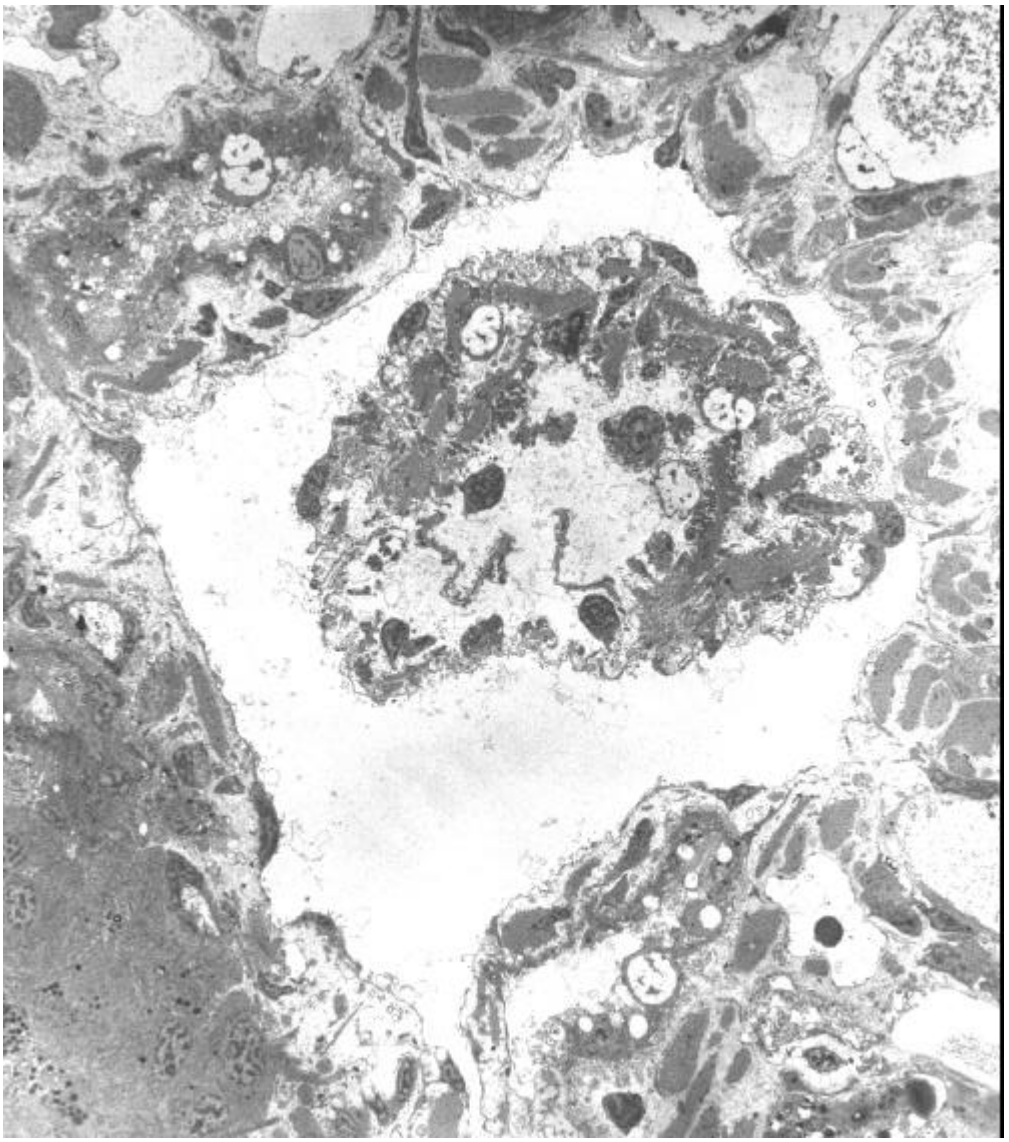
GENERAL REVIEW



SUMMARY

This comparative study comprises detailed anatomical and ultrastructural investigations of the excretory organs of opisthobranch gastropods by means of serial sectioning analyses, reconstruction techniques, and transmission electron microscopy (TEM). Representatives of major taxa, the Cephalaspidea, Thecosomata, Gymnosomata, Sacoglossa, Acochlidia, and of the anthobranch and cladobranch Nudibranchia, are examined to elucidate the basal condition of the excretory system of the Opisthobranchia. Particular reference is given to the ultrafiltration structures in the respective taxa and to possible modifications of the excretory system in species of special interest, i.e. without a pericardium or paedomorphic species. The results enable significant conclusions regarding the evolution of the molluscan excretory systems and the phylogenetic relationships within the Opisthobranchia.

In general, the adult Opisthobranchia show a metanephridial excretory system structurally consisting of podocytes in the pericardial epithelium and a single large kidney which is connected with the pericardium by a ciliated renopericardial duct. The podocytes with numerous basal processes and filtration slits, bridged by fine diaphragms, represent the ultrafiltration site. They are restricted to the epicardium of the auricle in the Thecosomata, Gymnosomata, Sacoglossa, and Acochlidia. In the Nudibranchia, podocytes additionally line the entire outer pericardial epithelium and, in the nudibranch subgroup Cladobranchia, also the ventricular wall. The Cephalaspidea lack true podocytes. Instead, special slashed cells (“podocyte-like cells” without diaphragms) with the capacity to form an ultrafiltration barrier adopted the podocyte function. These ultrafiltration cells form the entire epicardium and are also interspersed between the squamose cells of the outer pericadial epithelium in the investigated species *Runcina coronata.* In the heart-less sacoglossan species *Alderia modesta,* no podocytes or other epithelial cells with ultrafiltration capacity could be found at all. In most of the examined taxa the epithelium of the renopericardial duct is build up by two cell types: whereas multiciliated cells line the openings of the renopericardial duct towards the pericardium and the kidney, the cells of the central section with an apical microvillous border lack cilia. In the thecosome *Creseis virgula* and the nudibranch *Cuthona caerulea*, the pericardial cavity opens directly into the kidney via a ciliated funnel, a distinct renopericardial duct is absent.

The kidney epithelium of the Opisthobranchia is composed of cells of one single type that are characterized by large vacuoles, extensive basal infoldings, and an apical microvillous border, indicating both secretory and reabsorptive activity. Via a nephropore, the kidney opens directly to the exterior in most of the taxa or into a distinct mantle cavity in *Creseis*

virgula and the acochlidian *Hedylopsis* sp.. Solitary rhogocytes (pore cells) of the connective tissue and haemocoel could be detected in all species investigated except of the thecosome *Creseis virgula.* These cells represent additional loci of ultrafiltration with a fine-structure identical to that of the podocytes (slits between cytoplasmatic processes, bridged by fine diaphragms and covered by extracellular matrix).

The ultrastructural evidence on the renopericardial complex of the Opisthobranchia reveals that its structure and organization generally corresponds to that of other molluscs. Podocytes situated in the epicardial wall of the auricle as the sole site of ultrafiltration are regarded as plesiomorphic for the Mollusca and confirmed for the Opisthobranchia in this study, contradicting elder assumptions of the loss of podocytes in the ancestors of the Opisthobranchia. The absence of true podocytes and presence of a modified ultrafiltration cell-type in the Cephalaspidea s.s. does not reflect the basal condition of the Opisthobranchia, but the podocyte-like cells probably represent an autapomorphy of this taxon. The additional, extensive and separate ultrafiltration site in the pericardial wall of *Hypselodoris tricolor* and *Cuthona caerulea* is unique among the Gastropoda and represents a significant autapomorphy either of the Nudibranchia or of the Nudipleura. In contrast to other heart-less or paedomorphic species with pseudoprotonephridial or secondary protonephridial systems, the investigated heart-less *Alderia modesta* and the partly paedomorphic gymnosome and thecosome species show no further modifications of the metanephridial system. The organization of the excretory system of *A. modesta* proves that ultrafiltration is no prerequisite for effective excretion in the Mollusca. The presence of a reduced, yet distinct mantle cavity in *Hedylopsis* sp. has considerable implications on the reconstruction of the origin of the Acochlidia and puts the Hedylopsidae on the basis of the taxon.

1. INTRODUCTION

The structure and homology of the excretory organs among the Bilateria have been a matter of long lasting controversy (for historical review, see Goodrich 1945) that has been revived by the application of electron microscopy. Until recently, authors stressed fundamental differences between proto- and metanephridia and emphasized that the metanephridia evolved independently several times (Bartolomaeus and Ax 1992; Salvini-Plawen and Bartolomaeus 1995; Bartolomaeus 1997). In contrast, comparative ultrastructural studies suggested cytological homology between protonephridial terminal cells (cyrtocytes or solenocytes) and metanephridial podocytes (Ruppert and Smith 1988; Smith and Ruppert 1988; Smith 1992; Ruppert 1994). Haszprunar (1996) broadened this concept of a continuum between different types of ultrafiltration cells by including the nephrocytes of the Arthropoda and the molluscan rhogocytes (pore cells).

The Mollusca represent an ideal group to examine the variability and evolution of excretory systems, since (1) all taxa possess the solitary rhogocytes, (2) their larvae show protonephridial systems (Brandenburg 1966; Bartolomaeus 1989; Ruthensteiner and Schaefer 1991; Tardy and Dongard 1995; Haszprunar and Ruthensteiner 2000), and (3) the adults usually possess a metanephridial system in the sense of Ruppert and Smith (1988). Moreover, coelomatic characters, such as those that relate to the metanephridial system, are crucial concerning phylogenetic analyses of the origin and evolution of the Mollusca (Salvini-Plawen 1985; Ghiselin 1988; Willmer 1990). A general character of the Mollusca is the close ontogenetic and functional interrelation of the pericardium and the kidneys in excretion (Andrews 1988; Morse and Reynolds 1996). The so-called renopericardial complex (see Haszprunar 1992) consists of coelomatic (blastomere 4d) derivatives, the endothelially lined pericardium and, originally, two simple pericardial ducts leading to the exterior. The latter serve additionally as gonoducts in the aplacophoran Solenogastres and Caudofoveata. In the stem lineage of the Testaria (Polyplacophora and Conchifera), the distal parts of the pericardial ducts were enlarged and modified into the more complex, often sac-like kidneys. Pericardium and kidneys are interconnected to varying degrees in the different molluscan taxa (for review, see e.g. Martin 1983).

As has been demonstrated experimentally, the primary urine is produced initially by ultrafiltration of the haemolymph through the pericardial wall of the heart, the epicardium, into the pericardial cavity (Hevert 1984; Andrews and Taylor 1988). Via the renopericardial ducts, the ultrafiltrate drains off into the kidney where it is modified by reabsorption and

secretion (Martin 1983). Finally, a nephropore releases the urine into the mantle cavity, from where it is expelled by oriented water currents (Fretter and Graham, 1962; Morton, 1988). The site of ultrafiltration of the haemolymph, fine-structurally characterized by the presence of podocytes, extends from its plesiomorphic position at the auricular epicardium (Andrews 1988; Morse and Reynolds 1996) to the ventricular epicardium (Andrews 1988; Ruppert and Smith 1988; Bartolomaeus and Ax 1992) and to parts or appendages of the pericardial wall (Andrews and Jennings 1993; Meyhöfer *et al.* 1985; Schipp and Hevert 1981). Podocytes possess numerous basal processes between which ultrafiltration slits, bridged by fine diaphragms, provide a pathway for the primary filtrate molecules. The basal lamina underlying the slits has been shown to be the principal ultrafilter (Andrews 1981; Morse 1987; Meyhöfer and Morse 1996).

Numerous characters of the molluscan excretory system can only be discovered and elucidated by the application of transmission electron microscopy (TEM), i.e. the structural details and the position of the podocytes, the fine-structure of the extracellular matrix supporting the ultrafiltration site, and the cytomorphology of the kidney cells. Such studies of the excretory system at the ultrastructural level have been undertaken on all higher taxa of the Mollusca (see e.g. Andrews 1988; Morse and Reynolds 1996; Haszprunar and Schaefer 1997a). However, the extent of ultrastructural variation within these groups is still poorly known. Until recently, studies on the renopericardial complex of the Gastropoda have been focused largely on several groups of the Prosobranchia and the Pulmonata (for reviews, see Andrews 1981, 1988; Luchtel *et al.* 1997) while ultrastructural evidence from the Opisthobranchia has been absent (Martin 1983; Gosliner 1994). Andrews (1988) published some preliminary observations on the excretory system of two cephalaspidean species indicating the complete absence of podocytes and a simplification of the kidney cells (i.e. absence of basal infoldings). She therefore presumed that the primary site of ultrafiltration in the auricular epicardium probably was lost in the common ancestor of the Opisthobranchia and Pulmonata and that podocyte function has been adopted by other cell types (Andrews 1988). The only detailed data on the ultrastructure of opisthobranch excretory systems hitherto available referred to two small and aberrant species: (1) In the mesopsammic, partly paedomorphic, cephalaspidean *Philinoglossa helgolandica* Hertling, 1932, the basal excretory system of the Mollusca is modified in that the site of ultrafiltration moved to a part of the outer pericardial wall facing the kidney. In addition, true podocytes are absent; instead, other special slashed cells without diaphragms (“podocyte-like cells”) enable the filtration of the haemolymph in this species (Bartolomaeus 1997). (2) The worm-like, enigmatic *Rhodope*

transtrosa Salvini-Plawen, 1991 lacks a heart and shows an entirely new, pseudo-protonephridial ultrafiltration system (Haszprunar 1997). These data suggest that other opisthobranch taxa, in particular those that lack the primary organ of ultrafiltration, the pericardium, as well as mesopsammic or paedomorphic species, probably also exhibit significant modifications of the original excretory system.

This study presents an extensive comparative description of the anatomical and ultrastructural features associated with the excretory organs of the Opisthobranchia. Representatives of major taxa are examined (see Tab. 1), with particular regard to the following objectives: (1) To ascertain the basal condition of the ultrafiltration-structures (i.e. the presence or absence of podocytes, the position of ultrafiltration cells within the pericardium and their fine-structural details) in the respective taxa, establish a TEM database, and, from that, deduce a basal plan of the excretory system of the Opisthobranchia. (2) To describe modifications of the excretory organs and cell types in species without a pericardium. Do completely new structures such as the pseudo-protonephridium in *Rhodope transtrosa* occur? (3) To study the effects of paedomorphosis on the excretory system at the microanatomical and ultrastructural level. Can secondary protonephridia be found, as described for echiurids (dwarf males of *Bonellia*; see Schuchert 1990) and polychaetes (i.e. the progenetic *Hesionides arenaria*; see Westheide 1986)? (4) To evaluate the relevance and possible implications of ultrastructural features of the excretory system for phylogenetic relationships within the Opisthobranchia. Can new and significant apomorphies for higher taxa be obtained?

2. MATERIAL AND METHODS

Representatives of almost all major taxa of the Opisthobranchia were investigated by means of serial sectioning analyses and transmission electron microscopy (TEM). A brief overview on the species examined and their collection localities and data is given in Table 1. For detailed descriptions, see the relevant appendices.

All specimens collected were relaxed by slowly adding a solution of isotonic (about 7%) $MgCl_2$ to the seawater before they were processed for light microscopy (LM) and TEM. Fixation in 4 % seawater buffered formalin (LM) or 4 % glutardialdehyde (LM and TEM) buffered in 0.2 M sodium cacodylate (pH 7.2) was followed by a rinse in the same buffer in decreasing concentrations of the latter. After postfixation in buffered 1 % OsO_4 for two hours,

Table 1. List of the opisthobranch taxa investigated by TEM and their collecting data.

Major taxon	Species	Collection data	Appendix
Cephalaspidea	*Runcina coronata* (Quatrefages, 1844)	Mediterranean Sea and North Sea: Calvi, France; June 1992 and Plymouth, England; July 1993	VI
Thecosomata	*Creseis virgula* Rang, 1828	Mediterranean Sea: Elba, Italy; June 1998	I
Gymnosomata	*Pneumoderma* sp.	Mediterranean Sea: Calvi, France; June 1997	I
Sacoglossa	*Bosellia mimetica* Trinchese, 1890	Mediterranean Sea: Calvi, France; June 1997 and Elba, Italy; June 1998	II
	Alderia modesta (Lovén, 1844)	Eastern Pacific Ocean: San Diego, California, USA, Oct. 1996	
Acochlidia	*Hedylopsis* sp.	Indian Ocean, Red Sea: Gulf of Aqaba, Egypt; Oct. 1999	III
Nudibranchia, Doridoidea	*Hypselodoris tricolor* (Cantraine, 1835)	Mediterranean Sea: Rovinj, Croatia; July 1993 and Elba, Italy; June 1998 and July 2001	IV
Nudibranchia, Aeolidoidea	*Cuthona caerulea* (Montagu, 1804)	Mediterranean Sea: Banyuls-sur-Mer, France; June 1999	V

the specimens were rinsed again with cacodylate buffer and dehydrated in a graded series of ethanols. The fixed specimens were embedded overnight in paraplast or Araldit resin for LM and in Spurr`s (1969) low viscosity resin for TEM.

In order to examine the gross anatomy of the excretory systems, complete series of semithin sections (2µm) of all species were made with “Ralph” glass knifes and contact cement (“Pattex compact”) at the lower cutting edge (Henry 1977), then stained with methylene-blue – azure II according to Richardson *et al.* (1960). Serial sections of large,

paraplast-embedded specimens (8µm) were stained with Azan (see Romeis 1989). All histological slides are deposited at the malacology section of the Zoologische Staatssammlung München (see relevant appendices for registration numbers), selected slides were photographed on a Leica DM RBE compound microscope with a Kappa DX30 digital camera. For TEM, ultrathin sections (70 nm) of at least two specimens of each species were made with glass knives or a diamond knife and kept on formvar-covered, single slot copper grids. The sections were stained automatically with uranyl acetate and lead citrate (Reynolds 1963) and examined and photographed with a Philips CM 10 TEM at 80 kV.

Reconstructions of the examined excretory systems were prepared by hand, based on serial, semi-thin cross sections. The edges of the non-dissolved plastic sections or the outlines of the specimens, photographed prior to sectioning, served as reference scales for measurements.

3. RESULTS

This review provides a summary of the results given in detail in the Appendices I-VI, data are cited according to the following example: III: fig. 2B, p. 84 = Appendix III, Fig. 2B, page 84.

3.1. General anatomy

The excretory system of the Opisthobranchia (the renopericardial complex) consists of the pericardium, which is partly composed of podocytes, and a single, large kidney that is connected with the pericardium by a renopericardial duct. In the species examined (see Fig. 1 and Table 2), the thin spacious pericardium enclosing the single auricle and ventricle of the heart is either placed laterally, on the right side of the body in *Runcina coronata* (Quatrefages, 1844) (VI: figs. 1, p. 128; 2, p. 129), *Creseis virgula* Rang, 1828 (I: fig. 4, p. 54), *Pneumoderma* sp. (I: fig. 1, p. 50), and *Hedylopsis* sp. (III: figs. 1, p. 83; 2, p. 84), or mediodorsally in *Bosellia mimetica* Trinchese, 1890 (II: figs. 1, p. 67; 2, p. 68), *Hypselodoris tricolor* (Cantraine, 1835) (IV: figs. 1, p. 100; 2, p. 101), and *Cuthona caerulea* (Montagu, 1804) (V: fig. 1, p. 118). The pericardium occurs either anteriorly, in the vicinity of the anterior end of the kidney (*Bosellia mimetica, Hedylopsis* sp., and *Cuthona caerulea*), or in the posterior body half, close to the posterior end of the kidney (*Runcina coronata, Creseis virgula, Pneumoderma* sp., and *Hypselodoris tricolor*). However, the pericardium generally extends directly below the notum covering the underlying kidney. The typical orientation of the heart is along the longitudinal body axis, with the auricle lying posteriorly to the ventricle, only the thecosome *Creseis virgula* shows a vertically orientated heart with a ventral auricle and a dorsal ventricle lying side by side (I: fig. 4B, p. 54). The sacoglossan species *Alderia modesta* (Lovén, 1844) lacks heart and pericardium and its circulatory system shows only several haemocoelic sinuses.

The pericardial cavity opens ventrally in all taxa. It may drain into a long renopericardial duct that enters the kidney from dorsal or lateral (*Pneumoderma* sp., *Hedylopsis* sp., and *Hypselodoris tricolor*, see IV: fig. 2B,C, p. 101) or, alternatively, it opens directly into the kidney through a ciliated funnel, the nephrostome (*Creseis virgula, Bosellia mimetica* - see II: fig. 2E, p. 68 -, and *Cuthona caerulea*) (see Table 2). Thus, a distinct and long renopericardial duct is absent in the latter species. *Runcina coronata* is the only species that shows a short renopericardial duct. Whereas the ventral opening of the pericardium

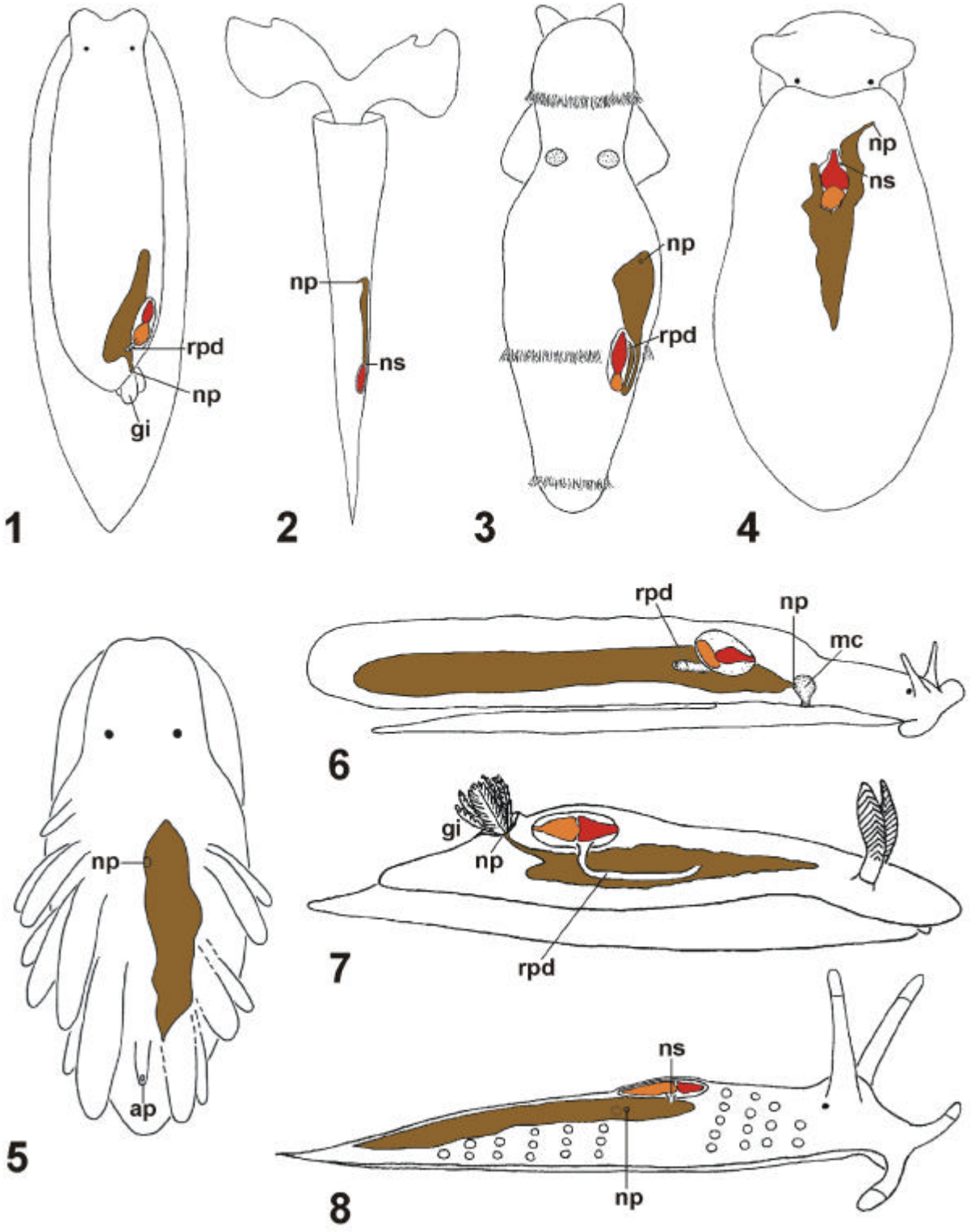


Fig. 1. Semi-schematic drawings of the species investigated (not to scale), showing the relative position and arrangement of the renopericardial complex. **1** *Runcina coronata* (2 mm), **2** *Creseis virgula* (3.5 mm), **3** *Pneumoderma* sp. (1.5 mm), **4** *Bosellia mimetica* (3 mm), **5** *Alderia modesta* (5 mm), **6** *Hedylopsis* sp. (2.5 mm), **7** *Hypselodoris tricolor* (15 mm), **8** *Cuthona caerulea* (10 mm).
Brown colour: kidney, orange-red colour: auricle, dark red colour: ventricle, *ap* anal papilla, *gi* gills, *mc* mantle cavity, *np* nephropore, *ns* nephrostome, *rpd* renopericardial duct.

Table 2. Distribution of characters of heart/pericardium and nephrostome/renopericardial duct in the investigated species. H/Pc: heart/pericardium; Nephrostome: direct opening of pericardium in kidney; Long rpd: long renopericardial duct composed of two cell types; Short rpd: short renopericardial duct composed of only one cell type; + present; - absent; longitudinal: along the longitudinal body axis, vertical: along the vertical body axis.

Taxon	H/Pc	Orientation of heart	Position of heart	Position in cross section	Nephro -stome	Long rpd	Short rpd
Runcina coronata	+	longitudinal	posterior	lateral, right	-	-	+
Creseis virgula	+	vertical	posterior	lateral, right	+	-	-
***Pneumoderma* sp.**	+	longitudinal	posterior	lateral, right	-	+	-
Bosellia mimetica	+	longitudinal	anterior	medio-dorsal	+	-	-
Alderia modesta	-	-	-	-	-	-	-
***Hedylopsis* sp.**	+	longitudinal	anterior	lateral, right	-	+	-
Hypselodoris tricolor	+	longitudinal	posterior	medio-dorsal	-	+	-
Cuthona caerulea	+	longitudinal	anterior	medio-dorsal	+	-	-

occurs in the region of the ventricle in *Pneumoderma* sp. and *Bosellia mimetica*, it is situated in the auricular region in *Creseis virgula, Hedylopsis* sp., and *Runcina coronata* and in the region of the transition between the two chambers of the heart in the two nudibranch species *Hypselodoris tricolor* and *Cuthona caerulea*.

Like the heart, also the single kidney of the Opisthobranchia is orientated along the longitudinal axis of the body and positioned on the right body side, under the notum (*Runcina coronata, Creseis virgula*, *Pneumoderma* sp., and *Hedylopsis* sp.), dorsolaterally (*Cuthona caerulea*), or mediodorsally (*Bosellia mimetica*, *Alderia modesta,* and *Hypselodoris tricolor*), but always touching the ventral surface of the pericardium. The large, tubular organ spreads almost over the entire surface of the visceral mass in some species (see Table 3). Its wall is heavily folded or pleated in larger specimens of the nudibranch species *Hypselodoris tricolor* and *Cuthona caerulea*, giving the appearance of several convoluted tubules in cross section (IV: fig. 2A,B, p. 101). In the sacoglossan *Bosellia mimetica*, the kidney splits into two branches in the anterior one-third, enclosing the heart ventrolaterally (II: figs. 1B, p. 67; 2D, p. 68). The wall of the kidney is composed of a single layer of glandular epithelium which is highly vacuolated in most taxa (II: figs. 2D,F, p. 68; 6B,C, p. 72; IV: fig. 2A,B p. 101; VI: fig. 2, p. 129).

The kidney opens to the exterior (II: fig. 2A,B, p. 68) or, where present, into the mantle cavity (*Creseis virgula* and *Hedylopsis* sp.) via the nephropore that is positioned either laterally, on the right side of the body (*Runcina coronata, Pneumoderma* sp., *Bosellia*

mimetica, Hedylopsis sp., and *Cuthona caerulea*), medially (*Creseis virgula*), or mediodorsally (*Alderia modesta* and *Hypselodoris tricolor*). The sphincter muscle around the nephropore is only weakly developed (I: fig. 6C, p. 56). Adjacent to the nephropore lies the anal opening, only in the heart-less sacoglossan *Alderia modesta* the nephropore opens to the exterior far away from the anal opening at the posterior end of the body (II: fig. 5, p. 71).

Table 3. Distribution of kidney characters in the investigated species. Kidney length: compared to length of entire visceral mass; Neph/A.: nephropore adjacent to anal opening; + present, - absent.

Taxon	Kidney	Position of kidney	Kidney legth	Position of nephropore	Neph/A
Runcina coronata	tubular	lateral, right	1/3	lateral, rigth	+
Creseis virgula	tubular	right	1/3	median	+
***Pneumoderma* sp.**	tubular	ventrolateral, right	1/3	ventrolateral, right	+
Bosellia mimetica	bifurcate anteriorly	medio-dorsal	1/2	ventrolateral, right	+
Alderia modesta	tubular	medio-dorsal	2/3	medio-dorsal	-
***Hedylopsis* sp.**	tubular	ventrolateral, right	1	ventrolateral, right	+
Hypselodoris tricolor	folded	medio-dorsal	2/3	medio-dorsal	+
Cuthona caerulea	folded	dorsolateral, right	2/3	dorsolateral, right	+

3.2. Pericardium and epicardium

Being a coelomatic cavity, the pericardium is completely lined by an endothelium that also builds up the outer wall of the heart, the epicardium (see Fig. 2). In the Opisthobranchia, the pericardial epithelium is generally comprised of two cell types, podocytes and epithelio-muscle cells. The podocytes of the investigated species show only little morphological diversity (see I: fig. 5B,C, p. 55; II: fig. 3D, p. 69; III: fig. 3E, p. 86; IV: fig. 3, p. 102; V: fig. 2, p. 120). They consist of the central cell body and numerous flat, foot-like projections, termed pedicels, which extend from the basal border of the cell body and interdigitate with those of adjacent cells (IV: fig. 3D, p. 102). The pedicels rest on a basal lamina that separates the haemocoel from the pericardial coelom. Gaps between the pedicels form fenestrations or slits that are bridged by fine diaphragms in the form of electron-opaque strands (IV: fig. 3E, p. 102; V: fig. 2E, p. 120). The width of these slits varies between 20 and 25 nm.

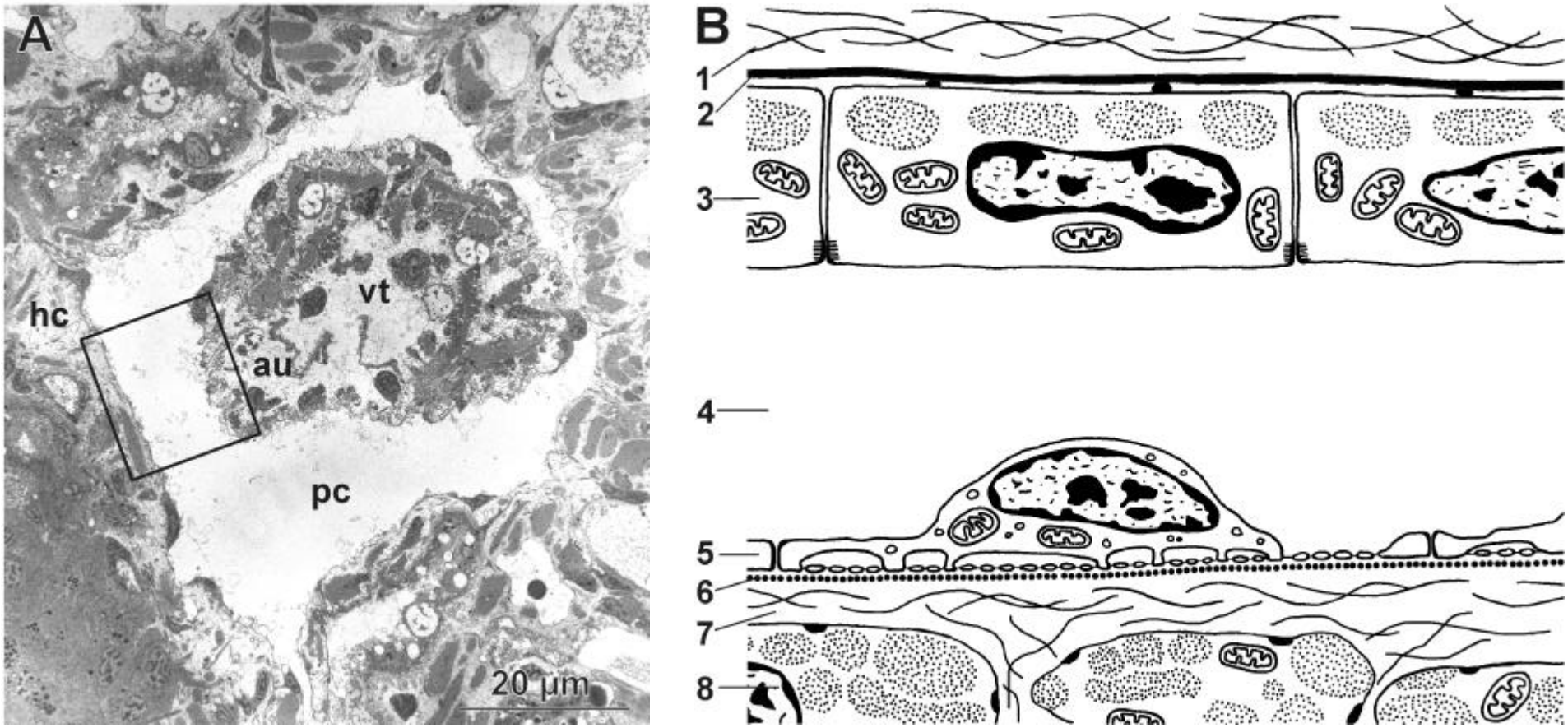


Fig. 2. General organization of pericardium and heart in the Opisthobranchia. **A.** TEM micrograph of the spacious pericardium enclosing the heart (*Bosellia mimetica*, see Appendix II). The boxed area is schematized and enlarged in B. *au* auricle, *hc* haemocoel, *pc* pericardial cavity, *vt* ventricle. **B.** Diagram of the typical composition of pericardium, epicardium and myocardium of the heart. 1 haemocoel with extracellular matrix (ECM), 2 basal lamina of outer pericardial wall, 3 outer pericardial wall (epithelial) composed of epithelio-muscle cells, 4 pericardial cavity, 5 epicardial wall of the auricle (epithelial) composed of podocytes, 6 basal lamina of epicardial wall, 7 ECM, 8 myocytes with hemidesmosomes forming the myocardium of the auricle (mesenchyme).

The cell body of the podocyte may either be flattened against the basal lamina (I: fig. 2A, p. 52; IV: fig. 3A, p. 102; V: fig. 3A, p. 121) or it may be elevated above it so that only the pedicels contact the basal lamina (IV: fig. 3D, p. 102; V: fig. 2A,B, p. 120). The cytoplasm contains a number of small vesicles, Golgi bodies, and few mitochondria. The epithelio-muscle cells of pericardium and epicardium generally contain basally located myofibrils (auricular cells fewer than ventricular cells), numerous mitochondria, and are apically connected by belt desmosomes (III: fig. 3B, p. 86; IV: fig. 3C, p. 102). Although pedicels are not present, certain epithelio-muscle cells form large, cytoplasmic, finger-like extensions apically into the pericardial cavity.

In general, the auricular epicardium of the Opisthobranchia is predominantly lined with podocytes, interspersed only by a few epithelio-muscle cells (see Table 4). In contrast, podocytes are absent from the epicardium of the ventricle and the outer pericardium, these epithelia exclusively consist of epithelio-muscle cells in most opisthobranch taxa. The Nudibranchia (*Hypselodoris tricolor* and *Cuthona caerulea*) differ significantly from this condition in that their entire outer pericardium consists of podocytes as well (IV: fig. 3A,C, p. 102; V: fig. 2A,C, p. 120). These podocytes of the outer pericardium are structurally identical to those of the epicardium, showing low cell bodies, isolated from their neighbours by expanses of pedicels, and only a few intercellular junctions. Epithelio-muscle cells are scattered between the podocytes of the auricular epicardium in *Hypselodoris tricolor* and additionally build up the ventricular epicardium. In *Cuthona caerulea*, also the ventricular epicardium consists exclusively of podocytes (V: fig. 2A,B, p. 120) and epithelio-muscle cells are completely absent. Thus, the entire pericardial epithelium is lined by podocytes in this species.

Pericardium and epicardium of the investigated cephalaspidean *Runcina coronata* are composed of two cell types different to those of the respective epithelia of all other Opisthobranchia. The outer pericardium is predominantely composed of very flat squamose cells with an electron-lucent cytoplasm containing numerous small vesicles (VI: fig. 3C, p. 131). These cells are interspersed by clusters of flat podocyte-like cells (VI: fig. 3A,C, p. 131) that are concentrated in certain areas, such as around the opening into the renopericardial duct. The podocyte-like-cells are characterized by cytoplasmic branches that extend from the cell body basally, are distinctly spherical in cross section, and form intervening slits of 20-75 nm width (VI: fig. 3B, p. 131). A basal lamina that might be apposed by a collagen layer of the ECM underlies the slits (VI: fig. 3A, p. 131), diaphragms bridging the slits are absent. The entire auricular and ventricular epicardium of *Runcina coronata* is composed of podocyte-

like-cells as well, epithelio-muscle cells are completely absent from the epicardium and outer pericardium.

Table 4. Sites of ultrafiltration in the investigated species; + present, - absent.

Species	Podocytes	Podocyte-like cells without diaphragms	Auricular epicardium	Ventricular epicardium	Outer pericardial epithelium
Runcina coronata	-	+	+	+	+
***Pneumoderma* sp.**	+	-	+	-	-
Creseis virgula	+	-	+	-	-
Bosellia mimetica	+	-	+	-	-
Alderia modesta	-	-	-	-	-
***Hedylopsis* sp.**	+	-	+	-	-
Hypselodoris tricolor	+	-	+	-	+
Cuthona caerulea	+	-	+	+	+

The myocardium of the heart itself (I: fig. 2B, p. 52; II: fig. 3C, p. 69; IV: fig. 3A,C, p. 102) consists of non-epithelial (mesenchymate) muscle fibers that are more loosely arranged in the auricular than in the ventricular portion. The muscles have thick and thin myofilaments and dense bodies are scattered among the filaments, whereas mitochondria, glycosomes, and nuclei are located peripherally. The basal lamina of the pericardium covers the myocardium. There are no belt-desmosomes but only hemi-desmosomes between the myocytes of the heart and the surrounding ECM.

3.3. Renopericardial duct and kidney

The ventral opening of the pericardium into the renopericardial duct or directly into the kidney is funnel-shaped in all opisthobranch taxa and therefore often termed pericardial funnel (or syrinx in the doridoid Nudibranchia). It is 5 µm (*Hedylopsis* sp.) to 40 µm (*Hypselodoris tricolor* and *Cuthona caerulea*) wide and lined with cuboidal, multiciliated cells (I: fig. 3A, p. 53; III: fig. 4C, p. 87; IV: fig. 4B, p. 104; V: fig. 3C, p. 121; VI: fig. 4B, p. 132). Short microvilli emanate from the apical surface of these cells (IV: fig. 4B, p. 104; V: fig. 3C, p. 121), the cytoplasm of which contains numerous mitochondria, a centrally located

nucleus, glycosomes (solitary organelles consisting of a glycogen-protein complex), and residual bodies (IV: fig. 4B, p. 104). In *Cuthona caerulea,* the ciliated cells of the nephrostome may show electron-lucent vacuoles identical to those of the kidney cells (V: fig. 3B, p. 121). The basal cell surface rests on an ECM and is not invaginated or folded in the Opisthobranchia examined, with the exception of *Cuthona caerulea*: the nephrostome cells of this species show weakly developed basal infoldings (V: fig. 3C, p. 121).

The same ciliated cells that line the opening of the pericardium into the renopericardial duct also occur at the opening of the renopericardial duct into the kidney. In species with direct opening of the pericardium into the kidney (see Table 2), the nephrostome epithelium is exclusively built up by the ciliated cells (I: fig. 6C, p. 56; V: fig. 3B, p. 121). The entire epithelium of the short, yet distinct renopericardial duct of *Runcina coronata* is formed only by this cell type as well (VI: fig 4, p. 132). In contrast, the central section of the long renopericardial duct of *Pneumoderma* sp., *Hedylopsis* sp., and *Hypselodoris tricolor* is composed of a second, non-ciliated cell type (showing weakly developed infoldings of the basal surface in *H. tricolor* alone). Apically, these cells bear numerous long microvilli and are connected with adjacent cells by belt desmosomes and septate junctions (III: fig. 4B, p. 87; IV: fig. 4A, p. 104). Cytoplasmic features similar to those of the ciliated cells of the pericardial funnel and of the opening into the kidney are the numerous mitochondria, the lyoglycosomes, and the centrally located nucleus.

A continuous epithelium of only one type of cuboidal excretory cells lines the kidney of the Opisthobranchia (see Fig. 3). These kidney cells show little morphological diversity in the investigated taxa (see II: fig. 4, p. 70; IV: fig. 5A, p. 105; V: fig. 4A, p. 122; VI: fig. 5B, p. 133), being mainly characterized by a dense microvillous apical border, a deeply infolded basal surface, and numerous mitochondria in the basal and central portion (IV: fig. 5D, p. 105; VI: fig. 5C, p. 133). Electron-lucent vacuoles of various sizes occur throughout the cytoplasm. Very rarely, some granular material could be detected within the otherwise transparent vacuoles (IV: fig. 5A, p. 105). Whereas the vacuoles are small (diameter up to 1.5 µm) and only sparsely scattered in *Pneumoderma* sp. (I: fig. 3A, p. 53) and *Creseis virgula* (I: fig. 6A, p.56), they are numerous and very large (diameter up to 10 µm) in *Runcina coronata* (VI: fig. 5B, p. 133) and *Alderia modesta* (II: fig. 7A,B, p. 73), and in the nudibranchs *Hypselodoris tricolor* (diameter up to 20 µm, see IV: fig. 5A, p. 105) and *Cuthona caerulea* (diameter up to 15µm, see V: fig. 4A,C, p. 122), often occupying almost the entire volume of the cell. Thus, the large vacuoles represent the most striking diagnostic feature of the kidney epithelium in the latter species, being clearly visible even in light microscopical observations. *Bosellia*

mimetica (II: fig. 4, p. 70) and *Hedylopsis* sp. (III: fig. 4A, p. 87) show numerous vacuoles of intermediate sizes (diameter up to 5 µm or 2 µm respectively). The prominent vacuoles of the kidney cells of the Nudibranchia seem to originate in the basal cytoplasm (V: fig. 4C,D, p. 122). They coalesce to form the largest vacuole apically, prior to fusion with the cell membrane.

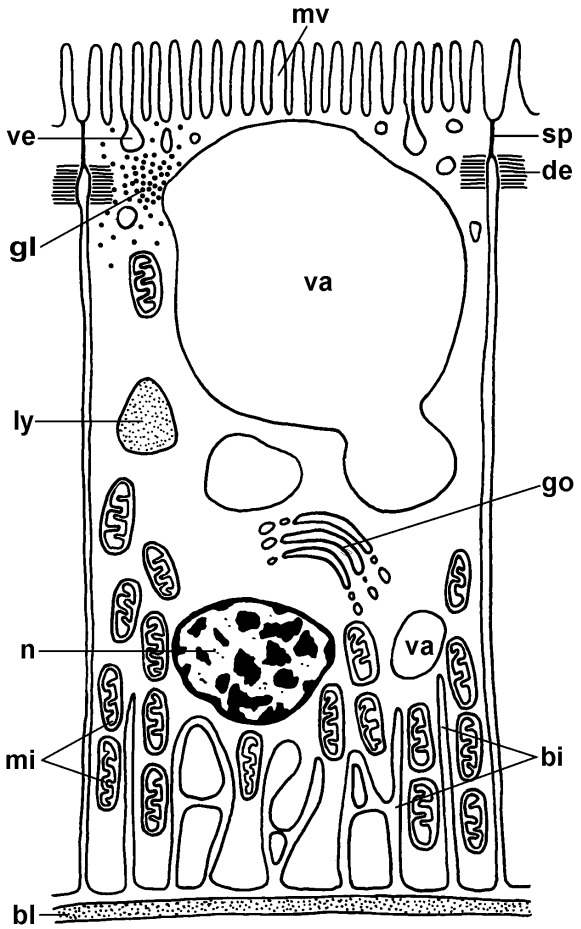


Fig. 3. Schematic drawing of an excretory cell of the kidney epithelium. *bi* basal infoldings of the cell membrane, *bl* basal lamina, *de* belt desmosome, *gl* glycosomes, *go* Golgi apparatus, *ly* lysosome, *mi* mitochondria, *mv* apical microvillous border, *n* nucleus, *sp* septate junction, *ve* vesicle.

Belt desmosomes and extensive septate junctions interconnect the kidney cells near their apices (III: fig. 4A, p. 87; IV: fig. 5A,D, p. 105; VI: fig. 5B, p. 133). Whereas the nucleus is located apically in *Pneumoderma* sp. and *Creseis virgula*, it is situated basally in *Alderia modesta,* centrally to basally in *Runcina coronata, Bosellia modesta,* and *Hedylopsis* sp., and varies in *Hypselodoris tricolor* and *Cuthona caerulea*, where it occurs from basally over centrally to apically. Except for the nucleus, the mitochondria, and the vacuoles, the content of the cytoplasm of the kidney cells varies both within different taxa, different

specimens of the same species, different areas of the kidney, and within individual cells: there may be endosomes, lysosomes, electron-dense granules, and residual bodies in the apical part of the cell. Glycosomes, distinct 20-30 nm small organelles consisting of a protein component and of glycogen (Rybicka 1996), may be absent (IV: fig. 5A, p. 105) or occupy almost the entire cytoplasm (IV: fig. 5B,C, p. 105). The lyoglycosomes lying freely in the cytoplasm may be scattered irregularly, aggregate into large clumps, or surround electron-lucent vacuoles (IV: fig. 5B, p. 105). Desmoglycosomes (glycosomes that are intimately associated with cellular structures, such as mitochondria, Golgi-bodies, polyribosomes, or endoplasmic reticulum) could only be observed very rarely.

The kidney cells of a juvenile specimen of *Runcina coronata* (VI: fig. 5D, p. 133) differ from those of adult specimens (VI: fig. 5B, p. 133) in that basal infoldings of the cell membrane are either completely absent or only very weakly developed. Furthermore, these cells also lack the large, electron-lucent vacuoles and few, much smaller ones (diameter up to 1 µm) occur at various positions of the cell. These vacuoles could frequently be observed to coalesce. Almost the entire volume of the excretory cells of the juvenile specimen is occupied by the prominent nucleus.

The cells of the distal part of the kidney, in the vicinity of the nephropore, generally resemble the excretory cells, but their basal infoldings are less extensively developed and vacuoles are often less large or absent (IV: fig. 6, p. 106). Only the cells of the nephropore are multiciliated.

3.4. Solitary rhogocytes

A second cell type with an ultrafiltration weir, the rhogocyte (IV: fig. 7, p. 108; V: fig. 5, p. 124; VI: fig. 6, p. 134), could be found in all opisthobranch species investigated, except in the thecosome *Creseis virgula*. Rhogocytes occur freely in the haemocoel or are embedded in the connective tissue. They can be situated in all parts of the body, although they seem to be concentrated in certain areas in some taxa. In the doridoid nudibranch *Hypselodoris tricolor*, a large number of rhogocytes could be traced in the connective tissue over the CNS, just below the dorsal notum (IV: fig. 7, p. 108). In the cephalaspidean species *Runcina coronata*, numerous rhogocytes are densely arranged covering the muscular layer that overlies the digestive gland and the gonoduct. In contrast to the epithelial podocytes of the epicardium and outer pericardium, rhogocytes are solitary cells that are completely surrounded by a distinct layer of ECM. They vary considerably in shape and form within one species and even within

one individual, reaching from 5 to 20 µm in diameter. In most cases rhogocytes are ovate to roundish cells (see I: fig. 2C, p. 52; II: fig. 3B, p. 69; 7C, p. 73; III: fig. 3C, p. 86; IV: fig. 7D, p. 108) but sometimes an irregular (II: Fig. 3B, p. 69; VI: fig. 6A, p. 134) or elongated shape (IV: fig. 7A, p. 108; V: fig. 5A, p. 123) occurs.

The most striking diagnostic character of the rhogocyte are the areas with slits scattered over the entire surface of the cell that are underlain by cisternae of different sizes. These cisternae are mostly flat, but in *Runcina coronata* alone they are as flattened and narrow (approx. 20 nm in width) that they are very inconspicuous (VI: fig. 6B, p. 134). The slits with a width of 20 to 25 nm occur between tiny cytoplasmatic bars and are spanned by fine, fibrillar diaphragms (I: fig. 2D, p. 52; II: fig. 7D, p. 73; III: fig. 3D, p. 86; IV: fig. 7B,C, p. 108; V: fig 5B, p. 123; VI: fig. 6B, p. 134). In *Hypselodoris tricolor* and *Runcina coronata*, phagocyte-like formation of vesicles at the base of the cisternae could be observed frequently (IV: fig. 7C, p. 108; VI: fig. 6B, p. 134). Further characteristic features of the rhogocyte are electron-dense granules (diameter 0.5 to 3 µm) which could be found in *Hedylopsis* sp. (III: fig. 3C, p. 86), *Hypselodoris tricolor* (IV: fig. 7A,D, p. 108), *Cuthona caerulea* (V: fig. 5A, p. 123), and *Runcina coronata* (VI: fig. 6A, p. 134), large, electron-lucent vacuoles (diameter up to 5 µm) present in *Pneumoderma* sp. (I: fig. 2C, p. 52), *Alderia modesta* (II: fig. 7A, p. 73), *Hedylopsis* sp. (III: fig. 3C, p. 86), and *Hypselodoris tricolor* (IV: fig. 7A,D, p. 108) and numerous small secretory vesicles. The prominent nucleus may be situated in various positions within the cell, but is mostly placed centrally (VI: fig. 6A, p. 134). Often, a well-developed rough endoplasmatic reticulum continuous with the nuclear membrane is present (V: fig. 5A, p. 123) and mitochondria are scattered throughout the entire cytoplasm.

4. DISCUSSION

The results of this study represent the first detailed and comparative account of the ultrastructure of the excretory systems of opisthobranch Gastropoda. Investigated taxa include the pelagic Gymnosomata and Thecosomata, the benthic Cephalaspidea, Sacoglossa, and Nudibranchia, and the interstitial Acochlidia. The data, obtained by transmission electron microscopy (TEM) and serial sectioning analyses, provide significant insights regarding the evolution of molluscan excretory systems and the phylogeny of several opisthobranch taxa; they enable the identification of traits that are shared among the Opisthobranchia and among all Mollusca studied to date and the proposal of a suite of coelom-derived characters as symplesiomorphies for the Opisthobranchia. In addition, the evidence of a distinct mantle cavity in one acochlidian species has far reaching implications for the elucidation of the origin of this aberrant taxon.

4.1. The site of ultrafiltration - the podocytes

4.1.1. *General aspects*

Podocytes, cells with fenestrations or slits between interdigitating, basal foot-processes, have been found to represent the cellular site of ultrafiltration and production of a primary filtrate in the metanephridial systems of a wide variety of coelomate animals (Kümmel 1973; Ruppert and Smith 1988; Haszprunar 1996). Fine diaphragms covering the slits, along with the underlying basal lamina, enable the selective transfer of molecules from one extracellular space (the haemocoel) to another (a coelomic cavity). Consistent with this model, it has been demonstrated in all major molluscan taxa that extracellular fluid from the haemolymph is initially filtered into the pericardial cavity through an ultrafiltration barrier formed by a peritoneal lining of podocytes (see Table 5).

In general, the podocytes are solely situated in the auricular epicardium of the heart, a condition that is considered as plesiomorphic for the Mollusca in general (Andrews 1988). In all opisthobranch species investigated, except for the cephalaspidean *Runcina coronata* and the heart-less sacoglossan *Alderia modesta,* podocytes could be detected. They were restricted to the auricular epicardium and absent from the ventricular epicardium and outer pericardial epithelium in *Pneumoderma* sp., *Creseis virgula, Bosellia mimetica,* and *Hedylopsis* sp..

Table 5. Sites of ultrafiltration in molluscs, based on ultrastructural investigations. Pod. = podocytes, P.l.c. = podocyte-like cells, + present, - absent.

System - *Species*	Site of ultrafiltration	Pod.	P.l.c.	Reference
SOLENOGASTRES				
Meiomenia sp.	auricular epicardium	+	-	Reynolds & Morse, 1991; Reynolds *et al*., 1993
POLYPLACOPHORA				
Lepidopleurus asellus	auricular epicardium	+	-	Økland, 1980
Tonicella marmorea	auricular epicardium	+	-	Økland, 1980
Cryptochiton stelleri	auricular epicardium	+	-	Morse & Reynolds, 1996
Mopalia lignosa	auricular epicardium	+	-	Morse & Reynolds, 1996
BIVALVIA – PROTOBRANCHIA				
Nucula nucleus	auricular epicardium	+	-	Andrews & Jennings, 1993
BIVALVIA – AUTOBRANCHIA				
Pteriomorpha (3 sp.)	auricular epicardium	+	-	Andrews & Jennings, 1993; Meyhöfer & Morse, 1996
Unionida (1 sp.)	outer pericardium over veins (“pericardial glands”)	+	-	Andrews & Jennings, 1993;
Heterodonta (3 sp.)	outer pericardium over veins (“pericardial glands”)	+	-	Andrews & Jennings, 1993; Meyhöfer & Morse, 1996
Heterodonta (4 sp.)	part of auricular epicardium and outer pericardium over veins	+	-	Andrews & Jennings, 1993
GASTROPODA – PATELLOGASTROPODA				
Patella vulgata	auricular and ventricular epicardium	+	-	Økland, 1982; Andrews, 1985
GASTROPODA – NERITIMORPHA				
Neritoidea (5 sp.)	auricular epicardium	+	-	Estabrooks *et al*., 1999
GASTROPODA – VETIGASTROPODA				
Emarginula reticulata	part of auricular epicardium and outer pericardium over veins	+	-	Andrews, 1985
Haliotis rufescens	auricular epicardium	+	-	Andrews, 1981
Monodonta lineata	auricular epicardium	+	-	Andrews, 1976b; Andrews, 1981; Andrews, 1985
Gibbula cineraria	auricular and ventricular epicardium	+	-	Andrews, 1976b; Andrews, 1981
GASTROPODA – CAENOGASTROPODA				
Cyclophoroidea (3 sp.)	auricular and ventricular epicardium	+	-	Andrews, 1981; Andrews & Little, 1982
Viviparus (3 sp.)	auricular epicardium	+	-	Andrews, 1976a; Andrews, 1979
Marisa cornuarietis	auricular epicardium	-	-	Andrews, 1976a

Assimineidae (3 sp.)	auricular epicardium	-	-	Little & Andrews, 1977; Andrews 1981
Cyclostomidae (1 sp.)	auricular epicardium	-	-	Andrews, 1981
Littorinimorpha (4 sp.)	parts of auricular epicardium	+	-	Andrews, 1981
Neogastropoda (2 sp.)	parts of auricular epicardium	+	-	Andrews, 1981; Andrews, 1988
GASTROPODA – OPISTHOBRANCHIA				
Philinoglossa helgolandica	part of outer pericardium over kidney	-	+	Bartolomaeus, 1997
Runcina coronata	auricular and ventricular epicardium and parts of outer pericardium	-	+	this study
Pneumoderma sp.	auricular epicardium	+	-	this study
Creseis virgula	auricular epicardium	+	-	this study
Bosellia mimetica	auricular epicardium	+	-	this study
Alderia modesta	-	-	-	this study
Hedylopsis sp.	auricular epicardium	+	-	this study
Hypselodoris tricolor	auricular epicardium and entire outer pericardium	+	-	this study
Cuthona caerulea	auricular and ventricular epicardium and entire outer pericardium	+	-	this study
Rhodope transtrosa	"warts" of protonephridial-like system	-	-	Haszprunar, 1997
GASTROPODA – PULMONATA				
BASOMMATOPHORA				
Lymnaea stagnalis	auricular epicardium	-	-	Andrews, 1976b
Helisoma duryi	distal part of kidney	-	-	Khan & Saleuddin, 1979a,b
Biomphalaria glabrata	part of kidney with arterial blood supply	-	-	Matricon-Gondran, 1990
STYLOMMATOPHORA				
Achatina achatina	parts of kidney	-	-	Skelding, 1973; Newell & Skelding, 1973
Helix pomatia	parts of kidney	-	-	Newell & Skelding, 1973
Helix aspersa	part of ventricular epicardium	-	-	Andrews, 1988
SCAPHOPODA				
Dentalium rectius	homolog of ventricular epicardium	+	-	Reynolds, 1990b
CEPHALOPODA				
Coleoidae	branchial heart appendages	+	-	Schipp & Hevert 1981; Schipp *et al.*, 1985

Accordingly, the auricular epicardium represents the sole site of ultrafiltration in these species. There are no significant differences between the podocytes of the Opisthobranchia, the components of the ultrafiltration membranes show the same pattern and dimensions in the species studied: the pedicels are elliptical in cross section, the ultrafiltration slits are approximately 20 nm in width, and the basal lamina is fairly uniform in thickness and structure. The presence of podocytes in all opisthobranch subtaxa, except the Cephalaspidea, clearly falsifies previous assumptions about the loss of this cell type in the common ancestor of Opisthobranchia and Pulmonata, *i.e.* the Euthyneura (Andrews 1988).

4.1.2. *The additional ultrafiltration site in the Nudibranchia*

The renopericardial complex of the two examined nudibranchs *Hypselodoris tricolor* and *Cuthona caerulea* is modified significantly such that podocytes do not only build up the auricular epicardium but also line the entire outer pericardial epithelium. In *C. caerulea* even the ventricular epicardium is formed by flat podocytes. Thus, the whole pericardial epithelium (outer pericardium plus auricular and ventricular epicardium) of this aeolid species is exclusively composed of podocytes; epithelio-muscle cells or other epithelial cells are absent. The presence of podocytes covering the auricular epicardium (and ventricular epicardium in *C. caerulea)* and, additionally, in a second, extensively developed ultrafiltration site in the outer pericardial wall as in the Nudibranchia has not been observed in any other molluscan species. Within certain molluscan taxa podocytes can be found in parts of the pericardium other than the auricular wall as well (see Table 5): some prosobranch gastropod species show additional podocytes in the epicardial surface of the ventricle (Økland 1982; Luchtel *et al.* 1997), while in the Cyclophoridae the ventricular epicardium represents the main site of ultrafiltration (Andrews and Little 1972). Scaphopoda with a reduced pericardium and lack of a heart have podocytes in the pericardial epithelium surrounding a muscular sinus that is either regarded as a perianal sinus (Reynolds 1990b) or as the rudimentary ventricle (Morse and Reynolds 1996; Shimek and Steiner 1997). A single ultrafiltration site in the outer pericardial wall is only known from Cephalopoda (in appendages of the branchial heart wall, see Schipp and Hevert 1981; Schipp *et al*. 1985) and particulary from unionidan and heterodont Bivalvia (as so-called pericardial glands, see Meyhöfer *et al*. 1985; Khan *et al.* 1988; Andrews and Jennings 1993; Meyhöfer and Morse 1996). Additionally, some heterodont Bivalvia (i.e. *Scrobicularia,* see Andrews and Jennings 1993) and vetigastropods

(i.e. *Emarginula,* see Andrews 1985) with podocytes in the outer pericardial epithelium show a few small clusters of podocytes scattered between the squamose cells of the auricular epicardium.

However, in contrast to the Nudibranchia, most of these other taxa with podocytes in the outer pericardial epithelium have completely removed the ultrafiltration site from the wall of the auricle and only a few show transitional stages. Andrews and Jennings (1993) proposed that in the Bivalvia, the plesiomorphic auricular site of ultrafiltration becomes less efficient with increasing body size and may impair the contractility of the auricle. The migration of the ultrafilter to a separate pericardial site, where constraints on size could be overcome by folding of the epithelium, should enable the increased rate of primary urine formation that must accompany the colonization of freshwater habitats. The mud-dwelling, often estuarine representatives of the genus *Scrobicularia* show features which may be regarded as intermediate between the plesiomorphic condition, exemplified by marine bivalves with an auricular ultrafiltration site, and freshwater species with pericardial glands. The auricular epicardium of *Scrobicularia* is mainly composed of squamous epithelial cells, interspersed only occasionally by small clusters of podocytes, whereas its outer pericardial wall is well differentiated into pericardial glands (Andrews and Jennings 1993). Such a migration of the ultrafiltration site has its parallels in the vetigastropod *Emarginula* with podocytes extending over the auricular epicardium and those parts of the veins lying in the pericardial cavity (Andrew 1985).

Thus, the Nudibranchia represent the only known molluscs with two extensively developed, separate sites of ultrafiltration in the epicardial and outer pericardial wall. This feature may represent a significant autapomorphy of the Nudibranchia, if future ultrastructural studies should demonstrate a restriction of podocytes to the epicadium in the sister group Pleurobranchomorpha. However, if the latter should show an ultrafiltration site in the outer pericardium as well, this would reflect an apomorphic state of the Nudipleura (Nudibranchia plus Pleurobranchomorpha). The microanatomical results of this study are not consistent with the consideration of a heart that is orientated along the longitudinal body axis as autapomorphic for the Nudibranchia as suggested by Wägele and Willan (2000). Such an arrangement could be observed in all higher opisthobranch taxa examined and is therefore considered as a highly homoplastic character for phylogenetic studies.

Apart from *Emarginula,* the Nudibranchia also represent the only gastropods with podocytes situated in the outer pericardial wall at all, a character that cannot be explained as an adaptation to estuarine or freshwater habitats as for bivalves (Andrews and Jennings 1993).

This contradicts the assumption of Andrews and Jennings (1993) that the development of a filtration site embedded in the outer pericardial wall is a character unique to the Bivalvia. It is likely, however, that the increase in the surface area of the ultrafiltration site in the carnivorous nudibranchs reflects a significant increase in the rate of filtration. The presence of distinct folds of the dorsal pericardial wall, again termed pericardial glands as in the Bivalvia, in several species of the Anthobranchia (bathydoridoid and doridoid Nudibranchia) (Wägele and Willan 2000) is highly indicative for the presence of podocytes at this site.

4.1.3. *The podocyte-like cells of the Cephalaspidea*

In the investigated cephalapidean species *Runcina coronata* true podocytes as the cellular ultrafiltration site are absent. Instead, other epithelial cells with basal foot processes and the capacity to form an ultrafiltration barrier have replaced the podocytes. These cells differ from podocytes in that they lack the characteristic diaphragms spanning the ultrafiltration slits, the slits are much wider (up to 70 nm), and the cytoplasmic pedicels are distinctly spherical in cross section. These ultrastructural features are consistent with the description and the TEM micrograph (Bartolomaeus 1997, Fig. 4B) of the so-called podocyte-like cells in the mesopsammic cephalaspidean *Philinoglossa helgolandica* Hertling, 1932. However, whereas the podocyte-like cells are restricted to a relatively small part of the outer pericardial epithelium, adjacent to the perinephridial sinus, in *P. helgolandica*, they line the entire auricular and ventricular epicardium and are additionally interspersed between the squamose epithelial cells of the outer pericardium in *R. coronata.* The only further, preliminary ultrastructural study on the metanephridial ultrafiltration system of a cephalaspidean species, (*i. e. Scaphander* sp.) suggests the auricular epicardium, again composed of cells without diaphragms, as the sole site of ultrafiltration (Andrews 1988). Accordingly, the site of ultrafiltration seems to be highly variable within the pericardial epithelium of the Cephalaspidea.

In contrast to true podocytes with slit diaphragms, the basal lamina underlying the slits between cytoplasmic branches is the only possible structure of the cephalaspidean podocyte-like cells which may serve as a molecular filter. This corroborates recent studies by means of tracer experiments and electron microscopy showing the basal lamina of the podocytes to be the principal ultrafilter in bivalves (Meyhöfer and Morse 1996) and contradicts previous reports from gastropods that implicated the substructure of the ultrafiltration slits (i.e. the

diaphragms) of the podocytes as principal molecular sieve (Boer and Sminia 1976). The presence of distinct slit diaphragms in the numerous solitary rhogocytes of *R. coronata* proves that the genetic basis for this ultrastructural feature still exists in the Cephalaspidea.

The podocyte-like cells are not known from any other molluscan taxon and, thus, represent an autapomorphy of the cephalaspidean subclade Philinoidea or, probably, of the Cephalaspidea s.s. (i.e. the Bullomorpha). Podocytes that lack slit diaphragms have also been described from the epicardium of a few prosobranch gastropods (Andrews 1981; Andrews and Little 1982), from two polyplacophoran species (Økland 1980), and also from some polychaete Annelida (Smith and Ruppert 1988). However, all other ultrastructural details of these cells are consistent with those of typical podocytes (i.e. the pedicels of these cells are clearly elliptical in cross section and the ultrafiltration slits are approximately 20 nm in width), indicating that these cells are in fact true podocytes. Furthermore, Morse and Reynolds (1996) found podocytes with distinct diaphragms in the two polyplacophoran species they investigated (see Table 5) and Økland`s (1980) TEM micrographs of podocytes (Fig. 1, 2A, 4F) all show quite distinct diaphragms covering the slits, in contrast to his interpretation.

It seems likely that the podocyte-like cells of the Cephalaspidea are homologous to the podocytes, as are the cells lining the small parts of the kidney supplied specifically with arterial haemolymph in some basommatophoran pulmonates, termed podocyte-like cells again (Matricon-Gondran 1990). True podocytes have not been found in any pulmonate yet, suggesting that there must have been a radical change in the location and organization of ultrafiltration structures in this group, possibly related to the colonization of freshwater and terrestrial habitats (Luchtel *et al.* 1997). The ultrafiltration site varies in the few further pulmonate species that have been studied ultrastructurally (see Table 5). There is general agreement that it occurs somewhere in the renopericadial complex and four different sites have been identified (see reviews by Andrews 1988 and Luchtel *et al.* 1997): in the auricular or ventricular epicardium of the heart, paracellular or transcellular in parts of the kidney, or restricted to a small specialized area of the kidney with arterial haemolymph supply.

4.1.4. *The loss of the podocytes in the sacoglossan Alderia modesta*

The sacoglossan *Alderia modesta* lacks heart and pericardium, and podocytes or other epithelial cells with the capacity to form an ultrafiltration barrier are therefore completely absent. Accordingly, the urine is formed directly in the kidney without a prior ultrafiltration step, a feature that can be presumed for the isolated left kidney in fissurellid vetigastropods (Andrews 1985) and right kidney of lepetelloid vetigastropods (Haszprunar and McLean 1996) and the kidneys of the likewise heart-less *Micropilina* species (Monoplacophora) as well (Haszprunar and Schäfer 1997a,b). In contrast to the enigmatic and heart-less opisthobranch *Rhodope transtrosa* Salvini-Plawen, 1991 (see Haszprunar 1997) that lacks podocytes and shows an entirely new pseudoprotonephridial system of ultrafiltration, the uniform epithelium of the large kidney of *A. modesta* is not modified at all. The cellular structures of the excretory cells give no indication for paracellular or transcellular ultrafiltration in (parts of) the kidney, as suggested for some pulmonate gastropods (see Luchtel *et al*. 1997). Thus, the organization of the excretory system of *A. modesta* shows that ultrafiltration is no prerequisite for effective excretion in the Mollusca.

4.2. Modification of the primary urine – the kidney epithelium

Primarily, the ultrafiltration site of the Mollusca in the pericardium was linked with a single pair of tubular, ciliated coelomoducts with a tendency to become U-shaped, each opening to the mantle cavity distally (Andrews 1988; Haszprunar 1992). Such an excretory system is exemplified by the aplacophoran taxa Solenogastres and Caudofoveata, where the coelomoducts additionally serve as gonoducts. In the Polyplacophora, the coelomoducts become more complex, with the distal portions modified into the kidneys that are connected with the pericardial cavity by the proximal, ciliated renopericardial ducts (Morse and Reynolds 1996). This arrangement can also be regarded as the basic plan for all further, major molluscan taxa other than the Monoplacophora (e.g. Reynolds 1990a). Primitive Gastropoda (Diotocardia) still show two kidneys, while in all other gastropods (Monotocardia), only the posttorsional left kidney is left as a functional excretory organ, the right becomes incorporated in the genital duct and loses all excretory activity (Johansson 1950; Andrews 1988).

The filtrate entering the pericardial cavity of the Mollusca, the primary urine, is modified by secretion and reabsorption as it sequentially passes through the renopericardial

ducts and the kidneys (see review by Andrews 1988). Podocytes of auricular and pericardial glands of bivalves (Andrews and Jennings 1993) and other cells of the pericardial epithelium of a few prosobranch gastropod species (Martoja 1975; Andrews 1979; Little 1979) participate in the reabsorption and transport of solutes as well. In general, however, the primary urine is conveyed unaltered to the molluscan kidneys which are often divisible into a proximal region involved in reabsorption of organic solutes (and in ion uptake in freshwater species), and a distal region responsible for nitrogenous excretion and elimination of other waste metabolites (Andrews 1988; Morse and Reynolds 1996). In patellogastropods and vetigastropods, only the right kidney is involved in excretion, while the left one is responsible for reabsorption (Harrison 1962; Andrews 1988). The epithelium of the single kidney of caenogastropods is generally composed of two basic cell types. Pigmented ciliated cells responsible for reabsorption occur in the proximal region, whereas vacuolated excretory cells line the distal region (Andrews 1981, 1988).

In contrast, the kidney of the Opisthobranchia shows no differentiated regions and is built up by only one type of epithelial cell. These aciliated kidney cells show very little morphological variability in the species examined: basally, extensive infoldings of the cell membrane increase the surface area across which material is exchanged with the haemolymph by pinocytosis. The apical surface is enlarged by a dense array of microvilli and pinocytotic activity is indicated by the presence of vesicles at the bases of the microvilli. One or several, often very large vacuoles are the most conspicuous feature of the cytoplasm that may additionally be occupied with lysosomes, and a large number of basally located mitochondria. Most of these features, i.e. the basal infoldings, numerous mitochondria, and extensive vacuolation, are indicative for a transcytotic activity which is characteristic for excretory cells of the distal regions of the kidneys in other molluscs (Morse and Reynolds 1996; Bartolomaeus 1997). However, the dense apical microvillous border and the small vesicles in the apical cytoplasm reflect an additional reabsorptive activity (Andrews 1988), as may the numerous glycosomes found in the kidney cells of several species. These small, electron-dense granules, often considered as particles of stored glycogen in the literature, represent dynamic cellular organelles (Rybicka 1996). They consist of a protein component, stainable with heavy metal, and of glycogen that does not react with uranium and lead. Most glycosomes found in the kidney cells of the Opisthobranchia lie freely in the cytoplasm (lyoglycosomes) and often aggregated into large clumps, whereas so-called desmoglycosomes that are intimately associated with different cellular structures could only be detected very rarely.

The ultrastructural data of this study corroborate Andrews (1988) who presumed that there has been a secondary simplification of the kidney in the Opisthobranchia, in which one type of epithelial cell subsumed both excretory and reabsorptive function. The above-mentioned, pigmented, ciliated cells responsible for reabsorption of organic solutes in prosobranchs are absent in opisthobranchs as they are in pulmonates (Luchtel *et al*. 1997). The only part of the kidney epithelium of the Opisthobranchia which is ciliated is in the immediate vicinity of its opening to the exterior. However, these cells lack pigmentation and are probably exclusively concerned with the circulation of urine. It may be that the simplification of the kidney is a result of the reduction and loss of the shell in the Opisthobranchia, allowing much more diffusion of ammonia through the body surface (often increased significantly by various outgrowths).

In a preliminary ultrastructural study of the cephalaspidean opisthobranch species *Philine aperta* (Linné, 1767) and *Scaphander* sp., Andrews (1988) described kidney cells that did not appear to be highly active, lacking basal infoldings and "glycogen-deposits" and showing only weakly developed apical microvilli. She therefore concluded that the excretory activity of the kidney cells of the Opisthobranchia is markedly reduced or even lost and that their function has been adopted by the cells of the digestive gland. As the kidney cells of all opisthobranch species investigated herein exhibit ultrastructural features typical for excretory cells, this assumption can be clearly falsified. However, cells of the kidney epithelium of a juvenile specimen of the cephalaspidean *Runcina coronata* show only very weakly elaborated or, partly, no basal infoldings and vacuolation, consistent with the excretory cells described by Andrews (1988) but in contrast to those of adult *R. coronata* specimens. If Andrews` specimens were juveniles as well, this would suggest a relatively late functional differentiation of the kidney cells in the ontogeny of the Opisthobranchia.

4.3. The connection of pericardium and kidney – renopericardial duct and nephrostome

In the excretory systems of most molluscs, a tubular, ciliated renopericardial duct is interpolated between the pericardial site of ultrafiltration and the kidney (Andrews 1988). Such an arrangement also characterizes the renopericardial complex of several of the opisthobranch species investigated (see Table 2). In other opisthobranchs, the pericardial cavity opens directly into the kidney via a ciliated funnel, the nephrostome. The long renoperiardial duct is composed of cells of two different types: cuboidal, aciliated cells with

long and dense apical microvilli line the central section, while the proximal and distal sections (the openings to the pericardium and the kidney) are built up by multiciliated cells. The latter also form the epithelium of the nephrostome and of the entire, short renopericardial duct of *Runcina coronata.* The beat of the cilia is always directed from the pericardium to the kidney (Luchtel *et al*. 1997) and has been interpreted by some authors as adding to the force available for filtration (Potts 1967; Witmer and Martin 1973; Andrews 1979, 1981).

The presence of large vacuoles in ciliated nephrostome cells of *Cuthona caerulea* and, in particular, the elaboration of weakly developed but distinct basal infoldings in the cells of the nephrostome and renopericardial duct of *C. caerulea* and *Hypselodoris tricolor* point to their origin from the kidney. Numerous glycosomes scattered throughout the cytoplasm of the cells of the nephrostome or renopericardial duct of most opisthobranch species provide additional evidence for this assumption. These ultrastructural data support earlier embryological evidence for a renal, and not a pericardial, origin of the molluscan renopericardial duct (Raven 1958).

4.4. Additional loci of ultrafiltration - the rhogocytes

Next to the epithelial podocytes, a second cell-type with an ultrafiltration weir could be found in all opisthobranch species investigated, except in *Creseis virgula*. The solitary rhogocytes occur throughout the primary body cavity, i.e. free in the haemocoel and embedded in the connective tissue and are characterized by slit areas on their surface that strongly resemble the fenestrations of the podocytes. Haszprunar (1996) previously outlined the striking similarity of the molecular sieves (slits bridged by diaphragms, covering ECM, underlying free lumen or cisternae) strongly suggesting a cytological homology between molluscan rhogocytes and metazoan podocytes, cyrtocytes, and nephrocytes. As indicated by the large number of vesicles that are formed at the base of the cisternae underlying the slit areas, filtration pressure is probably caused by endocytosis in rhogocytes. In contrast, muscular activity is the driving force in podocytes (Morse and Cooper 1993; Haszprunar 1996).

The presence of rhogocytes in *Cuthona caerulea* contradicts Wägele`s (1998) previous description of several cellular structures in opisthobranchs, reporting the absence of rhogocytes in *Cuthona* species and stating that the Doridoidea would be the only nudibranch taxon with recognizable rhogocytes. Since Wägele investigated the rhogocytes by histological techniques only, it seems obvious that she could not find them in all species. In fact, the only

reliable diagnostic feature of the rhogocytes are the ultrafiltration structures surrounding the cell surface which are exclusively detectable by electron microscopy. The data from *Hypselodoris tricolor* represent the first evidence of a striking variability of form and shape of the rhogocytes within one species and even within the same specimen. This proves that the shape of these cells may be independent from the physiological condition of the individual, as had been assumed (Haszprunar 1996). In *H. tricolor*, it is more likely that the shape of the rhogocytes varies according to the adjacent space available. Possible functions of the rhogocytes include a major role in the metabolism of metal ions and the detoxification of heavy metal ions (see review by Haszprunar 1996). Furthermore, it has been shown recently by means of electron microscopy and immunohistochemical experiments (Albrecht *et al.* 2001), that rhogocytes represent the site of haemocyanin biosynthesis in the vetigastropod *Haliotis tuberculata* Linné, 1758. However, haemocyanin molecules could not be identified in the vacuoles of the rhogocytes of the opisthobranch species investigated herein.

4.5. The renopericardial complex and mantle cavity of the acochlidian *Hedylopsis* sp.

The renopericardial complex of the acochlidian opisthobranch *Hedylopsis* sp. differs from the general anatomical diagnosis of the Hedylopsidae (Rankin 1979) in several details. The heart is composed of auricle and ventricle, the nephropore is situated adjacent to the anus and the genital opening, and the body openings lie ventrolaterally. In contrast, Rankin (1979) described the presence of a one-chambered heart, a nephropore which is situated distinctly closer to the anal opening than to the genital opening, and dextrolateral body openings in the Hedylopsidae and used these features to establish a new, highly ranked taxon (i.e. the Suborder Proprioneura) and to demarcate the Hedylopsidae from the Pseudunelidae. Since all characters mentioned above were considered to be of high diagnostic value, the validity of Rankin`s classification, which was based on literature data only, needs to be critically rechecked. A phylogenetic analysis of the Acochlidia is overdue.

The kidney of the acochlidian species *Hedylopsis* sp. does not open directly to the exterior but opens into a small, yet distinct, mantle cavity lined by an epithelium of squamous cells with microvillous borders. Ciliated cells that are interspersed between the regular epithelial cells of the mantle cavity in other molluscan taxa (e.g. Haszprunar and Schaefer 1997; Shimek and Steiner 1997) are restricted to the opening of the mantle cavity in *Hedylopsis* sp.. The special cells with a prominent microvillous pit that are scattered over the

mantle cavity epithelium in *Hedylopsis* sp. are not known from any other taxon. Both the position (much more common at the inner and posterior end of the mantle cavity than towards the opening) as well as their content (a large number of mitochondria and glycosomes) and the large, apical microvilli strongly indicate a reabsorptive capacity for these cells. Because of its small size, a significant role of the mantle cavity in respiration is unlikely.

The presence of a mantle cavity contrasts earlier descriptions of the Acochlidia (for review, see Rankin 1979). Originally, the Gastropoda possess a large, spacious mantle cavity into which the whole head and foot can be retreated. Within the Opisthobranchia, a trend to reduction and, finally, loss of the mantle cavity can be observed (Morton 1988). Rankin (1979) considered the absence of a permanent mantle cavity as a diagnostic character of the Acochlidia, only the formation of a "temporary mantle cavity" during complete withdrawl of the animal has been reported from some acochlidian taxa (see Rankin 1979). In contrast, Kudinskaja and Minichev (1978) pointed out that the species *Hedylopsis murmanica* Kudinskaja & Minichev, 1978 retained many primitive features, among them a mantle cavity. Accordingly, *Hedylopsis* sp. investigated in this study represents the second acochlidian species with a mantle cavity. This further supports the placement of the Hedylopsidae at the base of the Acochlidia, as suggested in the latest systematic review of the group by Arnaud *et al*. (1986) and Wawra (1987).

5. CONCLUSIONS

The data presented herein enable the following significant conclusions regarding the organization and evolution of opisthobranch and molluscan excretory systems:

(1) Symplesiomorphic features of the opisthobranch renopericardial complex are 1) the pericardium enclosing a monotocardian heart, 2) the auricular epicardium as sole site of ultrafiltration, characterized by the presence of podocytes with slit diaphragms between the pedicels and an underlying basal lamina, 3) the ciliated renopericardial duct or nephrostome connecting the pericardial cavity with the kidney, 4) the epithelium of the single kidney being composed of one single cell type with basal infoldings, apical microvillous border, and numerous vacuoles, indicating both excretory and reabsorptive activity. These characters clearly falsify previous assumptions on a significant modification of the excretory system at the base of the Opisthobranchia (*i.e.* the loss of the podocytes and of the excretory activity of the kidney cells).

(2) The ultrastructural data from the Opisthobranchia correspond to those of all higher molluscan taxa and are entirely consistent with the model of metanephridial systems proposed by Ruppert and Smith (1988). In documenting these coelomic features in an ultrastructurally poorly studied taxon and demonstrating widespread symplesiomorphy within the Mollusca, evidence for the shared coelomate nature of all molluscs is provided.

(3) Solitary rhogocytes in the haemocoel and connective tissue of the Opisthobranchia, as in other molluscs, represent additional loci of ultrafiltration showing filtration slits that are ultrastructurally identical to those of the podocytes.

(4) An extensive, additional ultrafiltration site (podocytes) in the outer pericardial epithelium of both doridoid and aeolidoid nudibranchs probably represents an autapomorphy of the Nudibranchia (alternatively of the Nudipleura).

(5) Podocyte-like cells without slit diaphragms and relatively wide (up to 70 nm) ultrafiltration slits between pedicels, that are distinctly spherical in cross section, are only known from the Cephalaspidea s.s. and regarded as significant autapomorphy of this group.

(6) The lack of podocytes in the heart-less sacoglossan *Alderia modesta* proves that ultrafiltration is no prerequisite for effective excretion in the Mollusca. In contrast to other, likewise heart-less taxa, *A. modesta* shows no further modifications of the excretory system.

(7) The presence of a small, yet distinct mantle cavity in the acochlidian *Hedylopsis* sp. is in contrast to earlier anatomical descriptions and indicates the placement of the Hedylopsidae at the base of the Acochlidia.

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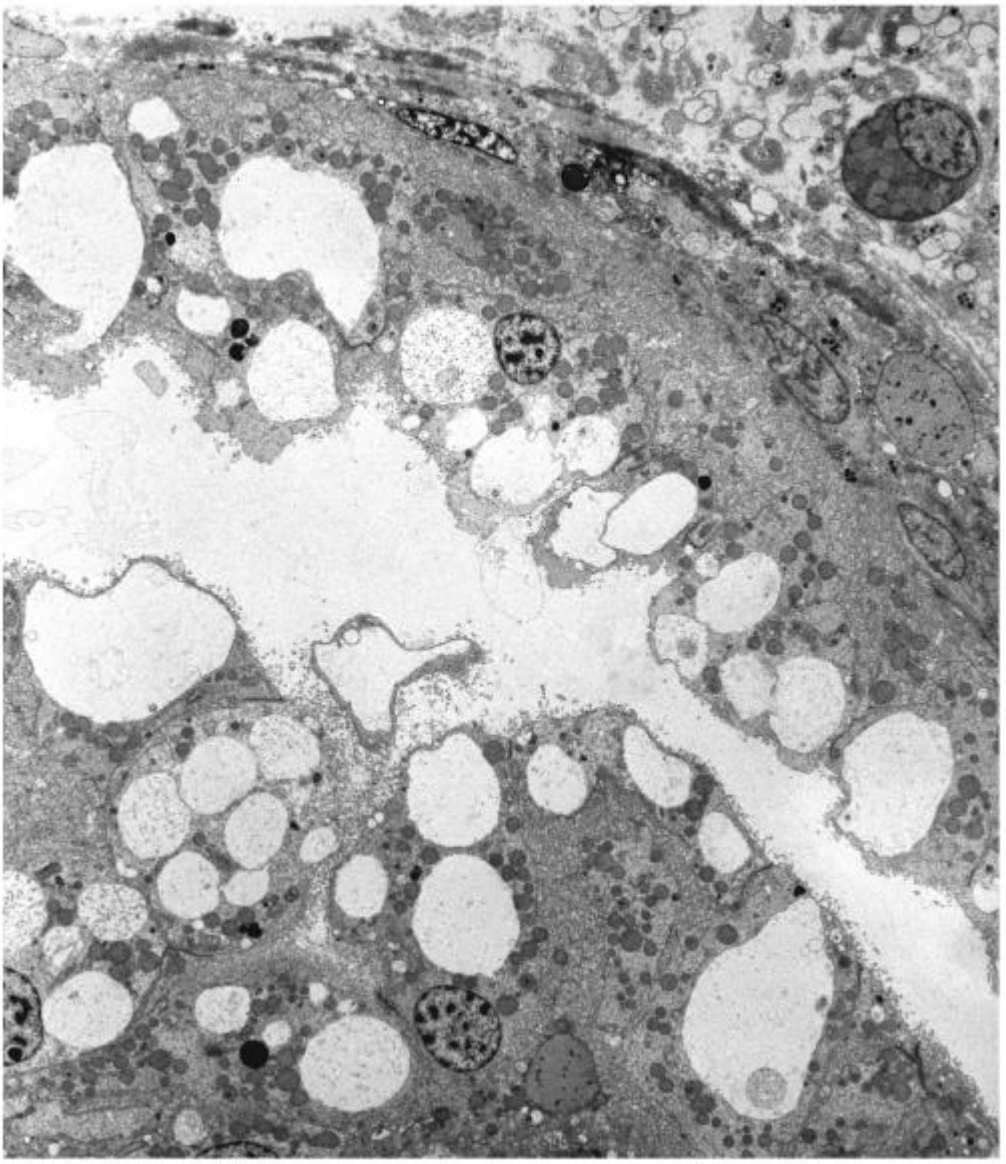
APPENDIX



APPENDIX I

Microanatomy and ultrastructure of the excretory system of two pelagic opisthobranch species (Gastropoda: Gymnosomata and Thecosomata)

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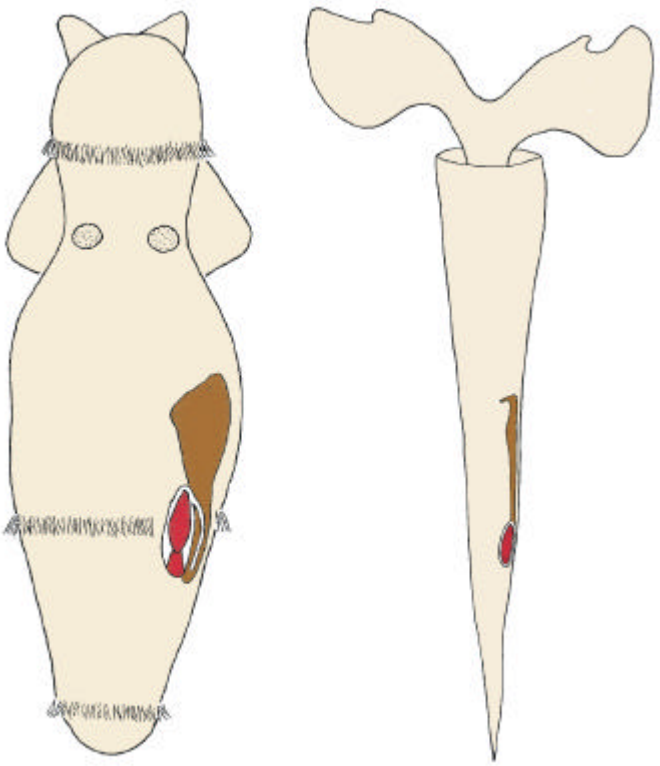

Abstract. The microanatomy and ultrastructure of the excretory system of *Pneumoderma* sp. (Gymnosomata) and *Creseis virgula* Rang, 1828 (Thecosomata) has been investigated by means of semithin serial sections, reconstructions and transmission electron microscopy. The studies revealed a functional metanephridial system consisting of a heart with a single ventricle and auricle in a pericardium and a single kidney in both species. Podocytes in the auricular wall of the pericardial epithelium are the site of ultrafiltration, whereas the flat epithelium of the kidney with numerous basal infoldings and a dense microvillous border on the luminal surface serves to modify the ultrafiltrate. In *Pneumoderma* sp., additional loci of ultrafiltration with identical finestructure (meandering slits with diaphragms covered by extracellular matrix) occur in the solitary rhogocytes (pore cells). The presence of podocytes situated on the auricular epicardium in representatives of two higher opisthobranch taxa contradicts former ideas on the loss of the primary site of ultrafiltration in the ancestors of the Opisthobranchia.

INTRODUCTION

The Mollusca represent an ideal group to examine nephridial variability and evolution. In general, molluscan larvae are characterized by the occurrence of protonephridial systems (Bartolomaeus, 1989; Ruthensteiner and Schaefer, 1991; Tardy and Dongard, 1995; Haszprunar and Ruthensteiner, 2000), whereas adults usually show metanephridial systems with own renoducts (Salvini-Plawen and Bartolomaeus, 1995) and podocytes (Ruppert and Smith, 1988). Moreover, solitary ultrafiltration cells (pore cells or rhogocytes) are diagnostic for all molluscs. These cells structurally resemble metanephridial podocytes and protonephridial cyrtocytes, therefore a common genetic basis and homology of these three cell types have been proposed recently (Haszprunar, 1996). The main excretory mechanism of the adult Mollusca is through ultra-filtration of the haemolymphic fluid by podocytes in the epicardium, resulting in an ultrafiltrate which is collected in the pericardial cavity. A pair of renopericardial ducts modify this ultrafiltrate before leading to the exterior environment (Andrews, 1988; Bartolomaeus and Ax, 1992; Salvini-Plawen and Bartolomaeus, 1995). Primarily, these renopericardial ducts were simple ciliated canals, but in higher evolved molluscan taxa, the distal portions of the ducts were modified into sac-like organs, the kidneys (Bartolomaeus, 1997). In the following, the endothelially lined pericardium,

enclosing the heart, plus the two renopericardial ducts will be called renopericardial complex (Haszprunar, 1992).

The ultrastructure and microanatomy of the excretory system have been investigated in representatives of almost all higher molluscan taxa (Andrews, 1988; Morse and Reynolds, 1996). However, within the gastropods, ultrastructural studies on the renopericardial complex have been focused almost exclusively on different prosobranch groups and the pulmonates (for reviews see Andrews, 1988; Morse and Reynolds, 1996; Luchtel *et al.*, 1997). There are only two ultrastructural studies dealing with the renopericardial complex of opisthobranch gastropods. In the partly paedomorphic, interstitial *Philinoglossa helgolandica*, this system is modified, in that the place of ultrafiltration moved from the epicardial wall to the pericardial wall (Bartolomaeus, 1997). The enigmatic and worm-like *Rhodope transtrosa* even lacks the heart and shows a completely new system of ultrafiltration (Haszprunar, 1997). These data suggest that other opisthobranch taxa also exhibit significant modifications of the original excretory system.

In this study, the microanatomy and ultrastructure of the renopericardial complex of representatives of the holoplanctonic Gymnosomata and Thecosomata (formerly combined in the order Pteropoda) are investigated in detail for the first time. *Pneumoderma* sp. and *Creseis virgula* Rang, 1828 are the first two opisthobranch species that are examined within the framework of a larger project, comprising all major taxa of the Opisthobranchia.

MATERIALS AND METHODS

Specimens of *Pneumoderma* sp. were obtained from plankton samples taken off the coast of Calvi (Corsica, France) in June 1997, using a net of 500 µm mesh-size for oblique hauls covering a depth range from 0 to 15 m. *Creseis virgula* was collected with a plankton net towed vertically from a depth of 25 m in Fetovaia Bay (Elba, Italy) in June 1998. The animals were removed by pipette from the samples, relaxed by a solution of 7% $MgCl_2$ (isotonic to local seawater) and fixed in 4 % glutardialdehyde buffered in 0,2 M sodium cacodylate (pH 7,2). After that the specimens were rinsed in the same buffer in decreasing concentrations. Postfixation in buffered 1 % OsO_4 for two hours was followed again by rinsing with cacodylate buffer in decreasing concentrations and dehydration in a graded ethanol series. After decalcification with EDTA, the fixed specimens were embedded overnight in Araldit resin for light microscopy and in Spurr`s (1969) low viscosity resin for electron microscopy.

To get an overall view on the heart position in situ, several specimens of both *Pneumoderma* sp. and *Creseis virgula* were cut into complete series of semithin (2µm) sections with glass knifes (Method Smith and Tyler, 1984). The sections were stained with methylene-blue – azure II according to Richardson *et al.* (1960) and will be deposited at the ZSM. For transmission electron microscopy, ultrathin sections (80 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyle acetate and lead citrate and examined and photographed with a Philips CM 10 transmission electron microscope.

Reconstructions of the renopericardial complexes were made by hand, based on cross serial semi-thin sections.

EXCRETORY SYSTEM OF *PNEUMODERMA* SP:

Anatomy and Histology

The renopericardial complex of *Pneumoderma* sp. is placed medio-laterally at the right side of the cylindrical body, covering the surface of the visceral envelope (Fig. 1 A, B).

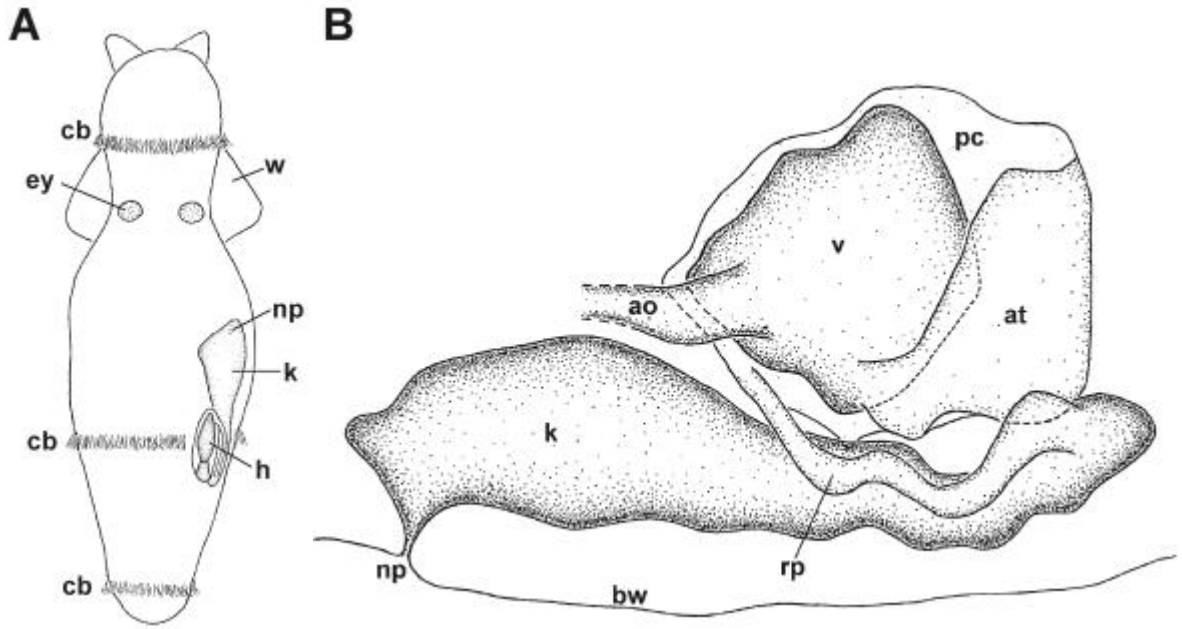

FIG. 1 *Pneumoderma* sp.. **A** Schematic drawing of *Pneumoderma* sp. (1.5 mm long) showing the position of the renopericardial complex. Dorsal view. *cb* locomotory ciliary bands, *ey* eye, *h* heart, *k* kidney, *np* nephropore, *w* wing. **B** Reconstruction of the renopericardial complex. Lateral view from left. *ao* aorta, *at* auricle, *bw* body wall, *k* kidney, *np* nephropore, *pc* pericardial cavity, *rp* renopericardial duct, *v* ventricle.

The heart comprises a single, thin-walled auricle and a thicker-walled ventricle and is enclosed in a wide pericardium. Near its anterior end, this coelomic cavity opens into a long and narrow renopericardial duct, leading to the posterior end of the kidney. Only the very proximal and distal parts of the duct are multiciliated, the central portion lacks ciliation. The elongated, tubiform kidney extends forwards from the posterior end of the heart and is characterized by a continuous, very flat, glandular epithelium. Anteriorly, the kidney is widened and it narrows towards its posterior end. Via a short and narrow duct, the kidney runs to the exterior. The ventro-laterally situated nephropore lies adjacent to the anal opening.

Fine structure

The epicardium of the ventricle and the auricle consists of epithelio-muscle cells (Fig. 2 B), bearing muscle fibers and many densely arranged mitochondria of the christae-type. The epithelial cells are interconnected by belt desmosomes and form full desmosomes to the underlying muscle cells of the atrial wall. The pericardial surface of the auricle is composed of a flat epithelium of interdigitating podocytes (Fig. 2 A). As is typical for true epithelia, the podocytes show obvious cell polarity. The extracellular matrix forms a grid and is restricted to the basal border of the cells, where also numerous foot-like projections, the pedicels, extend from the cell body. Aside from these diaphragmatic zones of the cell surface, longitudinal and transverse muscle fibers, many mitochondria and a prominent nucleus characterize the podocytes. Belt desmosomes occur between the cells, whereas true intercellular space is lacking. Podocytes are absent from the epicardial wall of the ventricle.

In contrast to the podocytes of the auricle, rhogocytes (Fig. 2 C, D) are solitary cells with an ultrafiltration weir, lying in the haemocoel (Haszprunar, 1996). They are variably shaped in *Pneumoderma* sp., 10-15 µm in diameter and entirely surrounded by the distinct grid formed by the extracellular matrix. Areas of slit-openings and the underlying cisternae are found at various positions of the cell surface. Accordingly, there is no cell polarity and there are no junctions to any other cell. Further diagnostic features of the rhogocyte are the granular cytoplasm with rough endoplasmatic reticulum, many electron-bright vacuoles (diameter up to 3 µm), the numerous small secretory vesicles and the prominent nucleus positioned in the center of the cell. Electron-dense granula that characterize the rhogocyte in other mollusc species (Haszprunar, 1996) are completely absent in *Pneumoderma* sp..

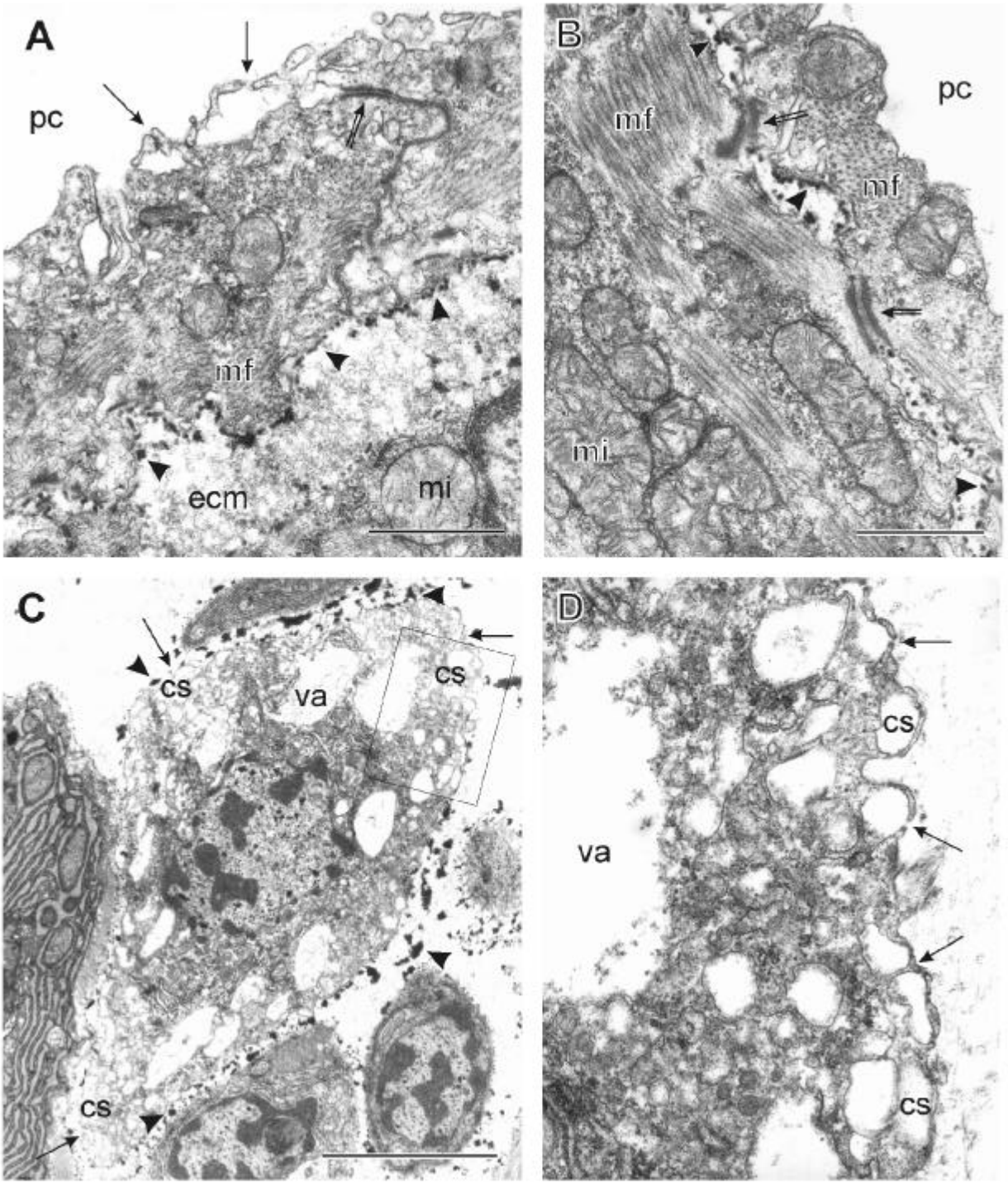

FIG. 2 *Pneumoderma* sp.. TEM-micrographs of the auricle and a rhogocyte. **A** Epithelial podocytes of the auricular epicardium. Aside from the extracellular matrix (*ecm*) forming a basal grid (arrowheads), note also the muscle fibers *(mf)*, the desmosome (double arrow) and the slit diaphragms between the pedicels (arrows). *mi* mitochondrium, *pc* pericardial cavity. Bar = 1 µm. **B** Myocytes of the auricle with two full desmosomes (double arrows), muscle fibers (*mf)*, numerous mitochondria (*mi*), and the basal grid (arrowheads). *pc* pericardial cavity. Bar = 1 µm. **C** Rhogocyte from the body cavity. Note the centrally located nucleus (*n*), the electron-bright vacuoles (*va*), the small cisternae (*cs*) which indicate the zone of slit openings (arrows) and the distinct grid of extracellular matrix (arrowheads) surrounding the cell. The rectangle marks the area shown in D. Bar = 4 µm. **D** Detail of slit area showing diaphragms (arrows), underlying cisternae (*cs*) and various vacuoles (*va*).

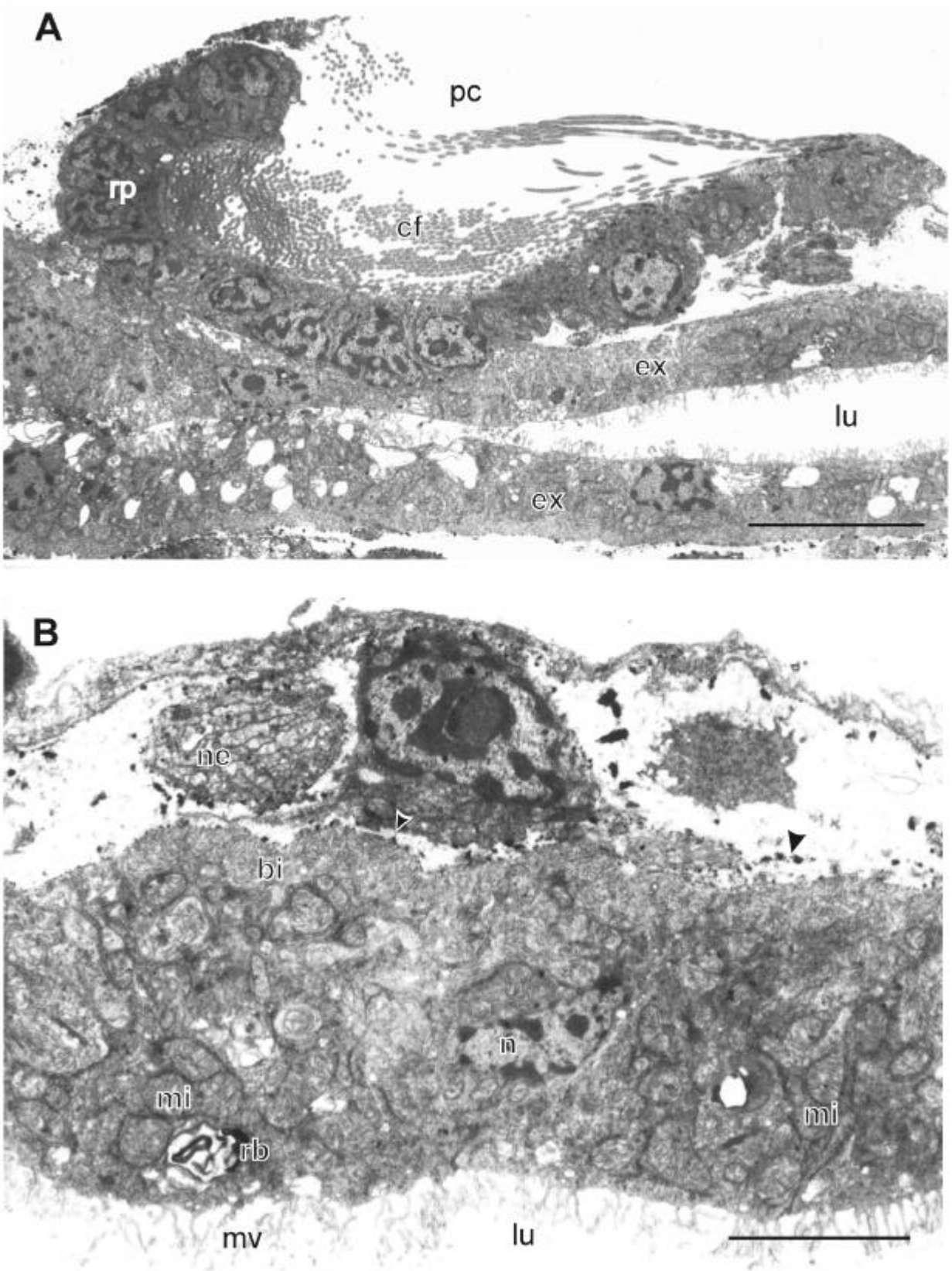

FIG. 3 *Pneumoderma* sp.. TEM-micrographs of kidney and renopericardial duct. **A** Opening of the pericardium (*pc*) into the renopericardial duct (*rp*). Note the ciliary flame (*cf*), as well as the excretory epithelium (*ex*) and the lumen (*lu*) of the adjacent kidney. Bar = 10 µm. **B** Nephrocytes of the kidney epithelium with basal infoldings (*bi*), apical microvillous border (*mv*) towards the lumen (*lu*), numerous mitochondria (*mi*), and a residual body (*rb*). Also note the surrounding basal lamina (arrowheads), the nucleus (*n*), and the nerve (*ne*) above the excretory epithelium. Bar = 4 µm.

The squamose cells of the renopericardial duct are characterized by numerous mitochondria and large nuclei. Only the proximal and distal parts of the duct are multiciliated (Fig. 3 A), whereas the central section is aciliated, possessing numerous microvilli. The renal cells (Fig. 3 A, B) are very flat with a large nucleus that occupies most of the height of the cell and form a continuous, simple epithelium. Numerous densely arranged mitochondria of the christae-type and some residual bodies occupy most parts of the cytoplasm. A dense microvillous border covers the luminal surface, whereas excessive infoldings characterize the surface towards the basal lamina.

EXCRETORY SYSTEM OF *CRESEIS VIRGULA* RANG, 1828:

Anatomy and Histology

As described correctly by Meisenheimer (1905) the renopericardial complex of *Creseis* species is situated on the right side of the body, covering the surface of the visceral envelope (Fig. 4 A, B).

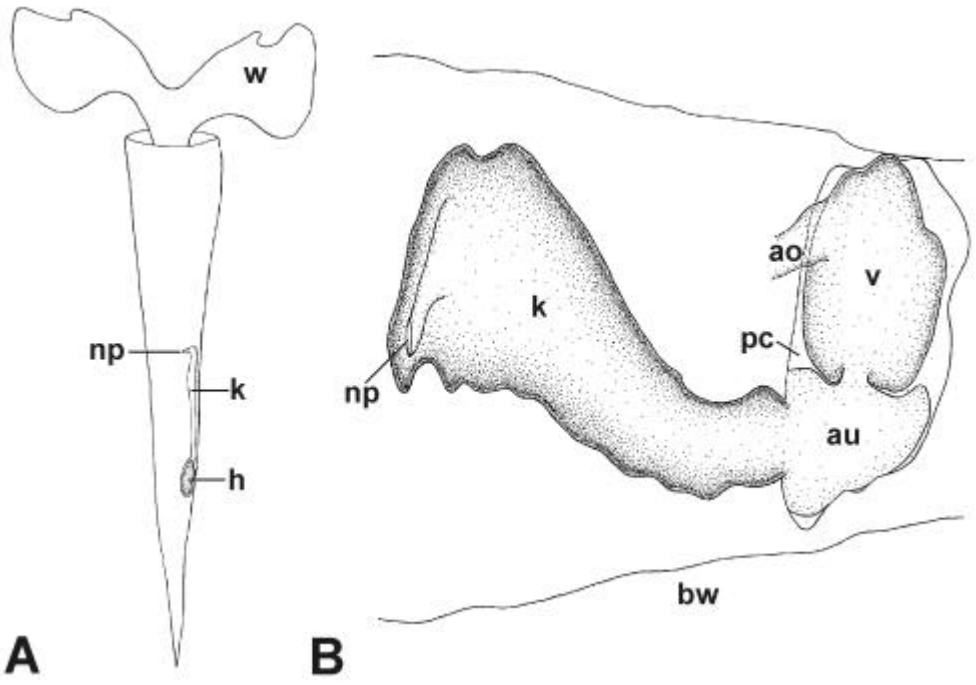

FIG. 4 *Creseis virgula*. **A** Schematic drawing of *Creseis virgula* (3.5 mm long) showing the position of the renopericardial complex. Dorsal view. *h* heart, *k* kidney, *np* nephropore, *w* wing. **B** Reconstruction of the renopericardial complex. Lateral view from the left. *ao* aorta, *au* auricle, *bw* body wall, *k* kidney, *np* nephropore, *pc* pericardial cavity, *v* ventricle.

The heart consists of a single auricle and ventricle lying side by side in a wide pericardial sac. Two auriculo-ventricular valves enable an unidirectional flow of the haemolymph into the ventricle. The myocardium of the auricle is much thinner than that of the ventricle. The elongated, tubiform kidney is located immediately anterior to the heart and is characterized by a homogeneous, flat glandular epithelium. Proximally, the pericardial cavity opens into the conical end of the kidney through a very short ciliated funnel, the nephrostome. A distinct renopericardial duct is absent. Forming a short duct, the kidney opens via a nephropore into the mantle cavity at its anterior end.

Fine structure

The outer pericardial epithelium and the epicardium of the ventricle consist of epithelio-muscle cells (Fig. 5A), while the auricular epicardium is composed of a homogenous flat epithelium of podocytes (Fig. 5 B, C).

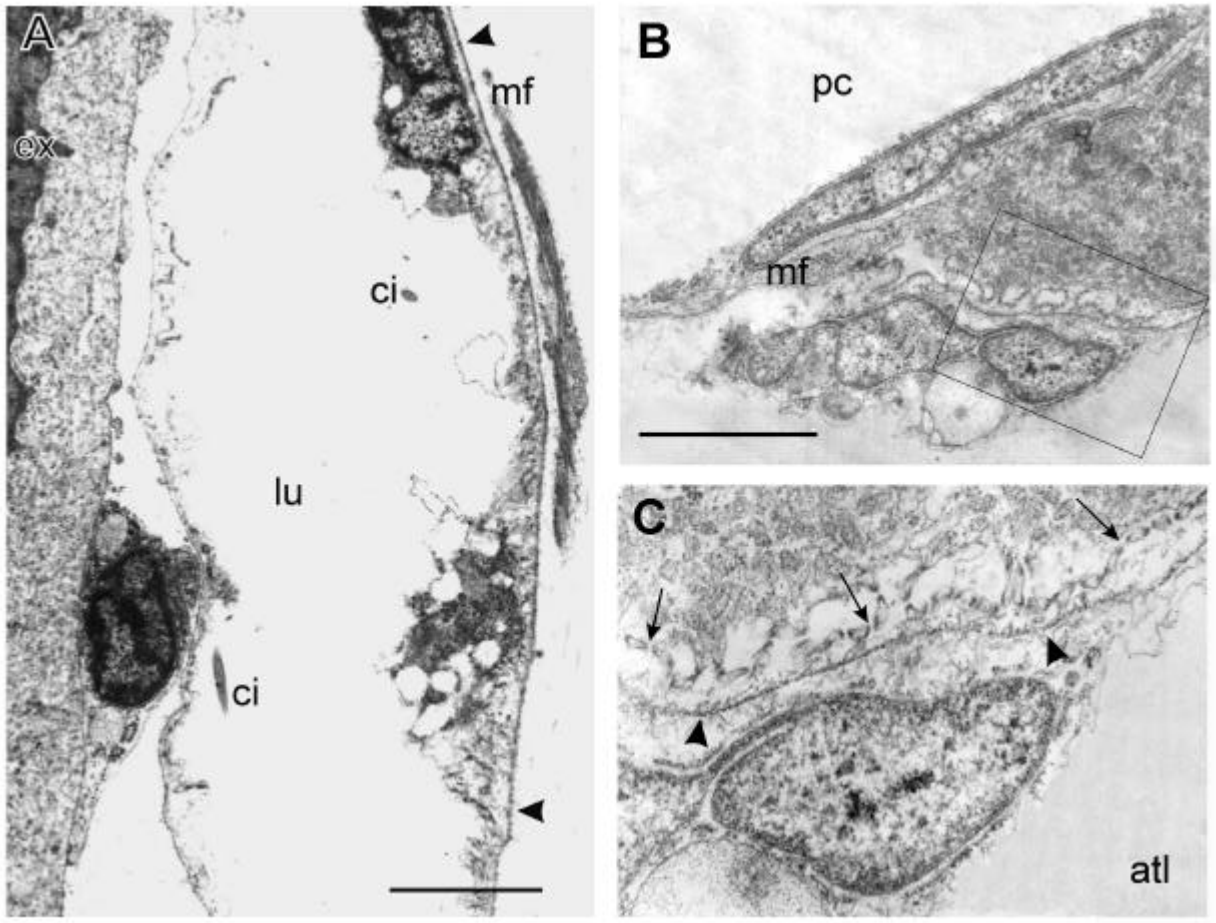

FIG. 5 *Creseis virgula.* TEM-micrographs of the auricle. **A** Overview on the auricle. *arrowheads* basal lamina, *ci* cilia, *ex* excretory epithelium of the adjacent kidney, *lu* kidney lumen, *mf* muscle fibers. Bar = 4 µm. **B** Podocyte of the auricular surface. *mf* muscle fiber, *pc* pericardial cavity. The rectangle marks the area shown in C. Bar = 2 µm. **C** Detail of B showing the slit diaphragms between the pedicels (arrows) and the basement membrane (arrowheads). *atl* auricle.

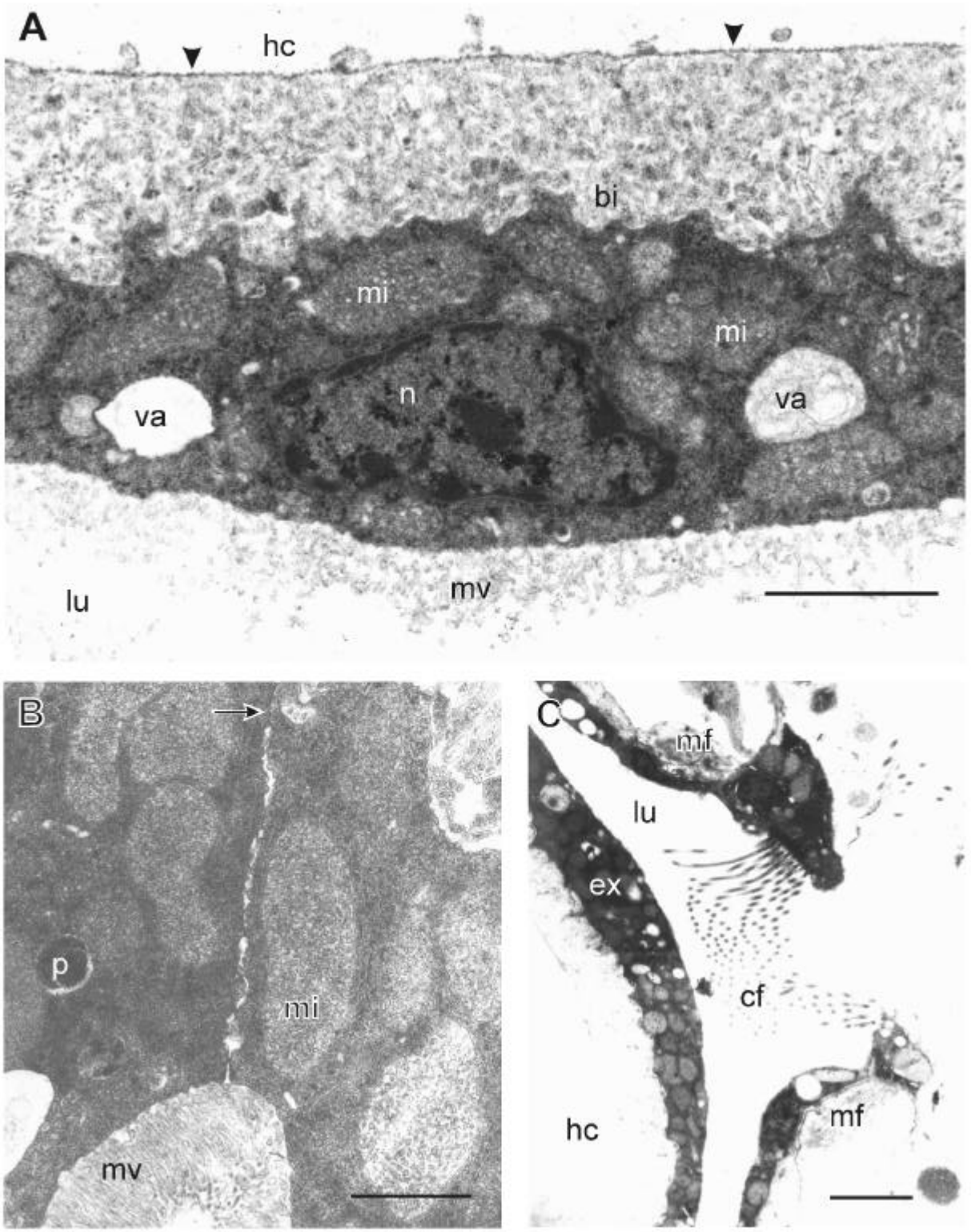

FIG. 6 *Creseis virgula*. TEM-micrographs of the kidney. **A** Nephrocytes of the kidney epithelium with apical microvillous border (*mv*) towards the lumen (*lu*). Note the basal infoldings of the cell surface (*bi*), the nucleus (*n*), the electron-bright vacuoles (*va*), the mitochondria (*mi*), and the surrounding basal lamina (arrowheads). *hc* haemocoel. Bar = 2 µm. **B** Nephrocytes showing several tubular mitochondria (*mi*), an electron-dense pigment granule (*p*), a spot desmosom (arrow) and the apical microvilli (*mv*). Bar = 8 µm. **C** Cells of the nephropore with ciliary flame *(cf)* and adjacent sphincter muscle *(mf)*. *ex* excretory epithelium of the kidney, *hc* haemocoel, *lu* kidney lumen. Bar = 1 µm.

The numerous pedicels are restricted to the basic border of the podocytes and rest on an underlying basal lamina formed by the extracellular matrix. Muscle fibers and many mitochondria further characterize the podocytes that are absent from the ventricular wall. Rhogocytes could not be detected in the body cavity or connective tissue of *Creseis virgula*.

The very homogeneous, flat epithelium of the kidney is composed of a single cell type. These excretory cells (Fig. 6 A, B) are 6-9 µm high and mainly characterized by a distal, dense microvillous border, a prominent nucleus and well developed infoldings of the basal cell membrane. Many mitochondria of the tubulous-type and various vacuoles and granulae are spread over the cytoplasm. The extracellular matrix forms a distinct basal lamina. Fine muscle-fibers inserting this basal lamina fix the kidney in the body cavity. The multiciliated cells of the nephropore (Fig. 6 C) are surrounded by a specialized sphincter muscle.

DISCUSSION

The histology and fine-structure of the renopericardial complex of *Pneumoderma* sp. and *Creseis virgula* generally correspond to that of other molluscs. In most of the taxa with available data on the excretory system, podocytes were identified as the site of ultrafiltration (Andrews, 1985; Andrews, 1988; Morse and Meyhöfer, 1990; Reynolds *et al.*, 1993; Morse and Reynolds, 1996). The slit areas with diaphragms between adjacent pedicels of the podocytes and the covering extracellular matrix enable a selective transfer of haemolymph molecules into the pericardial cavity (Andrews and Little, 1972; Andrews, 1988). Since in both *Pneumoderma* sp. and *Creseis virgula* podocytes could only be detected in the auricular epicardium but are absent in the ventricular and pericardial wall, the auricular wall has to be regarded as the sole site of ultrafiltration in these species. This condition is present in most of the molluscan taxa (Andrews, 1985; Morse and Reynolds, 1996; Bartolomaeus, 1997) and considered as plesiomorphic for the phylum.

The striking resemblance of the meandering pattern of diaphragmatic slits found in rhogocytes of *Pneumoderma* sp. to the ultrafiltration weir of the podocytes (Fig. 2A, D) suggests that rhogocytes also act as a molecular sieve. As previously outlined in detail (Haszprunar, 1996) this condition provides significant evidence for a cytological homology between molluscan rhogocytes and metazoan podocytes, cyrtocytes and nephrocytes. Whereas filtration pressure of the podocytes is by muscular activity, it is probably caused by endocytosis in rhogocytes (Morse and Cooper, 1993; Haszprunar 1996).

The epithelium of the kidney of both *Pneumoderma* sp. and *Creseis virgula* shows the typical fine-structure of excretory organs of marine molluscs (Andrews, 1985, 1988; Morse and Meyhöfer, 1990; Eernisse and Reynolds, 1994; Morse and Reynolds, 1996; Haszprunar and Schäfer, 1997). Numerous infoldings greatly increase its basal surface and a dense microvillous border is present on the luminal surface.

With a heart consisting of a single ventricle and auricle enclosed in a wide pericardium and a single kidney, *Pneumoderma* sp. and *Creseis virgula* show a situation typical for most of the caenogastropod and heterobranch species that have been examined so far (see Andrews, 1985; Luchtel *et al.*, 1997). The two species investigated belong to two different opisthobranch taxa that are highly specialized on a holoplanctonic life-cycle. Together with the shell-bearing Thecosomata (*Creseis virgula*) the shell-less Gymnosomata (*Pneumoderma* sp.) represent the “pteropods” that are mainly characterized by their wing-like locomotory organs. Both thecosome and gymnosome species frequently exhibit progenesis and paedomorphosis (Lalli and Gilmer, 1989). Whereas the shell of the Thecosomata is generally regarded as paedomorphic (Bandel *et al*., 1984), numerous gymnosome specimens show external larval features such as the locomotory ciliary bands (see Fig. 1A) while the velum and larval shell are already lost (Lalli and Gilmer, 1989; Barnich and Uthe, 1998). The *Pneumoderma* specimens examined in this study all possess three ciliary bands and a metanephridial excretory system, but only in the largest specimen (3 mm length) a fully developed hermaphroditic reproductive tract indicating progenesis is present.

Paedomorphic or progenetic representatives of other spiralian taxa like the Polychaeta or Echiura often exhibit unique modifications of their excretory system. Westheide (1986) described the paired excretory organs of the interstitial polychaete *Hesionides arenaria* closely resembling solenocytic protonephridia and argued that they might be derived from a metanephridium. The secondary protonephridium of the dwarf male of *Bonellia viridis* is presumably also derived from a metanephridium (Schuchert, 1990), since the female of this sexually dimorphic species possesses metanephridia. In contrast, both gymnosomes and thecosomes have retained a metanephridial excretory system that is regarded primary for adult Gastropoda (Ponder and Lindberg, 1997), despite of their numerous morphological and anatomical modifications and their paedomorphic tendencies.

Thus, the ultrastructural data of *Pneumoderma* sp. and *Creseis virgula* given in this study do not only represent the first detailed information on the excretory system of pteropods but are also the only example of opisthobranchs so far showing the original excretory system of molluscs with podocytes situated on the atrial wall. The present results contradict the

assumption of Andrews (1988) that the primary site of filtration of urine in the auricle probably has been lost in the ancestors of the opisthobranchs and that the function of podocytes has been adopted by other cell-types with a filtration barrier. Significant modifications of the excretory system such as the movement of the site of ultrafiltration to the pericardial wall in *Philinoglossa helgolandica* (cf. Bartolomaeus, 1997) and the loss of the heart and presence of a completely new system of ultrafiltration in *Rhodope transtrosa* (cf. Haszprunar, 1997) appear to be restricted to certain taxa and may be related to a special habitat or small body-size. The location of ultrafiltration also varies in the few pulmonate species that have been studied yet. There is general agreement that it occurs somewhere in the renopericadial complex and four different sites have been identified (see review by Luchtel *et al.*, 1997): ultrafiltration occurs in the heart, paracellular or transcellular in parts of the kidney or is restricted to a small specialized area of the kidney supplied specifically with aterial blood.

Further studies on the major opisthobranch taxa are necessary to increase our understanding of nephridial evolution in gastropods. Especially species like the enigmatic, worm-like *Helminthope psammobionta* Salvini-Plawen, 1991 that have lost the heart probably show other modifications of the primary excretory system of molluscs and will therefore be investigated within the framework of a larger project.

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APPENDIX II

Anatomy and ultrastructure of the excretory system of a heart-bearing and a heart-less sacoglossan gastropod (Opisthobranchia, Sacoglossa)

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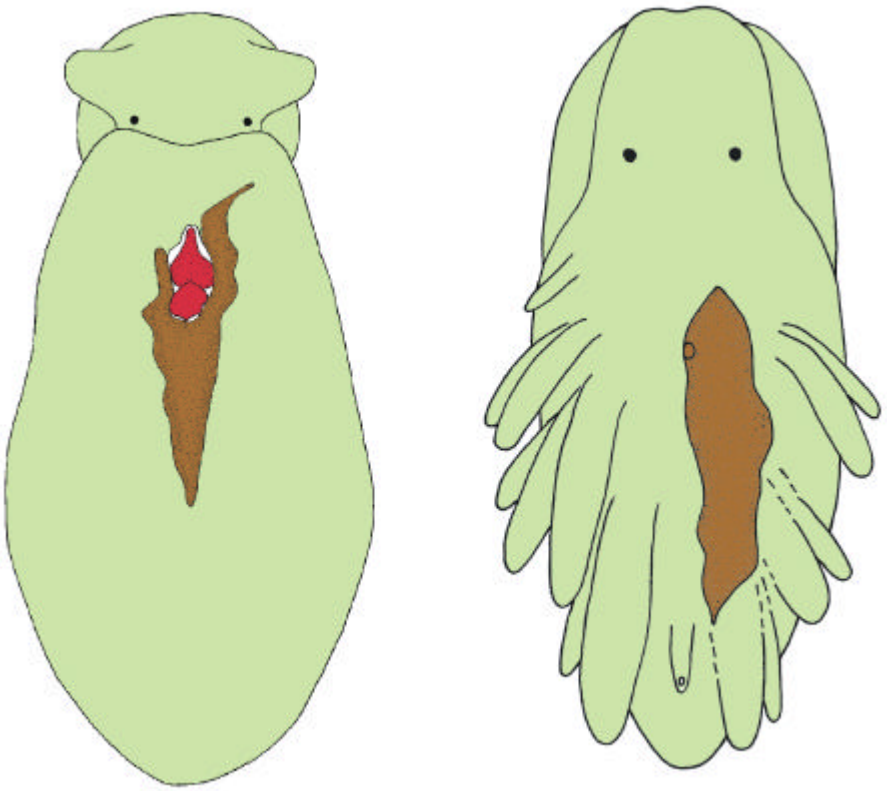

Abstract. The microanatomy and ultrastructure of the excretory system of the Sacoglossa have been investigated from two species by means of semithin serial sections, reconstructions, and transmission electron microscopy (TEM). Whereas *Bosellia mimetica* shows a functional metanephridial system consisting of a heart with ventricle and auricle in a pericardium and a single kidney, *Alderia modesta* lacks heart and pericardium, possessing only several haemocoelic sinuses and a very long kidney. In *Bosellia mimetica* podocytes as the site of ultrafiltration could be detected in the pericardial epithelium lining the auricular wall. The flat epithelium of the kidney with extensive basal infoldings and a dense microvillous border towards the luminal surface serves to modify the ultrafiltrate. In *Alderia modesta* podocytes are absent. Solitary rhogocytes (pore cells), the fine-structure of which strongly resembles podocytes (meandering slits with diaphragms covered by extracellular matrix) occur in *Bosellia mimetica* and *Alderia modesta,* representing additional loci of ultrafiltration. The presence of podocytes situated in the epicardial wall of the auricle is regarded as plesiomorphic for the Mollusca and confirmed for the Sacoglossa in this study, contradicting elder assumptions of the loss of the primary site of ultrafiltration in the ancestors of the Opisthobranchia. In contrast to the likewise heart-less Rhodopidae with a pseudoprotonephridial ultrafiltration system, *Alderia modesta* shows no further modifications of the excretory system.

INTRODUCTION

The excretory systems of the Mollusca show cellular structures to carry out three basic functions: ultrafiltration of the primary urine from the haemocoel, additional transport of waste products from the haemolymph into the urine, and reabsorption of useful metabolites from the primary urine (Luchtel *et al.* 1997). The larval protonephridial system (see Bartolomaeus 1989; Ruthensteiner and Schaefer 1991; Tardy and Dongard 1995; Haszprunar and Ruthensteiner 2000) is replaced in adults by derivates of the coelom that represent a functional metanephridial system in the sense of Ruppert and Smith (1988). A general character in the Mollusca is the close ontogenetic and functional interrelation of the pericardial complex and the nephridia in excretion (Andrews 1988, Morse and Reynolds 1996). The renopericardial complex of the Mollusca consists of the pericardium and, originally, of two pericardial ducts leading to the exterior (Haszprunar 1992). In the Testaria

(Polyplacophora and Conchifera), the distal parts of the renopericardial ducts were enlarged and modified into the sac-like kidneys.

In general, the wall of the heart is surrounded by the pericardial epithelium (called epicardium) with an underlying extracellular matrix (ECM), representing the location of ultrafiltration in molluscs. Podocytes of the epicardium that rest on the ECM are the cellular ultrafiltration barrier. The primary urine accumulates in the pericardial cavity and drains off into the excretory ducts that modify this ultrafiltrate (Andrews 1988; Ruppert and Smith 1988; Salvini-Plawen and Bartolomaeus 1995; Morse and Reynolds 1996; Bartolomaeus 1997). In addition, solitary cells with an ultrafiltration weir, the rhogocytes (pore cells), are diagnostic for all molluscs. As they structurally resemble metanephridial podocytes and protonephridial cyrtocytes (terminal cells), a common genetic basis and homology of these three cell types have been proposed (Haszprunar 1996).

The fine-structure of the excretory system has been investigated by TEM from representatives of almost all higher molluscan taxa (e.g., Andrews 1988; Morse and Reynolds 1996). However, within the Gastropoda fine-structural studies on the renopericardial complex have been restricted on several prosobranch groups and the Pulmonata (for reviews see Andrews 1988; Luchtel *et al.* 1997). Until recently, no detailed information has been provided for the Opisthobranchia (Gosliner 1994). The latest ultrastructural studies dealing with the renopericardial complex of opisthobranch gastropods refer exclusively to small and aberrant species: In the two holoplanktic species *Creseis virgula* Rang, 1828 and *Pneumoderma* sp., podocytes on the auricular wall as the original ultrafiltration site of the Mollusca are retained (Fahrner and Haszprunar 2000). In contrast, the excretory system of the partly paedomorphic, interstitial *Philinoglossa helgolandica* Hertling, 1932 is modified, in that podocyte-like cells in the pericardial wall are presumed to be the site of ultrafiltration (Bartolomaeus 1997). The enigmatic and worm-like *Rhodope transtrosa* Salvini-Plawen, 1991 lacks the heart and shows a completely new system of ultrafiltration (Haszprunar 1997).

These data suggest that other taxa of the Opisthobranchia, especially those that have lost the heart, may also exhibit significant modifications of the primary excretory system. Therefore, the ultrastructure of the renopericardial complex of the Sacoglossa is investigated in detail from two species, the heart-bearing *Bosellia mimetica* Trinchese, 1890 and the heart-less *Alderia modesta* (Lovén, 1844) in this study. The provided data are compared with the results of studies on the anatomy and ultrastructure of the excretory system of major opisthobranch taxa that are carried out within the framework of a larger, comparative project.

MATERIALS AND METHODS

The *Bosellia mimetica* specimens were collected in Calvi (Corsica, France) in September 1997 and in Fetovaia Bay (Elba, Italy) in June 1998 from *Halimeda* algae in 10 to 15m depth. *Alderia modesta* was found by P. Krug in October 1996 on a mudflat in the Kendall-Frost marine reserve in Mission Bay, San Diego (California, USA) on the alga *Vaucheria longicaulis*. The specimens were relaxed by a solution of 7% $MgCl_2$ and fixed in 4 % glutardialdehyde buffered in 0.2 M sodium cacodylate (pH 7.2) before rinsing in the same buffer in decreasing concentrations. Postfixation in buffered 1 % OsO_4 for two hours was followed again by rinsing the specimens with cacodylate buffer and dehydration in a graded ethanol series. The fixed specimens were embedded overnight in Araldit resin for light microscopy and in Spurr`s (1969) low viscosity resin for electron microscopy.

To enable an overall view on the *in situ*-position of the excretory system of the two species investigated, complete series of semithin sections (2µm) were made with glass knifes (Henry 1977). The sections were stained with methylene-blue – azure II according to Richardson et al (1960) and the slides are deposited at the ZSM (Nrs. 19971272/1, 20010664, 20010665). For transmission electron microscopy (TEM), ultrathin sections (70 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyl acetate and lead citrate and examined and photographed with a Philips CM 10 TEM. Reconstructions of the excretory systems were made by hand, based on serial, semi-thin, cross sections.

RESULTS

Excretory system of Bosellia mimetica

The renopericardial complex of *Bosellia mimetica* is placed medio-dorsally, overlying the gonad for most of its length (Fig. 2C,D,F). The heart lies in the second quarter of the body and comprises a thin-walled auricle and a thicker-walled ventricle enclosed in a wide pericardium. Two auriculo-ventricular valves cause an unidirectional flow of the haemolymph into the ventricle. Near its anterior end, the pericardium opens into the kidney via a short and narrow, ciliated nephrostome (Fig. 2E). The elongated, tubular kidney is characterized by a continuous, flat, and highly vacuolated epithelium (Fig. 2F). In the anterior third, the kidney splits into two arms enclosing the heart ventro-laterally (Fig. 2D). The right arm extends

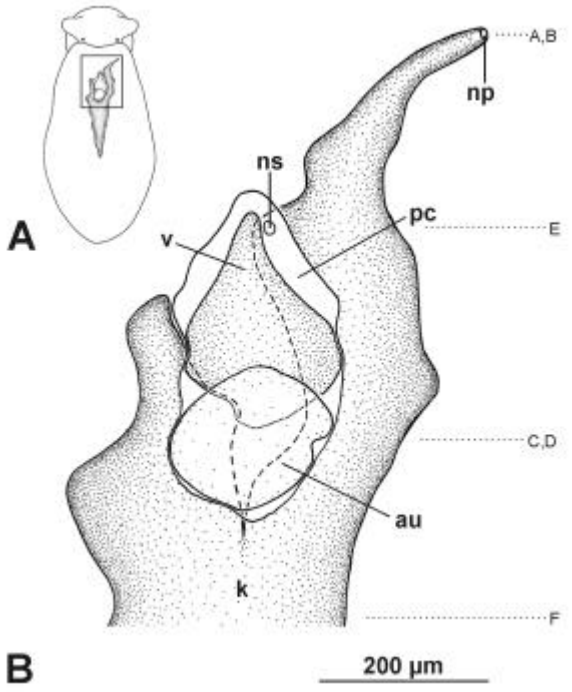

Fig. 1 A *Bosellia mimetica*, Reconstruction of a 3 mm specimen showing the position of the renopericardial complex. Dorsal view. Boxed area is enlarged in B. **B** Reconstruction of the renopericardial complex. Dorsal view. Posterior part of the kidney not shown. *au* auricle, *k* kidney, *np* nephropore, *ns* nephrostome, *pc* pericardium, *v* ventricle. Stippled lines mark the levels of the cross-sections shown in Fig. 2.

further anterior than the left arm, narrows, and opens to the exterior on the right side, in the upper region of the transverse fold separating head and body (Fig. 2A,B). The female genital pore and the anus lie closely associated, to the right of the nephropore (Fig. 2B).

The pericardial surface of the auricle is composed of a flat epithelium of epithelio-muscle cells and interdigitating podocytes (Fig. 3D). As is typical for true epithelia, the podocytes show belt desmosomes and obvious cell polarity. Numerous foot-like processes extending from the cell body, the pedicels, are restricted to the basal border of the cell. These pedicels rest on an underlying basement lamina formed by the extracellular matrix. The ultrafiltration-slits between the pedicels are spanned by fine diaphragms. A further diagnostic feature of the podocytes are muscle fibers. Podocytes are absent from the epicardial wall of the ventricle and the outer pericardial wall.

Aside from the epithelial podocytes of the auricle, solitary rhogocytes (Fig. 3B) represent a second cell type with an ultrafiltration weir (Haszprunar 1996). Rhogocytes are irregulary shaped in *Bosellia mimetica*, depending on the space available. They are 5-10 µm in diameter and entirely surrounded by a thin layer of extracellular matrix. Zones of slit-diaphragms and the underlying small cisternae occur at various positions of the cell surface.

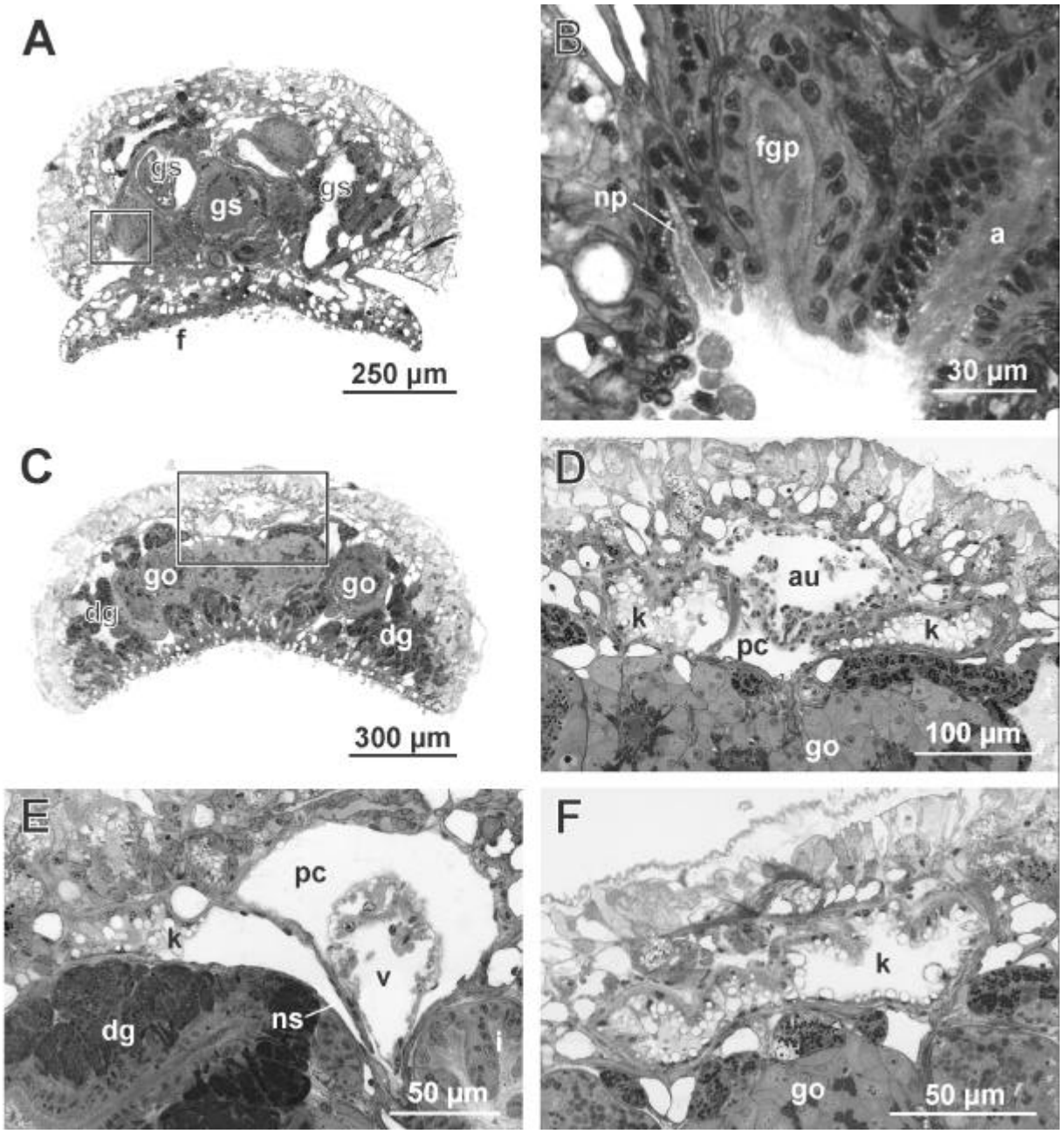

Fig. 2 *Bosellia mimetica*, Histology of the excretory system based on serial semi-thin cross sections, frontal view. **A** Cross section of entire body showing the position of the closely associated nephropore, female genital pore, and anal opening (boxed area, enlarged in B) on the right side, in the upper region of the transverse fold separating head and body. Note that the genital system (*gs*) occupies nearly the entire body space. *f* foot. **B** Nephropore (*np*) and adjacent female genital pore (*fgp*) and anal opening (*a*). **C** Cross section of entire body showing the dorso-median position of the renopericardial complex (boxed area, enlarged in D). *dg* digestive gland, *go* gonad. **D** Cross section through the anterior third of the renopericardial complex, overlying the gonad (*go*). The two branches of the kidney (*k*), characterized by a highly vacuolated epithelium, enclose the heart ventro-laterally. *au* auricle, *pc* pericardial cavity. **E** Opening of the pericardium (*pc*) into the kidney (*k*) via the narrow nephrostome (*ns*). *dg* digestive gland, *i* intestine, *v* ventricle. **F** Cross section through the posterior part of the kidney (*k*) after fusion of the two arms. *go* gonad.

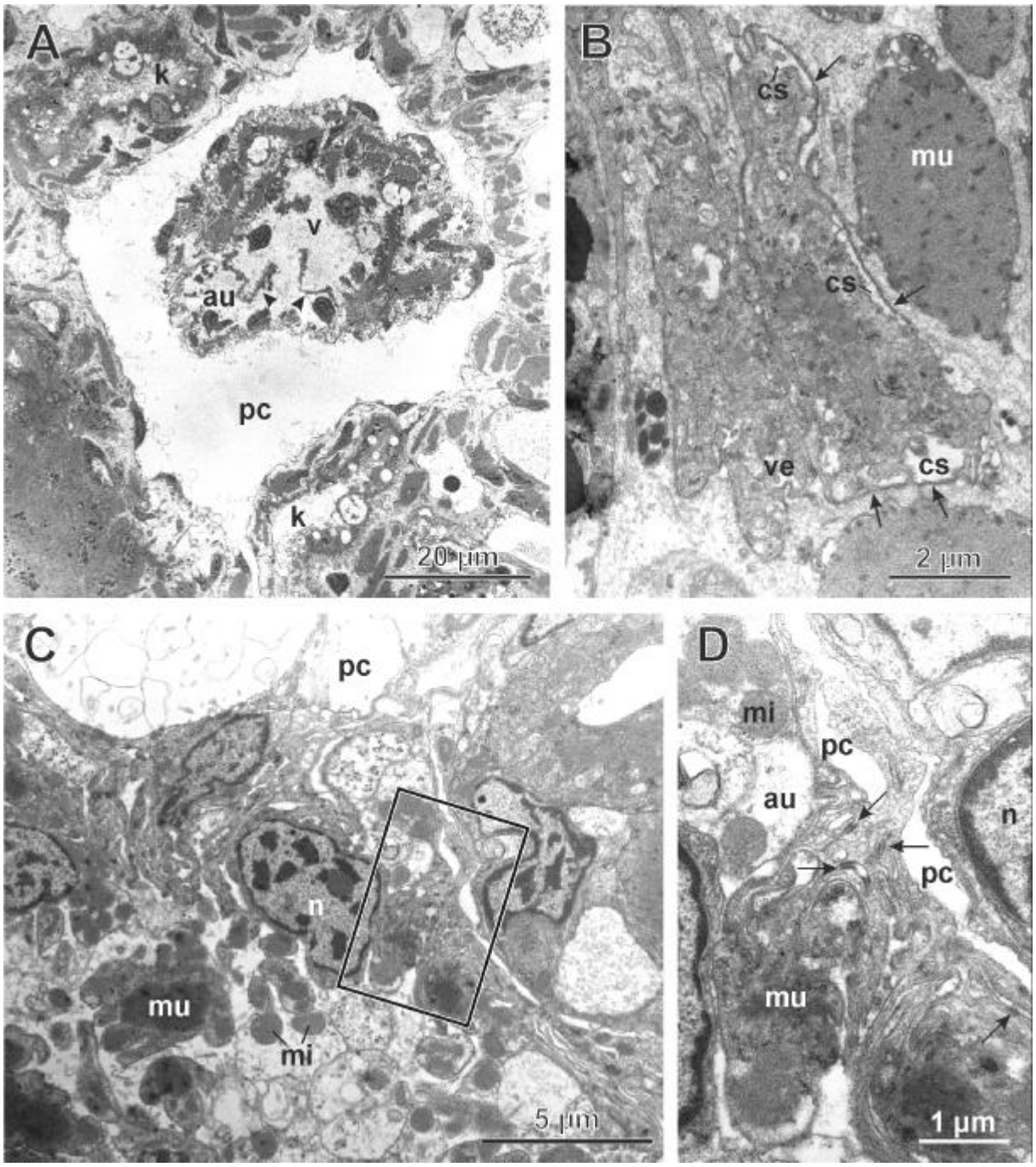

Fig. 3 *Bosellia mimetica*, TEM micrographs of the heart and a rhogocyte. **A** Overview of the heart showing the transition from ventricle (*v*) to auricle (*au*). Note the two auriculo-ventricular valves (arrowheads), the wide pericardial cavity (*pc*), and the two branches of the kidney (*k*) lying adjacent to the heart. **B** Rhogocyte with numerous vesicles (*ve*) and small cisternae (*cs*) indicating the zone of slit openings (arrows) surrounding the cell. The nucleus is not visible. *mu* muscle fiber. **C** Auricular wall. Boxed area is enlarged in D. *mi* mitochondria, *mu* auricular muscle fiber, *n* nucleus, *pc* pericardial cavity. **D** Interdigitating, epithelial podocytes of the auricular surface with narrow slits between the pedicels (arrows). *au* auricle lumen, *mi* mitochondrium, *mu* muscle fiber, *n* nucleus, *pc* pericardial cavity.

Accordingly, there is no cell polarity and there are no junctions to other cells. The rhogocytes are further characterized by their granular cytoplasm with rough endoplasmatic reticulum, several mitochondria, and numerous small secretory vesicles.

The renal cells of the kidney (Fig. 4) are flat with a large nucleus that occupies most of the height of the cell and form a continuous, simple epithelium. A dense microvillous border covers the luminal surface, whereas excessive infoldings characterize the surface towards the basement membrane. The cells lack any ciliation. Numerous mitochondria are accumulated close to the basal infoldings. Coated and uncoated vesicles, lysosomes, and some large, electron-lucent vacuoles (diameter up to 5 µm) further occupy the cytoplasm.

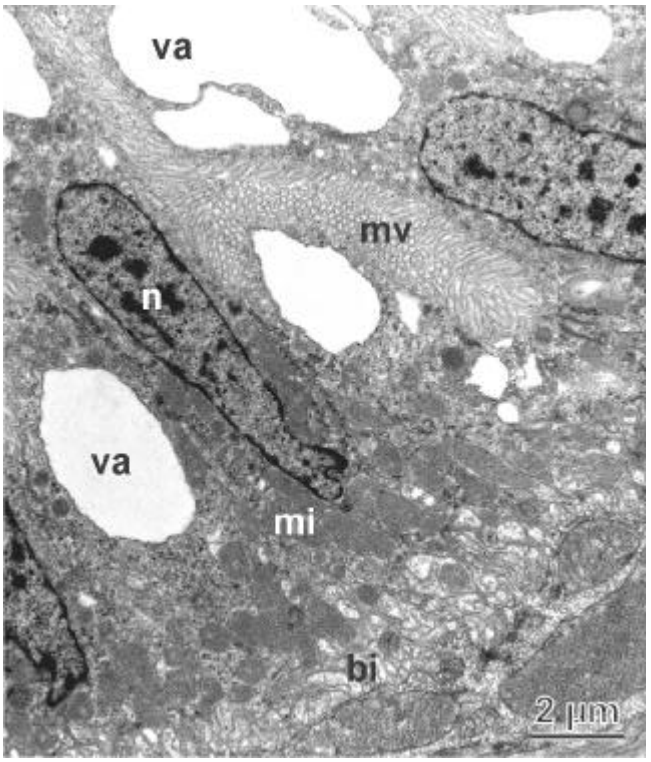

Fig. 4 *Bosellia mimetica*, TEM micrograph of excretory cells of the kidney showing excessive basal infoldings (*bi*), numerous mitochondria (*mi*), large, electron-lucent vacuoles (*va*), an elongated nucleus (*n*), and the dense, distal microvillous border to the collapsed lumen (*mv*).

Excretory system of Alderia modesta

Alderia modesta lacks heart and pericardium, its vascular system consists of haemocoelic spaces and lacunes within the ECM only (Fig. 6A,B). The viscera, including the long, tubular, medio-dorsally placed kidney, receive their haemolymph supply from a pattern of sinuses giving branches to them. In the anterior quarter of the kidney the nephropore (Fig. 6C) opens to the exterior, showing a prominent ciliary flame. The anal opening is positioned far posterior, at the end of a distinct anal papilla.

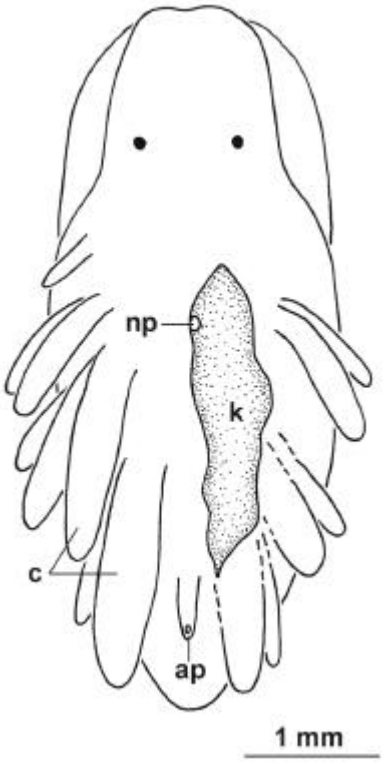

Fig. 5 *Alderia modesta*, Schematic drawing of a 5 mm specimen showing the position of the kidney. Dorsal view. *ap* anal papilla, *c* cerata, *k* kidney, *np* nephropore.

A single cell type composes the flat, homogenous epithelium of the kidney. These excretory cells (Fig. 7A,B) are characterized mainly by infoldings of the basal cell membrane, various, very large vacuoles (diameter up to 10 µm), a prominent nucleus, and a dense, apical microvillous border. Numerous apical, coated and uncoated vesicles, mitochondria, and basal lysosomes are spread over the cytoplasm.

The solitary rhogocytes (Fig. 7C,D) represent the only cell-type with an ultrafiltration weir in *Alderia modesta.* They are more or less round, 10-15 µm in diameter, and completely surrounded by a thin layer of a homogeneous extracellular matrix. Areas of slit-openings and the underlying small cisternae are spread over the entire cell surface. Further features of the rhogocyte are its granular cytoplasm with rough endoplasmatic reticulum, very large, electron-lucent vacuoles, and the numerous, small vesicles. Electron-dense granulae that are characteristic for rhogocytes in other mollusc species (Haszprunar 1996) are absent in *Alderia modesta.*

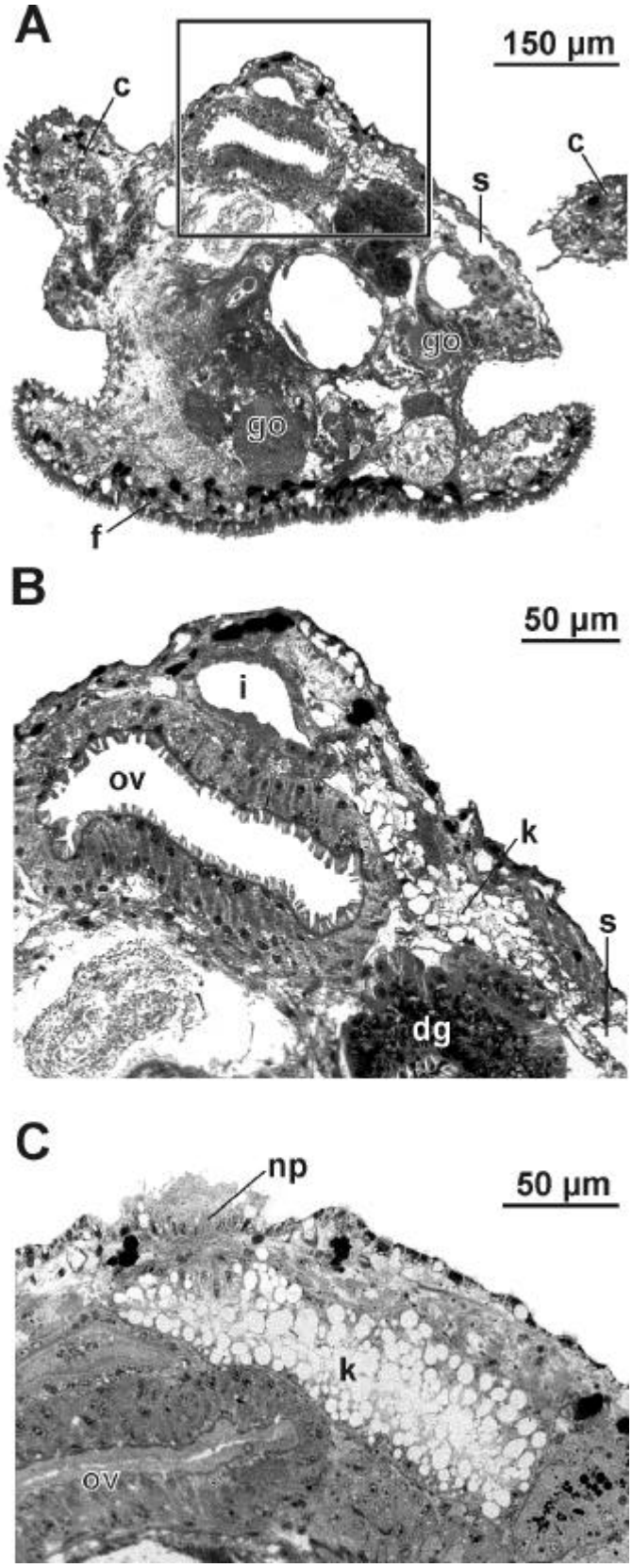

Fig. 6 *Alderia modesta*, Histology of the kidney based on serial semi-thin cross sections, frontal view. **A** Cross section of entire body showing the dorso-median position of the kidney (boxed area, enlarged in B). Also note the blood sinus (s) that touches the kidney ventrolaterally. *c* cerata, *f* foot, *go* gonad. **B** Kidney (*k*) with highly vacuolated epithelium and collapsed lumen. *dg* digestive gland, *i* intestine, *ov* oviduct, *s* blood sinus. **C** Opening of the kidney (*k*) via the nephropore (*np*). ov oviduct.

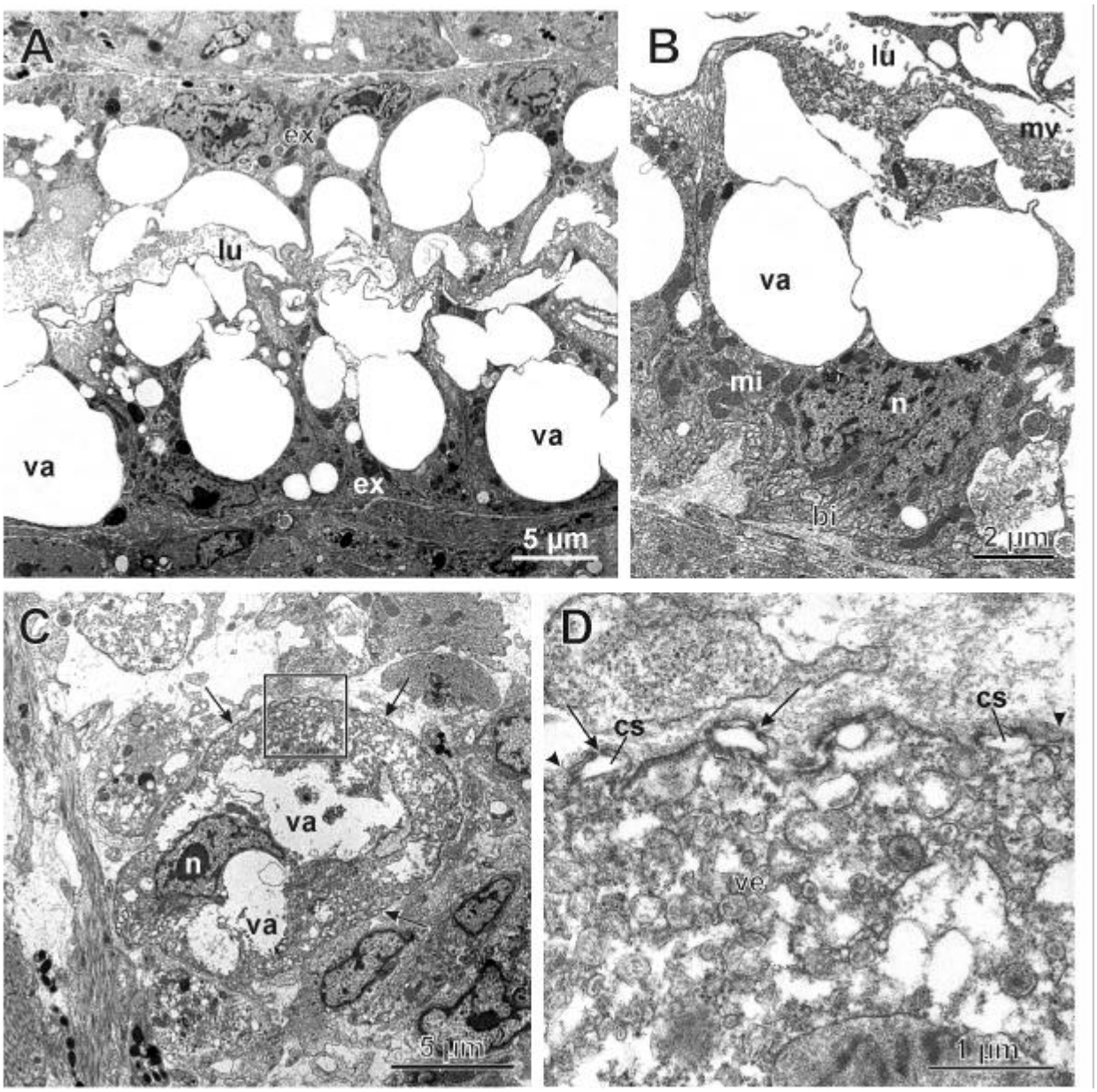

Fig. 7 *Alderia modesta*, TEM-micrographs of kidney and rhogocytes. **A** Excretory epithelium of the kidney (*ex*) with numerous very large, electron-lucent vacuoles (*va*). Lumen (*lu*) collapsed. **B** Cells of the excretory epithelium of the kidney showing basal infoldings (*bi*), mitochondria (*mi*), very large, electron-lucent vacuoles (*va*), and a dense microvillous border (*mv*) to the lumen (*lu*). *n* nucleus. **C** Rhogocyte with large electron-lucent vacuoles (*va*), nucleus (*n*), and small cisternae indicating the zone of slit openings (arrows) almost completely surrounding the cell-surface. Rectangle marks the area enlarged in D. **D** Detail of slit area showing the extracellular matrix (arrowheads), the diaphragmatic slits (arrows) with underlying cisternae (*cs*), and numerous vesicles (*ve*).

DISCUSSION

The histological and fine-structural data presented herein reveal that the excretory system of the Sacoglossa basically corresponds to that of other Mollusca. Podocytes were identified as the cellular site of ultrafiltration and production of a primary filtrate in most of the taxa in

which the excretory system has been investigated (Andrews 1985; Andrews 1988; Morse and Meyhöfer 1990; Reynolds et al. 1993; Morse and Reynolds 1996; Fahrner and Haszprunar 2000). The slit openings with diaphragms between adjacent foot processes of the podocytes covered by extracellular matrix enable a selective transfer of molecules from the haemolymph into the pericardial cavity (Andrews and Little 1972; Andrews 1988). In *Bosellia mimetica* podocytes could only be detected in the epicardial wall of the auricle but are absent from the ventricular or outer wall of the pericardial epithelium. Hence, the wall of the auricle is regarded as the sole site of ultrafiltration in this species. This condition is also present in most of the molluscan taxa studied so far (Andrews 1985; Morse and Reynolds 1996; Fahrner and Haszprunar 2000) and is considered as plesiomorphic for the Mollusca. In certain species of the Gastropoda, additional podocytes occur in the surface of the ventricle (Økland 1982; Luchtel et al. 1997), while in the Cyclophorida the ventricular wall probably represents the main site of ultrafiltration (Andrews and Little 1972). Scaphopoda with a reduced heart show podocytes in the epithelium of the existing pericardium (Reynolds 1990). The absence of podocytes in *Alderia modesta* is due to the complete loss of the heart and the pericardium in this genus. Accordingly, the urine is formed directly in the excretory duct without a prior ultrafiltration step in *Alderia modesta,* a situation that can be presumed for the likewise heart-less *Micropilina* species (Monoplacophora) as well (Haszprunar and Schäfer 1997a,b).

Rhogocytes with diaphragmatic slit-areas on their surface could be found both in *Bosellia mimetica* and *Alderia modesta.* The occurence of slit-regions on the surface of these cells, which strongly resemble the ultrafiltration weir of the podocytes, indicates, that rhogocytes also serve as molecular sieves. As previously outlined (Haszprunar 1996), this condition provides significant evidence for a cytological homology between molluscan rhogocytes and metazoan podocytes, cyrtocytes, and nephrocytes. Whereas in podocytes the filtration pressure is caused by muscular activity, this is probably due to endocytosis in rhogocytes (Morse and Cooper 1993; Haszprunar 1996). The presence of rhogocytes in *Alderia modesta* proves that this species in principle has the capacity to form a cellular ultrafiltration barrier, despite of the lack of podocytes.

The nephridial cells of the kidney of *Bosellia mimetica* and *Alderia modesta* show the characteristic fine-structure of excretory organs of the marine Mollusca (Andrews 1985, 1988; Morse and Meyhöfer 1990; Eernisse and Reynolds 1994; Morse and Reynolds 1996; Haszprunar and Schäfer 1997a). Extensive basal infoldings and a dense, apical microvillous border increase their surface significantly and numerous vesicles occur inside these cells, indicating their nephridial function, the modification of the primary urine.

As described correctly by Portmann (1958), the excretory system of *Bosellia mimetica* consists of a heart with a single ventricle and auricle enclosed in a wide pericardium, as well as a single kidney. This situation is typical for most of the Caenogastropoda, Opisthobranchia, and Pulmonata that have been examined so far (see Luchtel et al. 1997; Estabrooks *et al.* 1999, Fahrner and Haszprunar 2000). Jensen (1996) reported that in the Placobranchoidea (=Elysioidea), in which she includes the Boselliidae, an elongate extension of the pericardium contains the kidney. This so-called renopericardial prominence could not be found in *Bosellia mimetica*. Instead, the kidney is clearly situated outside the pericardium (see Fig. 3A). The secondary loss of the heart and pericardium in the intertidal and estuarine *Alderia* species represents a synapomorphy of the genus (Jensen 1996). Circulation of the haemocoelic fluid in these species is accomplished by muscular pulsations of the cerata. We confirm earlier observations by Evans (1953) that the vascular system of *Alderia modesta* consists of haemocoelic spaces and lacunes only. However, contrary to the description of Marcus and Marcus (1956), the kidney of *Alderia modesta* does not extend into the anal papilla with two posterior diverticulae, but ends closely in front of the latter without splitting into blind canals (see Fig. 5).

The ultrastructural data of *Bosellia mimetica* and *Alderia modesta* given herein represent the first detailed information on the excretory system of the Sacoglossa. Next to the pelagic Gymnosomata and Thecosomata (Fahrner and Haszprunar 2000), the Sacoglossa are the third major taxon that has been investigated within the framework of a larger study on opisthobranch excretory systems. Representatives of all three taxa show the ancestral molluscan condition with podocytes situated on the atrial wall as the site of ultrafiltration. These results contradict the assumption of Andrews (1988) that the primary site of urine filtration in the auricle has been lost in the ancestors of the Opisthobranchia and that the function of podocytes has been adopted by other cell-types with a filtration weir.

The organization of the excretory system of *Alderia modesta* shows that ultrafiltration is no prerequisite for effective excretion in the Mollusca. Significant modifications of the excretory system of the Opisthobranchia such as the movement of the ultrafiltration-site to the pericardial wall in *Philinoglossa helgolandica* (see Bartolomaeus 1997) and the loss of the heart as well as the presence of an entirely new system of ultrafiltration in *Rhodope transtrosa* (see Haszprunar 1997) appear to be restricted to certain taxa and are probably related to a special habitat or small body-size.

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APPENDIX III

Microanatomy, ultrastructure, and systematic significance of the excretory system and mantle cavity of an acochlidian gastropod (Opisthobranchia)

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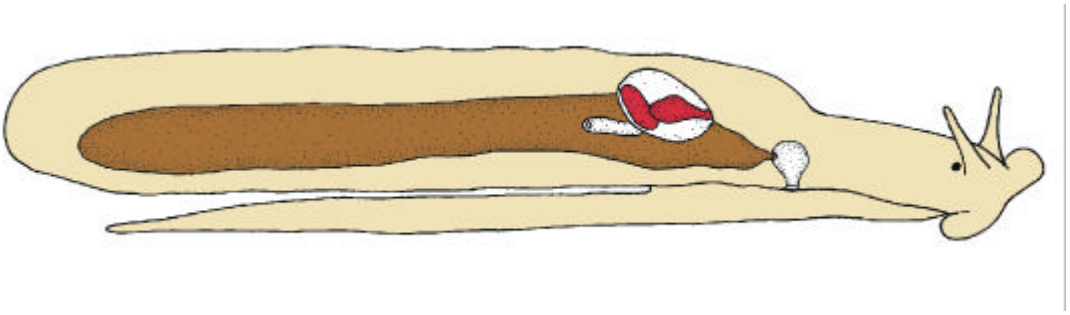

Abstract. The microanatomy and ultrastructure of the excretory system of an undescribed mesopsammic gastropod of the genus *Hedylopsis* have been examined by means of semithin serial sections, reconstructions, and transmission electron microscopy. The functional metanephridial system comprises a monotocardian heart with a single ventricle and auricle in a spacious pericardium as well as a single, large kidney. Podocytes in the auricular epicardium represent the site of ultrafiltration and formation of the primary urine, whereas the flat epithelium of the kidney with extensive basal infoldings, large vacuoles and the apical microvillous border indicates modification of the primary filtrate. Solitary rhogocytes (pore cells) represent additional loci of ultrafiltration with an identical fine-structure as those of the podocytes (meandering slits with diaphragms covered by extracellular matrix).

The presence of podocytes situated in the epicardial wall of the auricle is regarded as plesiomorphic for the Opisthobranchia and is confirmed for the Acochlidia for the first time. Kidney and rectum both open into a small, yet distinct mantle cavity. Within the Acochlidia this condition represents a plesiomorphic character only known from one further *Hedylopsis* species until now. Special cells (here termed microvillous pit-cells) with a presumed absorptive function are interspersed between the epithelial cells of the mantle cavity. They are mainly characterized by a prominent invagination of the apical border with densely arranged, very large microvilli. The presence of a mantle cavity that has been lost in all other acochlidian genera supports the systematic placement of the Hedylopsidae at the base of the Achochlidia.

INTRODUCTION

The excretory system of the adult Mollusca consists of coelomatic derivates, the endothelially lined pericardium and, originally, two pericardial ducts leading to the exterior via the mantle cavity (Andrews, 1988; Haszprunar, 1992). This functionally metanephridial system in the sense of Ruppert & Smith (1988) will be called renopericardial complex in the following. In the Testaria (Polyplacophora and Conchifera), the distal parts of the pericardial ducts were enlarged and modified into the sac-like kidneys. Pericardium and kidneys remain in communication with one another to varying degrees in the different molluscan taxa (for review, see Martin, 1983). As has been demonstrated experimentally, the primary urine is formed initially by ultrafiltration of the haemolymph through the epicardial wall of the auricle into the pericardial cavity (Martin & Aldrich, 1970; Hevert, 1984; Andrews & Taylor, 1988).

The ultrafiltrate drains off into the kidney by way of renopericardial ducts, where it is modified by reabsorption and secretion (Martin, 1983). Finally, the urine is released into the mantle cavity from where it is expelled by oriented water currents (Fretter & Graham, 1962; Morton, 1988).

The ultrafiltration of the haemolymph is associated with podocytes of the pericardial epithelium surrounding the heart, the epicardium (Andrews, 1988; Ruppert & Smith, 1988; Bartolomaeus & Ax, 1992). Podocytes possess numerous basal processes between which ultrafiltration slits, bridged by fine diaphragms, provide a pathway for the primary filtrate. The basal lamina, underlying the slits, has been shown to be the functional ultrafilter (Andrews, 1981; Morse, 1987). In addition, solitary rhogocytes (pore cells) with an ultrafiltration weir are diagnostic for all molluscs. Their striking structural resemblance to metanephridial podocytes and protonephridial cyrtocytes (terminal cells) lends strong support for a common genetic basis and the homology of these three cell types (Haszprunar, 1996).

The fine-structure of the excretory system is known from representatives of almost all higher molluscan taxa (see Andrews, 1988; Morse & Reynolds, 1996). However, until recently fine-structural studies on the renopericardial complex of the Gastropoda have been restricted to several groups of the Prosobranchia and the Pulmonata (for reviews see Andrews, 1988; Luchtel *et al.*, 1997) while no ultrastructural evidence from the Opisthobranchia had been available (Gosliner, 1994). Most recent TEM-based studies now showed significant differences in the organization of the excretory system of the Opisthobranchia: Whereas some taxa have retained podocytes on the auricular wall as the original ultrafiltration site of the Mollusca (Fahrner & Haszprunar, 2000, 2001), certain small opisthobranch species showed remarkable modifications of the renopericardial complex (Bartolomaeus, 1997; Haszprunar, 1997.

These data suggest that other Opisthobranchia may also exhibit considerable modifications of the original excretory system. Especially interstitial species seem to be promising in this sense, since also representatives of other phyla that inhabit the mesopsammic environment (e.g. the polychaete *Hesionides*, see Westheide, 1986) show unique, modified excretory systems. As an adaptation to the specific ecological factors in their habitat, the mesopsammic Acochlidia have reduced several organs, among them the shell, the gill, and the mantle cavity (Odhner, 1937; Rankin, 1979; Arnaud *et al.*, 1986). Thus, their excretory system opens via the nephropore directly to the exterior, as does the anus.

In this paper, we present the first ultrastructural details of the renopericardial complex of the Acochlidia from an undescribed, mesopsammic species of the genus *Hedylopsis*. These

data also have systematic significance since several features of the heart and kidney (e.g. number of chambers of the heart, size of kidney, position of nephropore in relation to other body openings) are considered to be diagnostic for higher taxa of the Acochlidia (Rankin, 1979). The present data on *Hedylopsis* sp. are compared with the results of investigations on the anatomy and ultrastructure of the excretory system of major opisthobranch taxa that are carried out within the framework of a larger, comparative project.

MATERIAL AND METHODS

Specimens of *Hedylopsis* sp., 3 mm to 5 mm long, were extracted from coarse coral sand samples (diameter 2mm) taken at 15m depth from the bottom of the fringing reef in Dahab, Gulf of Aqaba (Red Sea, Egypt) in October 1999. The mesopsammic animals were removed from the sediment samples by anaesthesia with a solution of 7% $MgCl_2$ (isotonic to local seawater). Living acochlidian gastropods were sorted out with a pipette and processed for light- and electron microscopy. After fixation in 4% glutardialdehyde buffered in 0.2 M sodium cacodylate (pH 7.2), the specimens were rinsed several times in the same buffer. Postfixation in buffered 1% OsO_4 for two hours was followed again by rinsing the specimens with cacodylate buffer in decreasing concentrations and dehydration in a graded ethanol series. Decalcification of the subepidermal spicules was achieved by using 2% EDTA. The fixed specimens were embedded overnight in Araldit resin for light microscopy and in Spurr`s (1969) low viscosity resin for electron microscopy. To enable an overall view on the *in situ*-position of the excretory system of *Hedylopsis* sp., complete series of semithin cross sections (2µm) were made with glass knifes (Henry, 1977) and stained with methylene-blue–azure II according to Richardson *et al.* (1960). The section slides are deposited at the Zoologische Staatssammlung München (ZSM-Nrs.: 20004766/1, 20004767, 20004768, 20004769). For transmission electron microscopy (TEM), ultrathin sections (70 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyle acetate and lead citrate and examined and photographed with a Philips CM 10 TEM. The reconstruction of the renopericardial complex of *Hedylopsis* sp. was made by hand, based on serial semithin cross sections.

RESULTS

General Anatomy

The excretory system of *Hedylopsis* sp. is placed at the right side of the body and comprises the heart being enclosed in a thin, spacious pericardium and the very long, tubular kidney (Figs 1A,B, 2A). The monotocardian heart, consisting of auricle and ventricle (Figs 2C,D), lies medio-laterally at the anterior end of the visceral hump, adjacent to the digestive gland. At the anterior end of the ventricle, the thick aorta arises. Pericardium and kidney are connected via a renopericardial duct that emerges ventro-laterally, under the auricular region, in the middle of the pericardial cavity (Fig. 2D). The duct runs posteriorly and enters the kidney laterally.

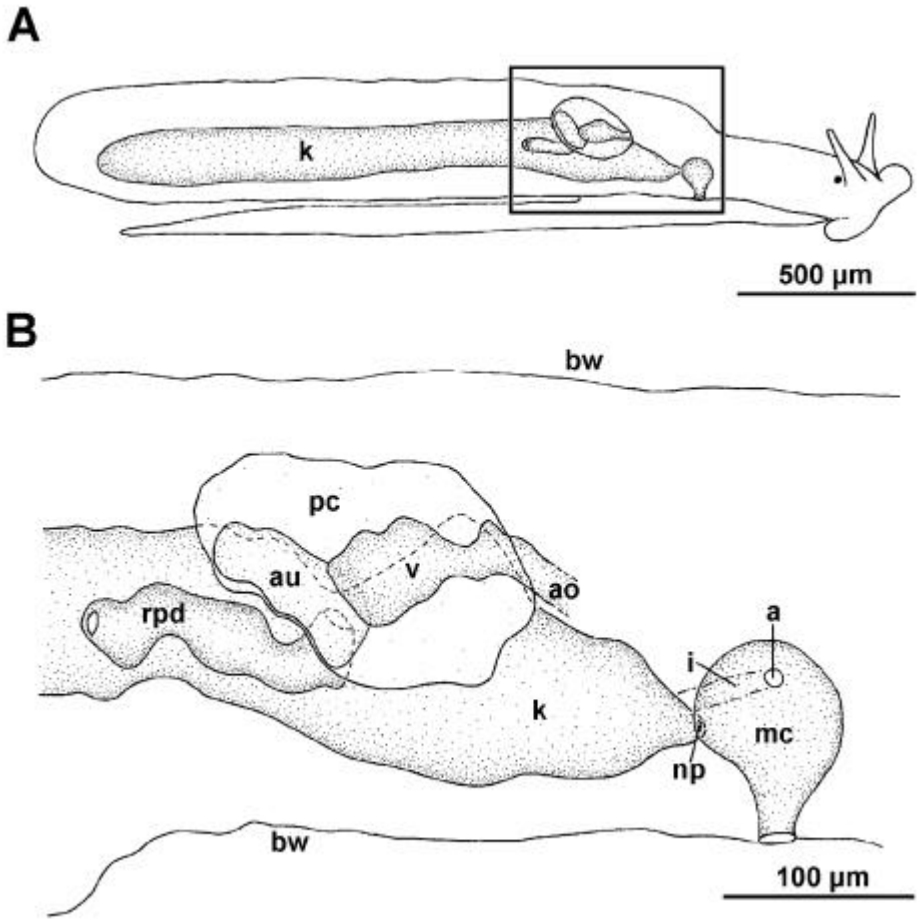

Fig. 1 A Lateral view of *Hedylopsis* sp. showing the relative position of the excretory system. Boxed area is enlarged in B. **B** Reconstruction of the renopericardial complex. Lateral view from the right. *a* anal opening, *ao* aorta, *au* auricle, *bw* body wall, *i* intestine, *k* kidney, *mc* mantle cavity, *np* nephropore, *pc* pericardial cavity, *rpd* renopericardial duct, *v* ventricle.

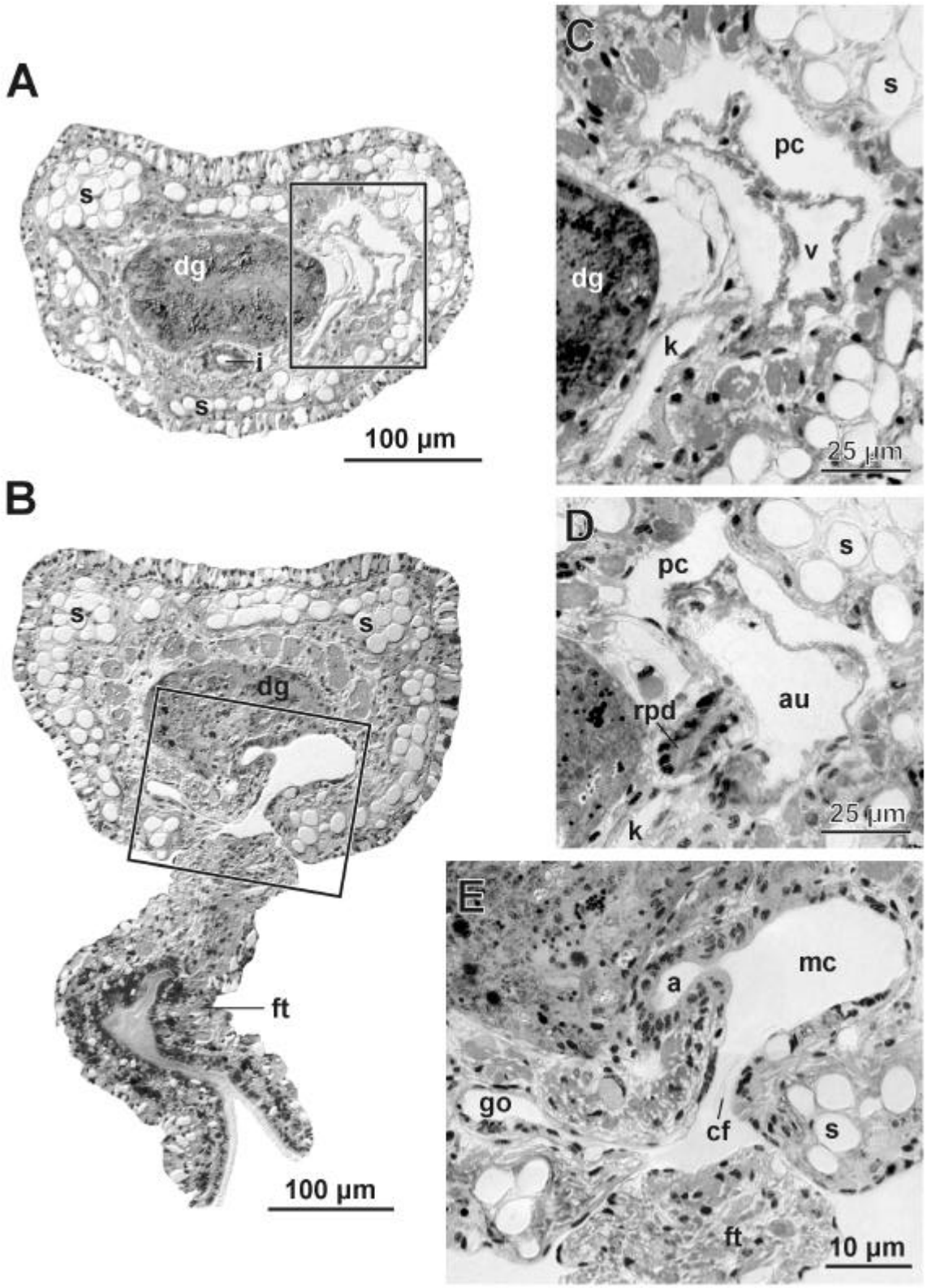

Fig. 2 Histology of the excretory system and the mantle cavity based on semithin serial sections. Dorsal faces upwards and right to the right. **A** Cross section of entire body showing the position of heart and kidney at the right side of the body (boxed area, enlarged in C). Also note the numerous large spicule cells in the visceral hump. **B** Cross section of entire body showing the ventral position of the mantle cavity- and the genital system opening in the boxed area (enlarged in E). **C** Renopericardial complex with heart (ventricle), pericardial cavity, and kidney. **D** Opening of the pericardium into the renopericardial duct. **E** Opening of the mantle cavity into the groove between visceral hump and foot. The closely associated, separate genital opening lies adjacent, to the left of the mantle cavity opening. The anus opens from the left side into the mantle cavity. *a* anal opening, *au* auricle, *cf* ciliary flame in the opening of the mantle cavity, *dg* digestive gland, *ft* foot, *go* genital opening, *i* intestine, *k* kidney, *mc* mantle cavity, *pc* pericardium, *rpd* renopericardial duct, *s* spicule cells.

The tubiform kidney extends almost over the whole length of the visceral hump and is characterized by a continuous, very flat, glandular and highly vacuolated epithelium. Anteriorly, the kidney opens into the posterior end of the small, spherical mantle cavity. A prominent ciliary flame characterizes the broad, medio-ventral opening of the mantle cavity to the exterior, into the cephalo-pedal groove between visceral hump and foot (Figs 2B,E). The closely associated, separate genital opening lies to the left of the mantle cavity opening, while the anus opens into the mantle cavity from the left side.

Fine-structure

The myocardium of the ventricular and auricular portions of the heart (Fig. 3A) consists of a loose network of muscle bundles and is lined with a basement membrane formed by ECM. The epithelio-muscle cells of the epicardium (Fig. 3B) that rest on this basal lamina are characterized by basally located myofibrils and are connected by belt desmosomes apically, an intercellular ECM is lacking. In the auricular region the squamous pericardial epithelium is composed of a second cell-type next to the pure epithelio-muscle cells, the podocytes (Fig. 2 E). Numerous foot-processes (i.e. the pedicels) extend from the basal border of the podocytes. Fine, fibrillar diaphragms bridge the ultrafiltration slits between these pedicels which interdigitate with those of adjacent cells. True intercellular spaces are very narrow or entirely lacking. The content of the podocytes is mainly characterized by muscle fibers. Podocytes are absent from the epicardial wall of the ventricle and the outer pericardial epithelium.

The opening of the pericardium into the renopericardial duct is about 5 µm wide. The renopericardial duct is composed of very flat cells with an irregularly shaped nucleus occupying most of the cytoplasm. Apically, there are belt desmosomes and septate junctions between adjacent cells. Whereas the cells of the central section of the duct lack cilia and bear numerous, long microvilli apically (Fig. 4B), the cells of its proximal and distal parts are multiciliated (Fig. 4C). The cilia of the proximal renopericardial duct do not extend into the pericardium. The nephridial cells of the kidney (Fig. 4A) form a continuous, flat, and simple epithelium and are mainly charcterized by a dense, apical microvillous border and a deeply infolded basal surface that rests on a basal lamina. Belt desmosomes and extensive septate junctions interconnect the nephridial cells near their apices. Besides the basally located nucleus, there are many mitochondria, in addition to coated vesicles, endosomes, and

lysosomes. The one to several, electron-lucent vacuoles in the cell originate in the basal cytoplasm and become largest apically, prior to fusion with the cell membrane.

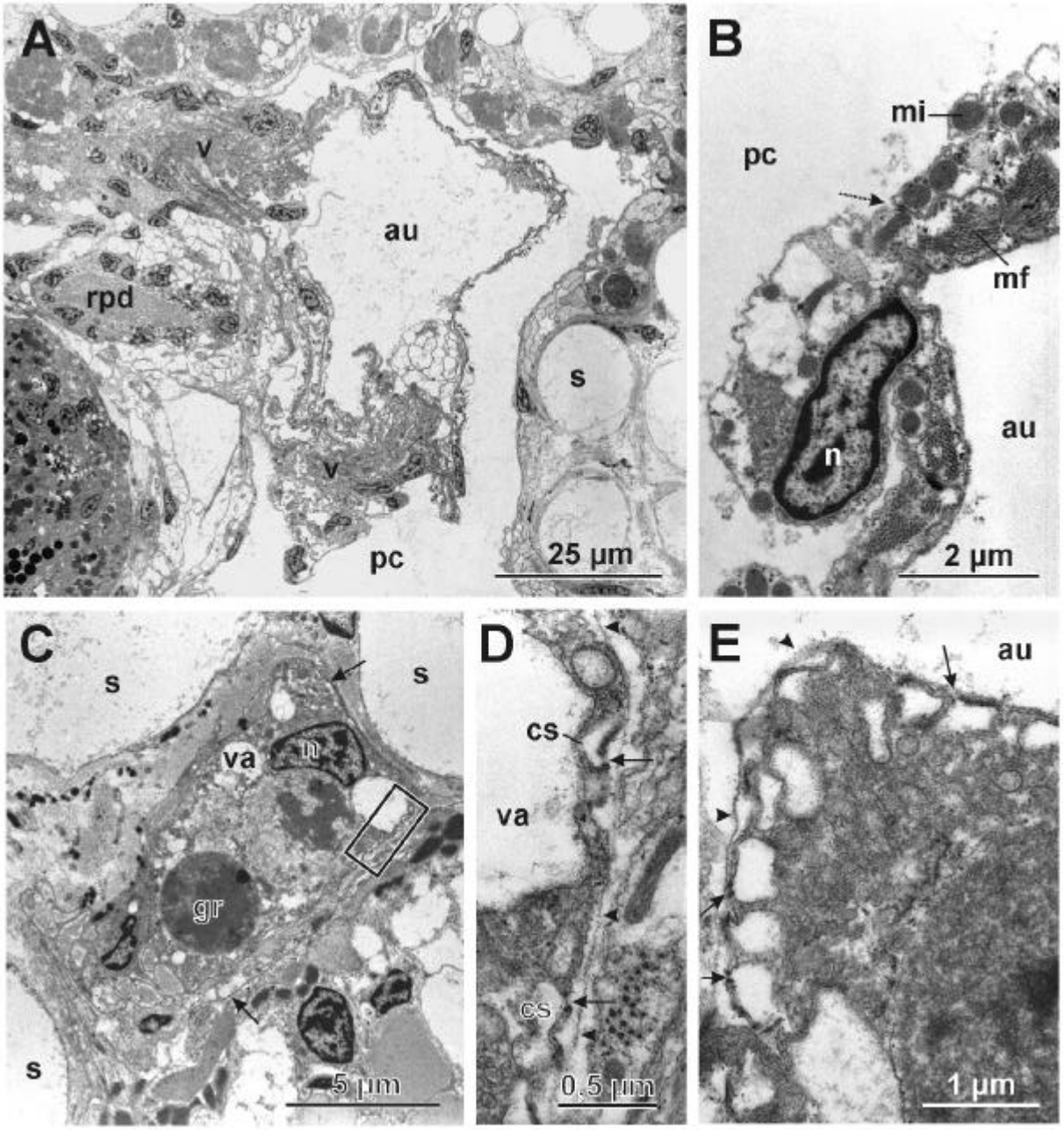

Fig. 3 TEM micrographs of the heart and a rhogocyte. **A** Overview of the heart [transition from ventricle (*v*) to auricle (*au*)] close to the opening of the pericardial cavity (*pc*) into the renopericardial duct (*rpd*). Note the large spicule cells (*s*). **B** Epithelio-muscle cells of the auricular epicardium with muscle fibers (*mf*), mitochondria (*mi*), and nucleus (*n*), interconnected by belt desmosomes (broken arrow). *au* lumen of the auricle, *pc* pericardial cavity. **C** Rhogocyte surrounded by spicule cells (*s*) with electron-lucent vacuoles (*va*), a very large electron-dense granule (*gr*), and excentrically situated nucleus (*n*). Areas of diaphragmatic slits are indicated by arrows, the boxed area is enlarged in D. **D** Detail of slit area showing diaphragms (arrows) with underlying small cisternae (*cs*) and the extracellular matrix (arrowheads), surrounding the cell. *va* vacuole. **E** Podocyte of the auricular surface with slit diaphragms between pedicels (arrows) resting on the extracellular matrix (arrowheads). *au* lumen of the auricle.

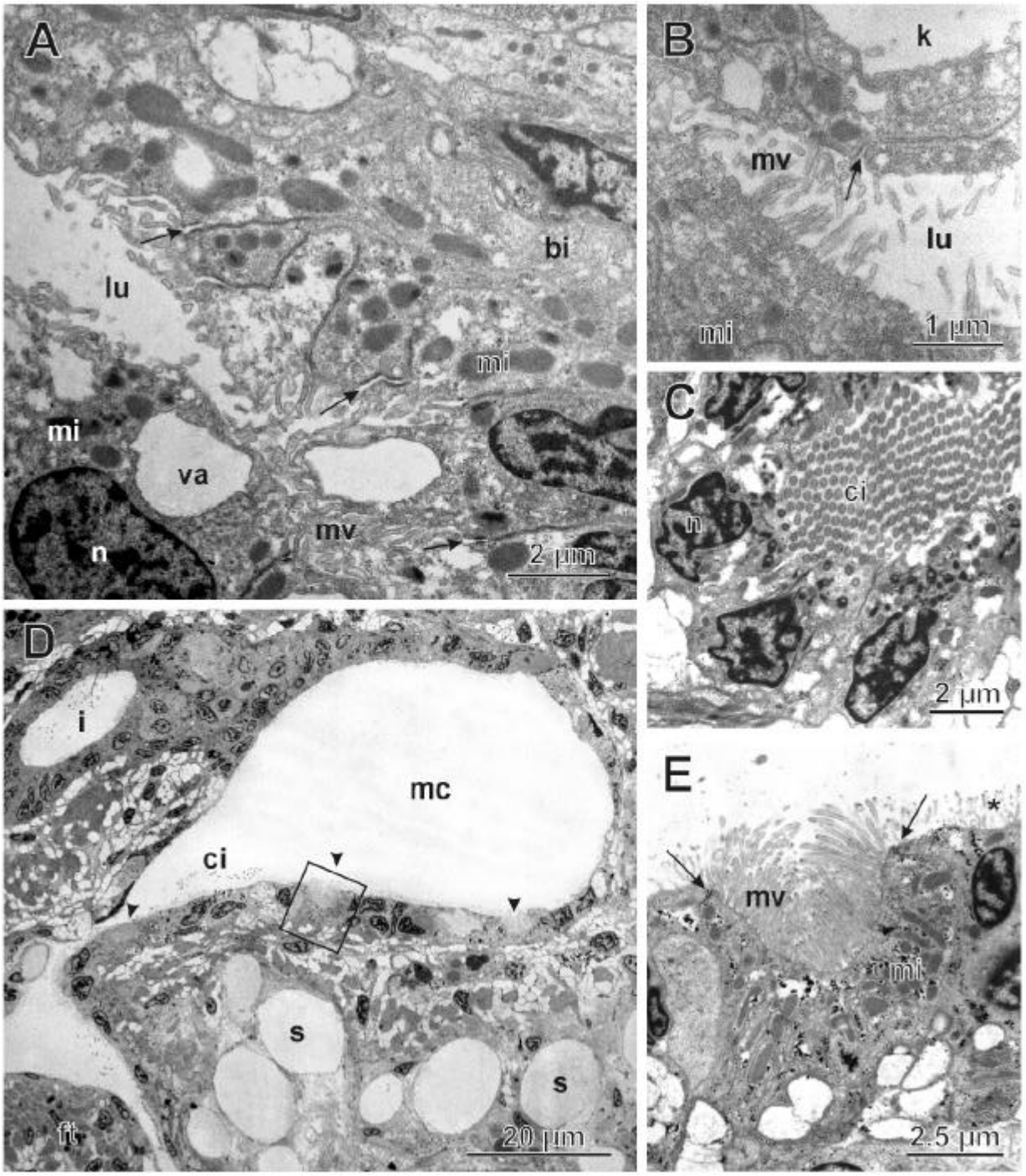

Fig. 4 TEM micrographs of kidney, renopericardial duct, and mantle cavity. **A** Excretory epithelium of the kidney, showing basal infoldings *(bi*), several mitochondria (*mi*), large, electron-lucent vacuoles (*va*), prominent nuclei (*n*), and a dense, apical microvillous border (*mv*) to the partly collapsed lumen (*lu*). The nephridial cells are connected apically by belt desmosomes (arrows) and extensive septate junctions. **B** Cells of the central, aciliated region of the renopericardial duct with microvillous border (*mv*) to its lumen (*lu*). arrow, belt desmosome; *k* lumen of the adjacent kidney, *mi* mitochondria. **C** Cells of the proximal region of the renopericardial duct (prior to opening of the pericardium) with irregular shaped nuclei (*n*) and numerous cilia (*ci*) occupying the entire lumen. **D** Beginning of the opening of the mantle cavity (*mc*) into the groove between mantle and foot (*ft*). Boxed area is enlarged in E. Note the position of the three prominent microvillous-pit cells (arrowheads). *ci* cilia, *i* intestine; *s* spicule cells. **E** Microvillous-pit cell from the epithelium of the mantle cavity showing the characteristic invagination of the apical surface with densely arranged, large microvilli (*mv*). Also note the numerous mitochondria (*mi*), the belt desmosomes (arrows), and the much smaller, regular microvilli of the adjacent cells (asterisk).

A second cell type with an ultrafiltration weir, the rhogocyte (Figs 3C,D), occurs in the connective tissue, between the spicule cells. In contrast to the epithelial podocytes of the auricle, rhogocytes are solitary cells that are completely surrounded by a thin layer of ECM in *Hedylopsis* sp.. They are irregularly shaped, depending on the space available and 5-15 µm in diameter. Areas of diaphragmatic slits and the underlying small cisternae are scattered over the entire surface of the cell. Accordingly, there is no cell polarity and there are no junctions to any other cell. Further features of the rhogocyte are the electron-lucent vacuoles, the numerous small secretory vesicles, the large electron-dense granules (diameter up to 3 µm), and the prominent, often excentrically situated nucleus.

The small mantle cavity (diameter: 80 µm) is lined by a flat epithelium covered by a low microvillous border (Fig. 4D). Only the cells at the opening of the mantle cavity to the exterior bear cilia. Special cells with microvillous pits (Fig. 4E) are interspersed between the regular epithelial cells. These cells are very common at the posterior end of the mantle cavity, but are subsequently less frequent towards the mantle cavity opening, where they are entirely lacking. Pit cells are well characterized by their prominent, deep, apical invagination of the surface with densely arranged, very large and thick microvilli. Their cytoplasm contains a large number of densely arranged mitochondria and numerous, small glycosomes. Usually the microvillous-pit cells occur solitary, but sometimes two cells of this type lie directly adjacent to each other.

DISCUSSION

Comparative cytology and histology

The data presented in this study reveal that the fine-structure of the excretory system of the Acochlidia basically corresponds to that of other molluscs. In most of the taxa with available TEM-data, podocytes of the epicardial epithelium were identified as the site of ultrafiltration and production of the primary urine (Andrews, 1985; Andrews, 1988; Morse & Meyhöfer, 1990; Reynolds *et al*., 1993; Morse & Reynolds, 1996; Fahrner & Haszprunar, 2000, 2001). The slits between interdigitating foot processes of the podocytes, covered by extracellular matrix serve to filter large molecules from the haemolymph into the pericardial cavity (Andrews & Little, 1972; Andrews, 1988).

In *Hedylopsis* sp., the epicardial wall of the auricle is regarded as the sole site of

ultrafiltration since podocytes could only be detected there but are absent from the ventricular wall or outer wall of the pericardial epithelium. This condition is also present in most of the molluscan taxa that have been investigated so far (Andrews, 1985; Reynolds *et al*., 1993; Morse & Reynolds, 1996; Bartolomaeus, 1997; Estabrooks *et al*., 1999; Fahrner & Haszprunar, 2000, 2001) and is considered as plesiomorphic for the phylum. In some gastropod species, additional podocytes occur in the surface of the ventricle (Økland, 1982; Luchtel *et al*., 1997), while in the Cyclophoridae the ventricular wall probably represents the main site of ultrafiltration (Andrews & Little, 1972). Scaphopoda with a reduced heart and a lost auricle show podocytes in the epicardium surrounding a muscular sinus that is either regarded as perianal sinus (Reynolds, 1990) or as the rudimentary ventricle (Morse & Reynolds, 1996; Shimek & Steiner, 1997). The absence of podocytes in *Micropilina* species (Monoplacophora, see Haszprunar & Schäfer, 1997a,b) and the sacoglossan gastropod *Alderia modesta* (Fahrner & Haszprunar, 2001) is a result of the complete loss of the heart and the pericardium. Accordingly, the primary urine is formed without a prior ultrafiltration step in these taxa.

Besides the podocytes, a second cell-type with an ultrafiltration weir is present in *Hedylopsis* sp.. Solitary rhogocytes that are situated freely within the connective tissue are characterized by slit areas on their surface that strongly resemble the fenestrations of the podocytes. As previously outlined in detail (Haszprunar, 1996), the great similarity of the molecular sieves (slits bridged by diaphragmans, covering ECM, underlying free lumen respectively cisternae) provides significant evidence for a cytological homology between molluscan rhogocytes and metazoan podocytes, cyrtocytes, and nephrocytes. In contrast to podocytes, where filtration pressure is caused by muscular activity, probably endocytosis is the driving force in rhogocytes (Morse & Cooper, 1993; Haszprunar, 1996).

Also the ultrastructure of the well-developed, reabsorptive kidney epithelium of *Hedylopsis* sp. generally corresponds to that of other marine molluscs (see Andrews, 1985, 1988; Morse & Meyhöfer, 1990; Eernisse & Reynolds, 1994; Morse & Reynolds, 1996; Haszprunar & Schäfer, 1997). Among features that are common to all species investigated are numerous mitochondria and the extensive basal infoldings, as well as the dense, apical microvillous border that increases significantly the surface of the nephridial cells. The large number of endosomes and vacuoles inside these cells and the extensive septate junctions between them further indicate their transcytotic activity and excretory function, the modification of the primary urine.

The epithelium of the mantle cavity of *Hedylopsis* sp. mainly consists of squamous cells with microvillous border. Ciliated cells, that are interspersed between the common epithelial cells in other molluscan taxa (e.g. Haszprunar & Schaefer, 1997; Shimek & Steiner, 1997), are restricted to the opening of the mantle cavity in *Hedylopsis* sp.. The special cells with prominent microvillous pit that are scattered over the mantle cavity epithelium in *Hedylopsis* sp. are not known from any other taxon. Both the position (much more common at the inner and posterior end of the mantle cavity than towards the opening) as well as their content (a large number of mitochondria and glycosomes) and the large, apical microvilli lend strong support for an reabsorptive capacity of these cells. Because of its small size, a significant role of the mantle cavity in respiration is unlikely.

The ultrastructural data of *Hedylopsis* sp. given herein represent the first detailed information on the excretory system of the Acochlidia. Next to the pelagic Gymnosomata and Thecosomata (Fahrner & Haszprunar, 2000) and the Sacoglossa (Fahrner & Haszprunar, 2001), the Acochlidia are the fourth major taxon that has been investigated within the framework of a larger study on opisthobranch excretory systems. Representatives of all four taxa show a single kidney with an extensive reabsorptive epithelium as well as the ancestral molluscan condition with podocytes situated on the atrial wall as the site of ultrafiltration. These results contradict the assumption of Andrews (1988) that the primary site of urine filtration in the auricle has been lost in the ancestors of the opisthobranchs and that the function of podocytes has been adopted by other cell-types with a filtration weir. Significant modifications of the excretory system in certain opisthobranch taxa, such as the movement of the ultrafiltration-site to the pericardial wall in the mesopsammic *Philinoglossa helgolandica* (cf. Bartolomaeus, 1997) and the loss of the heart as well as the presence of an entirely new, pseudo-protonephridial system of ultrafiltration in *Rhodope transtrosa* (cf. Haszprunar, 1997) are probably related to their habitat and small body-size but appear to be restricted to these taxa.

Anatomy and systematic considerations

The renopericardial complex of *Hedylopsis* sp. consists of a wide pericardium, containing the two-chambered heart, and a single kidney that opens into the mantle cavity. This condition is typical for most of the Caenogastropoda, Opisthobranchia, and Pulmonata that have been studied so far (e.g. Luchtel *et al*., 1997; Estabrooks *et al*., 1999; Fahrner & Haszprunar, 2000, 2001). However, especially the presence of a small, yet distinct mantle

cavity is in contrast to earlier descriptions of the excretory system of the Acochlidia (for review, see Rankin, 1979).

Originally, the Gastropoda possess a large, spacious mantle cavity into which the whole head and foot can be retreated. Within the Opisthobranchia, a trend to reduction and, finally, elimination of the mantle cavity can be observed (Morton, 1988). Rankin (1979) described the absence of a permanent mantle cavity as diagnostic character of the Acochlidia, only the formation of a temporary one during complete withdrawel of the animal has been reported from several acochlidian taxa. In contrast, Kudinskaja & Minichev (1978) pointed out that the species *Hedylopsis murmanica* Kudinskaja & Minichev (1978) retained many primitive features, among them the mantle cavity. *Hedylopsis* sp. investigated in this study accordingly represents the second acochlidian species with a mantle cavity. This further supports the placement of the Hedylopsidae at the base of the Acochlidia, as suggested in the latest review of the group by Arnaud *et al.* (1986) and Wawra (1987).

The renopericardial complex of *Hedylopsis* sp. differs from the general anatomical diagnosis of the Hedylopsidae (Rankin, 1979) in some further details. As usual, the heart is composed of auricle and ventricle and not one-chambered, the nephropore is not situated distinctly closer to the anus than to the genital opening, and the body openings lie ventrolaterally, not dextrolaterally. All these features were used by Rankin (1979) to establish a new, highly ranked taxon (i.e. the Suborder Proprioneura) and to demarcate the Hedylopsidae from the Pseudunelidae. Since all characters mentioned above were considered to be of high diagnostic value, the validity of Rankin`s classification, that was based on literature data only, needs to be critically rechecked and a phylogenetic analysis of the Acochlidia is overdue.

ACKNOWLEDGEMENTS

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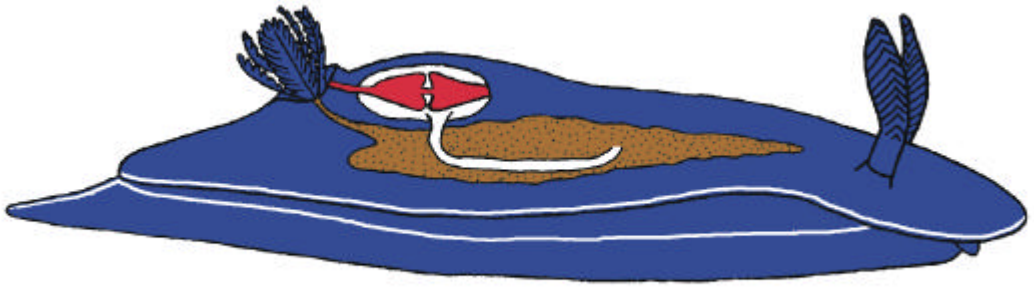
APPENDIX IV

Ultrastructure of the renopericardial complex in *Hypselodoris tricolor* (Gastropoda, Nudibranchia, Doridoidea)

Published in: *Zoomorphology* (in press)



Abstract. The histology and ultrastructure of the renopericardial complex of the doridoid nudibranch *Hypselodoris tricolor* have been investigated by means of semithin serial sections and transmission electron microscopy (TEM). The examinations revealed a functional metanephridial system comprising a monotocardian heart with ventricle and auricle in a spacious pericardium that is linked with the single, large kidney by a renopericardial duct with prominent ciliation towards its opening. Podocytes as the site of ultrafiltration were not only detected in the auricular epicardium, but also line the entire outer pericardial epithelium. The cuboidal, highly vacuolated excretory cells of the kidney epithelium with extensive basal infoldings and an apical microvillous border indicate secretory and reabsorptive activity. Solitary rhogocytes (pore cells) of the connective tissue and haemocoel represent additional loci of ultrafiltration with a fine-structure identical to that of the podocytes (slits between cytoplasmatic processes, bridged by fine diaphragms and covered by extracellular matrix). The presence of podocytes situated in the epicardial wall of the auricle is regarded as plesiomorphic for the Mollusca and is confirmed for the Nudibranchia. An additional, extensive and separate ultrafiltration site in the outer pericardial wall is not known from any other taxon of the Mollusca and strongly suggests a significantly increased ultrafiltration activity in *Hypselodoris tricolor*.

INTRODUCTION

The excretory system of adult Mollusca represents a functional metanephridial system in the sense of Ruppert and Smith (1988). With few exceptions it originally consists of coelomatic derivatives, the endothelially lined pericardium and one or two pericardial ducts leading to the exterior (Andrews 1988; Haszprunar 1992, 2000). In the Testaria (Polyplacophora and Conchifera), the distal parts of the pericardial ducts were enlarged and modified into the sac-like kidneys. Pericardium and kidneys are interconnected to varying degrees in different molluscan taxa (for review, see Martin 1983). As has been demonstrated experimentally, the primary urine is produced initially by ultrafiltration of the haemolymph through the pericardial wall of the heart, the epicardium, into the pericardial cavity (Hevert 1984; Andrews and Taylor 1988). The ultrafiltrate drains off into the kidney by way of renopericardial ducts, where it is modified by reabsorption and secretion (Martin 1983) before it is finally released to the external environment.

The site of ultrafiltration of the haemolymph, fine-structurally characterized by the presence of podocytes, varies from the auricular or ventricular epicardium (Andrews 1988; Ruppert and Smith 1988; Bartolomaeus and Ax 1992) to parts or appendages of the pericardial wall (Andrews and Jennings 1993; Meyhöfer *et al.* 1985; Schipp and Hevert 1981). Podocytes possess numerous basal processes between which ultrafiltration slits, bridged by fine diaphragms, provide a pathway for the primary filtrate molecules. Also the basal lamina, underlying the slits, has been shown to be a functional ultrafilter (Andrews 1981; Morse 1987). In addition, solitary rhogocytes (pore cells) with an ultrafiltration weir are diagnostic for all molluscs. Their striking structural resemblance to metanephridial podocytes and protonephridial cyrtocytes (terminal cells) indicates strong support for a common genetic basis and the homology of these three cell types (Haszprunar 1996).

The excretory system has been described at the ultrastructural level in all higher taxa of the Mollusca (see Andrews 1988; Morse and Reynolds 1996; Haszprunar and Schaefer 1997a). However, the extent of ultrastructural variation within these groups is still poorly known. Until recently, fine-structural studies on the renopericardial complex of the Gastropoda have been focused largely on several groups of the Prosobranchia and the Pulmonata (for reviews see Andrews 1988; Luchtel et al. 1997). Evidence from the Opisthobranchia had been restricted to two small and aberrant species that both show significant modifications of the excretory system (Bartolomaeus 1997; Haszprunar 1997). With a comparative analysis of the ultrastructure of the excretory system of major taxa of the Opisthobranchia, we aim to elucidate differences and similarities that might be of importance for a better understanding of excretion within the Gastropoda resp. the Mollusca. Previous studies within the framework of this project (Fahrner and Haszprunar 2000, 2001, 2002) revealed that the structure and organization of the opisthobranch excretory system generally corresponds to that of other Mollusca. In particular, podocytes were restricted to the auricular wall in representatives of all higher taxa investigated. In order to obtain ultrastructural details of the renopericardial complex and to clarify the distribution of podocytes in a representative of the highly derived Nudibranchia we herein examined the common Mediterranean species *Hypselodoris tricolor* (Cantraine, 1835).

MATERIALS AND METHODS

Specimens of *Hypselodoris tricolor* were collected by SCUBA off Rovinj (Croatia, Istria) in July 1993 and in Fetovaia Bay and Pomonto Bay (Elba, Italy) in June 1998 and July 2001. The animals were relaxed by slowly adding a solution of isotonic (about 7%) $MgCl_2$ to the seawater before they were processed for light microscopy (LM) and transmission electron microscopy (TEM). Fixation in 4 % seawater buffered formalin (LM) or 4 % glutardialdehyde (LM and TEM) buffered in 0.2 M sodium cacodylate (pH 7.2) was followed by a rinse in the same buffer in decreasing concentrations in the latter. After postfixation in buffered 1 % OsO_4 for two hours, the specimens were rinsed again with cacodylate buffer and dehydrated in a graded series of ethanols. The fixed specimens were embedded overnight in paraplast or Araldit resin for LM and in Spurr`s (1969) low viscosity resin for TEM.

In order to examine the gross anatomy of the excretory system, two complete series of semithin sections (2µm) were made with glass knifes (Henry 1977) and stained with methylene-blue – azure II according to Richardson et al. (1960). Serial sections of two very large, paraplast-embedded specimens (8 µm thick) were stained with Heidenhain`s azan. The histological slides were photographed on a Leica DM RBE compound microscope with a Kappa DX30 digital camera and are deposited at the Zoologische Staatssammlung München (ZSM; Malacology section; Nrs. 20020001, 20020002, 20000003/1, 20000003/2).

For TEM, ultrathin sections (70 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyl acetate and lead citrate and examined and photographed with a Philips CM 10 TEM at 80 kV.

RESULTS

Pericardium and epicardium

The heart-complex is orientated along the longitudinal axis of the body, with the auricle lying posteriorly to the ventricle (Fig. 1). The thin, spacious pericardium enclosing the heart is placed dorso-medially, in the posterior third of the body (right anterior to the gills), overlying the kidney and the digestive gland (Fig. 2). It is completely lined by an endothelium and its outer wall consists of only one single cell type, the podocyte (Fig. 3A,C,D). These flattened and peripherally slashed cells rest on a basal lamina that is underlain by a loose network of

collagen fibers of the extracellular matrix (ECM). The podocytes are attached to each other by belt desmosomes between cytoplasmic extensions of the cell and their isolated cell bodies often bulge into the lumen of the pericardial cavity. Numerous thin foot-processes, the pedicels, extend from the basal border of the podocytes and interdigitate with those of adjacent cells. Fine diaphragms consisting of electron-opaque strands bridge the ultrafiltration slits (approx. 20 nm in width) between these pedicels that overlie the basal lamina (Fig. 3E). The cytoplasm of the podocytes contains a number of small vesicles, Golgi bodies, few mitochondria, and the centrally located nucleus.

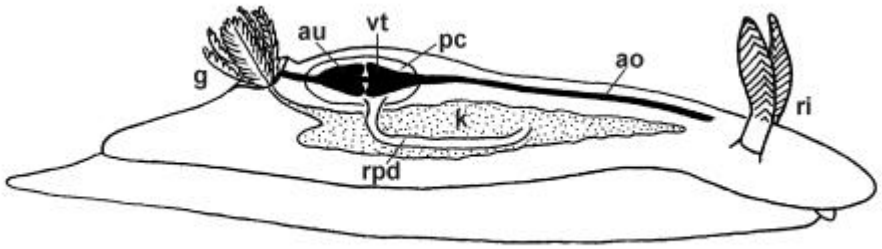


Fig. 1 Scheme of *Hypselodoris tricolor* (15 mm long), lateral view, showing the relative position of the excretory system. *ao* aorta, *au* auricle, *g* gill circle, *k* kidney; *pc* pericardium; *ri* rhinophores, *rpd* renopericardial duct, *vt* ventricle.

Whereas the epicardium of the ventricle consists exclusively of epithelio-muscle cells, the auricular epicardium is predominantly lined with podocytes, with only a few epithelio-muscle cells interspersed. The epithelio-muscle cells of the auricular and ventricular epicardium (Fig. 3C) contain basally located myofibrils (auricular cells fewer than ventricular cells), numerous mitochondria, and are connected by belt desmosomes apically. Basal pedicels are not present, but some epithelio-muscle cells form large, cytoplasmic, finger-like extensions apically into the pericardial cavity. The podocytes of the auricular epicardium (Fig. 3A,B) are structurally identical to those of the outer pericardial wall. They have low cell bodies, isolated from their neighbors by expanses of pedicels and only a few intercellular junctions. The main attachment sites are those of the pedicels to the underlying basal lamina.

The myocardium of the heart itself consists of non-epithelial muscle bundles that are more loosely arranged in the auricular (Fig. 3A) than in the ventricular portion (Fig. 3C). Mitochondria and glycosomes are scattered along the outer edges of the muscles and a basal lamina of the ECM lines the myocardium. There are no belt-desmosomes but only spot-desmosomes between the myocytes of the heart, hemi-desmosomes provide the connection with the basement membrane of the epicardium.

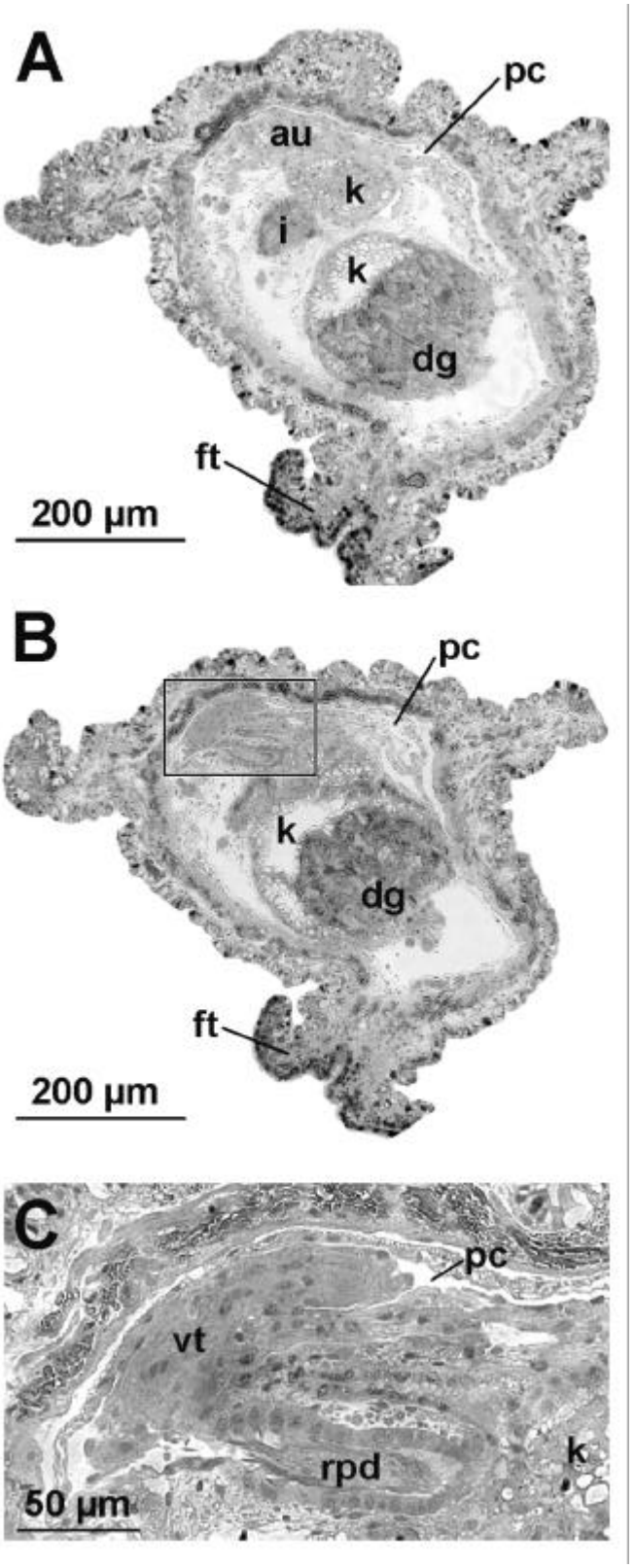


Fig. 2 A-C Histology of the renopericardial complex based on semithin serial sections. Frontal view, dorsal faces upwards. **A** Cross section of the entire body showing the dorsal position of the heart (auricular portion) and two separated parts of the kidney with highly vacuolated epithelium. **B** Cross section of the entire body showing the opening of the pericardium to the renopericardial duct in the boxed area (enlarged in C) and the heavily folded kidney overlying the digestive gland. **C** Ventro-lateral opening of the pericardium to the renopericardial duct in the region of the ventricle.
au auricle, *dg* digestive gland; *ft* foot; *i* intestine; *k* kidney; *pc* pericardium; *rpd* renopericardial duct, *vt* ventricle.

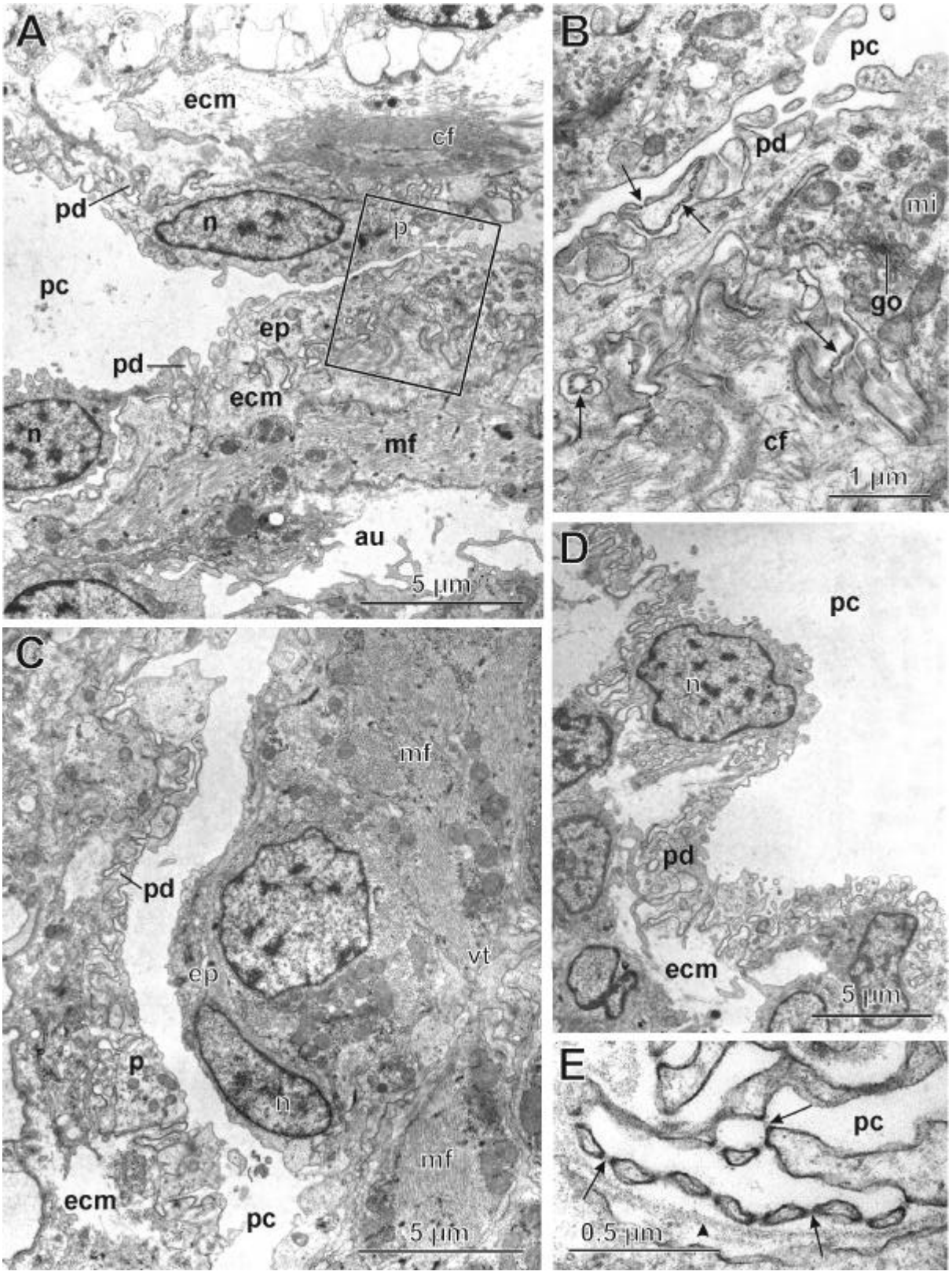


Fig. 3 A-E TEM micrographs of outer pericardium and epicardium. **A** Auricular epicardium (*ep*) and outer pericardium (*p*). Note that both epithelia are composed of structurally identical, flat podocytes with interdigitating basal pedicels (*pd*) resting on an underlying extracellular matrix (*ecm*). *au* auricle, *cf* collagen fibers, *mf* muscle fibers, *n* nuclei, *pc* pericardial cavity. The rectangle marks the area enlarged in B. **B** Detail of epicardial podocyte showing the diaphragmatic slits (arrows) between the pedicels (pd). *cf* collagen fibers of the ECM, *go* golgi apparatus, *mi* mitochondria, *pc* pericardial cavity. **C** Epithelio-muscle cell of the ventricular epicardium (*ep*) and podocyte of the outer pericardium (*p*). *ecm* extracellular matrix, *mf* muscle fibers, *n* nucleus, *pc* pericardial cavity, *pd* pedicels, *vt* ventricle. Boxed area is enlarged in E. **D** Podocyte from the outer

pericardial wall with extensive pedicels (pd), connected with the adjacent cell by a belt desmosome (broken arrow). *ecm* extracellular matrix, *n* nucleus, *pc* pericardial cavity. **E** Pedicels of outer pericardial podocyte showing slits bridged by fine diaphragms (arrows) and apposed by basal lamina (arrowhead). *pc* pericardial cavity.

Renopericardial duct and kidney

The pericardial cavity is connected with the lumen of the kidney via a long and narrow renopericardial duct (Fig. 1). The funnel-shaped opening of the pericardium to the renopericardial duct (often termed pericardial funnel or syrinx) is situated ventro-laterally, on the right side, in the region of the ventricle, immediately anterior to the transition between the two chambers of the heart (Fig. 2B,C). It is approximately 40 µm wide and lined with cuboidal, multiciliated cells (Fig. 4B,C). Short microvilli emanate from the apical surface of these cells and their cytoplasm contains numerous mitochondria, a centrally located nucleus, and solitary lyoglycosomes (cf. Rybicka 1996: distinct organelles, consisting of a glycogen-protein complex, that are not associated with other cellular structures). The basal cell surface is not invaginated or folded and rests on an ECM.

The renopericardial duct runs anteriorly, medially or laterally attached to the kidney, into which it enters ventrally, midway along its length. In contrast to the cells of the pericardial funnel, the cells of the long, central section of the renopericardial duct are non-ciliated and show weakly developed infoldings of the basal surface (Fig. 4A). Apically, they bear numerous, long microvilli and are interconnected with adjacent cells by belt desmosomes and septate junctions. Cytoplasmic features similar to the cells of the pericardial funnel are the numerous mitochondria, the lyoglycosomes, and the centrally located nucleus.

The large, sac-like kidney spreads almost over the entire dorsolateral surface of the visceral mass, covering the digestive gland and touching the ventral surface of the pericardium (Fig. 1, 2A,B). It reaches from the stomach backwards to the gills, where it narrows and opens to the exterior in the center of the gill circle, close to the anal opening. In larger specimens, the kidney is divided into several lobes and its wall may be heavily folded (Fig. 2A,B). A continuous, cuboidal epithelium of one single type of excretory cells lines the kidney (Fig. 5A,B). These cells are mainly charcterized by a dense, microvillous apical border, a deeply infolded basal portion, and electron-lucent, often very large vacuoles in the cytoplasm. Belt desmosomes and extensive septate junctions interconnect the excretory cells near their apices. Except for the nucleus and the numerous mitochondria, the content of the cytoplasm varies both within different areas of the kidney and within individual cells: there

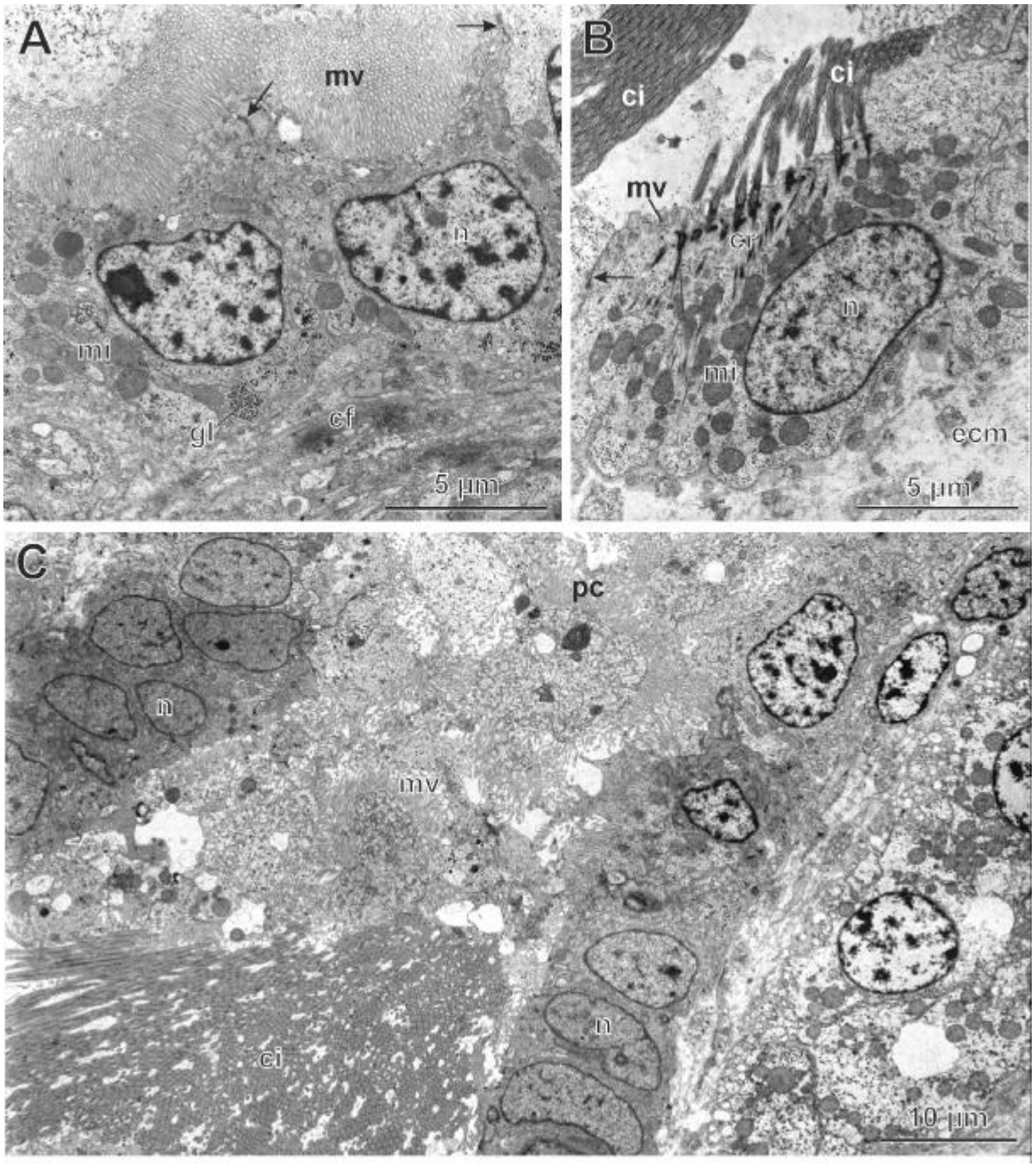


Fig. 4 A-C TEM micrographs of renopericardial duct. **A** Two cuboidal cells of the central section with long apical microvilli (*mv*), prominent nuclei (*n*), mitochondria (*mi*), and glycosomes (*gl*) scattered between the weakly developed basal infoldings. Also note the belt desmosomes (arrows) apically and the collagen fibers *(cf)* of the ECM underlying the epithelium. **B** Epithelial cell of the pericardial funnel showing numerous cilia (*ci*), short microvilli (*mv*), and belt desmosomes (arrow) apically and a large number of mitochondria (*mi*) and a prominent nucleus (*n*) occupying almost the entire cytoplasm. *cr* ciliary rootlets, *ecm* extracellular matrix. **C** Overview of the opening of the pericardium into the renopericardial duct. *ci* ciliary flame, *mv* microvilli, *n* nuclei, *pc* pericardial cavity.

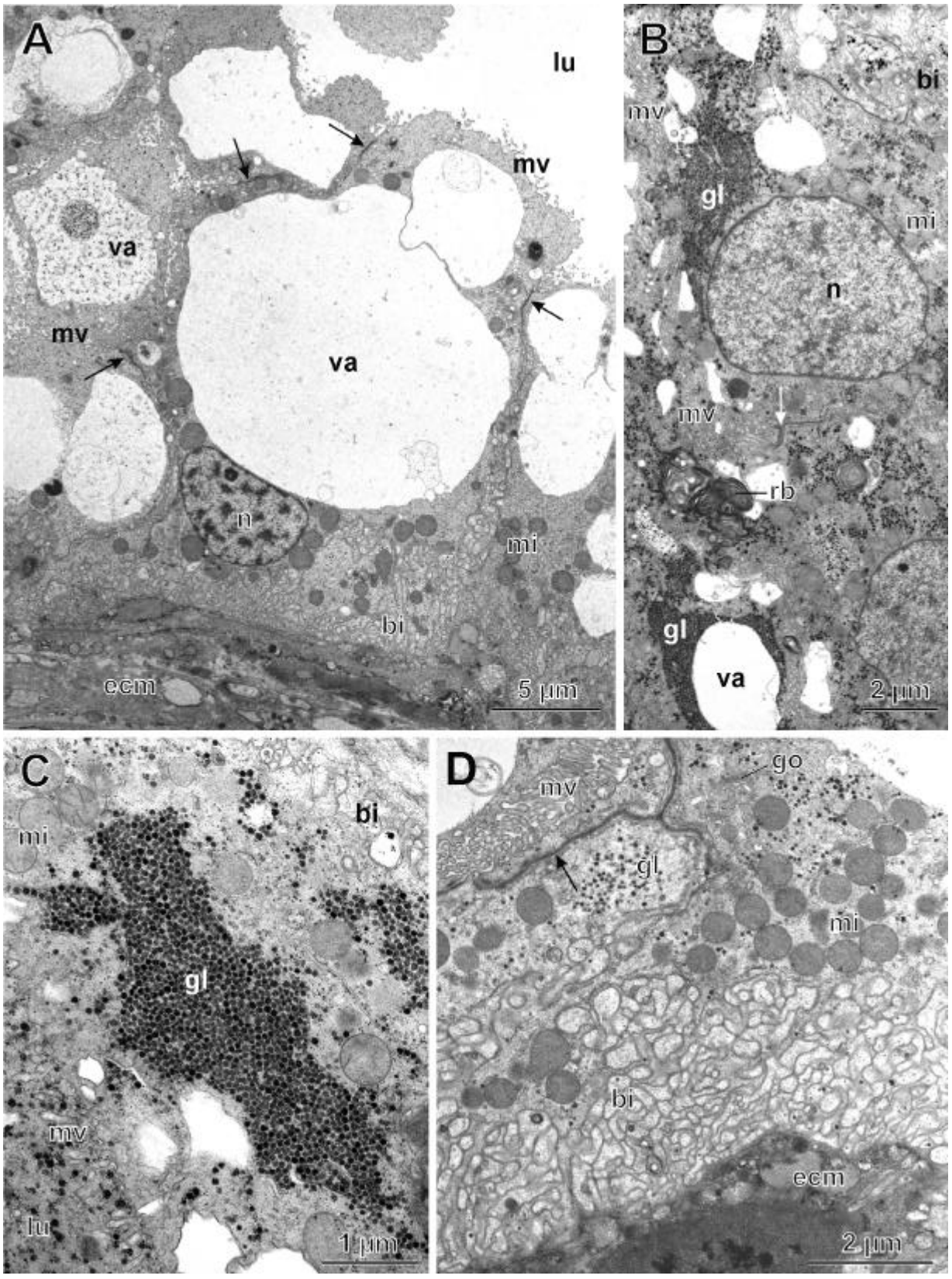


Fig. 5 A-D TEM micrographs of kidney epithelium. **A** Excretory cells from the posterior section of the kidney with very large electron-lucent vacuoles (*va*), a weak microvillous border (*mv*) towards the lumen (*lu*), and infoldings of the basal cell surface (*bi*). Also note the basally located nucleus (*n*) and mitochondria (*mi*), as well as the apical belt desmosomes between adjacent cells (arrows). Extracellular matrix (*ecm*) underlies the epithelium. **B** Excretory cells from the central portion of the kidney with much smaller vacuoles (*va*), centrally located nuclei (*n*), several residual bodies (*rb*), and numerous glycosomes (*gl*) scattered throughout the cytoplasm, aggregated into clumps, or arranged around vacuoles. *bi* basal infoldings, *mi* mitochondria,

mv microvilli, arrow septate junction. **C** Large cluster of lyoglycosomes (gl) occupying most of a excretory cells cytoplasm. *bi* basal infoldings, *mi* mitochondria, *mv* apical microvillous border towards the kidney lumen (*lu*). **D** Extensive basal infoldings (*bi*) of a nephrocyte from the anterior section of the kidney. Note also the single lyoglycosomes (*gl*), the golgi body (*go*), the mitochondria (*mi*), the apical microvilli (*mv*) and the septate junction (arrow). *ecm* extracellular matrix.

may be endosomes, lysosomes, and residual bodies (Fig. 5B) and lyoglycosomes (20-30 nm granules) may be absent (Fig. 5A) or occupy almost the entire cytoplasm (Fig. 5C). They may be scattered irregularly throughout the cytoplasm, aggregate into large clumps, or surround electron-lucent vacuoles (Fig. 5B). However, no further associations of glycosomes with cellular structures, such as mitochondria, Golgi-bodies, polyribosomes, or endoplasmic reticulum, i.e. true desmoglycosomes (cf. Rybicka 1996 for terminology), could be found. In addition, there are only one or two very large vacuoles (up to 20 µm in diameter) in some cells, whereas several smaller vacuoles of various sizes occur in others (Fig. 5B). The cells of the distal, narrow part of the kidney generally resemble the excretory cells but their basal infoldings are less extensively developed and vacuoles are less large or absent. Only the cells in the immediate vicinity of the nephropore are multiciliated (Fig. 6).

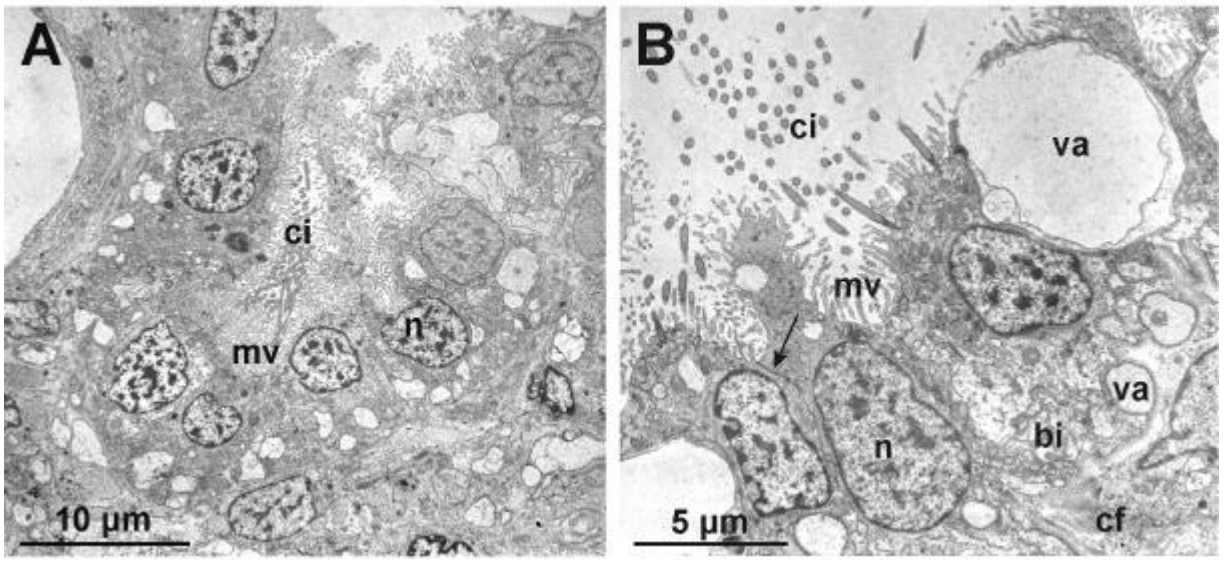


Fig. 6 TEM micrographs of nephropore. **A** Overview of the nephropore situated in the center of the gill circle. *ci* ciliary flame, *mv* microvilli, *n* nucleus. **B** Nephridial cells of the immediate vicinity of the kidney opening with apical cilia (*ci*) and microvilli (*mv*), basal infoldings of the cell surface (*bi*), electron-lucent vacuoles of various sizes (*va*), and centrally located nuclei (*n*). The arrow indicates a septate junction between two adjacent cells. *cf* collagen fibers of the ECM.

Solitary rhogocytes

A second cell type with an ultrafiltration weir, the rhogocyte (Fig. 7), occurs freely in the haemocoel or is embedded in the connective tissue. In contrast to the epithelial podocytes of the auricular epicardium and the outer pericardium, rhogocytes are solitary cells that are completely surrounded by a distinct layer of ECM. They vary considerably in shape and form even within one individual; in most cases they are roundish cells (Fig. 7D), but sometimes a very elongated or irregulary shape occurs (Fig. 7A). Areas with meandering slits are scattered over the entire surface of the cell and are underlain by flat cisternae. These slits (20-25 nm wide) occur between tiny cytoplasmatic bars and are spanned by fine, fibrillar diaphragms (Fig. 7B,C). Frequently, phagocyte-like formation of vesicles at the base of the cisternae could be observed (Fig. 7C). Further characteristic features of the rhogocyte in *Hypselodoris tricolor* are the large electron-dense granules (diameter up to 3 µm), the numerous small secretory vesicles, and, in particular, the large electron-lucent vacuoles (diameter 1-5 µm) that are scattered throughout the cytoplasm. The prominent nucleus can be found in various positions within the cell, but is generally situated centrally.

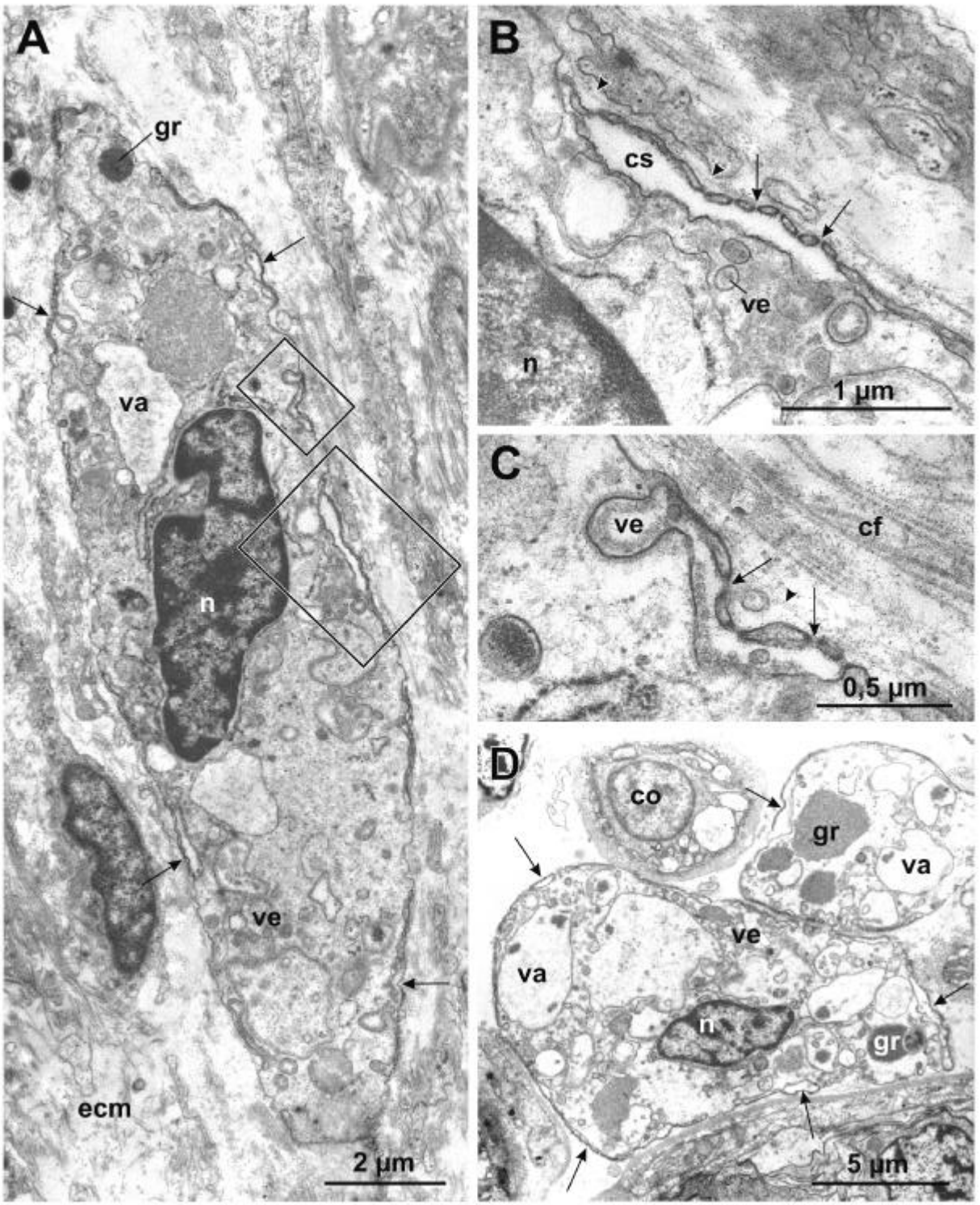


Fig. 7 A-D TEM micrographs of rhogocytes from the connective tissue surrounding the CNS. **A** Elongate rhogocyte with prominent, centrally located nucleus (*n*), electron-lucent vacuoles (*va*), numerous, small secretory vesicles (*ve*), and an electron-dense granulum (*gr*). Flat cisternae indicate the zone of slit openings (arrows) almost completely surrounding the cell-surface. *ecm* extracellular matrix. Rectangles mark the areas enlarged in B and C. **B** Large slit area with underlying cistern (*cs*). Arrows point to the diaphragmatic slits and arrowheads the covering lamina of the ECM. *n* nucleus, *ve* vesicle. **C** Phagocyte-like formation of a vesicle (ve) at the base of a cistern and overlying diaphragmatic slits (arrows). Note also the covering lamina (arrowhead) and the collagen fibers (*cf)* of the ECM. **D** Two spherical rhogocytes with large, electron-lucent vacuoles (*va*) and small secretory vesicles (*ve*) occupying almost the entire cytoplasm. Note the large, irregular shaped, electron-dense granules (*gr*), the slit areas (arrows) and the adjacent colloblast (*co*). The nucleus (*n*) is only visible in one rhogocyte.

DISCUSSION

General

The Nudibranchia are the fifth major taxon that has been investigated within the framework of a comparative study on opisthobranch excretory systems. Investigated taxa include the pelagic Gymnosomata and Thecosomata (Fahrner and Haszprunar 2000), the benthic Sacoglossa (Fahrner and Haszprunar 2001), and the interstitial Acochlidia (Fahrner and Haszprunar 2002). Our data on the renopericardial complex of the *Hypselodoris tricolor* reveal that its fine-structure basically corresponds to that of other Mollusca (for review, see Andrews 1988). Shared features of all the higher taxa of the Opisthobranchia examined so far are (1) the auricular epicardium as ultrafiltration site, characterized by the presence of podocytes with slit diaphragms between the pedicels and an underlying basal lamina, (2) the renopericardial duct connecting the pericardial cavity with the kidney, (3) the kidney with an excretory epithelium, composed of one single type of nephrocyte with both secretory and reabsorptive function, and (4) the presence of additional loci of ultrafiltration in the solitary rhogocytes of the haemocoel and connective tissue with a fine-structure identical to that of the podocytes.

The site of ultrafiltration and the podocytes

Compared to the excretory systems of other taxa of the Opisthobranchia described up to now, that of *Hypselodoris tricolor* is modified significantly in that the auricular wall does not represent the sole site of ultrafiltration, but that flat podocytes also build up the entire outer pericardial endothelium. The presence of such an additional, extensively developed ultrafiltration site in the outer pericardial wall as in *H. tricolor* has not been observed in any other species of the Mollusca.

In most of the taxa with available TEM data podocytes are restricted to the auricular epicardium (Pirie and George 1979, Andrews 1981, 1985; Morse and Meyhöfer 1990; Reynolds *et al.* 1993; Morse and Reynolds 1996; Luchtel *et al.* 1997; Estabrooks *et al.* 1999; Fahrner and Haszprunar 2000, 2001, 2002;), a condition that is considered to be symplesiomorphic for the Mollusca. Some species of the Prosobranchia show additional podocytes in the epicardial surface of the ventricle (Økland 1982; Luchtel *et al.* 1997), while in the Cyclophoridae the ventricular epicardium represents the main site of ultrafiltration (Andrews and Little 1972). In *Micropilina* species (Monoplacophora, see Haszprunar and

Schäfer 1997a,b) and in *Alderia modesta* (Lovén, 1844) (Gastropoda, Sacoglossa, see Fahrner & Haszprunar, 2001), podocytes are completely absent due to the complete loss of the heart and the pericardium. The likewise heart-less *Rhodope* species (Opisthobranchia, see Haszprunar 1997) lack podocytes as well, but show an entirely new, pseudoprotonephridial system of ultrafiltration that appears to be restricted to this taxon.

Podocytes in the outer pericardial wall are only known from Cephalopoda (in appendages of the branchial heart wall, see Schipp and Hevert 1981; Schipp *et al.* 1985) and particulary from heterodont Bivalvia (as so-called pericardial glands, see Meyhöfer *et al.* 1985; Khan *et al.* 1988; Andrews and Jennings 1993; Meyhöfer and Morse 1996). However, in contrast to *Hypselodoris tricolor*, most of these other taxa with podocytes in the outer pericardial epithelium have completely removed the ultrafiltration site from the wall of the auricle and only a few show transitional stages (Andrews and Jennings 1993). In the small and interstitial species *Philinoglossa helgolandica* Hertling, 1932 (Opisthobranchia), the sole ultrafiltration site has also moved to a part of the outer pericardial wall facing the kidney, but no true podocytes could be found (Bartolomaeus 1997). Instead, other special slashed cells (podocyte-like cells) enable the filtration of the haemolymph fluid into the pericardium in this species.

Thus, *H. tricolor* represents the only known representative of the Gastropoda with true podocytes situated in the outer pericardial wall. This feature can not be explained as an adaptation to estuarine or freshwater habitats as for the taxa of the Bivalvia with pericardial glands (Andrews and Jennings 1993) and contradicts Andrews and Jennings (1993) assumption that the development of a filtration site embedded in the outer pericardial wall is a character unique to the Bivalvia. It is likely that the increase in the surface area of the ultrafiltration site in the carnivorous *H. tricolor* reflects an increased need of filtration. Differences between the pressure of the haemolymph and the pericardial fluid may result from the action of the muscular system that forces to pass the pericardial podocytes.

Excretory epithelia

The ultrastructure of the excretory epithelia of renopericardial duct and kidney of *Hypselodoris tricolor* clearly corresponds to that of other Opisthobranchia (see Fahrner and Haszprunar 2000, 2001, 2002). In particular, there is only one single cell type that exhibits features associated with nitrogenous excretion as well as reabsorption of organic solutes. Microvilli at the apical border and extensive basal infoldings greatly enlarge the cell surface

and one or several large vacuoles occupy the cytoplasm. Additionally, the excretory cells contain endosomes, lysosomes, residual bodies, and a large number of mitochondria and glycogen granules. These data corroborate Andrews (1988) who presumed that there has been a secondary simplification of the kidney in the ancestors of the Opisthobranchia, in which one type of epithelial cell subsumed both secretory and reabsorptive function.

The small, electron-dense granules, often considered as particles of stored glycogen in the literature, represent dynamic cellular organelles called glycosomes (Rybicka 1996). They consist of a protein component, stainable with heavy metal, and of glycogen that does not react with uranium and lead. All glycosomes found in the excretory cells of *H. tricolor* were deposited free in the cytoplasm (lyoglycosomes) and often aggregated into large clumps, whereas so-called desmoglycosomes that are intimately associated with different cellular structures could not be detected. The varying number of the glycosomes, as well as of the electron-lucent vacuoles, may reflect a cytological turnover of the excretory cells.

Additional loci of ultrafiltration – the rhogocytes

Apart from the podocytes, a second cell-type with an ultrafiltration weir is present in *Hypselodoris tricolor*. The solitary rhogocytes occur throughout the primary body cavity, i.e. free in the haemocoel and embedded in the connective tissue and are characterized by slit areas on their surface that strongly resemble the fenestrations of the podocytes. Haszprunar (1996) previously outlined the high similarity of the molecular sieves (slits bridged by diaphragms, covering ECM, underlying free lumen or cisternae) that provides significant evidence for a cytological homology between molluscan rhogocytes and metazoan podocytes, cyrtocytes, and excretory cells. As indicated by the large number of vesicles that are formed at the base of the cisternae underlying the slit areas (see Fig. 7C), filtration pressure is probably caused by endocytosis in rhogocytes. In contrast, muscular activity is the driving force in podocytes (Morse and Cooper 1993; Haszprunar 1996).

The data from *H. tricolor* represent the first evidence of a striking variability of form and shape of the rhogocytes within one species and even within the same specimen. This proves that the shape of these cells may be independent from the physiological condition of the individual, as had been assumed (Haszprunar 1996). In the present case, it is more likely that the shape of the rhogocytes varies according to the adjacent space available. Possible functions of the rhogocytes include a major role in the metabolism of metal ions and the detoxification of heavy metal ions (see review by Haszprunar 1996). Furthermore, it has been

shown recently by means of electron microscopy and immunohistochemical experiments (Albrecht *et al.* 2001), that rhogocytes represent the site of haemocyanin biosynthesis in *Haliotis tuberculata* Linné, 1758 (Vetigastropoda). However, in the present investigation we were unable to identify haemocyanin molecules in the vacuoles of the rhogocytes.

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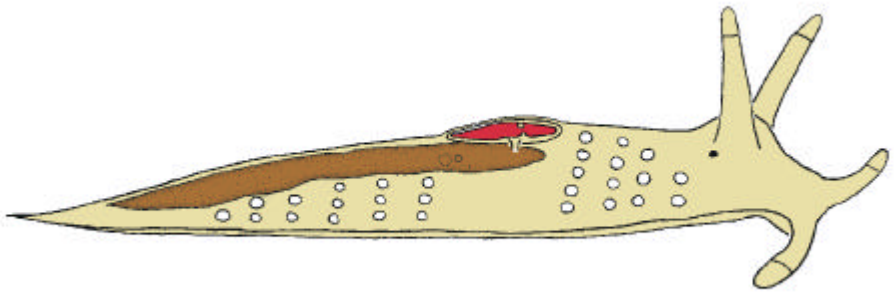
APPENDIX V

Ultrastructure of the renopericardial complex in *Cuthona caerulea* (Gastropoda, Nudibranchia, Aeolidoidea)



Abstract. The ultrastructure of the renopericardial complex of the aeolid nudibranch *Cuthona carulea* has been examined by means of serial sectioning analyses and transmission electron microscopy (TEM). The investigations revealed a functional metanephridial system consisting of podocytes in the epithelium of the pericardium that is linked with the single, large kidney by a ciliated nephrostome. Podocytes as the site of ultrafiltration were not only detected in the auricular epicardium, but also line the entire ventricular epicardium and outer pericardial epithelium. The presence of only one single type of cuboidal epithelial cell with large vacuoles, basal infoldings, and an apical microvillous border in the kidney indicates both secretory and reabsorptive activity. Solitary rhogocytes (pore cells) of the connective tissue and haemocoel represent additional loci of ultrafiltration with a fine-structure identical to that of the podocytes (slits between cytoplasmatic processes, bridged by fine diaphragms and covered by extracellular matrix). The presence of podocytes situated in the epicardial wall of the auricle is regarded as plesiomorphic for the Mollusca and is confirmed for the aeolidioid Nudibranchia. An additional, extensive and separate ultrafiltration site in the outer pericardial wall is a common feature of *Cuthona caerulea* and the doridoid nudibranch *Hypselodoris tricolor* that is unique among molluscs and most probably represents a significant autapomorphy, either of the Nudibranchia or the Nudipleura (Pleurobranchia and Nudibranchia). An epicardium and outer pericardium exclusively composed of podocytes and entirely devoid of epithelio-muscle cells are not known from any other taxon of the Mollusca and strongly suggest a significantly increased ultrafiltration activity in *Cuthona caerulea*.

MATERIAL AND METHODS

Specimens of *Cuthona caerulea* (Montagu, 1804) were collected by SCUBA in June 1999 off Banyuls-sur-Mer (France) from hydrozoans in a *Posidonia* meadow at 8m depth. The animals were anaesthetized by adding a solution of isotonic (about 7%) $MgCl_2$ to the seawater before they were processed for light microscopy (LM) and transmission electron microscopy (TEM). Fixation in 4 % seawater buffered formalin (LM) or 4 % glutardialdehyde (LM and TEM) buffered in 0.2 M sodium cacodylate (pH 7.2) was followed by a rinse in the same buffer in decreasing concentrations in the latter. After postfixation in buffered 1 % OsO_4 for two hours, the specimens were rinsed again with cacodylate buffer and dehydrated in a graded series of ethanols. The fixed specimens were embedded overnight in Spurr`s (1969) low viscosity resin.

In order to enable an overall view on the *in situ*- position of the excretory system, a complete series of semithin sections (2µm) was made with glass knifes (Henry 1977) and stained with methylene-blue – azure II according to Richardson et al. (1960). The histological slides are deposited at the Zoologische Staatssammlung München (ZSM; Malacology section; Nr. 20020344). For TEM, ultrathin sections (70 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyl acetate and lead citrate and examined and photographed with a Philips CM 10 TEM at 80 kV.

RESULTS

General anatomy

The heart of *Cuthona caerulea* (Montagu, 1804) is placed dorso-medially, at the end of the first body-half (in the large adanal space between the fourth and the fifth row of cerata), overlying the posterior part of the stomach and the anterior part of the kidney (Fig. 1). It is orientated along the longitudinal axis of the body, with the auricle lying posteriorly to the ventricle, and is enclosed in a thin, spacious pericardium. The dorsally situated pericardial cavity opens directly into the kidney via the ciliated, funnel-shaped nephrostome that is situated dorsolaterally, on the right side of the body. A distinct renopericardial duct is absent. The large, sac-like kidney spreads over the dorsolateral surface of the visceral mass, reaching backwards from the region of the ventricle, almost until the posterior end of the body.

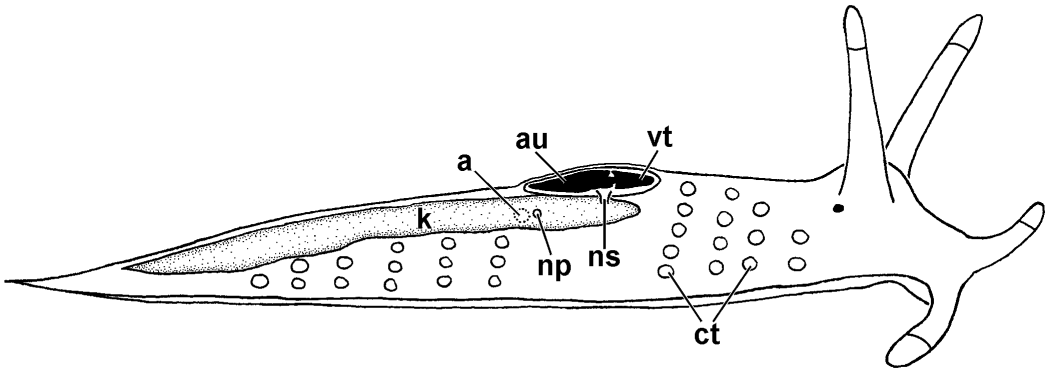


Fig. 1 Scheme of *Cuthona caerulea* (10 mm long), lateral view, showing the relative position of the excretory system. The cerata are not drawn, only their insertions are indicated. *a* anal opening, *au* auricle, *ct* rows of cerata, *k* kidney; *ns* nephrostome, *np* nephropore, *vt* ventricle.

The kidney touches the ventral surface of the pericardium and opens to the exterior dorsolaterally, immediately posterior of the heart, just in front of the anal opening and the right, innermost ceras of the fifth row (first postanal row) of cerata.

Pericardium and epicardium

The outer wall of the pericardium of *Cuthona caerulea* consists exclusively of podocytes (Figs. 2A,C, 3A). They are peripherally slashed and rest on a basal lamina that is underlain by a loose network of collagen fibers of the extracellular matrix (ECM). The podocytes are attached to each other by belt desmosomes between cytoplasmic extensions of the cell and their isolated cell bodies bulge into the lumen of the pericardial cavity (Fig. 2A). Numerous thin pedicels extend from the basal border of the podocytes and interdigitate with those of adjacent cells. Fine diaphragms span the ultrafiltration slits (approx. 20 nm in width) between the pedicels that overlie the basal lamina (Fig. 2E). The cytoplasm of the podocytes contains a number of small vesicles, few mitochondria, and the centrally located nucleus.

The entire epicardia of both ventricle (Fig. 2A,B) and auricle of *C. caerulea* (Fig. 2C,D) are composed of podocytes as well, epithelio-muscle cells are absent. These epicardial podocytes are structurally identical to those of the outer pericardial wall: they have flat cell bodies and are isolated from adjacent cells by excessively developed pedicels. The myocardium of the heart consists of loosely arranged, non-epithelial muscle bundles (Fig. 2A,C) and is covered by the basal lamina of the pericardium. Numerous mitochondria are scattered along the outer edges of the myocytes which are connected to the surrounding ECM by hemi desmosomes.

Nephrostome and kidney

The prominent, approximately 40 µm wide nephrostome is lined with cuboidal, multiciliated cells (Fig. 3B,C). Short microvilli emanate from the apical surface of these cells and their cytoplasm contains a centrally located nucleus and numerous mitochondria with some intimately associated desmoglycosomes. Several of the epithelial cells of the nephrostome show electron-lucent vacuoles (Fig. 3B), just like those occuring in the cells of the kidney epithelium (see below). The basal cell surface is not invaginated or folded and rests on an ECM.

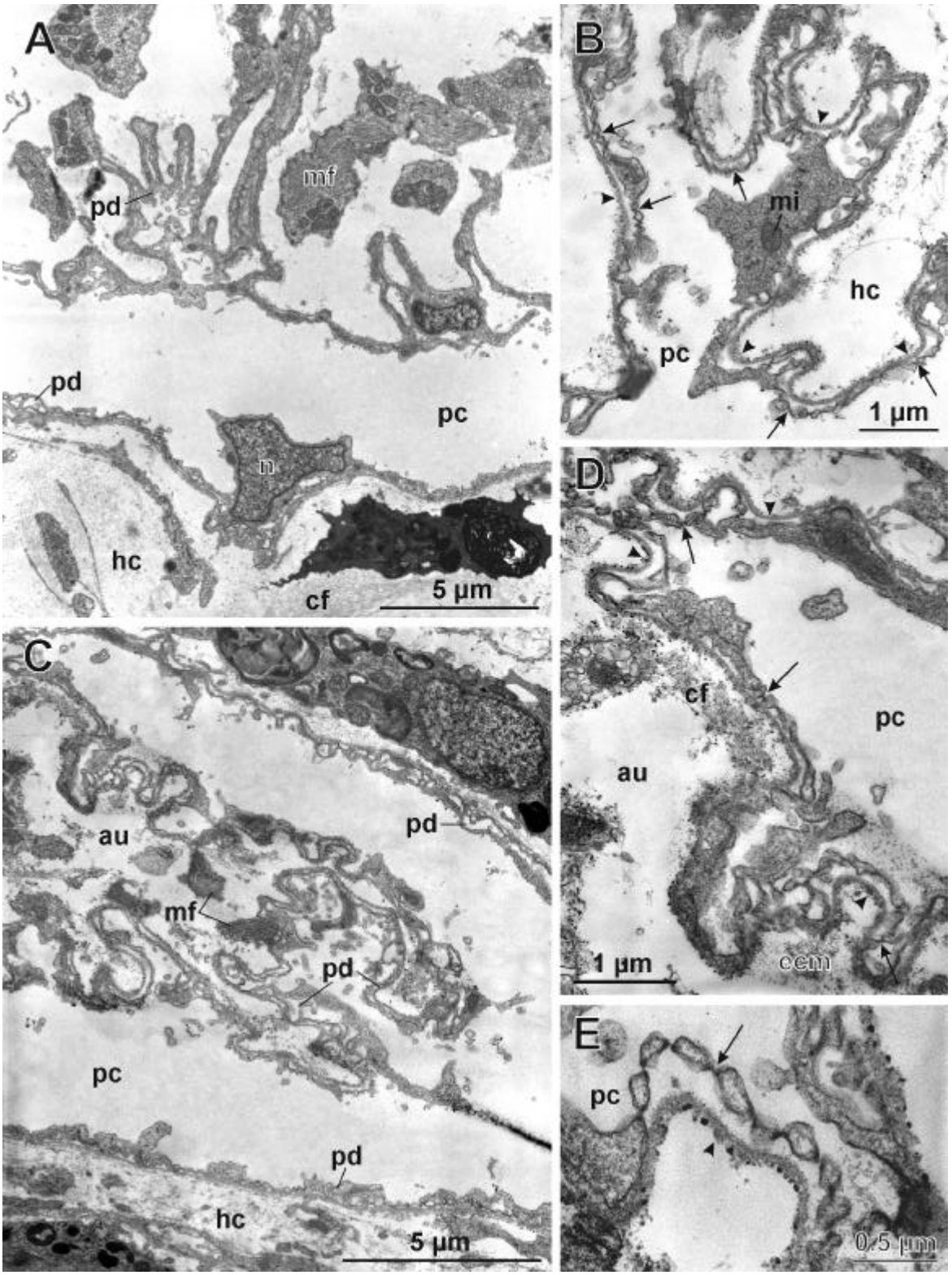


Fig. 2 A-E TEM micrographs of pericardium and heart. **A** Ventricular portion of the heart. Note that both ventricular epicardium and outer pericardial epithelium are composed of structurally identical, flat podocytes with interdigitating basal pedicels (*pd*). **B** Podocyte from the ventricular epicardium showing diaphragmatic slits (arrows) between the pedicels and the underlying basal lamina (arrowheads). **C** Auricular portion of the heart (*au*). Podocytes with basal pedicels (*pd*) line the entire auricular epicardium and the outer pericardium. **D** Pedicels of epicardial and pericardial podocytes with diaphragmatic slits (arrows) and underlying basal lamina (arrowheads). **E** Detail of pedicels of epicardial podocyte showing slits bridged by fine diaphragms (arrow) and apposed by basal lamina (arrowhead). *cf* collagen fibers, *ecm* extracellular matrix, *hc* haemocoel, *mf* muscle fibers, *mi* mitochondrion, *n* nucleus, *pc* pericardial cavity.

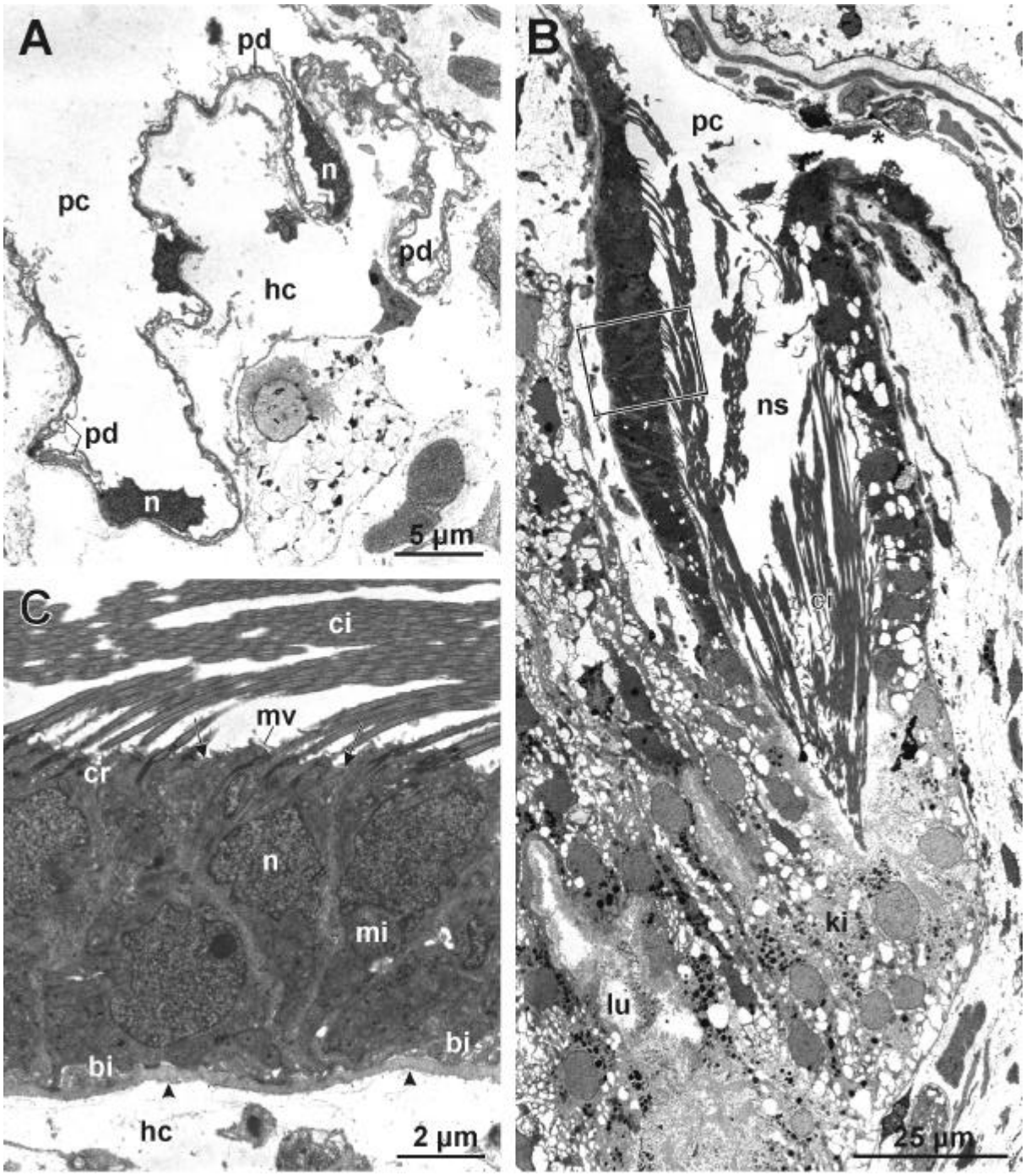


Fig. 3 A-C TEM micrographs of pericardium and nephrostome. **A** Pericardial cavity (*pc*) and outer pericardial epithelium, entirely composed of flat podocytes with pedicels (*pd*). **B** Funnel-shaped ciliated nephrostome (*ns*) connecting the pericardial cavity (*pc*) and the kidney (*ki*). Asterisk points to the body of a pericardial podocyte. The boxed area is enlarged in C. **C** Epithelial cells of the nephrostome with numerous cilia (*ci*) and their rootlets (*cr*), short microvilli (*mv*), and belt desmosomes (arrows) apically and mitochondria (*mi*), weakly developed infoldings (*bi*) of the cell surface, and the underlying basal lamina (arrowheads) basally. The prominent nucleus (*n*) occupies almost the entire cytoplasm of the cells. *ci* cilia, *hc* ,haemocoel, *lu* collapsed lumen of the kidney, *n* nuclei.

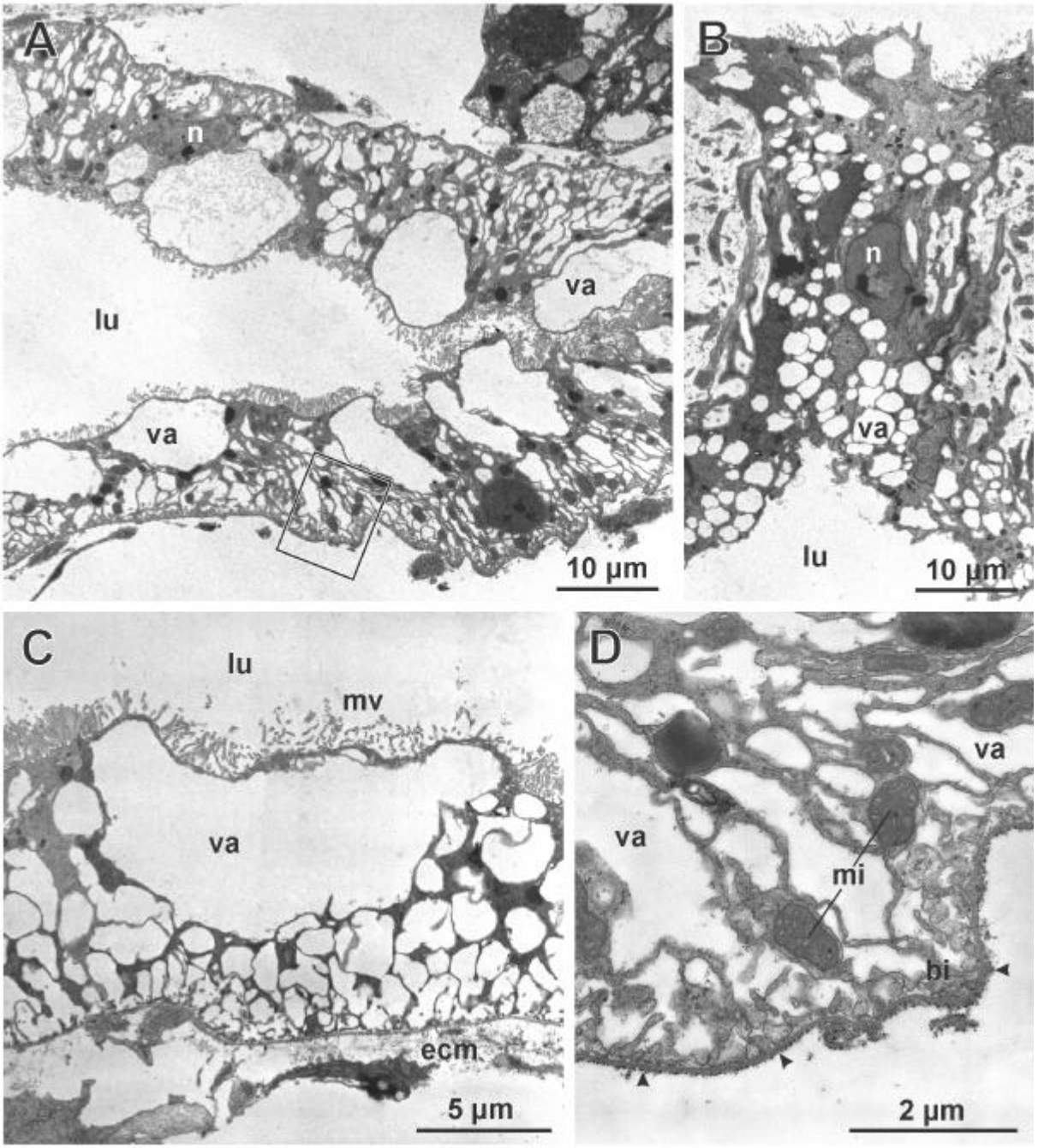


Fig. 4 A-D TEM micrographs of kidney epithelium. **A** Highly vacuolated cells of the kidney epithelium. Boxed area is enlarged in D. **B** Nephropore. **C** Excretory cell of the kidney epithelium with microvillous border (*mv*) towards the lumen (*lu*) and one very large, apical, and numerous smaller, basally located, electron-lucent vacuoles (*va*) that occupy almost the entire cytoplasm. **D** Basal part of excretory cell with weakly developed basal infoldings (*bi*) and mitochondria (*mi*), interspersed between the small vacuoles (*va*). Also note the basal lamina of the extracellular matrix, forming a grid (arrowheads). *ecm* extracellular matrix, *lu* kidney lumen, *va* electron-lucent vacuoles, *n* nuclei.

The epithelium of the kidney of *C. caerulea* is composed of one single cuboidal type of excretory cell (Fig. 4A) that is characterized by a dense, apical microvillous border and numerous electron-lucent, often very large vacuoles (up to 15 µm in diameter) in the cytoplasm (Fig. 4C). Belt desmosomes and extensive septate junctions interconnect the excretory cells near their apices. The basal portions of the excretory cells show weakly developed infoldings with some interspersed mitochondria and numerous small vacuoles (Fig. 4D). Some cells are completely devoid of basal infoldings and their cytoplasm seems to contain only vacuoles increasing in size from the basal to the apical border. No glycosomes, endosomes, or lysosomes could be detected. The cells forming the small nephropore (approx. 25 µm in diameter) generally resemble the excretory cells but lack basal infoldings and their vacuoles are distinctly smaller (Fig. 4B).

Solitary rhogocytes

Rhogocytes, solitary cells of the haemocoel and the connective tissue (Fig. 5A), represent a second cell type with an ultrafiltration weir in *Cuthona caerulea*. They are entirely covered by a basal lamina of the ECM and vary considerably in shape and form even within one individual. However, most of the rhogocytes in *Cuthona caerulea* show a distinctly elongated shape and reach up to 15 µm in length. The most striking diagnostic character of the rhogocyte are the areas with slits, scattered over the entire surface of the cell, that are underlain by flat cisternae. The slits with a width of 20 to 25 nm occur between tiny cytoplasmatic bars and are bridged by fine, electron-opaque diaphragms (Fig. 5B). A well-developed, rough endoplasmatic reticulum continuous with the nuclear membrane, mitochondria, and some electron-opaque granules (diameter 1 µm) further characterize the rhogocyte.

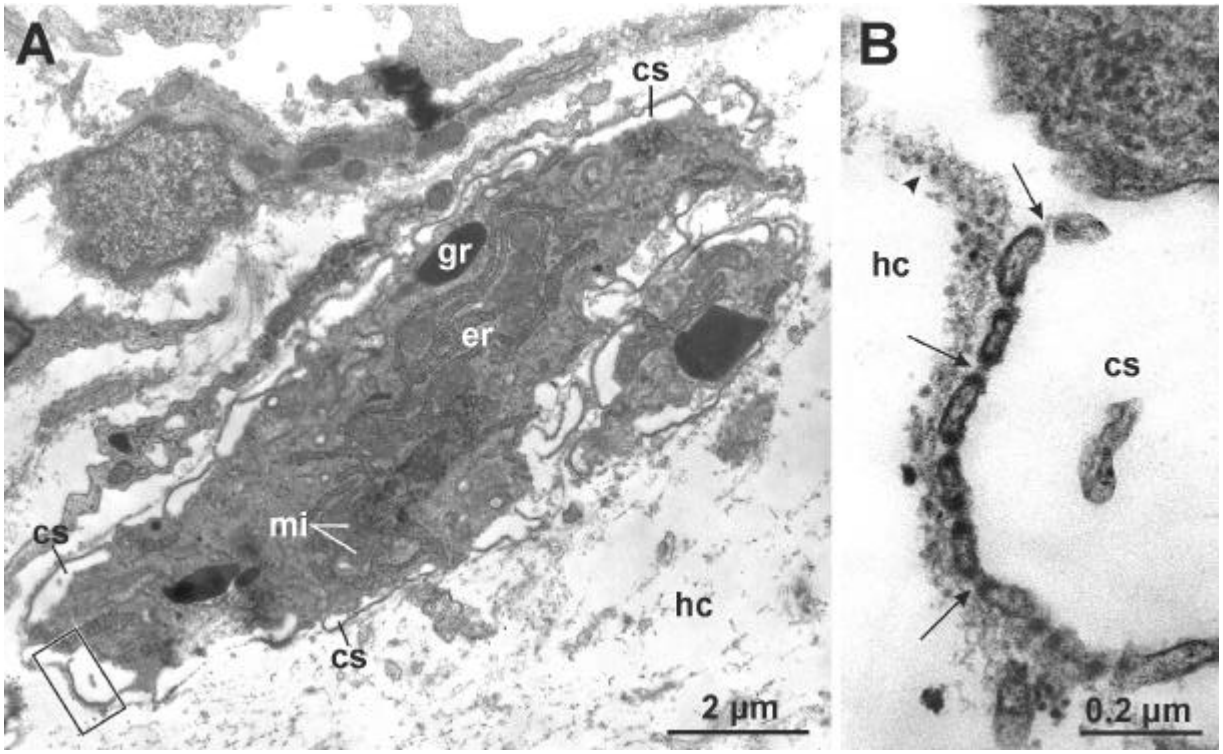
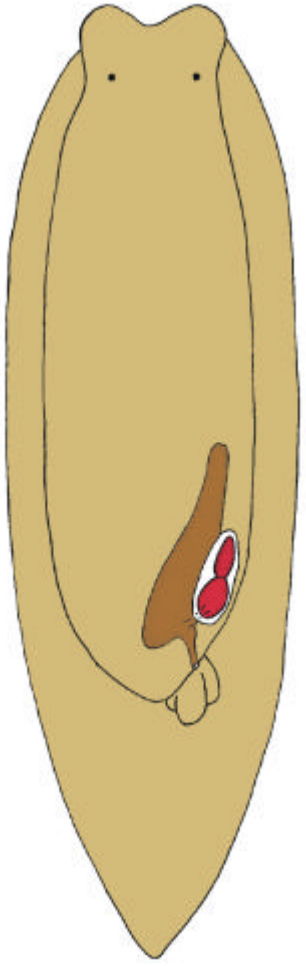


Fig. 5 A, B TEM micrographs of a rhogocyte from the haemocoel (hc). **A** Elongate rhogocyte with electron-dense cytoplasm containing tubular rough endoplasmatic reticulum (*er*), several mitochondria (*mi*), and some electron-dense granules (*gr*). The slit areas are mainly indicated by the underlying flat cisternae (*cs*). Rectangle marks the area enlarged in D. The nucleus is not visible. **B** Detail of slit area showing a cistern (*cs*), the diaphragms (arrows) that span the slits between the overlying cytoplasmic bars, and the covering basal lamina (arrowhead).

APPENDIX VI

Microanatomy and ultrastructure of the excretory system in *Runcina coronata* (Gastropoda, Cephalaspidea)



Abstract. The microanatomy and ultrastructure of the renopericardial complex of the bullomorph species *Runcina coronata* have been examined by means of serial sectioning analyses and transmission electron microscopy (TEM). The metanephridial excretory system consists of "podocyte-like-cells" in the pericardial epithelium and a single kidney which is connected with the pericardium by a short, ciliated, renopericardial duct. True podocytes as the typical site of ultrafiltration in molluscs are absent in *R. coronata.* Instead, special slashed cells (podocyte-like-cells) with the capacity to form an ultrafiltration barrier build up the entire auricular and ventricular epicardium of the heart and occur also between the squamose epithelial cells of the outer pericardium. These cells differ from podocytes in that they lack diaphragms spanning the ultrafiltration slits, the slits are much wider (up to 70 nm), and the cytoplasmic pedicels are distinctly spherical in cross section. In one juvenile specimen, pericardium and heart could not be detected, even though a distinct renopericardial duct was present. The kidney epithelium is composed of only one single type of cuboidal cell with large vacuoles, excessive basal infoldings, and an apical microvillous border in the kidney, indicating both secretory and reabsorptive activity. In kidney cells of a juvenile specimen, these morphological features are only weakly developed. Solitary rhogocytes (pore cells) of the connective tissue and haemocoel represent additional loci of ultrafiltration with a fine-structure identical to that of typical podocytes (slits between cytoplasmatic processes, bridged by fine diaphragms and covered by extracellular matrix). Numerous densely arranged rhogocytes overlie the muscular layer covering the digestive gland and the gonoduct, a feature not known from any other molluscan taxon. The data presented herein contradict earlier assumptions on the loss of the plesiomorphic site of ultrafiltration in the auricular epicardium in the Cephalaspidea. Next to *R. coronata*, the presence of podocyte-like-cells has only been described from the aberrant cephalaspidean species *Philinoglossa helgolandica*, suggesting a possible autapomorphy of the Cephalaspidea *s. str.* (i.e. the Bullomorpha).

MATERIAL AND METHODS

Specimens of *Runcina coronata* (Quatrefages, 1844) were collected off Calvi (Corsica, France) in June 1992 from samples of small algae taken in 5m to 25m depth by SCUBA. The algae were left undisturbed for several hours, up to one day, in small tanks, forcing the slugs to crawl to the water surface in search for oxygen. One additional specimen of *R. coronata* was found in an aquarium in Plymouth, England in July 1982. The animals were relaxed by slowly adding an isotonic (about 7%) solution of $MgCl_2$ to the seawater before they were

processed for light microscopy (LM) and transmission electron microscopy (TEM). Fixation in 4 % seawater buffered formalin (LM) or 4 % glutardialdehyde (LM and TEM) buffered in 0.2 M sodium cacodylate (pH 7.2) was followed by a rinse in the same buffer in decreasing concentrations in the latter. After postfixation in buffered 1 % OsO_4 for two hours, the specimens were rinsed again with cacodylate buffer and dehydrated in a graded series of ethanols. The fixed specimens were embedded overnight in Spurr`s (1969) low viscosity resin.

In order to examine the gross anatomy of the excretory system, two complete series of semithin sections (2µm) were made with glass knifes (Henry 1977) and stained with methylene-blue – azure II according to Richardson et al. (1960). They are deposited at the Zoologische Staatssammlung München (ZSM; Malacology section; Nrs. 20020342, 20020343). Selected histological slides were photographed on a Leica DM RBE compound microscope with a Kappa DX30 digital camera. For TEM, ultrathin sections (70 nm) were made with a diamond knife and kept on formvar-covered single slot copper grids. The sections were stained automatically with uranyl acetate and lead citrate and examined and photographed with a Philips CM 10 TEM at 80 kV.

RESULTS

General anatomy

The entire renopericardial complex of *Runcina coronata* is situated far posteriorly, on the right side of the body (Figs. 1, 2). The wide pericardium and the enclosed heart stretch along the longitudinal axis of the body, with the auricle being orientated posterior to the ventricle. A short, ciliated renopericardial duct connects the pericardial cavity in the posterior region of the auricle (Fig. 2C,D) with the posterior region of the kidney (Fig. 2E,F). The elongated, tubular kidney lies to the left of the heart, reaching both further anteriorly and posteriorly than the latter. Via a small nephropore, the kidney opens to the exterior on the right side of the body, close to the anterior insertion of the gill (Fig. 1).

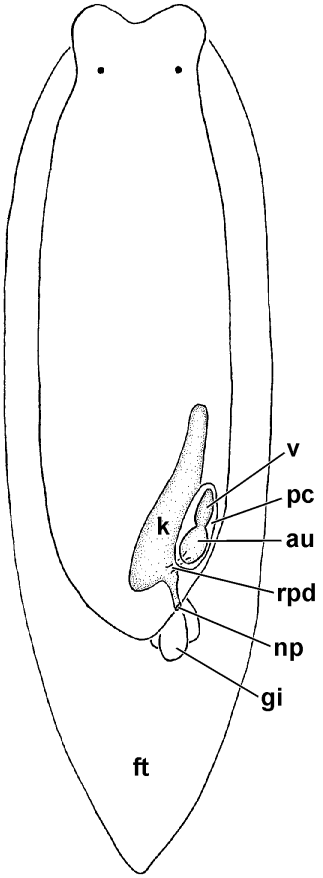


Fig. 1 Scheme of *Runcina coronata* (2 mm long), dorsal view, showing the relative position of the excretory system. *au* auricle, *ft* foot, *gi* gills, *k* kidney; *np* nephropore, *pc* pericardial cavity, *rpd* renopericardial duct, *v* ventricle.

In one juvenile specimen examined (1 mm long), a heart could not be detected, even though a distinct renopericardial duct is present (see Fig. 4C,D).

Pericardium and epicardium

Runcina coronata lacks true podocytes with diaphragms that bridge the ultrafiltration slits. The outer pericardial epithelium is predominantely composed of very flat squamose cells with an electron-lucent cytoplasm containing few mitochondria and numerous small vesicles (Fig. 3C). These cells are interspersed by flat podocyte-like cells (Fig. 3A) that are concentrated in some areas of the outer pericardium, such as around the opening into the renopericardial duct. Moreover, the podocyte-like cells form the entire auricular and ventricular epicardium (Fig. 3A,C). They are characterized by the presence of cytoplasmic branches that extend from the

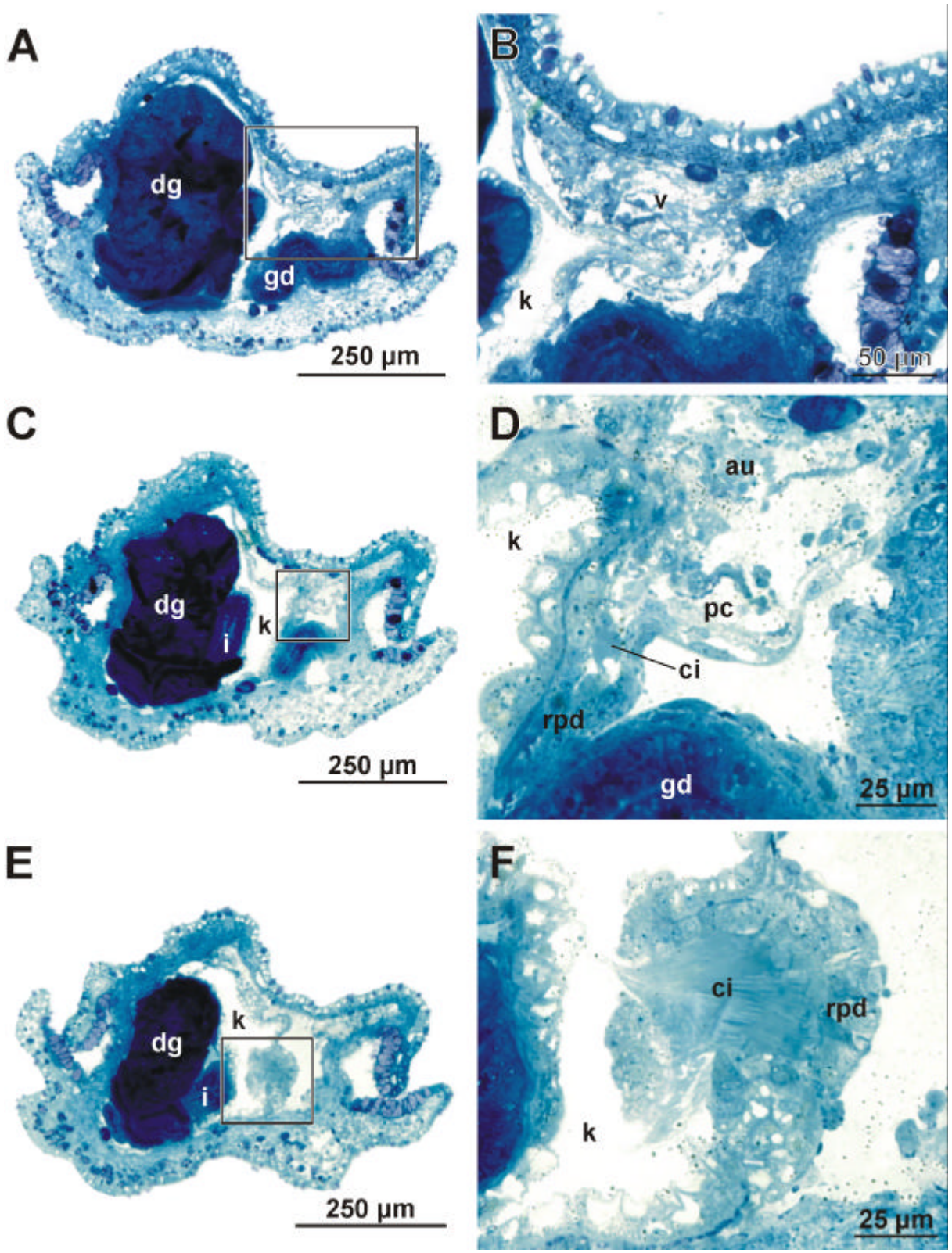


Fig. 2 A-F Histology of the excretory system based on serial semithin cross-sections, dorsal faces upwards and right to the right. **A** Cross section of entire body in the region of the gonoduct (*gd*) opening, showing the position of heart and kidney on the right side (boxed area, enlarged in B). **B** Ventricle (*v*) of the heart and adjacent kidney (*k*). **C** Cross section of entire body in the region of the auricle. Boxed area is enlarged in D. **D** Ventral opening of the pericardium (*pc*) into the renopericardial duct (*rpd*). **E** Cross section of entire body indicating the position of the opening of the renopericardial duct into the kidney on the right side (boxed area, enlarged in F). **F** Lateral opening of the renopericardial duct (*rpd*) into the kidney (*k*). Note the numerous cilia (*ci*) of the epithelial cells of the rpd. *au* auricle, *dg* digestive gland, *i* intestine.

cell body basally and form intervening ultrafiltration slits (20-75 nm in width, see Fig. 3B). Special filtration diaphragms covering these fenestrations are absent, but the slits are in all cases underlain by a basal lamina that might be apposed by a collagen layer of the ECM (Fig. 3A). The cytoplasmic extensions between the ultrafiltration slits are distinctly spherical in cross section (Fig. 3B). Mitochondria, rough ER and the centrally located nucleus occupy the perikarya of the podocyte-like cells that project into the lumen of the pericardial cavity. Epithelio-muscle cells are completely absent from the outer pericardium and the epicardium of *Runcina coronata.*

The myocardium of the auricular and ventricular portions of the heart is a loose network of muscle bundles (Fig. 3A,C). Mitochondria and glycosomes are scattered along the outer edges of the myocytes and the basal lamina of the pericardium surrounds the periphery of the cell. There is no evidence of intercellular junctions like belt desmosomes.

Renopericardial duct and kidney

The whole epithelium of the renopericardial duct of *Runcina coronata* (Fig. 4) is composed of cuboidal multiciliated cells. Numerous mitochondria and lyoglycosomes, few desmoglycosomes, and the centrally located nucleus occupy the cytoplasm of the epithelial cells (Fig. 4B). Their apical surface is characterized by short microvilli, the basal cell surface rests on an ECM and is devoid of infoldings (Fig. 4D). The openings of the renopericardial duct to the pericardium (Fig. 4A) and kidney (Fig. 5A) are funnel-shaped and approximately 25 µm wide. Whereas the pericardial opening is situated ventrally, in the posterior region of the auricle, (Fig. 2C,D), the renopericardial duct enters the kidney laterally, from the right side (Fig. 2E,F).

There are no differentiated regions of the kidney epithelium in *Runcina coronata.* The single type of excretory cell (Fig. 5B) has a highly infolded basal cell membrane and contains a centrally located nucleus and, in general, one very large transparent vacuole (up to 10 µm in diameter). Numerous mitochondria are scattered in the basal portion of the cell (Fig. 5C), whereas the apical border is characterized by the presence of long, thin microvilli and by septate junctions between adjacent cells (Fig. 5B). The excretory cells of the kidney of a juvenile specimen investigated differ from those of the kidney of adult specimens in that basal infoldings of the cell membrane are either completely absent or only very weakly developed (Fig. 5D). Furthermore, the large, prominent electron-lucent vacuoles are absent as well. Few,

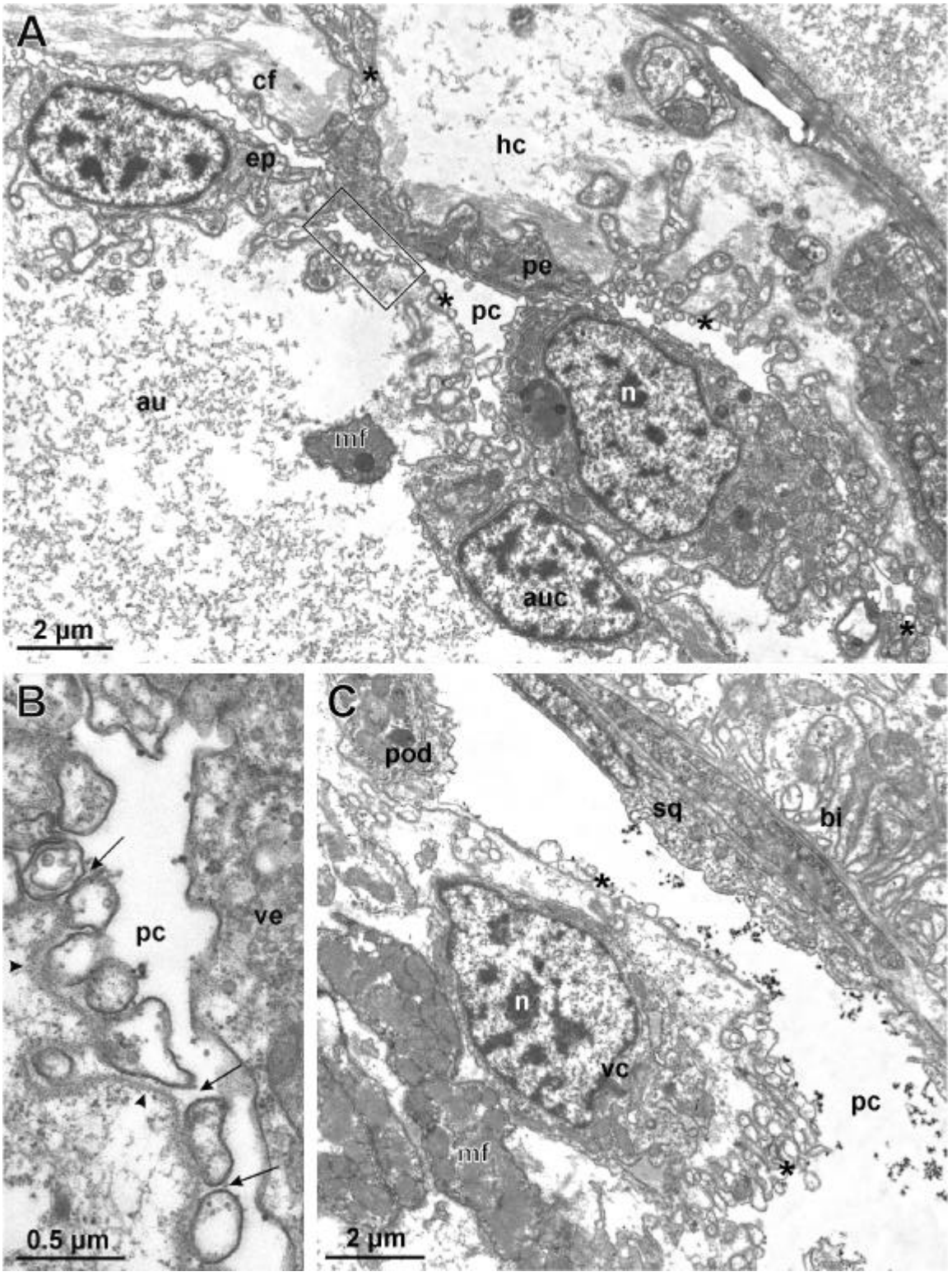


Fig. 3 A-C TEM micrographs of pericardium and heart. **A** Auricular epicardium (*ep*) and outer pericardium (*pe*), composed of podocyte-like cells with characteristic basal pedicels (asterisks) and cell bodies bulging into the lumen of the pericardial cavity (*pc*). An auricular cell (*auc*) is intimately attached to the basal lamina underlying the epicardium. The rectangle marks the area enlarged in B. **B** Slits between pedicels of an epicardial podocyte-like cell (arrows) apposed by basal lamina (arrowhead). **C** Ventricular epicardium, lined by a podocyte-like cell (*pod*) with excessively developed pedicels (asterisks) and outer pericardium with a very flat squamose cell (*sq*). Also note the ventricular cell (*vc*) with its prominent nucleus (*n*), the muscle fibers *(mf)* of the ventricular myocardium, and the basal infoldings (*bi*) of the adjacent kidney. *au* auricle, *cf* collagen fibers, *hc* haemocoel, *mf* muscle fibers, *n* nuclei, *pc* pericardial cavity, *ve* small vesicles in the cytoplasm of a pericardial podocyte-like cell.

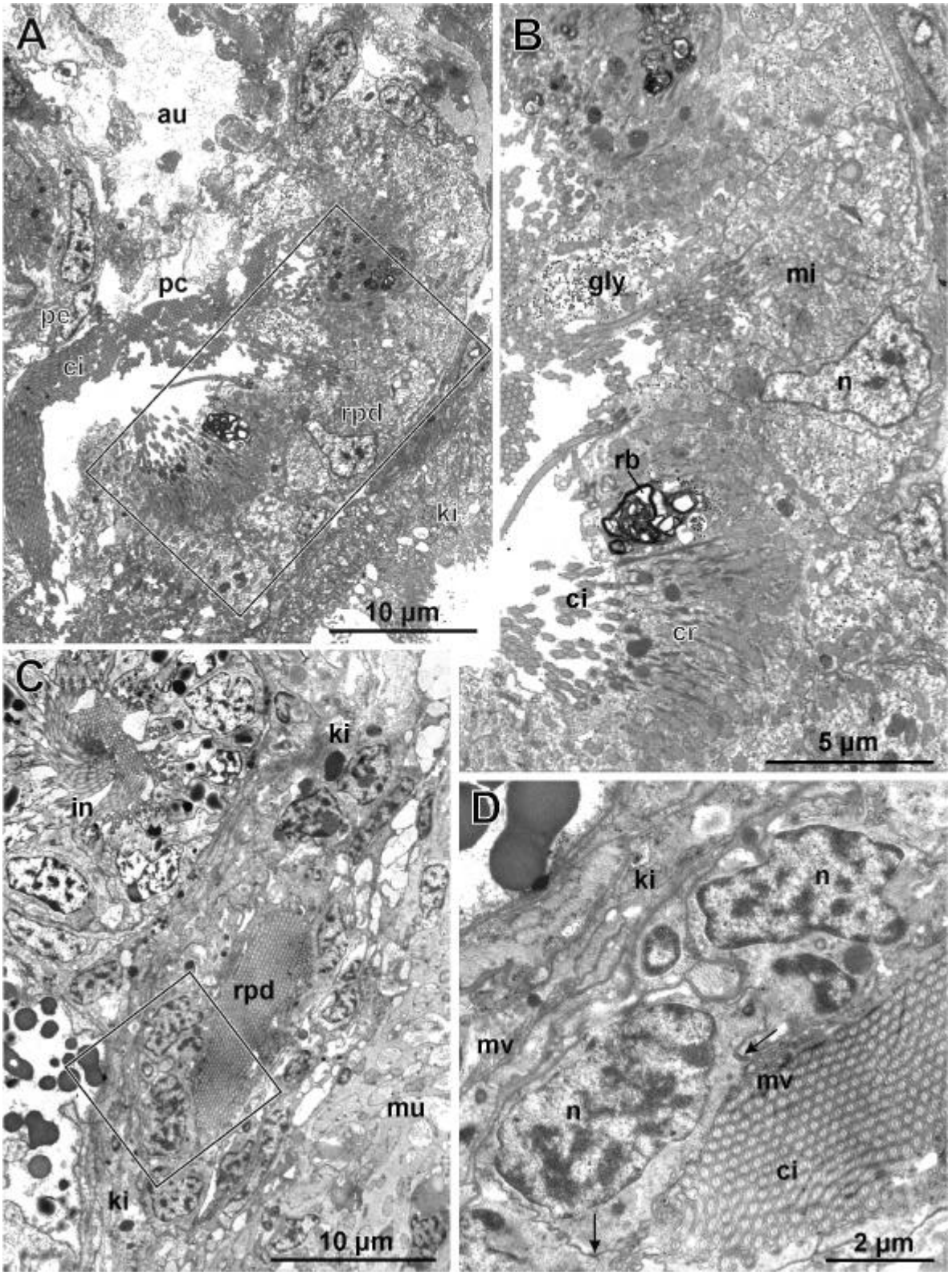


Fig. 4 A-D TEM micrographs of renopericardial duct. **A** Overview of the ciliary opening of the pericardium (*pe*) into the renopericardial duct (*rpd*) in the region of the auricle (*au*) (adult specimen). Boxed area is enlarged in B. **B** Detail of A showing epithelial cells with numerous mitochondria (*mi*), glycosomes (*gly*), cilia (*ci*) with rootlets (*cr*), a residual body with associated desmoglycosomes (*rb*), and a nucleus (*nu*). **C** Central section of the renopericardial duct (*rpd*) situated between the muscle layer of the epidermis (*mu*) to the right and the inconspicuos kidney (*ki*) and the intestine (*in*) to the left. (juvenile specimen). Boxed area is enlarged in D. **D** Two cuboidal cells of the central section with apical microvilli (*mv*), numerous cilia (*ci*), and prominent nuclei (*n*) that occupy almost the entire cytoplasm. Also note the adjacent kidney (*ki*) with microvilli (*mv*) towards the entirely collapsed lumen. *pc* pericardial cavity.

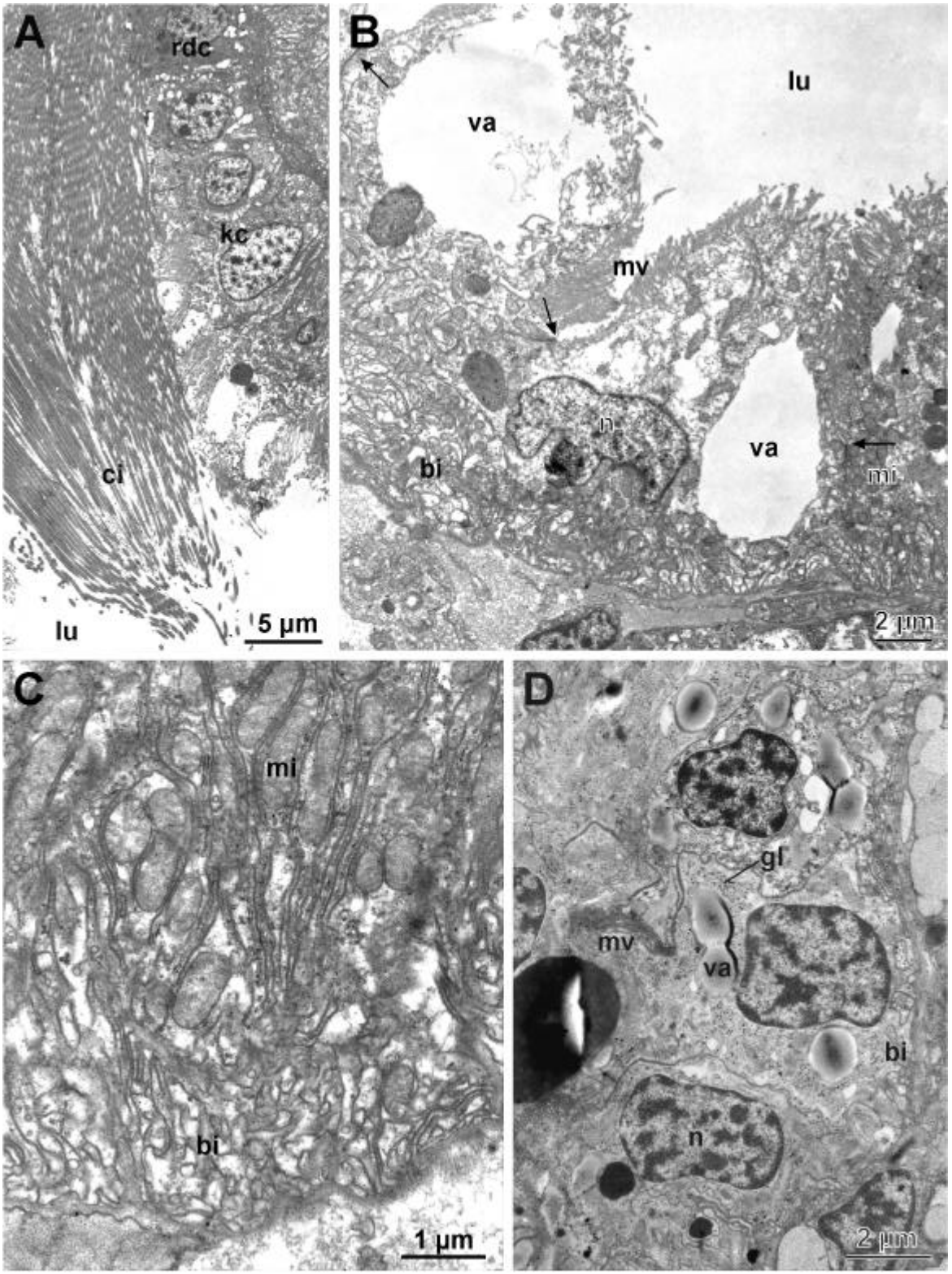


Fig. 5 A-D TEM micrographs of kidney epithelium. **A** Funnel-shaped opening of the renopericardial duct into the kidney. Note the numerous cilia (*ci*) reaching into the kidney lumen (*lu*), the ciliated renopericardial duct cells (*rdc*) and the non-ciliated neighbouring kidney cells (*kc*). **B** Excretory cells of the kidney of an adult specimen with very large electron-lucent vacuoles (*va*), a dense apical microvillous border (*mv*) towards the lumen (*lu*), and excesssively developed infoldings of the basal cell surface (*bi*). Also note the centrally located nucleus (*n*), the mitochondria (*mi*), as well as the apical belt desmosomes between adjacent cells (arrows). **C** Basal portion of an excretory cell showing the basal infoldings (*bi*) and numerous interspersed mitochondria (*mi*). **D** Excretory cells of the kidney of a juvenile specimen with weakly developed basal infoldings (*bi*), small vacuoles that may coalesce (*va*), and central nuclei (*n*). Lyoglycosomes (*gl*) are scattered over the entire cytoplasm of the cells and microvilli (*mv*) indicate the position of the collapsed lumen.

much smaller ones (diameter up to 1 µm) occur at various positions of the cell. These vacuoles could frequently be observed to coalesce. Numerous lyoglycosomes are scattered throughout the cytoplasm, that is predominantely occupied by the centrally placed nucleus.

Solitary rhogocytes

A second cell type with an ultrafiltration weir, the rhogocyte (Fig. 5), occurs in the haemocoel and connective tissue of *Runcina coronata.* In contrast to the epithelial podocyte-like cells of the pericardium and epicardium, rhogocytes are solitary cells completely surrounded by a thin layer of ECM that forms a basal lamina. They are mostly elongate or irregularly shaped and up to 15 µm long (respectively in diameter). Numerous rhogocytes are very densely arranged, covering the muscular layer that overlies the digestive gland and the gonoduct. Areas with slits and the underlying cisternae are scattered over the entire surface of the cell. The slits

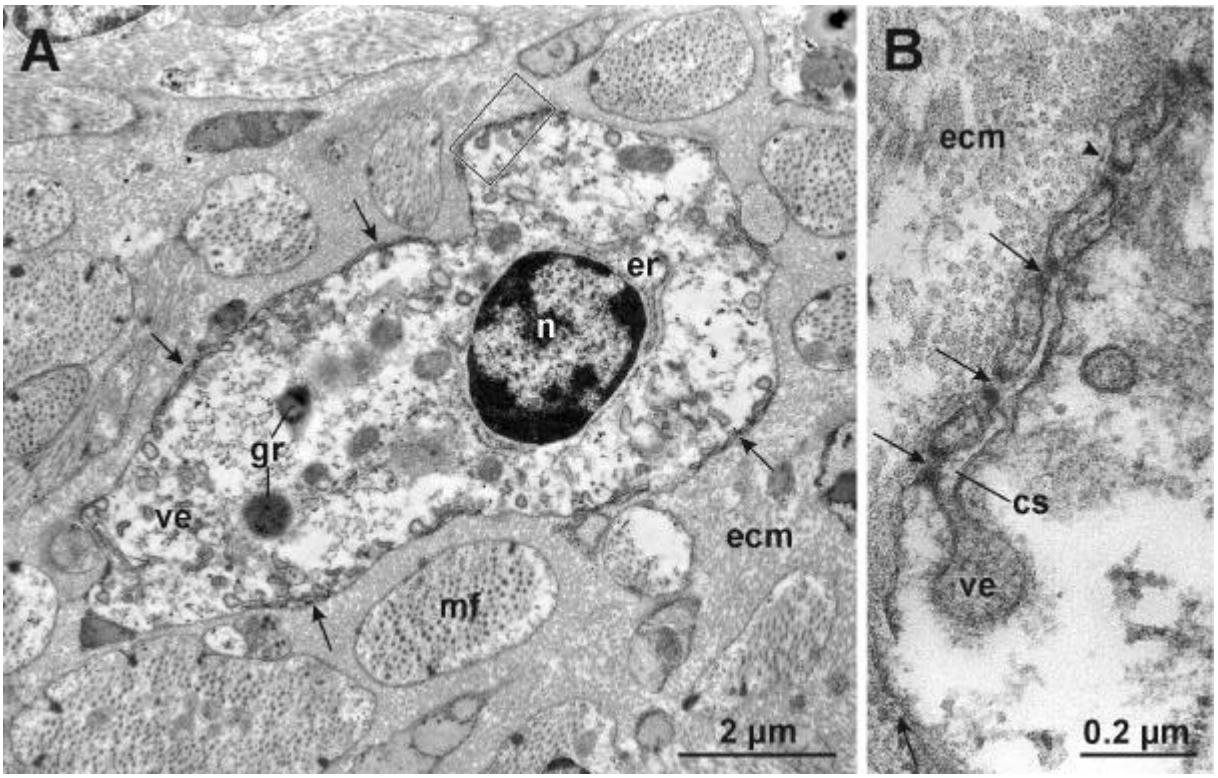


Fig. 6 A, B TEM micrographs of a rhogocyte from the connective tissue. **A** *Runcina coronata*. Irregularly shaped rhogocyte situated between the muscle fibers *(mf)* of the body wall. Note the electron-dense granules (*gr*), the small vesicles (*ve*) scattered throughout the electron-lucent cytoplasm, and the rough endoplasmatic reticulum (*er*) around the roundish nucleus (*n*). Zones of slit openings (arrows) surround almost the entire cell-surface. Rectangle marks the area enlarged in B. **B** Detail of slit area with underlying, extremely flat cistern (*cs*). Arrows point the diaphragms bridging the slits, the arrowhead indicates the covering basal lamina of the extracellular matrix (*ecm*). Also note the pinocyte-like formation of a vesicle (*ve*) at the base of the cistern.

with a width of 20 to 25 nm occur between tiny cytoplasmatic bars and are spanned by fine, fibrillar diaphragms (Fig. 6B). The cisternae are extremely flat and narrow (approx. 20 nm in width) and at their bases, phagocyte-like formation of vesicles could be observed frequently (Fig. 6B). Further features of the rhogocyte of *Runcina coronata* are electron-dense granules (diameter approximately 0.5 µm), numerous small secretory vesicles, and a rough endoplasmatic reticulum continuous with the membrane of the prominent nucleus.

ZUSAMMENFASSUNG

Die vorliegende Arbeit beinhaltet detaillierte, vergleichende Studien zur Morphologie und Ultrastruktur der Exkretionsorgane opisthobrancher Gastropoden, die mittels histologischer Semidünnschnitt-Serien, graphischer Rekonstruktionstechniken und insbesondere Transmisionselektronenmikroskopie (TEM) durchgeführt wurden. Um das Grundmuster der Exkretionsorgane der Opisthobranchia sowie Daten über deren Variabilität zu ermitteln, wurden Vertreter fast aller höherer Subgruppen, der Cephalaspidea, Thecosomata, Gymnosomata, Sacoglossa, Acochlidia und der anthobranchen und cladobranchen Nudibranchia, untersucht. Besondere Aufmerksamkeit galt dabei den Ultrafiltrationsstrukturen der jeweiligen Taxa sowie möglichen Abwandlungen der Exkretionsorgane bei evolutionär und funktional besonders interessanten Arten (ohne primäres Ultrafiltrationsorgan Perikard bzw. paedomorphe Arten). Die Ergebnisse ermöglichen weitreichende Schlußfolgerungen hinsichtlich der Evolution der Exkretionsorgane der Mollusken und der phylogenetischen Beziehungen innerhalb der Opisthobranchia.

Adulte Opisthobranchia besitzen generell ein metanephridiales Exkretionssystem (den Renoperikardialkomplex), das aus Podocyten des Perikardepithels und einer einzelnen, großen Niere, die durch einen bewimperten Renoperikardiodukt mit dem Perikard verbunden ist, besteht. Die Podocyten mit ihren zahlreichen, durch feine Diaphragmen überspannten Filtrations-Schlitzen zwischen den basalen cytoplasmatischen Fortsätzen stellen die Ultrafiltrationsloci dar. Sie sind bei den Thecosomata, Gymnosomata, Sacoglossa und Acochlidia in ihrem Vorkommen auf das Epikard des Atriums beschränkt. Bei den Nudibranchia besteht zusätzlich das gesamte Epithel des äußeren Perikards, und bei aeolidoiden Nudibranchia auch das Epikard des Ventrikels, ausschließlich aus Podocyten. Den Cephalaspidea *s. str.* (den Bullomorpha) fehlen echte Podocyten und andere, basal verzweigte Zellen („podocytenartige Zellen“ ohne Diaphragmenbildung des Ultrafilters) mit der Fähigkeit, eine Ultrafiltrationsbarriere zu bilden, übernehmen statt dessen deren Funktion. Die „podocytenartigen Zellen“ kleiden das gesamte Epikard des Herzens aus und kommen bei der untersuchten Art *Runcina coronata* auch vereinzelt zwischen den Plattenepithelzellen der äußeren Perikardwand vor. Bei der herzlosen sacoglossen *Alderia modesta* konnten keine Podocyten oder andere, epitheliale Zellen, die eine Ultrafiltration ermöglichen, gefunden werden. Das Epithel des Renoperikardiodukt besteht bei den meisten untersuchten Arten aus

zwei unterschiedlichen Zelltypen: multiciliäre Zellen finden sich an den Öffnungen des Renoperikardiodukts zum Perikard und in die Niere, während der mittlere Teil mit aciliären Zellen mit apikalem Mikrovillisaum ausgekleidet ist. Bei *Creseis virgula* (Thecosomata) und *Cuthona caerulea* (Nudibranchia) öffnet sich die Perikardhöhle über einen Wimpertrichter direkt in die Niere, ein echter Renoperikardiodukt fehlt diesen Arten.

Der Aufbau des Nierenepithels der Opisthobranchia erfolgt durch Zellen eines einzigen Typs, der durch große Vakuolen, ausgeprägte basale Einfaltungen der Zellmembran und apikale Mikrovilli charakterisiert ist. Diese Strukturen deuteten auf eine sowohl sekretorische, als auch resorptive Aktivität hin. Über den Nephroporus öffnet sich die Niere in den meisten Taxa direkt nach außen, nur in *C. virgula* und *Hedylopsis* sp (Acochlidia) mündet die Niere in eine Mantelhöhle. Rhogocyten (Porenzellen) konnten im Haemocoel und Bindegewebe aller untersuchter Arten, bis auf *C. virgula*, nachgewiesen werden. Diese Einzelzellen stellen zusätzliche Ultrafiltrationsloci mit einer identischen Feinstruktur wie die der Podocyten dar (Schlitze zwischen cytoplasmatischen Fortsätzen, die durch feine Diaphragmen überspannt und durch eine Basallamina unterlegt sind).

Die ultrastrukturellen Daten über den Renoperikardialkomplex der Opisthobranchia zeigen, daß dieser in seiner Struktur und Organisation grundsätzlich dem anderer Mollusken entspricht. Ultrafiltration in der epikardialen Wand des Atriums mittels Podocyten wird als plesiomorph für die Mollusken angesehen und hier erstmals für die Opisthobranchia nachgewiesen. Damit werden ältere Spekulationen über den Verlustes der Podocyten an der Basis der Opisthobranchia widerlegt. Das Fehlen echter Podocyten und Auftreten eines modifizierten Ultrafiltrations-Zelltyps bei Bullomorphen spiegelt keinen ursprünglichen Zustand wieder, sondern kann wahrscheinlich als Autapomorphie des Taxons angesehen werden. Die ausgeprägten Ultrafiltrationsstellen in der äußeren Perikardwand bei *Hypselodoris tricolor* und *Cuthona caerulea*, zusätzlich zur atrialen Wand, sind von keinem anderen Molluskentaxon bekannt und stellen eine signifikante Autapomorphie der Nudibranchia (alternativ der Nudipleura) dar. Im Gegensatz zu anderen herzlosen oder paedomorphen Arten mit pseudoprotonephridialen (*Rhodope*) oder sekundären protonephridialen Systemen (einige Polychaeten) zeigten das Nierenepithel der herzlosen *Alderia modesta* und der teilweise paedomorphen Vertreter der Gymnosomata und Thecosomata keinerlei Modifikationen. Der Aufbau des Exkretionssystems von *A. modesta* zeigt, daß Ultrafiltration bei Mollusken keine Voraussetzung für effektive Exkretion zu sein scheint. Der Nachweis einer zwar reduzierten, aber eindeutig erhaltenen Mantelhöhle in

Hedylopsis sp. hat wesentliche Auswirkungen auf die Rekonstruktion der Acochlidia und stellt die Hedylopsidae an die Basis dieses Taxons.

LIST OF PUBLICATIONS

(1) FAHRNER, A. & BECK, L.A. 2000. Identification key for the Indo-Pacific species of the nudibranch family Phyllidiidae RAFINESQUE, 1814, including the description of two new species (Gastropoda: Opisthobranchia). *Archiv für Molluskenkunde* **128**: 189-211.

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(3) FAHRNER, A. & SCHRÖDL, M. 2000b. Description of *Phyllidia schupporum*, a new nudibranch species from the northern Red Sea (Gastropoda, Nudibranchia, Phyllidiidae). *Spixiana* **23**: 55-60.

(4) FAHRNER, A. & SCHRÖDL, M. 2000c. Redescription of *Phyllidiopsis sinaiensis* (Yonow, 1988) (Nudibranchia: Doridoidea: Phyllidiidae), with a review of the Red Sea Phyllidiidae. *Journal of Molluscan Studies* **66**: 467-476.

(5) FAHRNER, A. & HASZPRUNAR, G. 2000. Microanatomy and ultrastructure of the excretory system of two pelagic opisthobranch species (Gastropoda: Gymnosomata and Thecosomata). *Journal of Submicroscopic Cytology and Pathology* **32**: 185-194.

(6) FAHRNER, A. & HASZPRUNAR, G. 2001. Anatomy and ultrastructure of the excretory system of a heart-bearing and a heart-less sacoglossan gastropod (Opisthobranchia, Sacoglossa). *Zoomorphology* **121**: 85-93.

(7) FAHRNER, A. & HASZPRUNAR, G. 2002a. Microanatomy, ultrastructure, and systematic significance of the excretory system and mantle cavity of an acochlidian gastropod (Opisthobranchia). *Journal of Molluscan Studies* **68**: 87-94.

(8) FAHRNER, A. & HASZPRUNAR, G. 2002b. Ultrastructure of the renopericardial complex in *Hypselodoris tricolor* (Gastropoda, Nudibranchia). *Zoomorphology* (in press).

(9) SCHRÖDL, M. & FAHRNER, A. 2002. Mollusca: Opisthobranchia. In: *Das Mittelmeer: Fauna, Flora, Ökologie. Band II/2: Marine Fauna.* Hofrichter, R. ed., Spektrum Akademischer Verlag, Heidelberg, Berlin (in press).

CURRICULUM VITAE

Personal data

Name	Alexander Fahrner
Date of birth	11. 05. 1970
Place of birth	Augsburg, Germany
Marital status	married, one daugther (born 06.06.2001)
Address	Maistr. 44 80337 München
Telephone	089 / 53819985
E-mail	kld1130@mail.lrz-muenchen.de

School

1976 - 1980	Elementary school, Augsburg
1980 - 1989	High School, “Holbein Gymnasium” Augsburg
24.06.1989	University Entrance Level ("Abitur"). Mark: 2,0

University studies

Nov. 1989 - May 1992	Biology studies, basic course education at the Ludwig-Maximilians-Universität München (LMU)
11. 05. 1992	Vordiplom-degree. Mark: 1,7
May 1992 - June 1996	Biology studies, Master`s degree education at the Philipps-Universität Marburg Major subject: Zoology; additional subjects: Natural conservation, Ecology, Geography
03. 07. 1996	Master of science degree (Diplom-Biologe). Mark: 1.1
	Master thesis "Phylogeny and Systematics of marine slugs of the family Phyllidiidae RAFINESQUE, 1814 (Nudibranchia)“. Mark: 1,0

Ph.D. studies

Since Nov. 1997	Ph.D. thesis "Comparative microanatomy and ultrastructure of the excretory systems of opisthobranch Gastropoda“. Supervisor Prof. Dr. G. Haszprunar, LMU
July 1998 - June 2000	Doctoral fellowship („Graduiertenstipendium“) of the LMU
July 2000 - Nov. 2001	Scientific employee of the LMU

Practical activities

April 1994 - March 1996	Teaching assistance in several zoological courses: Archicoelomata, beginners course Zoomorphology, zoological identification practice for advanced students
Jan. 1997 - Sep. 1997	Stay in Denmark. Danish course for foreigners at AOF-Aalborg Sprogskole
Feb. 1999 - March 2000	Scientific employee at the Zoologische Staatssammlung München, collection of molluscs